Essentials of Stem Cell Biology

Essentials of Stem Cell Biology

Third Edition

Edited by

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Anthony Atala

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Foreword

It is with great pleasure that I pen this foreword to the third edition of the *Essentials of Stem Cell Biology*. The field of stem cell biology is moving extremely rapidly as the concept and potential practical applications have entered the mainstream. Despite this worldwide intensity and diversity of endeavor, there remain a smaller number of definable leaders in the field, and this volume brings most of them together.

Although the concept of stem and progenitor cells has been known for a long time, it was the progress towards embryonic stem cells which lit the field. Mouse embryonic stem (ES) cells originally came from work aimed at understanding the control and progress of embryonic differentiation, but their *in vitro* differentiation, despite being magnificent, was overshadowed experimentally by their use as a vector to the germline, and hence as a vehicle for experimental mammalian genetics. This now has led to studies of targeted mutation in up to one third of gene loci, and an ongoing international program to provide mutation in every locus of the mouse. These studies greatly illuminate our understanding of human genetics.

Jamie Thomson, reporting the advent of the equivalent human embryonic stem cells, very clearly signaled that their utility would be neither in genetic studies (impractical and unethical in man), nor in fundamental studies of embryonic development (already catered for by mouse ES cells), but, by providing a universal source of a diversity of tissue-specific precursors, as a resource for tissue repair and regenerative medicine.

Progress towards the understanding of pluripotentiality and the control of cellular differentiation, that is basic fundamental developmental biology at the cell and molecular level, now stands as a gateway to major future clinical applications. This volume provides a timely, up-to-date state-of-the-art reference.

The ideas behind regenerative medicine, powered by the products of embryonic stem cells, reinvigorated study of committed stem and precursor cells within the adult body. The use of such stem cells in regenerative medicine

already has a long history, for example in bone marrow transplantation and skin grafting. In both of these examples not only gross tissue transplantation, but also purified or cultured stem cells may be used. They have been extensively applied in clinical treatment, and have most clearly demonstrated the problems which arise with histoincompatibility. Ideally, in most cases, a patient is better treated with his own – autologous – cells than with partially matching allogeneic cells. An ideal future would be isolation, manipulation, or generation of suitable committed stem or precursor cell populations from the patient for the patient. The amazing advances of induced pluripotential stem cells point to the possibilities of patient-specific *ad hominem* treatment. This personalized medicine would be an ideal scenario, but as yet the costs of the technologies may not allow it to be a commercial way forward. The timelines are, however, likely to be long before the full promise of these technologies is realized, and there is every possibility that such hurdles will be circumvented. Quite properly, much of this book concentrates on the fundamental developmental and cell biology from which the solid applications will arise.

This is a knowledge-based field in which we have come a long way, but are still relatively ignorant. We know many of the major principles of cell differentiation, but as yet need to understand more in detail, more about developmental niches, more about the details of cell–cell and cell growth-factor interaction, and more about the epigenetic programming which maintains the stability of the differentiated state.

Professor Sir Martin Evans

Sir Martin Evans, PhD, FRS
Nobel Prize for Medicine 2007

Sir Martin is credited with discovering embryonic stem cells, and is considered one of the chief architects of the field of stem cell research. His ground-breaking discoveries have enabled gene targeting in mice, a technology that has revolutionized genetics and developmental biology, and have been applied in virtually all areas of biomedicine – from basic research to the development of new medical therapies. Among other things, his research inspired the effort of Ian Wilmut and his team to create Dolly the cloned sheep, and Jamie Thomson's efforts to isolate embryonic stem cells from human embryos, another of the great medical milestones in the field of stem cell research. Professor Evans was knighted in 2004 by Queen Elizabeth for his services to medical science. He studied at Cambridge University and University College London before leaving to become director of bioscience at Cardiff University.

Preface

Much has happened since the first edition of *Essentials of Stem Cell Biology* was published. Sir Martin Evans, who is credited with discovering embryonic stem cells, received the Nobel Prize for Physiology or Medicine in 2007; and Shinya Yamanaka, who discovered how to reprogram differentiated cells into induced pluripotent stem (iPS) cells, won the Nobel Prize in 2012 for the achievement. The third edition of *Essentials* includes chapters by both of these groundbreaking pioneers, as well as by dozens of other scientists whose pioneering work has defined our understanding of stem cell biology. The volume covers the latest advances in stem cell research, with updated chapters on pluripotent, adult, and fetal stem cells. While it offers a comprehensive – and much needed – update of the rapid progress that has been achieved in the field in the last several years, we have retained those facts and subject matter which, while not new, is pertinent to the understanding of this exciting area of biology.

As in previous editions, the third edition of *Essentials* is presented in an accessible format suitable for students and general readers interested in following the latest advances in stem cells. The organization of the book remains largely unchanged, combining the prerequisites for a general understanding of pluripotent and adult stem cells; the tools and methods needed to study and characterize stem cells and progenitor populations; as well as a presentation by the world's leading scientists of what is currently known about each specific organ system. Sections include basic biology/mechanisms, tissue and organ development (ectoderm, mesoderm, and endoderm), methods (such as detailed descriptions of how to generate both iPS and embryonic stem cells), application of stem cells to specific human diseases, regulation and ethics, and a patient perspective by Mary Tyler Moore. For the third edition, Anthony Atala joins me as a new Editor to the book. The result is a comprehensive reference that we believe will be useful to students and experts alike.

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CHAPTER 1

Why Stem Cell Research? Advances in the Field

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1.1 THE ORIGINS OF STEM CELL TECHNOLOGY

Stem cell research, which aims to develop new cell therapies, has accelerated at an astonishing pace; both in terms of the breadth of interests and the discoveries that continue to evolve. Research in stem cell biology is opening new platforms to launch even more spectacular developments, crowding the pages of major journals each month. One might wonder why the field took so long to explode in such an incredible fashion.

The studies of John Gurdon and colleagues on reprogramming amphibian cells using oocytes stand as a very significant milestone that was emphatically amplified by Ian Wilmut and colleagues, who unexpectedly reprogrammed mammalian somatic cell nuclei into totipotent embryos when the nuclei were introduced into oocytes of the same species. Martin Evans and colleagues showed that cells isolated from the blastocyst stage of an embryo could be converted to pluripotent embryonic stem cells. Traveling on an independent plane of discovery were many great scientists, among whom Irv Weismann stands out for his discoveries of adult hematopoietic stem cells in mice and humans. Bone marrow transplants have a well-established history as a therapeutic strategy for cancer and other diseases of the blood.

What a melting pot of ingredients for James Thomson to launch the discovery of human embryonic stem cell lines, cloning for stem cells in the mouse by members of my own group, and most significantly the demonstration by Shinya Yamanaka of the ability to reprogram somatic cells to pluripotency (induced pluripotent stem cells) using four critical transcription factors. Again independently, Arthur Caplan isolated mesenchymal stem cells from bone marrow, showing their multipotent capacities to form bone, cartilage, and adipose tissue. Now we have the ingredients to explore the possibility of applying stem cell discoveries to regenerative medicine. The potential

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for using living cells to regenerate whole organs was quickly underscored by Anthony Atala's demonstration of engineering bladders for patients.

1.2 ORGANIZATIONS THAT ADVOCATE AND SUPPORT THE GROWTH OF THE STEM CELL SECTOR

Basic scientists gathered around Len Zon to form and launch the International Society of Stem Cell Research. Cell therapy and tissue transplant scientists have remained largely separate but have become another effective science and therapeutic organization under the International Society for Cell Therapy. Separately, the stem cell biotechnology industry has joined together under the umbrella of the Alliance for Regenerative Medicine to become an effective advocate for the emerging industry interests in cell and tissue therapies.

The Bush administration in the USA raised concerns within the fledgling stem cell science community by restricting the funding of embryonic stem cell research and limiting the number of embryonic stem cell lines that could be studied with federal funding. Key scientists in California coopted Robert Klein, a financier and lawyer, to their cause and he was able to galvanize the Californian voters to pass Proposition 71 (with 59% support) – a game-changing state bond initiative that required California to sell general obligation bonds up to $3 billion to fund pluripotent stem and progenitor cell research. This extremely clever approach to funding intellectual capital was supported by the Republican Governor Arnold Schwarzenegger, and established the Californian Institute for Regenerative Medicine (CIRM).

California has since become a major hub for stem cell research, attracting many of the world's best scientists and rivaling the well-established biotechnology hubs around Boston and New York. Twelve new research institutes have been built in California under CIRM sponsorship, assembling a critical mass of intellectual excellence and driving an incredible productivity of discovery research. Both Thompson and Yamanaka have appointments in California institutions. Two clusters of biotechnology companies involved in cell therapies have evolved in the Bay Area and San Diego, with a third forming in Los Angeles. Companies are relocating to California and are actively opening offices and labs to contribute to the energized environment there. CIRM has also developed a very major network of collaborations with 12 international countries and states, a number of US states, foundations, and, most recently, with the US National Institutes of Health. These collaborations are driving globally a vast array of basic research and translational medicine, and changing the quality and depth of global research to find solutions to the world's most feared and intractable diseases.

1.3 APPLICATIONS OF STEM CELLS IN MEDICINE

At the forefront of applying stem cell research are critical studies to find the means to eradicate the most dangerous cell of all – the malignancy seeding cancer stem cell in blood and solid tumors. There are also rapidly evolving strategies for curing HIV/AIDS, recovering sight from blindness, potentially curing Type I diabetes, using stem cells for delivering gene therapy, reversing spinal cord injuries, and curing other motor neuron and demyelinating diseases. The list of potential therapies is exhaustive and needs to be addressed as science opens an understanding of these diseases. Surprisingly, induced pluripotent stem (iPS) cell studies are exposing new insights into mental retardation, autism, epilepsy, and schizophrenia. Hope remains strong that cell therapies can offer substantial benefits to neurodegenerative conditions such as Parkinson's, Alzheimer's, and Huntington's diseases.

Meanwhile the biotechnology industry has begun to deliver clinical trials using therapies derived from adult cells. The majority of trials are employing mesenchymal stem cells, adipose-derived stromal cells, and adult or fetal neural stem cells to evaluate safety and efficacy of cell-based therapies in indications ranging from disorders of soft tissue and bone to chronic conditions of heart disease, diabetes, and stroke. Adult cell-based therapies are even being evaluated for their ability to reverse or ameliorate genetic diseases.

Why would there not be a strong move of scientists towards stem cell research with the tools and critical technologies that have evolved? It appears that endogenous cell lineages may be manipulated by judicious use of tissue targeting of key transcription factors. Converting stromal phenotypes to endocrine, muscle, or neural cell types that have been lost in disease and injury could be the next major platform of stem and progenitor cell research. Could these developments sidestep the need to develop transplantation tolerance strategies for enabling effective grafting of allogeneic cellular therapies?

1.4 CHALLENGES TO THE USE OF STEM CELLS

There remain very vocal and manipulative conservative and religious interest groups that decry the potential benefits of embryonic stem cell science, despite very strong overall community support in the US and elsewhere. They exclusively support adult stem cell therapies, including those where there is little scientific evidence of benefit and a lack of safety regulation. There is an important consideration that often fails in these commentaries that ignore science – *do no harm.*

Stem cell science will ultimately prevail despite the opposition from some quarters, because researchers will derive the evidence for understanding

disease. By rigorous design and adequately controlled experimentation the true value of stem cell-based treatments will be demonstrated. If not, the hypotheses will fail and we will move on.

I wish I were starting again in stem cell research.

FOR FURTHER STUDY

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CHAPTER 2

'Stemness': Definitions, Criteria, and Standards

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2.1 WHAT IS A STEM CELL?

Stem cells are functionally defined as having the capacity to self-renew and the ability to generate differentiated cells. More explicitly, stem cells can generate daughter cells identical to their mother (self-renewal), as well as produce progeny with more restricted potential (differentiated cells). Such a simple and broad definition may be satisfactory for embryonic or fetal stem cells that do not persist for the lifetime of an organism but it breaks down when trying to describe other types of stem cells (e.g., adult stem cells). Another functional parameter that should be included in a definition of stem cells is potency, or its potential to produce differentiated progeny. Does the stem cell generate multiple differentiated cell types (multipotent or pluripotent) or is it only capable of producing one type of differentiated cell (unipotent)? Thus, a more complete functional definition of a stem cell includes a description of its replication capacity and potency.

2.2 SELF-RENEWAL

Stem cell literature is replete with terms such as 'immortal,' 'unlimited,' 'continuous,' to describe a cell's replication capacity. These rather extreme and vague terms are not very helpful, as it can be noted that experiments designed to test the 'immortality' of a stem cell would by necessity outlast authors and readers alike. Such terms are probably best avoided or used sparingly.

Most somatic cells cultured *in vitro* display a finite number (less than 80) of population doublings prior to replicative arrest or senescence, in contrast to the seemingly unlimited proliferative capacity of stem cells cultured *in vitro*. Therefore, it is reasonable to say that a cell that can undergo more than twice this number of population doublings (i.e. 160) without oncogenic transformation can be termed 'capable of extensive proliferation.' In a few cases, this

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criterion has been met, most notably in embryonic stem (ES) cells derived from either humans or mice, as well as in adult neural stem cells (NSCs).

For adult stem cells, an incomplete understanding of the factors required for self-renewal *ex vivo* exists, thus the ability to establish similar proliferative criteria based on *in vitro* culture is limited. Therefore, the proliferative capacity of adult stem cells is currently best defined *in vivo*, where they should display sufficient proliferative capacity to last throughout the lifetime of the animal. In some cases, a rigorous assessment of the capacity for self-renewal of certain adult stem cells has been obtained by single cell or serial transfer into acceptable hosts, an excellent example of which is adult hematopoietic stem cells (HSCs).

2.3 POTENCY

The issue of potency may be the most difficult parameter to incorporate into a widely accepted definition of stem cells. A multipotent stem cell sits atop a lineage hierarchy and can generate multiple types of differentiated cells, the latter being cells with distinct morphologies and gene expression patterns. At the same time, many would argue that a self-renewing cell that can only produce one type of differentiated descendant is nonetheless a stem cell. A case can be made that a unipotent cell is best described as a progenitor cell for clarity of terminology. Progenitors are typically the descendants of stem cells, only they are more constrained in their differentiation potential or capacity for self-renewal, and are often more limited in both senses.

2.4 CLONALITY

Replicative capacity and potency are functional parameters. Clonality is a characteristic that describes how a population of cells, usually in culture, was derived. A clonal population is generated from a single cell, such as a stem cell, with the capacity for self-renewal. Clonality becomes very important when one asks what constitutes a cell line. Although the clonal 'gold standard' is well understood, there remain several confounding issues when applied to stem cells.

The lowest standard of defining a cell line would be to include any population of cells that can be grown in culture, frozen, thawed, and subsequently repassaged *in vitro*. A higher standard would be to limit the definition to a clonal or apparently homogeneous population of cells, but it must be recognized that cellular preparations that are not derived from a single cell may be a mixed population. Such preparations may contain both stem cells and other cells, some of which may be required to support the propagation of the purported stem cells. Hence, any reference to a stem cell line should include an explanation of its derivation. For example, it can be misleading to report on stem cells or stem cell lines if they were prepared from a tissue containing

multiple cell types because the possibility that the culture is contaminated with stem cells from another tissue (e.g., blood vessels) exists.

2.5 DEFINITION

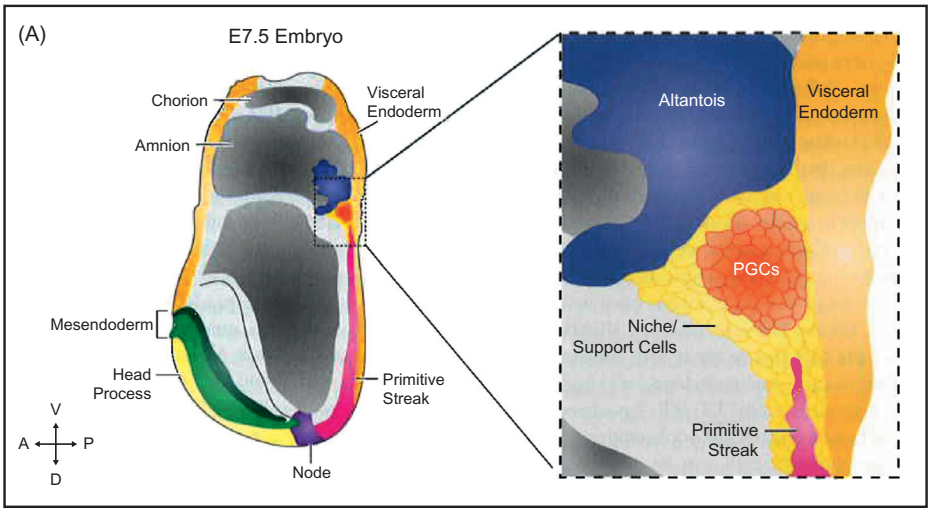
In conclusion, a working definition of a stem cell line is a clonal, self-renewing cell population that is multipotent and thus can generate several differentiated cell types. Admittedly, this definition is not applicable in all instances and is best used as a guide to help describe cellular attributes.

2.6 WHERE DO STEM CELLS COME FROM?

The origin or lineage of stem cells is well understood for ES cells, but the origin of adult stem cells is less clear and in some cases controversial. Of significance may be the observation that ES cells originate prior to germ layer commitment, raising the intriguing possibility that avoidance of commitment to a developmental pathway may be a mechanism by which multipotent stem cells arise. The lack of information on the developmental origins of adult stems cells leaves open the possibility that they too escape lineage restriction in the early embryo and subsequently colonize specialized niches, which function to both maintain their potency as well as restrict their lineage potential. Alternatively, the prevailing model for the origin of adult stem cells hypothesizes that they arise after somatic lineage specification and then colonize their respective cellular niches. In this section, I briefly summarize the origin of stem cells from the early embryo and explain what is known about the ontogeny of adult stem cells focusing attention on HSCs and NSCs.

2.7 STEM CELLS OF THE EARLY EMBRYO

Mouse and human ES cells are derived directly from the inner cell mass of preimplantation embryos after the formation of a blastocyst. This population of cells would normally produce the epiblast and eventually all adult tissues, which may help to explain the developmental plasticity exhibited by ES cells. In fact, ES cells appear to be the *in vitro* equivalent of the epiblast, as they have the capacity to contribute to all somatic lineages and in mice to produce germ-line chimeras. By the time the zygote has reached the blastocyst stage, the developmental potential of certain cells has been restricted. The outer cells of the embryo have begun to differentiate to form trophectoderm, from which a population of embryonic trophoblast stem cells has also been derived in mice. These specialized cells can generate all cell types of the trophectoderm lineage, including differentiated giant trophoblast cells. At the egg cylinder stage of embryonic development, commonly referred to as embryonic day 6.5 (E6.5) in mice, a population of cells near the epiblast (Figure 2.1) can be identified as primordial germ cells (PGCs), which



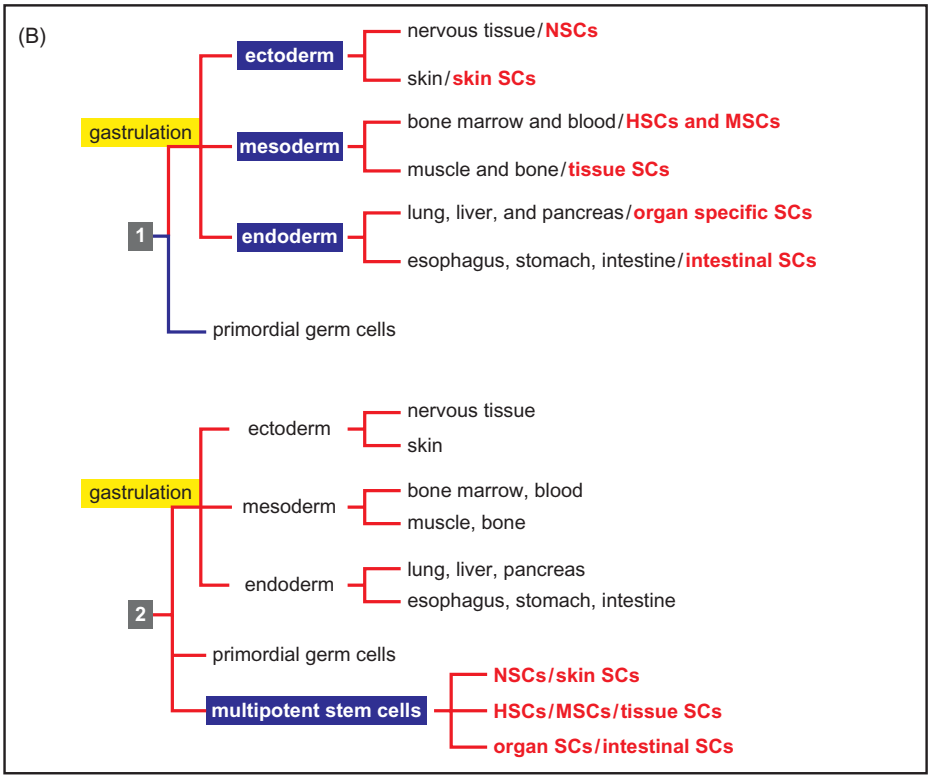


FIGURE 2.1

(A) Development of primordial germ cells. A schematic of an embryonic day 7.5 mouse embryo highlights the position of the developing primordial germ cells (PGCs) proximal to the epiblast. The expanded view on the right serves to illustrate the point that PGCs escape lineage commitment/restriction by avoiding the morphogenetic

are subsequently excluded from somatic specification or restriction. PGCs migrate to and colonize the genital ridges, where they produce mature germ cells and generate functional adult gametes. PGCs can be isolated either prior or subsequent to their arrival in the genital ridges and, when cultured with appropriate factors *in vitro*, can generate embryonic germ (EG) cells. EG cells have many of the characteristics of ES cells with respect to their differentiation potential and their contribution to the germ-line of chimeric mice. The most notable difference between ES and EG cells is that the latter may display (depending upon the developmental stage of their derivation) considerable imprinting of specific genes. Consequently, certain EG cell lines are incapable of producing normal chimeric mice.

Importantly, no totipotent stem cell has been isolated from the early embryo. ES and EG cells generate all somatic lineages as well as germ cells but rarely if ever contribute to the trophectoderm, extraembryonic endoderm, or extraembryonic mesoderm. Trophectoderm stem (TS) cells have been isolated, and these only generate cells of the trophectoderm lineage. It remains to be seen whether cells can be derived and maintained from totipotent embryonic stages. Although our understanding of cell fates in the early embryo is incomplete, it appears that the only pluripotent stem cells found after gastrulation are PGCs (with the possible exceptions of multipotential adult progenitor cells and teratocarcinomas). It may be that PGCs escape germ layer commitment during gastrulation by developing near the epiblast and subsequently migrate to positions inside the embryo proper. This developmental strategy may not be unique to PGCs, and it raises the interesting possibility that other stem cells might have similar developmental origins. Alternatively, it may be the case that adult stem cells are derived from PGCs. Although intriguing, it is important to stress that this idea lacks experimental evidence.

2.8 ONTOGENY OF ADULT STEM CELLS

The origin of most adult stem cells is poorly understood. With the issue of adult stem cell plasticity at the forefront, as described in this section, studies designed to elucidate the ontogeny of adult stem cells may help to reveal their specific lineage relationships and shed light on their plasticity and potential. Information on the origins of adult stem cells would also help to

◀ effects of migrating through the primitive streak during gastrulation. (B) Putative developmental ontogeny of stem cells. In lineage tree 1, the development of stem cells occurs after the formation of germ layers. These stem cells are thus restricted by germ layer commitment to their respective lineage (e.g., mesoderm is formed, giving rise to hematopoietic progenitors that become hematopoietic stem cells). Lineage tree 2 illustrates the idea that stem cells might develop similarly to PGCs, in that they avoid the lineage commitments during gastrulation and subsequently migrate to specific tissue and organ niches.

define the molecular programs involved in lineage determination, which may in turn provide insights into methods for manipulating their differentiation. To this end, I summarize what is known about the development of adult stem cells within the context of the hematopoietic and neural systems.

The development of hematopoietic cells in mice occurs soon after gastrulation (E7.5), although HSCs with the same activities as those in the adult have only been observed and isolated at midgestational stages (E10.5). These observations suggest that the embryo has a unique hematopoietic lineage hierarchy, which may not be founded by an adult type HSC. Thus, hematopoiesis appears to occur at multiple times or in successive waves within the embryo, and the emergence of an HSC may not precede or be concomitant with the appearance of differentiated hematopoietic cells.

The first site of hematopoiesis in the mouse is the extraembryonic yolk sac, soon followed by the intraembryonic aorta–gonad–mesonephros (AGM) region. Which of these sites leads to the generation of the adult hematopoietic system and, importantly, HSCs is still unclear. Results from non-mammalian embryo-grafting experiments, with various findings in the mouse, suggest that the mammalian embryo, specifically the AGM, generates the adult hematopoietic system and HSCs. Interestingly, the midgestational AGM is also the region that harbors migrating PGCs and is thought to produce populations of mesenchymal stem cells, vascular progenitors, and perhaps hemangioblasts.

In the absence of studies designed to clonally evaluate the lineage potential of cells from the AGM, and without similarly accurate fate mapping of this region, it remains possible that all of the adult stem cell types thought to emerge within the AGM arise from a common unrestricted precursor. This hypothetical precursor could help explain reports of nonfusion-based adult stem cell plasticity. The observed lineage specificity of most adult stem cells could likewise be attributed to the high-fidelity lineage restriction imposed on them by the specific niche they colonize or are derived from. Simple ideas such as these have not been ruled out by experimental evidence, underscoring both the opportunity and the necessity for further study of the developmental origins of adult stem cells.

A key lesson from studies of the developing hematopoietic system is that the appearance of differentiated cells does not tell us where or when the corresponding adult stem cells originate. Definitive lineage tracing, with assays of clonogenic potential, remains the method of choice for identifying the origin of stem cells. Another potential pitfall revealed by these studies is that the definition of the stem cell can influence whether and how it is identified.

The development of NSCs begins with the formation of nervous tissue from embryonic ectoderm following gastrulation. Induction of the neural plate is

thought to coincide with the appearance of NSCs as well as restricted progenitor types. The exact frequency and location of stem cells within the developing neuroepithelium remains unknown; specific markers must be discovered to fully unravel this question. An emerging view in the field is that embryonic neuroepithelia generate radial glia that subsequently develop into periventricular astrocytes, and that these cells are the embryonic and adult NSCs within the central nervous system. Developing and adult NSCs also appear to acquire positional and temporal information. For example, stem cells isolated from different neural regions generate region-appropriate progeny. In addition, several studies suggest that temporal information is encoded within NSCs, that earlier stem cells give rise more frequently to neurons, and that more mature stem cells preferentially differentiate into glia. Moreover, more mature NSCs appear incapable of making cells appropriate for younger stages when transplanted into the early cerebral cortex.

Taken together, observations to date suggest that the nervous system follows a classical lineage hierarchy, with a common progenitor cell generating most if not all differentiated cell types in a spatially- and temporally-specific manner. Rare stem cells may also exist in the nervous system, perhaps not of neural origin, which have greater plasticity in terms of producing diverse somatic cell types and lacking temporal and spatial constraints. Several caveats must be considered when describing the developmental origins of NSCs. First, disrupting the neuroepithelia to purify NSCs may have the undesirable effect of dysregulating the spatial patterning normally acquired by these cells. Second, growth of purified NSCs *in vitro* may reprogram the cells through exposure to non-physiological conditions during culture. Both of these problems can be addressed either by *in vivo* lineage tracing or by prospectively isolating NSCs and transplanting them into acceptable hosts without intervening culture. Such experiments, carefully done, might answer questions important for stem cell biology but also for neuroembryology and development. These questions include which features of the developmental program are intrinsic to individual cells, which differentiation or patterning signals act exclusively to instruct specific cell fates, and how developmental changes in cell-intrinsic programs restrict the responses of progenitors to cell-extrinsic signals.

2.9 HOW ARE STEM CELLS IDENTIFIED, ISOLATED, AND CHARACTERIZED?

The ways that stem cells are identified, isolated, and characterized are the key methodological questions in stem cell biology, so much so that subsequent chapters are devoted to addressing these problems in detail. Here, I briefly

outline standards and criteria that may be employed when approaching the challenge of identifying, isolating, and characterizing a stem cell.

2.10 EMBRYONIC STEM CELLS

The basic characteristics of an ES cell include self-renewal, multilineage differentiation *in vitro* and *in vivo*, clonogenicity, a normal karyotype, extensive proliferation *in vitro* under well-defined culture conditions, and the ability to be frozen and thawed. In animal species, *in vivo* differentiation can be assessed rigorously by observing whether transferred ES cells contribute to all somatic lineages and produce germ-line chimerism. However, experimentation ethics prohibit the same experiments being performed with human ES cells; consequently, human ES cells are assayed for their ability to generate embryoid bodies and teratomas containing differentiated cells of all three germ layers. Moreover, because a stringent *in vivo* assessment of pluripotency is impossible, human ES cells must exhibit expression of well-known molecular markers of pluripotent cells. Such markers are factors that are expressed consistently and are enriched in human ES cells.

Another experimental substitute for whole-animal chimerism is to evaluate the ability of human ES cells to contribute to the development of specific tissues when transplanted into discrete developmental regions of nonhuman adults or embryos. A complementary analysis might include transplanting human ES cells into nonhuman blastocysts and evaluating their contribution to various organs and tissues of the resulting embryo, although producing human/nonhuman chimeras at this earliest stage of development has raised ethical concerns.

Finally, a practical consideration for all ES cells is the number of times the cells have been passaged *in vitro*. Although it is important to establish the capacity of ES cells to proliferate extensively, it is equally important to preserve stocks of cells that have been passaged only a few times so that experimental findings observed on working stocks of ES cells can be verified with low-passage cells to screen for artifacts that can be introduced during *ex vivo* expansion.

2.11 ADULT STEM CELLS

The basic definition of an adult stem cell is that the culture is derived from a single cell (clonal) that self-renews and generates differentiated cells. The most rigorous assessment of these characteristics is to prospectively purify a population of cells (usually by cell surface markers), transplant a single cell

into an acceptable host without any intervening *in vitro* culture, and observe self-renewal and reconstitution of either a tissue, organ, or lineage as appropriate for the adult stem cell type. Admittedly, this type of *in vivo* reconstitution assay is not well defined for many types of adult stem cells. Thus, it is important to define a set of functional assays that accurately reflect the cells' developmental potential and can be performed on *in vitro* cultures. Above all, clonal assays should be the standard by which fetal and adult stem cells are evaluated because this assay removes any doubt that an observation is the result of contamination of a culture with other cell types.

Two concepts about the fate or potential of stem cells have moved to the forefront of adult stem cell research. The first is plasticity; the idea that restrictions in cell fates are not permanent but are flexible and reversible. The most obvious and extreme example of reversing a committed cell fate comes from experiments in which a terminally differentiated somatic cell generates an entire animal following nuclear transfer, or cloning. Nuclear transfer experiments have demonstrated that differentiated cells, under the appropriate conditions, can be returned to their most primal state. Thus, it may not be surprising if conditions are found for more committed or specified cells to dedifferentiate and gain a broader potential. A related concept is that of transdifferentiation. Transdifferentiation is the generation of functional cells of a tissue, organ, or lineage that are distinct from that of the founding stem cell. Important issues here are whether the cells proposed to transdifferentiate are clonal, and whether the mechanism by which they form the resulting cell type requires fusion. Experiments designed to carefully evaluate these possibilities will yield further insight into the basic nature of stem cells.

2.12 STEMNESS: PROGRESS TOWARD A MOLECULAR DEFINITION OF STEM CELLS

Stemness refers to common molecular processes underlying the core stem cell properties of self-renewal and the generation of differentiated progeny. Although stems cells in different cellular microenvironments or niches will by necessity have different physiological demands and therefore distinct molecular programs, there are likely to be certain genetic characteristics that are both specific to and shared by all stem cells. Through transcriptional profiling, many genes that are enriched in ES cell, TS cell, HSC, and NSC populations have been identified. By extending transcriptional profiling to other stem cells and more organisms, it may be possible to develop a molecular fingerprint for stem cells. Such a fingerprint could form the basis of a molecular definition of stem cells that, when combined with functional characteristics, would provide a more comprehensive set of criteria for understanding their

unique biology. Perhaps more importantly, transcriptional profiling could eventually become the primary tool by which new stem cells are identified and isolated.

The goal of having a comprehensive definition of stemness is far from being accomplished, but preliminary findings for specific stem cells have been described. Transcriptional profiling of stem cells has suggested that several distinct molecular characteristics are shared. Stem cells appear to have the capacity to sense a broad range of growth factors and signaling molecules and to express many of the downstream signaling components involved in the transduction of these signals. Signal transduction pathways present and perhaps active in stem cells include TGF, Notch, Wnt, and Jak/Stat family members. Stem cells also express many components involved in establishing their specialized cell cycles, either related to maintaining cell cycle arrest in G1 (for most quiescent adult stem cells) or connected to progression through cell cycle checkpoints promoting rapid cycling (as is the case for ES cells and mobilized adult stem cells).

Most stem cells also express molecules involved in telomere maintenance and display elevated levels of telomerase activity. Considerable evidence exists that stem cells have significantly remodeled chromatin due to the activity of DNA methylases, transcriptional repressors of histone deacetylase, and Groucho family members. Another common molecular feature is the expression of specialized posttranscriptional regulatory machinery that is influenced by RNA helicases of the Vasa type. Finally, a shared molecular and functional characteristic of stem cells appears to be their resistance to stress, mediated by multidrug resistance transporters, protein-folding machinery, ubiquitin, and detoxifier systems.

Although in its infancy, the search for a molecular signature to define stem cells continues. We have begun to understand in general terms what molecular components are most often associated with stem cells. In the future, it may be possible to precisely define stem cells as a whole and individually by their tell-tale molecular identities. Until that time, stemness remains a concept of limited utility with tremendous potential.

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FOR FURTHER STUDY

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CHAPTER 3

Pluripotent Stem Cells from Vertebrate Embryos: Present Perspective and Future Challenges

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3.1 INTRODUCTION

Many have contributed to the various discoveries that brought recognition of the enormous potential of cells of early embryonic origin for genetic modification of organisms, regenerative medicine, and investigation of facets of development that are difficult to explore *in vivo*. Historically, the work of two researchers stands out as forming a foundation for our current understanding of embryonic stem cells and their potential: Leroy Stevens and Barry Pierce Stevens developed and exploited mouse strains with high incidences of testicular tumors to determine their cellular origins. Pierce focused his attention on the nature of the cell that endowed teratocarcinomas with the potential for indefinite growth, which the more common teratomas lacked. Conversion of solid teratocarcinomas to an ascites form proved to be a significant advance for enriching a sub-population of morphologically undifferentiated cells in such tumors, among which it was expected that their stem cells could be isolated. Then, an impressive experiment by Pierce and a colleague showed unequivocally that a single morphologically undifferentiated cell could form a self-sustaining teratocarcinoma containing as rich a variety of differentiated tissues as its parent tumor when transplanted into a histocompatible adult host. Hence, the embryonal carcinoma (EC) cell, as the stem cell of teratocarcinomas has come to be known, was the first self-perpetuating pluripotential cell to be characterized.

Although teratocarcinomas were initially obtained as a result of mutations that affected the differentiation of male or female germ cells, later it was observed that teratocarcinomas could also be established in certain genotypes of mice by the ectopic grafting of early embryos into adult mice. Culture conditions were soon identified that allowed the propagation of EC cells in an undifferentiated state, or induced EC cells to differentiate *in vitro*. Although

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the range of differentiation observed during *in vitro* culture was more limited than what could be observed by *in vivo* transplantation, it was nevertheless impressive. Research on murine EC cells provided the impetus for obtaining and harnessing human EC cells.

One outstanding question regarding the use of murine EC cells as a model system for studying aspects of development remained; namely, the basis of their malignancy. Was this malignancy a consequence of genetic changes, or did it emerge because such 'embryonic' cells failed to relate to the ectopic sites into which they were transplanted? One obvious way of answering this question was to observe how EC cells behaved when transplanted into an embryonic rather than an adult environment. Three laboratories independently injected EC cells into blastocysts and the results each observed were consistent with the same rather striking conclusion: EC cells – which, if injected into an adult, would grow progressively and kill it – were able to participate in normal development following their introduction into the blastocyst. Using genetic differences to distinguish between donor and host cells, EC cells were found integrated into most if not all organs and tissues of the resulting offspring. Most intriguingly, according to reports from one laboratory, EC cells integrated into the germ-line. The potential significance of this finding was considerable because it implied that it was possible to manipulate the mammalian genome. If transplanted EC cells could integrate into all tissues of an offspring, including the germ-line, then the prospect of selecting for rare events was more likely and genetic manipulation in mammals could possibly become as routine as the genetic manipulation of microorganisms had become.

There were problems, however. One was that the EC contribution in chimeric offspring was typically both more modest and more patchy than that of cells transplanted directly between blastocysts. Also, chimeras frequently formed tumors; those that proved to be teratocarcinomas were often evident already at birth, suggesting that the growth regulation conferred by the embryonic environment failed completely in at least some of the transplanted EC cells. Other chimeras developed more tissue-specific tumors (e.g., rhabdomyosarcomas) as they aged that were clearly of donor origin, thereby revealing that some transplanted EC cells could progress along various lineages before their differentiation went awry. In extreme cases, the transplanted EC cells disrupted development altogether, so that fetuses did not survive to birth.

Although the best EC lines could contribute to all or most tissues of the body of chimeras, the penetrance of donor integration was inconsistent. In the end, it became clear to researchers that the frequency with which colonization of the germ-line occurred with EC cells was too low to enable them to be harnessed for genetic modification. One explanation was that the EC 'stem cells'

obtained by the protracted and complex process of generating teratocarcinomas *in vivo* and then adapting them to *in vitro* culture rarely if ever retained a normal genetic constitution. If this was indeed the case, one alternative was to see if such stem cells could be obtained in a less circuitous manner.

Researchers began investigating what happens when murine blastocysts were explanted directly on growth-inactivated feeder cells in an enriched culture medium. The result was the derivation of cell lines that were indistinguishable from EC cells in both their morphology and the expression of various antigenic and other markers, as well as in the appearance of the colonies they formed during growth. Like EC cells, these self-perpetuating, blastocyst-derived stem cells formed aggressive teratocarcinomas when transplanted into both syngeneic and immunologically compromised nonsyngeneic adult hosts. Unlike EC cells, self-perpetuating blastocyst-derived stem cells transplanted into embryonic environments generated chimeric offspring with more frequent and widespread integration into tissues and organs, including the germ-line. Moreover, when combined with host conceptuses whose development was compromised by tetraploidy, self-perpetuating blastocyst-derived stem cells could sometimes form offspring in which no host-derived cells were discernible. Thus, these cells, which exhibited all the desirable characteristics of EC cells and few of their shortcomings, came to be called embryonic stem (ES) cells.

Once it had been shown that ES cells could retain their ability to colonize the germ-line after *in vitro* transfection and selection, their future was assured. Surprisingly, however, despite the wealth of studies demonstrating their capacity for differentiation *in vitro*, particularly in the mouse, it was a long time before the idea of harnessing ES cells for therapeutic purposes took root. Edwards had proposed that ES cells might be used to develop new treatments in the early 1980s, but it was only after Thomson derived the first ES cell lines from human blastocysts in the late 1990s that the idea gained momentum.

3.1.1 Terminology

The literature contains some confusing terminology when referring to the range of different types of cells that ES cells are able to form, an attribute that, in embryological parlance, is called 'potency.' ES cells have been called 'totipotent' because, at least in the mouse, they have been shown to be able to generate all types of fetal cells and, under certain conditions, entire offspring. The term 'totipotent' is inappropriate when applied to ES cells, however. Totipotent in classical embryology is a term that is reserved for cells that retain the capacity to form an entire conceptus and thus produce a new individual unaided. The only cells that have so far been shown to be able to do this are blastomeres from early cleavage stages. Murine ES cells fail to meet

the classical definition of totipotency because they do not form all the different types of cell of which the conceptus is composed. Following injection into blastocysts, murine ES cells most frequently generate cell types that are products of the epiblast lineage. Murine ES have been observed to form derivatives of the primitive endoderm lineage, but such lineages are derived more readily *in vitro* than *in vivo*. In contrast, murine ES cells have never been shown to contribute to the trophectodermal lineage. A more accurate term to describe ES cell potency is 'pluripotent' to distinguish them from stem cells like those of the hematopoietic system, which have a narrower but nevertheless impressive range of differentiation potential. Although the pluripotent label has become widely adopted in the literature, putative ES cells from mammals other than mouse continue to be called 'totipotent' because their nuclei have been shown to be able to support development to term when used for reproductive cloning.

Another facet of terminology relates to the definition of an ES cell, which again is not employed in a consistent manner. One view, to which the author subscribes, is that use of the term 'ES cell' should be restricted to pluripotent cells derived from pre- or peri-implantation conceptuses that can form functional gametes in addition to having the ability to form the full range of somatic cell types in the offspring. Although there are considerable differences among strains of mice in the ease with which morphologically undifferentiated cell lines can be obtained from early conceptuses, competence to colonize the germ-line as well as somatic tissues seems to be common to ES cell lines from every strain from which cell lines have been derived. This is true, for example, even for ES cell lines derived from the non-obese diabetic (NOD) strain, where the lines have so far been found to grow too poorly to enable genetic modification.

3.1.2 ES-Like Cells in other Species

As shown in Table 3.1, cell lines that can be maintained for variable periods *in vitro* in a morphologically undifferentiated state have been obtained from morulae or blastocysts in a variety of species of mammals in addition to the mouse. They have also been obtained from the stage X blastoderm in the chick and from blastulae in three species of teleost fish. The criteria employed to support claims that such lines are counterparts of murine ES cells are quite varied and often far from unequivocal. The criteria range from maintenance of an undifferentiated morphology during propagation or expression of at least some ES cell markers, through differentiation into a variety of cell types *in vitro*, to production of histologically diverse teratomas or chimerism *in vivo*.

What such ES-like (ESL) cells lines have in common with murine ES cells, in addition to a morphologically undifferentiated appearance, is a high nuclear-cytoplasmic ratio. Variable morphology of the growing colonies complicates

Table 3.1 Vertebrates from Which ES-Like Cells Have Been Obtained

Species	Basis of Validation[a]
Rat	CP but mouse ES contamination
	M&M
	CP
	M&M
	M&M
Golden hamster	IVD
Rabbit	M&M, IVD
	CP
Mink	T (but limited range of cell types),
	T (wide range of cell types)
	IVD
	M&M
Pig	IVD
	M&M
	CP
	CP
Sheep	M&M
	?[b]
	?[b]
Cow	M&M
	IVD
	IVD
	?
	CP
	CP
	IVD
Horse	IVD
Marmoset	IVD
Rhesus monkey	T
Human	T
Chicken	IVD (and CP including germ-line but only with passage 1–3 cells)
Medaka	IVD
	CP
Zebra fish	IVD (limited) and CP (with short-term cultured cells)
Gilthead sea bream	IVD and (CP with short-term cultured cells)

[a]*M&M: morphology and ES cell markers, IVD: differentiation* ***in vitro****, T: teratoma production* ***in vivo****, CP: chimera production by morula aggregation or blastocyst injection.*
[b]*Exhibited an ES-like morphology initially but rapidly acquired a more epithelial one thereafter.*

the assessment of ESL cell lines derived from different species. Although colonies of ESL cells derived from hamster and rabbit are very similar to colonies of murine ES cells, those derived from most other mammals are not. This is particularly true in the human, where undifferentiated ESL cell colonies closely resemble those formed by human EC cells of testicular origin, as do ESL cell colonies from other primates. In the marmoset, rhesus monkey, and human, ESL cells not only form relatively flattened colonies but also exhibit different expression patterns for ES cell marker genes. Because they closely resemble human EC cells in all these respects, the differences may be more species-specific than indicative of cell type.

In two studies in the sheep, colonies are reported to initially look like those formed by murine ES cells, but adopt a more epithelial-like appearance soon thereafter. This change in morphology bears an intriguing similarity to the transition in conditioned medium of murine ES cells to so-called epiblast-like cells, which is accompanied by loss of their ability to colonize the blastocyst. Given that this transition is reversible, the question of whether a comparable one is occurring spontaneously in sheep clearly warrants further investigation.

In no species has the production of chimeras with ESL cells rivaled that obtained with murine ES cells. Where chimera production with ESL cells has been attempted, both the rates and the levels of chimerism are typically much lower than found with murine ES cells. An apparent exception is one report for the pig, in which 72% of offspring were judged to be chimeric. However, this finding was presented in an overview of work that remains unpublished and no details were provided regarding the number of times the donor cells were passaged before they were injected into blastocysts. A subsequent study in pigs used ESL cells that had passaged 11 times and one chimera was observed among 34 offspring. However, as the authors of this latter study point out, chimerism rates of only 10–12% have been obtained following direct transfer of inner cell mass cells to blastocysts in the pig. Hence, technical limitations may have contributed to the infrequent success with ESL cells in pigs.

The only species listed in Table 3.1 in which colonization of the germ-line has been demonstrated is the chicken, but only with cells that had been passaged only 1–3 times prior to being injected into host embryos. Hence, those chicken cells do not really qualify as stem cells that can be propagated indefinitely *in vitro*. Consequently, to conform with the terminology discussed earlier, morphologically undifferentiated cell lines in all species listed in Table 3.1 should be assigned the status of ESL cells rather than ES cells.

Generally, the strategy for deriving ES cell lines in species other than mice has been to mimic the conditions that are successful in the mouse, namely using enriched medium in conjunction with growth-inactivated feeder cells

and either leukemia inhibitory factor (LIF) or a related cytokine. Later, modifications were introduced, such as using same-species feeder cells instead of murine feeder cells and, in several species including the human, dispensing with LIF. Optimal conditions for *deriving* cell lines may differ from those for *maintaining* them. For example, in one study in the pig, the use of same-species feeder cells was found to be necessary to obtain cell lines, although murine STO cells were adequate as feeder cells during propagation. Feeder-free conditions were found to work best in both the medaka and the gilthead sea bream and the cloning efficiency of human ESL lines was improved under serum-free culture conditions.

Unexpectedly, despite its close relation to the mouse, deriving ES cell lines from the rat has proved particularly difficult (Table 3.1). So far, the sustainable rat cell lines that have been isolated seem to lack all properties of mouse ES cells, including differentiation potential. Only morphology is common between the mouse and rat ES cell lines. Indeed, except for the 129 strain of mouse, establishing cells lines that can be propagated *in vitro* in a morphologically undifferentiated state seems almost more difficult in rodents than in most of the other vertebrates in which it has been attempted.

Overall, one is struck by the species variability in the growth factors, status of conceptus or embryo, and other requirements for obtaining pluripotent cell lines in species other than the mouse. So far, one can discern no clear recipe for success.

Of course, obtaining cells that retain the capacity to colonize the germ-line following long-term culture is essential only for genetically modifying animals in a controlled manner. Having cells that fall short of this one property, but retain the ability to differentiate into a range of distinct types of cells *in vitro*, may suffice for many other purposes.

3.1.3 Embryonic Germ Cells

The preimplantation conceptus is not the only source of pluripotent stem cells in the mouse. Sustainable cultures of undifferentiated cells that bear a striking resemblance to ES cells in their colony morphology have also been obtained from primordial germ cells and very early gonocytes. Such cells, called embryonic germ (EG) cells, have been shown capable of yielding high rates of both somatic and germ-line chimerism following injection into blastocysts.

These findings have prompted those struggling to derive ES cell lines in other species to explore primordial germ cells as an alternative for achieving controlled genetic modification of the germ-line. As shown in Table 3.2, EG-like (EGL) cells have been obtained from several mammals as well as the chick, but as with ESL cells, their ability to participate in chimera formation has,

Table 3.2 Vertebrates from Which Embryonic Germ Cells Have Been Obtained

Species	Basis of Validation[a]
Mouse	M&M
	CP
	CP (including germ-line)
	CP (including germ-line)
	IVD
Pig	CP
	CP (with transfected cells)
Cow	IVD (and short-term CP)
Human	IVD
Chicken	CP (including germ-line, but cells cultured for only 5 days)
	CP

[a]*Abbreviations as listed in the footnote to Table 3.1.*

with one exception, only been demonstrated at low passage. Moreover, although donor cells have been detected in the gonad of a chimera obtained from low passage EGL cells in the pig, no case of germ-line colonization has been reported, except with cells from chick genital ridges that were cultured for only five days. Even here, the proportion of offspring carrying the donor type was very low.

It is, however, noteworthy that, even in the mouse, higher rates of malformation and perinatal mortality are observed in EG cell chimeras than in ES cell chimeras. This may relate to erasure of imprinting in the germ-line, which seems to have already begun by the time primordial germ cells colonize the genital ridges. For certain genes, imprinting appears to occur even earlier. It is perhaps because of such concerns that the potential of EG cells for transgenesis in strains of mice that have failed to yield ES cells has not been explored. Interestingly, unlike in the mouse, EGL cell lines derived from genital ridges and the associated mesentery of 5- to 11-week human fetuses seem not to have erased imprinting. Obviously, it is important to confirm that this is the case before contemplating the use of such cells as grafts for repairing tissue damage in humans.

3.1.4 Future Challenges

The value of ES and ESL cells as resources for both basic and applied research is now acknowledged almost universally. Present barriers to realizing their full potential in both areas are considered in the next sections of this chapter,

together with possible solutions. Fundamental to progress is gaining a better understanding of both the nature and the basic biology of these cells.

3.2 BIOLOGY OF ES AND ESL CELLS

3.2.1 Germ-Line Competence

Although murine ES cells have been used extensively for modifying the genome, there are still several problems that limit their usefulness. One is the loss of competence to colonize the germ-line, a common and frustrating problem whose basis remains elusive. The cause cannot be attributed merely to the occurrence of sufficient chromosomal change to disrupt gametogenesis, because such competence has been lost in lines and clones that were karyotypically normal. At present, it is not known whether the competence for colonizing the germ-line is lost because the cells are not included in the pool of primordial germ cells, or if they are unable to undergo appropriate differentiation thereafter, possibly because imprinting is perturbed or erased. Even within cloned ES lines, individual cells are observed to have heterogeneous expression of imprinted genes. Given that many ES cell lines are likely to have originated polyclonally from several epiblast founder cells, it is possible that they might, *ab initio*, consist of a mixture of germ-line-competent and noncompetent subpopulations. Results from studies on the role of bone morphogenetic protein signaling in inducing primordial germ cells have been interpreted as evidence against a specific germ cell lineage in mammals. Particular significance has been attached to experiments in which distal epiblast, which does not usually produce primordial germ cells, was found to do so when grafted to the proximal site whence these cells normally originate. However, because of the extraordinary degree of cell mixing that occurs in the epiblast before gastrulation, descendants of all epiblast founder cells are likely to be present throughout the tissue by the time of primordial germ cell induction. Hence, the possibility remains that competence for induction is lineage dependent, and segregates to only some epiblast founder cells. Because ES cell lines are typically produced by pooling all colonies derived from a single blastocyst, they might originate from a mixture of germ-line-competent and -noncompetent founder cells.

Male ES cell lines have almost invariably been used in gene-targeting studies, even though this complicates work on X-linked genes whose inactivation may lead to cell-autonomous early lethality or compromise viability in the hemizygous state. Here, female (XX) lines would, in principle, offer a simpler alternative except that they are generally believed to suffer partial deletion or complete loss of one X-chromosome after relatively few passages. However, the data supporting this belief is weak, because few references to their use have been published since the early reports, in which consistent loss of all

or part of one X was first documented. More recently, one of only two female lines tested was found to be germ-line competent, but the entirely donor-derived litters were unusually small, raising the possibility that the line in question was XO, but this explanation was not entertained by the authors. Interestingly, female human ESL cell lines seem not to show a similar propensity for X-chromosome loss.

3.2.2 Origin and Properties of ES and ESL Cells

It is evident from the earlier overview that there is considerable diversity even among eutherian mammals in the characteristics of cells from early conceptuses that can be perpetuated *in vitro* in a morphologically undifferentiated state. The reason for this is far from clear, particularly because most such cell lines have been derived at a corresponding stage – namely, the preimplantation blastocyst – often using inner cell mass tissue. Murine ES cells have not been obtained from postimplantation stages, in contrast to their EC counterparts, arguing that there is a rather narrow window during which ES derivation is possible. What this relates to in developmental terms remains obscure, although the finding that ES cells can shift reversibly to a condition of altered colony morphology and gene expression, together with loss of ability to generate chimeras following blastocyst injection, offers a possible approach for addressing this problem. Whether the late blastocyst stage sets the limit for obtaining ESL cell lines in other mammals has not yet been addressed critically.

Just as ES cell lines have been obtained from preblastocyst stages in the mouse, ESL cell lines have been obtained from such stages in other mammals. However, neither in the mouse nor in other species have the properties of cell lines derived from morulae been compared with those derived from blastocysts to see if they show consistent differences. Indeed, it remains to be ascertained whether the lines from morulae originate at an earlier stage in development rather than progressing to blastocyst or, more specifically, epiblast formation. Although it has been claimed that lines isolated from morulae have an advantage over those isolated from blastocysts in being able to produce trophoblast, this has not actually been conclusively demonstrated. However, species-, as opposed to stage-related, differences in the ability of cell lines to produce trophoblast tissue have been encountered. Early claims that mouse ES cells can form trophoblastic giant cells are almost certainly attributable to the short-term persistence of contaminating polar trophectoderm tissue. Thus, the production of such cells seems to be limited to the early passage of ES lines derived from entire blastocysts. Trophoblast tissue has never been observed with lines established from microsurgically isolated epiblasts. Although the situation is not clear in many species, in primates, differentiation of trophoblast has been observed routinely in ESL cell lines established from immunosurgically isolated inner cell masses. Moreover, differentiation

of human cell lines to the stage of syncytiotrophoblast formation has been induced efficiently by exposing them to bone morphogenic protein 4 (BMP4).

3.2.3 Pluripotency

A seminal characteristic of ES or ESL cells is pluripotency. The most critical test of this – not practicable in some species, particularly the human – is the ability to form the entire complement of cells of normal offspring. This assay, originally developed in the mouse, entails introducing clusters of ES cells into conceptuses whose development has been compromised by making them tetraploid, either by suppressing cytokinesis or by fusing sister blastomeres electrically at the two-cell stage. ES cells are then either aggregated with the tetraploid cleavage stages or injected into tetraploid blastocysts. Some resulting offspring contain no discernible host cells. It seems likely that host epiblast cells are present initially and play an essential role in 'entraining' the donor ES cells before being outcompeted, because groups of ES cells on their own cannot substitute for the epiblast or inner cell mass. Selection against tetraploid cells is already evident by the late blastocyst stage in chimeras made between diploid and tetraploid morulae. Aggregating ESL cells between pairs of tetraploid morulae has been tried in cattle, but resulted in their contributing only very modestly to fetuses and neonates.

The second most critical test is whether the cells yield widespread if not ubiquitous chimerism in offspring following introduction into the early conceptus, either by injection into blastocysts or by aggregation with morulae. The third is the formation of teratomas in ectopic grafts to histocompatible or immunosuppressed adult hosts, since it is clear from earlier experience with murine and human EC cells that a wider range of differentiation can be obtained in these circumstances than *in vitro*. For such an assay to be conclusive, it is necessary to use clonal cell lines to ensure that the diversity of differentiation observed originates from one type of stem cell rather than from a medley of cells with more limited developmental potential. Although teratoma formation has been demonstrated with clonal ESL cells in the human, this is not true for corresponding cell lines in other species. A note of caution regarding the use of teratomas for assessing pluripotency comes from the discovery that hepatocyte differentiation depended not only on the site where mouse ES cells were inoculated but also on the status of the host. Thus, positive results were obtained with spleen rather than hind-limb grafts, and only when using nude rather than syngeneic mice as hosts.

3.2.4 Conditions of Culture

ES and ESL cells are usually propagated in complex culture conditions that are poorly defined because they include both growth-inactivated feeder cells and serum. This complicates the task of determining the specific growth

factor and other requirements necessary for their maintenance induction of differentiation. Although differentiation of murine ES cells in a chemically defined medium has been achieved, their maintenance under such conditions has not. Murine ES cells can be both derived and maintained independently of feeder cells, provided that a cytokine that signals via the gp 130 receptor is present in the medium. However, whether the relatively high incidence of early aneuploidy recorded in the two studies in which LIF was used throughout in place of feeders is significant or coincidental is not clear. It is important to resolve this question in order to understand whether feeder cells serve a function beyond that of being a source of LIF or related cytokines. Production of extracellular matrix is one possibility. However, species variability is also a factor here since LIF is not required for maintaining human ESL lines, whose cloning efficiency is actually improved by omission of serum, though feeder cells are required. The norm has been to use murine feeder cells both for obtaining and for perpetuating ESL cell lines in other mammals, including the human. However, there has been a move to use feeders of human origin for human ESL cells. This is a notable development, because it would not be acceptable to employ xenogeneic cells for growing human ESL cell lines destined for therapeutic rather than laboratory use. The situation is somewhat confusing in the case of the pig; in one study, but not in others, porcine feeders were found to be necessary for deriving ESL cell lines that could then be perpetuated on murine STO cells. Moreover, among teleost fish, feeder-free conditions seem to be optimal for maintaining ESL cells in both the medaka and the sea bream but possibly not in the zebra fish.

3.2.5 Susceptibility versus Resistance to Derivation

An area whose further investigation could be informative in facilitating the establishment of pluripotent stem cell lines in other species is the basis of susceptibility versus resistance to ES cell derivation in the mouse. ES cell lines can be obtained easily in 129 mice and somewhat less easily in C57BL/6 and a few additional strains (Table 3.3), but other genotypes are more resistant. Notable among the resistant strains is the NOD strain from which, despite considerable effort, genetically manipulatable lines have not yet been obtained. This resistance is not simply related to the strain's susceptibility to insulin-dependent diabetes, because the ICR strain from which NOD was derived has proved to be equally refractory. The difficulty in obtaining ES lines from NOD and ICR strains seems to be a recessive trait because excellent lines with high competence to colonize the germ-line have been obtained from [NOD×129]F1 epiblasts. Moreover, this is not the only example in which resistance to the establishment of ES lines has been overcome by intercrossing. Interestingly, marked differences in the permissiveness for ESL cell derivation have also been found among inbred strains of the medaka.

Table 3.3 Genotypes of ES Cells Other Than 129 for Which Germ-Line Transmission Has Been Demonstrated

Genotype	
C57BL/6	C3H/He
C57BL/6N	C3H/Hen
C57BL/6JOla	FVB/N
[C57BL/6?CBA]F1	CD1[a]
CBA/CaOla	NOD
BALB/c	[NOD × 129/Ola]F1
DBA/1lacJ	129 × [129 × DDK]F1
DBA/1Ola	PO[a]
DBA/2N	

[a]*Outbred strains.*

3.2.6 Human ESL Cell

Mouse EC and ES cells have been used extensively to study aspects of development that, for various reasons, are difficult to investigate in the intact conceptus. Exploiting EC and ES cells derived from human conceptuses could allow us to develop a better understanding of early human development, especially given the relative scarcity of material, ethical concerns about experimenting on conceptuses, and statutory or technical limitations on the duration of successful maintenance *in vitro*. Because of their origin, human ESL cells are likely to provide a more suitable model system than human EC cells. One concern here is that so-called spare conceptuses (i.e., those surplus to the needs of infertility treatment) are the sole source of material for producing human ESL cell lines. Because the highest quality conceptuses generated *in vitro* by in vitro fertilization (IVF) or related techniques are selected for treating infertility, those available for deriving ESL lines tend to be of lower quality. Does this matter so far as the properties of the resulting cell lines are concerned, particularly if the goal is therapeutic? Is the ability to form a morphologically satisfactory blastocyst sufficient, or will it become more socially and morally acceptable to produce conceptuses specifically for generating ESL cell lines, so that the quality of conceptuses used to derive these cells is less of a concern?

3.2.7 ES Cell Transgenesis

One important use of ES cell transgenesis is to obtain animal models of human genetic diseases. Because few would claim that the mouse is the ideal species for this purpose, the incentive for being able to undertake such studies in more appropriate or experimentally tractable mammals should

remain high. For example, given its widespread use as an animal model for respiratory physiology, the sheep would be more desirable than the mouse as a model system for cystic fibrosis. However, unless pluripotent cells able to colonize the germ-line can be obtained in other species, ES cell transgenesis experiments will continue to be limited to the mouse. Although the feasibility of an alternative strategy – namely, genetic modification of cells that are not germ-line competent, such as fetal fibroblasts, then transferring their nuclei to oocytes – has been demonstrated, it is technically very demanding and involves considerable fetal attrition.

3.3 STEM CELL THERAPY

3.3.1 Potential Hurdles

Interest in ESL cells is high because of their therapeutic potential for repairing damage to tissues or organs resulting from disease or injury. This poses a host of new challenges, not all of which have received the attention they deserve. Perhaps the most obvious one is whether it will be possible to obtain efficient directed differentiation of stem cells to yield pure cultures of the desired phenotype as opposed to a mixed population. If the latter proves to be the case, rigorous purging of residual undifferentiated or inappropriately differentiated cells will be necessary. How this is approached will depend on the level to which contamination of grafts with undifferentiated or inappropriately differentiated cells is acceptable. One way in which this particular problem has been circumvented in murine model systems where ES cells are differentiated *in vitro* is to introduce a gene for antibiotic resistance or a fluorescent protein coupled to a promoter that is expressed only in the desired type of differentiated cell. Advances have made it possible to carry out similar genetic modification of human ESL cells. Although effective selection of the desired type of differentiated cell may be achieved with this approach, it remains to be seen whether these genetically modified cells will be approved for use in patients and not just for laboratory research.

Another important issue when considering stem cell therapy is the cell-cycle status of the desired cell type. It may be undesirable or unsafe to transfer cells that are not postmitotic into patients under certain circumstances. In other cases, the presence of such cells may be essential to meet the demands of tissue growth or turnover. In the latter, success would depend on differentiating ESL cells to stem cells rather than to fully differentiated cells of the desired type. There is growing evidence that certain tissue-specific stem cells are maintained in a distinct niche *in vivo*, which could prove difficult to replicate *in vitro*. Sorting out these issues clearly depends on gaining better knowledge of the normal biology of individual tissues.

Yet another important issue is whether engrafted cells will survive and function properly when placed in a damaged tissue or organ. If the function of the donor cells is to provide a hormone, neural transmitter, or soluble growth factor, it may be possible to place them at some distance from the site of damage. However, in cases where the need is structural or dependent on cell-cell iterations, the question arises whether transplanted cells will fare better than native ones in a tissue or organ seriously damaged by disease or injury. If they do not, how can one circumvent this difficulty, bearing in mind that achieving organogenesis *in vitro* is still a rather remote prospect? Regarding neurodegenerative disease, some progress has been made in 'cleaning up' sites of tissue damage. For example, antibody-mediated clearance of plaques from the brain in transgenic mice overexpressing amyloid precursor protein has been demonstrated. However, such intervention may not be necessary in all cases. Transplanting differentiated murine ES cells enriched for putative cardiomyocytes to a damaged region of the left ventricle in rats led concomitantly to a reduction in size of this region and an improvement in the performance of the heart.

3.3.2 Therapeutic Cloning

Establishing ESL cell lines from blastocysts derived by nuclear replacement, so-called therapeutic cloning, has been widely advocated as a way of tailoring grafts to individual patients, thereby circumventing the problem of graft rejection. Although the feasibility of producing ES cells in this way has been demonstrated in the mouse, there is sharp division of opinion within the biomedical research community about whether such cells would be safe to use therapeutically. Particular concern centers on the normality of the donor genome regarding the epigenetic status of imprinted genes. Moreover, observations on chromosome segregation during mitosis in early cloned primate embryos has raised doubts about whether cloning by nuclear replacement will work in the human.

3.3.3 Embryonic versus Adult Stem Cells

Concern about the use of early human conceptuses as a source of stem cells focused much attention on studies that suggest so-called adult stem cells are more versatile in their range of differentiation than has generally been supposed. There is a continuing lively debate about the interpretation of many findings, which do not at present justify the common assertion that adult cells render the use of ESL cells for therapeutic purposes unnecessary. Of particular concern is a growing body of evidence that adult cells may be changing their differentiated state not as independent entities but through fusing with cells of the type to which they are claimed to have converted.

For most tissues – the hematopoietic system is the clearest exception, but there may be others – evidence is lacking that cells propagated *ex vivo* truly functioned as stem cells in the native tissue from which they were isolated. Hence, the use of the term 'stem cell' for such cells is questionable. It is possible, if not likely, that cells which are strictly postmitotic in their native tissue can be induced to resume cycling when removed, dissociated from the tissue's extracellular matrix, and placed in an enriched culture medium, which may contain growth factors to which they would not otherwise be exposed. Such cells might lack features of true stem cells, such as accurate proofing of DNA replication, conservation of turnover through transient amplification of differentiating progeny, and maintenance of telomere length. They might therefore be severely compromised in their ability to function in grafts.

3.4 SUMMARY

Since the pioneering studies of Steven and Pierce pointed the way in the 1950s and 1960s, impressive progress has been made in harnessing stem cells of embryonic as opposed to fetal or adult origin for basic research and for exploring new approaches to regenerative medicine. There is, however, still a great deal to be learned about the origin and properties of such cells, as well as the control of their self-renewal versus differentiation, if we are to take full advantage of what they have to offer. The effort of acquiring the necessary knowledge will undoubtedly provide us with the further reward of gaining deeper insight into the biology of stem cells in general.

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CHAPTER 4

Embryonic Stem Cells in Perspective

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4.1 EMBRYONIC STEM CELLS IN PERSPECTIVE

Biologists have explored the development of embryos of all sorts, from worms to humans, in search of the answer to the question of how a complex organism derives from a single cell, the fertilized egg. We now know many of the genes involved in regulating development in different species and find remarkable conservation of genetic pathways across evolution. We also have a good understanding of the logic of development – how the embryo repeatedly uses the same kinds of strategies to achieve cellular specialization, tissue patterning, and organogenesis. One common developmental strategy is the use of stem cells to help generate and maintain a given tissue or organ. A stem cell is a cell that, when it divides, can produce a copy of itself as well as a differentiated cell progeny. This self-renewal capacity is essential to the function of adult stem cells, such as hematopoietic stem cells and spermatogonial stem cells – to continually renew tissues that turn over rapidly in the adult. The concept of the stem cell arose from the pioneering studies of Till and McCullogh on the hematopoietic stem cell and those of Leblond on spermatogenesis and the intestinal crypt. Even in tissues like the brain, where cells do not turn over so rapidly in the adult, scientists have discovered long-lived quiescent stem cells that may be reactivated to repair damage.

Much current research is focused on the identification, characterization, and isolation of stem cells from the adult, with the hope that such cells may be useful for therapeutic repair of adult tissues, either by exogenous cell therapy or by reactivation of endogenous stem cells. However, to date, most adult stem cells have been found to have restricted potential, and achieving indefinite proliferation and expansion of the stem cells in culture is still not routine. During embryogenesis, cells are initially proliferative and pluripotent; they only gradually become restricted to different cell fates. The question of

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whether pluripotent stem cells exist in the embryo has been of interest for years. In mammals, it was known in the 1960s and 1970s that early mouse embryos, up to late gastrulation stages, could produce tumors known as teratocarcinomas when transplanted to ectopic sites, such as the kidney capsule. These tumors contain a variety of differentiated cells types, including muscle, nerve, and skin, as well as an undifferentiated cell type, the embryonal carcinoma (EC) cell. EC cells could be propagated in the undifferentiated state *in vitro*. Importantly, Pierce in 1964 showed that a single EC cell could regenerate a tumor containing both EC cells and differentiated progeny, demonstrating that EC cells are the stem cells of the tumor.

What was the relevance of these tumor cells to normal development? Many studies were carried out in the 1970s showing that EC cells revealed their pluripotency when injected back into early embryos. The best, most karyotypically normal EC cells could contribute to many different cell types in the resulting chimeras, including, in rare instances, the germ-line. This observation generated excitement in the idea that these cells might be useful for introducing new genetic alterations into the mouse and that normalization of tumorigenicity could be achieved by promoting differentiation of tumor cells. However, the degree of chimerism achieved was often weak, and EC-derived tumors were a common feature of the chimeras. Thus, although EC cells had remarkable properties of differentiation, they were still clearly tumor cells. In 1981, Martin and Evans and Kaufman discovered that stably pluripotent cell lines, known as embryonic stem (ES) cells, could be derived directly from the blastocyst. This finding changed the whole perspective of scientists in the field. The differentiation of ES cells, although they could form teratomatous tumors in ectopic sites, was easier to control than that of EC cells. Dramatically, ES cells grown for many passages in culture could still make an entire mouse when supported by tetraploid extraembryonic tissues. When such mice were made from robust hybrid cell lines, they showed no enhanced tumor susceptibility and appeared normal in all respects. All of these properties have made mouse ES cells an incredibly powerful tool for introducing modifications into the mouse genome and analyzing the effect of those alterations.

Are ES cells true stem cells? The *in vivo* equivalent of the ES cell is unclear. ES cells resemble the cells of the primitive ectoderm or epiblast in their gene expression patterns and their pattern of tissue contribution in chimeras. Transcription factors, such as Oct4 and Nanog, which are required for formation and survival of the pluripotent cells in the embryo, are also needed for ES survival. However, *in vivo*, the epiblast has only a limited period of possible stem cell expansion before all cells differentiate into the three germ layers at gastrulation. Germ cells, which are set aside at gastrulation, go on to provide the gametes that will impart pluripotency to the zygotes of the next generation. However, there is no evidence that germ cells are a special stem

cell pool in the epiblast, which could be the ES equivalent. Rather, it appears that all epiblast cells have the capacity to form germ cells in the right environment. Thus, the germ cell is just one of the differentiation options of epiblast.

In vitro, it is clear that ES cells can be expanded indefinitely in the undifferentiated state and still retain the capacity for differentiation. In this regard, ES cells certainly exhibit stem cell properties. However, no conclusive demonstration that a single ES cell can both self-renew and differentiate has been published, in contrast to the literature on EC cells. It has been shown that single ES cells are fully pluripotent, since chimeras made by injecting a single ES cell into a blastocyst have yielded ES contributions to all fetal cell types analyzed. However, in some ways ES cells seem more like progenitor cells, where the population can be expanded by the right growth factor environment but all cells will differentiate when the supportive environment for self-renewal is removed. Practically, the difference is probably not important, but if true, it may be misleading to extrapolate what we know about how ES cells maintain the proliferative state to other stem cells. The search for 'stemness' genes and proteins may not be a useful undertaking until we agree on how to define different stem cell populations.

So much of the excitement about mouse ES cells has focused on their use as a tool for germ-line transmission of genetic alterations that the remarkable differentiation properties of these cells in culture have been underexplored. The derivation of cell lines from early human embryos that seem to share many of the properties of mouse ES cells has refocused attention on the *in vitro* properties of ES cells. Many questions remain before ES cells transition from being an interesting biological system to being a robust therapeutic modality for degenerative diseases. How similar are mouse and human ES cells and how valid is it to use data from one to drive research in the other? How can ES cells be maintained through many passages in a truly stable state, where all cells are stem (or progenitor) cells, epigenetic programming is stable, and genetic abnormalities are minimal? How can ES cells be directed to differentiate reproducibly into given cell types, and how can differentiated progenitors be isolated and maintained? How can we ensure that ES cells will not be tumorigenic *in vivo*?

Answering these questions through research with ES cells, both mouse and human, will certainly provide new insights into embryonic development and new clues as to how to isolate and characterize new stem cells from different embryonic or adult tissues. Conversely, research on how normal embryonic development is regulated will provide new clues as to how to maintain and differentiate stem-progenitor cells in culture. The interplay between developmental biologists and stem cell biologists will be a key interface for defining a fundamental understanding of stem cell development and its translation into therapeutic outcomes.

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CHAPTER 5

The Development of Epithelial Stem Cell Concepts

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5.1 INTRODUCTION

Till and McCulloch's groundbreaking work identified and studied the cells that were capable of repopulating hematopoietic tissues, depleting the replacing tissue of cells by exposure to a cytotoxic agent – radiation. Once the endogenous hematopoietic precursors were eliminated by irradiation, the mice were injected with bone-marrow-derived precursors obtained from another animal. The exogenous cells were subject to a variety of treatments prior to transplant. It was discovered that the hematopoietic precursors first circulated in the host, then engrafted into various hematopoietic tissues, including the spleen. Those cells that engrafted into the spleen and possessed extensive regenerative and differentiative potential grew by a process of clonal expansion to form macroscopically visible nodules of hematopoietic tissue 10 to 14 days after transplant. Genetic or chromosome tracking (marking) demonstrated that these nodules were derived from single cells (i.e., they were clones) and that further clonogenic cells were produced within the clones. The colonies were referred to as spleen colonies, and the cells that form the colonies were called colony-forming units (CFUs).

These experiments provided the theoretical basis for subsequent human bone marrow transplant studies. Through a variety of pre-irradiation manipulations and pre- and post-transplantation variables, this technique led to our current understanding of the bone marrow hierarchies or cell lineages and their stem cells. These studies showed that the bone marrow contained undifferentiated self-maintaining precursor cells that generated a variety of cell types representing a range of differentiated lineages. Subsequent studies have suggested that these CFUs are not the ultimate hematopoietic stem cells, but are part of a stem cell hierarchy in the bone marrow.

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Clonal regeneration approaches have subsequently been developed for a variety of other tissues, notably for the epidermis, intestine, kidney, and testis. These studies implicated hierarchical organizations within the proliferative compartments of many tissues. The stringency of the criteria used for defining a clone varied enormously, depending on the number of cell divisions required to produce detectable clones. For epidermis and intestine, the stringency was high since the clones could be large and macroscopic, appearing similar to spleen colony nodules.

One difficulty with interpreting and applying the results of clonal regeneration studies of bone marrow and other tissues was the need to disturb the host tissue to detect regenerating clones, usually by exposing the host tissue to irradiation. Such a disturbance may alter the cellular hierarchies that one wishes to study, and will almost certainly alter the nature (e.g., cell cycle status, responsiveness to signals, susceptibility to subsequent treatment) of the stem cell compartment. This has been referred to as the biological equivalent of the Heisenberg uncertainty principle defined in quantum physics. However, clonal regeneration assays still provide valuable and sometimes unique opportunities to study some aspects of stem cell biology *in vivo*, such as stem cell survival and functional competence under a variety of conditions.

5.2 A DEFINITION OF STEM CELLS

Relatively few attempts have been made to standardize the definition of the term stem cells, which has resulted in some confusion in the literature. A variety of terms are seen and the relationship between them is often obscure. Terms include precursors, progenitors, founder cells, and so on. The concept of what constitutes a stem cell is further complicated by the addition of modifiers (e.g., committed precursors or progenitors) and the sometimes confusing use of the term differentiation. One inherent difficulty in standardizing the definition of a stem cell is that it is often dependent on the perspective of the viewer, so different criteria are brought into the use of a given term by embryologists, hematologists, dermatologists, gastroenterologists, and other specialists.

In a 1990 paper published in *Development*, we attempted to define a stem cell. This definition was, admittedly, formulated within the context of the gastrointestinal epithelium, but we felt it had a broader application. The definition still largely holds and can be summarized as follows. Within adult replacing tissues of the body, the stem cells can be defined as a small subpopulation of the proliferating compartment, consisting of relatively undifferentiated proliferative cells that maintain their population size when

they divide, while at the same time producing progeny that enter a dividing transit population within which further rounds of cell division occur, together with differentiation events, resulting in the production of the various differentiated functional cells required of the tissue. The stem cells persist throughout the animal's lifetime in the tissue, dividing a large number of times; as a probable consequence of this large division potential, these cells are the most efficient repopulators of the tissue following injury. If this repopulation requires a re-establishment of the full stem cell compartment, the self-maintenance probability of the stem cells at division will be raised from the steady state value of 0.5 to a value between 0.5 and 1, which enables the stem cell population to be re-established, while at the same time maintaining the production of differentiated cells to ensure the functional integrity of the tissue.

The consequences of this definition are obvious, namely, that stem cells are:

- Rare cells in the tissue, vastly outnumbered by the dividing transit population, and are the cells upon which the entire lineage and ultimately the tissue are dependent;
- The only permanent long-term residents of the tissue;
- Cells at the origin of any cell lineages or migratory pathways that can be identified in the tissue.

The concept of differentiation enters into the definition of stem cells, and this, too, often leads to confusion. In our view, differentiation is a qualitative and relative phenomenon. Cells tend to be differentiated relative to other cells, and hence adult tissue stem cells may, or may not, be differentiated relative to embryonic stem cells (a point of current debate, bearing in mind the controversy in the literature concerning bone marrow stem cell plasticity). Stem cells produce progeny that may differentiate down a variety of pathways, leading to the concept of totipotency and pluripotency of stem cells in terms of their differentiation potential. Potency is actually a strange concept to apply to a stem cell, since it is the progeny that differentiate and not the stem cell itself. The fact that the progeny can differentiate down more than one lineage, as is very obviously the case in the bone marrow, results in bone marrow stem cells being referred to as pluripotent, and the initial dividing transit cells that initiate a lineage ultimately leading to specific differentiated cells are referred to as committed precursors for that lineage.

Some of the instructive signals for differentiation in the hematopoietic cell lineage are now well understood, but such signals for other tissues organized on a cell lineage basis have yet to be determined. There is much debate in the literature concerning the extent to which stem cells may be instructed to produce progeny of specific differentiated types, and whether this is limited

or unlimited. This topic is referred to as the degree of plasticity for stem cells. There are two very distinct issues here:

- The first is whether a stem cell, such as a bone marrow stem cell, is ever instructed by its environment in nature, or in laboratory or clinical situations, to make an apparently unrelated tissue cell type such as a liver, intestinal, or skin cell, and whether it can regenerate these tissues if they are injured. A subsidiary question is not whether this ever happens normally in nature, but whether we, as experimentalists or clinicians, can provide the necessary instructions or environment for this to happen in a controlled situation.
- The second issue relates not only to the stem cells, but also to the early progeny of stem cells from, for example, the bone marrow, and whether these cells, which circulate around the body, might end up in a distant tissue and ultimately express differentiation markers unrelated to the bone marrow cell lineages, but specific to the tissue in which the cell ultimately resides.

The former issue is one of the plasticity of the bone marrow stem cells, and the latter may be more an issue of the plasticity of the bone-marrow-derived cell lineages. If a bone marrow stem cell could ever be instructed to be a gastrointestinal stem cell, it should be capable of undertaking all the functional duties of a gastrointestinal stem cell, including the regeneration of the gastrointestinal epithelium if it is subsequently injured. The cloning of animals by nuclear transfer technology into egg cytoplasm clearly demonstrates that all nuclei of the body contain a full complement of DNA, and that under the right environmental conditions the way that the DNA is expressed can be reprogrammed (or unmasked) by environmental signals to make all the tissues of the body. It should be remembered, however, that such cloning outcomes, such as the one that resulted in Dolly the sheep, are rare and inefficiently produced events. They do, however, clearly indicate the enormous potential that can be achieved if the necessary instructive reprogramming signals are provided. These rare events fuel the belief that, in the future, reproducible instruction can be administered to any adult tissue stem cell and yield any tissue of the body. If and when this becomes the case, the distinction between embryonic stem cells and adult tissue stem cells may disappear.

5.3 HIERARCHICALLY ORGANIZED STEM CELL POPULATIONS

The issue here is what determines the difference between a dividing transit cell and a stem cell, and whether that transition is an abrupt or a gradual one. One can think of this transition as being a differentiation event that

distinguishes a dividing transit cell from a stem cell. This is an old argument. Do differentiation signals act on pre-existing stem cells, removing on average half the cells produced by previous symmetric divisions, or do the stem cells divide asymmetrically, to produce a differentiated progeny at division and a stem cell? One possibility is that this distinction is made at the time that a stem cell divides. Indeed, do they need to divide to differentiate? In this case, such divisions must be regarded as asymmetric, with the dividing stem cell producing one stem cell (i.e., for self-maintenance) and one dividing transit cell. This type of asymmetric division may occur in some tissues, such as the epidermis. If this is the case, however, the stem cell must also retain the potential to alter its self-maintenance probability, which for an asymmetric division is 0.5 in steady state, and adopt a value somewhat higher than this if stem cells are killed and require to be repopulated.

The current view regarding the bone marrow stem cells is that the transition between a stem cell and a dividing transit cell is a gradual one that occurs over a series of divisions within a cell lineage, which inevitably implies that one has a population of stem cells with a varying degree of stemness or, conversely, a varying degree of differentiation. For the bone marrow, one issue is whether experimentalists have ever identified the presence of the truly ancestral ultimate bone marrow stem cell. The difficulty here may be one of identifying and extracting such cells, the location of which is probably in the bone where they will be present in increasingly diminishing numbers, as one looks for the increasingly primitive cells.

We have attempted to accommodate as much experimental data as possible into a working model for gastrointestinal cellular organization. Our current working model is that the commitment to differentiation, which produces the dividing transit cells, does not occur at the level of the ultimate stem cell in the lineage, but at a position two or three generations along the cell lineage. If such a concept is drawn as a cell lineage diagram, the proliferative units in the intestine, the crypts, each contain four to six cell lineages and, hence, four to six lineage ancestor stem cells, but up to 30 second- and third-tier stem cells, which under steady state circumstances are inevitably displaced and moved toward the dividing transit compartment. But, if damage occurs in one or more of the ultimate stem cells, they can assume the mantle of the ultimate stem cell and repopulate the lineage. This gives rise to the concept of actual and potential stem cells (see Figure 5.1), which is discussed later in this chapter.

An analogy can be drawn here with the hierarchical personnel structure within an organization such as the army, a concept that was discussed at the time we were formulating the text for the 1990 paper in which we defined stem cells. In a military battlefield environment, the hierarchically organized

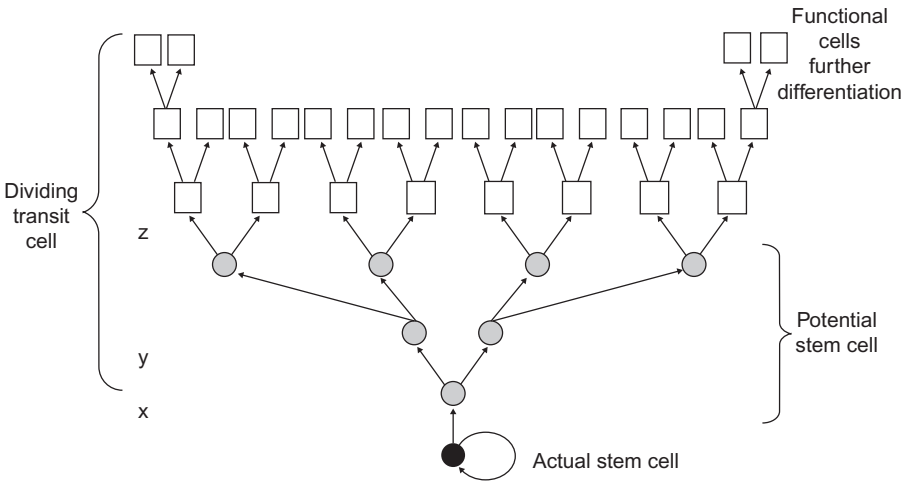

FIGURE 5.1 **A typical stem-cell-derived cell lineage that may be applicable to most epithelial tissues of the body.**

The lineage is characterized by a self-maintaining lineage ancestor actual stem cell (black) which divides and produces a progeny that enters a dividing transit population. The number of cell generations in the dividing transit population varies from tissue to tissue. The commitment to differentiation that separates the stem cell from the dividing transit population can occur at the point of the actual stem cell division (*x*), in which case the stem cells are dividing asymmetrically on average. This commitment may be delayed to point (*y*) or (*z*), generating a population of potential stem cells that can replace the actual stem cell if it is killed. Under normal steady state circumstances, the potential stem cells form part of the dividing transit population and are gradually displaced down the lineage, undergoing further differentiation events if required to produce the functional mature cells of the tissue.

army is under the control and ultimately dependent upon the highly trained (or so one hopes) general. In the event that the general is killed on the battlefield, there may be a reasonably well-trained captain who can take over command and assume the insignia and uniform, as well as the function of the general. In the event that the captain, too, should be killed, there may be less well-trained officers who will attempt to assume the mantle of command. Ultimately, the vast majority of the troops, the privates, would be insufficiently trained or experienced to be able to adopt the functional role of the commander. However, the Dolly the sheep scenario suggests that occasionally a private, given a crash course in military strategy, might function as the officer-in-command. The analogy could be taken even further to relate to the apoptosis sensitivity that is seen in the gastrointestinal ultimate stem cells. These cells appear to adopt a strategy with such complete intolerance to any genetic damage and a reluctance to undertake repair, since this may be associated with inherent genetic risk, that they commit an altruistic suicide – the

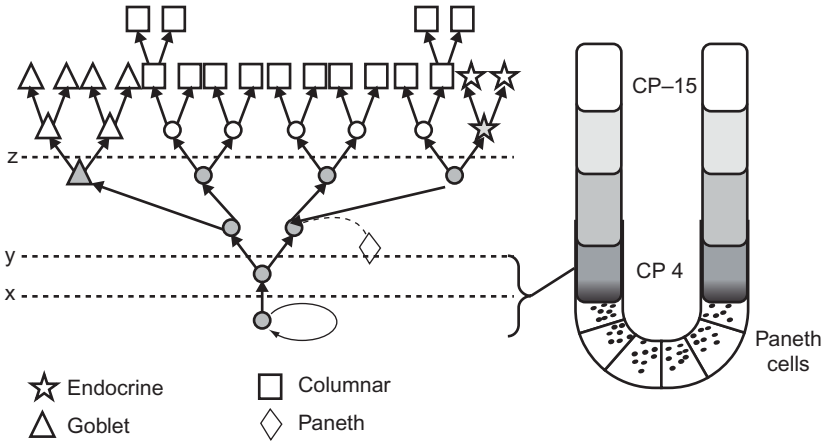

FIGURE 5.2 **The cell lineage for the small-intestinal crypts.**
It is postulated that each crypt contains four to six such lineages and, hence, four to six lineage ancestor actual stem cells, and there are about six cell generations in each lineage with at least four distinct differentiated cell types being produced. The attractive feature of this cell biological model system is that the position of a cell in a lineage can be related to its topographical position in a longitudinal section through the crypt, as shown on the right.

general who undergoes a nervous breakdown or serious injury and has to be removed from command.

In the small-intestinal crypts, no useful markers yet exist that permit the stem cells to be identified and, hence, studied; such markers are only now being identified. However, even in the absence of markers, the small intestine has proven to be an invaluable biological model system to study stem cells, because the cells of the intestinal cell lineage are arranged spatially along the long axis of the crypt. This spatial localization can be confirmed by cell migration tracking and mutational marker studies. As a consequence, the stem cells are known to be located at very specific positions in the tissue (crypts): at the fourth or fifth cell position from the crypt base in the small intestine and at the very base of the crypt in the mid-colon of the large intestine (see Figure 5.2).

5.4 SKIN STEM CELLS

The first suggestion that the proliferative compartment of the epidermis, the basal layer, was heterogeneous and contained only a small subpopulation of stem cells came with the development of the skin macrocolony clonal regeneration assay developed by Withers. This was soon combined with other cell kinetic and tissue organization data to formulate the concept of the

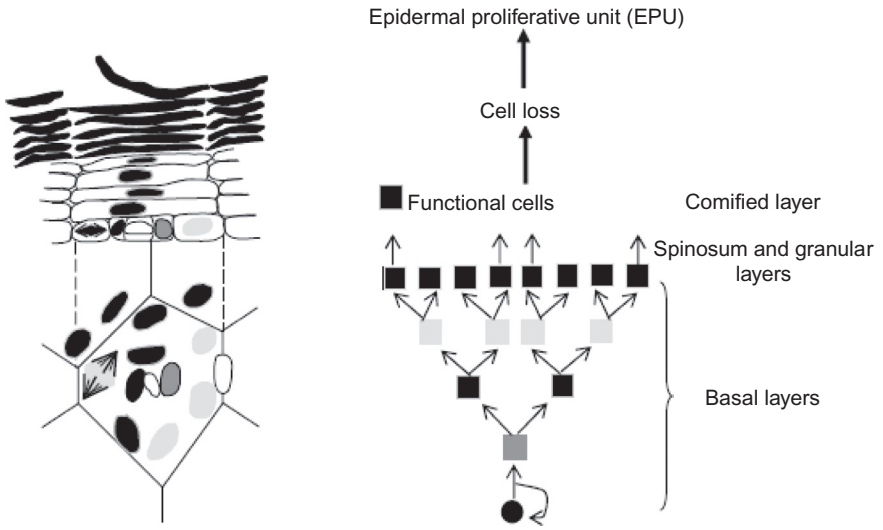

FIGURE 5.3

Diagrammatic representation of the cell lineage seen in the interfollicular epidermis and the relationship between the cell lineage and the spatial organization characterized as the epidermal proliferative unit (EPU), as seen in section view (upper portion of the figure on the left) and in surface view in epidermal sheets (lower portion of the figure on the left).

epidermal proliferative unit (EPU) (see Figure 5.3). This suggested that the basal layer consisted of a series of small, functionally and cell lineage-related cells, with a spatial organization that related directly to the superficial functional cells of the epidermis, the stratum corneum. The concept viewed the epidermis as being a series of functional proliferative units. Each EPU had a centrally placed self-maintaining stem cell and a short stem-cell-derived cell lineage (with three generations). The differentiated cells produced at the end of the lineage migrated out of the basal layer into the suprabasal layers in an ordered fashion, where further maturation events occurred, eventually producing the thin, flattened, cornified cells at the skin surface that were stacked into columns (like a pile of plates), with cell loss occurring at a constant rate from the surface of the column (Figure 5.3).

Such an organization is clearly evident in the body skin epidermis of the mouse, its ears, and a modified version of the EPU can be clearly identified in the dorsal surface of the tongue. Debate as to whether this concept applies to human epidermis still occurs. The human body contains many sites where a similar columnar organization can be seen in the superficial corneal layers of the epidermis. What is more difficult in humans is to correlate this superficial structure with a spatial organization in the basal layer. However, the spatial

organization seen in the superficial layers must have an organizing system at a level lower in the epidermis, and it is reasonable to assume that this system is in the basal layer, as is the case for mouse epidermis.

Two techniques used to study the basal organization of epidermis observe clonal regeneration at a macroscopic and a microscopic level; the resulting nodules are very similar in appearance to spleen colonies. The microscopic assay requires less time between irradiation and tissue sampling, but both techniques are fairly labor intensive and have not been used extensively. Together, studies using these two clonal regeneration assays yielded data indicating that only about 10% (or less) of the basal cells have a regenerative capacity (i.e., are stem cells).

The EPU stem cells must have an asymmetric division mode under steady state cell kinetics, because there is only one such cell per EPU. The microscopic epidermal clonal regeneration assay suggests that following injury, such as irradiation, surviving EPU stem cells can change their division mode from asymmetric to symmetric for a period of time to repopulate the epidermis (i.e., change their self-maintenance probability from 0.5 to a value higher than 0.5). These studies also indicated that a significant contribution to re-epithelialization could come from the upper regions of the hair follicles. Studies on the epidermal structure following injury yielded clear data that the epidermis undergoes a reorganization involving hyperplasia to re-establish the spatial distribution of stem cells, in which stem cells are redistributed and eventually re-established into EPU spatial configurations.

The skin contains another important stem cell population associated with hair follicles. Hair is produced over a protracted period of time by rapid divisions in the germinal region of the hair follicle (termed an 'anagen follicle'). Hair growth can continue for fairly sustained periods of time – three weeks in a mouse, months to years in humans, and more indefinite periods for some animal species such as Angora rabbits and Moreno sheep. The germinal matrix of a hair follicle, has considerable spatial polarity much like the intestinal crypt; therefore, it is presumed to have a fixed stem cell population residing in the lowest regions of the germinal matrix that maintains cell division during active hair growth. Very little is known about these stem cells. One complication with hair follicles is that, in mouse and human, hair follicles eventually produce a mature hair, and cell proliferation ceases. The follicle shrinks and becomes quiescent (a telogen follicle). The simplest explanation here is that the telogen follicle, which consists of far fewer total cells than an anagen follicle, contains a few quiescent hair follicle stem cells that can be triggered back into proliferation at the onset of a new hair growth cycle. However, as discussed below, there is some controversy concerning this concept.

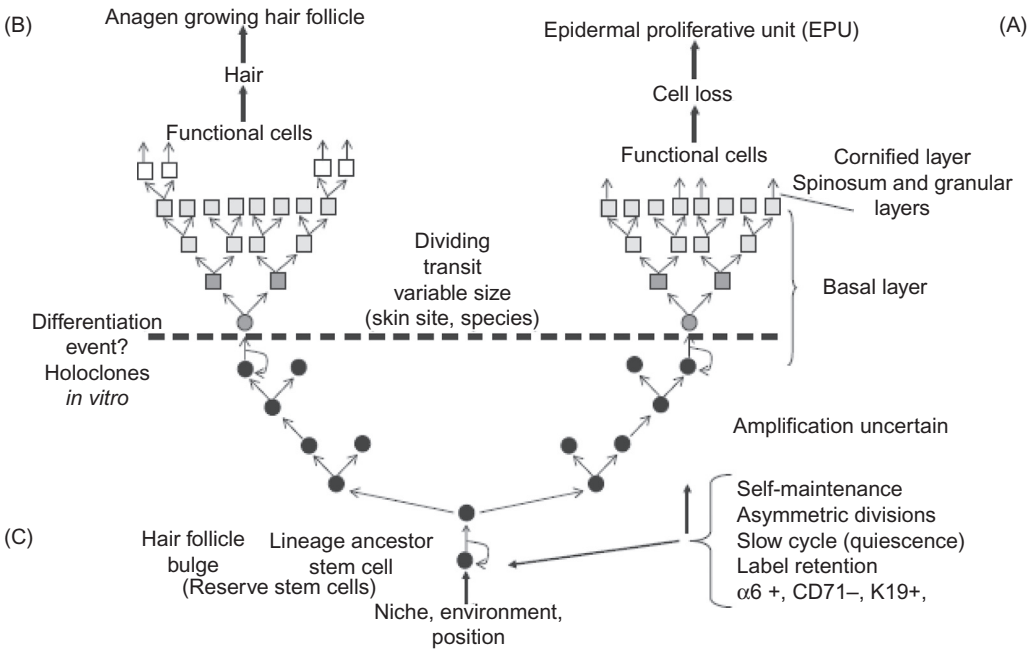

FIGURE 5.4 **The complexity of the stem cell populations in mammalian skin as characterized in the mouse.**

A distinct cell lineage is proposed (A) for the interfollicular epidermis (EPU); (B) another for the matrix region of the growing hair follicle (anagen follicle); and (C) a potent reserve regenerative stem cell compartment which resides in the upper/outer root sheath or bulge region of the hair follicle. The stem cells in the bulge region can regenerate the epidermis, the hair follicle, and probably other structures such as the sebaceous glands.

It is now very clear that the skin contains a third stem cell compartment located in the upper outer sheath of the hair follicle below the sebaceous glands. This compartment is sometimes visible as a small bulge in the outer root sheath, and so these cells are often called 'bulge cells.' A whole series of extremely elegant, but complicated, experiments have shown that these bulge cells possess the ability, under specialized conditions, to reform the hair follicle if it is damaged and also to contribute to the re-epithelialization of the epidermis. Cells from this region of the follicle were probably responsible for the epidermal re-epithelialization from follicles seen by Al-Barwari. Cells from the bulge can make follicles during development of the skin and also re-establish follicles if they are injured.

The controversy concerns the issue of whether bulge stem cells, which are predominantly quiescent cells, ever contribute to the re-establishment of an anagen follicle under normal undamaged situations. The simplest interpretation is that bulge cells are not normally required to re-establish anagen

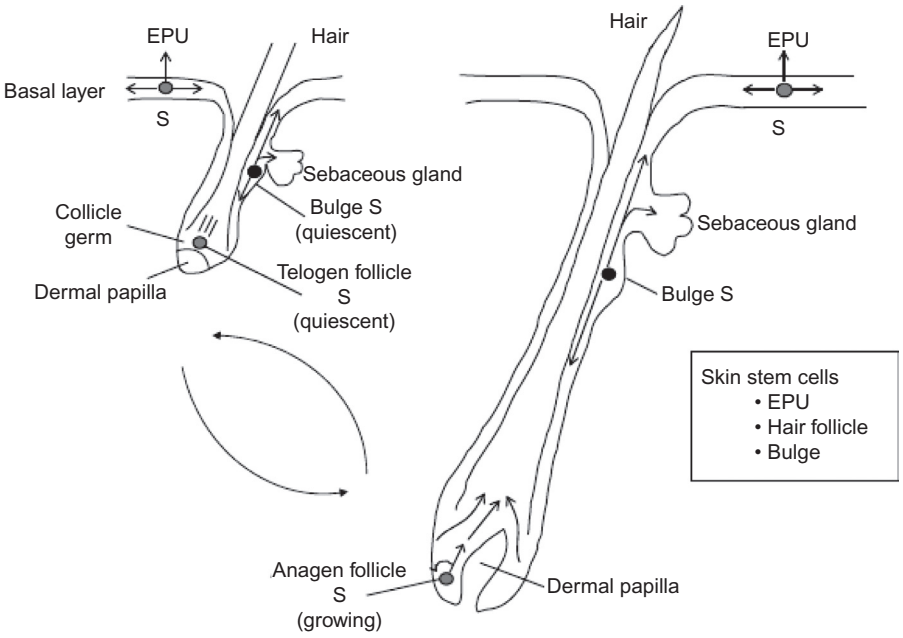

FIGURE 5.5
Diagrammatic representation of a growing anagen hair follicle and a resting or quiescent telogen follicle. The diagram shows the spatial distribution for the stem cell compartments shown in Figure 5.4.

follicles because such an event would involve activating some very complex cell division and cell migratory pathways, which goes somewhat against the concept of stem cells being fixed or anchored, and also against the concept of keratinizing epithelia being a tightly bound, strong and impervious barrier. What seems likely for the skin is that the EPU stem cell and the hair follicle stem cell have a common origin during the development of the skin from the bulge stem cells, which then become quiescent and are present as a versatile reserve stem cell population that can be called into action if the skin is injured and requires re-epithelialization (see Figures 5.4 and 5.5).

5.5 THE INTESTINAL STEM CELL SYSTEM

The intestinal epithelium, like all epithelia, is highly polarized and divided into discrete units of proliferation and differentiation. In the small intestine, the differentiated units are the finger-like villi protruding into the lumen of the intestine. These structures are covered by a simple columnar epithelium consisting of several thousand cells, which perform their specific function, become worn out, and are shed predominantly from the tip of the villus. There is no proliferation anywhere on the villus. The cell loss from the villus tip is precisely balanced in steady state by cell proliferation from the base of the villi – the crypts.

Each villus is served by about six crypts, and each crypt can produce cells that migrate onto more than one villus. The crypts in the mouse contain about 250 cells in total, 150 of which are proliferating rapidly and have an average cell cycle time of 12 hours. The cells move from the mouth of the crypt at a velocity of about one cell diameter per hour, and all this movement can be traced in the small intestine, back to a cell position about four cell diameters from the base of the crypt. The very base of the crypt, in mice and humans, is occupied by a small population of functional differentiated cells, called Paneth cells. Cell migration tracking and innumerable cell kinetic experiments suggest that the stem cells from which all this cell movement originates are located at the fourth position from the base of the crypt in the small intestine, and right at the base of the crypt in some regions of the large bowel.

The crypt is a flask-shaped structure with about 16 cells in the circumferential dimensions. Mathematical modeling suggests that each crypt contains about five cell lineages and, hence, five cell lineage ancestor stem cells. Under steady state kinetics, these cells are responsible for all the cell production, producing daughters that enter a dividing transit lineage of between six and eight generations in the small and large bowel, respectively (see Figures 5.1 and 5.2). The stem cells in the small intestine divide with a cycle time of approximately 24 hours and, hence, in the lifetime of a laboratory mouse divide about 1,000 times. It is assumed that these cells are anchored or fixed in a micro-environmental niche that helps determine their function and behavior. The uniquely attractive feature of this model system, from a cell biological point of view, is that, in the absence of stem cell specific markers, the behavior and characteristics and response to treatment of these crucial lineage ancestor cells can be studied by studying the behavior of cells at the fourth position from the bottom of the crypt in the small intestine. When this is done, one of the features that seem to characterize a small population of cells at this position (about five cells) is that they express an exquisite sensitivity to genotoxic damage, such as is delivered by small doses of radiation. They appear to tolerate no DNA damage and activate a p53-dependent altruistic suicide (apoptosis). It is believed that this is part of the genome protection mechanisms that operate in the small intestine, and accounts for the very low incidence of cancer in this large mass of rapidly proliferating tissue.

Macroscopic clonal regeneration techniques have been used extensively to study the intestinal crypts and suggest the presence of a second compartment of clonogenic or potential stem cells (about 30 per crypt) that possess a higher resistance to radiation and a good ability to repair DNA damage. These observations, together with others, suggest a stem cell hierarchy of the

sort illustrated in Figures 5.1 and 5.2, with the commitment to differentiation that distinguishes dividing transit cells from stem cells occurring about three generations along the lineage. Virtually identical lineage structures can be inferred for the colonic crypts.

There has been an absence of stem cell specific markers in the past, but some may now be available. Antibodies to Musashi-1, an RNA binding protein identified as playing a role in asymmetric division control in neural stem cells, appears to be expressed in very early lineage cells in the small intestine (see Figure 5.6).

Studies have indicated that the ultimate stem cells in the crypt possess the ability to selectively segregate old and new strands of DNA at division and retain the old template strands in the daughter cell destined to remain a stem cell. The newly synthesized strands which may contain replication-induced errors are passed to the daughter cell destined to enter the dividing transit population and to be shed from the tip of the villus five to seven days after birth from division. Cairns developed the concept of selective DNA segregation in 1975. Selective DNA segregation provides a second level of genome protection for the stem cells in the small intestine, and providing further support for the low cancer incidence in this tissue (see Box 5.1). When template strands are labeled with DNA synthesis markers at times of stem cell expansion (i.e., during late tissue development and during tissue regeneration after injury), the label persists and provides a truly specific marker for lineage ancestor cells (see Figure 5.6). Figure 5.6 also illustrates some other ways in which intestinal stem cells may be distinguished from their rapidly dividing progeny.

5.6 STEM CELL ORGANIZATION ON THE TONGUE

Oral mucosae are keratinizing, stratified epithelia, similar to skin epidermis in their structural organization. The dorsal surface of the tongue is composed of many small, filiform papillae that have a very uniform shape and size. Detailed histological investigations, together with cell kinetic studies performed by Hume, showed that each papilla is composed of four columns of cells: two dominant and two buttressing columns. The dominant anterior and posterior columns represent modified versions of the EPU and are called tongue proliferative units. Cell migratory pathways in the tongue were mapped using techniques that were applied to intestinal crypts and identified the position from which all migration originated, (i.e., the presumed location of the stem cell compartment). The lineage characterizing tongue epithelium is similar to that of the dorsal epidermis of the mouse – that is, self-replacing, asymmetrically dividing stem cells, occurring at a specific position in the

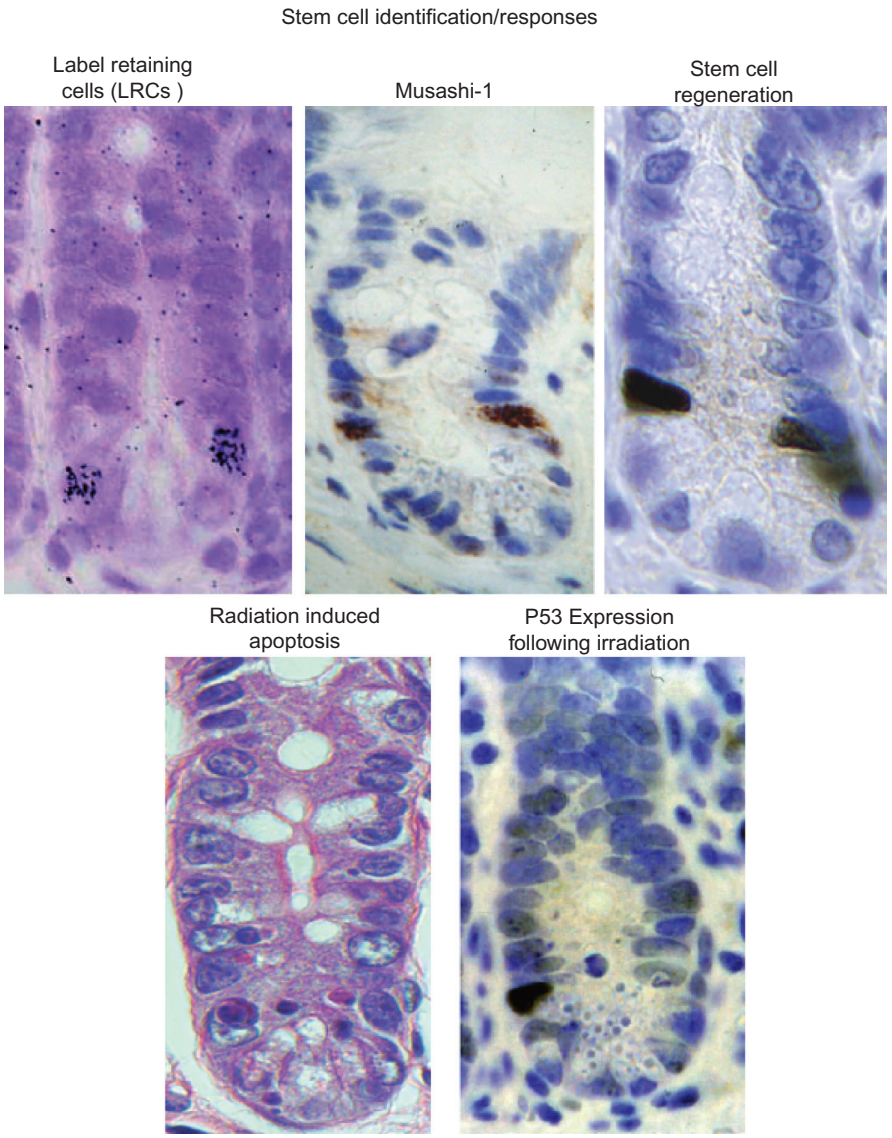

FIGURE 5.6 Photomicrographs of longitudinal sections of the small-intestinal crypts from the mouse illustrating a range of possible ways of identifying the stem cell compartment. Making use of the selective strand, segregation hypothesis template strands of DNA can be labeled, generating label-retaining cells at the fourth position from the bottom of crypts. Musashi-1, an RNA binding protein, is expressed in early lineage cells and under some labeling conditions can show specificity for individual cells at around cell position 4. Part of the regenerative or potential stem cell compartment can be seen by S-phase labeling (bromodeoyuridine labeling) at critical phases following cytotoxic injury when these cells are called into regenerative mode. The example shown here is a labeling pattern at 24 hours after two doses of 5-fluorouracil when the only cells in S-phase are a few cells scattered around the

BOX 5.1 WHY DON'T SMALL-INTESTINAL STEM CELLS DEVELOP MORE CANCERS?

When one considers that, relative to the large intestine, the small intestine has:

- 3–4 times greater mass (length)
- 1.5 times more rapid proliferation
- 2–3 times more total stem cells
- 3–4 times more stem cell divisions in a lifetime

it is quite remarkable that cancers of the small intestine occur 70 times less frequently.

tissue and producing a cell lineage that has approximately three generations (Figure 5.7). The stem cells in tongue have a particularly pronounced circadian rhythm.

5.7 GENERALIZED SCHEME

For the major replacing tissues of the body, hierarchical or cell lineage schemes appear to explain the cell replacement processes. These schemes may involve isolated, single stem cells that under steady state circumstances must be presumed to divide asymmetrically, producing a dividing transit population. The size of the dividing transit population differs dramatically from tissue to tissue, the number of generations defining the degree of amplification that the transit population provides for each stem cell division. This amplification is inversely proportional to the frequency that stem cells will be found within the proliferating compartment (see Figure 5.8).

For some systems, such as the bone marrow and the intestine, the commitment to differentiation that separates the dividing transit compartment from the stem cell compartment appears to be delayed until a few generations along the lineage. This generates a stem cell hierarchy with cells of changing (decreasing) stemness or, conversely, increasing commitment, leading to the concept of committed precursor cells. In the small intestine, this delay in the

◀ fourth position from the base of the crypt. As part of the genome protective mechanism, it is postulated that the ultimate lineage ancestor stem cells have an exquisite sensitivity to radiation and the induction of genome damage. When this happens, the cells commit suicide via apoptosis, which can be easily recognized and occurs at about the fourth position from the base of the crypt. These cells do not express p53 protein, at least at the times studied and as detectable by immunohistochemistry. However, some cells do express p53 protein at high levels following radiation exposure, and it is postulated that these are the surviving potential stem cells in cell cycle arrest to allow for repair prior to entering rapid regenerative cell cycles. Under appropriate immunohistochemical preparative procedures, individual wild-type p53 protein expressing cells can be seen at around cell position 4.

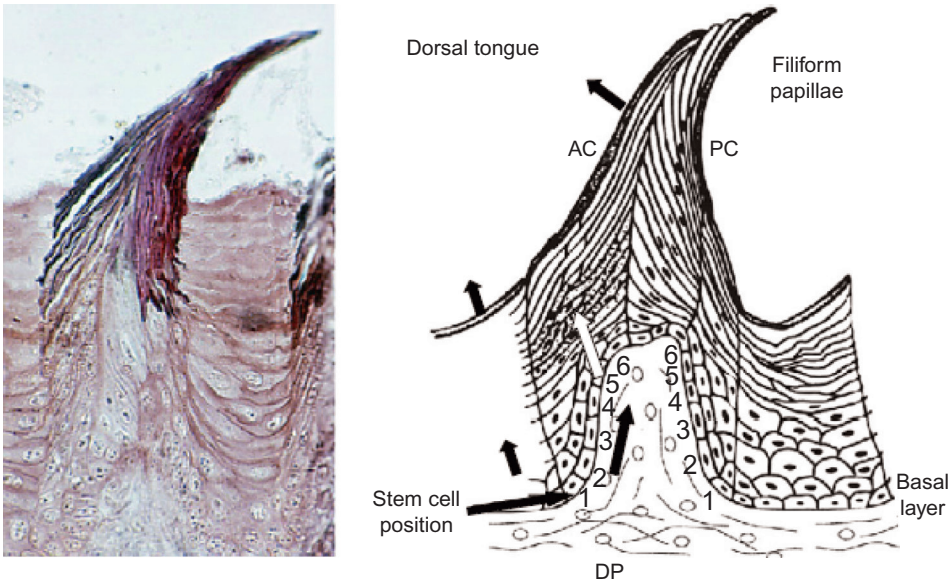

FIGURE 5.7

A histological section through the dorsal surface of the tongue (left panel) and a diagrammatic representation of this tissue showing the tongue proliferative units (the dominant anterior column AC, and posterior column PC). Cell migratory pathways have been identified based on cell positional analyses and cell marking and the location of the stem cells identified in the basal layer. The stem cells in this tissue express one of the strongest circadian rhythms in proliferation seen anywhere in the body.

commitment to differentiation to a dividing transit population provides the tissue with a reserve population of potential stem cells that can repopulate the tissue if the lineage ancestor cells are destroyed; providing an added level of tissue protection in this extremely well-protected tissue.

With regard to the bone marrow, committed precursors or even earlier cells appear to circulate in the blood and may lodge in various tissues. Given appropriate microenvironments and local signals, some of these lodged cells may be instructed to differentiate down unusual pathways. This has prompted research into using such cells to repopulate the liver of patients with specific gene defects that result in life-threatening, hepatic metabolic deficiencies.

Although the transdifferentiation theory is attractive, research indicates that the apparent plasticity of stem cells may be less clear-cut. Transplantation experiments in mice with specific gene disorders suggest that transplanted bone marrow cells may 'fuse' with liver cells, and hence complement any gene deficiency in the hepatocytes. These hybrid cells will be viable and undergo clonal expansion. Experimental findings do, indeed, show that

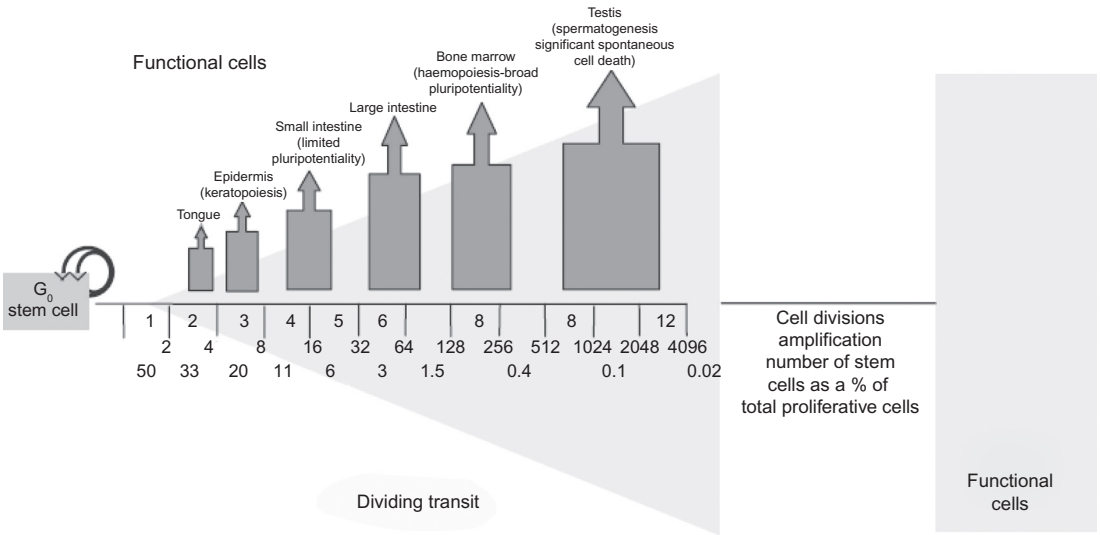

FIGURE 5.8 **A diagrammatic representation of a stem-cell-derived cell lineage showing the approximate positions for the number of cell generations in the dividing transit population for a range of murine tissues.**
Stratified keratinizing epithelia such as the tongue and epidermis tend to have the shortest lineages, and the bone marrow and the testis tend to have the longest lineages. Also shown is the degree of theoretical amplification that the dividing transit lineage provides for each stem cell division and the inverse relationship between the degree of amplification and the proportion of the proliferative compartment that the stem cells occupy.

cells forming functional liver tissue in the gene-deficient animals have specific genetic markers for both the donor and the host animal. Our concepts of stem cells clearly require further development and refinement.

5.8 SUMMARY

Stem cell concepts have evolved dramatically over the last few years, culminating in a rapid expansion of interest in both embryonic and adult tissue stem cells. This chapter explores the evolution of stem cell concepts as applied to adult epithelial tissues. These tissues are characterized by a high degree of polarization and very distinct cell maturation and migration pathways, which permit the identification of specific locations in the tissues which represent the origins of all this cell movement. Cells at the origin of the migratory pathways must represent the cells on which the tissue is ultimately dependent, and the cells that have a long-term (permanent) residence in the tissue, i.e., the stem cells. A variety of cell kinetic studies, together with lineage tracking experiments, have indicated that, in the intestine, the dorsal surface of the tongue, and the interfollicular epidermis, the proliferative compartment of the tissue is divided into discrete units of proliferation, each with

its own stem cell compartment. In the skin, the evolving stem cell studies suggest at least three distinct stem cell populations providing a source of cells for the epidermis, for the growing hair follicle, and a reserve regenerative, highly potent population in the upper follicle region. In the small intestine there are indications that the stem cell compartment itself is hierarchical, with a commitment to differentiation occurring two to three generations down the lineage, resulting in a population of actual stem cells that perform their function in steady state, and a population of potential stem cells that can be called into action if the actual stem cells are killed. Whereas previously, there were no reliable markers for adult intestinal stem cells, new findings have yielded potential markers for identifying these cells. Cancer is rare in the small-intestinal epithelium, which is surprising since this tissue represents a large mass with many stem cells dividing many times. This suggests that effective genome protective mechanisms have evolved, and some aspects of these mechanisms have now been identified.

FOR FURTHER STUDY

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CHAPTER 6

Stem Cell Niches

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6.1 STEM CELL NICHE HYPOTHESIS

In classical developmental biology, daughter cells that differentiate along distinct lineages arise by one of two mechanisms: asymmetric partitioning of cell fate determinants within the mother cell; or orientation of the plane of cell division so that each daughter cell is placed in a different microenvironment. The first strategy relies on intracellular signaling, the second on extracellular signals. However, in reality, the balance between self-renewal and differentiation is likely to be regulated by the integration of intrinsic factors and extrinsic cues provided by the surrounding microenvironment, now known as the stem cell niche.

The concept of the stem cell niche arose from observations that many adult stem cells, such as hematopoietic stem cells, lose the potential for continued self-renewal when removed from their normal cellular environment, and that different signaling microenvironments can direct daughter cells to adopt different fates. In the stem cell niche hypothesis, self-renewal signals are limited to a finite space. If space within the niche were limited, one daughter cell would be placed outside the niche, where it would initiate differentiation due to lack of self-renewal factors. If space within the niche is available, then both daughters of a stem cell division could retain stem cell identity (i.e., self-renewal). Therefore, the stem cell niche hypothesis predicts that stem cell numbers are controlled by the availability of niches with the necessary signals for self-renewal and survival.

The precise spatial organization of the stem cells and surrounding support cells defines the ability of the niche to provide adequate proliferative and antiapoptotic signals and exclude factors that promote differentiation. The surrounding support cells serve as a source of critical signals controlling stem cell behavior. Adhesion between stem cells and either an underlying basement

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membrane or support cells themselves appears to play an important role in anchoring stem cells within the niche. In addition, the niche could provide polarity cues to orient daughter cells such that one cell is placed outside of the niche and into an alternative environment that encourages differentiation.

In this chapter, we review the role of the niche and its control of stem cell self-renewal using the *Drosophila* male and female germ lines as model systems. We then extrapolate from these models to suggest paradigms for controlling stem cell behavior within other adult stem cell systems.

6.2 STEM CELL NICHES IN THE *DROSOPHILA* GERM-LINE

The *Drosophila* germ-line has provided insight into the regulation of stem cell behavior and the importance of the stem cell niche. The precise identity and location of the germ-line stem cells (GSCs) in the *Drosophila* ovary and testis are known. In addition, the availability of many mutants, a sequenced genome, and powerful genetic tools for cell type-specific ectopic expression in *Drosophila* have provided the opportunity to address essential questions regarding how stem cells interact with their surrounding microenvironment.

Lineage tracing by clonal marking analysis has led to the identification of GSCs in both the male and the female germ lines *in vivo*, within their normal environment. These genetically marked GSCs can be observed to continually produce a series of differentiating germ cells. Clonal analysis also allows the generation of mutant GSCs in an otherwise wild-type animal, allowing the analysis of a specific gene's function on stem cell maintenance, self-renewal, and survival.

In *Drosophila*, both male and female GSCs normally divide with invariant asymmetry, producing precisely one daughter stem cell and one daughter cell that will initiate differentiation. In both the ovary and the testis, GSCs are in intimate contact with surrounding support cells that provide critical self-renewal signals, maintenance signals, or both, thereby constituting a stem cell niche. Oriented division of stem cells is important for placing one daughter cell within the niche while displacing the other daughter cell destined to initiate differentiation outside of the germ-line stem cell niche.

6.3 THE GERM-LINE STEM CELL NICHE IN THE *DROSOPHILA* OVARY

The adult *Drosophila* ovary consists of approximately 15 ovarioles, each with a specialized structure, the germarium, at the most anterior tip (Figure 6.1A). Two to three GSCs lie at the anterior tip of the germarium, close to several

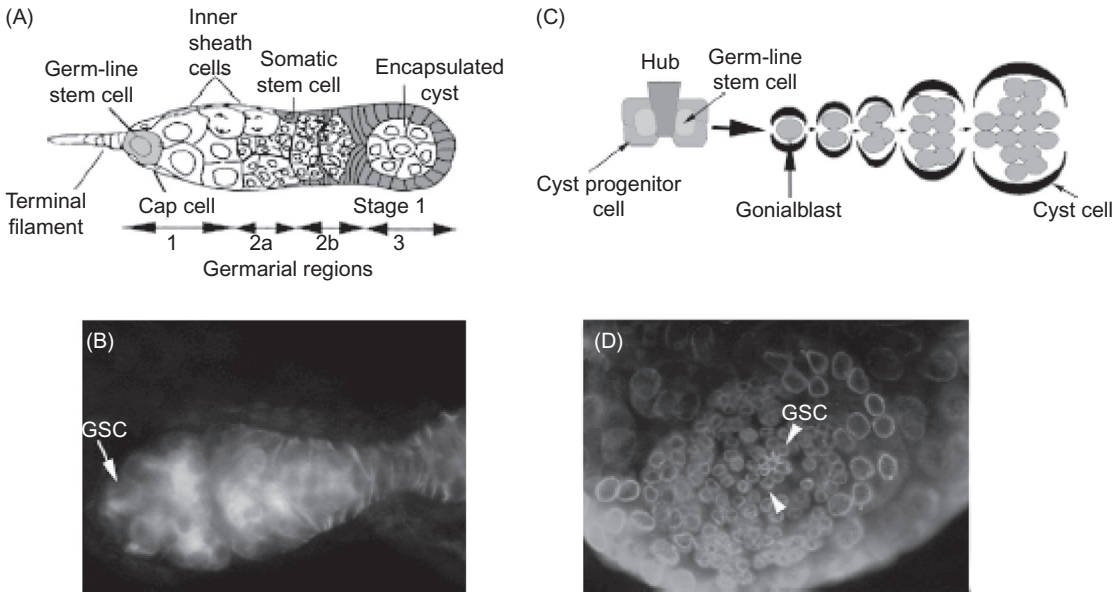

FIGURE 6.1 **Germ-line stem cell niches in the *Drosophila* ovary and testis.**
(A) In the schematic of a *Drosophila* germarium, which houses the germ-line stem cells (GSCs), the anterior is to the left and the posterior is to the right. The terminal filament, cap, and inner sheath cells express molecules important for the maintenance and self-renewal of female GSCs and comprise the stem cell niche. GSCs undergo asymmetric cell division, producing one daughter cell that will retain stem cell identity and one daughter cell, a cystoblast, that will initiate differentiation. As these divisions take place, the more mature cysts are displaced toward the posterior of the germarium. Cyst encapsulation by the somatic stem cell (SSC) derivatives occurs in region 2A–2B. Mature encapsulated cysts budding from the germarium make up region 3. (B) In the immunofluorescence image of a *Drosophila* germarium, germ cells are labeled with an antibody to the germ cell-specific protein, Vasa. Antibodies to the membrane protein α-spectrin label the somatic cells within the germarium, as well as a vesiculated, cytoplasmic, ball-shaped structure known as the spectrosome in GSCs (arrow) and cystoblasts. (C) In the schematic of the early steps in *Drosophila* spermatogenesis, the GSCs surround and are in contact with a cluster of postmitotic, somatic cells known as the apical hub. The hub cells are a primary component of the male GSC niche. Each GSC is surrounded by two somatic stem cells, the cyst progenitor cells. The GSC undergoes asymmetric cell division, generating one daughter cell that will retain stem cell identity and one daughter cell, a gonialblast, that will undergo four rounds of cell division with incomplete cytokinesis to produce 16 spermatogonia. The gonialblast is surrounded by cyst cells, which ensure spermatogonial differentiation. (D) In the immunofluorescence image of the apical tip of a *Drosophila* testis, the germ cells are labeled with an antibody to Vasa, and the somatic hub is labeled with an antibody to the membrane-associated protein, Fasciclin III. Eight GSCs (arrowheads) surround the apical hub.

groups of differentiated somatic cell types, including the terminal filament, cap cells, and inner germarial sheath cells (Figure 6.1A, B). When a female GSC divides, the daughter cell that lies closer to the terminal filament and cap cells retains stem cell identity; the daughter cell that is displaced away from the cap cells initiates differentiation as a cystoblast. The cystoblast and its progeny undergo four rounds of cell division with incomplete cytokinesis. Of the 16 germ cells, only one will become the oocyte; the other 15 cells

become nurse cells, which support the growth of the oocyte. The terminal filament, cap cells, and inner germarial sheath cells express molecules that regulate critical aspects of GSC behavior, constituting the germ-line stem cell niche in the ovary.

Xie and Spradling directly demonstrated the existence of a functional stem cell niche that can program cells to assume stem cell identity in the ovary. Taking advantage of a mutation that increases the rate at which stem cells are lost, an empty stem cell niche was observed to fill quickly the division of a neighboring stem cell. In this case, the mitotic spindle of the GSC became reoriented parallel to the terminal filament and cap cells, and the stem cell divided symmetrically such that both daughters of the stem cell division became stem cells. The analogous assay has not been performed for the *Drosophila* testis due to the much larger number of stem cells in that organ.

In the ovary, the vertebrate bone morphogenetic protein (BMP) 2/4 homolog Decapentaplegic (Dpp) is required for maintenance of GSCs. The cap cells and inner germarial sheath cells express dpp, which activates the Dpp signaling pathway in adjacent GSCs. Dpp binds and facilitates the association of type I and type II serine/threonine kinase receptors, allowing the type II receptor to phosphorylate and activate the type I receptor, which in turn phosphorylates the downstream mediator Mothers against dpp (Mad). Mad facilitates nuclear translocation of Medea (Med), a transcriptional activator that stimulates Dpp target gene expression.

Excessive Dpp signaling can block germ cell differentiation in the *Drosophila* ovary. Overexpression of dpp results in enlarged germaria filled with cells resembling GSCs. TGF-β pathway signaling is also required for long-term maintenance of GSCs. Loss of function mutations in the type I receptor saxophone (sax) shorten the half-life of GSCs from one month to one week, and slow the rate of GSC divisions. Clonal analysis revealed that the downstream signaling components Mad and Med are required cell-autonomously in the germ-line for maintaining the normal half-life of GSCs. In the current working model, it is thought that Dpp, secreted from cap cells and inner germarial sheath cells, signals to regulate the maintenance and rate of division of female GSCs. As differentiating germ cells are also in contact with inner germarial sheath cells, which also express dpp mRNA, the model may require an additional mechanism to ensure germ cell differentiation even in the presence of Dpp. This raises the possibility that Dpp signaling from the niche may play a permissive rather than an instructive role in specifying stem cell maintenance.

The Piwi protein is also expressed in the terminal filament and cap cells and has been shown to act nonautonomously to support GSC maintenance in

the *Drosophila* ovary. The piwi gene family, implicated in RNA silencing and translational regulation, plays crucial roles in stem cell maintenance in many organisms. In *Drosophila*, piwi mutant ovaries contain a normal number of primordial germ cells (PGCs) at the third instar larval stage, but adult ovaries contained only a few differentiating germ cells. Overexpression of piwi in the soma leads to an increase in GSCs in the germarium, suggesting a role in GSC self-renewal. Piwi is also expressed in the germ-line, where it appears to play a cell-autonomous role in controlling the rate of GSC division in the ovary. Null mutations in piwi also result in a failure to maintain *Drosophila* male GSCs, although the mechanism by which Piwi acts on GSCs in the male has not been established.

6.4 GERM-LINE STEM CELL NICHE IN THE *DROSOPHILA* TESTIS

The *Drosophila* adult testis is a long, coiled tube filled with cells at all stages of spermatogenesis. In adult *Drosophila melanogaster*, approximately nine GSCs lie at the apical tip of the testis, forming a ring that closely surrounds a cluster of postmitotic somatic cells called the hub (Figure 6.1C, D). When a male GSC divides, it normally produces one cell that will retain stem cell identity and one cell, called a gonialblast, that is displaced away from the hub and will initiate differentiation (Figure 6.1C). The gonialblast and its progeny undergo four rounds of transit-amplifying mitotic divisions with incomplete cytokinesis, creating a cluster of 16 interconnected spermatogonia.

In the *Drosophila* testis, signaling through the Janus kinase–Signal Transducer and Activator of Transcription (JAK–STAT) pathway has been shown to specify stem cell self-renewal of male GSCs. The somatic apical hub cells are a major component of the GSC niche in the testis. Hub cells express the ligand Unpaired (Upd), which activates the JAK–STAT pathway in the adjacent stem cells and specifies stem cell self-renewal. *Drosophila* melanogaster has one known JAK, encoded by the hopscotch (hop) gene and one known STAT, Stat92E. In males carrying a viable, male sterile hop allele, the initial round of germ cell differentiation occurs, but GSCs are lost soon after the first rounds of definitive stem cell divisions at the onset of spermatogenesis. Mosaic analysis of homozygous mutant germ cells demonstrated that Stat92E activity is required cell autonomously in the germ-line for stem cell self-renewal. Upd is normally expressed exclusively in the hub cells, and ectopic expression of Upd in early germ cells resulted in an enlarged testis tip filled with thousands of small cells resembling GSCs and gonialblasts. Together, these data suggest that the hub cells contribute to the germ-line stem cell niche by secreting the ligand Upd, which specifies stem cell self-renewal by activating the JAK–STAT pathway in GSCs.

Experiments in tissue culture suggest that Upd protein associates with the extracellular matrix upon secretion. If binding to the extracellular matrix restricts Upd diffusion *in vivo*, then only cells that maintain direct contact with the hub may receive sufficient levels of Upd to retain stem cell identity. Consistent with this hypothesis, activation of Stat92E, the sole *Drosophila* STAT homolog, is observed only in hub cells and the adjacent GSCs. *In situ* analysis showed that the gene encoding the receptor for Upd, domeless, is broadly expressed in the testis, excluded only from postmeiotic spermatocytes and spermatids.

6.5 COORDINATE CONTROL OF GERM-LINE STEM CELL AND SOMATIC STEM CELL MAINTENANCE AND PROLIFERATION

Multiple stem cell populations can reside within a common anatomical location – for example, hematopoietic and mesenchymal stem cells in the bone marrow. Coordinated control of the proliferation of different stem cell types may be especially important when the two stem cell types generate differentiated cell populations that must work together to maintain a tissue. The *Drosophila* female and male gonads provide excellent systems in which to study how the behavior of two such stem cell populations, somatic and germline, can be coordinately controlled.

In the *Drosophila* ovary, the germarium houses a second population of stem cells in addition to GSCs. These somatic stem cells (SSCs) (Figure 6.1A, B) produce the many specialized follicle cells that cover each developing egg chamber. Lineage tracing, achieved through clonal analysis, demonstrated that SSCs are located several cell diameters from the female GSCs in the ovariole (Figure 6.1A). Each cyst of interconnected germ cells is encapsulated by somatic follicle cells in region 2A–2B before budding from the posterior end of the germarium (Figure 6.1A, B).

The Hedgehog (Hh) signal transduction pathway has been implicated in controlling the proliferation and differentiation of the SSCs and their progeny. Hh is strongly expressed in terminal filament and cap cells at the tip of the germarium (Figure 6.1A). Loss of hh activity reduced the number of somatic cells in the germarium. Consequently, fewer follicle cells are available to intercalate between adjacent germ-line cysts, resulting in an accumulation of unencapsulated cysts in the germarium. Overexpression of hh in the ovary leads to hyperproliferation of somatic cells, resulting in increased numbers of cells that separate adjacent egg chambers and increased numbers of specialized follicle cells at the poles of developing egg chambers. At this time it is not clear how hh, expressed in the terminal filament and cap cells,

might regulate SSC proliferation, as the SSCs lie several cell diameters away. It is possible that the SSCs receive the Hh signal directly, or that Hh is also signaling through some other somatic cell type – the inner sheath cells, for example – to control the proliferation of SSCs indirectly (Figure 6.1A).

In the ovary, the fs(1)Yb gene may serve as an upstream regulator of both GSC and SSC proliferation in the *Drosophila* ovary. Mutations in fs(1)Yb lead to precocious differentiation of GSCs without apparent self-renewal. Consequently, ovarioles consist of several differentiating germ-line cysts and a germarium devoid of germ cells. There is a concomitant reduction in the number of somatic cells. Conversely, overexpression of Yb leads to increased numbers of both GSCs and somatic cells in the germarium.

Yb protein is expressed in the terminal filament and cap cells, and Yb mutants exhibit reduced expression of Hh and Piwi protein in cap cells and somewhat reduced expression in terminal filament cells. Loss of function of Yb results in loss of GSCs, similar to that observed in piwi mutants. Also, the phenotypes resulting from overexpression of Yb are similar to those seen upon ectopic expression of piwi and hh. Based on these observations, Yb may regulate the expression of piwi and hh within the GSC niche and, in doing so, coordinately control the behavior of GSCs and SSCs in parallel pathways.

In the *Drosophila* testis, a population of SSCs, called cyst progenitor cells (CPCs), self-renew and generate the somatic cyst cells. The CPCs flank the male GSCs and directly contact the apical hub cells using thin cytoplasmic extensions (Figure 6.1C). As for GSCs, the daughter cell that remains adjacent to the hub retains its stem cell identity, and the daughter cell displaced from the hub becomes a cyst cell and does not divide again. Two somatic cyst cells, which may be the functional equivalent of mammalian Sertoli cells, enclose each gonialblast and its progeny and play a major role in ensuring spermatogonial differentiation (Figure 6.1C).

In the testis, self-renewal of both GSCs and CPCs may be regulated by the same signal; the Upd ligand. In the testis, both the GSCs and somatic CPCs reside adjacent to the apical hub cells. The number of early somatic cells at the testis tip decreases dramatically in hop mutant testes. Reciprocally, the number of early somatic cells increases in response to ectopic expression of Upd in early germ cells. Upd secreted from the apical hub could signal directly to the somatic as well as to the GSC populations to specify stem cell self-renewal. Alternatively, Upd signaling to the germ-line could control somatic stem cell proliferation indirectly by causing germ cells to send a second signal to neighboring CPCs to specify stem cell identity. CPCs and cyst cells are present in agametic testes, which supports the first model over the second. If Upd signals directly to both GSCs and CPCs to specify stem cell identity, then a requirement for a signal from the apical hub to direct stem

cell self-renewal may serve to spatially coordinate an asymmetric outcome to stem cell divisions in both the somatic and germ-line lineages.

6.6 STRUCTURAL COMPONENTS OF THE NICHE

In *Drosophila* gonads, adhesion between GSCs and the surrounding support cells is important for holding stem cells within the niche, close to self-renewal signals and away from differentiation cues GSCs in the ovary and testis appear to make direct cell-cell contact with surrounding support cells. Clusters of adherens junctions are observed between female GSCs and cap cells, as well as between male GSCs and the adjacent hub cells. Immunofluorescence analysis revealed that the *Drosophila* E-cadherin homolog Shotgun (Shg) and β-catenin homolog Armadillo (Arm) are highly concentrated at the interface between the GSCs and the cap cells in females and between the GSCs and the hub cells in the male. Recruitment into and maintenance of female GSCs within the niche requires the activity of both shg and arm. Removal of shg activity from the germ-line using clonal analysis resulted in failure of female GSCs to be efficiently recruited into their niches in the developing ovary. Furthermore, shg mutant GSCs that were recruited to the niche were not maintained, suggesting that DE-cadherin-mediated cell adhesion is required for holding GSCs in their niche in the germarium, which in turn is required for efficient stem cell self-renewal.

Gap junctional intercellular communication via transfer of small molecules may also be involved in the survival and differentiation of early germ cells in the *Drosophila* ovary. Mutations in the zero population growth (zpg) gene, which encodes a germ-line-specific gap junction protein, result in loss of early germ cells at the beginning of differentiation in both males and females. Zpg protein is concentrated on the germ cell-soma interface in males and females and between adjacent germ cells in developing egg chambers. Transfer of small molecules, nutrients, or both from surrounding support cells to germ cells via gap junctions may be essential for the survival of early germ cells undergoing differentiation. The presence of gap junctions between female GSCs and adjacent support cells, coupled with the eventual loss of GSCs in zpg mutants, also suggest that signaling via gap junctions may play a role in stem cell maintenance or may help physically maintain GSCs in their niche.

In summary, the male and female germ lines of *Drosophila* have provided a genetic system in which to test the principles and investigate the basic underlying mechanisms of the stem cell niche theory. Clonal marking experiments have conclusively identified GSCs *in situ* in both the testis and ovary, allowing the study of the relationship between these stem cells and their surrounding

microenvironment. Several themes arising from the analysis of *Drosophila* male and female GSCs offer potential paradigms for analysis of stem cell niches in mammalian systems. First, stem cells are usually located adjacent to support cells that secrete factors required for maintaining stem cell identity: the hub cells at the apical tip of the testis and the cap cells in the germarium at the tip of the ovary. The signal transduction pathways involved in stem cell maintenance may not be conserved between male and female GSC systems, nor are they necessarily conserved between GSC and SSC populations within the gonads. Second, cell-cell adhesion between GSCs and niche cells is required for stem cell maintenance, physically maintaining stem cells within the niche and ensuring that GSCs are held close to self-renewal signals emanating from the microenvironment.

6.7 STEM CELL NICHES WITHIN MAMMALIAN TISSUES

Specialized niches have been proposed to regulate the behavior of stem cells in several mammalian tissues maintained by stem cell populations, including the male germ-line, the hematopoietic system, the epidermis, and the intestinal epithelium. Many of these niches share several characteristics with stem cell niches in the *Drosophila* germ-line, specifically signaling molecules secreted from the surrounding microenvironment and cell adhesion molecules required for anchoring stem cells within the niche. Here, we briefly describe the proposed niches for each tissue. For more detailed information on each individual tissue and stem cell population, see individual chapters within this volume.

6.7.1 Mammalian Testis

The seminiferous tubules of the mammalian testis are the site of ongoing spermatogenesis in the adult. In the embryo, PGCs divide and migrate to the genital ridges. In males, the PGCs, known as gonocytes, home to the basement membrane of the seminiferous tubules and differentiate into spermatogonial stem cells. The As (single) spermatogonia, the presumptive stem cells, are found close to several groups of supporting somatic cells, including the peritubular myoid and Sertoli cells, that may contribute to the stem cell niche (Figure 6.2).

Spermatogonial stem cells taken from a fertile mouse or rat can be transplanted into the seminiferous tubules of an immunodeficient mouse recipient. These exogenous stem cells are able to migrate through layers of differentiating germ cells and Sertoli cell tight junctions to find the stem cell niche along the basement membrane and establish colonies of donor-derived

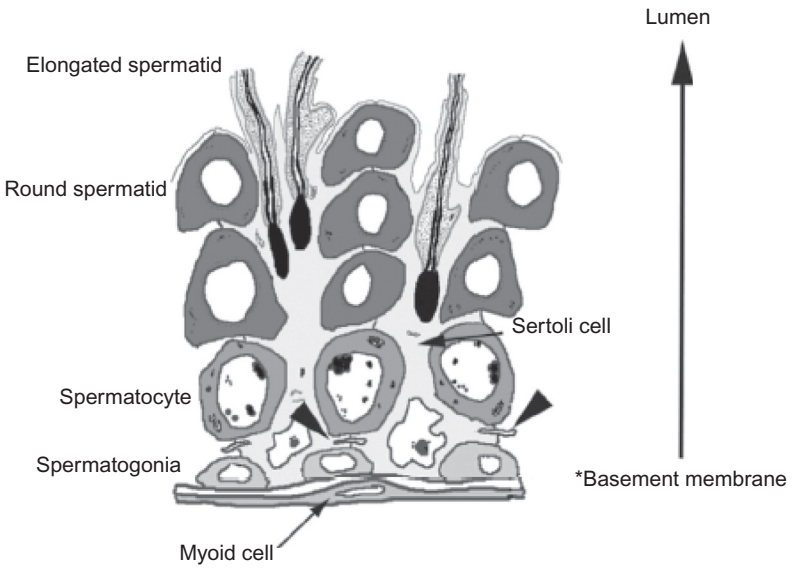

FIGURE 6.2 **Organization of germ cells and somatic cells within a seminiferous tubule.** Spermatogenesis occurs inside the seminiferous tubules that make up the testis. The mammalian male germ-line stem cells (as spermatogonia) lie at the periphery of the seminiferous tubules adjacent to the basement membrane (asterisk), and differentiation proceeds through multiple stages, creating spermatogonia, spermatocytes, spermatids, and spermatozoa, which are released into the lumen of the tubule. The spermatogonia are in close association with several somatic cell types, including the peritubular myoid cells and Sertoli cells. Sertoli cells flank germ cells of all stages and are joined continuously around the tubule by tight junctions (arrowheads). Sertoli cells and myoid cells are strong candidates for cellular components of the stem cell niche within the testis.

spermatogenesis. The availability of a spermatogonial stem cell transplantation assay has allowed characterization of the stem cell niche in the mammalian testis. For example, both the number of stem cells and the available niches increase with age and testis growth; the microenvironments within testes from immature pups were better at allowing colonization events, whether the donor stem cell was from an adult or from a pup.

Secreted signaling molecules that specifically direct self-renewal of mammalian male GSCs have not yet been identified. However, Sertoli cells produce a growth factor, glial cell line-derived neurotrophic factor (GDNF), which affects the proliferation of premeiotic germ cells, including stem cells. Depletion of stem cell reserves is observed in mice lacking one copy of GDNF. Conversely, mice overexpressing GDNF under the control of a promoter that drives preferential expression in the germ-line show accumulation of undifferentiated spermatogonia that neither differentiate nor undergo apoptosis. Older GDNF-overexpressing mice regularly form nonmetastatic testicular tumors,

thus suggesting that GDNF contributes to paracrine regulation of spermatogonial proliferation and differentiation.

The development of a transplantation assay to test the function of mammalian male GSCs has provided a framework on which to begin the molecular characterization of spermatogonial stem cells and of the stem cell niche in the adult mammalian testis. Enrichment of stem cells using Fluorescence-activated cell sorting (FACS) and monoclonal antibodies for specific surface markers, followed by transplantation of sorted populations, has led to identification of α6 integrin as a candidate surface marker for spermatogonial stem cells, raising the possibility that attachment of stem cells to the extracellular matrix (ECM) may be important for stem cell maintenance. The association of spermatogonia and differentiating spermatocytes with Sertoli cells is likely to be mediated in part by adherens junctions, although the precise cadherin and cadherin-like molecules involved in this cell-cell interaction have not been conclusively identified. Meanwhile, Sertoli cells lining the tubules are continuously joined by tight junctions that regulate the movement of cells and large molecules between the basal compartment and the lumen of the seminiferous tubules (Figure 6.2).

6.7.2 Hematopoietic System

The major anatomical sites of hematopoiesis change during ontogeny. Hematopoietic stem cells (HSCs) are first present in the embryonic yolk sac and the aorta–gonad–mesonephros (AGM) region, followed by the fetal liver and spleen. Just before birth, HSCs migrate to the bone marrow, where blood formation is maintained throughout the lifetime of the animal.

Characterization of the HSC niche and of the signaling molecules that influence HSC maintenance and self-renewal is in its initial stages. HSCs reside along the inner surface of the bone, and differentiating cells migrate toward the center of the bone marrow cavity. The bone-forming osteoblasts have been proposed to be a major component of the HSC niche, since increases in the number of osteoblasts led to a concomitant increase in the number of long-term HSCs. These osteoblasts secrete an elevated level of the Notch ligand Jagged-1, suggesting that activation of the Notch signaling transduction pathway in HSCs may support HSC proliferation. Furthermore, the cell adhesion molecule N-cadherin, which is expressed by the spindle-shaped N-cadherin+ CD45− osteoblasts, may be responsible for holding HSCs within the niche and close to self-renewal and survival signals.

A stromal cell line that can maintain highly purified murine and human HSCs *in vitro* has been isolated and molecularly characterized. The AFT024 cell line was derived from murine fetal liver and can support HSC growth from four to seven weeks. These cultured stem cells retain the ability to

reconstitute hematopoiesis *in vivo* after transplantation comparable to freshly purified HSCs. Studies of the growth factors secreted by this cell line, combined with the cell-cell and cell-ECM adhesion molecules present on the surface of these cells, may also serve as a source for candidate molecules that will be important components of the HSC niche in the bone marrow.

Studies have shown that signaling through the canonical Wnt pathway can direct HSC self-renewal *in vitro* and *in vivo*. Wnt is a secreted growth factor that binds to members of the Frizzled (Fz) family of cell surface receptors. The β-catenin molecule serves as a positive regulator of the pathway by mediating transcription in cooperation with members of the Lef-TCF transcription factor family. In the absence of a Wnt signal, cytoplasmic β-catenin is quickly degraded through the ubiquitin-proteasome pathway.

Reya et al. (2003) have demonstrated that transduction of HSCs with a retrovirus encoding a constitutively active β-catenin molecule results in self-renewal and expansion of HSCs in culture for at least four weeks and in some cases as long as 1–2 months under conditions in which control HSCs do not survive in culture beyond 48 hours. The cultured cells resemble HSCs morphologically and phenotypically and are capable of reconstituting the entire hematopoietic system of lethally irradiated mice when transplanted in limiting numbers. Proliferation of wild-type HSCs cultured in the presence of growth factors was blocked by a soluble form of the ligand-binding domain of the Fz receptor, suggesting that Wnt signaling is required for the proliferation response of HSCs to cytokines within their niche. Because no other cell types were present in these cultures, this result raises the possibility that a Wnt secreted from HSCs may act as an autocrine signal to promote HSC proliferation.

The cell-cell and cell-ECM adhesion molecules involved in anchoring HSCs within the bone marrow have not yet been identified. Interestingly, HSCs are mobile and detectable in the peripheral blood, spleen, and liver, suggesting that HSCs can migrate out of the niche. Although circulating HSCs and progenitor cells are quickly cleared from the peripheral blood, the number of blood-borne HSCs is fairly stable, suggesting that the flux of HSCs into and out of the blood is roughly equivalent. The mechanisms that recruit HSCs back into the niche after migration or homing of HSCs to the bone marrow after transplantation have not been clearly elucidated, although cellular adhesion molecules and chemokine receptors are likely involved. However, the mobility of HSCs suggests that adhesion between HSCs and niche cells may be highly regulated.

6.7.3 Mammalian Epidermis

The mammalian epidermis is comprised primarily of keratinocytes, a subpopulation of which is stem cells. Epidermal stem cells are multipotent; they

produce progeny that differentiate into interfollicular epidermis and sebocytes and contribute to all the differentiated cell types involved in the formation of the hair follicle, including the outer root sheath, inner root sheath, and hair shaft.

It is not yet understood whether one 'primordial' epidermal stem cell creates the stem-progenitor cell populations that maintain the interfollicular epidermis, the hair follicle, and sebaceous gland, or whether the stem cells that maintain each of these specific cell types are equivalent, with their fate determined by the local environment. However, accumulating evidence supports a model whereby the microenvironment, or niche, affects differentiation toward particular lineages. For example, cultured rat dermal papillae cells can induce hair follicle formation by rat footpad epidermis, in which follicles are not normally found. These data suggest that stem cells that normally maintain the interfollicular epidermis can be reprogrammed to act as hair follicle stem cells by signals emanating from the surrounding microenvironment. For this chapter, we consider the stem cells that generate the hair follicle and the interfollicular epidermis separately.

6.7.4 Hair Follicle

After placement and formation of the hair placode during mammalian embryonic development, the lower portion of the hair follicle cycles through periods of growth (anagen), regression (catagen), and quiescence (telogen). The proliferative cells that generate the inner root sheath and hair shaft are called matrix cells, a transiently dividing population of epithelial cells at the base of the hair follicle that engulfs a pocket of specialized mesenchymal cells, called the dermal papilla (Figure 6.3A).

Using multiple strategies, a stem cell niche for the mammalian epidermis has been located along the upper portion of the hair follicle in a region called the bulge. Specifically, the bulge is located along the outer root sheath, which is contiguous with the interfollicular epidermis (Figure 6.3A). As the hair follicle regresses during catagen, the dermal papilla comes into close proximity with the follicular bulge. It has been suggested that one or more signals from the dermal papilla may cause stem cells, transit-amplifying cells, or both in the bulge to migrate out and begin proliferating to regenerate the hair follicle.

Both in the human and mouse epidermis, β1 integrin expression is enriched in cells within the bulge region of the outer root sheath. Targeted disruption of the β1 integrin gene in the outer root sheath cells did not disrupt the first hair cycle; however, proliferation of matrix cells was severely impaired, resulting in progressive hair loss and dramatic hair follicle abnormalities. Proliferation of interfollicular keratinocytes was also significantly reduced, and by seven weeks these mice completely lacked hair follicles and sebaceous

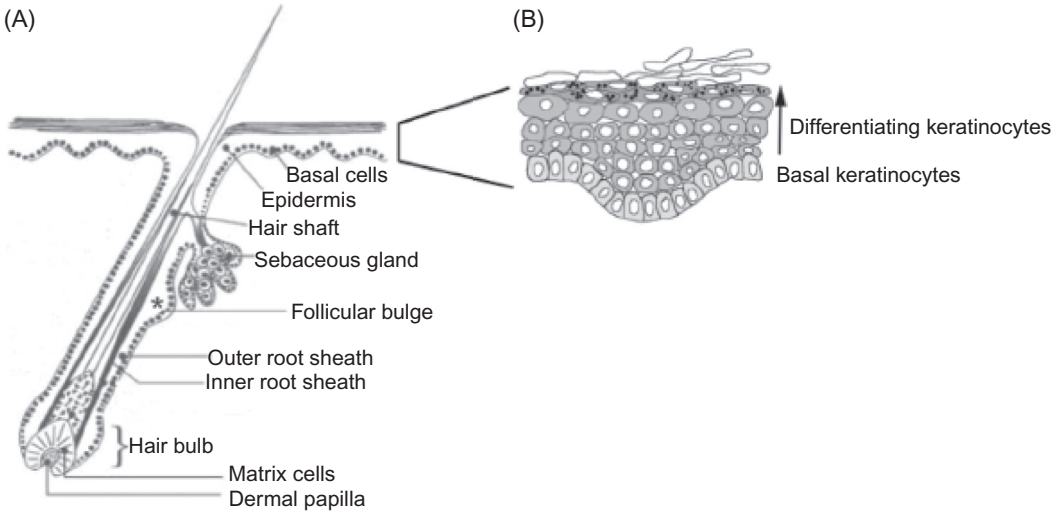

FIGURE 6.3

(A) Schematic of the components of a hair follicle (modified from Gat et al. 1998, Cell, 95, 605). The follicular bulge (asterisk) has been proposed to act as a stem cell niche, which houses cells that can contribute to all the differentiated cell types involved in the formation of the hair follicle, including the outer root sheath, inner root sheath, and hair shaft. Stem cells within the bulge can also generate sebocytes and the cells that maintain the interfollicular epidermis. The bulge is located along the outer root sheath, which is contiguous with the interfollicular epidermis (dotted line). (B) Drawing of a cross-section of the interfollicular epidermis. The stem cells that maintain the interfollicular epidermis are within the basal layer of the epidermis and divide to produce the transit-amplifying cells, which undergo a process of terminal differentiation as they migrate toward the surface of the skin. Dead squamae are shed from the surface of the skin. Interfollicular epidermal stem cells are found in patches, surrounded by transit-amplifying cells that form an interconnecting network between the stem cell clusters (Kore-eda et al. 1998, Am J Dermatopathol, 20, 362).

glands, suggesting that β1 integrin function is required for normal epidermal proliferation. It is possible that one role of β1 integrin is to anchor stem cells within the bulge, close to self-renewal signals.

To date, no candidate growth factors that might be secreted by cells in and around the bulge to control stem cell self-renewal have been definitively identified. However, both the Sonic Hedgehog (Shh) and the Wnt/β-catenin signaling pathways have been shown to affect some aspects of cell proliferation and differentiation in the epidermis and epidermal appendages. Shh signaling appears to specify hair follicle placement and growth during embryogenesis, as well as postnatal follicle regeneration. Shh is expressed at the distal portion of the growing hair follicle, along one side of the matrix closest to the skin surface (Figure 6.3A). Interestingly, fibroblast growth factors (FGFs) and BMPs, expressed in the dermal papilla, affect hair follicle growth by regulating Shh expression in matrix cells (Figure 6.3A).

Loss of Shh function leads to disruption in hair follicle growth while ectopic expression of Shh target genes induces follicular tumors. In addition, basal cell carcinomas, caused by mutations in downstream components of the Shh pathway, are composed of cells similar to hair follicle precursor cells. Shh expression in the epithelium results in the expression of target genes such as Patched (Ptc) in both the proliferating matrix cells and the adjacent dermal papilla. Because Shh targets are expressed in both the epidermis and the underlying dermal tissue, it is unclear whether the effect of Shh on epithelial cell proliferation is direct or indirect.

Wnt signaling also plays an important role in the formation of hair follicles during embryogenesis and postnatal specification of matrix-derived cells into follicular keratinocytes. Overexpression of a stabilized form of β-catenin in murine skin leads to the formation of ectopic hair follicles and hair follicle-derived tumors. Alternatively, a skin-specific knockout of β-catenin attenuated hair germ formation during embryogenesis and dramatically restricted specification of cell fates by the multipotent bulge stem cells after completion of the initial hair cycle. At the initiation of the second growth phase, β-catenin-deficient stem cells were incapable of differentiating into follicular epithelial cells and were restricted to producing interfollicular keratinocytes.

Several members of the Lef1-Tcf family, as well as numerous Wnts, are expressed in the skin. Cells within the bulge and the lower outer root sheath express Tcf3, while Lef1 is highly expressed in the proliferative matrix cells and differentiating hair shaft precursor cells. Experiments suggest that Tcf3 may act as a repressor to maintain characteristics of the bulge and the lower outer root sheath cells and may do so independently of binding to β-catenin. This implies that Tcf3 likely acts in a Wnt independent manner to direct the differentiation of cells within the bulge and lower outer root sheath. Lef1, on the other hand, requires binding to β-catenin and presumably activation by one or more Wnts to mediate its effects on hair follicle differentiation. Although these data re-emphasize a role for Wnt signaling in hair follicle generation, there is still no evidence that Wnts directly control the proliferation, maintenance, or self-renewal of epidermal stem cells.

Stabilization of β-catenin through Wnt signaling acts in concert with the activation of Lef1 transcription by repression of BMP signaling by noggin to repress E-cadherin expression and to drive follicle morphogenesis. Conditional removal of α-catenin also results in the arrest of hair follicle formation and the subsequent failure of sebaceous gland formation. Together, these results highlight the importance of Wnt signaling and of the regulation of adherens junction formation in the development and maintenance of hair follicles.

6.7.5 Interfollicular Epidermis

Stem cells in the bulge can migrate superficially to maintain the interfollicular epidermis. The stem cells that maintain the interfollicular epidermis are within pockets in the basal layer of the epidermis and divide to generate the transit-amplifying cells, which undergo a process of terminal differentiation as they migrate toward the surface of the skin (Figure 6.3B). Interfollicular epidermal stem cells are found in patches, surrounded by transit-amplifying cells that form an interconnecting network between the stem cell clusters.

Adhesion to the extracellular matrix promotes stem cell identity and prevents differentiation of keratinocytes. Human or mouse basal keratinocytes can be grown and cultured *in vitro*, and cultured adult human keratinocytes have been used as autografts in the treatment of burn victims for the past 20 years. The ability to culture keratinocytes *in vitro* has also allowed the development of potential strategies to use cutaneous gene therapy to correct various skin disorders and chronic wounds.

When cultured keratinocytes are placed in suspension, they immediately cease cell division and initiate differentiation. Keratinocytes express a variety of integrins, and although some are generally expressed, others are induced only during development, wounding, and disease. All cells within the basal layer of the epidermis express β1 integrin. However, the cells most likely to be stem cells within the interfollicular epidermis express surface levels of β1 integrin two- to three-fold higher than those of transit-amplifying cells. High levels of β1 integrin with signaling through the mitogen-activated protein kinase cascade were demonstrated to promote stem cell identity in basal keratinocytes. As a result, a high level of β1 integrin is commonly used as a visual marker for stem cells within the interfollicular epidermis.

Activated β-catenin and signaling through Lefs Tcfs has been demonstrated to increase the proportion of stem cells in keratinocyte cultures. Cultured mouse keratinocytes exposed to Wnt3a- and noggin-conditioned media showed a significant increase in β-catenin and Lef1 levels and in localization of Lef1 to the nucleus. These two signaling pathways could act to reduce E-cadherin expression in interfollicular basal keratinocytes, similar to the manner in which they act to regulate E-cadherin expression during hair follicle morphogenesis. Interestingly, stem cells in the interfollicular epidermis have surface levels of E-cadherin lower than those of transit-amplifying cells.

Notch signaling has been suggested to promote differentiation of interfollicular keratinocytes. High levels of the Notch ligand, Delta1, in cultured human epidermal keratinocytes signal to adjacent cells to differentiate while the stem cells are protected from this signal. Notch signaling has also been demonstrated to stimulate differentiation in mouse epidermal cells. Accordingly, conditional removal of Notch from basal keratinocytes results in epidermal hyperplasia, suggesting that Notch negatively regulates epithelial stem

cell proliferation and may act as a tumor suppressor in mouse epidermis. Interestingly, Notch1 deficiency also results in increased levels of β-catenin and Lef1 and in the formation of basal cell carcinoma-like tumors.

6.7.6 GUT Epithelium

The inner lining of the colon and small intestine is a simple columnar epithelium constantly renewed by the proliferation of stem cells residing within pockets, or crypts, along the intestinal wall. Intestinal cells leave the crypt at a rate of 200–300 cells/day and migrate onto ciliated villi that protrude into the gut lumen (Figure 6.4A). No cell-specific marker has been characterized that allows conclusive identification and characterization of intestinal stem cells. However, lineage tracing experiments have located the presumptive stem cells of both the small intestine and the colon near the base of each crypt. Within a crypt, approximately 4–5 stem cells generate transit-amplifying cells, which are capable of up to six transit divisions. Migration of these transit-amplifying cells out of the proliferative zone is required for the onset of differentiation (Figure 6.4B). The stem cells may be maintained at the base of crypts, embedded in the intestinal wall, for protection from toxins passing through the gut lumen.

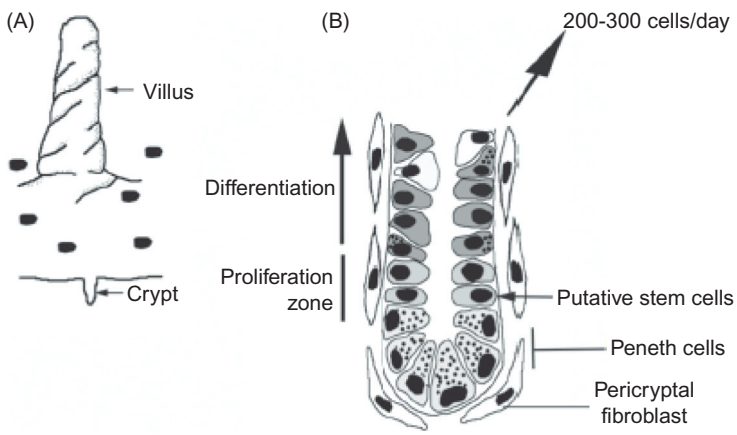

FIGURE 6.4

(A) Drawing showing the relationship between small intestinal villi and crypts. (B) Schematic representation of a small intestinal crypt. Lineage tracing experiments in both the small intestine and the colon have led to the conclusion that stem cells reside near the crypt base. The approximate location of stem cells in the small intestine is at position 4 from the crypt base above the differentiated Paneth cells. Colonic crypts do not contain Paneth cells, and the stem cells in the colon have been localized to the base of the crypt (Korinek et al.1997, Science, 275, 1784). Other differentiated cells migrate out toward the lumen of the gut. Factors secreted by the pericryptal fibroblasts could contribute to stem cell maintenance and proliferation; therefore, these cells are likely candidates for cellular components of the stem cell niche within the gut.

Wnt signaling is involved in controlling the proliferation and differentiation of intestinal epithelial cells. In humans, mutations in the adenomatous polyposis coli (APC) gene, a negative regulator of Wnt signaling, are etiologically linked to the development of colorectal cancers. Also, constitutively active nuclear complexes of Tcf4-β-catenin are found in APC −/− colon carcinoma cell lines or in cell lines that have a stable form of β-catenin, suggesting that hyperactivation of Tcf4 may contribute to cellular transformation. Loss of the Tcf4 transcription factor, which is expressed in the intestinal epithelium, leads to the depletion of stem cells and the failure to maintain the proliferative compartments in the intervillus pockets of the neonatal small intestine. This phenotype was not evident in the colon, suggesting that another Tcf family member may act redundantly with or instead of Tcf4 in the large intestine.

If crypts serve as a niche to support the self-renewal of intestinal stem cells, this would imply that cells near the intestinal stem cells may be the source of a secreted self-renewal signal. Nuclear β-catenin is found only in the cells at the base of the crypts within the adult mouse small intestine, suggesting activation of the Wnt pathway in these cells (Figure 6.4B). Therefore, mesenchymal cells underlying the crypt epithelium could be a source for a secreted Wnt ligand that could act as a paracrine signal to direct the proliferation of stem cells, progenitor cells, or both in the intestinal epithelium.

6.7.7 Neural Stem Cells

Neurogenesis persists in particular regions of the adult brain, occurring in both the subventricular zone (SVZ) of the lateral ventricle and the hippocampus. Neural stem cells that have the capacity to self-renew and produce precursors that will differentiate into both neurons and glia can be cultured from the SVZ and hippocampus. When cultured *in vitro*, cells from these tissues can generate free-floating, spherical clusters called neurospheres which contain mixed populations of stem cells and precursor cells. Although growth factors such as fibroblast growth factor 2 (FGF 2) and epidermal growth factor (EGF) can support the growth of neurospheres in culture, the physiologically relevant signaling molecules that support stem cell self-renewal in the adult brain have not yet been identified.

Cells isolated from many regions in the adult brain, including nonneurogenic regions, can generate neurons both *in vitro* and, after grafting back to neurogenic regions, *in vivo*. These data suggest that neural stem cells may be distributed throughout the adult central nervous system and that the local environment, or niche, may determine their developmental fate.

Astrocytes from both the SVZ and the hippocampus can provide neurogenic signals to progenitor cells, suggesting that astrocytes may be a critical component of the neural stem cell niche. Astrocytes from the adult spinal cord

have no effect on the growth of neural stem cells in culture, indicating that astrocytes from different regions of the central nervous system exhibit different capabilities for regulating the fate choice of adult stem cells.

6.8 SUMMARY

Stem cell niches have been proposed to play a critical role in the maintenance of stem cells in the male germ-line, the hematopoietic system, the epidermis, the intestinal epithelium, and the adult nervous system. Characterization of these stem cell niches depends on the ability to identify stem cells *in vivo* in their normal environment. Through comparison of different stem cell systems, some themes emerge that indicate possible general characteristics of the relationship between stem cells and their supporting niche.

First, secreted factors elaborated by or induced by cells composing the stem cell niche can function to direct stem cell fate decisions. However, the precise signaling pathway or pathways may be different for each stem cell type and within each stem cell niche. Studies in *Drosophila* indicate that support cells adjacent to stem cells secrete factors required for maintaining stem cell identity and for specifying stem cell self-renewal. Both JAK-STAT signaling and TGF-β signaling have been implicated in the regulation of stem cell behavior by surrounding support cells in *Drosophila*. In mammals, the Wnt signal transduction pathway has been demonstrated to play a role in specifying stem cell self-renewal in HSCs, although the Wnt signal may be secreted from the stem cells themselves and may act in an autocrine loop to control stem cell proliferation. Wnt signaling may also be involved in directing the proliferation of stem cells, transit-amplifying cells, or both in the intestinal epithelium. However, the same signaling pathway may be exploited for distinct purposes in different stem cell systems. In the mammalian epidermis, Wnt signaling is likely involved in specifying the fate of hair follicle precursors rather than in specifying self-renewal of the multipotent stem cells in the bulge.

Cell adhesion is also emerging as an important characteristic of the interactions of stem cells with the niche. Adhesion between stem cells and niche cells is required for stem cell maintenance in the *Drosophila* male and female germ-line, ensuring that GSCs are held close to self-renewal signals emanating from the niche. Attachment to niche cells or to a basal lamina may also be important for stem cell maintenance within adult mammalian tissues, hence the high levels of the β1 integrin characteristic of stem cells in the interfollicular epidermis and in the multipotent stem cells within the bulge region of the outer root sheath. Interestingly, targeted disruption of β1 integrin in cells within the bulge region of the outer root sheath severely impaired the proliferation of precursor cells that contributed to the

interfollicular epidermis, hair follicle, and sebaceous glands. Thus, similar to the role of adherens junctions in maintaining *Drosophila* GSCs in the niche, β1 integrin-mediated adhesion may be required to hold multipotent epidermal stem cells within the niche and close to self-renewal signals. In the mammalian testis, α6 integrin has been identified as a cell surface marker for the enrichment of spermatogonial stem cells, although a specific role for α6 integrin in spermatogonial stem cell maintenance has not yet been directly demonstrated. Similarly, α6 integrin is expressed by basal keratinocytes in the epidermis; however, there is no strong correlation between α6 expression and proliferative potential. Therefore, although cell adhesion is frequently a conserved feature of stem cell maintenance in supportive niches, the specific types of junctions and cell adhesion molecules that play roles may differ among different stem cell niche systems.

Third, the precise cellular organization of stem cells with respect to surrounding support cells may play an important role in the regulation of appropriate stem cell numbers. In the *Drosophila* ovary and testis, where the stem cells normally divide with invariant asymmetry, the mitotic spindle is oriented to place the daughter cell that will retain stem cell identity within the stem cell niche; the daughter cell destined to differentiate is placed outside of the niche and away from self-renewal signals. Either attachment to niche cells or the extracellular matrix via junctional complexes or localized signals within the niche may provide polarity cues toward which stem cells can orient during division. This stereotyped division plane can in turn specify an asymmetric outcome to stem cell divisions, in which one daughter cell retains attachment to niche cells and the other is displaced out of the stem cell niche. As stem cells are definitively identified *in vivo*, in the context of their normal support cell microenvironment, it will be interesting to determine if stem cell divisions are likewise oriented in the seminiferous tubules, bone marrow, follicular bulge, and intestinal crypts, within neurogenic regions of the adult brain.

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FOR FURTHER STUDY

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CHAPTER 7

Mechanisms of Stem Cell Self-Renewal

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7.1 SELF-RENEWAL OF PLURIPOTENT STEM CELLS

The capacities for self-renewal and differentiation are the two most prominent characteristics of stem cells. Self-renewal can be defined as making a complete phenocopy of stem cells through mitosis, which means at least one daughter cell generated by mitosis possesses the same capacity of self-renewal and differentiation. In stem cell self-renewal, symmetric cell division generates two stem cells; asymmetric cell division results in one stem cell and either one differentiated progeny or a stem cell with a restricted capacity for differentiation. Self-renewal by symmetric cell division is often observed in transient stem cells appearing in early embryonic development to increase body size. In contrast, self-renewal by asymmetric cell division can be found in permanent stem cells in embryos in later developmental stages, and also in adults to maintain the homeostasis of the established body plan.

Stem cells are categorized by their abilities for differentiation. Pluripotency is defined as the ability of a cell to differentiate into any of three germ layers. Pluripotent stem cells can be found in both pre- and postimplantation embryos. Mouse ES cells are authentic pluripotent stem cells established from the inner cell mass (ICM) of blastocyst-stage embryos. Mouse ES cells continue self-renewal in a leukemia inhibitory factor (LIF)-dependent manner, and retain the ability to contribute to embryonic development after injection into blastocysts, which is the characteristic now designated as naïve pluripotency. In contrast, mouse epiblast stem cells (EpiSCs) are derived from epiblasts or primitive ectoderm of postimplantation embryos. Mouse EpiSCs maintain self-renewal in the presence of fibroblast growth factor 2 (Fgf2) and Activin and they rarely contribute to chimeric embryos after blastocyst injection, which is the characteristic now designated as primed pluripotency.

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X chromosome inactivation occurs in female mouse embryos immediately after implantation, and it was reported that mouse ES cells retain two active X chromosomes whereas EpiSCs have one inactive X chromosome, indicating a difference in their developmental stages. Human ES cells are derived from ICM but demonstrate characteristics of primed pluripotent stem cells. It was also demonstrated that mouse ICMs are capable of giving rise to EpiSCs in the presence of Fgf2 and Activin, suggesting that pluripotency is determined by culture conditions rather than the origin of the stem cells.

7.1.1 Molecular Mechanisms to Retain ES Cell Self-Renewal

The ability for continuous self-renewal *in vitro* is one of the characteristic phenotypes of ES cells. As found in other cellular phenotypes, continuous self-renewal should be regulated via transcription in the nucleus, which in turn is triggered by extracellular signals. In this section, the minimal molecular mechanism to maintain naïve pluripotency in mouse ES cells will be described, based on functional analysis data (see Figure 7.1).

7.1.1.1 Extracellular Signals for ES Self-Renewal

7.1.1.1a Leukemia Inhibitory Factor

LIF is a soluble factor that is sufficient for maintaining the pluripotency of mouse ES cells. LIF belongs to the interleukin (IL)-6 cytokine family, whose members share the transmembrane glycoprotein Gp130 as a common component for signal transduction of their receptors. The high-affinity LIF receptor consists of a heterodimer of Gp130 and LIF receptor β (Lifrβ). One major signal transduction pathway mediated by Gp130 is JAK–STAT, and it has been shown that the activation of Stat3 is necessary and sufficient to maintain pluripotency. The phosphatidylinositol-3-OH kinase (PI3K)–Akt pathway is also activated by LIF under Gp130, and artificial activation of Akt was able to replace the requirement for LIF. These two intracellular pathways integrate the LIF signal into the nuclei in parallel, which activates the transcription factors responsible for maintaining pluripotency. In contrast, the Grb2-mitogen-activated protein kinase (MAPK) pathway is also activated by LIF, but acts as a negative regulator of pluripotency. Among these intracellular signal pathways, JAK–STAT is exclusively activated by LIF in ES cells, whereas the PI3K–Akt and the Grb2–MAPK pathways are regulated by LIF as well as other extracellular signals such as insulin/insulin-like growth factor (IGF) and Fgf4, respectively.

Although LIF sustains self-renewal of mouse ES cells in media containing fetal calf serum, or in serum-free N2B27 media in combination with bone morphogenetic protein (BMP) 4, its physiological action during development appears to be restricted. Elimination of the function of *Lif*, *Gp130*, *Lifrβ*, or *Stat3* by gene targeting did not interfere with self-renewal of pluripotent

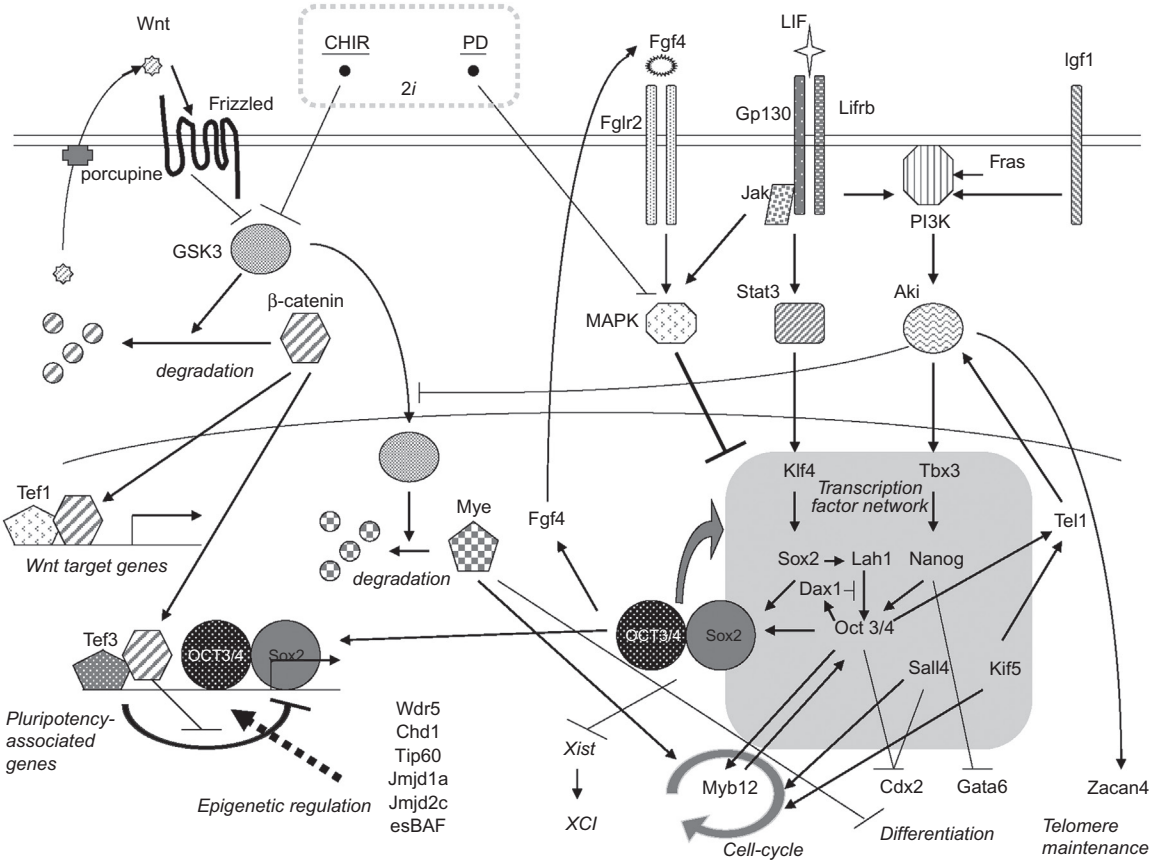

FIGURE 7.1 **A bird's-eye view of the molecular mechanism working in mouse ES cells to maintain pluripotency.**
See text for the abbreviations in the figure.

stem cells during early embryogenesis. The role of Gp130 in the pluripotent cell phenotype was only evident when the delayed blastocysts were carefully analyzed. The ICM of delayed blastocysts normally maintains pluripotency, but *Gp130*-null blastocysts could not maintain pluripotency during the delayed period. Since the maintenance of blastocysts in the uterus without implantation is a characteristic feature in rodents, the responsiveness of ES cells to Gp130 signaling has its origin in this adaptive physiological function. Moreover, it may be the reason why LIF does not show obvious effects on ES cells of other species, especially primates. However, the function of the Gp130–Stat3 pathway in germ cell development is evolutionally conserved, since it can be found in invertebrates, which may suggest that the role of this system in rodent ES cells is a small evolutionary cooption derived from the maintenance of germ cells.

7.1.1.1b Fibroblast Growth Factor 4

Fgf4 is an autocrine factor that promotes the differentiation of mouse ES cells. Either elimination of Fgf4 secretion by gene targeting or blockage of Fgf receptor kinase activity by a specific inhibitor promotes self-renewal and inhibits spontaneous differentiation in the medium containing LIF and serum. The negative signal for pluripotency from Fgf4–Fgfr2 is transduced by the Grb2–MAPK pathway, and the targeted disruption of Grb2 or blockage of the MAPK activity by a specific inhibitor also promotes self-renewal. Since *Fgf4* expression is regulated by the pluripotency-associated transcription factors Oct3/4 and Sox2, destabilization of pluripotency also seems to be one of the important contradictive effector pathways of pluripotency-coupled transcription factor network. Nuclear localization of Tbx3 is negatively regulated by MAPK, but there might be other targets to mediate this negative effect.

7.1.1.1c Wnt

Wnt is also an autocrine factor that stabilizes naïve pluripotency. Inhibiting the secretion of Wnt family ligands by inhibiting the activity of porcupine causes mouse ES cells to change to EpiSC-like cells. In contrast, inhibiting glycogen synthase kinase (GSK) 3 activity, which is negatively regulated in the canonical Wnt signaling pathway, stabilizes self-renewal when combined with inhibition of MAPK in serum and LIF-free culture media (also called *2i* culture conditions). However, neither addition of recombinant Wnt ligand nor inhibition of GSK3 is sufficient to support self-renewal of mouse ES cells without inhibition of MAPK activity or LIF. Since LIF shows a synergistic effect with inhibition of GSK3 and MAPK, they may have different integration points in the pluripotency-associated transcription factor network. Inhibition of GSK3 causes stabilization of β-catenin and promotes its nuclear localization. Nuclear-localized β-catenin interacts with Tcf1 to activate its transcriptional activity as well as with Tcf3 to inhibit its repressor function. GSK3 might not be regulated exclusively by Wnt and it was suggested that the PI3K–Akt pathway negatively regulates its nuclear localization.

7.1.1.2 Transcriptional Regulation for ES Cell Self-Renewal

7.1.1.2a Oct3/4

Oct3/4 encoded by *Pou5f1* was initially identified as Oct3 or Oct4 for undifferentiated, state-specific expression in embryonal carcinoma cells. Oct3/4 is absolutely essential for establishing and maintaining pluripotency in embryonic development, since *Oct3/4*-null embryos die immediately after implantation and ICM isolated from *Oct3/4*-null blastocysts undergoes differentiation toward trophectoderm. Inducible loss of *Oct3/4* in ES cells also causes their differentiation toward trophectoderm, indicating that Oct3/4 is essential for self-renewal of mouse ES cells. Oct3/4 forms a heterodimer with Sox2, and regulates multiple pluripotency-associated genes including

both *Oct3/4* and *Sox2*, indicating that a positive feedback mechanism may be involved in the maintenance of ES cell self-renewal. Oct3/4 acts as a transcriptional repressor for the genes involved in the induction of differentiation toward trophectoderm such as *Cdx2*, which explains the reason why loss of Oct3/4 function leads exclusively to trophectoderm differentiation. Overexpression of Oct3/4 induces differentiation to a mixture of differentiated cells other than trophectoderm, which might be due to the retention of the repressor function that blocks trophectoderm differentiation.

7.1.1.2b Sox2

Sox2 is a member of the Sry-related HMG-box transcription factor family. It acts as a partner of Oct3/4 to direct transcriptional activation, and is essential for maintaining a pluripotent cell population in embryos, since *Sox2*-null embryos die after implantation. Inducible loss of *Sox2* in ES cells causes their differentiation toward trophectoderm, indicating that Sox2 is essential to maintain ES cell self-renewal. However, artificial maintenance of *Oct3/4* expression from a transgene at physiological expression level restores self-renewal of *Sox2*-null ES cells with keeping the expression of the known target genes of Oct3/4–Sox2 complex. Therefore, the primary role of Sox2 is deduced to the indirect transcriptional activation of Oct3/4 and the activator function with Oct3/4 can be compensated by other Sox family members.

7.1.1.2c Nanog

Nanog encodes an NK2-family homeobox transcription factor. *Nanog* comes from *Tir na nÓg*, the name of the land of the ever-young in Celtic mythology, because its forced expression in mouse ES cells allows self-renewal in the absence of LIF. This is a quite unique feature of *Nanog*, because artificial maintenance of either *Oct3/4* or *Sox2* cannot replace the requirement of LIF in ES cell self-renewal. Nanog should be required for normal preimplantation development since *Nanog*-null embryos die after implantation. However, inducible deletion of *Nanog* in ES cells gives rise to *Nanog*-null ES cells that are still able to continue self-renewal, although they show higher incidence of spontaneous differentiation, indicating that Nanog is not essential for ES cell self-renewal. Since Nanog co-localizes to the target sites in the genome with Oct3/4 and Sox2, it may contribute to stabilizing their binding or enhancing their function. It was reported that *Nanog* expression is regulated by LIF via the PI3K–Akt pathway.

7.1.1.2d Klf4

Klf4 is a Krüppel-type zinc-finger transcription factor. Forced expression of *Klf4* maintains ES cell self-renewal in the absence of LIF as found in the case of *Nanog*. However, *Klf4*-null embryos pass through early developmental stages without abnormality and die immediately after birth due to the defect

of the epithelial seal in skin and colon. Although the establishment of *Klf4*-null ES cells has not yet been reported, siRNA-mediated knockdown suggests that Klf4 shares its function with other Klf family members Klf2 and Klf5 to maintain ES cell self-renewal. No single gene knockout showed any defect, whereas triple knockdown of all three Klf factors caused prevention of ES cell self-renewal. However, since *Klf5*-null ES cells showed defective proliferation, each Klf factor has unique functions, as well as functions in common with other Klf factors. It was reported that *Klf4* is regulated by LIF via the JAK–STAT pathway whereas *Klf2* is mainly regulated by Oct3/4, suggesting different regulatory functions in ES cell self-renewal. Klf4 cooperates with Oct3/4 and Sox2 to activate the transcription of a particular set of target genes including *Lefty1*.

7.1.1.2e Tbx3

Tbx3 belongs to the T-box transcription factor family. Knockdown of *Tbx3* in mouse ES cells induces differentiation, whereas its forced expression maintains LIF-independent self-renewal. However, *Tbx3*-null embryos die at E13.5 due to a defect in the yolk sac, indicating that its function is not required for the maintenance of pluripotent cell population *in vivo*. *Tbx3* expression is positively regulated by LIF via the PI3K–Akt pathway, while the nuclear localization of Tbx3 protein is negatively regulated by MAPK.

7.1.1.2f Tcf3

The T-cell factor (Tcf) family is regarded as a target of the canonical Wnt pathway. In mouse ES cells, Tcf1 acts as a conventional target to mediate transcriptional activation of nuclear-localized β-catenin. In contrast, Tcf3 works with a unique mode of action in mouse ES cells. Tcf3 co-localizes to the target sites of Oct3/4, Sox2, and Nanog and negatively regulates their transcriptional activation, which is competed by nuclear-localized β-catenin. This model is supported by the evidence that *Tcf3*-null ES cells show stabilized self-renewing ability and that *Tcf3* overexpression induces differentiation.

7.1.1.2g Myc

Myc is known as a common regulator of cellular proliferation and metabolism in various stem cells. Forced expression of *Myc*, especially its mutant form *MycT58A*, supports ES cell self-renewal without LIF. It was reported that nuclear-localized GSK3 phosphorylates Myc at T58 leading to its degradation, and nuclear localization of GSK3 is negatively regulated by PI3K–Akt under the LIF signal. Although *Myc*-null ES cells continue self-renewal, double knockout of *Myc* and *Mycn* induces differentiation to primitive endoderm, suggesting a shared function of Myc and Mycn to prevent differentiation in mouse ES cells. However, their function is context-dependent, because ES cells lacking *Max*, which is a common dimerization partner of the Myc family, can be maintained in *2i* culture although they die in fetal bovine serum-containing culture. Therefore, the requirement for Myc in mouse ES cells seems to prevent

the differentiation to primitive endoderm which is enforced by MAPK activation, and its function in cellular proliferation is dispensable.

7.1.1.3 Nuclear Receptors

Nuclear receptors are suggested as being involved in the pluripotency-associated transcription factor network, especially in the regulation of *Oct3/4*. SF1/Nr5a1 is known to bind to the *Oct3/4* promoter with RAR to activate its transcription. Lrh1/Nr5a2 is reported to bind to the SF1 binding sites in mouse ES cells, and *Lrh1*-null ES cells showed rapid downregulation of *Oct3/4* in primitive ectoderm, suggesting Lrh1 has a major function in the primed pluripotent state. Tr2/Nr2c1 and Tr4/Nr2c2 are also suggested to be involved in the transcriptional activation of *Oct3/4* in ES cells. The repressive nuclear receptor family members such as Gcnf/Nr6a1 and Couptfs (Nr2f1–3) occupy SF1 binding sites in somatic cells, which may contribute to the blocking of aberrant activation of *Oct3/4* in differentiated cells. Dax1/Nr0b1 encodes a negative regulator of Nr5a1 and Nr5a2 and is specifically expressed in naïve pluripotent stem cells. *Dax1* expression is positively regulated by Oct3/4 and Stat3, and Dax1 inhibits the activity of Nr5a1 and Nr5a2 as well as Oct3/4 to repress *Oct3/4* expression, suggesting a role in fine-tuning *Oct3/4* gene expression within an appropriate range. Indeed, forced expression of *Dax1* in ES cells causes induction of differentiation toward trophectoderm along with repression of *Oct3/4*.

7.1.1.3a Stat3

Stat3 is a pivotal mediator of the LIF signal. Artificial activation of Stat3 using the Stat3–ER fusion is sufficient to substitute the requirement of LIF for ES cell self-renewal. However, it is not absolutely required for maintaining pluripotency because *Stat3*-null ES cells can be propagated in *2i* culture as in the case of *Myc:Mycn*-null ES cells. Therefore, the function of Stat3 is limited to the transduction of the LIF signal into the core transcription factor network.

7.1.1.3b Rex1

Rex1 encodes a C2H2 zinc-finger transcription factor that is also known as *Zfp42*. Although it is specifically expressed in pluripotent stem cells both *in vitro* and *in vivo*, its function is absolutely dispensable for maintaining pluripotency. Indeed, *Rex1*-null ES cells maintain self-renewal and an ability to contribute to chimeric embryos. A similar unexpected observation was reported for Esg1 and Rest.

7.1.1.4 Mediator Complex

The transcriptional activation by tissue-specific transcription factors is transmitted to the recruitment of RNA polymerase II to the promoter site via mediator complex. This general mechanism is shared in mouse ES cells for transcriptional activation by pluripotency-associated transcription factors. Mediator complex

also contributes to chromatin architecture modulation by the interaction with cohesin, which is also a ubiquitous mechanism.

7.1.1.5 Epigenetic Regulation of ES Cell Self-Renewal

A unique epigenetic feature of mouse ES cells provides the basis of the pluripotency-associated transcription factor network. In general, all epigenetic regulation in mouse ES cells aims to keep genome-wide open chromatin conformation.

7.1.1.5a Histone Marks

Polycomb group complex (PRC) 2 consists of Suz12, Eed, and Ezh2 and mediates tri-methylation at Lys27 of histone H3, which contributes to gene silencing by establishing bivalent domains at the regulatory elements of the developmentally regulated genes. On the other hand, it was also shown that PRC2 is dispensable in maintaining ES cell self-renewal, since *Suz12*-null, *Eed*-null, and *Ezh2*-null ES cell lines all continue to self-renew. PRC1, which consists of Ring1a and Ring1b, mediates histone ubiquitination. ES cell self-renewal is abolished by disruption of both *Ring1a* and *Ring1b* loci. In general, PRC2 recruits PRC1 to the target sites but they may have shared function since only *Eed:Ring1b*-null ES cells, but not ES cells lacking either PRC1 or PRC2 function, lose the ability to differentiate properly. It is known that the trithorax group (trxG) mediates methylation of Lys4 of H3 to counteract the silencing effect by PRC. Recently, Wdr5 has been identified as a member of trxG, and is involved in the maintenance of ES cell self-renewal.

Methylation of Lys9 of histone H3 is involved in heterochromation formation by the recruitment of heterochromatin protein (HP)-1. Although H3K9 di- and tri-methyl transferases G9a and Glp are dispensable for ES cell self-renewal, knockout of H3K9 mono-methyl transferase *Eset* in ES cells causes de-repression of endogenous retroviruses. A similar phenotype was observed in *Kap1/Trim28*-null ES cells probably because Kap1 is required for the recruitment of Eset to the target sites.

Jumonji-family proteins mediate demethylation of methylated histones. Jumonji/Jarid2 is not required for ES cell self-renewal, but it is involved in fine-tuning H3K27 methylation by interacting with PRC2. Knockdown of *Jmjd1a* or *Jmjd2c* leads to the differentiation of ES cells, suggesting their involvement in the positive regulation of pluripotency-associated genes via demethylation of H3K9.

7.1.1.5b DNA Methylation

Methylation of cytosine residues in DNA, which generates 5-methylcytosine, is a major heritable epigenetic mark. Dnmt3a and Dnmt3b act as a *de*

novo methyltransferase, while Dnmt1 works for the maintenance of methyltransferase during DNA replication. Although mouse ES cells express high levels of *Dnmt3a* and *Dnmt3b*, double knockout ES cells of both *de novo* methyltransferases continue self-renewal. However, these ES cells lose DNA methylation during long-term culture and then show a higher incidence of spontaneous differentiation toward trophectoderm than wild-type ES cells. Triple knockout ES cells lacking all three DNA methyltransferases are still viable, indicating that DNA methylation is not required for ES cell self-renewal.

5-Hydroxymethylcytosine is a demethylation-product of 5-methylcytosine by tet-family DNA demethylase. *Tet1* is strongly expressed in ES cells, resulting in abundant distribution of 5-hydroxymethylcytosine in mouse ES cell genome. However, it was demonstrated that *Tet1*-null ES cells, although showing a partial reduction of 5-hydroxymethylcytosine, maintained pluripotency.

7.1.1.5c Chromatin Remodeling

Chromatin remodeling is involved in the regulation of proper transcriptional activation at the promoter region by altering the histone-DNA contact. Chromatin remodeling proteins can be divided into four families: SWI/SNF (switch/sucrose nonfermentable), CHD (chromodomain helicase DNA binding), ISWI (imitation switch), and INO80 (inositol-requiring 80). It was reported that mouse ES cells express a specific type of SWI/SNF complex designated as esBAF. Knockdown of its major component *Brg1* causes defects in proliferation and differentiation, and knockout of *BAF250a*/*Arid1a* or *Baf250b*/*Arid1b* affect proliferation and differentiation of ES cells, indicating its functional significance. Recently, it was reported that esBAF co-localizes with Stat3 in the genome to exclude PRC2 from its target sites. In contrast, disruption of *Mbd3*, that is a component of NuRD complex of CHD family, results in stable self-renewal in the absence of LIF, suggesting its negative role in ES cell self-renewal. Tip60 of the INO80 family mediates acetylation of H3K4 and was suggested to be involved in ES cell self-renewal, since its knockdown causes prevention of self-renewal, and Chd1 of the CHD family was suggested to be required for the maintenance of proper pluripotency by knockdown experiment. Both of these hypotheses are awaiting confirmation by gene targeting.

7.1.1.6 miRNA in ES Cell Self-Renewal

miRNA regulates a set of gene expression by binding to its target sequence. Processing of miRNA is mediated by Drosha/Dgcr8, and *Dgcr8*-null ES cells show slow proliferation ratios with a lack of all miRNA, indicating that the role of miRNA in ES cells is reduced to the positive regulation of their proliferation.

7.2 PREVENTION OF DIFFERENTIATION

To maintain ES cell self-renewal by keeping pluripotency, pluripotency-associated transcription factors must counteract the induction of differentiation. Mouse ES cells directly differentiate into *only* three cell lineages; primitive ectoderm, primitive endoderm, and trophectoderm. Mouse ES cells rarely differentiate to trophectoderm after withdrawal of LIF or by formation of embryoid body. However, when *Oct3/4* expression is artificially reduced, ES cells are homogeneously converted into trophectoderm, indicating that Oct3/4 blocks the differentiation program toward trophectoderm. *Cdx2* and *Eomes* are upregulated immediately after repression of *Oct3/4*, and their forced expression is sufficient to induce trophectoderm differentiation, suggesting that these trophectoderm-associated transcription factors are critical repressive targets of Oct3/4 in ES cells. It was revealed that Oct3/4 and Cdx2 form a repressor complex that inhibits their own expression, suggesting the reciprocal inhibitory loop between pluripotency- and trophectoderm-associated transcription factors involves a sharp segregation of these cell lineages.

In the case of primitive endoderm, the Gata-family transcription factors Gata4 and Gata6 have a pivotal role to determine this cell fate. Primitive endoderm differentiation is induced in embryoid bodies as well as in culture without LIF. Induction of *Gata4* and *Gata6* is coupled with these differentiation events, and forced expression of either *Gata4* or *Gata6* in mouse ES cells triggers differentiation toward primitive endoderm which resembles the character of eXtraembryonic ENdoderm stem (XEN) cells derived from blastocysts. Since *Gata4* and *Gata6* link via cross auto-regulation, their activities should be repressed in ES cells. It was suggested that Nanog works as a repressor of *Gata6* expression, because the Nanog expression pattern is reciprocal to Gata6 in preimplantation embryos, and also because *Gata6* is directly repressed by Nanog *in vitro*. In addition, the original report of *Nanog*-null ES cells showed that they acquire an XEN cell-like character, although later reports showed no such phenotype in *Nanog*-null ES cells. To establish a reciprocal expression pattern, *Nanog* and *Gata6* may form a reciprocal inhibitory loop as in the case of *Oct3/4* and *Cdx2*, but so far there is no direct evidence of repression of *Nanog* by Gata6. The repression of *Nanog* might be indirect via activation of the repressive nuclear receptors such as Gcnf and Couptfs.

Conversion of mouse ES cells to primitive ectoderm occurs naturally in embryoid body formation. However, no transcription factor that acts as a trigger of this conversion, as Cdx2 in trophectoderm and Gata6 in primitive endoderm differentiation, has been identified. When the expression of pluripotency-associated transcription factors is compared in naïve and primed pluripotent stem cells, many transcription factors such as *Klf4*, *Tbx3*, *Dax1*, and *Rex1* are expressed at higher levels in the naïve state than in the primed

state. These naïve-specific genes may have functional significance to trigger conversion to the primed state by their downregulation, but there is no direct evidence to support this idea to date.

7.3 MAINTENANCE OF STEM CELL PROLIFERATION

Mouse ES cells proliferate continuously, with a doubling time of 12–14 hours in the conventional culture with LIF. Such rapid proliferation is based on the characteristic cell-cycle regulation, which allows unlimited transition from G1 to S phase. It was shown that Rb, which acts as a major regulator of G1-S checkpoint by inhibition of E2F transcriptional activity, is constitutively inactivated by phosphorylation, and mouse ES cells lacking all three *Rb*-related genes proliferate normally, although the precise mechanism mediating hyperphosphorylation of Rb is unknown.

Mybl2 is a pleiotropic regulator of cell-cycle promotion, and one of its major functions is the promotion of G2-M transition. It was demonstrated that Mybl2 (also known as B-Myb) is required for preimplantation development and normal ES cell proliferation. Interestingly, *Mybl2* is a putative target of Oct3/4, and Mybl2 activates the transcription of *Oct3/4*, suggesting a positive regulatory loop between cell-cycle promotion and pluripotency-associated transcription factor network.

The PI3K–Akt pathway has a dual role in mouse ES cells. One is the transduction of the LIF signal to activate the pluripotency-associated transcription factors such as *Tbx3* and *Nanog*. Another is the promotion of proliferation as found in many other cell types. Eras is a member of the Ras family small-GTP binding protein and is specifically expressed in ES cells. *Eras*-null ES cells show reduced proliferation ratio whereas its forced expression promotes proliferation, indicating its positive function in ES cell proliferation. Eras constitutively activates PI3K but not the Raf–MAPK pathway, suggesting that its function is mediated by the PI3K–Akt pathway. Tcl1 encodes an adaptor protein that promotes dimerization of Akt and their phosphorylation. *Tcl1* is a direct target of Oct3/4 and knockdown of *Tcl1* causes reduction of proliferation of ES cells, suggesting the positive regulation of the PI3K–Akt pathway by the pluripotency-associated transcription factors to promote proliferation.

Sall4- and *Klf5*-null ES cells show slower proliferation than wild-type ES cells, suggesting their positive role on ES cell proliferation. Sall4 belongs to the spalt-like zinc-finger transcription factor family. *Sall4*-null embryos die after implantation, and ICM isolated from *Sall4*-null blastocysts shows defective growth *in vitro*, indicating its function in proliferation of pluripotent cell

population *in vivo*. *Sall4*-null ES cells still possess pluripotency, as shown by their contribution to three germ layers after blastocyst injection. However, they show higher incidence of differentiation toward trophectoderm, indicating its function in repressing trophectoderm-associated transcription factors, which could be mediated by interaction with Oct3/4. Klf5 encodes a Krüppple-type, zinc-finger transcription factor, and *Klf5*-null ES cells show a fairly similar phenotype to that of *Sall4*-null ES cells. It is speculated that the function of Klf5 in promoting proliferation is partly mediated by transcriptional activation of *Tcl1*.

7.4 MAINTENANCE OF TELOMERE LENGTH

Mouse ES cells are supposed to have the ability to proliferate indefinitely. It was demonstrated that mouse ES cells can be maintained up to about 250 cumulative doublings with no indication of crisis or transformation. They indeed have high telomerase activity, and telomerase RNA component (*Terc*)-null ES cells show gradual loss of telomere length as well as reduction of proliferation rate after 300 divisions followed by virtual termination of cell growth at 450 divisions.

An additional mechanism for maintaining the telomere length of mouse ES cells was reported. Zscan4 (zinc-finger and SCAN domain containing 4) was first noticed for its 2-cell-stage-specific expression in embryos, and its heterogeneous expression in ES cells. When *Zscan4* was knocked down, telomeres were shortened and karyotype abnormalities accumulated, resulting in crisis by passage eight (~50 divisions), although telomerase activity was maintained. Zscan4 forms a complex with meiosis-specific homologous recombination proteins such as Dmc1 and Spo11, and mediates the maintenance of telomere length by homologous recombination between telomeres when *Zscan4* expression is transiently activated. Interestingly, *Zscan4* is listed as a putative target of the PI3K–Akt pathway as are Tbx3 and Nanog, suggesting a function as a mediator of the effect of this pathway on cell proliferation.

7.5 X CHROMOSOME INACTIVATION

In female somatic cells, one of the X chromosomes is randomly inactivated for dosage compensation. This event, termed random X chromosome inactivation (XCI), occurs in the epiblast of postimplantation embryos at around E5.0. As mentioned earlier, female naïve pluripotent stem cells, such as female mouse ES cells, possess two active X chromosomes, whereas female primed pluripotent stem cells such as EpiSCs carry one inactive X chromosome, indicating that the maintenance of active X chromosomes by inhibiting random XCI is a unique feature of mouse ES cells. XCI is initiated by

the expression of a non-coding RNA on the X chromosome, *Xist*. *Xist* mRNA coats the X chromosome carrying transcriptionally active *Xist* and mediates the recruitment of epigenetic machinery such as PRC2 to silence the entire chromosome. It was reported that *Xist* expression is negatively regulated by Oct3/4, Sox2, and Nanog, and that Rnf12, the positive regulator of *Xist*, is also negatively regulated by these three transcription factors. In contrast, *Tsix*, a negative regulator of *Xist*, is positively regulated by Rex1, Klf4 and Myc. Moreover, the involvement of Oct3/4 was demonstrated in X chromosome pairing and counting in cooperation with Ctcf. This evidence indicates that the pluripotency-associated transcription factor network is tightly coupled with XCI regulation to inhibit its activity in the undifferentiated state and promote its activity immediately after differentiation.

Imprinted XCI is a characteristic event found in extra-embryonic cell lineages. In female embryos, the paternal X chromosome is reactivated after fertilization, but selectively inactivated in 4- to 8-cell stages. This state is kept in the trophectoderm and primitive endoderm, whereas reactivation occurs in pluripotent stem cells in the ICM. Imprinted XCI is mediated by the epigenetic marks on both paternal and maternal X chromosomes. Although imprinted XCI is tightly coupled with lineage restriction toward extra-embryonic cell lineages, female ES cells undergo random X inactivation when they are directed to differentiate into primitive endoderm and trophectoderm by forced expression of *Gata6* and *Cdx2*, respectively, suggesting the complete erasure of the epigenetic marks for imprinted XCI in female ES cells.

7.6 SUMMARY

Mouse ES cells are the best analyzed among stem cells. Pluripotency is a unique stem cell state that allows the generation of all cell types which appear in development. The artificial activation of particular transcription factors triggers the induction of homogeneous differentiation, providing a good model system for studying how self-renewal switches to differentiation. Extensive analyses of ES cell self-renewal and differentiation at the molecular level will provide a basic concept of how stem cell self-renewal is regulated in general.

FOR FURTHER STUDY

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CHAPTER 8

Cell Cycle Regulators in Stem Cells

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8.1 INTRODUCTION

As mammals transition from the developing to the mature organism, there is often a shift in stem cell kinetics towards greater quiescence, a state associated with protection from premature exhaustion. However, this limited proliferation constrains the therapeutic use of adult stem cells and may restrict endogenous tissue repair. In this chapter, we describe the distinct cell cycle kinetics of stem cells *in vivo* and *ex vivo*, briefly introduce the current understanding of general cell cycle regulation in mammalian cells, and focus on the specific impact of some cell cycle regulators on stem cell functions. As examples, two cyclin-dependent kinase (CDK) inhibitors (CKIs), $p21^{Cip1/Waf1}$ (p21) and $p27^{kip1}$ (p27), which have been shown to govern the pool sizes of hematopoietic stem and progenitor cells, respectively, are discussed. Their inhibitory role in primitive hematopoietic cells, distinct from the action of the inhibitory cytokine transforming growth factor β-1 (TGFβ-1), is also explored. The distinct impacts of these CKIs in HSCs and other stem cell types underscore the crucial role of cell cycle regulators with respect to stem cell potentiality and offer new paradigms for their therapeutic manipulation.

8.2 CELL CYCLE KINETICS OF STEM CELLS *IN VIVO*

As largely modeled in the hematopoietic system, maintenance of mature cell production requires a cytokine-responsive progenitor cell pool with prodigious proliferative capacity and a much smaller population of stem cells intermittently giving rise to daughter cells, some of which constitute the proliferative progenitor compartment. Under activating conditions (e.g., after transplantation), an increase in stem cell divisions takes place as evident by

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depletion of cycling cells using the S-phase toxin (5-fluorouracil [5-FU] or hydroxyurea). However, relative quiescence or slow cycling in the stem cell pool appears to be essential to prevent premature depletion under conditions of physiologic stress over the lifetime of the organism. Therefore, the highly regulated proliferation of HSCs occurs at a very limited rate under homeostatic conditions. Stem cell proliferation has been directly measured by bromodeoxyuridine (BrdU) labeling experiments, and cell cycle lengths have been estimated at approximately 30 days in small rodents, which means that only about 8% of the cells cycle during a 24-hour period. Similar analyses using population kinetics have estimated that stem cells replicate once per 10 weeks in cats. In higher order primates, the frequency of cell division in the stem cell pool has been estimated to occur once per year. However, it is still not clear whether the relative quiescence reflects a complete cell cycle arrest of most cells in the stem cell compartment, termed the 'clonal succession model,' or a very prolonged G_1 or G_2 phase of cycling stem cells. Although retrovirus-based clonal marking studies indicated that most stem cells are dormant at any given time, an observation that supports the clonal succession model, this view has been challenged by the competitive repopulation model and by BrdU incorporation in defined stem cell pools.

In contrast, the essential feature of the progenitor population is that it irreversibly develops into maturing cells and in the process undergoes multiple, rapid cell divisions. The progenitor cell pool essentially operates as a cellular amplification machine, generating many differentiated cells from the few cells entering the system; therefore, it is directly responsible for the number of terminally differentiated cells. The progenitor cell pool is also called the transit or amplifying cell pool. Differences between the stem and progenitor cell populations at different stages have been regarded as a phenotypic distinction marking the stage of a cell within the hematopoietic cascade. However, an alternative model is that the specific position in a cell cycle determines whether a primitive cell functions as a stem or a progenitor cell. In this alternative model, stimuli received at distinct points in the cell cycle do or do not provoke proliferation-differentiation and yield either stem or progenitor cell outcomes, thereby challenging the traditional view of 'hierarchy' within the hematopoietic differentiation. In either model, 'stemness' associates with limited proliferation.

Relative arrest of the cell cycle distinguishes stem from progenitor cells in other tissue systems as well. In the central nervous system (CNS), for example, evidence suggests that the proliferative pools of adult neural progenitors are derived from a quiescent multipotent precursor or neuronal stem cell (NSC). For example, ablation of the proliferative zone containing the lineage-committed neuronal progenitor cell (NPC) can be repopulated from a small number of quiescent NSCs. Perhaps it is because of this quiescence

that endogenous NSCs do not produce complete recovery in cases of severe injury, although they do participate in self-repair after brain damage. Another example is found in the mouse dermal stem cell population. There are about four-fold fewer cells in S-G_2/M in the stem cell population compared with the progenitor pool, although both cell populations constantly proceed through the cell cycle. In summary, the dichotomy of relative resistance to proliferative signals by stem cells and the brisk responsiveness by progenitor cells is generally believed to be a central feature of tissue maintenance although the distinctions between stem and progenitor cells in many nonhematopoietic organs are yet to be fully defined.

8.3 STEM CELL EXPANSION *EX VIVO*

The relative quiescence of stem cells may prevent their premature exhaustion, but it is problematic in the context of the *in vitro* expansion necessary for transplantation and gene therapy. Methods for inducing stem cell proliferation have long been sought as a means to expand the population of cells capable of repopulating the marrow of ablated hosts and to render stem cells transducible with virus-based gene transfer vectors. Although great effort has been made to directly expand stem cells using different combinations of hematopoietic growth factors (cytokine cocktails), few culture systems have been applied in the clinical setting at least in part because of the lack of proof that any of the culture conditions support expansion of long-term repopulating HSCs in humans. Gene-marking studies in large animals, including primates and humans, indicate poor transduction in the stem cell compartment during long-term engraftment. These cytokine-based efforts to expand stem cells have often resulted in increased cell numbers at the expense of multipotentiality and homing ability. Although data suggest that under some specific conditions murine HSCs may divide *in vitro*, net expansion is achieved in limited fashion and is always associated with, and often dominated by, cellular differentiation. Studies on the potent effect of Notch ligands and Wnt proteins on stem cell expansion *in vitro* are promising. However, importantly, whether such 'successful' protocols can be adapted to clinically useful human HSC expansion remains to be determined.

Alternative approaches aimed at targeting the negative regulatory cytokines have also been sought to activate or expand stem cells. Factors such as TGFβ-1 and macrophage inhibitory protein-1α (MIP-1α) have been noted to play a role in dampening hematopoietic cell growth kinetics. Particularly, TGFβ-1 has been shown to be able to selectively inhibit the growth of HSCs and progenitor cells. Antisense oligonucleotides or specific neutralizing antibodies have been shown to permit primitive hematopoietic cell entry into the cell cycle and to enhance the efficiency of retroviral transduction into those cells.

However, long-term engraftment after *in vitro* manipulation by these methods remains to be defined in an *in vivo* model. Furthermore, more efficient and specific methods, such as RNA interference technology, aimed at knocking down the essential elements in the inhibitory circuits of stem cell expansion, have yet to be developed.

Although HSCs may be induced to divide *in vitro*, it remains unclear which combination of factors is specific for stem cell proliferation without differentiation. The complex microenvironments ('niches') in which the stem cells reside and the intrinsic properties of HSCs in relation to environmental cues are largely unknown. The ultimate success of stem cell expansion *in vitro* will require greater understanding of the interplay between stem cell and microenvironment, and the signaling circuitry involved in achieving self-renewal.

8.4 MAMMALIAN CELL CYCLE REGULATION AND CYCLIN-DEPENDENT KINASE INHIBITORS

The molecular principles of cell cycle regulation have been largely defined in yeast. A number of surveillance checkpoints monitor the cell cycle and halt its progression, mainly via the p53 pathway, when DNA damage occurs and cannot be repaired. In eukaryotic cells, factors that determine whether cells will continue proliferating or cease dividing and differentiate appear to operate mainly in the G_1 phase of the cell cycle (Figure 8.1). Cell cycle progression is regulated by the sequential activation and inactivation of CDKs. In somatic cells, movement through G_1 and into S phase is driven by the active form of the Cyclin D1, 2,3/CDK4, 6 complex and the subsequent phosphorylation retinoblastoma (Rb) protein. Once Rb is phosphorylated, the critical transcription factor, E2F-1, is partially released from an inhibited state and turns on a series of genes including cyclin A and cyclin E, which form a complex with CDK2 and cdc25A phosphatase. The cdc25A is able to remove the inhibitory phosphates from CDK2 and the resultant cyclin E/CDK2 complex then further phosphorylates Rb, leading to a complete release of E2F and the transcription of multiple other genes essential for entry into S-phase and DNA synthesis. In parallel, the c-myc pathway also directly contributes to the G1–S transition by elevating the transcription of genes for cyclin E and cdc25A (Figure 8.1). CDK activity is strictly dependent on cyclin levels which are regulated by ubiquitination

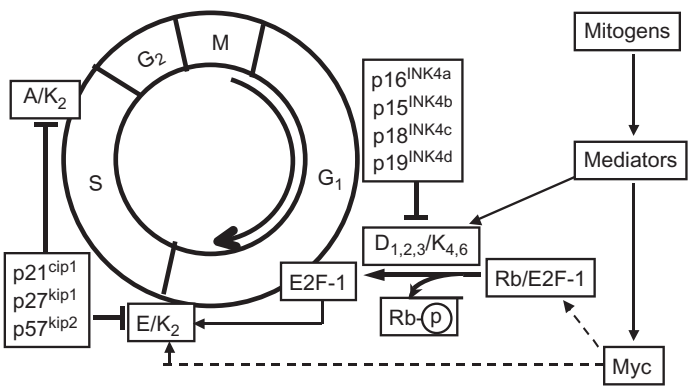

FIGURE 8.1
Cell cycle regulators in G1 and S phases (description detailed in the text).

and subsequent proteolysis. On mitogenic stimulation, cyclin D serves as an essential sensor in the cell cycle machinery and interacts with the CDK4/6-Rb-E2F pathway. In addition to regulation by cyclins and phosphorylation/dephosphorylation of the catalytic subunit, CDKs are largely controlled by CKIs. Two families of low molecular weight CKIs, Cip/Kip and INK4, are capable of interacting with CDKs to impair progression through G_1. The Cip/Kip family, which includes $p21^{Cip1/Waf1}$, $p27^{kip1}$, and $p57^{Kip2}$ (p21, p27, and p57 hereafter), may interact with broad range of cyclin–CDK complexes, whereas the INK4 family, $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, $p19^{INK4D}$ (p16, p15, p18, and p19 hereafter), specifically inhibit CDK4 and CDK6 kinases. Both families have been shown to have essential roles in arresting cell cycle progression in a number of model systems. Studies using antisense strategies have been able to release the cells in G_0 stage into the cell cycle. Knockout analysis in rodent models has provided a strong basis for further exploring those molecules in stem cell biology. Interestingly, the CKIs p27 and p18 have a profound effect on overall cellularity and organ size resulting in a larger whole animal when either gene is knocked out.

In addition there appears to be a distinct cell cycle control operating in stem cells to maintain their stemness. This has been shown in mouse embryonic stem (ES) cells with a 'defective' Rb pathway and a nonresponsive p53 pathway. Finally, because stem, progenitor, and more differentiated cells share many common cytokine receptors, it is likely that the distinct cell cycle profile in stem cells must be mediated by either distinct upstream intracellular mediators or unique combinatorial relationships of common biochemical mediators limiting the intensity of signals to enter into cell cycle. Defining the basis for the participating mechanisms in the stem cell response requires step-wise analysis of individual cell cycle regulators and ultimately a systems approach to define how these cell cycle regulators interact with one another and intersecting signaling pathways.

8.5 ROLES OF CYCLIN-DEPENDENT KINASE INHIBITORS IN STEM CELL REGULATION

Although significant progress has been made in our understanding of how the cell cycle is regulated in a variety of other model systems, little is known about how the cell cycle is molecularly controlled in stem cells. Given the relative quiescence of stem cells *in vivo*, a reasonable starting point is the analysis of cell cycle inhibitors and whether reduction of their cell cycle blockade may be a mechanism for enabling stem cell entry into the cell cycle (Figure 8.2).

CKIs have been demonstrated to be involved in a number of stem and progenitor cell systems. *Dipio*, an analog of p21/p27 in *Drosophila*, has been

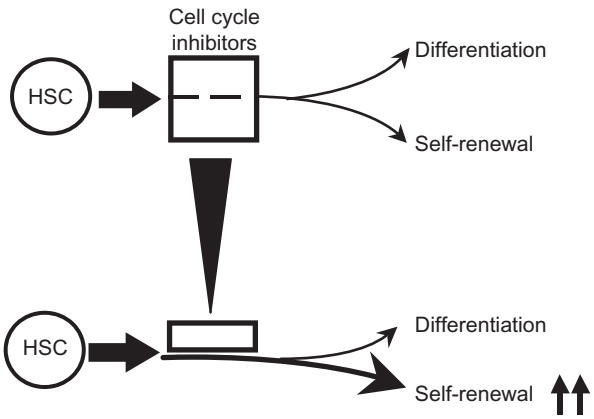

FIGURE 8.2 Model of targeting cell cycle inhibitors for enhancement of stem cell self-renewal.
Top: Under the homeostatic condition. Bottom: Potential novel approach for stem cell expansion by targeting the cell cycle inhibitors.

reported to control embryonic progenitor proliferation. Studies of knockout mice and cells that lack a specific CKI have begun to clarify their unique activity in stem cell populations. An increased stem or progenitor cell potential has been found in p21−/− or p27−/− mice when dermal, neural, or otic tissues were assessed. In the hematopoietic system, most CKI family members have been found to be expressed in $CD34^+$ cells, although the expression patterns differ. One CKI with abundant mRNA in quiescent HSCs with reduced levels in progenitor populations is p21. Functional assessment of this CKI in stem cell biology has been carried out using p21−/− mice.

8.6 ROLES OF p21 IN STEM CELL REGULATION

In the absence of p21, HSC proliferation and absolute number were increased under normal homeostatic conditions. Exposing the animals to cell cycle-specific myelotoxic injury resulted in premature death because of hematopoietic cell depletion. Furthermore, self-renewal of primitive cells was impaired in serially transplanted bone marrow from p21−/− mice leading to hematopoietic failure. Therefore, p21 governs cell cycle entry of stem cells, and, in its absence, increased cell cycling leads to stem cell exhaustion. Under conditions of stress, restricted cell cycling is crucial to prevent premature stem cell depletion and hematopoietic death. These findings have been extended to human cells and to a nondevelopmental context. Using postnatal CD34 + CD38− human cells, it was shown that interrupting p21 expression with lentivectors *ex vivo* resulted in expanded stem cell number, which was validated by increased function in the transplantation assay using irradiated NOD/SCID mice recipients. Such a study further supports an alternative paradigm for increasing HSC numbers by releasing the brake on cell cycle entry, rather than focusing on combination of pro-proliferative cytokines. Importantly, these data further supported the notion that postnatal human stem cell proliferation can be uncoupled from differentiation in *ex vivo* settings.

Interestingly, the role of p21 has been paradoxically noted to positively affect proliferation capability following cytokine stimulation in progenitor cell pools. This may be due to the requirement for p21 to promote the association of CDK4 with D-type cyclins. Low concentrations of p21 promote the assembly of active kinase complexes and thereby entry into cycle, whereas higher concentrations are inhibitory. The stoichiometry of p21 and

cyclin-CDK complexes appears to be crucial in determining the relative effect on movement of the cell through late G_1 into S phase. This was further confirmed in a study showing that p21 and p27 are essential activators of cyclin D-dependent kinases in murine fibroblasts. Bone marrow progenitor cells from mice proliferated poorly and formed few colonies with thymidine treatment except when transduced with a p21-encoding retroviral vector. Similarly, p21 transiently rises immediately following release of cell cycle arrest in 32D cells. Therefore, as observed in other systems, p21 has a dual function in the hematopoietic system depending on the differentiation stage and CDK complex type and status. In addition, complex roles of p21 in apoptosis or differentiation may participate in stem cell regulation, although these functions are yet to be thoroughly investigated.

Why p21 expression is elevated in HSCs is unclear, but two upstream regulators have been assessed. Wilms' Tumor suppressor (WT1) is known to induce p21 transcription, and overexpression of WT1 results in altered stem cell cycling and differentiation in primary hematopoietic cells. However, null mutant mice for WT1 do not appear to have a stem cell defect. It is well known that p21 is also transcriptionally regulated by p53, and serves as a downstream mediator of cell cycle arrest induced by the p53 pathway. It would be logical to expect the HSC phenotype of the p53−/− animal to be similar to that of p21−/−. Interestingly, in the absence of p53, HSC function has been reported to be significantly enhanced under stress conditions in a manner opposite to that in the absence of p21. Because p53 mediates apoptosis in many cell types, the enhanced function of HSCs in the absence of p53 suggests that in some settings, increased survival may dominate over accelerated proliferation of HSCs.

8.7 ROLES OF p27 IN STEM CELL REGULATION

Of particular interest to tissue regeneration is p27 because of its direct involvement in cell cycle-mediated hyperplasia. Direct flow cytometric analysis shows p27 expression in primitive cells and in more mature progenitors, supporting the hypothesis that p27 has a role in hematopoiesis. This role is also supported indirectly by improved retroviral transduction following knockdown of p27 with an antisense oligonucleotide. The p27 appears to accumulate at points in which signals for mitosis affect cell cycle regulators and has been shown to serve as an important regulator at a restriction point for mitogenic signals. Because progenitor cells have a robust response to growth factors, p27 likely plays a specific role in progenitor cell pools. Its role may be quite distinct from that of p21, which is shown to be a molecular switch for stem cell cycle control. Disruption of the p27 gene has resulted in a mouse with hyperplasia of a number of organs (including hematopoietic tissues) and spontaneously generated tumors of specific type. Using p27−/− mice,

it has been reported that p27 does not affect stem cell number, cell cycling, or self-renewal but markedly alters progenitor proliferation and pool size. When competitively transplanted, p27-deficient stem cells generated progenitors that eventually dominated blood cell production. Thus, modulating p27 expression in a small number of stem cells may translate into effects on most mature cells, thereby providing a strategy for potentiating the impact of transduced cells in stem cell gene therapy. Such a dramatic effect of p27 absence on hematopoietic reconstitution was also observed in liver regeneration, and a specific role of p27 in the committed progenitor cells not at the stem cell level was also reported in the mouse CNS. Therefore, distinct roles for p27 and p21 have been defined in hematopoiesis and indirect evidence suggests that these distinctions may be preserved across stem and progenitor pools from multiple different adult tissues.

8.8 OTHER CYCLIN-DEPENDENT KINASE INHIBITORS AND THE RETINOBLASTOMA PATHWAY IN STEM CELL REGULATION

One of the best studied pathways in cell cycle regulation is that of Rb, which directly interacts with Cyclin D and the INK4 proteins in early G_1 phase and serves as a critical and initial interface between mitogenic stimuli and cell fate commitment following division. A role for Rb in stem cell regulation is indirectly supported by the finding that ES cells do not have intact G_1 machinery but that acquisition of Rb pathway products induces the transition from symmetric to asymmetric cell division, which is a critical feature of mature stem cell function. Mice deficient in Rb are not viable and show defects in multiple tissue types including the hematopoietic lineage. Although deficient hematopoiesis in Rb−/− mice indicated that this protein might be critical to stem cell function, more definitive studies in the stem cell compartment have not been reported, likely because of the early lethality of the Rb null embryo. Instead, INK4 proteins closely associated with Rb have been studied in the context of stem cell biology. These studies include a report indicating that Bmi-1, an upstream inhibitor of $p16^{INK4AINKIdffdsddasdfda}$ (p16) and $p19^{INK4D}$ (p19) expression, is critical for HSC self-renewal. In the absence of Bmi-1, self-renewal of HSCs and neural stem cells is diminished, an effect dependent on the expression of p16. In mice engineered to be devoid of p16, a complex HSC phenotype has been observed with a decreased number of stem cells in younger mice but enhanced self-renewal in serially transplanted animals.

The INK4 family member, $p18^{INK4C}$ (p18 hereafter), is expressed in multiple tissue types including hematopoietic cells, the loss of which in mice results in organomegaly with higher cellularity and an increase in the incidence of

tumors with advanced age or in the presence of carcinogens. Furthermore, p18 has been suggested to be involved in the symmetric division of precursor cells in the developing brain of the mouse. It has been reported that the absence of p18 increases HSCs. Similar to the p21 null setting, but unlike p21, an increase in stem cell self-renewal is observed.

Systematic evaluation of proximate molecular regulators of cell cycling are, therefore, yielding a complex picture of how each influences primitive cell function. Different members of the CKI subfamilies appear to play distinct roles in stem or progenitor cell populations. The function of these different CKIs appears to be highly differentiation-stage specific and confers an important level of regulation in stem or progenitor cells to maintain homeostasis. Cooperative effects between members of the two CKI subfamilies are likely with evidence of such interplay between p15 and p27 now documented. How the CKIs exert distinct effects and the pathways converging on these regulators are the subject of ongoing study and will potentially provide further insight for manipulation of stem and progenitor populations. Whether these pathways are shared among primitive populations of cells in all tissues is not yet clear but preliminary data are suggestive that such is the case.

8.9 RELATION BETWEEN CYCLIN-DEPENDENT KINASE INHIBITORS AND TRANSFORMING GROWTH FACTOR β-1

TGFβ-1 has been documented to have varied effects on hematopoietic cells including enhancement of granulocytes proliferation in response to granulocyte–macrophage colony-stimulating factor or inhibition of progenitor cell responsiveness to other growth promoting cytokines. The detailed roles of TGFβ-1 in signaling pathways and in hematopoiesis have been extensively reviewed elsewhere. TGFβ-1 has been extensively characterized as a dominant negative regulator of hematopoietic cell proliferation including inhibiting primitive progenitor cells. Antisense TGFβ-1 or neutralizing antibodies of TGFβ-1 have been used to induce quiescent stem cells into the cell cycle and to augment retroviral gene transduction in conjunction with down-regulation of p27 in human $CD34^+$ subsets. Based on the roles of CKIs in hematopoietic cells as described previously, the link between TGFβ-1 and CKIs in stem cell regulation has been addressed. TGFβ-1-induced cell cycle arrest has been shown to be mediated through p15, p21, or p27 in multiple cell lines or cell types, including human epithelial cell lines, fibroblast cells, and colon and ovary cancer cell lines. It was proposed that p21 and p27 are key downstream mediators for TGFβ-1 in hematopoietic cells, and a study examined whether p21 or p27 was a proximal mediator for TGFβ-1 induced

cell cycle exit in primary hematopoietic cells. Using fine mapping of gene expression in individual cells, TGFβ-1 and p21 were documented as being upregulated in quiescent, cytokine-resistant HSCs and also in terminally differentiated mature blood cells as compared with immature, proliferating progenitor cell populations. Type II TGFβ-1 receptors were expressed ubiquitously in these subsets of cells without apparent modulation. To provide further biochemical analysis of whether the coordinate regulation of TGFβ-1 and p21 or p27 represented a dependent link between them, the cytokine-responsive 32D cell line was analyzed for p21 or p27 upregulation following cell cycle synchronization and release in the presence or absence of TGFβ-1. Despite marked antiproliferative effects of TGFβ-1, neither the transcription of p21 mRNA nor the expression of p21 or p27 was altered. To corroborate these observations in primary cells, bone marrow mononuclear cells derived from mice engineered to be deficient in p21 or p27 were assessed. Both progenitor and primitive cell function was inhibited by TGFβ-1 equivalently in knockout and wild-type littermate controls. These data indicated that TGFβ-1 exerts its inhibition on cell cycling independent of p21 and p27 in primitive hematopoietic cells. Other data have reported examining the Cip/Kip CKI family member p57 in hematopoietic progenitors. The absence of p57 was associated with a lack of responsiveness to TGFβ, failing to arrest cell cycling. Furthermore, TGFβ was noted to induce p57 expression – arguing for a direct link between TGFβ and the cell cycle regulatory function of p57. In addition, blocking TGFβ-1 can downregulate p15 expression in human CD34+ cells, indicating that TGFβ-1 may act through the INK4 family and the Cip/Kip family in hematopoietic cells. However, extensive biochemical analysis in primary hematopoietic cell subsets is needed to further address this question.

8.10 CKIs AND NOTCH

Notch1 has been well defined as a mediator of decisions at multiple steps in the hematopoietic cascade, including stem cell self-renewal versus differentiation. Variable effects on cell cycling have been reported, including inhibition of proliferation and, in contrast, maintenance of proliferation but with a decreased interval in the G_1 phase of the cell cycle. The latter observation has been followed up by more extensive analysis of an interaction between Notch1 and CKI regulation. It has been reported that the basis for Notch influencing G_1 may be through alteration in G1-S checkpoint regulator stability, specifically affecting the proteasome degradation of CKI, p27. The links of receptor-mediated effectors of stem cell function and cell cycle regulators are, therefore, beginning to emerge and provide an essential component of the larger regulatory network.

8.11 SUMMARY AND FUTURE DIRECTIONS

Further therapeutic potential of stem cells is envisioned to be broadened if the biology of the stem cells can be exploited to permit efficient *ex vivo* manipulation and enhance repopulation *in vivo*. Given the relative quiescence of HSCs, which has not been satisfactorily overcome by cytokine manipulation *in vitro*, direct intervention in the control of the cell cycle has been sought as an approach to disassociate cell proliferation from cell differentiation, thereby potentially bypassing a major hurdle in current stem cell expansion strategies. CKIs appear to be compelling candidates for the latter approach. In particular, we have learned that p21 and p27 govern the pool size of hematopoietic stem or progenitor cells, respectively, and their inhibitory roles in hematopoietic cells are not dependent on the action of TGFβ-1. Therefore, targeting specific CKIs together with TGFβ-1 may provide complementary strategies for enhancing hematopoietic stem or progenitor cell expansion and gene transduction. Controlled manipulation of specific CKIs directly or through their upstream mediators may also be relevant for the expansion or possible regeneration of other non-HSC pools.

As the roles of cell cycle regulators in the molecular control of stem cells are explored, many issues remain to be addressed. Much work is needed to delineate the roles of individual members of the cell cycle machinery within the context of tissue-specific stem cell types. Furthermore, how these relate to one another and to signal transduction pathways that operate uniquely in specific stem cell populations are yet to be elucidated. Coupling extrinsic signals to cycle control will be essential first steps before understanding how a stem responds to the complicated setting of its microenvironment. Piecing together the components and their interactions by either a reductionist or systems approach will offer targets for a more rational manipulation of the stem cell.

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CHAPTER 9

How Cells Change Their Phenotype

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9.1 METAPLASIA AND TRANSDIFFERENTIATION

9.1.1 Definitions and Theoretical Implications

'Metaplasia' is defined as the conversion of one cell type to another, and it can include conversions between tissue-specific stem cells. 'Transdifferentiation,' on the other hand, refers to the conversion of one differentiated cell type to another, and should therefore be considered a subset of metaplasia. Historically, metaplasia has been the term used by pathologists, but in recent years transdifferentiation has become the favored term, even when discussing the conversion of tissue-specific stem cells to unexpected lineages. Within the medical community, the idea of metaplasia is not controversial, but in the scientific community, some skepticism still surrounds the phenomenon of transdifferentiation and it is often attributed to tissue culture artifacts or cell fusion. Nevertheless, it is important to study metaplasia and transdifferentiation to gain a better understanding of the regulation of cellular differentiation, which may lead to new therapies for a variety of diseases, including cancer.

9.1.2 Why Study Transdifferentiation?

Regardless of which definition is applied, we consider the study of transdifferentiation and metaplasia to be important for four reasons. First, it allows us to understand the normal developmental biology of the tissues that interconvert. Most transdifferentiations occur in tissues that arise from neighboring regions in the developing embryo, and are therefore likely to differ in the expression of only one or two transcription factors. If the genes involved in transdifferentiation can be identified, then this might shed some light on the developmental differences that exist among adjacent regions of the embryo.

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Second, it is important to study metaplasia because it predisposes to certain pathological conditions, such as Barrett's metaplasia (see later sections for further details). In this condition, the lower end of the esophagus contains cells characteristic of the intestine, and there is a strong predisposition to adenocarcinoma. Therefore, understanding the molecular signals in the development of Barrett's metaplasia will help to identify the key steps in neoplasia and may provide us with potential therapeutic targets, as well as diagnostic tools.

Third, understanding transdifferentiation will help to identify the master switch genes and thus allow us to reprogram stem cells or differentiated cells for therapeutic purposes. The fourth reason is that we will be able to identify the molecular signals for inducing regeneration, and therefore promote regeneration in tissues that otherwise do not regenerate (e.g., limb regeneration).

9.2 EXAMPLES OF TRANSDIFFERENTIATION

Despite the controversy surrounding transdifferentiation, there are numerous examples that exist in both humans and animals; here we will focus on a select few. The examples we have chosen to examine in detail include the conversions of pancreas to liver, liver to pancreas, esophagus to intestine, iris to lens, and bone marrow to other cell types. Other chapters in this book also describe some of these, as well as additional examples, in more detail.

9.2.1 Pancreas to Liver

The conversion of pancreas to liver is one well-documented example of transdifferentiation. This type of conversion is not surprising, since both organs arise from the same region of the endoderm and both are thought to arise from bipotential cells in the foregut endoderm. In addition, the organs share many transcription factors, displaying their close developmental relationship. The appearance of hepatocytes in the pancreas can be induced by different protocols, including feeding rats a copper-deficient diet with the copper chelator Trien, overexpressing keratinocyte growth factor in the islets of the pancreas, or feeding animals a methionine-deficient diet and exposing them to a carcinogen. It has also been observed naturally in a primate, the vervet monkey. Although the functional nature of the hepatocytes has been examined in detail, until recently the molecular and cellular basis of the switch from pancreas to liver was poorly understood.

Two *in vitro* models have been produced for the transdifferentiation of pancreas to liver. The first model uses the pancreatic cell line AR42J, and the second uses mouse embryonic pancreas tissue in culture: both rely on

the addition of glucocorticoid to induce transdifferentiation. AR42J cells are amphicrine cells derived from azaserine-treated rats, and they express both exocrine and neuroendocrine properties – that is, they are able to synthesize digestive enzymes and express neurofilament. The dual nature of this cell line is evident in that, when exposed to glucocorticoid, they initially enhance the exocrine phenotype by producing more amylase, but when cultured with hepatocyte growth factor and activin A, the cells convert to insulin-secreting β-cells. These properties of the AR42J cells suggest that they are an endodermal progenitor cell type with the potential to become exocrine or endocrine cell types.

The transdifferentiated hepatocytes formed from pancreatic AR42J cells express many of the proteins normally found in adult liver – for example, albumin, transferrin, and transthyretin. They also function as normal hepatocytes; in particular, they are able to respond to xenobiotics (e.g., they increase their catalase content after treatment with the peroxisomal proliferator, ciprofibrate). Although the mouse embryonic pancreas also expresses liver proteins after culture with dexamethasone, it is not clear whether the same cellular and molecular mechanisms are in operation as in the AR42J cells. It is possible that, rather than the hepatocytes arising from already differentiated cell types, the liver-like cells are derived from a subpopulation of pancreatic stem cells.

To determine the cell lineage of hepatocyte formation from pancreatic AR42J cells, we performed a lineage experiment based on the perdurance of green fluorescent protein (GFP) and used the pancreatic elastase promoter. After the transdifferentiation, some cells that expressed GFP also contained liver proteins (e.g., glucose-6-phosphatase). This result suggests that the nascent hepatocytes must have once had an active elastase promoter; therefore, they were differentiated exocrine cells. To elucidate the molecular basis of the switch in cell phenotype, we determined the expression of several liver-enriched transcription factors. Following treatment with dexamethasone, C/EBPβ became induced, the expression of the exocrine enzyme amylase was lost, and liver genes (e.g., glucose-6-phosphatase) were induced. These properties of C/EBPβ make it a good candidate for an essential factor involved in the transdifferentiation of pancreas to liver. Indeed, C/EBPβ is sufficient to transdifferentiate AR42J cells to hepatocytes. Therefore, C/EBPβ appears to be a good candidate for the master switch gene distinguishing liver and pancreas.

9.2.2 Liver to Pancreas

The numerous examples of pancreas to liver transdifferentiation suggest that the reverse switch should also occur readily; nevertheless, examples of this type of conversion are infrequent. The presence of pancreatic tissue in an abnormal

location is known as heterotopic, accessory, or aberrant pancreas, and the frequency has been reported to range from 0.6 to 5.6%. In most cases (70–90%), the heterotopic pancreas is found in the stomach or intestine and is considered to be an embryological anomaly. In contrast, intrahepatic pancreatic heterotopia has only been reported in six individuals, comprising less than 0.5% of all cases of heterotopic pancreas. In general, heterotopic pancreatic tissue can be composed of exocrine, endocrine, or both types of cells. In almost every case of pancreatic heterotopia in the liver, however, only exocrine cells are present; only one case describes the presence of endocrine cells. Unlike the other cases of accessory pancreas, these rare incidents of intrahepatic pancreatic tissue cannot be explained as the result of a developmental error. In fact, in most of these cases, the patients were diagnosed with cirrhosis, suggesting that the heterotopic pancreas arose as a metaplastic process. Results with the animal models concur.

In other animals, pancreatic exocrine tissue can be induced in the liver by feeding rats polychlorinated biphenyls or by exposing trout to various carcinogens, such as diethylnitrosamine, aflatoxin B_1, or cyclopropenoid fatty acid. In these examples, the hepatic exocrine tissue is most often associated with tumors or injury, such as hepatocellular carcinomas (which arise from hepatocytes) or cholangiolar neoplasms (which arise from the bile duct), or adenofibrosis. Much like the human cases, these results suggest that, during carcinogenesis, a metaplastic event occurs that generates pancreatic tissue. Indeed, pancreatic metaplasia in trout can be inhibited by the addition of the glucosinolate indole-3-carbinol, a known anticancer agent. Whether inhibiting metaplasia prevents neoplasia remains to be determined. The ability of one cell (liver) to transdifferentiate into another (pancreas), no matter how rare, suggests that it should be possible to identify the molecular signals involved in switching a cell's phenotype, and thus to learn how to control and direct this conversion for therapeutic purposes.

Two recent reports have shown that it is possible to convert liver cells experimentally into pancreatic cells. Each has used a different approach to bring about transdifferentiation – by changing either the extracellular or the intracellular environment. In the first example, hepatic oval cells were isolated and maintained in tissue culture media supplemented with leukemia inhibitory factor (LIF). Upon the removal of LIF and the addition of high concentrations of glucose (23 mM) in the medium, the oval cells transdifferentiated to pancreatic cells. The oval cells were converted into a variety of pancreatic cell types, including glucagon, insulin, and pancreatic polypeptide-expressing cells. Functionally, these oval cell-derived endocrine cells were able to reverse hyperglycemia in streptozotocin-induced diabetes. The mechanism whereby glucose induces the transdifferentiation is not known, though previously it

was shown that glucose can promote the growth and differentiation of β-cells in the normal pancreas; perhaps a similar mechanism operates here.

In the second example, hepatic cells (either *in vivo* or *in vitro*) were induced to transdifferentiate by overexpression of a superactive form of a known pancreatic transcription factor, Pdx1. Pdx1 is expressed early in the endoderm prior to overt morphological development of the pancreas, and has been shown to play a fundamental role in the development of the entire pancreas. Although a previous study showed that continuous overexpression of the unmodified Pdx1 in the liver increased hepatic insulin production, it is not known if this represents a true transdifferentiation or simply the activation of the insulin gene.

In the study using modified Pdx1, the transdifferentiation appears to be relatively complete in that both exocrine cells and endocrine cells were produced, including insulin, glucagon, and amylase-expressing cells. On its own, Pdx1 is unable to convert liver to pancreas and requires an extra activation domain, VP16. This might be because of the lack of appropriate protein partners or the presence of some inhibitory proteins in the liver cells, since sequence-specific transcription factors are known to require other tissue-specific coactivators to stimulate transcription. To overcome this problem, the VP16 activation domain was fused to Pdx1 – the VP16 activation being able to activate transcription directly by binding to various coactivators, as well as the basal transcription machinery, eliminating the need for other tissue-specific proteins. When Pdx1-VP16 was overexpressed in the liver, using the liver-specific promoter transthyretin, it was able to induce the transdifferentiation of liver to pancreas. This is an example in which a known transcription factor essential for pancreas development was engineered to act as a master switch gene.

In conclusion, the preceding results demonstrate that it is possible to transdifferentiate liver cells, whether fully differentiated or not, into pancreatic cells. Since the liver has the ability to regenerate, this tissue can provide an abundant resource for the production of pancreatic cells with the aim of curing diabetes. The first study shows that an extracellular factor, glucose, can be used; the second reveals that a modified intracellular tissue-specific transcription factor, Pdx1-VP16, is sufficient. These studies suggest that if we can identify the key factors involved in the physiological regulation of the adult pancreas, as well as in embryonic development, this will play an important role in understanding and promoting the transdifferentiation of various cell types into pancreas. However, overexpression of a single transcription factor may not be sufficient; the ability to change a cell's phenotype may therefore require the use of modified or engineered transcription factors (e.g., Pdx1-VP16) that can artificially recruit the transcriptional machinery.

9.3 BARRETT'S METAPLASIA

Barrett's metaplasia (or Barrett's esophagus, as it is sometimes called) is the clinical situation in which intestinal cells are found in the tissue of the lower end of the esophagus. In the strictest terms, it is the conversion of stratified squamous epithelium to columnar epithelium, and is characterized by the presence in biopsy material of acid mucin-containing goblet cells. The importance of Barrett's metaplasia stems from the rise in apparent incidence of the disease, and its risk associated with the development of adenocarcinoma of the esophagus. It is not known why metaplastic cells have a greater disposition toward neoplastic progression.

One of the proposed mechanisms for the development of Barrett's metaplasia is gastroesophageal reflux. It is assumed that prolonged acid reflux from the stomach (generally with bile acids) promotes damage to the epithelia at the end of the esophagus. Presumably, in the early stages of the disease, the normal stratified squamous epithelium is replaced. Eventually, re-epithelialization results in the formation of columnar, as opposed to stratified, squamous epithelium, most likely because of repeated exposure to an acid environment. It is not clear whether the different intestinal cell types formed in the esophagus arise from the same stem cell in the basal layer, or whether there is a transdifferentiation of columnar cells to goblet cells. It is also not understood why some patients with efflux disease do not go on to develop Barrett's metaplasia.

The molecular events underlying Barrett's metaplasia are not well understood. However, good candidate genes include the *caudal*-related homeobox genes *cdx1* and *cdx2*. Several lines of evidence support this statement. First, both genes are expressed in the intestine (but not in the stomach or in the esophagus) and are known to be important in regulating intestine-specific gene expression. Second, colonic epithelium is transformed to squamous epithelium (similar to the esophagus) in mice haploinsufficient for *cdx2*. Third, and more recently, it was found that ectopic expression of *cdx2* in the stomach can induce intestinal metaplasia, and there is some evidence for early expression of cdx2 in patients with Barrett's metaplasia. These results suggest that *cdx* genes may provide a target for therapeutic intervention in Barrett's metaplasia.

9.4 REGENERATION

The idea of regenerative medicine or tissue engineering mainly implies using tissue-specific stem cells to replace damaged or 'lost' organs. However, as more examples of the plasticity of differentiated cells become known, it may

be more feasible to use them than to use stem cells. Historically, the classical example of transdifferentiation occurs during lens regeneration in newts, where the dorsal iris-pigmented epithelium (IPE) is converted to lens. In other species, it may be a different source of cells that undergoes transdifferentiation to lens, such as the outer cornea in *Xenopus laevis*. In a similar fashion, regeneration of the neural retina occurs by transdifferentiation of the retinal-pigmented epithelium (RPE) in various vertebrates. In both cases, the regeneration has been shown to occur in adult newts, as well as in other vertebrate embryos, including chick, fish, and rat.

On removal of the lens (lentectomy) only the dorsal IPE transdifferentiates into lens, in three phases – dedifferentiation, proliferation, and transdifferentiation – even though both the dorsal and ventral IPE have the potential to become lens. It has been shown that the dorsal, and not the ventral, IPE expresses Pax6, Prox1, and FGFR-1, suggesting that these factors may play an important role in inducing the transdifferentiation. Indeed, inhibition of FGFR-1 will block the transdifferentiation of IPE to lens, and in *Xenopus* it has been shown that FGF-1 can induce the transdifferentiation of outer cornea to lens. In agreement with this, retinal regeneration in chick embryos can be promoted by the addition of either FGF-1 or FGF-2, but not other growth factors such as TGFβ. These results show that the microenvironment in which a cell resides plays a critical role in regulating transdifferentiation.

Why one cell changes its phenotype (dorsal IPE), but another does not (ventral IPE) in response to exogenous growth factors may depend on the competence of each cell (e.g., the presence of the appropriate receptors). It is possible that FGFs may be responsible for inducing dedifferentiation of the tissue, and that the expression of particular transcription factors induces both proliferation and transdifferentiation. This classical model of lens regeneration illustrates that the molecular signals involved in transdifferentiation can be identified and used to promote regeneration of cells normally considered unable to alter their phenotype.

9.5 BONE MARROW TO OTHER CELL TYPES

Some cell type conversions using bone marrow-derived stem cells have been shown to occur across what was previously considered to be germ-line boundaries (i.e., mesoderm to endoderm). In this situation, it is not evident whether the cell must first become a different stem cell and then differentiate along a different pathway or if it directly transdifferentiates to another phenotype. However, some doubt has been cast on these observations, and it has been suggested that the result is caused by an artifact from fusion of the circulating hematopoietic stem cells with resident cells.

9.6 DEDIFFERENTIATION AS A PREREQUISITE FOR TRANSDIFFERENTIATION

A question arises: If transdifferentiation is to occur, must the parent cell lose its phenotype before acquiring a new identity? In some examples (IPE to lens), there is an intermediate phenotype in which the cells do not express markers for either cell type (Figure 9.1B). However, examples of direct transdifferentiation do occur. Perhaps the best example is the transdifferentiation of pancreas to liver (pancreatic exocrine to hepatocyte) (Figure 9.1A). Whether a cell undergoes transdifferentiation directly, through a dedifferentiated state, or through a stem cell, may vary depending on which cell types are being studied (Figure 9.1). In other words, does the parent cell contain the necessary information to change its phenotype directly, or does it require the synthesis of new proteins? In direct transdifferentiation, the cell's competency is already established, and it is the removal of an inhibitor or the

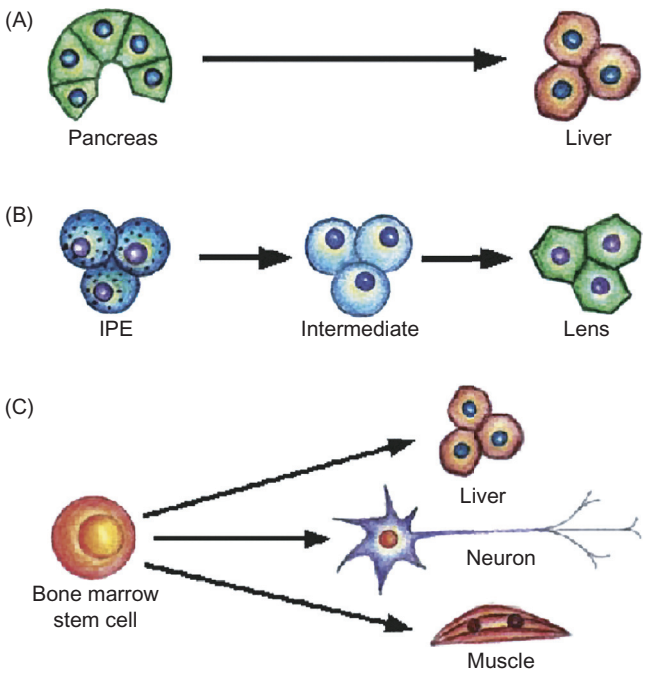

FIGURE 9.1 **Examples of transdifferentiation.**
Transdifferentiation can occur in different stages: (A) Transdifferentiation of pancreas to liver can occur without cell division or an intermediate phenotype. (B) Transdifferentiation of pigment epithelium to lens requires an intermediate stage in which the cell does not possess the characteristics of either phenotype. (C) The pluripotency of stem cells is shown by their ability to convert to different cell lineages. In this example, the switch is direct and there is no conversion to another tissue-specific stem cell.

addition of an activator that pushes the fate of the cell over the final hurdle. For dedifferentiation and stem cell intermediates, it may be necessary to establish the competency of the parent cell before it can undergo transdifferentiation. Further studies examining the transdifferentiation potential of individual transcription factors and various cell types will help to bring about an understanding of the rules of transdifferentiation.

9.7 HOW TO CHANGE A CELL'S PHENOTYPE EXPERIMENTALLY

The ability to change a cell's phenotype will greatly facilitate the design of therapies for diseases such as diabetes, liver failure, and neurodegenerative disorders (e.g., Parkinson's disease). We suggest six steps to follow to try to change a cell's phenotype experimentally.

1. Identify Potential Factors to Induce Transdifferentiation
   Transdifferentiation may be achieved in several ways using extracellular growth factors, individual transcription factors, or combinations of the two. An understanding of how individual organs or cell types form will help to identify those molecular factors that can be used to direct the transdifferentiation of other cell types. We believe that those factors essential for the initial development of an organ will work best, since they sit at the top of the hierarchy of the signaling cascade. Functional screens, such as those used previously to identify novel mesoderm-inducing factors, should aid the identification of new factors with the potential to direct transdifferentiation.
2. Choose a Cell Type to Convert
   In many cases of transdifferentiation, only certain cells can undergo a specific transdifferentiation, suggesting that there are restrictions on whether a cell has the competence to undergo transdifferentiation. Therefore, choosing the cell type initially is important. We suggest that using closely-related cell types will greatly improve the chances for transdifferentiation. Examples include the use of pancreatic AR42J cells in the conversion of pancreas to liver. Ultimately, either primary cultures of well-defined cell types or *in vivo* experiments will be necessary for this to be of therapeutic use.
3. Choose the Method of Overexpression
   It is important to determine whether continuous or limited overexpression of a particular factor is required. On the one hand, tissue-specific promoters will allow the factor to be expressed for only a relatively short time; upon transdifferentiation, the promoter will no longer be active. Ubiquitous promoters, on the other hand, will express the chosen factor continuously and may produce undesired results.

Many transcriptional regulators are expressed only transiently, and require a strict temporal regulation for proper development to occur. For example, continuous overexpression of *Hlxb9* in the normal pancreas interferes with the differentiation of both exocrine and endocrine cells. Thus, the use of the constitutive promoters, such as cytomegalovirus, may not be suitable. We believe that the use of tissue-specific promoters is best suited to the type of experiments described here, to prevent the chosen factor from interfering with the proper differentiation of the new phenotype.

4. Identify Whether a Modification of the Factor is Required
   It is possible that after identifying the factor, no transdifferentiation occurs. There may be several reasons for this, but we suggest testing a superactive version of the same factor before dismissing it. The easiest way to do this is to make use of a strong activation domain that has already been characterized, such as VP16. Whether it is fused to the N- or C-terminus should not matter, but we suggest that the VP16 be fused to the whole open reading frame of the transcription factor, and not just to the DNA-binding domain.
5. Characterize the New Phenotype
   Initially, use of a reporter construct will greatly help the identification of a successful conversion – for example, the use of the elastase promoter driving GFP in the conversion of liver to pancreas. It is best initially to use a promoter expressed in all cell types in a particular organ rather than one specific to an individual cell type within that organ. Three questions can be addressed under this heading. First, is the transdifferentiation specific to producing a single cell type or, if the organ contains numerous cell types, are several types produced? In the case of the transdifferentiation of liver to pancreas, ectopic expression of Pdx1–VP16 produces more than one type of pancreatic cell. Second, what is the identity of the new cell type, and can the loss of the other phenotype be demonstrated? Third, is the transdifferentiation stable? For a cell type conversion to be a true transdifferentiation, the phenotype of the new cell must be stable.
6. Test the Transdifferentiation Activity in Other Cell Types
   As mentioned previously, in many instances only a subset of cells can undergo a particular transdifferentiation. Therefore, it is essential to identify which cells can be transdifferentiated, and why one cell is able to respond but another is not. An understanding of the competence of individual cells will lead to a greater understanding of what is necessary for transdifferentiation to occur – for example, is dedifferentiation a necessary prerequisite? The question of whether it will be possible to direct the transdifferentiation of cells into one particular cell type, such as an insulin-secreting β-cell, remains to be seen.

9.8 SUMMARY

The recent demonstration that adult stem cells, and even differentiated cells, are more versatile than previously thought means that abundant tissue can be obtained from these cell types for therapeutic purposes. Since some scientists and members of the public are averse to the use of embryonic stem cells for any form of clinical treatment, using either adult stem cells or differentiated cells induced to transdifferentiate may obviate the use of embryonic tissue. In the end, the ethical issues raised by some may not apply to transdifferentiated cells.

ACKNOWLEDGMENTS

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FOR FURTHER STUDY

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CHAPTER 10

Differentiation in Early Development

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10.1 PREIMPLANTATION DEVELOPMENT

In mammals, fertilization occurs in the oviduct where sperm encounters and fuses with the oocyte. As a result, the oocyte nucleus, which had been arrested in metaphase II, completes meiosis, and the two parental pronuclei fuse to form the diploid zygotic nucleus (Figure 10.1A). Progressive DNA demethylation of first the paternal and then the maternal genomes (excluding the genomic imprints) begins after fertilization as part of the epigenetic reprogramming that takes place during preimplantation development. Transcription of the embryonic genome starts at the two-cell stage in mice and at the four- to eight-cell stage in humans. Until then, the embryo relies solely on maternal mRNA but after activation of the embryonic genome, maternal transcripts are rapidly degraded, although maternally encoded proteins may still be present and functionally important. The embryo continues cleavage divisions without visible growth (Figure 10.1) as it travels inside a protective glycoprotein coat, the zona pellucida, through the oviduct into the uterus.

10.2 CELL POLARIZATION OCCURS DURING COMPACTION

At the eight-cell stage in mice and the eight to 16-cell stage in humans, the embryo undergoes a process known as compaction to become a morula; a compact smooth spherical structure (Figure 10.1D). All blastomeres flatten, maximize their contacts, and become polarized. Their cytoplasm forms two distinct zones: the apical domain accumulates endosomes, microtubules, and microfilaments, whereas the nucleus moves to the basal domain. Furthermore,

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gap junctions form basally, ensuring communication between blastomeres and numerous microvilli and tight junctions are formed apically.

The next cleavage plane of some of the blastomeres is perpendicular to their axis of polarity, resulting in two cells of different phenotypes (asymmetric division). One daughter cell is located inside the embryo ('inner' cell), is small and apolar, and contains only basolateral elements. The other daughter cell is located at the surface of the embryo ('outer' cell), is larger and polar, and contains the entire apical domain of the progenitor cell and some basolateral elements. These polar cells inherit the region containing the tight junctions, thereby creating a physical barrier between the inner apolar cells and the maternal environment.

10.2.1 Blastocyst Formation (Cavitation)

After compaction, the presumptive trophectoderm (TE) cells form the outer layer of the embryo. Intercellular contacts strengthen between these cells, and a true epithelium is formed. This thin single cell layer develops a continuum of junctional complexes, including gap junctions, desmosomes, and tight junctions. Furthermore, the composition of the basal and apical cell membranes of the TE cells becomes more distinct, with Na^+/K^+-ATPases accumulating in the basal membrane. These ion pumps actively transport sodium ions into the embryo, which leads to accumulation of water molecules, possibly via aquaporins. A fluid-filled cavity, the blastocoelic cavity, is thus created on one side of the embryo in a process known as cavitation (Figure 10.1E). The presumptive inner cell mass (ICM) cells stay closely associated during this process, not only because of gap junctions, tight junctions, and interdigitating microvilli between the cells, but also because processes from TE cells fix the ICM to one pole of the embryo and partially isolate it from the blastocoel. The intercellular permeability seal of the TE cells prevents fluid loss, and as a consequence the blastocoelic cavity gradually expands to occupy most of the blastocyst between the 64-cell and 128-cell stages (Figures 10.1E to G). At this point, the embryo is not radially symmetrical around the embryonic-abembryonic axis, but is bilaterally symmetrical because the zona pellucida is slightly oval.

The outer TE layer and the ICM are composed of descendants of the 'outer' and 'inner' cell population of the morula, respectively. The TE in turn consists of two subpopulations: the polar TE that contacts the ICM and the mural TE that surrounds the blastocoelic cavity. The TE descendants give rise to extra-embryonic structures such as the placenta and do not contribute to the embryo proper. The cells of the ICM also consist of two subpopulations mixed initially distributed in a salt-and-pepper fashion. However, due to adherence differences, one of the subpopulations (the GATA6-positive cells)

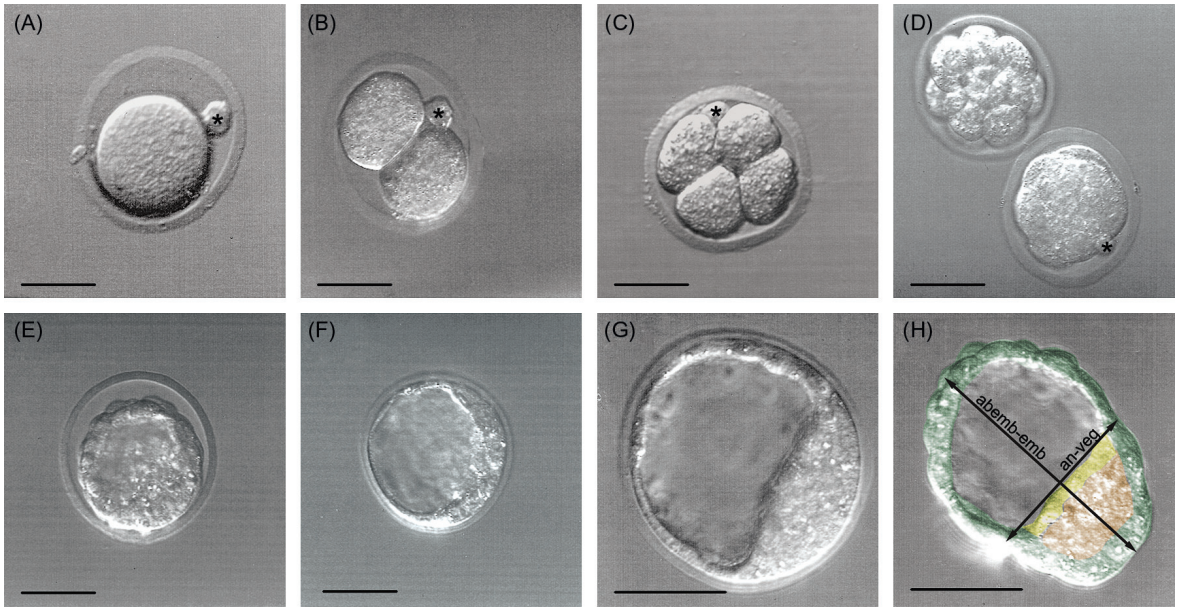

FIGURE 10.1 **Mouse preimplantation development.**
After fertilization the two parental pronuclei fuse to form the zygote (A). The embryo cleaves, forming a two-cell (B), four-cell (C), and eight-cell embryo. The embryo then undergoes compaction to become a smooth spherical structure, the morula (D). Note that the second polar body remains attached to the embryo (*). The blastocoelic cavity then develops on one side of the embryo to form an early blastocyst (E). The cavity enlarges, occupying most of the expanded blastocyst (F, G). Around embryonic day (E) 4.5, the late blastocyst reaches the uterus, 'hatches' from the zona pellucida, and is ready to implant (H). The late blastocyst consists of three cell subpopulations: the trophectoderm (green), the inner cell mass (orange), and the primitive endoderm (yellow). In the blastocyst three axes can be defined: the embryonic–abembryonic (abemb–emb), the animal–vegetal (an–veg), and a third axis on the same plane but perpendicular to the an–veg axis. *(Photomicrographs courtesy of B. Roelen.)*

segregates to the surface of the ICM where it contacts the blastocoelic cavity and differentiates into primitive endoderm, also an extra-embryonic tissue. The ICM gives rise not only to the embryo proper, but also to extra-embryonic mesoderm which contributes cells to the visceral yolk sac, amnion, chorion and the allantois, a structure that will later develop into the umbilical cord. An overview of cell lineage relationships in the early mouse embryo is shown in Figure 10.2. During preimplantation development (three to four days in mice, five to seven days in humans), the embryo has remained inside the zona pellucida, which prevents its premature implantation while still in the oviduct. Reaching the uterus, the blastocyst 'hatches' from the zona pellucida using the enzyme strypsin and is then ready to implant in the uterine wall (Figure 10.1H). Stages of mouse and human preimplantation development are summarized in Table 10.1.

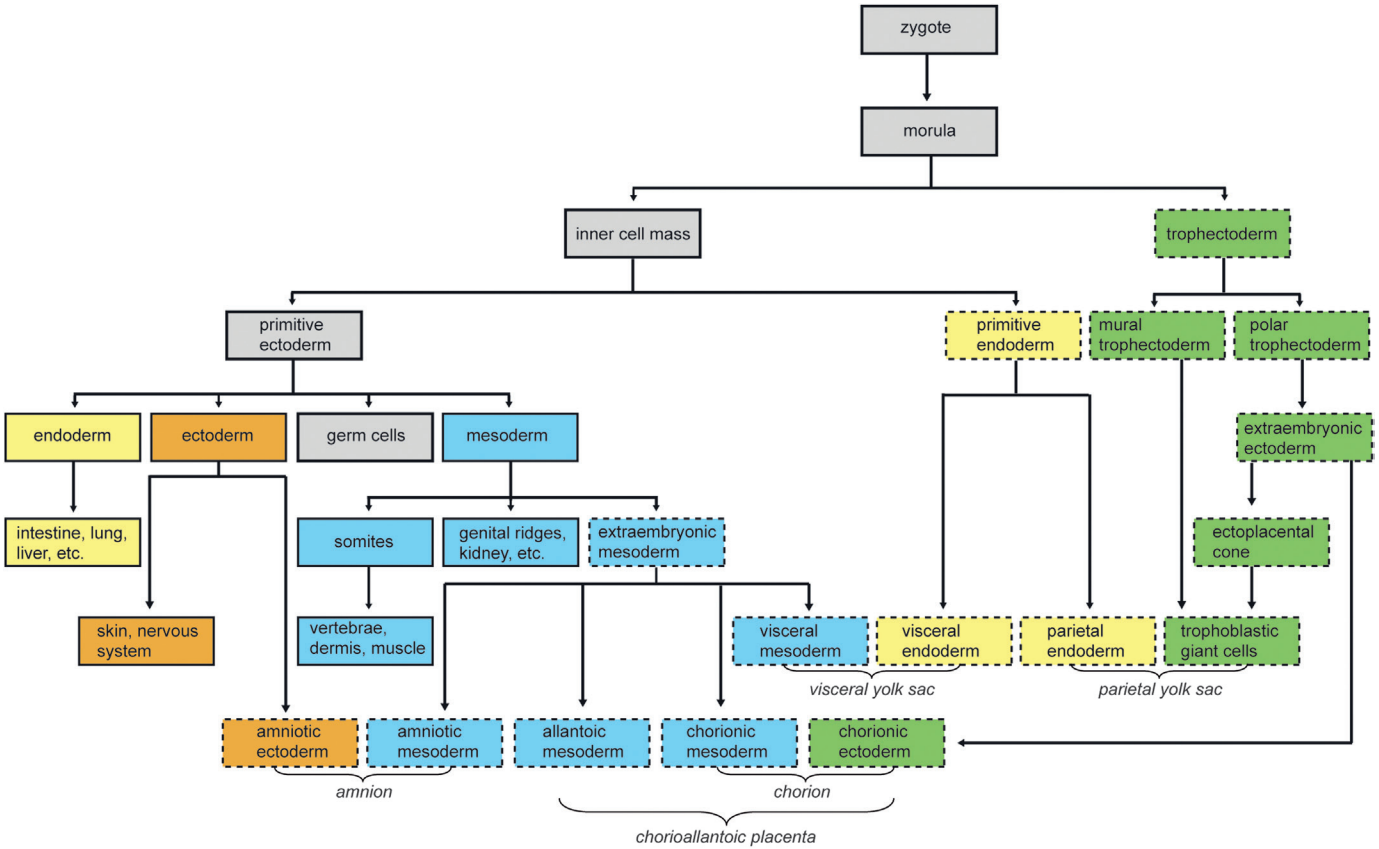

FIGURE 10.2 **Cell lineages in mouse development.**

Trophectoderm-derived tissues are depicted in green, endoderm-derived tissues in yellow, ectoderm-derived tissues in orange, and mesoderm-derived in blue. The cell/tissues represented in gray are regarded as pluripotent. All extra-embryonic tissues are enclosed by hatched lines, whereas embryonic tissues are enclosed by solid lines.

Table 10.1 Summary of Mouse and Human Preimplantation Development

Stage (M)	Time (M)	Stage (H)	Time (H)	Developmental Processes
Zygote	0–20 h	Zygote-2-cell	0–60 h	Axis determination?
2-cell	20–38 h	4–8-cell	60–72 h	Activation of embryo genome
4-cell	38–50 h			Lineage determination?
8-cell	50–62 h	8–16-cell	~3.5 d	Compaction
16-cell	62–74 h		~4.0 d	Two phenotypically different cells emerge
32-cell	~3.0 d	32-cell	~4.5 d	Blastocoelic cavity forms (cavitation)
64-cell	~3.5 d		~5.5 d	Blastocyst consist of two cell populations (ICM and TE)
128–256-cell	~4.5 d	166–286-cell	~6.0 d	Part of ICM differentiates to PrE; hatching, followed by implantation

Adapted from Nagy, A., Gertsenstein, M., Vintersten, K., Behringer, R. (2003) Manipulating the Mouse Embryo. New York: Cold Spring Harbor Laboratory Press and from Larsen, W.J. (1997) Human Embryology. New York: Churchill Livingstone. d, days; h, hours; H, human; ICM, inner cell mass; M, mouse; PrE, primitive endoderm; TE, trophectoderm.
During mouse and human development, the timing of each cleavage division is dependent on environmental factors (in vitro versus in vivo), individual variation, and mouse strain. The cleavage times presented here are ranges from several published sources.

10.3 AXIS SPECIFICATION DURING PREIMPLANTATION IN THE MOUSE

In lower vertebrates, the body axes are already specified (determined) in the undivided egg or very soon thereafter, whereas in mammalian embryos, axis specification was thought to be completed only at gastrulation, with the appearance of the primitive streak. However, the first morphological sign of the axis determination (anterior–posterior) is now considered to be the migration of the slightly cuboidal visceral endoderm at the distal tip of the embryo towards the more anterior part, forming the anterior visceral endoderm (AVE) at embryonic day (E) 5.5–6.0. Data suggest that the anterior–posterior axis of the embryo is molecularly determined even earlier; at E4.0–4.5, by asymmetric expression of *Lefty1* on one side of the primitive endoderm which will eventually correspond to the 'tilt' (see the section on implantation). This view of relatively late axis determination is supported by the observation that the mammalian embryo is extremely plastic and able to ignore disturbances such as the removal or reaggregation of blastomeres. The prevailing concept, therefore, became one of no embryonic prepatterning before implantation.

However, several studies challenge this view, suggesting that the mammalian zygote may in fact be polarized and that the body axes could somehow be specified at the time of fertilization in a fashion similar to that in lower vertebrates. In the mouse zygote, the position of the animal pole, marked by the second polar body, or the sperm entry point, which triggers Ca^{2+} waves or the plane defined the two pronuclei in the zygote, have all been discussed as defining the

plane of first cleavage. However, it is still unclear whether the position of these cues is directly responsible for the zygote polarity and the subsequent position of the first cleavage plane. Alternatively, zygote polarity, the position of the second polar body and the sperm entry point (and of the paternal pronucleus) might be determined by an intrinsic asymmetry already present in the oocyte. The oocyte has been shown, for example, to have an asymmetric distribution of mitochondria and other factors including Leptin and Stat3.

The first cleavage plane coincides with the embryonic–abembryonic boundary of the blastocyst. However, it is still unsettled whether the fates of the two blastomeres are distinguishable and can be anticipated. The blastomere containing the sperm entry site generally divides first and contributes preferentially to the embryonic region of the blastocyst, whereas its sister cell preferentially forms the abembryonic region. Parthenogenetic eggs that do not contain a sperm entry point are able to divide and develop into blastocysts, although there is no tendency for the two blastomeres to follow different fates. This indicates that, although during normal development the site of sperm penetration correlates with the later spatial arrangement of the blastocyst, it is not essential for patterning the embryo. Moreover, several studies have claimed that the two blastomeres are similar but it is the ellipsoidal shape of the zona pellucida that defines the embryonic–abembryonic axis. The topographic relationship between the zygote and the blastocyst axes and between the blastocyst and body axes of the future fetus remains a matter of debate, although the prevalent view is that of existing cues, but those could be easily overruled.

10.4 DEVELOPMENTAL POTENCY OF THE EARLY MOUSE EMBRYO

In the mouse, both blastomeres of a two-cell stage embryo transplanted separately into foster mothers develop into identical mice. Isolated blastomeres were combined with genetically distinguishable blastomeres of the same age to engineer chimeric composites and assess the developmental potential of each blastomere of four- and eight-cell mouse embryos. Each blastomere was shown to contribute extensively to both embryonic and extra-embryonic tissues (TE and visceral yolk sac) and to generate viable and fertile mice. These results indicate that at these developmental stages all blastomeres are still totipotent. However, single isolated four- and eight-cell stage blastomeres were able to develop only to blastocysts and implant but they were incapable of generating viable concepti. This may be explained by the fact that a defined number of cell divisions (five) occurs before blastocyst formation. Thus, in contrast to the normal 32-cell blastocyst, isolated blastomeres from four- and eight-cell embryos resulted in 16-cell and eight-cell blastocysts, respectively. Blastocysts generated from isolated four- and eight-cell blastomeres contain

progressively fewer cells in the ICM, making it likely that a minimum number of ICM cells is necessary for survival beyond the blastocyst stage.

It is the position of a cell in the blastocyst that determines its fate: cells at the surface of the embryo become TE, whereas cells enclosed in the embryo become ICM. Recent studies have revealed that molecular heterogeneity already detectable at the four-cell stage could direct a developmental bias towards TE or ICM, perhaps by dictating the preferential axis of division (giving rise to two 'outer' cells by symmetric division or one 'inner' and one 'outer' cell by asymmetric division).

Although different phenotypically, the two-cell subpopulations in the 16-cell morula are still plastic and able to produce cells of the other lineage provided they are at the correct position in the embryo, that is, inside or at its surface. Cells of the ICM of 32-cell and 64-cell embryos are also still capable of contributing to all tissues of the conceptus (embryonic and extra-embryonic) and are thus still totipotent. The potency of TE cells has been difficult to determine because TE cells are not easy to isolate (tightly connected with each other) and because they are not readily integrated inside the embryo (low adhesiveness). After the 64-cell stage, the ICM loses totipotency.

Once the mouse embryo has implanted (up to E7.0), the embryonic cells (including the primordial germ cells formed slightly later in development) lose their ability to contribute to the embryo when introduced directly into a host blastocyst to give rise to a chimeric embryo or *chimera*. In agreement, stem cells isolated from E5.5 and E6.5 embryonic (or epiblast) cells, the so-called EpiSCs, are also unable to form chimeras. Remarkably, when introduced directly into genetically identical adult mice, epiblast cells are able to generate teratocarcinomas, tumors containing a spectrum of differentiated tissues derived from the three germ layers (endoderm, mesoderm, and ectoderm) and a stem cell population called embryonal carcinoma (EC) cells. These epiblast-derived EC cells are also able to form mouse chimeras (but unable to go germ-line) when introduced into blastocysts, suggesting that, although pluripotency is lost in the epiblast, it can be regained to a certain extent. Similarly, primordial germ cells isolated from E8.5 mouse embryos cultured to become embryonic germ (EG) cells and also adult hematopoietic and neural stem cells are able to regain pluripotency and can contribute to the embryo when introduced into blastocysts.

10.5 GENES IMPORTANT DURING PREIMPLANTATION MOUSE DEVELOPMENT

Before implantation, the embryo is relatively self-sufficient and can, for example, develop *in vitro* in simple culture media without growth factors supplements.

Of particular importance during preimplantation development are genes that regulate activation of the embryonic genome, genome DNA demethylation and chromatin remodeling, the cell cycle, compaction, cavitation, and hatching. However, only relatively few mutations (specific gene deletions, insertions, and more extensive genetic abnormalities) have been reported to result in preimplantation lethality (Table 10.2). The reasons for this are not clear, but one

Table 10.2 Lethal Mouse Mutations Affecting Differentiation During Early Development

Gene/Locus	Mutant Phenotype
Wt1 (Wilms' tumor 1)	Zygotes fail to undergo mitosis
Faf1	2-cell stage arrest
Tgfb1	2- to 4-cell arrest
C^{25H} *(pid)*	2- to 6-cell stage embryos fail to undergo mitosis
L2dtl	4- to 8-cell stage arrest
Geminin	4- to 8-cell stage arrest
E-cadherin (uvomorulin, Cdh1)	Defects in compaction
Trb (Traube)	Defects in compaction
Cdk8	Defects in compaction
Mdn (morula decompaction)	Defects in compaction
Om (ovum mutant)	Failure to form blastocysts
SRp20	Failure to form blastocysts
Rbm19	Failure to form blastocysts
Wdr36	Failure to form blastocysts
t^{12}, t^{W32}	Failure to form blastocysts
T^{hp} *(hairpin)*	Failure to form blastocysts
Ts (Tail short)	Failure to form blastocysts
a-E-catenin	Failure to form blastocysts (TE defect)
SNEV (Prp 19, Pso4, NMP200)	Failure to form blastocysts
Emi1	Failure to form blastocysts
Vav	Blastocysts fail to hatch
Os (oligosyndactyly)	Mitaphase arrest st the early blastocyst
Brg1	Abnormal blastocyst development
A^{x} *(lethal nonagouti)*	Abnormal blastocyst development
l(5)-1	Abnormal blastocyst development
$t^{wPa\text{-}1}$	Abnormal blastocyst development
PL16	Abnormal blastocyst development
CpG binding protein (CGBP)	Abnormal blastocyst development
Mbd3	Abnormal blastocyst development
Thioredoxin (Txn)	Abnormal blastocyst development
Gpt	Abnormal blastocyst development

(*Continued*)

Table 10.2 (Continued)

Gene/Locus	Mutant Phenotype
Ltbp2	Abnormal blastocyst development
Wdr74	Abnormal blastocyst development
Hba$^{th\text{-}j}$	Decreased TE cell number
A^{y} (lethal yellow)	Defects in TE formation
Evx1	Defects in TE formation
Eomes (Eomesodermin)	Defects in TE formation
Cdx2	Defects in TE formation
Tead4	Defects in TE formation
Arp3	Defects in TE formation
Egfr	Defects in ICM formation
b1 integrin	Defects in ICM formation
Lamc1	Defects in ICM formation
B-myb	Defects in ICM formation
Fgf4	Defects in ICM formation
Fgfr2	Defects in ICM formation
Taube Nuss (Tbn)	Defects in ICM formation
Oct4 (Oct3,Pou5f1)	Defects in ICM formation
Nanog	Defects in ICM formation
Eset	Defects in ICM formation
Ronin	Defects in ICM formation
Sall4	Defects in ICM formation
Grb4	Defects in ICM formation

The table is divided in two sections. The top includes genes and loci that, when deleted, cause embryonic lethality before implantation. The lower section incudes genes and loci that, when deleted, causes embryonic lethality during implantation but before the formation of the egg cylinder. Embryos deficient in most of these genes develop to normal blastocysts and are able to hatch and implant, but the whole embryo or selectively the TE or ICM (ICM- or primitive endoderm-derived cells) degenerates soon thereafter, leading to resorption. ICM, inner cell mass; TE, trophectoderm.

may be that the initial presence of maternal transcripts in the zygote effectively results in maternal rescue. Ablation of specific maternal transcripts in the zygote is not always feasible using conventional knockout techniques because deficiency in candidate genes often results in lethality before adulthood. However, a growing number of maternal-effect genes involved in preimplantation development are being identified (see Table 10.3). Interestingly, most genes transcribed during preimplantation development are detected immediately after embryonic genome activation and continue to be transcribed, resulting in mRNA accumulation. Therefore, to trigger the different specific developmental events during preimplantation development, post-transcriptional regulation may play an important role.

Table 10.3 Maternal Effect Mutations Affecting Preimplantation Embryonic Development

Gene/Locus	Mutant Phenotype
Zar1	Zygote arrest
Dicer	Zygote arrest
Brwd1	Zygote arrest
Hsf1	Zygote to 2-cell stage arrest
Ago2	Zygote to 2-cell stage arrest
Npm2	Zygote to 2-cell stage arrest
Mater (Nalp5)	2-cell stage arrest
Hr6a (Ube2a)	2-cell stage arrest
E-cadherin (uvomorulin,Cdh1)	2-cell stage arrest
Padi6	2-cell stage arrest
Floped (Ooep)	2-cell stage arrest
Basonuclin (Bnc1)	2-cell stage arrest
Pdk1 (Pdpk1,Pkb kinase)	2-cell stage arrest
Zfp3612	2-cell stage arrest
Importin a7	2-cell stage arrest
Brg1	2- to 4-cell stage arrest
Tcl1	4- to 8-cell stage arrest
Atg5	4- to 8-cell stage arrest
Dppa3 (Stella, PGC7)	Failure to form blastocysts
Uchl1	Defects in compaction
CTCF	Failure to form blastocysts

The table lists a growing number of maternal effect genes. Embryos lacking these genes develop normally at least until implantation/gastrulation in heterozygous, but not in homozygous, mothers. If the gene of interest is homozygous lethal, the deletion of the maternal pool of transcript is achieved by the conditional deletion of the gene by crossing mice carrying a 'floxed' allel (fl/fl or fl-) with transgenic mice expressing a zone pellucida 3 (ZP3) promoter mediated CRE recombinase [de Vries et al. (2000) Genes is 26, 110], which deletes the gene specifically in growing oocytes.

ES cells are derived from the ICM; therefore, it is not surprising that ES and ICM cells express common genes. Some of these genes have been described as being necessary for maintaining the undifferentiated phenotype of ES cells and could be expected to play important roles in the segregation of the pluripotent ICM from the differentiated TE cell population. However, when deleted in the mouse, most of those genes appear to be crucial during implantation or gastrulation but not during the preimplantation period when both ICM and TE are formed. Most pertinent in this respect are the genes for leukemia inhibitory factor (LIF) and LIF receptors. Although mouse ES cells are highly dependent on LIF for maintenance of pluripotency in culture, deletion of neither receptor nor ligand genes appears to affect the pluripotency of the

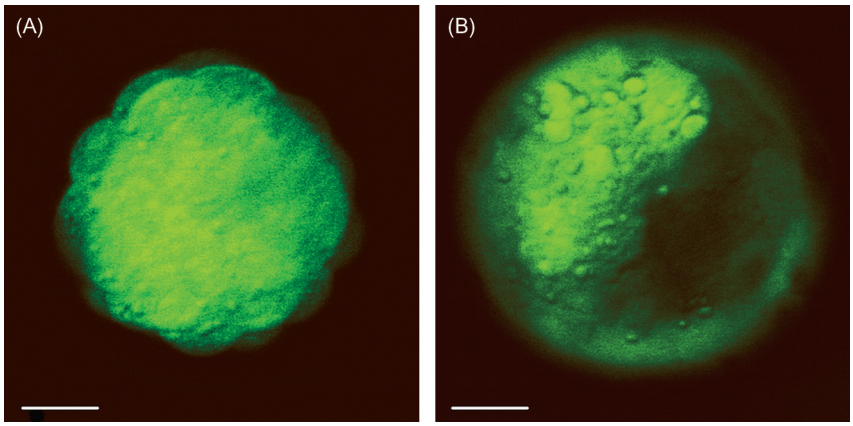

FIGURE 10.3 **Oct4 expression at morula and blastocyst stages.**
GFP expression driven by distal elements of the Oct4 promoter was used here to mimic endogenous Oct4 expression. In the morula, all blastomeres express high levels of Oct4 (A). In the early blastocyst, the inner cell mass expresses high levels of Oct4, whereas weaker expression is observed in trophectoderm cells (B).

ICM at the blastocyst stage. Interestingly, *in vivo* LIF signaling appears important for regulation of implantation (see the section on implantation).

The POU transcription factor Octamer-binding transcription factor 4 (Oct4) has the best-characterized involvement in regulating potency in mammals. Oct4 is initially expressed by all blastomeres but expression becomes restricted to the ICM as the blastocyst forms (Figure 10.3). Thereafter, a transient upregulation of Oct4 occurs in the ICM cells that differentiate to primitive endoderm. Interestingly, expression levels of Oct4 in mouse ES cells also regulate early differentiation choices, mimicking events in the blastocyst: mouse ES cells lacking *Oct4* differentiate to TE, whereas a twofold increase in Oct4 expression leads to endoderm and mesoderm formation. Mouse embryos deficient in *Oct4* are unable to form mature ICM and die around the time of implantation. Other genes described as being involved in cell fate determination during preimplantation development include *Taube nuss*, *B-myb*, *Nanog*, *Cdx2*, and *Eomes* (see Table 10.2). Both *Taube nuss* and *B-myb* homozygous-deficient mice develop to normal blastocysts. At the time of implantation, however, *Taube nuss* −/− ICM cells undergo massive apoptosis and the embryo becomes a ball of trophoblast cells; in *B-myb* knockout mice, the ICM also degenerates although the reason for this is unclear. *Taube nuss* and *B-myb* seem to be necessary for ICM survival, whereas *Oct4* is required for establishment and maintenance of the ICM identity but not cell survival. *Nanog* is expressed exclusively in the ICM and, whereas *Oct4* prevents TE differentiation, *Nanog* prevents differentiation of ICM to primitive endoderm. In agreement, *Nanog* −/− blastocysts are formed

but derivative ICM in culture differentiates into endoderm. In contrast, *Cdx2* and *Eomes* are involved in trophoblast development; embryos lacking these genes die soon after implantation because of defects in the trophoblast lineage.

10.6 FROM IMPLANTATION TO GASTRULATION

The mechanisms used by the mammalian embryo to implant are species dependent, contrasting with the general developmental steps during the preimplantation period. In addition, an intimate and highly regulated cross-talk between mother and embryo makes implantation in mammals a complex process.

Upon reaching the uterus, the blastocyst hatches from the zona pellucida and the TE cells become adhesive, expressing integrins that enable the embryo to bind the extracellular matrix (ECM) of the uterine wall. The mouse embryo adheres to the uterine wall via the mural TE cells of the abembryonic region and is slightly tilted. In contrast, human embryos bind through the embryonic region. Once attached to the uterus, trophoblast cells secrete enzymes that digest the ECM, allowing them to infiltrate and start uterine invasion. At the same time, the uterine tissues surrounding the embryo undergo a series of changes collectively known as the decidual response. These include the formation of a spongy structure known as deciduum in mice (decidua in humans), vascular changes leading to the recruitment of inflammatory and endothelial cells to the implantation site, and apoptosis of the uterine epithelium.

10.7 THE MOUSE TROPHECTODERM AND PRIMITIVE ENDODERM CELLS

Apoptosis occurring in the uterine wall gives TE cells the opportunity to invade the deciduum by phagocytosing dead epithelial cells. At about E5.0, the mural TE cells cease division but continue endoreduplicating their DNA to become primary trophoblastic giant cells. This cell population is joined by polar TE cells that migrate around the embryo and similarly become polytene (secondary trophoblastic giant cells). However, other polar TE cells continue dividing and remain diploid, giving rise to the ectoplacental cone and the extra-embryonic ectoderm that pushes the ICM into the blastocoelic cavity (Figure 10.4). These proliferative TE cells when cultured in the presence of fibroblast growth factor 4 (FGF4) and heparin give rise to the so-called trophoblast stem (TS) cells, which are able to either self-renew or differentiate into trophoblastic giant cells.

During implantation, the primitive endoderm layer forms two subpopulations: the visceral endoderm (VE) and the parietal endoderm (PE), both of which are extra-embryonic tissues. The VE is a polarized epithelium closely associated with the extra-embryonic ectoderm and the ICM/epiblast

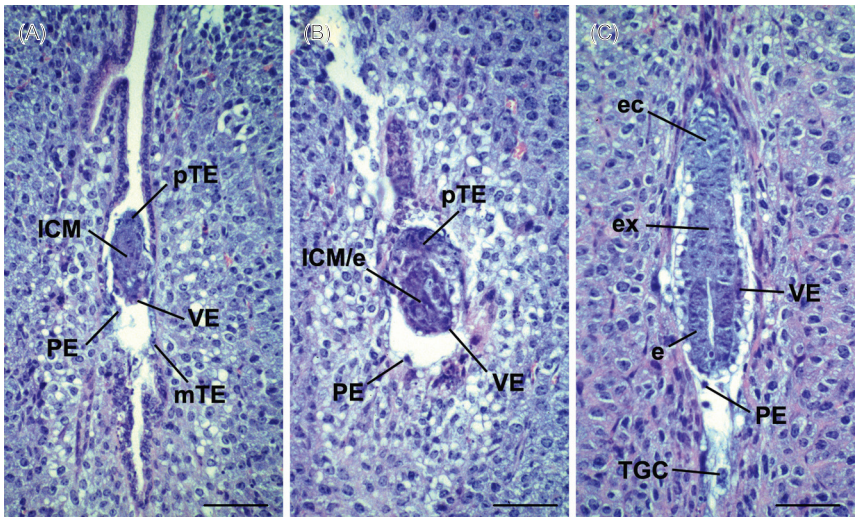

FIGURE 10.4 **Tissue formation and movements during and shortly after implantation of the mouse embryo (E5.0–E5.5).**

During implantation, cell division rate in the embryo increases, leading to rapid growth (A–C). The primitive endoderm cells segregate into visceral endoderm (VE) and parietal endoderm (PE). The polar trophectoderm cells (pTE) form the ectoplacental cone (ec) and the extra-embryonic ectoderm (ex). pTE cells together with mural trophectoderm cells (mTE) contribute to form the trophoblastic giant cells (TGC). The inner cell mass (ICM) cavitates and organizes into an epithelium known as the epiblast (e).

(Figures 10.4 and 10.5) which is heterogeneous in character (with prominent vacuolization in the extra-embryonic VE). When in culture, the primitive/visceral endoderm is also able to give rise to a self-renewing stem cell population of extra-embryonic endoderm (XEN) cells.

Later in development, the VE contributes to the formation of the visceral yolk sac but some VE cells may end up intercalated in the definitive gut. PE cells migrate largely as individual cells over the TE (Figures 10.4 and 10.5) and secrete large amounts of ECM to form a thick basement membrane known as Reichert's membrane. The PE cells, together with the trophoblastic giant cells and Reichert's membrane, form the parietal yolk sac.

10.8 DEVELOPMENT OF THE MOUSE INNER CELL MASS TO THE EPIBLAST

The ICM located between the TE-derived extra-embryonic ectoderm and the primitive endoderm-derived VE gives rise to all cells of the embryo proper. During implantation, the ICM organizes into a pseudostratified columnar epithelium (also referred to as primitive or embryonic ectoderm, epiblast, or egg

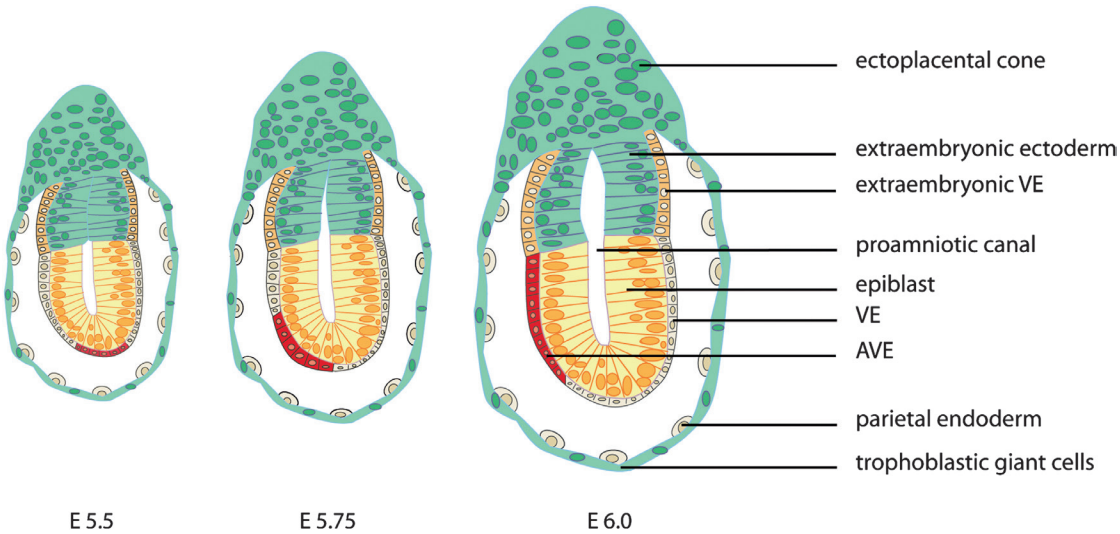

FIGURE 10.5 **Tissue formation and movements in the pregastrulation mouse embryo (E5.5–E6.0).**
During this period, the extra-embryonic ectoderm organizes into an epithelium. The proamniotic cavity initially restricted to the epiblast now expands into the extra-embryonic ectoderm, forming the proamniotic canal. At E5.5, the most distal visceral endoderm cells (red) express a different set of markers then the surrounding visceral endoderm (VE). These (or other) distal VE cells move from the distal tip to surround the prospective anterior part of the epiblast and form the anterior visceral endoderm (AVE). The VE surrounding the extra-embryonic ectoderm consists of a columnar epithelium, whereas the VE cells surrounding the epiblast are more flattened.

cylinder) surrounding a central cavity, the proamniotic cavity (Figure 10.4). Signals from the VE (and perhaps also from the extra-embryonic ectoderm), including bone morphogenetic proteins (BMPs), are responsible for apoptosis in the core of the epiblast leading to its cavitation. Between E5.5 and E6.0, the proamniotic cavity expands to the extra-embryonic ectoderm, forming the proamniotic canal (Figure 10.5).

After implantation, a wave of *de novo* DNA methylation occurs, leading to epigenetic reprogramming (finished by E6.5). This affects the entire genome to a different extent in embryonic and extra-embryonic lineages and may be responsible for the observed loss of the ability to contribute to chimeras. After implantation, the rate of cell division increases, followed by rapid growth. At E4.5, the ICM consists of approximately 20 to 25 cells, at E5.5 the epiblast has about 120 cells, and at E6.5 it consists of 660 cells.

At E6.5, the embryo is not cylindrical but has already a long and a short axis. Gastrulation starts with the formation of a morphologically visible structure (the primitive streak) that marks the future posterior side of the embryo. Surprisingly, the primitive streak forms at one side of the short axis. However,

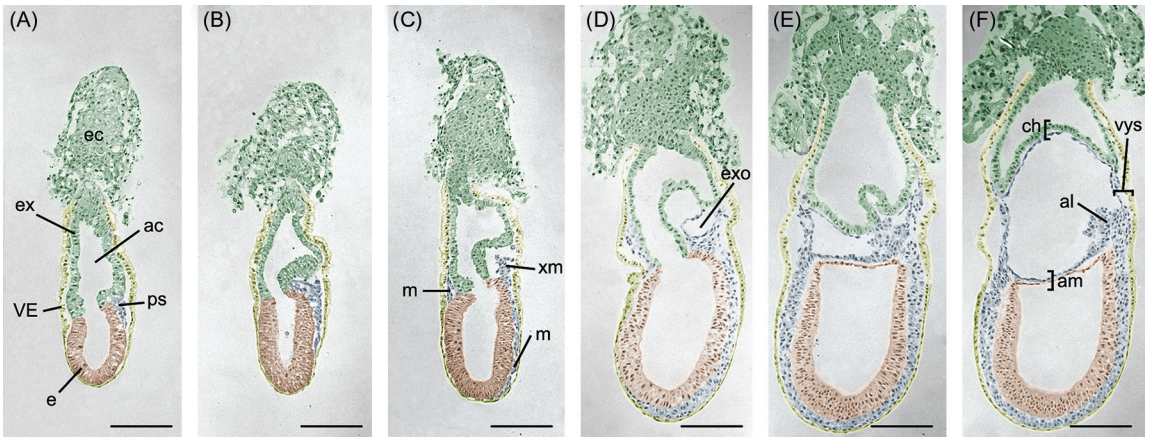

FIGURE 10.6 **Tissue formation and movements during the gastrulation of the mouse embryo (E6.5–E7.5).** Gastrulation begins with the formation of the primitive streak (ps) in the posterior side of the E6.5 embryo at the junction of the extra-embryonic ectoderm (ex) and epiblast (e) (A). As more cells ingress through the streak, it elongates toward the distal tip of the embryo, between epiblast and visceral endoderm (VE) (B). While the newly formed embryonic mesoderm (m) moves distally and laterally to surround the whole epiblast, the extra-embryonic mesoderm (xm) pushes the extra-embryonic ectoderm upwards and to the center (C, D). The extra-embryonic mesoderm develops lacunae, creating a mesoderm-lined cavity known as exocoelom (exo). The exocoelom enlarges and as a consequence, the tissue at the border of extra-embryonic and embryonic ectoderm fuses, dividing the proamniotic cavity (ac) in two and forming the amnion (am) and the chorion (ch) (E). The layer of extra-embryonic mesoderm and the visceral endoderm together form the visceral yolk sac (vys). At the posterior side of the embryo the allantois (al) and the primordial germ cells are formed (E, F). The tissues colored green (the extra-embryonic ectoderm and ectoplacental cone (ec)) are derived from the trophectoderm. The tissues in yellow are derived from the primitive endoderm and epiblast cells that passed through the streak, generating the definitive endoderm. The definitive endoderm cells intercalate with the visceral endoderm in the embryonic part of the embryo. The tissues colored orange are derived from the inner cell mass and remain ectoderm. The tissues colored blue are formed during gastrulation and represent primitive streak and mesoderm-derived tissues (excluding the primordial germ cells present at the basis of the allantois). For the lineages of early mouse development see Figure 10.2.

the embryo undergoes an apparent shift in orientation inside the deciduum and the primitive streak ends up at one side of the long axis. In addition, during gastrulation the three definitive germ layers are formed, the germ-line is set aside, and the extra-embryonic mesoderm that contributes to the visceral yolk sac, placenta, and umbilical cord is generated. An overview of tissue formation and movement during mouse gastrulation is shown in Figure 10.6.

10.9 THE HUMAN EMBRYO

Human development during implantation and gastrulation is significantly different from that of the mouse. Briefly, the human trophoblast cells invade the uterine tissue and form the syncytiotrophoblast, a syncytial tissue. The

trophoblast cells that contact the ICM and the blastocoelic cavity stay as single cells, remain diploid, and are known as cytotrophoblasts. These cells proliferate and can fuse with the syncytiotrophoblast or develop into the column cytotrophoblast or the (to some extent polyploid) extravillous cytotrophoblast. In humans, a structure equivalent to the mouse extra-embryonic ectoderm is not thought to form.

Human primitive endoderm cells, also known as hypoblast cells, segregate on the surface of the ICM and proliferate. Some of these cells migrate to line the blastocoelic cavity leading to the formation of the exocoelomic membrane (or Heuser's membrane). Analogous to the formation of the mouse Reichert's membrane, a spongy layer of acellular material known as the extra-embryonic reticulum is formed between the cytotrophoblast and the exocoelomic membrane. Thereafter, the extra-embryonic reticulum is invaded by extra-embryonic mesoderm. The origin of this tissue in humans is still uncertain (epiblast derived or hypoblast derived). The extra-embryonic mesoderm proliferates to line both Heuser's membrane (forming the primary yolk sac) and cytotrophoblast (forming the chorion). The extra-embryonic reticulum then breaks down and is replaced by a fluid-filled cavity, the chorionic cavity. The human primary yolk sac is thus not equivalent to the mouse parietal yolk sac, although both are transient structures. Moreover, it is still unclear whether human embryos develop a PE-like cell type.

A new wave of hypoblast proliferation generates cells that contribute to the formation of the definitive yolk sac. This new structure displaces the primary yolk sac, which buds off and breaks up into small vesicles that remain present in the abembryonic pole. The definitive yolk sac in humans is equivalent to the visceral yolk sac in the mouse.

The human ICM organizes into a pseudostratified columnar epithelium and cavitates, producing the amniotic cavity. The ICM cells that lie on the hypoblast are known as the epiblast and will give rise to the embryo proper. The ICM cells that contact the trophoblast form the amnion. The human embryo forms a bilaminar embryonic disc, similar the chick embryo and the patterns of cell movement during gastrulation are relatively conserved between chick and human.

With such diversity in extra-embryonic structures supporting the development of the ICM in mice and humans, it is not surprising that ES cells derived from mice and humans are not equivalent. They differ in developmental potency, for example, in their ability to differentiate to TE-like cells. Human ES cells can form TE in culture but under normal circumstances mouse ES cells do not. Furthermore, mouse ES cells in culture have been shown to develop into cells with some properties of mature germ cells (sperm- and oocyte-like cells). The potential of these cells to fertilize or to be fertilized and

generate viable mice is still unclear. It is not yet known whether human ES cells have the potential to form mature gamete-like cells in culture. Mouse and human ES cells also express different cell surface markers, have different requirements in culture for self-renewal and respond differently to growth and differentiation cues although their expression profile of core pluripotency genes is similar.

Recent literature has shown that mouse ES cells are a heterogeneous population in terms of expression of markers, containing cells that resemble either ICM or epiblast cells. Mouse ES cells that are most related to the epiblast cells resemble mouse EpiSCs. In contrast to ICM-like mouse ES cells, EpiSCs and human ES cells have similar characteristics. Current thought suggests that the unusual characteristics of mouse ES cells arise from the fact that the mouse uses a reproductive strategy known as facultative embryonic diapause. This means that the mouse blastocyst has the capacity to wait (temporarily arrested in development) in the uterus until the conditions for implantation become favorable (for example, the embryos wait until the mother stops lactating). The mouse ES cells would reflect this 'arrested' stage in culture. In humans and most other mammals, the blastocyst is not able to arrest its development and when in the uterus it either implants and develops or degenerates.

10.10 IMPLANTATION: MATERNAL VERSUS EMBRYONIC FACTORS

In mice, the presence of the blastocyst in the uterus is sufficient to trigger ovarian production of progesterone and estrogen. These two hormones are absolutely required for embryo survival because they prime the uterus for implantation and decidualization. The uterus starts producing LIF and members of the epidermal growth factor (EGF) family, including EGF, heparin-binding EGF, transforming growth factor-alpha (TGFalpha), and amphiregulin. Those molecules, together with HoxA10, induce the production of cyclo-oxygenase (COX) enzymes, the rate-limiting enzymes in the production of prostaglandins. These factors and corresponding receptors play crucial roles during the 'window of implantation' and when genetically deleted or mutated lead to female infertility due to a defective uterine response and embryonic lethality during or soon after implantation. The embryo, on the other hand, also produces important molecules including interleukin-1β, TGFα, and insulin growth factor (IGF) that act in autocrine and paracrine ways to stimulate embryo-uterine cross-talk leading to implantation.

The suppression of the maternal immune response is also essential during implantation but is still incompletely understood. TE cells, the only cell

population of the conceptus that physically contacts maternal cells, have developed several mechanisms to avoid rejection. Examples are the production of numerous factors and enzymes, including indoleamine 2,3-dioxygenase (IDO) by the TE cells that suppress the maternal immune system and the lack of polymorphic class I and II major histocompatibility complex (MHC) antigens in TE cells.

10.11 THE ROLE OF EXTRA-EMBRYONIC TISSUES IN PATTERNING THE MOUSE EMBRYO

Extra-embryonic tissues not only are necessary for nutrition and regulating implantation during development, but also play crucial roles in patterning the embryo before and during gastrulation. Unequivocal evidence for this comes from the analysis of chimeric embryos generated from blastocysts colonized with ES cells. In chimeras, ES cells preferentially colonize epiblast-derived tissues. It is, therefore, possible to generate embryos with extra-embryonic tissues of one genotype and epiblast-derived tissues of another genotype. For example, Nodal is expressed embryonically and extra-embryonically (depending on the developmental stage). Furthermore, *Nodal*-deficient embryos fail to gastrulate. It was, thus, initially difficult to distinguish embryonic from extra-embryonic functions. However, when *Nodal* −/− ES cells were introduced into wild-type blastocysts, the extra-embryonic tissues were wild-type, whereas epiblast-derived tissue lacked *Nodal*. The developing chimera was essentially normal until mid-gestation, suggesting that the presence of *Nodal* (exclusively) in the extra-embryonic tissues was sufficient to rescue embryonic patterning.

In contrast to the extensive mixing of epiblast cells, labeled primitive endoderm cells develop as more coherent clones, consistent with the function of the VE in embryo patterning. The primitive endoderm cells in the vicinity of the second polar body preferentially form VE cells surrounding the epiblast, whereas cells away from the second polar body preferentially form VE cells surrounding the extra-embryonic ectoderm.

At E5.5, the most distal VE (DVE) cells are characterized by the expression of the genes *Hex* and *Lefty1*. Until recently it was thought that this cell population migrated towards the prospective anterior side of the embryo during the next day of development, producing an endodermal stripe known as the AVE (Figure 10.5). However, recent data proposes that the migratory AVE may not directly descend from DVE cells, but constitute a newly formed population. The AVE is responsible for the production of innumerous secreted signaling molecules. Of particular interest is the production of antagonists of the

Nodal (Lefty1 and Cer1) and the Wnt (Dkk1) signaling pathways, which play important roles in the specification of anterior fate in the embryo. There is less known about specific gene products produced by the posterior part of the VE (PVE), but expression of Wnt3, Wnt2b, and BMP2 by the PVE is important for posterior embryonic patterning and development. Before gastrulation, the extra-embryonic ectoderm also signals to the proximal epiblast, inducing expression of several genes important for posterior proximal identity, in particular via BMP4 and BMP8b. By controlling the levels of activity of the Wnt and Nodal/BMP signaling pathways, the extra-embryonic tissues VE and extra-embryonic ectoderm determine both anterior and posterior fate in the embryo.

The same two signaling pathways will also play further roles in dorsal-ventral patterning and organogenesis. Both VE and VE-like cell lines secrete signals that are able to induce differentiation of mouse and human ES cells at least towards cardiomyocytes. Making use of the tissues or sequences of signal transduction pathways used by the embryo for its own patterning and differentiation seems to be the most efficient way to direct ES cell differentiation and therefore it is paramount to understand the events that take place early during embryonic development to define differentiation signals more precisely.

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CHAPTER 11

Stem Cells Derived from Amniotic Fluid

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11.1 AMNIOTIC FLUID – FUNCTION, ORIGIN, AND COMPOSITION

The amniotic fluid (AF) is the clear, watery liquid that surrounds the growing fetus within the amniotic cavity. It allows the fetus to freely grow and move inside the uterus, protects it from outside injuries by cushioning sudden blows or movements by maintaining consistent pressure and temperature, and acts as a vehicle for the exchange of body chemicals with the mother.

In humans, the AF starts to appear at the beginning of the second week of gestation as a small film of liquid between the cells of the epiblast. Between days 8 and 10 after fertilization, this fluid gradually expands and separates the epiblast (i.e., the future embryo) from the amnioblasts (i.e., the future amnion), thus forming the amniotic cavity. Thereafter, it progressively increases in volume, completely surrounding the embryo after the fourth week of pregnancy. Over the course of gestation, AF volume markedly changes from 20 mL in the seventh week to 600 mL in the 25th week, 1000 mL in the 34th week, and 800 mL at birth. During the first half of gestation, the AF results from active sodium and chloride transport across the amniotic membrane and the non-keratinized fetal skin, with concomitant passive movement of water. In the second half of gestation, the AF is constituted by fetal urine, gastrointestinal excretions, respiratory secretions, and substances exchanged through the sac membranes.

The AF is primarily composed of water and electrolytes (98–99%) but also contains chemical substances (e.g., glucose, lipids, proteins, hormones, and enzymes), suspended materials (e.g., vernix caseosa, lanugo hair, and meconium), and cells. AF cells derive both from extra-embryonic structures (i.e., placenta and fetal membranes) and from embryonic and fetal tissues.

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Although AF cells are known to express markers of all three germ layers, their exact origin still represents a matter of discussion; the consensus is that they mainly consist of cells shed in the amniotic cavity from the developing skin, respiratory apparatus, and urinary and gastrointestinal tracts. AF cells display a broad range of morphologies and behaviors, which vary with gestational age and fetal development. In normal conditions, the number of AF cells increases with advancing gestation; if a fetal disease is present, AF cell counts can be either dramatically reduced (e.g., intrauterine death, urogenital atresia) or abnormally elevated (e.g., anencephaly, spina bifida, exomphalos). Based on their morphological and growth characteristics, viable adherent cells from the AF are classified into three main groups: epithelioid (33.7%), amniotic fluid (60.8%), and fibroblastic type (5.5%). In the event of fetal abnormalities, other types of cells can be found in the AF, e.g., neural cells in the presence of neural tube defects and peritoneal cells in the case of abdominal wall malformations.

The majority of cells present in the AF are terminally differentiated and have limited proliferative capabilities. In the 1990s, however, two groups demonstrated the presence in the AF of small subsets of cells harboring a proliferation and differentiation potential. First, Torricelli reported the presence of hematopoietic progenitors in the AF collected before the 12th week of gestation. Then Streubel was able to differentiate AF cells into myocytes, thus suggesting the presence in the AF of non-hematopoietic precursors. These results initiated a new interest in the AF as an alternative source of cells for therapeutic applications.

11.2 AMNIOTIC FLUID MESENCHYMAL STEM CELLS

Mesenchymal stem cells (MSCs) represent a population of multipotent stem cells able to differentiate towards mesoderm-derived lineages (i.e., adipogenic, chondrogenic, myogenic, and osteogenic). Initially identified in adult bone marrow, where they represent 0.001–0.01% of total nucleated cells, MSCs have since been isolated from several adult (e.g., adipose tissue, skeletal muscle, liver, brain), fetal (i.e., bone marrow, liver, blood), and extraembryonic tissues (i.e., placenta, amnion).

The presence of a subpopulation of AF cells with mesenchymal features, able to proliferate *in vitro* more rapidly than comparable fetal and adult cells, was described for the first time in 2001. In 2003, In 't Anker demonstrated that the AF can be an abundant source of fetal cells that exhibit a phenotype and a multilineage differentiation potential similar to that of bone-marrow-derived MSCs; these cells were named AF mesenchymal stem cells (AFMSCs).

11.2.1 Isolation and Culture

AFMSCs can be easily obtained: in humans, from small volumes (2–5 mL) of second and third trimester AF, where their percentage is estimated to be 0.9–1.5% of the total AF cells, and in rodents, from the AF collected during the second or third week of pregnancy. Various protocols have been proposed for their isolation; all are based on the expansion of unselected populations of AF cells in serum-rich conditions without feeder layers, allowing cell selection by culture conditions. The success rate of the isolation of AFMSCs is reported by different authors to be 100%. AFMSCs grow in basic medium containing fetal bovine serum (20%) and fibroblast growth factor (5 ng/mL). Importantly, it has been very recently shown that human AFMSCs can be also cultured in the absence of animal serum without losing their properties; this finding is a fundamental prerequisite for the beginning of clinical trials in humans.

11.2.2 Characterization

The fetal versus maternal origin of AFMSCs has been investigated by different authors. Molecular histocompatibility locus antigen (HLA) typing and amplification of the sex determining region Y (*SRY*) gene in AF samples collected from male fetuses demonstrated the exclusively fetal derivation of these cells. However, whether AFMSCs originate from the fetus or from the fetal portion of extra-embryonic tissues is still a matter of debate.

AFMSCs display a uniform, spindle-shaped, fibroblast-like morphology similar to that of other MSC populations and expand rapidly in culture. Human cells derived from a single 2 mL AF sample can increase up to 180×10^6 cells within 4 weeks (three passages) and, as demonstrated by growth kinetics assays, possess a greater proliferative potential (average doubling time 25–38 hours) in comparison with that of bone-marrow-derived MSCs (average doubling time 30–90 hours). Moreover, AFMSCs' clonogenic potential has been proved to exceed that of MSCs isolated from bone marrow (86 ± 4.3 vs. 70 ± 5.1 colonies). Despite their high proliferation rate, AFMSCs retain a normal karyotype and do not display tumorigenic potential even after extensive expansion in culture.

Analysis of the AFMSC transcriptome demonstrated that:

1. AFMSCs' gene expression profile, as well as that of other MSC populations, remains stable between passages in culture, enduring cryopreservation and thawing well;
2. AFMSCs share with MSCs derived from other sources a core set of genes involved in extracellular matrix remodeling, cytoskeletal organization, chemokine regulation, plasmin activation, TGF-β and Wnt signaling pathways;

Table 11.1 Immunophenotype of Culture Expanded Second and Third Trimester Human AFMSC: Results by Different Groups

Makers	Antigen	CD no.	You et al., 2009	Roubelakis et al., 2007	Tsai et al., 2004	In 't Anker et al., 2003
Mesenchymal	SH2, SH3, SH4	CD73	+	+	+	+
	Thy1	CD90	+	+	+	+
	Endoglin	CD105	+	+	+	+
	SB10/ALCAM	CD166	nt	+	nt	+
Endothelial and hematopoietic	LCA	CD14	nt	–	nt	–
	gp105-120	CD34	nt	–	–	–
	LPS-R	CD45	–	–	–	–
	Prominin-1	CD133	nt	–	nt	nt
Integrins	β1-integrin	CD29	+	+	+	nt
	β3-integrin	CD61	–	nt	nt	nt
	α4-integrin	CD49d	nt	–	nt	–
	α5-integrin	CD49e	nt	+	nt	+
	LFA-1	CD11a	nt	+	nt	–
Selectins	E-Selectin	CD62E	nt	+	nt	–
	P-selectin	CD62P	nt	+	nt	–
Ig-superfamily	PECAM-1	CD31	–	+	–	–
	ICAM-1	CD54	nt	+	nt	+
	ICAM-3	CD50	nt	–	nt	–
	VCAM-1	CD106	nt	+	nt	–
	HCAM-1	CD44	nt	+	+	+
MHC	I (HLA-ABC)	none	nt	+	+	+
	II (HLA-DR,DP,DQ)	none	nt	nt	–	–

nt = not tested.

3. In comparison to other MSCs, AFMSCs show a unique gene expression signature that consists of the upregulation of genes involved in signal transduction pathways (e.g., HHAT, F2R, F2RL) and in uterine maturation and contraction (e.g., OXTR, PLA2G10), thus suggesting a role of AFMSCs in modulating the interactions between the fetus and the uterus during pregnancy.

The cell-surface antigenic profile of human AFMSCs has been determined through flow cytometry by different investigators (Table 11.1). Cultured human AFMSCs are positive for mesenchymal markers (i.e., CD90, CD73, CD105, CD166), for several adhesion molecules (i.e., CD29, CD44, CD49e,

CD54), and for antigens belonging to the major histocompatibility complex I (MHC-I). They are negative for hematopoietic and endothelial markers (e.g., CD45, CD34, CD14, CD133, CD31).

AFMSCs exhibit a broad differentiation potential towards mesenchymal lineages. Under specific *in vitro* inducing conditions, they are able to differentiate towards the adipogenic, osteogenic, and chondrogenic lineage.

Despite not being pluripotent, AFMSCs can be efficiently reprogrammed into pluripotent stem cells (iPS) via retroviral transduction of defined transcription factors (Oct4, Sox2, Klf-4, c-Myc). Strikingly, AFMSC reprogramming capacity is significantly higher (100-fold) and much quicker (6 days vs. 16–30 days) in comparison with that of somatic cells such as skin fibroblasts. As iPS derived from adult cells, AF-derived iPS generate embryoid bodies (EBs) and differentiate towards all three germ layers *in vitro*, and *in vivo* form teratomas when injected into severe combined immunodeficient (SCID) mice.

11.2.3 Preclinical Studies

After AFMSC identification, various studies investigated their therapeutic potential in different experimental settings. Different groups demonstrated that AFMSCs are able not only to express cardiac and endothelial specific markers under specific culture conditions, but also to integrate into normal and ischemic cardiac tissue, where they differentiate into cardiomyocytes and endothelial cells. In a rat model of bladder cryo-injury, AFMSCs show the ability to differentiate into smooth muscle and to prevent the compensatory hypertrophy of surviving smooth muscle cells.

AFMSCs can be a suitable cell source for tissue engineering of congenital malformations. In an ovine model of diaphragmatic hernia, repair of the muscle deficit using grafts engineered with autologous mesenchymal amniocytes leads to better structural and functional results than equivalent fetal myoblast-based and acellular implants. Engineered cartilaginous grafts have been derived from AFMSCs grown on biodegradable meshes in serum-free chondrogenic conditions for at least 12 weeks; these grafts have been successfully used to repair tracheal defects in fetal lambs when implanted *in utero*. The surgical implantation of AFMSCs seeded on nanofibrous scaffolds and predifferentiated *in vitro* towards the osteogenic lineage into a leporine model of sternal defect leads to a complete bone repair in two months' time.

Intriguingly, recent studies suggest that AFMSCs can harbor trophic and protective effects in the central and peripheral nervous systems. Pan showed that AFMSCs facilitate peripheral nerve regeneration after injury and hypothesized that this can be determined by cell secretion of neurotrophic factors. After transplantation into the striatum, AFMSCs are capable of surviving and integrating in the rat adult brain and migrating towards areas of ischemic

damage. Moreover, the intraventricular administration of AFMSCs in mice with focal cerebral ischemia-reperfusion injuries significantly reverses neurological deficits in the treated animals.

Remarkably, it has also been observed that AFMSCs present *in vitro* an immunosuppressive effect similar to that of bone-marrow-derived MSCs. Following stimulation of peripheral blood mononuclear cells with anti-CD3, anti-CD28, or phytohemagglutinin, irradiated AFMSCs determine a significant inhibition of T-cell proliferation with dose-dependent kinetics.

11.3 AMNIOTIC FLUID STEM CELLS

The first evidence that the AF could contain pluripotent stem cells was provided in 2003, when Prusa described the presence of a distinct subpopulation of proliferating AF cells (0.1–0.5% of the cells present in the AF) expressing the pluripotency marker Oct4 at both transcriptional and proteic levels. Oct4 (i.e., octamer binding transcription factor 4) is a nuclear transcription factor that plays a critical role in maintaining embryonic stem (ES) cell differentiation potential and capacity of self-renewal. Other than by ES cells, Oct4 is specifically expressed by germ cells, where its inactivation results in apoptosis, and by embryonal carcinoma cells and tumors of germ cell origin, where it acts as an oncogenic fate determinant. While its role in stem cells of fetal origin has not been completely addressed, it has been recently demonstrated that Oct4 is neither expressed nor required by somatic stem cells or progenitors.

After Prusa, different groups confirmed the expression of Oct4 and of its transcriptional targets (e.g., Rex-1) in the AF. Remarkably, Karlmark transfected human AF cells with the green fluorescent protein gene under either the Oct4 or the Rex-1 promoter and established that some AF cells were able to activate these promoters. Several authors subsequently reported the possibility of harvesting AF cells displaying features of pluripotent stem cells. Thereafter, the presence of a cell population able to generate clonal cell lines capable of differentiating into lineages representative of all three embryonic germ layers was definitively demonstrated. These cells, named AF stem (AFS) cells, are characterized by the expression of the surface antigen c-kit (CD117), which is the type III tyrosine kinase receptor of the stem cell factor.

11.3.1 Isolation and Culture

The proportion of c-kit+ cells in the AF varies over the course of gestation, roughly describing a Gaussian curve; they appear at very early time points in gestation (i.e., at seven weeks of amenorrhea in humans and at E9.5 in mice) and present a peak at midgestation equal to 90×10^4 cells/fetus at 20 weeks of pregnancy in humans and to 10,000 cells/fetus at E12.5 in mice. Human AFS cells can be derived either from small volumes (5 mL) of second trimester

AF (14–22 weeks of gestation) or from confluent back-up amniocentesis cultures. Murine AFS cells are obtainable from the AF collected during the second week of gestation (E11.5–14.5). AFS cell isolation is based on a two-step protocol consisting of the prior immunological selection of c-kit positive cells from the AF (approximately 1% of total AF cells) and of the subsequent expansion of these cells in culture. Isolated AFS cells can be expanded in feeder-layer-free, serum-rich conditions without evidence of spontaneous differentiation *in vitro*. Cells are cultured in basic medium containing 15% of fetal bovine serum and Chang supplement.

11.3.2 Characterization

Karyotype analysis of human AFS cells deriving from pregnancies in which the fetus was male revealed the fetal origin of these cells.

AFS cells proliferate well during *ex vivo* expansion. When cultivated, they display a spectrum of morphologies ranging from a fibroblast-like to an oval-round shape (Figure 11.1A). As demonstrated by different authors, AFS cells possess a great clonogenic potential. Clonal AFS cell lines expand rapidly in culture (doubling time = 36 h) and, more interestingly, maintain a constant telomere length (20 kbp) between early and late passages (Figure 11.1B). Almost all clonal AFS cell lines express markers of a pluripotent undifferentiated state: Oct4 and NANOG. However, they have been proved not to form tumors when injected in SCID mice.

The cell-surface antigenic profile of AFS cells has been determined through flow cytometry by different investigators (Table 11.2). Cultured human AFS cells are positive for ES cell (e.g., SSEA-4) and mesenchymal markers (e.g., CD73, CD90, CD105), for several adhesion molecules (e.g., CD29, CD44), and for antigens belonging to the major histocompatibility complex I (MHC-I). They are negative for hematopoietic and endothelial markers (e.g., CD14, CD34, CD45, CD133, CD31) and for antigens belonging to the MHC-II.

As stability of cell lines is a fundamental prerequisite for basic and translational research, AFS cells' capacity of maintaining their baseline characteristics over passages has been evaluated based on multiple parameters. Despite their high proliferation rate, AFS cells and derived clonal lines show a homogeneous, diploid DNA content without evidence of chromosomal rearrangement even after expansion to 250 population doublings (Figure 11.1C). Moreover, AFS cells maintain constant morphology, doubling time, apoptosis rate, cell cycle distribution, and marker expression (e.g., Oct4, CD117, CD29, CD44) up to 25 passages. During *in vitro* expansion, however, cell volume tends to increase and significant fluctuations of proteins involved in different networks (i.e., signaling, antioxidant, proteasomal, cytoskeleton, connective tissue, and chaperone proteins) can be observed using a gel-based proteomic

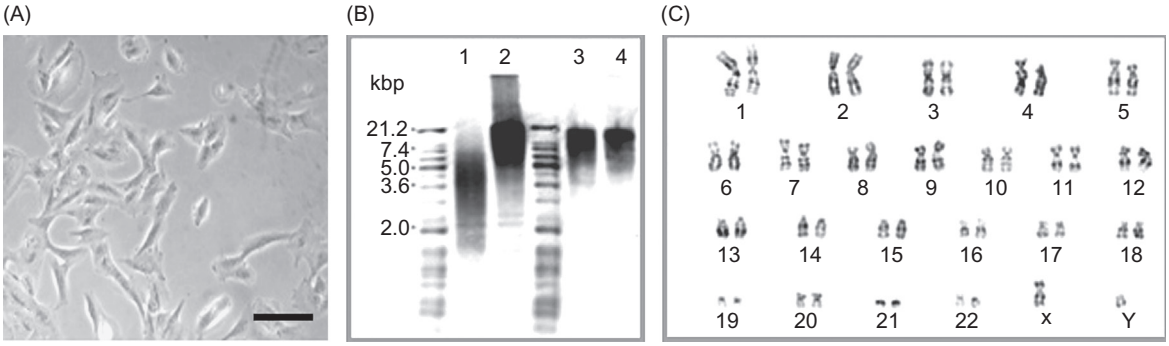

FIGURE 11.1

(A) Human AFS cells mainly display a spindle-shaped morphology during *in vitro* cultivation under feeder-layer-free, serum-rich conditions. (B–C) Clonal human AFS cell lines retain long telomeres and a normal karyotype after more than 250 cell divisions. (B) Conserved telomere length of AFS cells between early passage (20 population doublings, lane 3) and late passage (250 population doublings, lane 4). Short length (lane 1) and high length (lane 2) telomere standards provided in the assay kit. (C) Giemsa band karyogram showing chromosomes of late passage (250 population doublings) cells. *Adapted from de Coppi, P., et al., (2007). Isolation of amniotic stem cell lines with potential for therapy. Nat Biotechnol, 25(1), 100–6.*

approach; the significance of these modifications warrants further investigations but needs to be taken into consideration when interpreting experiments run over several passages and comparing results from different groups.

AFS cells and, more importantly, derived clonal cell lines are able to differentiate towards tissues representative of all three embryonic germ layers, both spontaneously, when cultured in suspension to form EBs, and when grown in specific differentiation conditions.

EBs consist of three-dimensional aggregates of ES cells, which recapitulate the first steps of early mammalian embryogenesis. As ES cells, when cultured in suspension and without anti-differentiation factors, AFS cells harbor the potential to form EBs with high efficiency: the incidence of EB formation (i.e., percentage of number of EBs recovered from 15 hanging drops) is estimated to be around 28% for AFS cell lines and around 67% for AFS cell clonal lines. Similarly to ES cells, EB generation by AFS cells is regulated by the mTor (i.e., mammalian target of rapamycin) pathway and is accompanied by a decrease of Oct4 and Nodal expression and by an induction of endodermal (GATA4), mesodermal (Brachyury, HBE1), and ectodermal (Nestin, Pax6) markers.

In specific mesenchymal differentiation conditions, AFS cells express molecular markers of adipose, bone, muscle, and endothelial differentiated cells (e.g., LPL, desmin, osteocalcin, and V-CAM1). In the adipogenic, chondrogenic, and osteogenic medium, AFS cells respectively develop intracellular lipid droplets,

Table 11.2 Surface Markers Expressed by Human c-kit + AF Stem Cell: Results by Different Groups

Markers	Antigen	CD no.	Ditadi et al., 2009	De Coppi et al., 2007b	Kim et al., 2007	Tsai et al., 2006
ES cells	SSEA-3	none	nt	–	+	nt
	SSEA-4	none	nt	+	+	nt
	Tra-1-60	none	nt	–	+	nt
	Tra-1-81	none	nt	–	nt	nt
Mesenchymal	SH2,SH3,SH4	CD73	nt	+	nt	+
	Thy1	CD90	+	+	nt	+
	Endoglin	CD105	nt	+	nt	+
Endothelial and hematopoietic	LCA	CD14	nt	nt	nt	–
	gp105-120	CD34	–	–	nt	–
	LPS-R	CD45	+	–	nt	nt
	Prominin-1	CD133	–	–	nt	nt
Integrins	β1-integrin	CD29	nt	+	nt	+
Ig-superfamily	PECAM-1	CD31	nt	nt	+	nt
	ICAM-1	CD54	nt	nt	+	nt
	VCAM-1	CD106	nt	nt	+	nt
	HCAM-1	CD44	+	+	+	+
MHC	I (HLA-ABC)	None	+	+	+	+
	II (HLA-DR,DP,DQ)	none	–	–	–	–

nt = not tested.

secrete glycosaminoglycans, and produce mineralized calcium. In conditions inducing cell differentiation towards the hepatic lineage, AFS cells express hepatocyte-specific transcripts (e.g., albumin, alpha-fetoprotein, multidrug resistance membrane transporter 1) and acquire the liver-specific function of urea secretion (Figure 11.2A). In neuronal conditions, AFS cells are capable of entering the neuroectodermal lineage. After induction, they express neuronal markers (e.g., GIRK potassium channels), exhibit barium-sensitive potassium current, and release glutamate after stimulation (Figure 11.2B). Ongoing studies are investigating AFS cell capacity to yield mature, functional neurons.

AFS cells can be easily manipulated *in vitro*. They can be transduced with viral vectors more efficiently than adult MSCs, and, after infection, maintain their antigenic profile and the ability to differentiate into different lineages. AFS cells labeled with superparamagnetic micrometer-sized iron oxide particles (MPIOs) retain their potency and can be noninvasively tracked by magnetic resonance imaging (MRI) for at least four weeks after injection *in vivo*.

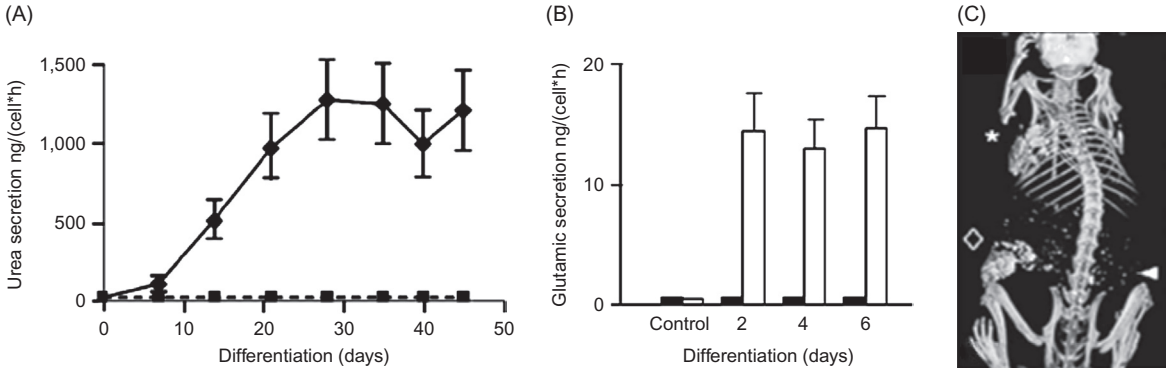

FIGURE 11.2 **AFS cells' differentiation into lineages representative of the three embryonic germ layers.**
(A) Hepatogenic differentiation: urea secretion by human AFS cells before (rectangles) and after (diamonds) hepatogenic *in vitro* differentiation. (B) Neurogenic differentiation: secretion of neurotransmitter glutamic acid in response to potassium ions. (C) Osteogenic differentiation: mouse micro computed tomography (CT) scan 18 weeks after implantation of printed constructs of engineered bone from human AFS cells; arrow head: region of implantation of control scaffold without AFS cells; rhombus: scaffolds seeded with AFS cells. *Adapted from de Coppi, P., et al., (2007). Isolation of amniotic stem cell lines with potential for therapy. Nat Biotechnol, 25(1), 100–6.*

11.3.3 Preclinical Studies

Despite the very recent identification of AFS cells, several reports have investigated their potential applications in different settings.

11.3.3.1 Bone

Critically-sized segmental bone defects are one of the most challenging problems faced by orthopedic surgeons. Autologous and heterologous bone grafting are limited respectively by the small amount of tissue available for transplantation and by high refracture rates. Tissue engineering strategies that combine biodegradable scaffolds with stem cells capable of osteogenesis have been indicated as promising alternatives to bone grafting; however, bone regeneration through cell-based therapies has been limited so far by the insufficient availability of osteogenic cells.

The potential of AFS cells to synthesize mineralized extracellular matrix within porous scaffolds has been investigated by different groups. After exposure to osteogenic conditions in static two-dimensional cultures, AFS cells differentiate into functional osteoblasts (i.e., activate the expression of osteogenic genes such as *Runx2*, *Osx*, *Bsp*, *Opn*, and *Ocn*, and produce alkaline phosphatase) and form dense layers of mineralized matrix. As demonstrated by clonogenic mineralization assays, 85% of AFS cells versus 50% of MSCs are capable of forming osteogenic colonies. When seeded into three-dimensional biodegradable scaffolds and stimulated by osteogenic supplements (i.e., rhBMP-7 or dexamethasone), AFS cells remain highly viable up for to several months in culture, and produce extensive mineralization throughout

the entire volume of the scaffold. *In vivo*, when subcutaneously injected into nude rodents, predifferentiated AFS cell-scaffold constructs are able to generate ectopic bone structures in four weeks' time (Figure 11.2C). AFS cells embedded in scaffolds, however, are not able to mineralize *in vivo* at ectopic sites unless previously predifferentiated *in vitro*. These studies demonstrate the potential of AFS cells to produce three-dimensional mineralized bioengineered constructs and suggest that AFS cells may be an effective cell source for functional repair of large bone defects. Further studies are needed to explore AFS cell osteogenic potential when injected into sites of bone injury.

11.3.3.2 Cartilage

Enhancing the regeneration potential of hyaline cartilage is one of the most significant challenges for treating damaged cartilage.

The capacity of AFS cells to differentiate into functional chondrocytes has been tested *in vitro*. Human AFS cells treated with TGF-β1 have been proven to produce significant amounts of cartilaginous matrix (i.e., sulfated glycosaminoglycans and type II collagen) both in pellet and alginate hydrogel cultures.

11.3.3.3 Skeletal Muscle

Stem cell therapy is an attractive method for treating muscular degenerative diseases because only a small number of cells, together with a stimulatory signal for expansion, are required to obtain a therapeutic effect. The identification of a stem cell population providing efficient muscle regeneration is critical for the progression of cell therapy for muscle diseases.

AFS cell capacity of differentiating into the myogenic lineage has recently started to be explored. Under the influence of specific induction media containing 5-Aza-2′-deoxycytidine, AFS cells are able to express myogenic-associated markers such as Mrf4, Myo-D, and desmin both at a molecular and proteic level. However, when transplanted undifferentiated into damaged skeletal muscles of SCID mice, despite displaying a good tissue engraftment, AFS cells did not differentiate towards the myogenic lineage. Further studies are needed to confirm the results of this single report.

11.3.3.4 Heart

Cardiovascular diseases are the primary cause of mortality in developed countries despite advances in pharmacological, interventional, and surgical therapies. Cell transplantation is an attractive strategy for replacing endogenous cardiomyocytes lost by myocardial infarction. Fetal and neonatal cardiomyocites are the ideal cells for cardiac regeneration as they have been shown to integrate structurally and functionally into the myocardium after transplantation. However, their application is limited by the ethical restrictions involved in the use of fetal and neonatal cardiac tissues.

Undifferentiated AFS cells express cardiac transcription factors at a molecular level (i.e., Nkx2.5 and GATA-4 mRNA) but do not produce any myocardial differentiation marker. Under *in vitro* cardiovascular inducing conditions (i.e., coculture with neonatal rat cardiomyocytes), AFS cells express differentiated cardiomyocyte markers such as cTnI, indicating that an *in vitro* cardiomyogenic-like medium can lead to a spontaneous differentiation of AFS cells into cardiomyocyte-like cells. *In vivo*, when xenotransplanted in the hearts of immunodeficient rats 20 minutes after creating a myocardial infarction, the differentiation capabilities of AFS cells were impaired by cell immune rejection. More recently, we have proved that we could activate the myocardial gene program in GFP-positive rat AFS (GFP-rAFS cells) by coculture with rat cardiomyocytes (rCMs). The differentiation attained via a paracrine/contact action was confirmed using immunofluorescence, RT-PCR, and single-cell electrophysiological tests. Moreover, despite only a small number of Endorem-labeled GFP-rAFS, cells acquired an endothelial or smooth muscle phenotype and to a lesser extent cardiomyocytes (CMs) in an allogeneic acute myocardial infarction (AMI) context, and there was still improvement of ejection fraction as measured by MRI three weeks after injection. This could be partially due to a paracrine action perhaps mediated by the secretion of thymosin β4.

11.3.3.5 Hematopoietic System

Hematopoietic stem cells (HSCs) lie at the top of hematopoietic ontogeny and, if engrafted in the right niche, can theoretically reconstitute the organism's entire blood supply. Thus, the generation of autologous HSCs from pluripotent, patient-specific stem cells offers real promise for cell therapy of both genetic and malignant blood disorders.

The hematopoietic potential of c-kit+ hematopoietic lineage negative cells present in the amniotic fluid (AFKL cells) has been recently explored. *In vitro*, human and murine AFKL cells exhibit strong multilineage hematopoietic potential. Cultured in semisolid medium, these cells are able to generate erythroid, myeloid, and lymphoid colonies. Moreover, murine cells exhibit the same clonogenic potential (0.03%) as hematopoietic progenitors present in the liver at the same stage of development. *In vivo*, mouse AFKL cells (i.e., 2×10^4 cells intravenously injected) are able to generate all three hematopoietic lineages after primary and secondary transplantation into immunocompromised hosts (i.e., sublethally irradiated Rag −/− mice), demonstrating their ability to self-renew. These results clearly show that c-kit+ cells present in the AF have true hematopoietic potential both *in vitro* and *in vivo*.

11.3.3.6 Kidney

The incidence and prevalence of end stage renal disease (ESRD) continues to increase worldwide. Although renal transplantation represents a good

treatment option, the shortage of compatible organs remains a critical issue for patients affected by ESRD. Therefore, the possibility of developing stem cell-based therapies for both glomerular and tubular repair has received intensive investigation in recent years. Different stem cell types have shown some potential in the generation of functional nephrons but the most appropriate cell type for transplantation is still to be established.

The potential of AFS cells in contributing to kidney development has been recently explored. Using a mesenchymal/epithelial differentiation protocol previously applied to demonstrate the renal differentiation potential of kidney stem cells, AFS cells and clonal-derived cell lines can differentiate towards the renal lineage; AFS cells sequentially grown in a mesenchymal differentiation medium containing epidermal growth factor (EGF) and platelet-derived growth factor, beta/beta homodimer (PDGF-BB), and in an epithelial differentiation medium containing hepatic growth factor (HGF) and fibroblast growth factor 4 (FGF4), reduce the expression of pluripotency markers (i.e., Oct4 and c-Kit) and switch on the expression of epithelial (i.e., CD51, ZO-1) and podocyte markers (i.e., CD2AP, NPHS2). AFS cells have also been shown to contribute to the development of primordial kidney structures during *in vitro* organogenesis; undifferentiated human AFS cells injected into a mouse embryonic kidney cultured *ex vivo* are able to integrate in the renal tissue, participate in all steps of nephrogenesis, and express molecular markers of early kidney differentiation such as ZO-1, claudin, and glia-derived neurotrophic factor (GDNF). Finally, very recent *in vivo* experiments show that AFS cells directly injected into damaged kidneys are able to survive, integrate into tubular structures, express mature kidney markers, and restore renal function. These studies demonstrate the nephrogenic potential of AFS cells and warrant further investigation of their potential use for cell-based kidney therapies.

11.3.3.7 Lung

Chronic lung diseases are common and debilitating; medical therapies have restricted efficacy, and lung transplantation is often the only effective treatment. The use of stem cells for lung repair and regeneration after injury holds promise as a potential therapeutic approach for many lung diseases; however, current studies are still in their infancy.

AFS cell ability to integrate into the lung and to differentiate into pulmonary lineages has been elegantly investigated in different experimental models of lung damage and development. *In vitro*, human AFS cells injected into mouse embryonic lung explants engraft into the epithelium and into the mesenchyme and express the early pulmonary differentiation marker TFF1. *In vivo*, in the absence of lung damage, systemically administered AFS cells show the capacity to home to the lung but not to differentiate into specialized cells; while, in the presence of lung injury, AFS cells not only exhibit a strong tissue

engraftment but also express specific alveolar and bronchiolar epithelial markers (e.g., TFF1, SPC, CC10). Remarkably, cell fusion phenomena were elegantly excluded and long-term experiments confirmed the absence of tumor formation in the treated animals up to seven months after AFS cell injection.

11.3.3.8 Intestine

To date, very few studies have considered the employment of stem cells in gastroenterological diseases. Although still at initial stages and associated with numerous problems, ever-increasing experimental evidence supports the intriguing hypothesis that stem cells may be possible candidates to treat and/or prevent intestinal diseases.

In a study evaluating AFS cell transplantation into healthy newborn rats after intraperitoneal injection, it was observed that AFS cells:

1. Diffuse systemically within a few hours from their administration in 90% of the animals,
2. Engraft in several organs of the abdominal and thoracic compartment and
3. Localize preferentially in the intestine colonizing the gut in 60% of the animals.

Preliminary *in vivo* experiments investigating the role of AFS cells in a neonatal rat model of necrotizing enterocolitis showed that intraperitoneal-injected AFS cells are able not only to integrate into all gut layers, but also to reduce bowel damage, improve rat clinical status, and lengthen animal survival.

11.4 CONCLUSIONS

Many stem cell populations (e.g., embryonic, adult, and fetal stem cells) as well as methods for generating pluripotent cells (e.g., nuclear reprogramming) have been described to date. All of them carry specific advantages and disadvantages and, at present, it has yet to be established which type of stem cell represents the best candidate for cell therapy. However, although it is likely that one cell type may be better than another, depending on the clinical scenario, the recent discovery of easily accessible cells of fetal derivation, not burdened by ethical concerns, in the AF has the potential to open new horizons in regenerative medicine. Amniocentesis, in fact, is routinely performed for the antenatal diagnosis of genetic diseases and its safety has been established by several studies documenting an extremely low overall fetal loss rate (0.06–0.83%) related to this procedure. Moreover, stem cells can be obtained from AF samples without interfering with diagnostic procedures.

Two stem cell populations have been isolated from the AF so far (i.e., AFMSCs and AFS cells) and both can be used as primary (not transformed or immortalized) cells without further technical manipulations. AFMSCs exhibit

typical MSC characteristics: fibroblastic-like morphology, clonogenic capacity, multilineage differentiation potential, immunosuppressive properties, and expression of a mesenchymal gene expression profile and of a mesenchymal set of surface antigens. However, ahead of other MSC sources, AFMSCs are easier to isolate and show better proliferation capacities. The harvest of bone marrow remains, in fact, a highly invasive and painful procedure, and the number, the proliferation, and the differentiation potential of these cells decline with increasing age. Similarly, UCB-derived MSCs exist at a low percentage and expand slowly in culture.

AFS cells, on the other hand, represent a novel class of pluripotent stem cells with intermediate characteristics between ES cells and AS cells. They express both embryonic and mesenchymal stem cell markers, are able to differentiate into lineages representative of all embryonic germ layers, and do not form tumors after implantation *in vivo*. However, AFS cells have only recently been identified, and many questions need to be answered concerning their origin, epigenetic state, immunological reactivity, and regeneration and differentiation potential *in vivo*. AFS cells, in fact, may not differentiate as promptly as ES cells, and their lack of tumorigenesis can be argued against their pluripotency.

Although further studies are needed to better understand their biologic properties and to define their therapeutic potential, stem cells present in the AF appear to be promising candidates for cell therapy and tissue engineering. In particular, they represent an attractive source for the treatment of perinatal disorders such as congenital malformations (e.g., congenital diaphragmatic hernia) and acquired neonatal diseases requiring tissue repair/regeneration (e.g., necrotizing enterocolitis). In a future clinical scenario, AF cells collected during a routinely performed amniocentesis could be banked and, in case of need, subsequently expanded in culture or engineered in acellular grafts. In this way, affected children could benefit from having autologous expanded/engineered cells ready for implantation either before birth or in the neonatal period.

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CHAPTER 12

Stem and Progenitor Cells Isolated from Cord Blood

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12.1 ADDRESSING DELAYED TIME TO ENGRAFTMENT AND GRAFT FAILURE WITH CB

Advantages to using cord blood (CB) compared with bone marrow (BM) or mobilized peripheral blood (MPB) include the ease of obtaining cells, access to CB stored as banked HLA-typed collections, and a lower incidence of graft versus host disease (GVHD), which allows for somewhat less stringency in HLA-matching for transplantation into both unrelated and related recipients. Disadvantages include the limited quantity of cells in a single CB collection, which can increase the incidence of graft failures, slow the time to neutrophil and platelet engraftment, and delay recovery of immune cells compared with using BM or MPB. These disadvantages may in part be related to fewer donor cells being available for engraftment in CB, although this may not be the only, or main, reason for this delayed time to engraftment. To overcome the first mentioned disadvantage, investigators have used double CB transplantation. This has had a positive impact on the field, and has resulted in greatly increased numbers of CB transplants performed to treat adults and higher weight children, and in decreased graft failures. However, double CB units have increased cost, which can be up to twice that of a single CB unit. Also, in the majority of the double CB transplants, only one of the units wins out to become the dominant or only unit engrafting longer term. There is no significant insight into why this phenomenon occurs. It does not appear that double CB units have significantly accelerated time to neutrophil, platelet, or immune cell reconstitution. Moreover, double CB transplants elicit increased levels of GVHD, negating in part the advantage of less GVHD noted with single CB unit transplantation.

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Efforts to expand HSC from CB *ex vivo* for clinical transplantation have not yet been rewarding. No one has significantly expanded long-term repopulating, self-renewing human HSCs. A study using Notch-ligand in combination with cytokine-induced stimulation resulted in clinically relevant enhancement in *ex vivo* expansion of CB HSCs. However, it was the short-, not long-term, HSCs that were expanded. One problem with expanding human HSCs is the lack of definitive phenotypic information on human HSCs; a problem that was not observed in analogous procedures in mice, where a single phenotyped mouse HSC could long-term engraft a lethally irradiated mouse recipient. Human HSCs have been functionally studied in the laboratory via their capacity to engraft sub-lethally irradiated immune-deficient mice. One traditional model has been a strain of mouse with non-obese diabetic (NOD)-severe combined immunodeficiency (SCID), but more recently a model having a IL-2 receptor gamma null mutation in addition to NOD-SCID has been found to be an even more efficient acceptor of human HSC engraftment. These models have allowed better definition of the the phenotype of human SCID repopulating cells (i.e., HSCs). This new information may accelerate our ability to *ex vivo* expand human HSCs, as they can now be more quickly identified phenotypically than if the *in vivo* engraftment assay is used. However, phenotype does not necessarily recapitulate function, especially after the stress of *ex vivo* culture, and any results from expanding a human HSC with a defined phenotype must verify that this cell fulfills the functional definition of an HSC: engraftment of primary and secondary immune-deficient mice.

Laboratory studies have suggested means for enhancing the engrafting capability of a limited numbers of cells, such as are found in single CB collections, by altering the proliferation, self-renewal, survival, and homing capacity of HSCs. These studies include, but are not limited to:

- modulation of HSC function by inhibition of CD26/dipeptidylpeptidase IV (DPPIV) *ex vivo* or *in vivo*
- stimulation of cells *ex vivo* with prostaglandin E (PGE)
- modifying cell surface fucosylation of target cells
- use of rapamycin *ex vivo* to inhibit the mTOR pathway

Clinical trials are either in progress or in preparation for the first three of these four scenarios.

CD26/DPPIV is found on the surface of a number of hematopoietic cells, including HSCs and hematopoietic progenitor cells (HPCs); DPPIV is also present in soluble form in the circulation. By truncating stromal-cell-derived factor-1 (SDF-1/CXCL12; a chemotactic and homing chemokine) at the N-terminus, the truncated SDF-1/CXCL12 no longer acts as a chemotactic agent, and the truncated molecule blocks the activity of full-length SDF-1/

CXCL12 for chemotaxis. With this information in mind, inhibition of CD26/DPPIV was used to enhance the engrafting capability of limiting numbers of mouse BM cells in mouse recipients, and human $CD34^+$ CB and MPB cells in NOD/SCID mice. Recent unpublished studies from my group demonstrate that CD26/DPPIV also truncates specific colony-stimulating factors (CSFs), and administration of an inhibitor of DPPIV *in vitro* enhances the functional activity of these CSFs; deletion of CD26 in CD26−/− mice allows enhanced hematopoietic recovery from non-lethal irradiation and cytotoxic drugs. Short-term pulse treatment of a population of cells containing HSCs and HPCs with PGE enhances the engrafting capability of mouse BM and human CB, respectively into lethally irradiated mice and sub-lethally irradiated immune-deficient mice, through effects on stimulating homing and division of pretreated donor cells. Modifying the fucosylation status of donor cells has resulted in enhanced homing of mouse BM HSCs in a mouse model. Studies using mouse BM cells with enforced expression of Rheb2, in which these transduced cells manifested enhanced expansion of HPCs at the expense of repopulating HSC activity, led to studies using rapamycin to inhibit the mTOR pathway, in combination with certain cytokines to enhance the functional capacity of human $CD34^+$ cells *ex vivo* for engraftment of NOD/SCID IL-2R gamma chain null mice.

It is hoped that the above treatment strategies, and others yet to be developed, will enhance engraftment. It may be that combinations of such treatments will be most efficacious, and correct timing and administration of these treatments must be carefully considered. Our ability to manipulate human HSCs for improved engraftment will require a more in-depth understanding of the cytokine and microenvironmental influences on HSCs and the intracellular signals elicited in HSCs for proliferation, self-renewal, survival, differentiation, and homing/migration. Two, of many possible intracellular signals to possibly focus on include SIRT1, and Tip110.

One clinical effort to enhance engraftment of limiting numbers of CB cells has used intrafemoral, instead of intravenous, donor cell delivery to the recipient, but it is too early to be sure if this will become a preferred cell infusion strategy.

12.2 CRYOPRESERVATION OF CB CELLS

CB banking has been instrumental in clinical CB transplantation. This area is undergoing enhanced scrutiny and governmental regulatory changes. One unknown in this area is the length of time for which a frozen CB unit can be stored with efficient recovery of HSCs and HPCs after thawing the sample. We previously demonstrated the efficient recovery of such cryopreserved

cells after 15 years in frozen storage. Recently, we found comparably efficient recovery after up to 23.5 years of storage of frozen CB.

There have been a number of different non-hematopoietic cell types found in CB. These include endothelial progenitor cells (EPCs = high proliferative endothelial colony forming cells) and mesenchymal stem/stromal cells. Interestingly, cryopreservation methods for HSCs and HPCs may not be optimal for EPCs, or other cell types; while EPCs can be cryopreserved and recovered, the efficiency of recovery is less than that noted for HSCs/HPCs. The key to determining the efficient recovery of any specific cell type is that one compares recovery to the exact same pre-freeze sample.

12.3 INDUCED PLURIPOTENT STEM CELLS GENERATED FROM CB

There has been excitement in the newly developed field of induced pluripotent stem cells (iPSCs). A wide variety of mature cell types have been reprogrammed, completely or partially, to an embryonic stem cell (ESC)-like state by enhanced/induced expression of certain critical transcription factors (including: Oct 4, Sox 2, KLF4, c-Myc) with subsequent differentiation down cells of the mesoderm, endoderm and ectoderm germ cell layers; this includes generation from immature (e.g., $CD34^+$) cells in CB. However, whether or not the generated iPSCs will be ready for 'prime-time' clinical use in the near future, or ever, is not known. Regardless, we can learn a great deal about normal and disordered cell regulatory processes from these generated iPSCs. Whether or not CB cells will be a preferred cell source for generating of iPSCs remains to be determined.

12.4 CONCLUDING COMMENTS

A year or so ago (2012) was the first time that more HSC/HPC transplants were performed with CB, than with BM. Increased knowledge of CB HSCs and HPCs and their regulation will allow more enhanced and efficacious use of CB to treat malignant and non-malignant disorders. We look forward to such advances.

FOR FURTHER STUDY

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CHAPTER 13

The Nervous System

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13.1 INTRODUCTION

The mammalian brain is one of the most complex biological structures known. The study of brain function in the normal and diseased brain is therefore a daunting task. However, an understanding of the basic elements that produce the extraordinary cell diversity in the brain is an important step toward that end. Developmental biology has taught us that all the cells of the CNS are derived from a small set of neuroepithelial cells, which sequentially generate the various neuronal and glial subtypes that comprise the CNS and peripheral nervous system (PNS). ES cells provide a powerful model system for studying developmental neurobiology, and for testing hypotheses regarding how neural identity and regional specification are established, and how neural stem cells decide to undergo neuronal or glial differentiation programs. The protocol for the directed differentiation of ES cells into specific neuronal or glial subtypes can serve as a cell-based developmental screen to identify novel genes involved in specific aspects of CNS development, and to assess the role of known genes under conditions that can bypass the embryonic lethality often observed when studying gene function *in vivo*. This interplay between ES cells and developmental biology is mutually beneficial and will, one hopes, not only result in novel insights for basic biology but also provide a comprehensive framework of differentiation strategies for cell-therapeutic applications. Although work with mouse ES cells has achieved several of these goals, human ES cell work significantly lags behind the mouse work. It will be essential to narrow this gap soon to fulfill some of the promises of stem cell research for medical applications.

This review provides a short introduction to neural development, followed by a section on neural stem cells, contrasting their advantages and limitations for both basic and clinical applications with the use of ES cells. Subsequently,

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the various strategies available for inducing neural differentiation in mouse ES cells are introduced, and various examples for neuronal, glial, and neural crest specification *in vitro* are given. This section is followed by a brief summary of published work on lineage selection using surface makers and cell type-specific promoters, a strategy that can be used as an alternative or in parallel to directed differentiation protocols. A synopsis of the current studies of neural differentiation in human and nonhuman primate ES cells is followed by more general remarks on the potential for ES cells to address developmental questions using cell-based screens. The final section summarizes work with ES cells for cell-therapeutic applications in preclinical models of neural disease.

13.2 NEURAL DEVELOPMENT

A basic understanding of neural development is a prerequisite for developing rational strategies of stem cell differentiation. The neural plate is derived from the dorsal ectoderm and the process is induced by 'organizer' signals derived from the underlying notochord. The dominant model of neural induction is the default hypothesis. This hypothesis states that neural tissue is formed spontaneously in the absence of bone morphogenetic protein (BMP) signaling during early gastrulation, and exposure to BMP signals causes epidermal differentiation. Accordingly, signals emanating from the organizer which are essential for neural induction are BMP inhibitors such as chordin, noggin, follistatin, and cerberus. However, there is also strong evidence that fibroblast growth factor (FGF) signals emanating from precursors of the organizer prior to gastrulation are essential for a 'prepattern' of neural induction through activation of Sox3 and early response to neural induction (ERNI). Studies have identified Churchill (ChCh), a zinc-finger transcriptional activator induced by low doses of FGF signaling, which inhibits the mesoendoderm-inducing effects of FGF and sensitizes cells to BMP signals, thereby acting as a switch from gastrulation to neurulation. Other essential players during neural induction are insulin-like growth factor (IGF) and Wnt signaling.

After the formation of the neural plate, cells undergo a well-defined set of morphological and molecular changes leading to the formation of neural folds and neural tube closure. This is followed by orchestrated waves of neural proliferation and differentiation. Of particular importance in determining specific neural fates are signals that provide regional identity both in the anterior–posterior (A–P) and in the dorsal–ventral (D–V) axis, and those that define domains of distinct expression of homeodomain proteins and basic helix-loop-helix (bHLH) transcription factors. The leading hypothesis of A–P axis specification states that anterior fates are established as defaults during early neural induction, and FGF, Wnt, and retinoid signals are essential for establishing posterior cell fates. D–V identity is determined by the

antagonistic action of Sonic hedgehog (SHH) secreted ventrally from the notochord and floor plate and of BMPs from the roof plate. There is ample evidence from explant studies – and more recently from ES cell differentiation studies – that confirm such a concentration-dependent role of SHH in defining specific progenitor domains within the neural tube by activating class II genes in the ventral spinal cord. However, genetic studies with SHH/Gli3, or SHH/Rab23, double-mutant mice have shown that D–V patterning can occur in the absence of SHH, questioning an exclusive role for SHH gradients in D–V patterning. It has been shown that the timing of D–V patterning is controlled by the antagonistic action of FGF and retinoid signals, with FGF signals inhibiting the establishment of D–V homeodomain transcription factors. Other findings have suggested a more specific role for FGFs in inhibiting dorsal gene expression, and identified retinoids as an activator of class I genes essential for D–V patterning. A SHH-antagonistic concentration-dependent role for BMPs in dorsal-neural patterning has been proposed from work with explants *in vitro*, and has partly been confirmed in ES cell differentiation studies. Although some *in vivo* data from genetic ablation studies and work in transgenic mice that overexpress the BMP receptor type 1a (BMPR1a/Alk3) under control of the regulatory elements of the *nestin* gene are compatible with a dorsal patterning role for BMPs, loss-of-function studies of BMPR1b suggested a much more limited role for BMPs in determining selectively choroids plexus fate. In addition to BMPs, Wnt signals might contribute to dorsal neural patterning, particularly in the establishment of the neural crest.

Subsequent differentiation of patterned neural precursor cells occurs in a stereotypic fashion with neurons being born first, followed by astroglial and oligodendroglial differentiation. The onset of neuronal differentiation is controlled through inhibition of the Notch pathway, which represses proneural bHLH genes. Astrocytic fate is established using activation of Jak/Stat signals, which exert an instructive role on multipotent neural progenitors to drive astrocytic differentiation. However, insights into the neurogenic properties of radial glial cells, as well as the identification of adult neural stem cells as cell-expressing astrocytic markers, suggest a more complex and dynamic interaction between neural stem cell and astrocytic fates. Oligodendrocytes were believed to derive from bipotent glial precursors (termed O2A progenitors) or from other glially committed precursors. However, other data suggest a lineage relationship between motor neurons and oligodendrocytes in the spinal cord as well as γ-aminobutyric acidergic (GABAergic) neurons and oligodendrocytes in the forebrain by their shared requirement for expression of the bHLH gene *Olig 2*. A review of the developmental signals that control the various neuronal subtypes is beyond the scope of this chapter, but some of the necessary signals will be described in the individual headings under the section 'Derivation of ES-cell-derived Neurons.'

13.3 NEURAL STEM CELLS

The isolation of neural stem cells provided a first step toward developing rational strategies of *in vitro* neuronal and glial differentiation. Neural stem cell culture systems had significant influence on protocols of directed neural differentiation from ES cells. I therefore briefly highlight some of the basic neural stem cell techniques with a view toward explaining commonalities and discrepancies between the two *in vitro* differentiation strategies. Neural stem cells have been isolated from both the developing and the adult brain. More than 10 years of intensive research has convincingly demonstrated the capacity of neural stem cells for self-renewal and multilineage differentiation into neurons, astrocytes, and oligodendrocytes; the three major CNS lineages. However, although many tissue-specific stem cells, such as hematopoietic stem cells, are capable of differentiation into all progeny within an organ system, neural stem cells do not efficiently generate all neuronal subtypes in the adult brain. They are largely limited to the production of Gamma-AminoButyric Acid (GABA) and glutamatergic neurons after long-term expansion. Early attempts at isolating and propagating neural stem cells *in vitro* were based purely on selective growth and proliferation conditions. The most common method is the neurosphere culture system. Under these conditions, neural precursors are grown at low density and allowed to proliferate as free-floating spheres in the presence of epidermal growth factor (EGF) and FGF-2. Human neurosphere cultures are typically supplemented with leukemia inhibitory factor (LIF) in addition to EGF and FGF-2. Neurospheres can be formed from single cells, and the capacity for neurosphere formation is often used as an assay to test stem cell properties of neural cells. For example, the isolation of prospectively identified neural stem cells based on surface markers such as AC133, Lex1, or combinations of surface markers was largely developed through the ability of the cells to form neurospheres *in vitro*. These data need to be interpreted cautiously, as neurosphere formation is not necessarily a true test of stemness, and neurospheres do contain many differentiated cells in addition to the presumptive progenitor-stem cell population. Studies have demonstrated that neurospheres are formed more efficiently from transit-amplifying populations than from true stem cells in the adult subventricular zone.

An alternative approach to the neurosphere technology is the growth of neural precursor-stem cells as monolayer culture attached on a matrix – typically fibronectin or laminin – in the presence of FGF-2. These conditions are more amenable to the study of the precise lineage relationship, and they allow precise manipulations at the single-cell level. Complete lineage trees for single cortical stem cells have been worked out under such conditions. Among the most important limitations of current neural stem cell technology are the limited *in vitro* control of neural patterning and neuronal subtype specification.

The derivation of midbrain dopamine neurons has served as a model for these difficulties. Functional midbrain dopamine neurons can be derived from short-term-expanded precursor cells isolated from the early rodent and human midbrain. However, long-term expansion causes a dramatic loss in the efficiency of midbrain dopamine neuron generation. Several strategies have been developed in an attempt to overcome these problems, ranging from exposure and complex growth factor cocktails to changes in oxygen levels and transgenic expression of Nurr1, a key transcription factor during midbrain dopamine neuron development. However, none of these approaches has succeeded in deriving midbrain dopamine neurons that exhibit full functionality *in vitro* and *in vivo* from naïve, long-term-expanded neural stem cells. Although the mechanisms of the restricted neuronal differentiation potential remain to be elucidated, the cell types most difficult to derive from neural stem cells are neurons born at developmental stages prior to stem cell isolation. This suggests that the competence of the precursor population to generate these neuronal subtypes might be lost, or that the environmental cofactors required for appropriate neuron subtype specification are missing. Alternatively, the mitogens used for propagation of the neural precursor cells might select for progeny incapable of generating early neuronal subtypes, bias competent precursors into a noncompetent state, or directly deregulate the neural patterning state as suggested for FGF-2-expanded precursors in the spinal cord. Possible solutions might consist of isolating neural stem cells at an earlier developmental state and defining conditions that allow these cells to retain early competency for patterning factors, identifying growth factors that do not bias neuronal subtype or that are able to reinduce competence in later precursors, or introducing novel patterning strategies that can bypass current limitations in neuronal subtype potential. One early example in this direction is the growth of midbrain-derived precursors in the absence of FGF2 but in the presence of SHH and FGF8 as well as TGFβ-3, a combination capable of increasing midbrain dopamine neurons generated *in vitro* but still with limited capacity for cell expansion. Although these problems continue to hamper *in vitro* differentiation studies with primary neural stem cells, ES cells offer a simple and efficient alternative solution to overcoming such concerns.

13.4 NEURAL DIFFERENTIATION OF MOUSE ES CELLS

ES cells are capable of virtually unlimited *in vitro* proliferation at the undifferentiated stage, overcoming many problems associated with the instability of stem cell phenotype observed in tissue-specific stem cells, including neural stem cells. In addition to proliferation potential, ES cells offer many important advantages for both basic and applied research, such as ease of genetic manipulation, access to the earliest stages of neural development, and

comprehensive differentiation potential. The cell fate potential of ES cells is most vividly illustrated upon injection of ES cells into the developing blastocyst, where ES progeny contributes to all tissues including the germ line. Neural differentiation has been one of the best-studied *in vitro* differentiation pathways in ES cell research. This is partly because of the ease by which ES cells generate neural progeny but also because of the potential of neural progeny for cell-therapeutic applications in the CNS. The use of ES cells in regenerative medicine received a significant boost some years ago when human ES cells and embryonic germ (EG) cells were first isolated.

13.4.1 Neural Induction

The initial requirement for generating defined neural subtypes from ES cells is appropriate control of neural induction. There are at least three main strategies for using ES cells *in vitro* to induced neural differentiation in mouse ES cells: embryoid body (EB)-based systems, stromal feeder-mediated neural induction, and protocols based on default differentiation into neural fates (Figure 13.1).

13.4.1.1 EB-Based Protocols

EBs are formed upon aggregation of ES cells in suspension cultures. The interactions of cells within the EB causes cell differentiation in a framework that mimics normal development, particularly the steps of gastrulation. Accordingly, derivatives of all three germ layers can be found in EBs, and EB culture is often used as a first screening tool to demonstrate pluripotency of putative ES cell lines. Although the derivation of neural progeny is inefficient under basic EB conditions, several protocols have been developed to enhance neural induction and to select and expand EB-derived neural precursors.

The first EB-mediated neural differentiation protocol was based on exposure to retinoic acid (RA) for 4 days after EBs had been formed for 4 days in the absence of RA – the so-called 4−/4+ protocol. RA is a vitamin A derivative, released primarily by surrounding mesodermal cells, and it exhibits a strong neural induction and patterning effect. Many variations on the basic protocol have been developed. However, no clear mechanistic understanding of the action of RA in EBs has emerged, and the overall cell composition under these conditions remains heterogeneous. One major additional concern in RA-based neural induction protocols is the concomitant effect of RA on A–P patterning mediated through activating the Hox gene cascade.

An alternative EB-based strategy is the exposure to conditioned medium derived from the hepatocarcinoma cell line (HepG2), which appears to induce neuroectodermal fate directly. Accordingly, HepG2-treated aggregates do not express endodermal or mesodermal markers but directly generate neural progeny expressing progenitor markers such as Sox1, Sox2, and

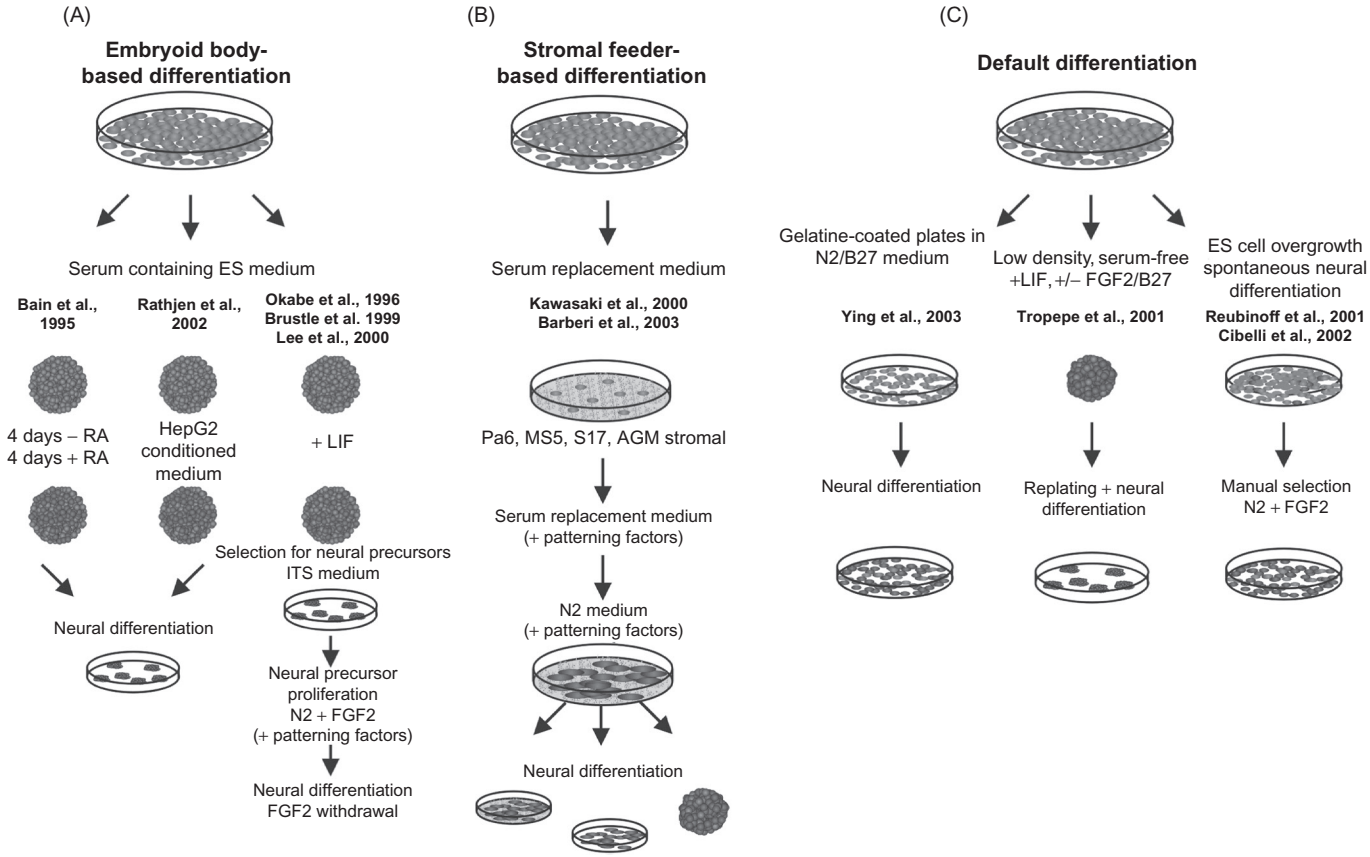

FIGURE 13.1 **Basic techniques for inducing neural differentiation in ES cells *in vitro*.**

(A) Embryoid body-based protocols are initiated by the aggregation of undifferentiated ES cells. Neural differentiation is promoted by exposure to RA, hepatocarcinoma cell line (HepG2)-conditioned medium, or neural selection in defined media. Classic studies are cited for each of the three main EB-based strategies. (B) Stromal feeder-mediated differentiation is obtained upon plating undifferentiated ES cells at low density on stromal feeder cell lines derived from the bone marrow of the AGM region of the embryo. Serum-free conditions are required throughout the protocol. Conditions can be readily adapted to achieve neural subtype-specific differentiation for a large number of CNS cell types. Classic studies are cited for PA6- and MS5-mediated differentiation. (C) Default neural differentiation is achieved by reducing endogenous BMP signals using plating cells at low density under minimal medium conditions or in the presence of the BMP antagonist noggin. Neurally committed cells can also be mechanically isolated and propagated from plates exhibiting spontaneous neural differentiation after overgrowth of ES cells.

nestin. The active component within HepG2-conditioned media remains to be isolated. Some evidence indicates that at least two separable components are responsible for this activity, and one of these components is apparently a known extracellular matrix molecule. Unlike the RA protocols, HepG2 conditions are not thought to bias neural subtype composition toward specific A–P or D–V fates. However, no detailed studies are available which have addressed this issue experimentally, or have developed neural-subtype-specific protocols.

A third strategy for achieving neural induction is an RA-free EB-based protocol that subjects EB progeny to neural-selective growth conditions. Neural selection from EB progeny is achieved under minimal growth conditions in a serum-free medium supplemented with insulin, transferrin, and selenite (ITS medium). Under these conditions, most EB-derived cells die, and a distinct population of immature cells emerges that expresses increasing levels of the intermediate filament nestin. These nestin$^+$ precursors can be replated and directed toward various neuronal and glial fates using a combination of patterning, survival, and lineage-promoting factors (see the sections 'Derivation of ES-cell-derived Neurons' and 'ES-cell-derived Glia'). This technique is quite robust and highly modular for generating a variety of neural subtypes (see later sections of this chapter). Moreover, commercially available kits provide a simple entry point for setting up the system. However, ES cell line-specific differences in the efficiency of neural induction can occur, particularly at stage III of differentiation (selection of nestin$^+$ precursors from EBs in ITS medium) and can be limiting for some applications.

13.4.1.2 Stromal Feeder-Mediated Neural Induction

Bone marrow-derived stromal cell lines have been used for years to support the growth of undifferentiated hematopoietic stem cells acting partly through expression of the membrane protein mKirre, a mammalian homolog of the *kirre* gene of *Drosophila melanogaster*.

More recently, it has been reported that many stromal cell lines that support hematopoietic stem cell growth exhibit neural-inducing properties in coculture with mouse ES cells. Stromal cell lines with the highest efficiencies of neural induction are typically at the preadipocytic stage of differentiation. Although most of these cell lines were isolated from the bone marrow (e.g., PA-6, MS5, and S17), stromal cells derived from the aorta–gonad–mesonephros (AGM) region were found to induce neural differentiation equally well in mouse ES and nuclear transfer ES cells. The inducing effect does not require the survival of the stromal cells, as similar or increased neural-inducing activity is observed upon paraformaldehyde fixation. The main activity resides within the cell surface of the stromal cells and does not diffuse efficiently over long distances. The molecular nature of this inducing activity

of stromal-derived cells remains unknown, but preliminary data suggest that it does not involve BMP or Wnt signaling. The efficiency and robustness of neural induction using stromal feeder cells is extremely high compared with alternative protocols, and differentiation appears to occur without any bias toward regional specification or neuronal-versus-glial fate choice.

13.4.1.3 Neural Differentiation by Default

Coculture-free, direct neural differentiation protocols are based on the default hypothesis proposing that in the absence of cell-cell signaling, particularly in the absence of exposure to BMPs, ectodermal cells will adopt a neural fate. Two independent studies with mouse ES cells confirmed that, under minimal conditions, in the absence of BMP but in the presence of a required endogenous FGF signal, neural induction does occur in nonadherent or adherent monocultures. Interference with FGF signaling using dominant-negative or gene targeting approaches prevents neural differentiation in either system, confirming the early role of FGF in neural induction that was suggested by *in vivo* studies. The development of more refined culture systems will be particularly useful for defining the minimal molecular requirements that drive neural fate specification in early development. Maintenance of undifferentiated mouse ES cells in the absence of serum but in the presence of BMP4 and LIF will be an additional important step toward that end. However, the efficacy of the default monoculture approach in comparison with EB and stromal feeder protocols has not been determined. Furthermore, although these systems appear highly defined at the time of plating, the heterogeneity of cell types starts to appear quickly after initiation of differentiation even under monoculture conditions.

13.4.2 Derivation of ES-Cell-Derived Neurons

Neuronal differentiation occurs rapidly upon neural induction of mouse ES cells. The efficiency of neuronal-versus-glial differentiation varies widely. Neuronal subtype specification can be influenced by the mode of neuronal induction. This is particularly the case for the RA induction protocol known to induce the *Hox* gene cascade in a dose-dependent fashion and to promote hindbrain and spinal cord fates at the expense of forebrain differentiation. The basic strategy for achieving neuronal subtype differentiation relies on the application of signals that mimic early patterning events in the embryo to define A–P and D–V domains within the body axis of the developing embryo. A–P patterning is thought to involve factors that actively posteriorize regional identity, including RA, FGFs, and Wnts. D–V patterning is thought to be controlled by the antagonistic action of SHH and BMP signals. Although early mouse ES cell differentiation strategies yielded neurons that were poorly characterized and typically of GABA or glutamatergic origin, more refined protocols have been developed for many neuronal subtypes. Protocols for a selection of neuronal subtypes are described in more detail later in this chapter.

13.4.2.1 Midbrain Dopaminergic Neurons

Derivation of midbrain dopamine neurons from ES cells has been of particular interest because of the clinical potential for dopamine neuron transplants in the treatment of Parkinson's disease. Protocols for the dopaminergic differentiation of mouse ES cells closely follow findings obtained in explant studies that identified FGF8 and SHH as critical factors in midbrain dopamine neuron specification. The effect of SHH/FGF8 on ES-cell-derived neural precursors was first described using an EB-based five-step differentiation protocol. Under these conditions, up to 34% of all neurons expressed tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of dopamine. However, the effect of SHH and FGF8 was relatively modest and did not exceed that of another dopamine neuron-promoting factor, ascorbic acid (AA, vitamin C). A further increase in dopamine neuron yield was obtained in ES cells overexpressing Nurr1. Nearly 80% of all neurons express TH under these conditions, and expression of midbrain dopaminergic markers remains stable even after transgenic expression of Nurr1 has been silenced. Midbrain dopaminergic differentiation was also obtained using coculture of ES cells on the stromal feeder cell line (PA6). PA6 cells mediated neural induction, yielding dopaminergic differentiation in up to 16% of all neurons without requiring exposure to exogenous SHH and FGF8 but in the presence of AA. These results were initially interpreted as PA6 exhibiting a specific action promoting dopamine neuron fate in addition to its neural-inducing properties. However, later studies demonstrated that there is no irreversible bias toward the generation of midbrain dopamine neurons using PA6 but that neural precursors derived under these conditions remain highly amenable to A–P and D–V patterning. When combining stromal feeder-mediated neural induction with exposure to SHH/FGF8, about 50% of all neurons express TH15 without requiring any transgene, such as Nurr1, to further push the dopamine neuron phenotype.

Numbers of TH neurons need to be interpreted carefully in all *in vitro* differentiation studies, as TH is an unreliable marker for identifying dopamine neurons. TH is also expressed in other catecholaminergic neurons, including noradrenergic and adrenergic cells, and it is induced in many cell types under various conditions, including cell stress, hypoxia, and exposure to a variety of growth hormones. It is therefore essential that studies reporting on the derivation of midbrain dopamine neurons provide additional markers and evidence of dopamine neuron function *in vitro* and *in vivo*. The derivation of TH neurons has also been achieved using a monolayer, default neural induction protocol. However, the efficiency of dopamine neuron induction, characterization of midbrain phenotype, and *in vitro* and *in vivo* functionality using a default neural induction protocol have not yet been reported.

13.4.2.2 Serotonergic Neurons

The developmental origin of serotonergic neurons is closely related to that of midbrain dopamine neurons. Both neuronal subtypes are dependent on signals emanating from the isthmic organizer. Accordingly, serotonergic neurons are a major 'contaminating' neuronal subtype in protocols aimed at the derivation of midbrain dopaminergic cells. Protocols specifically designed to increase serotonergic-versus-dopaminergic differentiation have been based on the exposure to FGF4. Application of exogenous FGF4 preceding FGF8 and SHH application ectopically induces serotonergic neurons in explant culture. Although application of FGF4 to neural precursors in the presence of FGF2 at stage IV (neural precursor cell proliferation) of the multistep EB differentiation protocol does not yield a significant increase in serotonergic differentiation, FGF4 added in the absence of FGF2 causes a dramatic shift from dopaminergic to serotonergic differentiation. Efficient derivation of hindbrain serotonergic neurons using stromal feeder-mediated neural induction also involves early FGF4 exposure in the absence of FGF2, followed by FGF2, FGF8, and SHH application. Novel strategies to refine serotonergic differentiation protocols might use novel, basic developmental insights that demonstrated the importance of the transcription factor Lmx1b in serotonergic differentiation. They also may use studies of zebra fish in which several novel genes have been isolated that affect the proportion of dopaminergic-versus-serotonergic neurons, including the elongation factor foggy and the zinc-finger protein named too few. Although cell therapy might be not a primary goal for optimizing serotonergic differentiation protocols, the derivation of ES-cell-derived serotonin neurons may provide insights into brain development and offer an unlimited source of cells for pharmacological screens in a neurotransmitter system involved in various psychiatric disorders.

13.4.2.3 Motor Neurons

Development of spinal motor neurons has been studied in great detail using a variety of mouse loss-of-function and chick gain-of-function models as well as explant culture systems. A more advanced understanding of the developmental signals involved in motor neuron specification and a wealth of reagents available for their phenotypic characterization make the derivation of motor neurons from ES cells an obvious target. Early studies have demonstrated that cells expressing markers of motor neurons can be generated using an EB induction protocol with RA exposure (2−/7+). More systematic approaches of using RA exposure with exogenous SHH to promote ventral fates have yielded ES-cell-derived motor neurons at high efficiency and have demonstrated how developmental pathways can be harnessed to direct ES cell fate *in vitro*. By creating an ES cell line, which expresses green fluorescent protein (GFP) under the control of the motor neuron-specific gene *HB9*, these

ES-cell-derived motor neurons could also be readily identified and purified. The *in vivo* properties of ES-cell-derived motor neurons were demonstrated upon transplantation into the spinal cord of early chick embryos. ES-cell-derived motor neurons were detected in the ventral spinal cord, extended axons, and innervated nearby muscle targets.

The efficient derivation of motor neurons has also been achieved using stromal feeders such as PA6 or MS5 cells with SHH and RA treatment. The next challenges for *in vitro* motor neuron differentiation protocols will be the selective generation of motor neurons of distinct A–P and columnar identity. Insights from developmental biology studies will provide a good starting point. The potential of ES-cell-derived motor neurons for future therapeutic applications in spinal cord injury or amyotrophic lateral sclerosis (ALS, Lou Gehrig's disease) will heavily rely on the ability to generate precise motor neuron subtypes *in vitro*. Additional requirements are controlling axonal outgrowth, target selection, and specificity of muscle innervation. One particularly useful approach might be the genetic or pharmacological manipulation of ES-cell-derived motor neurons to overcome growth inhibitors in an adult environment, a strategy used successfully to overcome growth inhibition of primary dorsal-root ganglion cells upon activation of the cyclic adenosine monophosphate pathway.

13.4.2.4 GABA Neurons

GABA cells are the main inhibitor neuron type within the brain, and are found at high densities in basal forebrain structures, particularly in the striatum. The presence of GABAergic neurons during ES cell differentiation *in vitro* has been reported under various conditions including the classic 4−/4+ EB-based differentiation protocols, which yield approximately 25% GABA neurons. Interestingly, protocols with shorter periods of RA-free EB formation followed by extended RA treatment (2−/7+) select for motor neurons rather than GABA neurons. This suggests that the timing of RA application might be crucial for neural fate specification. The presence of GABA neurons has also been reported under default neural induction conditions.

Directed differentiation to GABA neurons has been achieved using a stromal feeder-based approach. Neural induction on MS5 is followed by neural precursor proliferation in FGF2 and subsequent exposure to SHH and FGF8. The delayed application of FGF8 and SHH promotes ventral forebrain identities as determined by the expression of the forebrain-specific marker FOXG1B (BF-1), and increases GABAergic differentiation. In addition to forebrain striatal and cortical GABA neurons, there are many other types of GABA neurons in various brain regions, including the thalamus, midbrain, and cerebellum. It remains to be determined whether there will be common differentiation strategies that can be used to derive these various GABAergic subtypes from ES cells or

whether diversity in the expression of region-specific markers will be reflected in the culture conditions. GABA neurons are highly relevant for a variety of neurological disorders, including Huntington's disease, epilepsy, and stroke.

13.4.2.5 Glutamate Neurons

Glutamate neurons can be readily obtained at high efficiencies from mouse ES cells. Using several related isolation protocols, approximately 70% of all neurons are glutamatergic, and ES-cell-derived neurons with N-methyl-D-aspartate (NMDA) and non-NMDA receptor subtypes have been described. More detailed physiological data on glutamatergic neurons have been reported after coculture of ES-cell-derived neurons on hippocampal brain slices. Interestingly, this study suggested a possible bias toward establishing AMPA- over NMDA-type synaptic contacts. There is currently no detailed data on the derivation of glutamatergic neurons using stromal feeder or default neural differentiation protocols.

13.4.2.6 Other Neuronal and Neural Subtypes

The presence of about 5% glycinergic neurons has been reported using the classic 4−/4+ EB protocol. However, directed differentiation into this main inhibitory neuronal subtype in the spinal cord has not been demonstrated. Other interesting neural types generated from ES cells are precursors of the otic anlage. These precursors were obtained by culturing EBs for 10 days in EGF and IGF followed by bFGF expansion. After transplantation of these precursors *in vivo*, differentiation was observed into cells expressing markers of mature hair cells. Derivation of radial glial cells from mouse ES cells provides another interesting assay system to probe neuronal and glial lineage relationships in early neural development.

13.4.2.7 Neural Crest Differentiation

The neural crest is a transient structure formed from the most dorsal aspects of the neural tube of the vertebrate embryo. It contains migratory cells that form the PNS including sensory, sympathetic, and enteric ganglia; large parts of the facial skeleton; as well as various other cell types including Schwann cells, melanocytes, and adrenomedullary cells. ES cells provide a powerful assay to study neural crest development *in vitro*. The main strategy for deriving neural crest-like structures from ES cells is based on the exposure to BMPs (BMP2, BMP4, or BMP7) following neural induction. The feasibility of this approach was demonstrated for mouse and partly nonhuman primate ES cells using the PA6 stromal feeder cell system. This study showed the development of sensory as well as sympathetic neurons in a BMP dose-dependent manner. The derivation of smooth muscle cells required growth in chicken extract with BMP withdrawal. No melanocytes or Schwann cells could be obtained under these conditions.

Another study suggests efficient neural crest induction, including Schwann cell differentiation, using an EB-based, multistep differentiation protocol with BMP2 treatment. Neural crest formation has also been reported in HepG2-mediated neural differentiation protocols upon exposure to staurosporine, previously reported to induce avian neural crest development. However, characterization of neural crest progeny was limited to morphological observations and expression of Sox10, a marker expressed during neural crest development but also during glial development in the CNS. Future studies are required to define conditions for deriving all neural crest lineages *in vitro* and to validate stability and function of neural crest phenotypes *in vivo*.

13.4.3 Derivation of ES-Cell-Derived Glia

Neural progenitors derived from mouse ES cells can be readily differentiated into astrocytic and oligodendroglial progeny under conditions similar to those described from primary neural precursors. The first reports on the glial differentiation of mouse ES cells were based on the 4−/4+ EB protocols or multistep EB differentiation protocols. Most of the glial progeny under these conditions are astrocytes, with only few immature oligodendrocytes present. However, subsequent studies have defined conditions for the selective generation of both astrocytes and oligodendrocytes.

13.4.3.1 Oligodendrocytes

Highly efficient differentiation into oligodendrocytes was reported first using a modified, multistep, EB-based protocol. ES-cell-derived neural precursors were expanded with FGF2 followed by $FGF2^+$EGF and $FGF2^+$platelet-derived growth factor (PDGF). These conditions yielded a population of $A2B5^+$ glial precursors capable of differentiation into both astrocytic (~36% glial fibrillary acidic protein-positive, or GFAP+) and oligodendrocytic (~38% O4+) progeny upon mitogen withdrawal. The 4−/4+ EB–RA induction protocol has been optimized for the production of oligodendrocytic progeny. This study demonstrated efficient selection of neural progeny by both positive (Sox1-enhanced green fluorescent protein, or EGFP) and negative (Oct4-herpesvirus thymidine kinase) selection and oligodendrocytic differentiation in RA-induced EBs after expansion in FGF2 followed by dissociation and replating in serum-free medium containing FGF2 and SHH. The final step involved SHH and FGF2 withdrawal and the addition of PDGF and thyroid hormone (T3). Under these conditions, approximately 50% of all cells express oligodendroglial markers. Optimized conditions for oligodendrocyte differentiation using HepG2 or default neural differentiation protocols have not yet been reported. However, stromal feeder-mediated induction, initially thought to bias toward neuronal progeny can be readily adapted to derive oligodendrocytes at very high efficiencies and without requiring genetic selection.

13.4.3.2 Astrocytes

Highly efficient differentiation of ES cells into astrocytes has been reported using stromal feeder-mediated neural induction followed by sequential exposure to FGF2, bFGF/EGF, EGF/CNTF, and ciliary neurotrophic factor (CNTF). More than 90% of all cells expressed the astrocytic marker GFAP under these conditions. Significant numbers of GFAP cells were also obtained using HepG2-mediated neural differentiation or multistep EB protocols. Glial progenitors obtained with a multistep EB protocol were 'transplanted' *in vitro* into hippocampal slices and revealed that full physiological maturation of ES-cell-derived astrocytes can be achieved upon interaction with an appropriate host environment. Under these conditions, ES-cell-derived astrocytes integrated seamlessly into host astrocytic networks tightly coupled by gap junctions.

13.4.4 Lineage Selection

Lineage selection based on surface markers or the cell type-specific expression of promoter-driven selectable markers provides an alternative approach to directed *in vitro* differentiation protocols. The use of genetic markers in ES cells is particularly attractive because of ease of inducing stable genetic modifications and the availability of large libraries of transgenic and gene-targeted mice and ES cells. Efficient purification of neural progeny from mouse ES cells *in vitro* was demonstrated through positive selection using a Sox1-EGFP knockin cell line and combined with a negative selectable marker controlled by an endogenous Oct4 locus. Other ES lines successfully used for the genetic identification and purification of neural precursor and neuronal and glial progeny *in vitro* include a tau-EGFP knockin cell line, a GFAP transgenic line, GAD-lacZ knockin ES cells, BF1-lacZ knockin ES cells, and ES cell lines driven under the regulatory elements of the *Nestin* gene. Promoter-driven lineage selection for motor neuron fate *in vitro* includes ES cell lines expressing EGFP in the Olig2 locus or as a transgene under the *HB9* promoter.

13.5 NEURAL DIFFERENTIATION OF HUMAN AND NONHUMAN PRIMATE ES CELLS

Neural differentiation potential was readily observed when primate ES or ES-like cells were first established in both monkey and human ES cells as well as in human EG cells and monkey parthenogenetic stem cells. However, the derivation of purified populations of neural progeny from human ES cells required more systematic studies. A highly efficient protocol for the neural differentiation of human ES cells was based on a modified multistep EB approach. ES cells are aggregated for a short time (approximately four days) and subsequently replated under serum-free conditions in the presence of

FGF-2. Under these conditions, neural precursors can be readily identified on the basis of the formation of multilayered epithelia, termed rosettes. It was suggested that such rosettes might mimic neural tube-like structures *in vitro*. These neural precursor cells can be enzymatically separated from the surrounding cell types and grown to purity under neurosphere-like conditions in the presence of FGF-2. Differentiated neural progeny derived from these neurosphere-like structures are neurons and astrocytes. The main neuronal subtypes are GABAergic and glutamatergic phenotypes. A similar spectrum of differentiated progeny was obtained when human ES-cell-derived neural precursors were obtained after spontaneous neural differentiation by overgrowth of undifferentiated cells. Rosette-like structures were manually isolated under microscopic view and subsequently grown and passaged under neurosphere-like conditions. A third strategy that yielded differentiated cell populations enriched for neural precursors was based on a modified EB-RA induction protocol, followed by lineage selection in attached cultures using serum-free conditions and supplementation with bFGF.

Protocols that yield better control over neuronal subtype specification are still in development, but the first examples have been provided for deriving midbrain-like dopamine neurons from nonhuman primate ES cells. Interestingly, PA6-mediated neural induction caused differentiation into retinal-pigment epithelial cells in addition to neural differentiation and the derivation of TH^+ neurons. A bias toward eye differentiation was not observed with standard mouse ES cell protocols using stromal feeder cells. However, more systematic efforts have identified conditions that yield eye-like differentiation in mouse ES cells, suggesting that eye phenotypes can be obtained not only with human ES cells but also with mouse ES cells.

One interesting difference between human and mouse ES cell differentiation is the presence of large numbers of rosette-like structures during the early stages of neural differentiation in human ES cells. These structures are rarely observed during mouse ES cell differentiation. A better understanding of the developmental nature of these structures may give important hints about how to optimize neuronal subtype specification *in vitro*. D–V patterning in vertebrates occurs during the time of neural tube formation, and distinct transcription factor domains are established by the time of neural tube closure. The presence or absence of such domains in cultured rosettes will be an important factor in determining whether D–V identity has been established or whether these structures remain sensitive to patterning strategies.

13.6 DEVELOPMENTAL PERSPECTIVES

Highly reproducible *in vitro* differentiation protocols and an increasing number of available ES reporter cell lines provide powerful tools for establishing cell-based developmental screens. In addition, large-scale gene-trapping

approaches have yielded ES cells lines mutant for a large proportion of all the genes within the mouse genome. Several ES cell-based *in vitro* differentiation screens have been carried out to assay for genes involved in neural-neuronal induction. One such screen using kinase-directed combinatorial libraries has identified a molecule with potent inhibitory activity for GSK3β during neural differentiation of mouse ES and P19 cells. The result suggested that activation of Wnt signaling through increased β-catenin levels and activation of downstream targets, including LEF1/TCF1, increases neuronal differentiation. In agreement with these findings, previous studies have shown that Wnt1 is downstream target during RA-mediated neural differentiation of P19 cells and that Wnt1 is sufficient to trigger neural differentiation in these cells. In contrast, the Wnt antagonist Sfrp2 has been identified during neural differentiation of EBs exposed to RA. This study used functional gene screening of RA-treated and control EBs through subtraction hybridization followed by episomal expression of differentially expressed cDNAs. The discrepancy between the two approaches on the role of Wnt signaling during neural differentiation *in vitro* reflects differences in the assays used. It underscores the importance of using better-defined, developmentally based *in vitro* differentiation systems that allow careful interpretation of the findings in such screens. The availability of more comprehensive genomic tools will facilitate the establishment of detailed gene expression profiles under all *in vitro* ES cell differentiation conditions that will complement the few existing data sets. Early gene-trapping studies that used *in vitro* differentiation screens prior to or instead of *in vivo* studies may also see a revival using refined neural differentiation protocols. The availability of high-throughput functional genomic approaches such as RNAi-based gene knockdown screens in *Drosophila* and *C. elegans* could be readily adapted to ES cell *in vitro* differentiation systems and may provide unprecedented opportunities for studying neural development using cell-based differentiation systems. Such approaches could be of particular importance in studying human neural development when no appropriate *in vivo* alternatives exist.

13.7 THERAPEUTIC PERSPECTIVES

One of the driving forces behind deciphering the developmental program that controls cell fate specification is the hope that such insights could be harnessed for generating specialized cells for therapy. However, despite the excitement about the potential of ES cells in neural repair, there are few examples in which such approaches have been tested in animal models of disease.

13.8 PARKINSON'S DISEASE

One of the most widely discussed applications is the derivation of unlimited numbers of dopamine neurons from human ES cells for the treatment of

Parkinson's disease. This disease is a particularly attractive for cell transplantation because of the relatively defined pathology that mainly affects midbrain dopamine neurons and the largely unknown etiology that currently precludes causative treatment. At the onset of clinical symptoms, most midbrain dopamine neurons have already died, providing further rationale for a cell replacement approach. The first ES cell-based study that showed functional improvement in 6OHDA lesioned rats, an animal model of Parkinson's disease, was based on the transplantation of low numbers of largely undifferentiated mouse ES cells isolated after short-term differentiation in EB cultures. Spontaneous differentiation into large numbers of neurons with midbrain dopamine characteristics was observed. However, the clinical relevance of this approach is rather limited because of the high rate of tumor formation (>50% of the animals with surviving grafts developed teratomas).

Remarkable functional improvement was obtained after transplantation of dopamine neurons derived from mouse ES cells overexpressing Nurr1. This study was based on a multistep EB differentiation protocol. In addition to behavioral restoration in 6OHDA lesioned rats, this study demonstrated *in vivo* functionality using electrophysiological recordings from grafted dopamine neurons in acute brain slices obtained from the grafted animals. However, transgenic expression of Nurr1 raises safety concerns that may preclude clinical translation. Functional recovery with dopamine neurons derived from naïve mouse ES cells has been reported. This study demonstrated functional improvement *in vivo* after grafting dopamine neurons derived from ES and nuclear transfer ES cells using stromal feeder-mediated differentiation. Successful grafting of nuclear transfer ES-cell-derived dopamine neurons provided a first example of therapeutic cloning in neural disease. Differentiation of human ES cells into midbrain dopamine neurons has not yet been reported. However, it is likely such protocols will become available within the next few years to be tested in rodent and primate models of Parkinson's disease.

Several issues remain to be addressed before clinical trials with human ES-cell-derived dopamine neurons should be initiated. Twenty years of fetal tissue research have demonstrated that fetal midbrain dopamine neurons can survive and function long term (more than 10 years) in the brain of Parkinson's patients. However, these studies have also shown limited efficacy in placebo-controlled clinical trials and demonstrated potential for side effects. Stem cells will have to learn from the fetal tissue transplantation trials and better define and address the critical parameters that can take cell therapy in Parkinson's disease to the next level. The derivation of highly purified populations of substantia nigra-type dopamine neurons from human ES cells will be an important first step on this road.

13.9 HUNTINGTON'S DISEASE

Similar to Parkinson's disease, the pathology in Huntington's disease preferentially affects selective neuronal subtypes, particularly GABAergic medium spiny neurons, the main neuron subtype in the striatum. Fetal tissue transplantation trials provide experience on how to design stem cell-based approaches for the treatment of Huntington's disease. However, unlike in Parkinson's disease, grafted cells are required not to reconnect to local targets within the striatum, but to project from the striatum to the targets in the globus pallidus and the substantia nigra pars reticulata. Data on whether fetal tissue grafts have been able to re-establish such long-distance connections in patients remains controversial. Although the identification of the molecular defect in Huntington's disease as unstable expansion of cytosine-adenine-guanine repeats in the IT15 gene suggests many alternative therapeutic approaches to the use of cell transplantation, it also provides the research community with genetic models of the disease that allow careful evaluation of all strategies. ES-cell-derived GABAergic neurons have not yet been tested in any animal model of Huntington's disease, but the *in vitro* derivation of GABA neurons with forebrain characteristics has been achieved at high efficiencies. Another interesting avenue for stem cells in Huntington's disease is the derivation of human ES cells with Huntington's disease mutations from embryos discarded after pre-implantation diagnostics. Such Huntington's disease ES cell lines could provide invaluable insights into the selective vulnerability of the striatal GABAergic cell population.

13.9.1 Spinal Cord Injury and Other Motor Neuron Disorders

Traumatic or degenerative injuries to the spinal cord are often devastating and irreversible. Cell replacement using stem cells has been touted as a prime application of stem cell research. However, the complexity of cell therapy in spinal cord injury is enormous and far from resolved. Motor neurons are one of the main cell types affected by spinal cord injuries and by various degenerative diseases such as ALS. The efficient derivation of motor neurons from mouse ES cells has been demonstrated with both EB-based and stromal feeder protocols. However, the functionality of ES-cell-derived neurons *in vivo* has only been addressed using xenografts into the developing chick spinal cord. The behavior of ES-cell-derived motor neurons in the adult CNS and in animal models of spinal cord injury or disease has not been tested.

Prior to the availability of directed differentiation protocols, there have been reports of functional improvement in animal models of spinal cord injury after grafting ES-cell-derived progeny. In one such study, dissociated 4−/4+

mouse EBs derived from D3 or Rosa26 ES cells were transplanted into the spinal cord 9 days after a crush injury. The grafted cells differentiated *in vivo* into oligodendrocytes, neurons, and astrocytes, and they induced significant functional improvement in Basso, Beattie, and Bresnahan (BBB) scores over those of sham-injected animals. However, the mechanism by which functional improvement was obtained remains controversial. Based on the ability for efficient differentiation into oligodendrocytes *in vivo*, it was suggested that remyelination of denuded axons might be a key factor. Functional improvement was also reported after grafting EB-derived cells obtained from human EG cells. EB cell populations derived from human EG cells were implanted into the cerebrospinal fluid (CSF) of rats after virus-induced neuronopathy and motor neuron degeneration, a model of ALS. Although a very small number of transplanted cells started to express markers compatible with motor neuron fate, most cells differentiated into neural progenitor or glial cells. It was concluded that the functional improvement was caused by enhancing host neuron survival and function rather than reestablishing functional connections from graft-derived motor neurons. Future studies will have to bridge the gap between the increasing knowledge about controlling *in vitro* differentiation from ES cell to motor neuron progeny and the limited information from *in vivo* studies based on grafting poorly defined cell populations.

13.10 STROKE

Little work has been done with ES-cell-derived progeny in animal models of stroke. A study showed that grafted ES cells can survive in a rat stroke model induced by transient ischemia using occlusion of the middle cerebral artery. The goal of this study was noninvasive imaging of the grafted cells using high-resolution MRI after transfection with ultrasmall, superparamagnetic iron-oxide particles. The authors provided evidence for extensive migration of the grafted cells along the corpus callosum toward the ischemic lesion. However, no phenotypic analyses of the differentiated cell types were performed, and no functional effects were measured. Cell transplantation efforts for the treatment of stroke are complicated by the multiple cell types affected and the variability of the affected cell populations depending on stroke location.

13.11 DEMYELINATION

The capacity of mouse ES-cell-derived progeny to remyelinate *in vivo* has been demonstrated after transplantation of highly purified ES-cell-derived glial progenitors into the spinal cord of *md* rats that lack the X-linked gene encoding myelin proteolipid protein, an animal model of Pelizaeus-Merzbacher syndrome. This study showed impressive *in vivo* differentiation results and

yielded large grafts comprised of myelinating oligodendrocytes. However, the grafted cells were not able to extend the short life span of these animals, precluding detailed functional analyses. A second study demonstrated remyelination after grafting purified oligosphere cultures derived from 4−/4+ EBs into the spinal cord of shiverer mice or into the chemically demyelinated spinal cord.

Important challenges are the derivation of functional oligodendrocytes from human ES cells and the demonstration of functional benefits *in vivo*, including strategies to obtain remyelination over more extended CNS regions. Transplantation of ES-cell-derived oligodendrocytes into models of multiple sclerosis will be of particular clinical interest. However a successful approach will require sophisticated strategies to overcome host-mediated factors that prevent oligodendrocytes maturation as well as strategies that address the autoimmune nature of the disease.

13.11.1 Other Diseases

Several disease models approached with fetal neural progenitors have not yet been tested using ES cell-based approaches. These include epilepsy and enzymatic deficiencies such as lysosomal storage diseases. Other CNS disorders such as Alzheimer's disease have been touted as future applications for ES cell therapy. However, at the current stage of research, the challenges for cell therapy in Alzheimer's disease seem to be overwhelming, and it appears more likely that the role for ES cells might be in providing cellular models of disease rather than replacing cells. Some early attempts toward this goal have involved the study of neural differentiation in ES cells that exhibit the disease causing the mutant of amyloid precursor protein (APP) knocked into the endogenous APP locus.

13.12 SUMMARY

The development of protocols that allow the directed differentiation from ES cells to specific neural fates provides an essential basis for all cell-based approaches in neural repair. Although these protocols are becoming routine for mouse ES cells, work with human ES cells lags behind. However, it is likely that these difficulties will be overcome within the next few years and that some of the first clinical ES cell applications will be within the CNS. Beyond the role in regenerative medicine, ES cell *in vitro* differentiation protocols will become an essential tool for gene discovery and serve as a routine assay of neural development. The availability of libraries of ES cells with specific mutations or expressing specific transgenes will be a great asset for such studies. *In vitro* ES cell differentiation will also provide unlimited sources of defined neural subtypes for pharmacological assays in drug screening and

toxicology. However, one of the most important contributions might be the availability of a basic research tool that allows us to unravel step by step the complex signals that govern the development of single pluripotent ES cells to the amazing diversity of cell types that comprise the mammalian CNS.

FOR FURTHER STUDY

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CHAPTER 14

Sensory Epithelium of the Eye and Ear

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14.1 INTRODUCTION

Humans rely heavily on both vision and hearing. Unfortunately, both deteriorate with age, partly because of the death of cells in the primary sensory organs, the eye and the ear. In addition, the frequency of disease genes that affect one or both of these modalities is relatively high. Stem cells able to replace some of the dying cells, either *in situ* or through engraftment, have been a hope for some time. In part, this is because there are few effective therapies for diseases of these tissues. Work aimed at identifying retinal and otic stem cells has been undertaken with more energy in the last several years because of the exciting findings for stem cells elsewhere in the body. Here, we review these findings in the context of normal development.

14.2 INTRODUCTION TO PROGENITOR AND STEM CELLS IN THE RETINA

The retina has served as a model of central nervous system (CNS) anatomy, physiology, and development. Most studies aimed at understanding its development have concerned the production of the retinal neurons and glia from retinal progenitor cells. These cells were originally shown by lineage analysis to be multipotent throughout development, capable of generating both neurons and glia, even in a single, terminal cell division. Retinal progenitor cells do not appear to be totipotent except for the earliest progenitor cells, when clones can comprise all retinal cell types. Moreover, retinal progenitor cells do not appear to be able to proliferate extensively *in vivo* or following explantation and exposure to different culture conditions. More recent studies have been aimed at finding retinal stem cells. These studies have been conducted along the two lines established in the search for stem cells elsewhere in the

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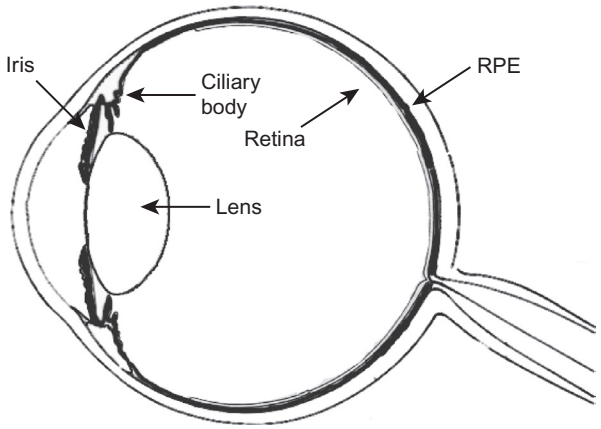

FIGURE 14.1

The eye is a complex tissue, developing from cells originating from the neural tube, the neural crest, the surface ectoderm, and the mesoderm. The retina is the neurosensory tissue that originates from the inner layer of the optic cup, and the RPE originates from the outer optic cup layer. Both the inner and outer optic cup layers contribute to the formation of the ciliary body and iris. The ciliary body also comprises cells from the neural crest, which form the ciliary muscle. The iris muscles derive from the outer layer of the optic cup. Stem cells have been isolated from the ciliary body and iris, and Muller glia in the retina have been found to divide and generate neurons in the early posthatch chick.

CNS. One approach has been to search for mitotic cells capable of generating retinal neurons in the adult *in vivo*. The other approach has been to culture cells in growth factors. Both types of experiments have begun to yield promising answers, but much more needs to be done.

14.3 THE OPTIC VESICLE GENERATES DIVERSE CELL TYPES THAT CAN UNDERGO TRANSDIFFERENTIATION

To appreciate some of the intriguing observations concerning retinal stem cells, a review of the tissues that derive from the optic vesicle is needed. The optic vesicle is an evagination of the neural tube where the diencephalon and telencephalon meet. The vesicle at first protrudes as a simple evagination when the neural tube forms. Soon thereafter, the vesicle undergoes an invagination to form a two-layered optic cup. The outer cup will form a non-neural structure, the retinal pigmented epithelium (RPE), as well as with other support structures of the eye (Figure 14.1). The RPE is a single layer of epithelial cells heavily pigmented to capture stray light that passes through the retina. It performs several support functions, including such highly specialized functions as the isomerization of *trans* to *cis* retinal to allow the photopigments,

the opsins, to continue to capture light. The RPE expresses many specific gene products. A transcriptome analysis conducted using serial analysis of gene expression (SAGE) showed that 40% of the RPE SAGE tags did not have a corresponding cDNA in GenBank. This is a much higher rate of unknowns than seen in other tissues (e.g., the human retina).

The inner wall of the optic cup forms the neural retina (Figure 14.1). The primary sensory cells are the photoreceptors (PRs), which comprise two types: the rods and the cones. The rods are active under dim light, and the cones are active under daylight conditions. In addition, there are several types of interneurons, horizontal cells, amacrine cells, and bipolar cells and an output neuron, the retinal ganglion cell. The retina also has one glial cell that spans the retinal layers; the Müller glial cell. During the early phase of retinal neurogenesis, retinal progenitor cells produce the various retinal neurons in a conserved fashion, typically beginning with production of ganglion cells and finishing with production of rod PRs, bipolar interneurons, and Müller glia. The production of these cells begins in the center of the retina and proceeds to the retinal periphery, or margin. In amphibians and teleost fish, there is continual growth at the margin throughout the life of the animal in a region termed the ciliary marginal zone, or CMZ. In addition, in fish, there is a late wave of production of rod PRs as the retina expands.

The developmental sequence at the periphery of the retina is complex and not at all understood at a molecular level, although it is an important region for stem cells. The margin is the fold that develops following invagination of the primary optic vesicle. Following the initial formation of the morphologically simple folds at the periphery, where the presumptive RPE and retina meet, some rather unusual morphogenetic events, including transdifferentiation, take place to form several anterior support structures for the eye. The ciliary body, with the associated pars plicata and pars plana, as well as the iris, form from this area (Figure 14.1). The pars plana and plicata each comprise two epithelial layers, one pigmented and one unpigmented. They are the site of attachment of the zonules, or suspensory ligaments of the lens. The unpigmented epithelial layer of the pars plicata and plana is continuous with the retina, and the pigmented layer is continuous with the RPE. The tight apposition of these two epithelial layers allows regulation of secretion from the ciliary body, as it is highly vascularized with a rather leaky type of blood vessel. Beyond secretion of aqueous (through the pars plicata) and vitreous (through the pars plana) humor, the ciliary body also controls the shape of the lens. Neural-crest-derived muscles form within the ciliary body and contract and relax the ligaments surrounding the lens during lens accommodation.

The iris is the shutter that opens and closes to allow more or less light to penetrate the eye. It includes a pigmented epithelial layer derived from the

margin of the optic cup that is continuous with the RPE. It also has an initially unpigmented epithelial layer, the inner or posterior layer of epithelium, which is continuous with the retina. This layer gradually becomes pigmented, however, and additional pigmentation of the iris is contributed by neural-crest-derived melanocytes. Remarkably, the pupil is opened and closed by muscles that derive from the margin of the optic cup, the only ectodermally derived muscles in the body. This occurs because of transdifferentiation, as initially pigmented cells separate from the epithelial sheet, proliferate, and form muscles. Thus, the retinal margin develops to serve several functions, and most of these diverse cell types derive from both the outer and the inner walls of the early optic cup.

Classical embryological experiments with birds, fish, amphibians, and mammals have revealed a great deal of plasticity among the ocular tissues. For example, extirpation of most – but importantly, not all – of the retina leads to transdifferentiation of the RPE into the retina. This capacity exists only until embryonic day (E) 4 in chickens and E14 in mice. In urodeles, it can occur throughout life. In chicks, it was found to be induced by fibroblast growth factor (FGF) in RPE cultures. *In vivo*, it is not clear whether FGF is involved in the initial distinction between the retina and RPE, but delivery of FGF8 *in vivo* can trigger transdifferentiation. Wolffian regeneration in newts is a remarkable process by which the dorsal iris can regenerate a lens, originally not derived from the optic vesicle but from the surface ectoderm. This type of regeneration has not been seen in birds or mammals. All of these examples reveal that end-stage differentiated cells are not necessarily committed or irreversibly differentiated. Perhaps most remarkably, as explained later in this chapter, the pigmented cells derived from this area of the optic cup display broad developmental potential in adult mammals in that they are the source of retinal stem cells.

14.4 *IN VIVO* NEUROGENESIS IN THE POSTHATCH CHICKEN

Retinal neurogenesis in most of the chick retina is complete by E12. In a search for stem cells in the developed retina, the posthatch chick (i.e., >E21) was examined for the incorporation of bromodeoxyuridine (BrdU). They found that two areas could be labeled. In a normal retina without injury, the P7 retina was labeled in the ciliary margin, reminiscent of the aforementioned findings for amphibians and fish. These cells were followed using their BrdU label and were found to incorporate into the inner nuclear layer (INL), generating bipolar and amacrine neurons. No cells were found in the outer nuclear layer (ONL), the layer containing PRs. Antigens consistent with the INL fates were also observed. The newly generated cells appeared

progressively more centrally as harvests were made later and later. These findings suggest that the CMZ cells were generating more retinal neurons to accommodate additional growth of the eye, previously thought to occur only through an expansion of the volume of the vitreous cavity, and stretching of the retinal tissue. However, this does not occur throughout the life of the chicken as it does in amphibians and fish, since the growth stops a few weeks posthatch. The number of mitotic cells within the CMZ was not increased following injection of a toxin, *N*-methyl-d-aspartate (NMDA), unlike the response seen in the Xenopus eye. However, injection of 100 ng doses of epidermal growth factor (EGF), insulin, or insulin-like growth factor 1 (IGF-1), but not FGF, did increase the mitotic activity in this area. In addition, if insulin was applied with FGF2, cells with gene expression profiles and processes consistent with the ganglion cell fate were observed.

Another site of BrdU incorporation in the posthatch chick could be identified following application of the toxin NMDA, which primarily targets amacrine cells. If BrdU was applied two days after injection of NMDA at P7, incorporation of BrdU into Müller glial cells in the central retina was observed. Labeling with BrdU at one or three days following toxin administration led to few BrdU-labeled cells. It thus appears that a process triggered by toxin administration requires approximately two days to stimulate one or two rounds of cell division. This response is also developmentally limited, as few BrdU-labeled cells were observed after P14, and the response was lost in a central-to-peripheral manner, similar to the initial wave of neurogenesis in the retina. Müller glial cells do possess the ability to undergo *reactive gliosis*, a phenomenon associated with various types of retinal damage in adult mammals and birds. Reactive gliosis occurs in astrocytes throughout the CNS and is characterized by: limited cell division; expression of intermediate filament proteins, such as vimentin and glial fibrillary acidic protein (GFAP); and increased process outgrowth. It has not been established whether this type of cell division leads to production of neurons elsewhere in the CNS or in the retina of adult birds.

The BrdU-labeled Müller glial cells induced by toxin treatment were found in the ONL and INL, whereas Müller glial nuclei are typically only found in the INL. Some of these BrdU-labeled cells coexpressed two markers of retinal progenitor cells, Chx10 and Pax6, and some expressed a bHLH gene, *CASH-1*, a marker of early retinal progenitor cells. A small percentage (<10%) of the $BrdU^+$ cells subsequently were found to have neuronal morphology and to express markers of amacrine cells and bipolar cells. Transiently, however, many cells expressed a neurofilament (NF) marker, normally expressed on horizontal and ganglion cells in the retina. This appeared to be transient as the number of NF^+ cells decreased, and markers and morphology consistent with mature ganglion and horizontal cells were not seen. Researchers did

not find markers of PR cells. Many of the BrdU-labeled cells persisted in what appeared to be an arrested state for at least 12 days. They explored the possibility that the *in vivo* environment was limited for production of PR cells by culturing the toxin-treated retinas, but they were similarly unable to observe the genesis of PR cells, though they did find that toxin-treated retinas proliferated *in vitro* more than the untreated controls.

The division of Müller glia, following by genesis of neurons in the chick, can also be stimulated by growth factors in the absence of added toxins. Application of both insulin and FGF2 by intraocular injection, starting at P7 and continuing for three days, led to the production of many mitotic Müller glial cells. Fourteen days after the last injection, some $BrdU^+$ cells showed markers of amacrine and perhaps ganglion cells, and others showed markers of Müller glia. Similar to the findings following NMDA injections, no markers of PR cells were observed. To explore whether the type of toxin, and thus the target cell killed by the toxin, combined with growth factor injection might give a more specific replacement of the targeted cells, researchers injected several types of toxins with insulin and FGF2. When ganglion cells were targeted by the toxins, kainic acid, or colchicine, more cells with ganglion cell markers were observed. These studies led to the hope that the specific cells that die in various retinal diseases, such as ganglion cells in glaucoma, might be effectively replaced following stimulation of stem cells with the right cocktail of factors.

In a search for proliferation within the uninjured retina of mammals, four-week-old rats were injected intraperitoneally with BrdU for five days. The only incorporation reported was in the ciliary margin – that is, no labeling of Müller glial cells was observed. Opossum and mouse (as well as quail) cells were also examined, and no incorporation in centrally located Müller glia was observed. Some incorporation of BrdU in the ciliary margin of quail was seen, though less than in the chicken, and a few labeled cells in the ciliary margin of the opossum, but no labeled cells in the ciliary margin in the mouse.

Radial glial cells, astrocytes, and Müller glia have been shown to share some antigens with progenitor cells. This resemblance is more extensive that previously appreciated. We systematically analyzed gene expression in the developing and mature murine retina. We found that there were 85 genes preferentially expressed in Müller glia in the mature retina. Of these genes, the majority were also found in retinal progenitor cells. Some of these genes, such as *cyclin D3*, undoubtedly reflect the fact that Müller cells retain the ability to divide; but other genes are more enigmatic. Nonetheless, the results of the previous studies and the SAGE data argue that Müller glia should be explored further as a source of cells that might replace dying

neurons. This notion is in keeping with the idea that radial glial cells elsewhere in the developing CNS, as well as astrocytes in the mature forebrain, can serve as neuronal progenitor cells and stem cells, respectively.

14.5 GROWTH OF RETINAL NEUROSPHERES FROM THE CILIARY MARGIN OF MAMMALS

Culturing CNS tissue in the presence of EGF, FGF, or both would result in balls of cells with indefinite proliferation capacity, or *neurospheres*. Neurospheres were subsequently shown to produce neurons, astrocytes, and oligodendrocytes and were thus identified as originating from neural stem cells. Several groups have applied these protocols to the retina. Cultures from ocular tissues of mouse, rat, human, and cow have been made using FGF and EGF. Retinal neurospheres have been recovered and appear to have cells with indefinite proliferation potential, multipotency, and possibly totipotency.

The ciliary body from adult mice has the most enriched source of retinal stem cells. E14 RPE and retina and adult retina, RPE, iris, and ciliary body were examined for production of neurospheres in the presence of FGF, EGF, or both. No neurospheres with the proliferative capacity of stem cells were recovered from the embryonic or adult retina or from the adult iris, RPE, and ciliary muscle. A few neurospheres were recovered from the embryonic RPE, which included the peripheral margin, the precursor to the ciliary body. In the adult, neurospheres were only recovered from the pigmented cells of the ciliary body (Figure 14.1), termed the pigmented ciliary margin (PCM). Although there was some recovery from the E14 RPE, the number per eye increased 10-fold in the adult PCM compared to the entire RPE of the E14 retina. This curious finding suggests that these cells are formed at the end of development and not the beginning, the opposite of what one might have predicted. Alternatively, during the maturation of the PCM, there is an expansion of a few early stem cells. Stem cells are either rare or hard to culture, as only 0.6% of the adult PCM-plated cells will produce a neurosphere in the presence of FGF2. The neurospheres that do arise in the absence of FGF, albeit at a reduced frequency, do not require exogenous FGF. This is presumably because of endogenous FGF, as addition of anti-FGF antibody reduced the formation of neurospheres.

As the PCM neurospheres originated with the pigmented cells of the ciliary body, it was of interest to determine if pigment was necessary for their ability to be stem cells. This was examined in albinos, where they were isolated at a comparable frequency to that of pigmented animals. The RPE and ciliary body normally do not express Chx10. However, upon the genesis of neurospheres, cells began to express this retinal marker. If cultured under conditions that favor differentiation, the cells turned on markers of PRs, bipolar

cells, and glia. However, no markers of amacrine and horizontal cell interneurons, or of ganglion cells, were seen. It is significant that rhodopsin, a definitive marker of a PR cell, can be expressed. Rhodopsin is not expressed by other CNS neurospheres or neural cell lines derived from other CNS locations. Retinal progenitor cells normally do not make oligodendrocytes, and the oligodendrocyte marker, O4, was not observed.

There is a naturally occurring null allele of the paired-type homeobox gene, *Chx10*, in mice (*orj*). This mutant has a retina and RPE approximately 10-fold smaller than wild-type mice, with an expanded ciliary margin. When cultures were made from the ciliary margin of *orj*, approximately five-fold more neurospheres were recovered. These spheres were approximately one third of the size of the wild-type spheres, in keeping with the finding that *Chx10* is required for the full proliferation of retinal progenitor cells. Thus, *Chx10* must be involved with the allocation or regulation of the number of stem cells in the PCM.

The isolation of retinal neurospheres from the ciliary body of rats and from postmortem human and bovine eyes has been reported. The rat neurospheres were dependent upon FGF2 and could not be recovered from the retina, the RPE, or the nonpigmented portion of the ciliary epithelium. Retinal stem cells were also isolated from the rat eye, but from iris tissue rather than ciliary margin tissue. As shown in Figure 14.1, the iris and ciliary body are adjacent to each other at the margin of the eye. The iris cells were cultured as an explant in the presence of FGF, rather than as dissociated cells. Unlike the ciliary body cells, dissociated cells from the iris did not generate neurospheres. Cells that migrated out of the iris explants were able to express some neural markers, such as NF200, but not PR-specific markers. However, if the cells were transduced with *crx*, a homeobox gene important in PR differentiation, approximately 10% of the cells expressed rhodopsin and recoverin, two markers of PR cells. The ciliary-body-derived neurospheres can generate cells bearing the same retinal markers, including rhodopsin, without transduction of *crx*. The difference may be caused by culture conditions. The ciliary-body-derived cells can be grown as spheres, and this environment may support PR development without a need for *crx* transduction. When ciliary-body-derived cells were cultured as monolayers, as were iris-derived cells, the ciliary-body-derived cells did not express PR markers.

Tissue from the iris can be readily obtained for autologous grafts. It is far more difficult to obtain tissue from the ciliary body, with an accompanying risk of damage to the ciliary body. However, postmortem human and bovine PCM could produce spheres at low frequency, so it is possible that humans could be used as a source of donor cells. Such cells would not be as immunologically compatible as an autologous graft, but nonetheless might suffice, as it is still not clear how well tolerated retinal grafts might be.

That the pigmented ciliary body and iris cells are the source of retinal stem cells is rather surprising. It was expected that the periphery of the eye might be the area where stem cells would reside, as this is the location of stem cells in amphibians and fish. In the CMZ of amphibians and fish, the nonpigmented cells contiguous with the retinal epithelium, located nearest to the retina, make more retinal cells. However, stem cells have not been recovered from this area in mammals. An alternative prediction might have been that retinal stem cells would be the RPE cells. As explained previously, the RPE is quite plastic early in development in mammals and chicks and throughout life in urodeles, maintaining the ability to make retinal cells in response to certain conditions. However, the adult mammalian RPE has not been shown to generate neurospheres when cultured under the conditions described previously. Moreover, even the embryonic RPE does not supply many neurospheres in the neurosphere culture conditions. In contrast to the preceding predictions, the most robust source of retinal stem cells in the adult mammal is the ciliary body pigmented cells. These cells are immediately adjacent to, and contiguous with, the RPE, but they are not RPE cells, at least in terms of function, as described previously. We do not have markers that would help to further define them. They derive from the outer walls of the optic cup, and the inner walls normally produce the retina in normal development. It should be noted, however, that whether the iris stem cells are derived from the inner or outer walls of the optic cup is not clear at this time. Although the iris stem cells are pigmented, both the inner and outer walls of the optic cup normally develop pigmentation in the iris. Both iris- and ciliary-body-derived stem cells are pigmented, which may provide a useful marker for their prospective isolation.

14.6 PROSPECTS FOR STEM CELL THERAPY IN THE RETINA

A large number of the diseases of the retina are caused by degeneration of PR cells. Approximately 40% of the genes identified as human disease genes that lead to blindness are rod specific. Many of these diseases nonetheless lead to the loss of cone PR cells. This nonautonomous death of cones is the reason for loss of daylight, high-acuity vision. Thus, replacement of dying rods, or the retardation of the death of rods, might prevent or slow the death of cones. Replacement of dying cones themselves is another potential therapeutic approach. This is particularly appropriate when the etiology of the disease is not clear, as is the case in the most prevalent disease; age-related macular degeneration. The source of either rod or cone PR cells could be the endogenous stem cells themselves. The best scenario would be the stimulation of the division of Müller glia, which are distributed through the retina, followed

by the induction of PR differentiation. Unfortunately, as noted previously, Müller glia have not been observed to generate PR cells in the chick or any mammal that has been investigated. Nonetheless, future studies might lead to a manipulation that would stimulate PR production by Müller glia. A second source of PR cells might be the endogenous stem cells in the ciliary body or iris, left *in situ*. Although these cells can produce PR cells when cultured, they have not been shown to generate PR cells *in situ*. In addition, unless the cells could be made to migrate and cover the central retina, where most of our high-acuity vision occurs, they would not lead to retention or recovery of high-acuity vision. Still, if they could lead to retention of peripheral vision, some therapeutic benefit would be realized. Finally, engraftment of stem cells or of PR cells generated *in vitro* by stem cells can be attempted. The problems of graft rejection, if not an autologous graft, would have to be confronted, but they might not be as difficult to overcome as engraftment to sites in the periphery. Furthermore, iris-derived cells might be used as an autologous graft. Preliminary data of engraftment of the neurospheres derived from the PCM are promising. Injection of such cells into the vitreous body of postnatal day 0 rats led to the formation of many PR cells, expressing rhodopsin, in the ONL. If such cells could be formed in a diseased retina, then two possible benefits might be realized. One would be to simply prevent further degeneration of endogenous PR cells, which, as mentioned previously, can die by nonautonomous processes. The second benefit might be that the engrafted PR cells synapse with second-order neurons and provide vision themselves. To date, this has not been achieved. The concern here is that the site of engraftment might not support synaptogenesis – particularly at the advanced stage when much of the retina has degenerated, likely the stage when such therapies would be attempted. Nonetheless, such strategies are worth pursuing, particularly now that there are stem cells that can be manipulated to generate retinal cells; our understanding of the processes of the development of retinal cells has similarly been advancing.

14.7 DEVELOPMENT AND REGENERATION OF TISSUES DERIVED FROM THE INNER EAR

The entire vertebrate inner ear derives from the otic placode, a thickening of the dorsolateral surface ectoderm immediately lateral to the hindbrain. Like the lens and olfactory placodes, the otic placode invaginates and pinches off to form a single-layered ball of cells, now called the otic vesicle. From this simple epithelium, a large variety of tissues and cells types arise. The otic ectoderm is neurogenic for the first-order neurons of the eighth cranial ganglion, the statoacoustic ganglion. The ganglion neuroblasts are the earliest recognizable cell type; they delaminate from the otic ectoderm at

the otic cup stage even before it completes vesicle formation. The otic vesicle is also sensorigenic, generating 6–8 different sensory patches. Inner ear sensory organs subserve hearing and balance and are differentiated according to their function. There are three major classes of sensory organs: macula, crista, and acoustic. Acoustic organs vary substantially in structure and sensitivity across the vertebrates, reaching their highest complexity and frequency selectivity in the mammalian organ of Corti. In all inner ear sensory organs, the mechanosensory hair cells are interspersed among a field of supporting cells essential for hair cell survival and function. Finally, beyond the sensory patches, several types of nonsensory tissues derive from the otic epithelium. The most highly differentiated is the tissue that secretes the extracellular fluid, called endolymph, which bathes the apical surfaces of all otic epithelial cells. Endolymph contains an unusually high concentration of potassium ions. The tissue responsible for endolymph production is anatomically complex, highly vascularized, and endowed with many ion pumps and channels. Other nonsensory epithelia flank the sensory organs and may contribute, with the supporting cells, to the secretion of the specialized extracellular matrices perched above the hair cells to enhance their mechanosensitivity.

Lineage studies have yet to reveal all possible relationships among the constellation of inner ear cells, so we do not yet know if individual otic placode cells are truly pluripotent for all inner ear cell types. We are confident that mechanoreceptors and their supporting cells share a common progenitor in the bird and zebra fish inner ear and in the regenerating salamander lateral line. There is also evidence that sensory and nonsensory cells can be clonally related in the chicken ear. Preliminary studies in mice include a single clone with members in both the sensory saccule and nonsensory utricle. Furthermore, otic neurons and sensory cells can be related in the bird. To date, there is no direct evidence for the existence of a true otic stem cell – that is, one that divides asymmetrically to replicate itself while generating a daughter with an alternative cell fate or fates. Nonetheless, lineage studies indicate that multipotent progenitor cells constitute a normal feature of inner ear development, leaving open the possibility that similar cells may lurk in mature ears, where they might be poised to expand with appropriate signals or culture conditions.

A variety of growth factors and growth factor receptors have been associated with developing inner ears. However, it is important to distinguish between growth factors that may regulate cell proliferation and those that may influence cell fate specification in other ways, such as the role of FGFs in otic induction. Furthermore, many of the growth factor assays are performed *in vitro*, with the inherent risk that cells and tissues can change their growth factor responsiveness depending upon culture conditions. For example, culturing of neonatal sensory epithelia leads to upregulation of FGFR1/2 and

insulin-like growth factor receptor 1 (IGF-1R) in macular supporting cells and downregulation of EFGR in the organ of Corti. This caveat notwithstanding, members of several growth factor families can enhance cell proliferation of developing otocyst or neonatal inner ear tissues, either alone or in combinations, when presented in culture. These include bombesin, EGF, FGFs, GGF2, heregulin, insulin, IGFs, PDGF, and TGFα.

14.8 *IN VIVO* NEUROGENESIS IN POSTEMBRYONIC ANIMALS

14.8.1 Proliferation in Normals (or After Growth Factor Treatment)

The vestibular maculae are involved in sensing gravity, and in fishes and amphibians these organs continue to increase in size throughout life. Here, we focus on the sensory organs of warm-blooded vertebrates, where there is a marked contrast between birds and mammals in the timing of inner ear organogenesis. In both classes, sensory organs stop generating new cells approximately midway through embryogenesis. A notable exception is the vestibular maculae of birds, where there is ongoing addition and death of cells well beyond hatching. Cell counts or BrdU labeling have led to estimates that hair cells turn over with half-lives of 20 days, 30 days, or 52 days in the chicken utricular macula. Within two weeks of hatching, nearly 500 cells in the saccular macula and 1,400 cells in the utricular macula may be added per day with the steady-state addition of 850 hair cells per utricle per day reached 60 days after hatching. However, these numbers are apparently achieved in the absence of an amplifying progenitor. Rather, most progenitors divide once to produce a sibling pair consisting of one hair cell and one nonhair cell (presumed to be the supporting cell). $BrdU^+$ clusters exceeding three cells are rare 2–4 months after labeling. Thus, if there is a self-renewing stem cell pool in the mature avian macula, it consists of cells that divide on an extremely slow timescale. Interestingly, the neuronal colony-forming cells of the olfactory epithelium, thought to be the true stem cell of this sensory organ, are also rare (1 in 3,600 purified progenitors) and divide at a very slow rate. Ongoing receptor cell turnover (and regeneration) in the olfactory epithelium utilizes a transient amplifying progenitor pool that in turn generates a population of immediate neuronal precursors; the latter will divide symmetrically to make differentiated olfactory receptor cells.

In contrast to lower vertebrates, in mammals the vestibular macula is quiescent from birth. So, too, are the auditory organs of both birds and mammals. For example, BrdU injections failed to label cells in the adult mouse organ of Corti. However, in mice lacking the cyclin-dependent kinase inhibitors $p27^{kip1}$ or $p19^{Ink4d}$, cell turnover continues in postnatal animals. Dividing

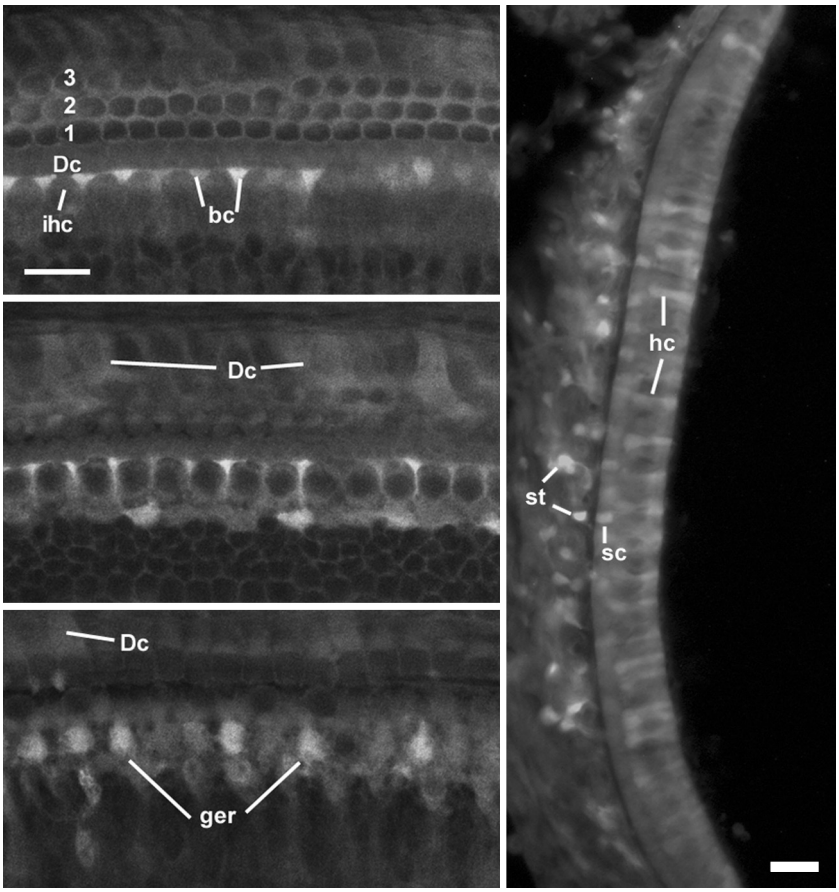

FIGURE 14.2

Nestin-GFP in organ of Corti (left) and utricular macula (right) of P5 mouse. In the cochlea, GFP is seen in border cells (bc) surrounding inner hair cells (ihc), Deiters cells (Dc) surrounding outer hair cells (outer hair cell rows 1, 2, and 3 are indicated), and greater epithelial ridge cells (ger). In the utricular macula, GFP is seen in stromal cells (st), supporting cells (sc) and hair cells (hc). *Figure courtesy of Ivan Lopez (UCLA).*

cells are observed several weeks after birth among hair cell (p19Ink4d−/−) or supporting cell (p27kip1−/−) layers. Prolonged mitosis in the p27kip1-null is accompanied by the differentiation of supernumerary hair cells and supporting cells. In both mutants, many hair cells eventually undergo apoptosis, leading to hearing loss. Nonetheless, these data suggest that the differentiated organ of Corti, which has never been shown to regenerate naturally, has the potential to harbor cells that can divide and differentiate under appropriate circumstances. In this context, it is intriguing that a subset of cells in both the auditory and vestibular organs of the neonatal mouse expresses

the neural stem cell marker, nestin (Figure 14.2). Nestin is rapidly downregulated about one week after birth, although it persists in nonsensory cochlear cells to day 15.

Several growth factors and cytokines can affect cell proliferation in undamaged, mature inner ear sensory organs or within sheets of sensory epithelial supporting cells. Insulin, IGF-1, or IL-1β enhanced proliferation of normal chicken utricular cells *in vitro*, and FGF-2 reduced proliferation. Rodent utricular cells proliferated in response to GGF2, EGF^{+}insulin, neu differentiation factor, TGFα, or TGFα+insulin but lost response to heregulin by adulthood.

14.8.2 Proliferation after the Destruction of Cells

The strongest evidence that progenitor cells reside in quiescent sensory organs of warm-blooded vertebrates comes from regeneration studies. Beginning with ground-breaking work in the late 1980s, a large number of studies have shown that damaged sensory organs will regenerate new hair cells in chickens, largely through a proliferative mechanism. Thus, despite the absence of ongoing turnover, supporting cells of the auditory organ (the basilar papilla) mount a vigorous mitotic response to damaging conditions. Up to 15% of them enter the cell cycle to generate both hair cells and new supporting cells. Whether supporting cells coexist alongside self-renewing stem cells remains an open question. Data suggest that only 1–4% of cycling cells in the regenerating basilar papilla will divide more than once within a three-day window after ototoxic drug treatment. Even among this pool, ongoing proliferation appears to be extremely modest, although lineage analysis has not yet been performed to provide an unequivocal measure of clonal expansion. Like the basilar papilla, chicken macular cells also divide and differentiate in response to hair cell loss. The proliferation index of the drug-damaged macula rises in the presence of TGFα or TNFα.

Once again, we contrast mammals with lower vertebrates. The mammalian macula has only a weak proliferative response to hair cell loss, with scant evidence that hair cells can be regenerated through a cycling intermediate. Instead, the maculae primarily restore their hair cells through direct transdifferentiation of supporting cells or through self-repair of sublethally damaged hair cells. The limited proliferation that accompanies recovery may serve to replace transdifferentiated supporting cells rather than hair cells. Hair cell recovery is promoted by the addition of TGFα, IGF-1, retinoic acid, and brain-derived neurotrophic factor *in vivo* or *in vitro*. The weak proliferative response of the cultured, drug-damaged macula averaged 26 $BrdU^{+}$ cells per sensory organ. This proliferation was enhanced 10-fold by addition of heregulin (a member of the neuregulin family) and to a lesser extent by EGF or TGFα alone or with insulin. Neither hair cells nor supporting cells of the

mammalian organ of Corti responded to heregulin, although more remote nonsensory epithelial cells of the cochlea did.

14.8.3 Transcription Factor Requirements

Although many cell types are generated from otic epithelium, relatively few transcription factors have been definitively associated with cell fate specification in the ear. *NeuroD*, *Neurogenin-1*, and *Eya1* are essential for otic ganglion cell fate. *Brn3a/Brn3.0* is needed for ganglion cell survival and differentiation. *Math-1* is required for hair cell development and survival, and *Pou4f3/Brn3c/Brn3.1* is needed for subsequent hair cell differentiation. Ectopic delivery of *Math1* leads to ectopic production of partially differentiated hair cells in the mammalian ear, both in the adult guinea pig and in cultures of postnatal rat sensory organs. Thus, some cells retain the capacity to switch to a hair cell fate even into adulthood.

14.9 *IN VITRO* EXPANSION OF OTIC PROGENITORS

Several groups have used immortalizing oncogenes to isolate cell lines from the developing inner ear and explore their differentiation potential. Efforts to expand unadulterated otic progenitor pools are just beginning, using either immature or differentiated otic epithelium as starting material. The work is presented in order, based on the age of the starting tissue. Several of the studies have been reported only in abstract form to date.

Culture conditions (EGF^+ periotic mesenchyme) that permit E13.5 cochlear progenitors to persist in culture long after they would normally become postmitotic have been elucidated. Mitotic progenitors generate islands of Math-1^+ hair cells, with numbers of hair cells continuing to increase two weeks after plating.

Cells from the newborn rat organ of Corti were dissociated, approximately 5–6 days after sensory progenitors become postmitotic *in vivo*. Filtering through fine (15 µm) nylon mesh resulted in the isolation of a population of small cells, 98% of which expressed nestin. Beginning with cell suspensions, spherical colonies called *otospheres* developed when cultured in the presence of EGF, FGF2, or both factors. After only two days of culturing, $BrdU^+$ progenitors expressing the hair cell marker myosin VIIA were observed, albeit in very small numbers (one per colony on average). About two cells per colony were immunopositive for the cell cycle inhibitor, $p27^{kip1}$, which normally labels the supporting cells of the organ of Corti. The number of myosin $VIIA^+$ cells increased to five cells per colony by 14 days, often appearing as a coherent islet. Some ultrastructural features of hair cells were evident in rare cells

after both two and 14 days *in vitro*. Hair cell differentiation was not enhanced by switching the otospheres to 'adherent' culture conditions that had been reported to induce neuronal differentiation in neurospheres. It is important to note that the method used in this study did not ensure that each otosphere originated from a single progenitor cell.

A preliminary study from another group reported successful generation of spherical cultures from mouse utricle or cochlea but only until postnatal day 6. Cells from 14- and 21-day-old sensory organs failed under similar culture conditions.

Spheres from the isolated organ of Corti of adult guinea pig have also been cultured. Initially the spheres did not express nestin or the supporting cell marker, cytokeratin. Serum or EGF supplements, or long-term culture, induced nestin expression and allowed differentiation of a small number of cells as hair cells (calretinin$^+$, myosin VIIA$^+$, prestin$^+$, or Brn3.1$^+$), supporting cells (cytokeratin+ or connexin+), neurons (NF+ or βIII-tubulin+), or astrocytes (GFAP+). The appearance of differentiated hair cells was extremely rare, at less than 1% of the cells in long-term cultures.

Most promising to date is the generation and differentiation of spherical cultures from single cells isolated from the utricular macula of the adult mouse. Taking their cue from the growth factor responsiveness of damaged vestibular sensory organs, Heller's laboratory used EGF, IGF-1, and bFGF to enhance sphere formation. The combination of EGF$^+$IGF-1 was most effective. Nestin$^+$, sphere-forming cells could be dissociated into single cells and then expanded into new spheres through several rounds. Approximately 2.5 spheres could be formed at each passage, suggesting that a small number of cells retain sphere-renewal capability. To induce differentiation, spheres were moved to adherent culture conditions in the presence of serum, but then grown for 14 days in serum-free conditions. The cells downregulated nestin and other markers of the early otic vesicle and upregulated markers of several differentiated cell types. Cells expressing hair cell markers were present in up to 15% of differentiated cells. Many showed features consistent with rudimentary stereociliary bundle formation and were surrounded by cells with an expression profile consistent with supporting cells. Math-1$^+$ cells colabeled with BrdU, indicating that these hair cells arose from a proliferative progenitor. Significantly, neurospheres grown from the subventricular zone of the mouse forebrain, cultured under identical conditions, did not generate hair cells. This suggests that the utricular stem cells have a special capacity to form inner ear mechanoreceptors. Macular-derived spheres also produced a significant percentage of cells with neuronal (6%) or astrocytic (35%) phenotypes, cell types normally absent from the macular epithelium. Spheres could also

generate an array of ectoderm, mesoderm, or endoderm derivatives when the cells were delivered into the amniotic cavity of stage 4 chicken embryos. The incidence of sphere-forming stem cells was rare even under optimal growth conditions: 0.07% of plated cells. This is consistent with the absence of BrdU labeling of adult sensory organs in the mouse and suggests that stem cells may be both rare and quiescent *in vivo*.

Heller's group also defined culture conditions that induced embryonic stem (ES) cells to form spheres containing many $BrdU^+$, $nestin^+$ cells. Under growth conditions the cells expressed nestin and markers of early otic vesicle (e.g., Pax2, BMP4, and BMP7). Under differentiating conditions, early otic markers plummeted, and markers of hair cells and supporting cells rose. This is extremely encouraging as it suggests that it may be unnecessary to start with endogenous ear tissue to generate otic progenitors for therapeutic purposes.

14.10 PROSPECTS FOR THERAPY

As methods for culturing otic stem cells become established, one can ask whether the addition of different transcription factors (such as Math1) can induce differentiation of one cell type over another. Stem cells or differentiated cells derived from various sources could then be implanted back into the animal to ask whether the cells will integrate and provide restoration of function in animal models of inner ear cell loss. The ear has some definite advantages for delivery of cells or gene transfer vectors, such as viruses. Surgical approaches to the fluid compartment of the inner ear provide access to the inner ear hair cells without requiring systemic delivery. For example, it is possible to inject through the round window, delivering substances, such as neurotrophins, that can influence survival of sensory tissues or ganglion cells. Delivery of cells that release soluble molecules, such as growth factors, could potentially provide functional restoration without necessarily restoring structural integrity. On the other hand, structural integration of replacement mechanoreceptors will probably be essential, even if extremely difficult, in view of the precision with which hair cell stereocilia must interact with the nonsensory matrices overlying them. Replacement of functional ganglion neurons, rather than sensory receptor cells, may be less problematic. We anticipate considerable progress in these and related therapeutic approaches over the next decade, although substantial technical hurdles remain.

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FOR FURTHER STUDY

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CHAPTER 15

Epithelial Skin Stem Cells

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15.1 A BRIEF INTRODUCTION TO MOUSE SKIN ORGANIZATION

The complex process of constructing the protective cover of the body starts around day 9 of mouse embryonic life (E9). Through a succession of signal exchanges between the ectoderm and mesoderm, a very structured tissue emerges, which is designed to seal and protect the body of the animal against a diverse range of environmental assaults. The barrier function, or the sealing of the body from the external environment, is essential for the survival of the animal and is fully completed at E18, the day before the mouse is born. Hair follicle morphogenesis starts around E13 and takes place in waves until just after birth. The hair follicles are specified embryologically, and, consequently, the maximum number of hair follicles that an animal will have for the rest of its life is determined before birth.

Mature skin is composed of two main tissues: the *epidermis* and its appendages, largely composed of specialized epithelial cells (keratinocytes), and the *dermis*, largely composed of mesenchymal cells. Epidermis consists of an innermost basal layer (BL) of mitotically active keratinocytes that express diagnostic keratins K5 and K14. As these cells withdraw from the cell cycle and commit to a program of terminal differentiation, they remain transcriptionally active and move upward toward the skin surface. As they enter the spinous layers, they first protect themselves by switching from expression of K5 and K14 to K1 and K10, which form keratin filaments that bundle and provide a robust inner strength to the cells. As they complete this process, they next go about producing the barrier by synthesizing and depositing proteins, such as involucrin, loricrin, SPRR, and others beneath the plasma membrane. In addition, as the cells enter the granular layer, they begin to produce

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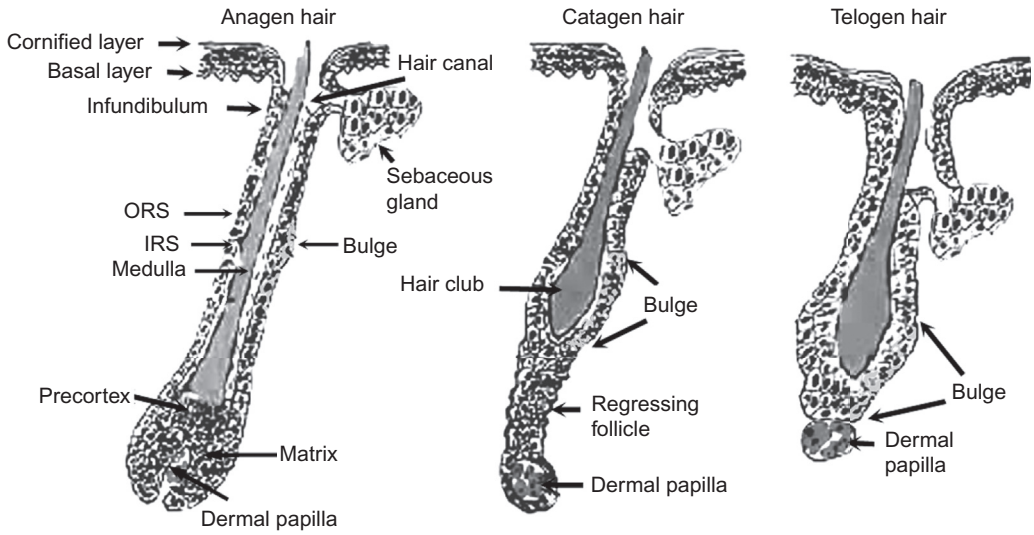

FIGURE 15.1 The hair follicle cycle in adult mouse skin.

and package lipids into lamellar granules, and they produce filaggrin, which bundles the keratin filaments even further to form cables. As the cells complete these tasks, an influx of calcium activates transglutaminases, which cross-link involucrin and its associates into a cornified envelope that serves as a scaffold for organization of the extruded lipids into external bilayers. Metabolically inert, these cells undergo an apoptotic-like loss of their nuclei and organelles, resulting in flattened, dead squames. These highly keratinized cells provide a seal to the body surface; from which they are eventually sloughed, replaced by differentiating cells that are continuously moving outward. Epidermis regenerates itself every few weeks and has a remarkable ability to heal, expand, or retract in response to environmental cues.

Epidermal appendages, including the hair follicle and the sebaceous gland (SG) (Figure 15.1), are inserted deep into the dermis. The hair follicle is a complex structure made of at least eight different cell types. The hair shaft is located in the middle of the follicle and grows upward, 'breaking' the surface of the skin. Concentric layers of cells surrounding the shaft form the outer root sheaths (ORS) and inner root sheaths (IRS) (Figure 15.1). The BL of the epidermis is contiguous with the ORS of the epidermis, which the IRS degenerates in the upper portion of the follicle, liberating the hair shaft.

The basal epidermal layer and the ORS share a number of common biochemical markers, including K5, K14, and α6β4 integrins, and they represent

potent proliferative compartments of the follicle. The highest proliferation, however, is seen in the matrix cells at the bulb of the follicle, which generate the IRS and the hair cells. Matrix cells surround a pocket of specialized mesenchymal cells, the dermal papilla (DP), with potential hair growth inductive properties. Matrix cells express a number of transcription factors (e.g., Lef1 and Msx-2) implicated in hair follicle differentiation. To some extent, IRS cells resemble epidermal granular cells, but their granules are uniquely composed of trichohyalin. In contrast, hair cells are more similar to stratum corneum because they are metabolically inert as they complete differentiation; however, they express a unique set of hair-specific keratins. Finally, the SG is located in the upper portion of the hair follicle, just above the arrector pili muscle and is made of fat-containing cells that will release their lipid content into the hair canal. The mitotically active cells of the SG express K5 and K14.

At birth, the morphogenesis of the hair follicle is almost complete. Certainly by day 4 of postnatal life all hair follicles in a mouse skin have reached maturity and hair shafts start to appear at the skin surface. In postnatal life, the ORS of maturing follicles widens a bit on one side, creating a bulge. Located below the SG, and near or at the juncture of the arrector pili muscle, this specialized region is thought to be the compartment where epithelial stem cells reside.

Stem cells in postnatal skin are required for self-renewal of the epithelial tissues. Like epidermis, the cells of the postnatal hair follicles are in a state of flux, undergoing perpetual cycles of growth, regression, and rest. In relative synchrony at least for the early hair cycles, mouse hairs continue to grow and complete anagen, the growth phase of the hair cycle. Around 17 days of postnatal development, anagen ceases and massive cell death occurs in the bulb of the hair follicle, the catagen phase. Follicle cells below the bulge area are destroyed, except for the DP, which moves upward by virtue of its attachment to a shrinking basement membrane that separates the epithelial and mesenchymal compartments. The DP comes to rest just beneath the bulge. At this stage, hair follicles enter their rest, or telogen, phase. The new anagen is re-initiated around 21 days of life, and hair follicles start again performing this precise choreography of changes. As yet unidentified signals from the surrounding environment and from the DP are thought to be necessary to reactivate stem cells to restart the process of follicle morphogenesis.

Adult mouse skin contains epithelial stem cells located in at least two different compartments: epidermis and hair follicle. This review focuses primarily on the hair follicle stem cells located in the bulge area of the hair follicle. Despite their location in the hair follicle ORS, cells within this compartment have been shown to give rise to all the different hair cell lineages, the SG, and the epidermis.

15.2 THE BULGE AS A RESIDENCE OF EPITHELIAL SKIN STEM CELLS

It has been postulated that stem cells should have evolved special mechanisms of protecting their DNA against accumulation of replication errors, which may otherwise result in a high rate of tissue cancer. These mechanisms may involve either slow or rare cell cycling and/or asymmetrically segregating the newly synthesized DNA into the non-stem cell progeny (the immortal DNA strand hypothesis). To date, the validity of these hypotheses remains uncertain. In the hair follicle, the cell cycle times of ESSCs relative to transit-amplifying (TA) (progeny) cells have been difficult to measure with precision. However, the concept of SCs as rarely dividing cells is in good agreement with the low mitotic activity detected in the bulge. Second, in a similar epithelial system, there are some suggestions that intestinal epithelial stem cells might be segregating their newly synthesized DNA strands asymmetrically such that adult stem cells always inherit the original strand of DNA. In the future, it will be important to assess whether asymmetrical divisions might also occur in the bulge. However, the lack of markers for SC and the apparent scarcity of SC divisions in postnatal skin have made this issue technically difficult to address.

What is certain is that a population of slow-cycling and/or asymmetrically dividing cells, likely to be the stem cells, is detected in the skin epithelium. When mouse pups are administered tritiated thymidine or BrdU from 3 to 6 days of postnatal life, the label is incorporated into the newly replicated DNA and is found abundantly distributed in the BL and ORS. When labeling is subsequently followed by 4 to 8 weeks of chase, transient amplifying cells quickly divide and dilute the label, as they move up into the differentiated layers of hair follicle and epidermis, eventually being eliminated from the tissue. In contrast, the slow-cycling cells retain the label and are kept within the tissue. Using this approach, label-retaining cells (LRCs) have been localized predominantly to the bulge area of the murine hair follicle, with fewer scattered cells in the BL of the epidermis or elsewhere in the epidermis or hair follicle. If the LRCs in the skin epithelium are stem cells, then the bulge represents a major stem cell compartment of postnatal skin epithelium. A strategy has been developed to fluorescently tag and isolate infrequently cycling stem cells in tissues. The method was based upon driving expression of a green fluorescent protein (GFP) fused to histone H2B in a cell-type-specific and tetracycline-regulatable fashion. When expression is shut off, all dividing and differentiating cells dilute out the label and/or are sloughed from the tissue, leaving only the infrequently cycling cells brightly labeled. The system was successfully used in skin to monitor the LRCs in the bulge and to isolate and characterize these cells.

With BrdU as a means of labeling the bulge cells, LRCs have been shown to contribute to the formation of both lower hair follicle and the upper region of the hair follicle ORS, called the infundibulum. This is based on the observation that LRCs can be found exclusively in the bulge area during the telogen phase of the hair cycle, but, on initiation of a new anagen, cells containing less BrdU signal, presumably derived from division of bulge cells, were also found in the lower and upper ORS, matrix, and even medulla (in the hair shaft). The correlation with the bulge was indirect with a single-label experiment. Using a double-label technique that marked the upper ORS cells based on distinct division time, a flux of the infundibular (upper follicle above the bulge) cells into the BL of the epidermis in neonates and in wounded adult skin was observed.

Using the tetracycline-regulatable H2B-GFP pulse and chase system, LRCs were both efficiently and sufficiently brightly labeled such that progeny could be tracked for eight divisions. At the start of the new hair cycle (early anagen), transiently amplifying progeny of bulge LRCs could be found in the matrix in the follicle bulb. Remarkably, even the terminally differentiating hair and inner root sheath cells could be assigned on the basis of fluorescence as being cellular derivatives of the bulge. Moreover, in response to a wounding stimulus, these H2B-GFP LRCs appeared to exit the bulge and migrate toward the wound site. In transit to replenish the damaged epidermis, these cells also appeared to proliferate, deposit a fresh basement membrane, and change some biochemical properties.

The bulge area of the rat vibrissae has been shown to contain multipotent cells that have tissue morphogenesis ability. Although clonal analyses will be required to establish the multipotency of individual bulge cells, microdissected rat vibrissae bulge tissue transplanted onto the back of athymic mice yielded entire hair follicles, SG, and epidermis, as further demonstrated by later studies. Bulge cells also exhibited the highest colony-forming ability when cells were placed in tissue culture, and this was true irrespective of hair follicle cycle stage. In other follicle regions, efficient, colony-forming cells were not found except for the bulb of the rat vibrissae, where such cells were detected only in early anagen. Although the mouse vibrissae and pelage follicle bulge did not function in these assays, this seemed to be due to technical difficulties arising from the size of the follicles, because human follicles displayed a distribution of clonal cells that was similar to that of rat vibrissae.

The rationale for attributing clone-forming ability to stem cells, at least in the hair follicle, is based on the assumption that the high tissue regenerative potential of stem cells will be manifested as an ability of these cells to give rise to large colonies of cells in tissue culture. Consistent with this notion is the fact that cultured skin keratinocytes are capable of long-term skin engraftment and regeneration of injured skin.

There is a paradox between the slow-cycling features of bulge stem cells in skin and the ability of cultured cells isolated from the bulge region to form large colonies over a two-week period. *A priori* it is possible that stem cells in culture divide more rapidly either through the trauma of the isolation process or through their exposure to culture medium. Alternatively, the large colony may arise not from expansion of stem cells *per se* but instead from a rare asymmetrical division of the original stem cells to produce a heterogeneous mixture of stem cells and TA cells. The authors' data suggest that a concentrated population of isolated stem cells may be necessary to achieve efficient colony formation *in vitro* or successful grafting *in vivo*.

It is not yet clear precisely what the relation is between LRCs identified in the bulge and the cells dissected from the bulge that give rise to cloned colonies or grafted follicles. There is some indication, however, that LRCs are the cells that form colonies in culture. One caution about the use of *in vitro* approaches for stem cell studies is that the study of stem cells outside of their tissue may be 'the biologic version of the Heisenberg principle.' In other words, properties attributed to stem cells, (i.e., colony-forming or grafting), cannot be studied 'without altering the tissue and in so doing altering the stem cell state of the tissue.'

Label retention ability seems to be a good marker for characterizing a slow-cycling and/or asymmetric dividing population of cells that are likely to be stem cells. However, it may be possible that in the tissue there are stem cells that do not possess LRC properties. Thus, although intriguing as a potential stem cell marker, label retention is circumstantial and clearly dependent on the label-chase scheme used. Thus, it is possible that researchers may be overlooking the existence of stem cells that are not label retaining but that are nevertheless critical in tissue regeneration. Future studies will be necessary to evaluate this possibility.

Studies using retroviral transduction of keratinocytes have revisited the concept that the bulge contains a population of stem cells that is the normal source of tissue regeneration. Dermabraded mouse skin was transduced with a retrovirus encoding the β-galactosidase reporter gene, followed by a 36-week chase with five cycles of depilation-induced hair follicle cycles. If the entire skin epithelium is generated from multipotent, long-lived stem cells within the bulge, then the distribution of β-Gal positive cells might be expected to be uniform across the different cell lineages in the hair follicle and epidermis. The observed result was puzzling because, even after the long chase and repeated stimulation of stem cells, only 30% of the hair follicles were uniformly blue. The rest of the follicles were positive in the ORS, IRS, or SG but not in all three locations. Moreover, there were defined units of blue in the epidermis far away from the hair follicle. Similar results were obtained

when the skin of mosaic mice obtained by aggregation of two stem cell types of different genetic background was analyzed. These results suggest that there may be multiple classes of stem cells that are long lived, each with restricted potency that may not even reside in a single niche (the bulge).

Although these data are difficult to reconcile solely on the basis of the multipotent bulge hypothesis, it does not necessarily rule out the hypothesis. It could be that the special slow-cycling properties of multipotent stem cells render them exceptionally difficult to infect. Those follicles with mosaic patterns of β-Gal expression may arise either from perhaps more easily infectible cells that have left the niche and are already committed to particular lineages. It is well established that mosaicism can arise from a chromatin inactivation mechanism, referred to as position effect variegation that often operates to spontaneously silence retroviral or transgene promoter activity. This mechanism may operate differently in stem cells and their committed progeny, leading to a nonrandom silencing pattern of gene expression of the reporter, in which the transduced retroviral DNA is silenced with higher efficiency in stem cells. This said, an equally plausible hypothesis is that lineage-specific and multipotent stem cells exist in the skin. Irrespective of resolving the issue of stem cell variability, it should be noted that again there is the underlying caveat that different kinds of injury (i.e., dermabrasion, depilation, cell grafting) have been used as representative models for the normal tissue homeostasis. Resolution of these various issues will require clonal analyses with single stem cells and/or their descendants.

Despite the controversy, the evidence is compelling that:

1. The bulge contains a large pool of LRCs that participate in the regeneration of the hair follicle and of the wounded skin;
2. Bulge cells form large colonies in culture; and
3. Bulge cells regenerate the entire hair follicle, SG, and epidermis after grafting.

These cells have many of the characteristics expected of ESSCs. Keeping the 'principle of uncertainty' in mind, we consider in the next sections the different models of stem cell activation and stem cell function in the skin epithelium.

15.3 MODELS OF EPITHELIAL STEM CELL ACTIVATION

Early data on hair follicle growth and stem cell function has suggested that the bulb of the hair follicle, containing matrix cells, is the residence of stem cells. This hypothesis was hard to explain because of the cycling nature of the bulb, which undergoes extensive apoptosis in catagen, leaving only a

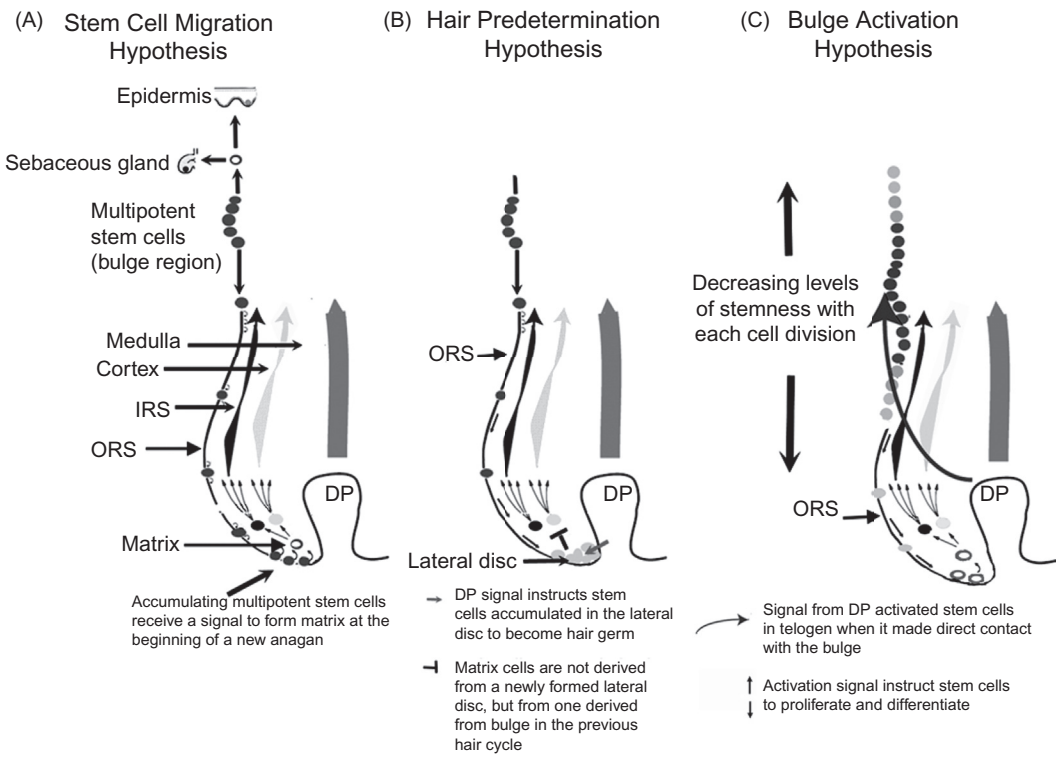

FIGURE 15.2 Models of stem cell activation in the hair follicle.

small strand of epithelial cells connecting the DP and the bulge. In addition, the hair follicle can completely regenerate even after the bulb is surgically removed.

Evidence that the bulge contains a large pool of LRCs, together with the appealing location of the bulge as a 'niche' at the base of the permanent portion of the hair follicle, suggested a novel model for hair follicle growth, the 'bulge activation hypothesis' (Figure 15.2C). In this model, stem cells are activated by as yet unidentified signals transmitted through direct contact with the DP. Such contact occurs at the end of each hair cycle, as the surrounding dermal sheath shrinks during the apoptotic phase, and drags the DP upward until it comes into contact with the bulge. Although this contact does not appear to be the sole source of stimulation, it seems to be a necessary stimulus to activate one or more bulge cells to divide, resulting in TA cells with decreasing levels of stemness.

At least in neonate and wounded adult skin, stem cell activation may be accompanied by a flux of upper ORS cells to the epidermis. When cells exit

the bulge and migrate upward, they take on the fate of a TA epidermal or SG cell. When they migrate downward during early anagen, they give rise to a population of TA ORS and matrix cells that in turn further specialize to form the IRS, cortex, and medulla. When matrix TA cells cease dividing, perhaps through exhaustion of their proliferative capacity, the bulb of the hair follicle undergoes apoptosis (catagen phase). Mutations in a number of genes, including those encoding the transcription factors hairless and RXRα, result in a failure of the DP to be dragged upward at the end of the first postnatal hair cycle. The consequence is a block in bulge activation and a loss of all subsequent hair cycles.

The bulge activation hypothesis on its own does not explain why bulge cells and DP can sometimes sit adjacent to each other for extended periods of time, without stem cell activation, but further experiments may bring more evidence in support of this model. Although a number of possibilities could be envisioned to account for this lag period, one model is that stem cells in the bulge may need to replenish themselves during telogen to reach a critical threshold density.

A second model for stem cell activation is the 'cell migration, or the traffic light hypothesis.' This model is based on the observation that clonogenic and morphogenic cells in the rat vibrissae are found in the bulge at any hair follicle stage, but they are found at the base of the bulb in late catagen and early anagen. The telogen phase is short if not absent from vibrissae, and a new vibrissae cycle is initiated before the DP has moved upward completely to come into contact with the bulge. Stem cells, or their more or less committed progeny, appeared to migrate along the ORS to come in contact with the DP, which then seemed to signal their pathway to differentiate. During catagen and early anagen, these bulge cell derivatives accumulated at the base of the hair follicle in a relatively undifferentiated state. Later in anagen, this 'traffic stop' appeared to be removed, and cells at the base of the bulb seemed to progress to become matrix and then to differentiate into IRS and hair shaft cells. While in transit along the basement membrane surrounding the ORS, these cells were not able to form colonies or engraft successfully, perhaps because of their low concentration. In this model, stem cells or their immediate progeny leave the niche and, with an estimated speed of ~100 μm/day, they migrate with minimal division along 2 mm of ORS to reach the bulb (Figure 15.2A).

A third model has come from an integrative reassessment of the literature, with a novel interpretation of the accumulated data. Known as 'hair follicle predetermination,' this model is based on multiple studies of hair follicle growth, and although speculative, it offers an alternative explanation for conflicting results. In this model, during mid and late anagen, SCs are stimulated

to leave the bulge niche and migrate away to form the ORS but not the matrix or the rest of the hair. It is postulated that these SC progeny then accumulate at the base of the follicle, adjacent to the DP, where they are modified to form a 'lateral disc' in the bulb (Figure 15.2B). During telogen, the lateral disc maintains contact with the DP, and, upon the next anagen, it proliferates (upward) and forms the new matrix and the inner layers of the hair follicle. During this phase, newly activated bulge cells give rise to the ORS and replenish the lateral disc. Therefore, the new hair follicle is formed from lateral disc cells that are predetermined for this role during the previous hair cycle. Based on the recent H2B-GFP LRC study, it would seem that a modification of the predetermination model is now warranted to explain how, possibly as a secondary step following the initial 'lateral disk' activation, the H2B-GFP LRCs are stimulated to exit the bulge area, proliferate, and participate in the formation of the matrix, all within a single anagen. Finally, some models postulate the presence of multiple populations of nonbulge skin epithelial stem cells that are long-lived and either unipotent or of interchangeable potential (hair cells can make epidermis and/or SG and vice versa). These epidermal cells must divide more frequently than bulge LRCs to account for why they were much less brightly labeled with H2B-GFP in the pulse and chase experiments outlined above.

15.4 MOLECULAR FINGERPRINT OF THE BULGE – PUTATIVE STEM CELL MARKERS

One of the major problems in the advancement of the stem cell field has been the lack of specific biochemical markers characteristic for stem cells despite extensive searches. This has raised the possibility that stem cells may have few if any unique features, but instead it is their niche that instructs stem cells to behave differently than their offspring. Infrequently cycling bulge cells have been isolated based on their H2B-GFP retention, and their transcriptional profile has been compared with progeny in the BL of the epidermis and ORS of the hair follicle. Probing a third of the mouse genome, 154 mRNAs scored as being upregulated by greater than two-fold in the bulge LRCs relative to the BL/ORS. Similar studies using a K15-GFP transgenic mouse to purify bulge cells have recently corroborated many of these results. Although detailed analyses of all these factors will take time to complete, immunofluorescence microscopy has already indicated that a number of the proteins encoded by these bulge-upregulated mRNAs are expressed within the bulge, but are not necessarily specific for the brightest LRCs within the bulge. Additionally, based upon the comparison made, these mRNAs cannot be considered as exclusive markers for the bulge, and indeed some are expressed in other cell types within the skin. This said, some appear to be specific for the

bulge, and a few appear to be better markers for the bulge than previously identified factors.

Traditionally, some of the best characterized markers relating to stem cell function are integrins. β1 and α6 integrins are increased in keratinocytes in culture with high proliferative capacity, and they also appear to be enhanced in the bulge area, relative to the lower and upper segments of the anagen phase follicle. Interestingly, both β1 and α6 integrin mRNAs are among the 200 mRNAs identified in three different populations of stem cells when compared with their TA progeny, suggesting that the correlation between integrin levels and 'stemness' may extend to stem cells beyond those of the skin.

The relation between integrin levels and stem cells is intriguing, because it suggests the possibility that stem cells may be kept tight within the niche by adherence to each other and to the basement membrane. The need for stem cell progeny to migrate along the basement membrane surrounding the epidermis and its appendages may be fulfilled by a reduction in cell-substratum anchorage through downregulation of integrins. Alternatively, however, it could be that, because cell proliferation is dependent on integrins, cells with high proliferative capacity by nature have elevated integrin levels. The extent to which regulation of integrins in the bulge is a reflection of hair growth mechanisms versus stem cell characteristics *per se* is an important issue, and one that has not yet been unequivocally resolved. Interestingly, upregulation of cell migration associated integrin β6 in the ORS during anagen, when bulge and ORS cells migrate downward to make the new hair follicle, seems to suggest the second possibility.

What are the cellular mechanisms that regulate differences in integrin levels? Are there specific stem cell cues that prompt these changes in integrin levels in TA cells to allow them to exit the niche and migrate? These questions remain to be addressed. In the meantime, integrins are thought to play a major role in the skin, in general, irrespective of stem cells in regulating epidermal adhesion, growth, and differentiation.

Proteins expressed differentially in the bulge include: keratins K15 and K19, both also present in a large fraction of BL cells; CD71 low; S100 proteins; E-cad low; p63; and CD34. Of these, CD34, S100A6, and S100A4 were all upregulated at the level of mRNA expression in the bulge.

Although it is found throughout the mitotically active cells of the skin and hence is not restricted to the bulge, a p53 homologue protein called p63 may play a role in stem cell function. P63 null mice exhibit defects in epidermal proliferation, leading to the possibility that p63 might function in repressing epidermal growth factor (EGF) receptor and other cell cycle-regulated genes. Interestingly, a naturally occurring dominant negative isoform of p63

is induced in the stratified epithelial layers, where it appears to promote cell cycle withdrawal and commitment to terminal differentiation.

Another putative regulator of stem cell function is c-myc. Transgenic mice with elevated c-myc levels in the BL and ORS, which encompass both stem and TA skin cells, show epidermal hyperproliferation, severely impaired wound healing, and loss of hair. Microarray profiling of bulge LRCs adds new candidates that may be involved in regulating the transition between infrequently cycling skin stem cells and their transit-amplifying progeny. As these candidates are systematically tested, new inroads into our understanding of stem cell activation are likely to emerge.

15.5 CELL SIGNALING IN MULTIPOTENT EPITHELIAL SKIN STEM CELLS

What coaxes stem cells to become hair follicles rather than epidermis is one of the most fascinating questions in the skin stem cell field. Many of the answers are still not in, but hints have begun to emerge in the past five years. It has long been known that in embryonic development, as in postnatal hair cycling, this decision to coax cells toward the hair pathway involves critical cross-talk between the epithelial and mesenchymal cells. What is interesting is that similar signals seem to be used in specification of not only hair but also nail, mammary glands, and teeth.

In embryonic development of skin and its appendages, wnt and bone morphogenic pathway (bmp) signaling have surfaced as two major players that are critical for normal morphogenesis. Gene targeting of Lef1, β-catenin, sonic hedgehog, and the noggin inhibitor of the bmp pathway results in reduction, loss, or developmental impairment of hair follicles.

The wnt signaling acts through a large family of soluble wnt morphogens that recognize a specific receptor family known as frizzles to stimulate β-catenin, a dual protein that acts at the crossroads between cell adhesion and cell signaling. A wnt signal results in an inhibition of β-catenin degradation, leading to an accumulation of the protein above and beyond what is required for adherens junctions. β-catenin can then complex with members of the Lef/Tcf family of HMG DNA binding proteins and affect expression of transcription of downstream target genes. A truncated, constitutively active form of β-catenin expressed at high levels in the ORS of BL results in excess skin and *de novo* initiation of hair follicle buds in postnatal skin, a characteristic normally specific to the embryonic skin. Taken together, these data suggest that wnt signaling may induce adult stem cells into a state more specific for their embryonic relatives or that the wnt signaling has a major role in specifying hair follicle fate to relatively undifferentiated cells.

In the adult hair follicle, two members of the Tcf/Lef1 family are expressed in strategic places. Lef1 is in the matrix, precortex, and DP, whereas Tcf3 is in the bulge and the ORS of skin epithelium. mRNA profiling revealed that Tcf3 mRNA is also preferentially upregulated in bulge LRCs, suggesting that this regulation is at the level of gene expression. The localization of Tcf3 in the bulge is intriguing, because a related protein Tcf4 is found in the intestinal stem cell compartment, or crypt, where it appears to play a role in intestine stem cell maintenance. *In vitro*, Tcf3 acts as a repressor in the absence of wnt signaling, and, in its presence, it can be converted to an activator. *In vivo*, transgenic expression of the repressor forms of Tcf3 both in stem cells and TA cells of the skin results in a lethal phenotype, in which epidermal basal cells are conferred ORS characteristics. Taken together, these findings suggest that Tcf3 in the bulge acts as a repressor. Although target genes for Tcf3 have not yet been identified in the skin, one candidate is c-Myc, an established Tcf/Lef1 target gene, which induces hyperproliferation and appears to deplete the stem cell compartment of the skin when c-Myc is overexpressed in transgenic mice.

In contrast to Tcf3, Lef1 is expressed in the matrix but accumulates more strongly in the nuclei of precortical cells. The progenitor cells of the hair shaft precortex express a bank of hair-specific keratin genes that possess Lef1 binding sites in their promoters. Precortex also shows nuclear β-catenin, and it expresses a wnt-responsive reporter gene TOPGAL. These cells are thus likely to receive a wnt signal, and wnt expression is seen in this region of the anagen hair follicle. Interestingly, when expressed in hair precursor cells in transgenic mice, dominant negative forms of Lef1 result in the production of SG cells while loss of β-catenin results in production of epidermal cells at the expense of hair differentiation (Figure 15.3). In contrast, the transcription factor GATA3 has been demonstrated to be indispensable for inner root sheath differentiation. Curiously, Lef1 and GATA3 have both been implicated in hematopoietic stem cell lineage determination.

Although wnts are clearly necessary for β-catenin stabilization, to obtain a wnt response, a cell must express Tcf/Lef factors. Noggin, a bmp inhibitor, can induce Lef1 expression in keratinocytes *in vitro*, a feature corroborated in keratinocytes and skin epithelium null for the Bmp receptor-1a. When these cells are also treated with Wnt3a, they affect the transcription of wnt-responsive reporter genes. In most cases, this combination leads to transactivation, but curiously, the E-cadherin promoter seems to be downregulated by these factors. *In vivo*, downregulation of the E-cadherin promoter accompanies induction of hair placodes in embryogenesis and secondary hair germs in cycling hair follicles, and transgenic elevation of E-cadherin blocks follicle morphogenesis. Taken together, these findings suggest a model whereby activation of wnt signaling and inhibition of bmp signaling work together to

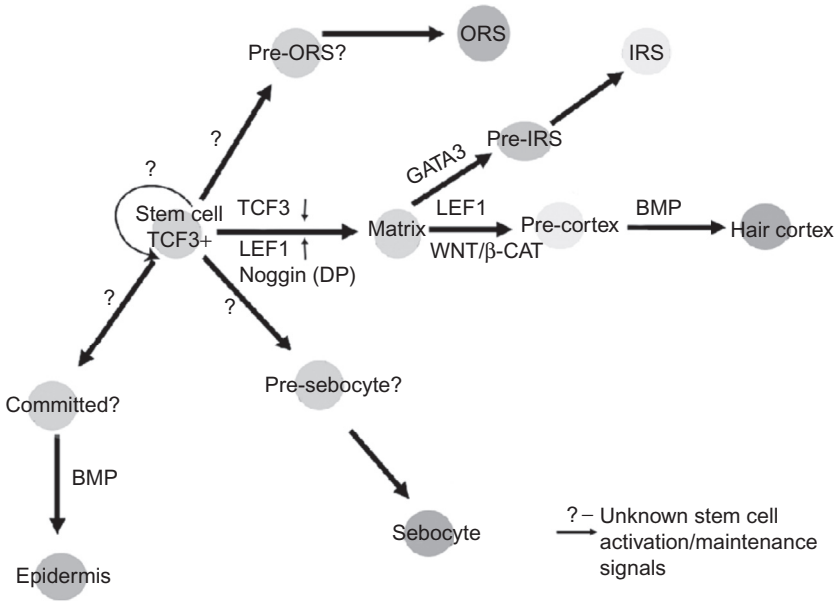

FIGURE 15.3

Schematics of signaling pathways in stem cell activation and fate choice.

maintain wnt responsiveness in skin epithelial cells and that, through down-regulation of E-cadherin, the cells are able to undergo the epithelial cell-cell remodeling, necessary for follicle morphogenesis. Moreover, the findings further suggest that adult and embryonic stem cells may use similar signals to control their differentiation.

An intriguing feature of the wnt signaling pathway is that, when constitutively activated, it leads to elevated levels of tissue progenitor cells in skin, brain and intestine. Additionally, purified Wnts stimulate isolated hematopoietic stem cells to proliferate in culture, and, following skeletal muscle injury, Wnts appear to mobilize resident stem cells during the regeneration process. Taken together, these findings implicate the wnt signaling pathway in the self-renewal of stem cells and/or their transit-amplifying progeny. Consistent with these observations is the finding that the growth-restricted environment of the bulge is associated with an upregulation of mRNAs more typically affiliated with wnt-inhibition. Future studies will be required to assess whether this tantalizing correlation is functionally significant.

15.6 COMMENTARY AND FUTURE DIRECTIONS

Probing more deeply into the molecular mechanisms of stem cell maintenance and lineage determination in the skin is now possible using factors that distinguish putative bulge stem cells from their progeny. Interestingly, a large fraction of these factors is either secreted or transmembrane factors likely involved in interactions with the environment. Some of these have been previously associated with stimulation of different cell types that coincidentally surround the bulge area, while others are likely involved in extracellular matrix and basement membrane organization. Still others are candidates for creating a local gradient of signaling molecules that could maintain stem cells within the bulge area in a growth and differentiation inhibited environment. These findings are in agreement with the bulge being an entity with tissue morphogenesis activity upon grafting, and begin to unravel the molecular mechanisms and the identity of the cells within the bulge that perform this activity. If future tests of these candidates support these predictions, then the concept of preformed niches, in which stem cells find their 'home,' may have to be reconsidered. In this regard, somatic stem cells may be even more potent than initially surmised, and may be actively involved in building and maintaining their own niches through adult life.

For skin stem cells in particular, many of the most interesting questions still remain unaddressed. Are there multipotent stem cells, or does the bulge contain a heterogenous mixture of unipotent stem cells? What specific genes are responsible for stem cell maintenance? How will their expression change during the hair cycle? How will their patterns change as stem cells either exit from their niche and migrate *in vivo* or are removed from their niche and cultured *in vitro*? How does wounding influence the status of stem cells, and what particular signals penetrate the bulge to instruct cells to exit and help replenish the epidermis? How strong is the correlation between slow-cycling cells and stem cells? Are there long-term and short-term stem cells, and, if so, what makes them different? Do stem cells of the skin undergo asymmetrical or symmetrical divisions?

Once we begin to learn more about the stem cells of the skin, and their spectacular self-renewing properties, it should be possible to begin to identify the niche cues that these cells respond to and the specific environmental cues that coax stem cells along specific lineages. As we gain further insights into these issues, we will begin to explore the relation between adult and embryonic skin stem cells and the parallels between skin stem cells and other stem cells of the body, including pluripotent embryonic stem cells. It is the

ultimate goal of the stem cell field to understand enough about the various unipotent, multipotent, and pluripotent cells of the body to make major inroads in the interface between stem cell biology and human medicine.

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CHAPTER 16

Hematopoietic Stem Cells

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16.1 EMBRYONIC STEM CELLS AND EMBRYONIC HEMATOPOIESIS

Over two decades of research in mice has established that embryonic stem cells (ESCs) can give rise to all differentiated cell types in the adult organism, and that ESCs are thus pluripotent. *In vitro*, ESCs undergo spontaneous aggregation and differentiate to form cystic embryoid bodies (EBs). These teratoma-like structures consist of semi-organized tissues, including contractile cardiac myocytes, striated skeletal muscle, neuronal rosettes, and hemoglobin-containing blood islands. In the last decade and a half, this *in vitro* system has been exploited to study differentiation events in a number of tissues, and has begun to be used for discovery and characterization of small-molecule pharmaceuticals. The availability of ES cells from the human might make it possible to produce specific differentiated cell types for replacement cell therapies to treat a host of degenerative diseases.

Stem cells from the embryo have fundamentally distinct properties when compared with stem cells from somatic tissues of the developed organism. The hematopoietic stem cell (HSC) is the best-characterized somatic stem cell in the adult. This rare cell, residing in the bone marrow, gives rise to all blood cell lineages and will reconstitute the lympho-hematopoietic system when transplanted into lethally irradiated animals. Bone marrow transplantation is widely employed for the treatment of congenital, malignant, and degenerative diseases. Although adult HSCs are an important target for genetic modification, success has been limited by difficulty expressing genes in HSCs, and by the challenge of maintaining and expanding HSCs in culture. Moreover, recent concerns have been raised about the safety of gene therapy with retroviruses. Thus, harnessing ES cells as a source of HSCs would make it easier to genetically modify stem cell populations *ex vivo*, to discover small molecules that impact blood development, to study genetic and epigenetic influences on hematopoietic cell fate, and to empower preclinical models for gene and cellular therapy.

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A critical question is whether the current methods for *in vitro* differentiation of ESCs produce HSCs capable of long-term blood formation in adults. All protocols for *in vitro* differentiation of ES cells published to date appear to recapitulate the yolk sac stage of hematopoietic commitment, with questionable developmental maturation into adult-type somatic HSCs. The first blood cells detected in the yolk sac of the embryo and in EBs *in vitro* are primitive nucleated erythrocytes. These cells express embryonic forms of hemoglobin with a left-shifted oxyhemoglobin dissociation curve, adapted to the low-oxygen environment of the embryo. Embryonic forms of hemoglobin serve as markers of primitive, embryonic erythropoiesis. Later in development, both yolk sac and EBs produce a variety of more differentiated myeloid cell types and enucleated red blood cells that express adult globins and are typical of circulating mature blood. This suggests that yolk sac precursors are capable of making the transition from the primitive to definitive hematopoietic programs, but the extent to which yolk-sac-derived progenitors contribute long-term to hematopoiesis in the adult remains controversial. Experimental manipulation of yolk sac blood progenitors can reveal a latent potential for hematopoietic engraftment in adults. When marked yolk sac cells from one embryo are transplanted into other embryos, yolk sac progenitors can contribute to blood formation in the adult. Yet yolk sac progenitors fail to engraft if injected directly into the adult. However, when directly injected into the liver of myeloablated newborn mice, highly purified CD341/c–Kit1 progenitors isolated from mouse yolk sac will provide long-term blood production throughout adulthood. Apparently, the newborn liver retains the embryonic hematopoietic microenvironment, and supports the developmental maturation of the yolk sac stem cells. Yolk sac cells can also engraft in irradiated mice if they are first cultured on a supportive stromal cell line taken from the aorta–gonad–mesonephros region of the embryo. Here once again, the yolk sac cells can be 'educated' by the stromal cells to adopt an adult profile. So indeed, hematopoietic progenitors from yolk sac appear capable of sustaining hematopoiesis in adults; but, based on recent evidence from a number of groups, it appears likely that yolk sac progenitors contribute to embryonic blood formation, and thereafter yield to a distinct source of definitive HSCs that arise within the embryo proper. Compelling evidence suggests that the definitive HSCs responsible for lifelong hematopoiesis arise chiefly from a separate and distinct source in the aorta–gonad–mesonephros region of the developing embryo.

16.2 BLOOD FORMATION IN EMBRYOID BODIES

As for yolk sac progenitors, it has proven exceedingly difficult to demonstrate that ES-derived hematopoietic progenitors can repopulate adult mice. This resistance of embryonic progenitors from EBs to engraft in the adult is

reminiscent of the block to engraftment observed for yolk sac progenitors, and likewise is believed to reflect the developmental immaturity of ES-derived HSCs, and the different microenvironments of the embryo and the adult. Whether *in vitro* differentiation of ESCs will promote formation of AGM-like HSCs remains unresolved. In order to model hematopoietic transplantation from ES cell sources, this challenge must be overcome.

Although EBs show a temporal wave of primitive, followed by definitive, hematopoiesis, the nature of the ES-derived HSCs has been a subject of great interest. The delineation of hematopoietic development within EBs has been pioneered by Keller and colleagues, whose seminal contributions have made the hematopoietic program among the most well-defined aspects of *in vitro* ES cell differentiation. Their work has defined the most primitive hematopoietic progenitor in EBs as the blast colony-forming cell (BL-CFC), a transient cell with both primitive erythroid potential and the capacity to generate definitive erythrocytes and multilineage myeloid colonies upon replating. It also has the potential to form endothelial cells and, as such, has been defined as the hemangioblast. To date there is no data demonstrating lymphoid potential for this cell type. Only one group has reported repopulation of irradiated adult mice with cells taken from EBs fortuitously timed to harbor the maximal number of BL-CFCs, but in this report there were no markers to demonstrate that lymphoid and myeloid cells developed from a single cell committed to the hematopoietic lineage. Thus, to date the relationship of the BL-CFC to definitive HSCs remains ill-defined, and whether EBs support the development of AGM-like HSCs remains an open question.

16.3 TRANSFORMATION OF AN EB-DERIVED HSC BY BCR/ABL

We set out to address the question of whether a lymphoid-myeloid HSC developed within differentiating EBs by attempting to transform that putative cell. We borrowed from our experience with the disease chronic myeloid leukemia (CML), the classical pathologic condition of adult HSCs that is caused by the BCR/ABL oncoprotein. Several biological properties unique to the BCR/ABL oncoprotein made it particularly well suited for addressing the nature of primitive blood progenitors within EBs. Induction of CML requires expression in pluripotent HSCs. Multilineage hematopoiesis is monoclonal in most patients at the time of CML diagnosis, demonstrating that the fusion oncoprotein BCR/ABL endows leukemic stem cells with a competitive repopulation advantage over normal stem cells. Despite expression in a wide array of tissues, transgenic mice that carry BCR/ABL in their germ line develop only hematopoietic malignancies, reflecting the tropism of BCR/ABL for hematopoietic cell types and the sparing of nonhematopoietic tissues. Patients with CML

harbor the Philadelphia chromosome translocation in both lymphoid and myeloid lineages, showing that the multilineage differentiation of stem cells is preserved despite BCR/ABL transformation.

We hypothesized that BCR/ABL would enable us to transform an ES-derived HSC, engraft mice, and determine the extent of lymphoid and myeloid differentiation *in vivo*. We introduced BCR/ABL into differentiated murine ES cells and cultured a primitive hematopoietic blast cell that generated nucleated erythroblasts *in vitro*, mimicking yolk sac blood formation. We picked and expanded single-cell clones of these cells, verified clonality by retroviral integration, injected irradiated mice, and observed successful lymphoid–myeloid engraftment in primary and secondary animals. The erythroid progenitors from engrafted mice expressed only adult globins, suggesting that the cells underwent developmental maturation to the definitive hematopoietic program *in vivo*. BCR/ABL expression enabled adult engraftment by altering the cell's homing properties, complementing a missing cytokine signal, or blocking apoptosis, allowing the ES-derivatives to acclimatize to the adult microenvironment and to differentiate into multiple hematopoietic lineages. These results provided the first definitive demonstration of the embryonic HSC (e-HSC) that arises *in vitro* during ES differentiation. This cell is a common progenitor of both primitive embryonic erythropoiesis (yolk sac type) and the definitive adult lymphoid–myeloid HSC. Although the BCR/ABL-transformed clones produce colonies with a comparable morphology to the BL-CFC and show primitive erythroid potential, their precise relationship to the hemangioblast is unclear, as the BCR/ABL-transformed clones do not appear to have endothelial potential.

16.4 PROMOTING HEMATOPOIETIC ENGRAFTMENT WITH STAT5 AND HOXB4

Although BCR/ABL transformation targets a rare cell in ES cell cultures with lymphoid–myeloid developmental potential, the engrafted mice succumbed to leukemia, prompting us to explore means for isolating the e-HSC without inducing the transformed phenotype. Our approach to generating normal, nontransformed blood progenitors from ES cells involved a combination of two strategies: (1) expression of single proteins in signaling pathways activated by BCR/ABL (e.g., STAT5), postulating that activation of downstream targets would be less disruptive to cell physiology than transformation by the complete oncoprotein; and (2) conditional expression of candidate genes using a novel ES cell line engineered to express the gene of interest from a tetracycline regulated promoter such that genetic effects could be induced and then reversed. The Ainv15 ESC line we created expresses the tet-dependent transcriptional transactivator protein from an active genomic locus (*ROSA26*).

Any gene of interest can be inserted with high targeting efficiency into an expression cassette located within the active *HPRT* gene locus. Genes that are targeted correctly become resistant to neomycin (G418), and the inserted gene is expressed only in the presence of the potent tetracycline analog doxycycline, and can be rapidly silenced following doxycycline removal. We chose to express the *STAT5* transcriptional regulator and the homeobox gene *HoxB4* in this system, owing to the central role of STAT5 in BCR/ABL and cytokine receptor signaling, and to extensive prior evidence from the Humphries group of the role of *Hox* genes in hematopoiesis and the unique properties of *HoxB4*, which was previously shown to enhance hematopoietic engraftment without inducing leukemia. We differentiated the modified ES cells into EBs and activated gene expression by adding doxycycline to the culture medium between differentiation days four and six, timed to coincide with the maximal generation of primitive multipotential hematopoietic colonies. After six days, the EBs were dissociated, and the cells were cultured on the OP9 stromal cell line, previously shown to enhance production of hematopoietic progenitors from mES cells. Expansion of hematopoietic blast cells was observed upon *STAT5* and *HoxB4* gene induction, and vigorously growing colonies of hematopoietic cells were detected only in the presence of doxycycline. These cells were harvested, plated in semisolid media plus cytokines, and shown to produce a variety of blood cell colony types, with only the most primitive multipotential colonies significantly expanded. The cells, which also express the green fluorescent protein (GFP), were injected intravenously into irradiated syngeneic or immunodeficient mice.

Contributions of GFP1 cells to the peripheral blood were then monitored by flow cytometry, and specific lymphoid and myeloid cell populations were scored by antibody staining against specific cell surface differentiation antigens, by forward and side scatter properties, and by direct microscopic examination of cells cyto-centrifuged onto cover slips. In these experiments, both *STAT5* and *HoxB4*-expressing cells engrafted in mice and generated both lymphoid and myeloid populations in circulating blood. Interestingly, contributions of the *STAT5*-stimulated cells appeared to be transient, despite continued gene induction *in vivo* (through inclusion of doxycycline in the drinking water of the mice). Engraftment with *HoxB4*-expressing cells persisted in primary animals, even in the absence of gene induction, and the cells of primary animals could be transplanted into secondary animals, suggesting self-renewal of a long-term reconstituting HSC. Examination of peripheral blood smears from engrafted mice showed no evidence of abnormal hematopoiesis, although rare animals succumbed to hematologic malignancies from donor cells, suggesting some tendency for the genetically modified ESCs to undergo transformation *in vivo*. Retroviral delivery of *HoxB4* directly to populations of cells dissociated from EBs after 4–6 days of differentiation also succeeded in generating

expanding cultures of hematopoietic cells that engrafted in irradiated mice. These data demonstrate that expression of the *STAT5* or *HoxB4* gene in differentiating cultures of ES cells yields hematopoietic engraftment in irradiated mice, with *HoxB4* showing the most promise for stable engraftment in primary and secondary animals.

The mechanisms by which *STAT5*, and particularly *HoxB4*, drive hematopoietic engraftment from ESCs remain unclear. By driving cell proliferation, both genes might serve to increase the numbers of otherwise vanishingly rare HSCs above a threshold of detection. Alternatively, *HoxB4* might be altering cell fate, a known effect of homeobox genes, by promoting a transition from primitive to definitive HSC fate. RT-PCR analysis of *HoxB4*-expressing EB-derived cells, in comparison with hematopoietic progenitors isolated from precirculation yolk sac, confirmed the detection of markers of definitive hematopoiesis in *HoxB4*-expressing cells, including adult-type globin. Moreover, *HoxB4*-transduced cells expressed *CXCR4*, the chemokine receptor implicated in HSC homing to the bone marrow, and *Tel*, the transcription factor implicated in migration from the fetal liver to the adult bone marrow microenvironment. *HoxB4* does not appear to be expressed in the precirculation yolk sac, but is detected in primitive populations of CD341 bone marrow cells from the adult. We therefore also tested whether expression of the *HoxB4* gene by retroviral infection in yolk sac progenitors would endow them with engraftment potential in adults. As for EB-derived cells, *HoxB4* expression in yolk sac progenitors induced dramatic expansion on OP9 stromal cultures and stable hematopoietic engraftment in adult mice. Yolk-sac-derived progenitors could also be transplanted into secondary animals. These data support the hypothesis that activation of *HoxB4* endows embryonic hematopoietic progenitors with the potential to engraft in adult hematopoietic microenvironments, and therefore may be critical to the transition from the embryonic to adult hematopoietic program.

Lymphoid potential and transient lymphoid reconstitution of engrafted immunodeficient mice has been demonstrated, showing convincingly that EB-derived cells can show full hematopoietic differentiation potential. Native EB-cell derived hematopoietic progenitors function at best inefficiently to reconstitute the adult host, or may develop in only limited numbers under idiosyncratic culture conditions. If indeed distinct progenitors contribute to primitive yolk sac and definitive AGM-type hematopoiesis in the embryo, then distinct progenitors might arise at spatially and temporally distinct sites during EB formation from ES cells *in vitro*. Identifying the precise culture conditions that promote differentiation of ESCs into robust adult-type definitive HSCs *in vitro* remains a critical goal.

To date, gene modification has been required to demonstrate development of lymphoid–myeloid HSCs from differentiating ES cells *in vitro*, yet even gene

modification results in inefficient engraftment. Given the technical challenges and risks inherent in genetic modification, it would be preferable to derive engraftable HSCs in as natural a process as possible, by mimicking the developmental pathways of the embryo. Applying principles that specify blood formation to *in vitro* systems might enable enhanced blood formation in a safe and efficient manner.

16.5 PROMOTING BLOOD FORMATION *IN VITRO* WITH EMBRYONIC MORPHOGENS

During gastrulation in the mouse, secreted signaling molecules induce distinct cell fates from developing mesoderm. The earliest stages of blood formation occur in the extraembryonic mesoderm of the yolk sac, where blood islands form surrounded by endothelial cells and closely opposed to the visceral (primitive) endoderm. Several of the players that regulate the transcriptional control of hematopoiesis have been identified (e.g., *SCL*, *AML1/CBFa2*, and several *Hox* genes), largely due to their involvement at translocation breakpoints in leukemia. Gene knockout studies of these factors have validated their role in hematopoiesis. More recently, a small number of secreted factors have been identified that act as early embryonic inducers of mesodermal fate in the hematopoietic lineage. The most interesting of these are hedgehog factors and bone morphogenetic protein 4 (BMP4).

Indian hedgehog (Ihh) is a member of the hedgehog family of signaling molecules that play diverse roles in patterning early embryonic events. Baron and colleagues have shown that early hematopoietic activity in the developing murine yolk sac is dependent on signals from the adjacent primitive/visceral endoderm. Recently, they demonstrated that Ihh was produced by visceral endoderm sufficient to mediate this induction. Ihh could respecify neuroectoderm to hematopoietic fate, and blocking Ihh function by anti-hh antibodies abrogated hematopoietic development. Baron and colleagues showed that Ihh induction of hematopoiesis in murine embryo explants led to expression of BMP4. BMP4, a member of the TGFβ family, has been implicated as a potent ventralizing factor and inducer of hematopoietic mesoderm in Xenopus development. A related hedgehog factor, Sonic hedgehog (Shh), has been shown by Bhatia and colleagues to enhance the expansion of human hematopoietic repopulating cells assayed by transplantation in immunodeficient mice. BMP4 and other members of the BMP family have also been implicated in regulation of proliferation and survival of primitive human hematopoietic populations. Antibodies to hh and noggin, a specific antagonist of BMP4 signaling, abrogated the proliferative effects of Shh in human hematopoietic cell culture. It has been shown that Ihh is expressed by visceral endoderm in developing EBs. There is a single report that addition of BMP4 to differentiating

cultures of rhesus ES cells augments formation of hematopoietic clusters. Most recently, Bhatia and colleagues have shown that BMP4 can enhance hematopoietic potential from human ES cells.

These studies offer the first hints that directed differentiation of human ES cells into HSCs might be feasible. However, numerous questions persist: will HSCs derived from hES cells function normally? Will they reconstitute normal immune function and remain nontumorigenic *in vivo*? Can immunologic issues be circumvented through nuclear replacement or gene modification? The hurdles for therapeutic applications of ES-derived cells remain high, but no matter what, the *in vitro* differentiation system will remain an important model for investigations of blood formation and embryonic development.

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CHAPTER 17

Peripheral Blood Stem Cells

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17.1 INTRODUCTION

The bone marrow is, most likely, the source of peripheral blood stem and progenitor cells. Hemangioblasts are the embryonic precursors of hematopoietic stem cells (HSCs), giving rise to committed hematopoietic progenitors such as lymphoids, thymocytes, myeloids, granulocytes – monocytes, megakaryocytes – erythrocytes, and mast cells. These progenitor cells complete their differentiation in the bone marrow, peripheral blood, and thymus and in the target tissues. Extensive research in hematology/oncology has resulted in the identification of a wide variety of cell surface markers which allow the characterization and isolation of HSCs at different stages of their differentiation. Initially, adult bone marrow mesenchymal cells (MSCs) were isolated, expanded *in vitro*, and examined for their multilineage differentiation potentials. These early studies were followed by extensive research on bone-marrow-derived (BM-derived) multipotent adult progenitor cells (MAPCs). This special cell population can proliferate long-term without senescence and can differentiate to multiple lineages *in vitro* and contribute to the regeneration of several tissues *in vivo*. Like HSCs, MSCs may leave the bone marrow environment and be found in peripheral blood. Identification and isolation of MSCs is based on the differential expression of cell surface markers that distinguish them from circulating HSCs. Endothelial progenitor cells (EPCs) are probably derived from the same hemangioblast precursors of HSCs, but they take a separate path of differentiation in the bone marrow. The identification of circulating EPCs suggests that the process of vasculogenesis, previously believed to be restricted to the embryonic stages, continues into adulthood. The circulating EPCs have specific cell surface markers that are not found on mature endothelial cells (ECs) and that are lost when the EPCs differentiate to ECs.

This chapter will briefly review the types and source of stem cells in peripheral blood, their specific cell surface markers, and factors that change their

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abundance in peripheral blood. We will focus on the isolation and *in vitro* expansion of peripheral blood-derived MSCs and EPCs, and describe their therapeutic applications in regenerative medicine. We will further describe the role of peripheral blood-derived stem cells in normal and pathological processes. Although much information was gathered in the past on the identification of different populations of peripheral blood stem cells, their clinical potential for therapy is only now being explored. Peripheral blood is readily obtainable and is a viable source of cells for regenerative medicine, and therefore deserves special attention.

17.2 TYPES AND SOURCE OF STEM CELLS IN THE PERIPHERAL BLOOD

It is well documented that the bone marrow is the source of cells in peripheral blood. HSCs are characteristically quiescent, multipotent cells with the capacity for both self-renewal and differentiation. After development in the fetus, HSCs reside in adult bone marrow and serve to replenish lymphoid, megakaryocytic, erythroid, and myeloid hematopoietic lineages throughout adulthood. Observations that systemically administrated MSCs could home back to the bone marrow have suggested that MSCs may also reside in bone marrow. Results of our recent studies indicate that mature cells, such as ECs, may enter the circulation. A different study tested the fate of muscle progenitor cells introduced into the circulation of lethally irradiated recipient mice together with distinguishable bone marrow cells. All recipients showed high-level engraftment of muscle-derived cells representing all major adult blood lineages. Collectively, these results indicate that there is a constant exchange of cells from the bone marrow to peripheral blood. On the other hand, bone marrow transplantation studies have indicated that this process may be reversed and cells from peripheral blood may repopulate the bone marrow.

17.2.1 Mobilization of Bone Marrow Cells

Stem cell numbers in peripheral blood are very low compared with those in the bone marrow. Although stem cells can be collected by apharesis, this requires the processing of large volumes of blood. Amplification of peripheral blood stem cells can facilitate collection and allows for rescuing autologous stem cells from the bone marrow. Mobilization of HSCs from bone marrow into peripheral blood can be achieved by hematopoietic growth factors. Recombinant human granulocyte (G)- and granulocyte-macrophage (GM) colony-stimulating factor (CSF) have been used as stimulators of hematopoiesis. Results of studies indicate higher numbers of circulating progenitor cells in patients receiving G-CSF or GM-CSF. In fact, transplantation of G-CSF-mobilized stem cells harvested from peripheral blood is replacing bone marrow biopsy, the method of

choice for collecting stem cells for autologous bone marrow transplantation. However, it is important to find better mobilizing techniques to provide more efficient harvesting and faster hematopoietic recovery. Recently, elegant studies were designed to prove the role of angiogenic factors in EPC mobilization. Mobilization of HSCs and EPCs from bone marrow is mediated through activation of metalloproteinases and adhesion molecules. In the bone marrow, vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) induce MMP-9 expression. Activation of MMP-9 results in the release of stem cell-active soluble kit ligand, which mobilizes quiescent HSCs and EPCs to the vascular zone, where they are released to the circulation. The results of these studies indicate that co-mobilization of EPCs and HSCs contributes to the revascularization processes.

17.3 ENDOTHELIAL PROGENITOR CELLS

Initial evidence that EPCs can be detected in peripheral blood came from research conducted mainly by the groups of Isner and Asahara in Boston, and Rafii in New York. They showed that cells with EC characteristics can be isolated from peripheral blood and expanded *in vitro*. They and others have shown that the numbers of EPCs in peripheral blood are significantly increased as a result of acute vascular injuries, angiogenic stimuli, estrogen, and nitric oxide synthase, but reduced by certain chronic disease states (e.g., coronary artery disease). Circulating EPCs originate primarily from the bone marrow and can be identified by differential expression of hematopoietic and endothelial cell markers. This is important because HSCs and EPCs probably share a common precursor, the hemangioblasts. Hemangioblasts reside mainly in the bone marrow and differentiate into HSCs and angioblasts. This process occurs mainly during early embryogenesis but was shown to exist in adults. Angioblasts will give rise to EPCs that upon stimulation with angiogenic factors such as VEGF and PlGF are mobilized from bone marrow to peripheral blood. Once in peripheral blood, EPCs can be recruited to sites of active neovascularization, as seen in wounds, diabetic retinopathy, and tumors. The role of EPCs in physiological and pathological neovascularization and their therapeutic applications are described below.

17.3.1 Identification and Isolation of EPCs

Marrow and peripheral blood cells expressing CD34 can give rise to EPCs. Although CD34 is commonly used to isolate EPCs, CD34 expression is also shared by HSCs and MSCs and cannot be used to distinguish between these populations. Likewise, VEGF receptor 2 (human KDR and mouse Flk-1), which is used to identify EPCs, is expressed also on HSCs. In humans, CD133 (AC133) is used to distinguish EPCs from mature ECs, since CD133 is not expressed by

Table 17.1 Cell Surface Makers Expressed on Progenitor and Mature Endothelial Cells

	EPC/ECFC	Vessel Wall-Derived CEC
Proliferative capacity:	High	Limited
Proposed source and mobilization	Bone marrow release and proliferation to CSF, VEGF, and other stimuli	EC damage, VEGF decrease, and apoptosis
Marker		
VEGFR-2 (KDR)	+	+
CD34	+	+
CD31 (PECAM)	+/−	+
CD133 (AC133)	+	−
CD146 (P1H12, MUC18)	−	+

CEC, circulating endothelial cell; ECFC, endothelial colony-forming cell; EPC, endothelial progenitor cell.

mature ECs. CD133 is a stem cell marker with as yet unrecognized functions. Additionally, Hebbel and colleagues have used P1H12 antibodies that recognize CD146 (MUC18) on circulating ECs in peripheral blood but not on monocytes, granulocytes, platelets, megacaryocytes, or T or B lymphocytes. Other markers common to progenitor and mature ECs are the cell surface receptors KDR and Tie2. Purified populations of $CD133^+/KDR^+$ EPCs proliferate *in vitro* in an anchorage-independent manner and can be induced to differentiate into mature adherent ECs. It is thought that $CD133^+/KDR^+$ EPCs are a population of immature ECs that are mobilized from the bone marrow to participate in neovascularization. As myelomonocytic cells have lost surface expression of CD133, this marker also provides an effective means to distinguish true EPCs from cells of myelomonocytic origin. Yet, recent studies showed that cells expressing CD14, considered as a typical monocytic lineage marker, can give rise to ECs. Collectively, these studies suggest that identification of circulating EPCs may be achieved using different markers that may define subpopulations of EPCs based on their differentiation stage and origin (Table 17.1).

The number of EPCs in bone marrow is very low (< 10 per 10^6 mononuclear cells) and the reported numbers vary a great deal, based on which identifying markers are used among the different studies. For practical applications, the EPC fraction may be enriched using cell surface markers such as CD34, CD133, and KDR. Stromal-cell-derived factor-1 stimulates the mobilization of EPCs via an enhancement of protein kinase B (Akt) and endothelial nitric oxide synthase activity. Interestingly, VEGF has been found to promote endothelial cells to express SDF-1 (also known as CXCL12) and CXCR4 (the SDF-1 receptor); on the other hand, SDF-1 has the potency to induce the

expression of VEGF. Thus, in the bone marrow vascular niche, the expression of VEGF and endothelial's SDF-1 possibly modulate the microenvironment through proteolytic enzymes (MMP2, MMP9) and controlling EPC mobilization. In addition to the above factors, recent data indicate that erythropoietin (Epo), platelet-derived growth factor (PDGF), and nitric oxide stimulate EPC mobilization as well.

One functional assay capitalizes on *in vitro* growth kinetics to discriminate BM-derived EPCs and circulating ECs from vessel-wall-derived mature ECs. In this assay, the isolated cells are incubated with VEGF, basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF), and fibronectin or collagen. EC colonies that appear early are derived from the recipient vessel wall circulating ECs, whereas late outgrowth cells or colonies originate mainly from BM-derived EPCs. Early EPCs (7-day cultures) are derived from $CD14^+$ cells (monocytes), while late outgrowing EPCs (4–6 weeks of culture) are derived from CD14− cells that bear endothelial markers (VE-$cardherin^+$$Flk\text{-}1^+$$vWF^+$$CD36^+$$CD146^+$). Total mononuclear cells obtained from human peripheral blood were cultured and also were derived from two EPC types. Some cells organized into clusters as early as three days after plating, and these were called early EPCs. These cells had limited proliferative capacity and disappeared after four weeks of culture. In contrast, another group of cells appeared later, at two to three weeks after plating. These cells demonstrated endothelial cell-like cobblestone morphology and were called late EPCs. Late EPCs proliferated robustly, and were positive for VE-cadherin, Flt-1, and KDR, but negative for CD45. The late EPCs produced nitric oxide and formed capillary tubes. Late outgrowths of cells from umbilical cord or circulating blood mononuclear cells were termed endothelial colony-forming cells (ECFCs). These cells appeared at 14–21 days after plating and formed adherent colonies with cobblestone morphology. ECFCs expressed the cell surface antigens CD31, CD105, CD144, CD146, vWF, and KDR and took up aceylated low-density lipoprotein (AcLDL). ECFCs did not express hematopoietic or monocyte/macrophage cell surface antigens such as CD14, CD45, or CD115. Whether isolated from cord or adult peripheral blood, ECFCs display clonal proliferative potential and relatively high levels of telomerase. Taken together, the recent consensus is that late outgrowth endothelial colonies may be considered as angioblast-like EPCs.

The cell surface antigen CD31 (also known as PECAM-1) has been used as a marker for endothelial cells for many years. Recently, it was suggested that CD31 could also be used as a marker for EPCs. However, since it has been shown that $CD31^+$ are fully differentiated ECs, it could be argued that CD31 may not be a suitable marker for identification of EPCs, since it may not distinguish between progenitor and fully differentiated endothelial cells. However, in these studies, CD31-selected EPCs had a typical EC/EPC phenotype, proliferated robustly and integrated into nascent vasculature. The close phenotypic similarity between

mature and progenitor ECs was highlighted by single cell colony-forming assays, demonstrating that populations of both human umbilical vein endothelial cells (HUVECs) and human aortic endothelial cells (HAECs), which were thought to be fully differentiated endothelial cells, contain a subpopulation of EPCs with different clonogenic and proliferative potential. This work suggested that HUVECs and HAECs are not homogeneous populations and appear to contain some fully differentiated ECs that are unable to proliferate as well as other cell fractions that demonstrate more 'stemness,' as shown by their robust ability to proliferate and form colonies. It is not yet clear why HUVEC and HAEC populations demonstrate heterogeneity in *ex vivo* culture conditions, but these findings may suggest that culturing and sorting for EPCs may help to standardize experimental data obtained from different research groups.

Isolation of EPCs is conventionally based on FACS or immuno-magnetic techniques, which are complex, time-consuming procedures and need experienced operators. New devices could be engineered to simplify operation and shorten the time for obtaining EPCs. Miniaturization of EPC isolation procedures by using similar strategies, adopted from microfluidic lab-on-a-chip devices, may significantly facilitate EPC isolation. A microfluidics chamber, coated with antibodies against CD34, VEGFR-2, CD31, CD146, or CD45, was made to capture EPCs. When cells were flowing through the 'EPC chip,' it demonstrated specific affinity to EPCs. Although this prototype device could only be coated with one antibody on one single chamber, it is potentially one alternative option for easy and rapid separation of EPCs. An extracorporeal cellular affinity (ECA) column that can recover CD133-expressing progenitor cells with high efficiency was also developed. In a sheep model, 1.8 L of blood was passed through a Sepharose-based column with affinity for CD133, and unbound cells and plasma were returned to the animal. The results show that this process has a minimal effect on the hematologic and physiologic parameters of the animal and EPC recovery was over 600-fold more efficient than conventional density centrifugation from a peripheral blood specimen. This technology may facilitate the generation of large numbers of progenitor-derived cells for clinical therapies and reduce the time required to attain clinically relevant cell numbers while minimizing loss of other important cell types to the donor.

17.3.2 *In vitro* Expansion of EPCs

The relative paucity of EPCs in the circulation likely contributes to the difficulty associated with EPC culture. Density gradient separation procedures, designed for separating mononuclear cells (MNCs) from blood, may also contribute to this problem. Thus, successful culture of EPCs usually requires a relatively large amount (50–100 mL) of peripheral or cord blood and, still, very few colony forming units (CFUs) or ECFCs can be obtained. The mononuclear fraction is placed in fibronectin-coated plates containing endothelial basal medium that

contains angiogenic growth factors such as vascular endothelia growth factor (VEGF) and bFGF. Other growth factors such as endothelial growth factor (EGF) and insulin-like growth factor (IGF) contribute to cell growth but not differentiation. VEGF but not bFGF is important for EPC differentiation and bFGF may be used by the differentiated ECs for subsequent proliferation. Inclusion of angiogenic factors in the media helps to prevent 'contamination' by other cell types, including lymphocytes, macrophages, and dendritic cells. VEGF appears to inhibit dendritic cell maturation from $CD34^+$ MNC fraction. Within 7–10 days of culture in fibronectin or collagen-coated dishes, colonies with spindle-shaped cells appear in the dish. These are 'slow growing' cells defined as 'late outgrowth' EPCs. They differ from the mature circulating ECs that readily proliferate *in vitro*. The whole blood culture method may be used to increase the likelihood of obtaining EPC cultures. This approach does not require MNC separation procedures, and thus may minimize cell damage caused by density gradient separation. Recently, whole blood culture yielded nearly eight-fold more ECFC colonies than density gradient separation. The whole blood culture approach may simplify EPC culture procedures and increase the possibility that EPCs will be used for clinical diagnostic and therapeutic purposes.

Commercially available media kits were introduced to facilitate EPC growth. These kits provide reagents and media for culturing EPCs from peripheral blood. However, these culture kits may not be optimal for EPC expansion and differentiation. Cells isolated with such kits displayed limited proliferative capacity and did not integrate into nascent vasculature. Further studies revealed that monocytes and T-cells can contribute to CFU-ECs and therefore it is reasonable to argue that some of these kits are not suitable for EPC culture and assays. Instead, ECFCs should be cultured in EGM-2 culture medium, which was developed from MCDB131 medium in the 1980s. ECFCs demonstrate late colony-forming, robust proliferation, endothelial morphology, expression of EPC/EC-related markers, and the ability to integrate into nascent vasculature. Regardless of the EPC source, the cells assume a typical flat EC morphology after 2–3 weeks and present mature EC markers such as CD31, VE-cadherin, and CD146 (P1H12). They metabolize acetylated low-density lipoprotein (acLDL), bind Ulex Europaeus agglutinin 1(UEA-1), and produce nitric oxide (NO), consistent with EC properties. Proper characterization of EPC-derived ECs requires the analysis of a combination of cell surface markers.

17.3.3 The Role of EPCs in Physiological and Pathological Neovascularization

Blood vessels form by two processes:

1. Angiogenesis – the sprouting of capillaries from pre-existing blood vessels; and
2. Vasculogenesis – the in situ assembly of capillaries from undifferentiated ECs.

Vasculogenesis takes place mostly during the early stages of embryogenesis. Vascular channels in the yolk sac originate from the mesoderm by differentiation of angioblasts, which subsequently generate primitive blood vessels. The early findings that EPCs can participate in angiogenic processes indicate that post-natal neovascularization does not rely only on sprouting from preexisting blood vessels (angiogenesis), but may be assisted by EPCs via postnatal vasculogenesis.

BM-derived EPCs contribute to adult tissue neovascularization in several models, including wound healing and cornea and tumor angiogenesis. BM-derived EPCs could be detected in normal organs including spleen, lung, liver, intestine, skin, hindlimb muscle, ovary, and uterus, indicating their participation in maintenance of physiological neovascularization. Hormonally induced ovulation cycles were also associated with localization of BM-derived EPCs in the corpus lutea and in the uterus endometrium and stroma. These findings indicate that EPCs contribute to physiological neovascularization associated with post-natal regenerative processes.

A recent study examined the presence of endothelial, smooth muscle, and Schwann cell chimerism in patients with sex-mismatched (female-to-male) heart transplants. The Y chromosome was used to determine chimerism. Biopsy specimens taken at increasing times after heart transplantation showed that endothelial cells had the highest degree of chimerism (24.3%), Schwann cells showed the next highest chimerism (11.2%), and vascular smooth muscle cells the lowest (3.4%). Results of this study indicate that circulating progenitor cells are capable of repopulating most major cell types in the heart, but they do so with varying frequency. The signals for endothelial progenitor recruitment occur early and could relate to the injury during the surgery.

Cumulative evidence shows that bone marrow cells may improve ischemic myocardium function by paracrine stimulation of angiogenesis rather than differentiation into contractile cardiomyocytes. In addition to directly and indirectly contributing to neovascularization, EPCs probably can also provide paracrine survival signals to cardiomyocytes. Clinical studies also suggested that cell-based therapy with EPCs can improve myocardial function.

In parallel, EPCs were found incorporated into the vasculature of pathological lesions such as atherosclerotic plaques, tumors, the retina, and ischemic brain tissue. Vascular smooth muscle cell (SMC) proliferation results in neointimal hyperplasia and the development of restenosis. BM-derived SMCs can integrate into the hyperplastic neointima and atherosclerotic plaques. Evidence for the contribution of BM-derived MSCs to human atherosclerotic plaques originated from a study showing donor-derived neointimal cells

within the plaques. Also, decreased EPCs in the circulation have been correlated with a higher risk of cardiovascular complications. It was hypothesized that lower levels of peripheral blood-EPCs were associated with an impaired capacity to repair the damaged vessels, but the pathophysiological role of BM-derived EPCs remains unclear. Recruitment of peripheral blood EPCs to damaged or diseased tissues is dependent on the underlying pathology and is probably due to the release of specific growth factors and chemokines by these tissues. Abnormal retinal neovascularization contributes to the pathogenesis of proliferative retinopathy in diabetes and age-related prematurity and macular degeneration. BM-derived hemangioblasts were shown to contribute to retinal neovascularization in models of proliferative retinopathy. This study documented the incorporation of EPCs into mature endothelium of the retinal blood vessels. Cerebral infarction is associated with neovascularization of the ischemic zone and new vessel growth. Bone marrow transplantation studies showed that EPCs could be detected in the neovessels at the repair sites after 3 days.

Compelling evidence for the role of EPCs in tumor vascularization comes from a study by Lyden and colleagues using an angiogenesis-defective mouse model. Mice lacking both alleles of Id1 (*id1*−/−) and Id3 (*id3*−/−) died by embryonic day 13.5 and exhibited massive vascular malformation. The *Id3*−/−/*id1*+/− mice survived but could not support the growth of several tumor types due to insufficient tumor vascularization. However, transplantation of *id3*−/−/*id1*+/− mutant mice with bone marrow from wild-type mice gave rise to tumors that were indistinguishable from tumors grown on wild-type mice. Furthermore, 90% of the tumor vessels contained BM-derived ECs, indicating the contribution of EPCs to tumor neovascularization. VEGF treatment failed to elevate the number of EPCs in *id3*−/−/*id1*+/− mutant mice but not in *id3*−/−/*id1*+/− transplanted with wild-type bone marrow. Further evidence is provided by a model in which transplantation of human BM-derived MAPCs into tumor xenograft-bearing mice resulted in the incorporation of human cells as 40% of the tumor vessel endothelium, indicating the importance of circulating endothelial cells (CECs) for tumor neovascularization. Different tumors secrete different types and concentrations of angiogenic factors that may have a different capability to induce the mobilization of EPCs. Although a formal correlation between tumor type/stage/size and number of EPCs has not been established in human cancer, some tumor types may be more dependent than others on CECs as a source of endothelium.

Taken together, the results of these studies indicate that EPCs' contribution to neovascularization is not restricted to normal healing processes and they contribute significantly to several pathological processes.

17.4 MESENCHYMAL STEM CELLS

MSCs are multipotent cells that can differentiate into various mesenchymal lineages including bone, cartilage, fat, and muscle. MSCs were initially found in adult bone marrow, and were first identified as osteogenic progenitors capable of forming bone-like structures *in vitro*. These early studies suggested that bone marrow MSCs are also fat cell progenitors. Further studies report that MSCs may be found in every mesenchymal tissue that has regeneration capacity. In addition to bone marrow, MSCs were isolated from muscle, fat, skin, cartilage, bone, and blood vessels. MSCs have some of the basic properties of stem cells, including self-renewal, multilineage differentiation capacity, clonality, and the ability to regenerate tissues *in vivo*. In addition, adult bone marrow MSCs proliferate for many passages without senescence. The authors analyzed telomere length in these cells and showed that it was longer than in neutrophils and lymphocytes and was not different between young or old donors. Their results indicated that bone marrow MSCs have high telomerase activity *in vivo* and came from a population of quiescent cells.

17.4.1 Identification and Isolation

Because of the multiple sources and methods of isolation of MSCs, their identifying markers vary between studies. Some of the 'classical' markers of BM-derived MSCs include CD34, CD44, CD45, c-Kit, Sca-1 (murine), CD133 (human), and CD105 (Thy-1), and higher concentrations of CD13 and stage-specific antigen I (SSEA-1). As stated above, MSCs were isolated from multiple sources but only a few studies have analyzed their presence in peripheral blood. Systemic infusion of MSCs showed that they may be engrafted in various mesenchymal tissues. These results suggest that MSCs may be present in peripheral blood. In fact, MSCs were isolated from peripheral blood of cancer patients given G-CSF and GM-CSF. The cells were grown *in vitro* and had a fibroblast-like phenotype. The cells were negative for hematopoietic markers and CD34, but expressed CD105, SH3, I-CAM, and V-CAM. MSCs were also isolated from normal human peripheral blood without 'mobilization.' The cells were isolated by gradient centrifugation and plated in growth media. After two weeks, adherent fibroblast-like cells appear in the culture. These cells were positive for CD105, Stro-1, vimentin, and BMP receptors, but were negative for CD34. Taken together, these results indicate that a small population of MSCs exists in peripheral blood. These cells are difficult to isolate, but may be identified by their morphology and the expression of a subset of MSC markers.

17.4.2 *In vitro* Expansion

Peripheral blood-derived MSCs are obtained through density centrifugation using Histopaque™ or Ficoll™. There are several factors that are important for

successful maintenance of MSCs, including cell density, pH of the medium, source of sera, and the type of culture dishes. Human MSCs require densities of 1,500–3,000 cells/cm^2 in order to prevent spontaneous differentiation at higher cell densities. The basal media may be Dulbecco's modified Eagle's medium (DMEM) or α-modified Eagle's medium (MEM) with 10% fetal serum. Collectively, the methods used for MSC expansion *in vitro* do not differ from those used to expand BM-derived MSCs. Following expansion, MSCs can be differentiated *in vitro* into the mesenchymal lineages and tested *in vivo*. Interestingly, marrow-derived MSCs were induced to differentiate into cells with functional properties of endothelial cells, hepatocytes, and neuroectodermal cells. *In vitro*-differentiated cells may be used for future therapeutic applications. However, we need to define the appropriate phenotype and functional properties of the differentiated cells before they can be used clinically.

17.5 THERAPEUTIC APPLICATIONS OF PERIPHERAL BLOOD STEM CELLS

The physiological role of MSCs in tissue regeneration has prompted researchers to evaluate their use in therapeutic applications. The ethical discussions regarding embryonic stem cells underscore the need to explore the clinical applications of adult stem cells, including MSCs. MSCs were first tested in several animal models and have recently been used in clinical studies. Although the results of the animal experiments are promising, the mechanisms behind the regenerative potential of peripheral blood MSCs are not fully understood. The therapeutic applications can be divided into three groups:

1. Tissue engineering,
2. Cell delivery applications, and
3. MSCs as a vehicle for gene therapy.

The main advantage of MSCs for clinical use is their presence in peripheral blood. However, as discussed above, further work is needed to evaluate their culture and expansion properties.

17.5.1 EPCs

In many cases, organ and tissue regeneration requires the re-establishment of the vascular network. There are two possible sources of endothelialization:

1. Mature endothelial cells that migrate from pre-existing vessels; and
2. Circulating EPCs from peripheral blood.

Cultured EPCs offer a robust cell source for tissue engineering and cell delivery applications. EPCs can be obtained from the same patient to avoid

immune rejection. Although EPCs were shown to contribute to tissue revascularization, their function in a clinical setting has not been established. The use of EPCs for tissue engineering requires *ex vivo* expansion that is not optimal for clinical use because of animal products and inadequate tissue culture environment.

Two concerns must be addressed before EPCs can be used for therapeutic applications. First, there is the possibility that, when allogeneic cells are implanted, a graft-versus-host immune reaction, caused by a residual T-cell fraction derived from $CD34^+$ cells, could occur. Studies showed that BM-derived $CD34^+$ cells can be differentiated into T-cells both *in vitro* and *in vivo*. Currently, CD34 is one of the surface markers most widely used for EPC isolation. It is important to use CD34 in combination with other surface markers to identify the subsets of EPCs that will be suitable for clinical use and reduce the risk of subsequent T-cell differentiation. Second, in order to develop cells for clinical use, it is necessary to remove all animal substances, such as serum, from the culture environment, because they might be pathogenic or immunogenic. For clinical cell therapy applications, EPC culture conditions must be free of animal products to meet good manufacturing practices (GMP) standards. Efforts were made to develop clinical-grade human embryonic stem cell lines and culture conditions. Recently published were results of clinically approved culture conditions to expand peripheral blood EPCs. By using pooled human platelet lysate (pHPL) to replace fetal bovine serum (FBS) in the culture environment, they reported successful recovery of EPCs (ECFCs) from peripheral blood. The ECFCs were characterized by robust proliferative potential (more than 30 population doublings), normal karyotype, and vascular network-forming ability.

17.5.1.1 Tissue Engineering

Vascular diseases are the leading causes of morbidity and mortality in the USA each year. Over 500,000 coronary bypass grafts and 50,000 peripheral bypass grafts are performed annually in this country. However, up to 30% of the patients who require arterial bypass surgery lack suitable or sufficient amounts of suitable autologous conduits such as small-caliber arteries or saphenous veins. Synthetic grafts, such as polytetrafluoroethylene (PTFE) or Dacron (polyethylene terephthalate fiber), have been used successfully to bypass large-caliber, high-flow blood vessels. However, these grafts invariably fail when used to bypass small-caliber, low-flow blood vessels, because of increased thrombogenicity and accelerated intimal thickening leading to early graft stenosis and occlusion. It has been shown that a confluent EC monolayer on small-caliber prosthetic grafts may provide immediate protection from thrombus formation following implantation. However, the use of allogeneic endothelial cells is limited by rejection, whereas the use of autologous human endothelial cells

for the construction of vascular grafts has not been widely explored. The idea of using EPCs to seed the lumen of engineered blood vessels came from the observations that MSCs contributed to the lining of vascular grafts *in vivo*. We have shown that EPCs might be an ideal source of autologous ECs for seeding small-diameter grafts, eliminating the need to remove native vessel from which to culture ECs. By seeding EPC-derived ECs onto a scaffold, a non-thrombogenic barrier between blood and vessel wall is created, thereby promoting patency *in vivo*. EPC-seeded collagen matrices derived from decellularized porcine arteries were used for carotid artery reconstruction in sheep. These bioengineered arteries remained patent for more than four months, whereas control grafts without autologous ECs occluded within 15 days. Thus, functional vessels can be engineered using decellularized arteries and EPCs. Moreover, we have shown that these bioengineered blood vessels, after a brief period of healing *in vivo*, develop a fully cellularized wall of three distinct layers analogous to normal adventitia, media, and intima. Although these are exciting results, bioengineered grafts will need to be constructed in a mechanically relevant environment.

In vitro engineering of blood vessels should mimic the flow conditions that exist *in vivo* in order to enhance tissue formation. Local blood flow properties induce changes in endothelial cell morphology and orientation. Further studies showed that the levels and duration of shear stress induced different changes in EC morphology, proliferation, and differentiation. EPCs cultured under biologic-like shear conditions expressed higher levels of VE-cadherin than those cultured under static conditions.

Two endothelial cell types – valve-derived mature ECs and EPCs from peripheral blood – were assessed for use in bioengineered heart valves. The study showed that both sources of endothelial cells, when seeded on PGA/P4HB scaffolds, proliferate in response to VEGF. The EPCs could be induced to transdifferentiate to a mesenchymal phenotype on PGA/P4HB in response to TGF-β1. These results indicate that EPCs can respond to soluble signals that induce events that occur during valvulogenesis (Figure 17.1).

One problem with all of these studies is that heterogeneous cell populations are being expanded for seeding onto vascular scaffolds. As mentioned previously, one solution is to isolate MSCs and to differentiate them to EPCs. Another general problem with these bioengineered vascular grafts is immediate availability. For instance, when an emergency bypass needs to be performed, growth of an artificial vessel and preparation for implantation would take too much time if autologous cells are to be implemented. Alternatively, these bioengineered grafts could be seeded with stem cells that were differentiated into ECs.

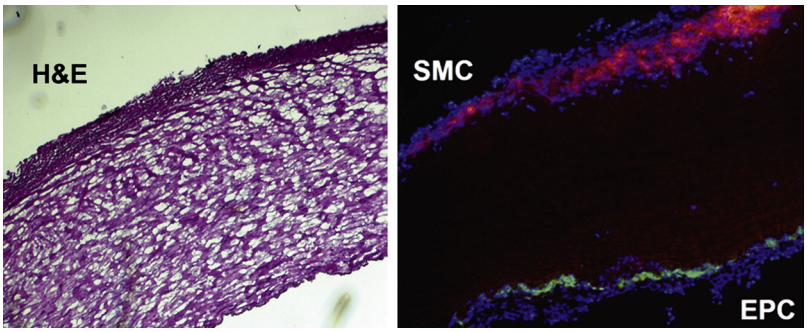

FIGURE 17.1

Acellular porcine arterial segment (stained with hematoxyline and eosin (H&E)) and seeded with peripheral blood-derived smooth muscle cell (SMC) (dyed red with PKH26) and EPC (dyed green with PKH27).

17.5.1.2 Tissue Regeneration

Several studies have suggested that EPCs participate in the vascular healing process, in part by recruitment of EPCs to the regenerated site. Genetically labeled EPCs were detected in ischemic limbs of mice and shown to accelerate the revascularization process. Administration of cytokines such as G-CSF and GM-SCF appear to enhance mobilization of EPCs and revascularization. In humans, EPCs contributed to wound healing of patients implanted with a left ventricular-assisted device. The EPCs adhered to the device and formed a non-thrombogenic surface. These studies suggested that EPCs may be recruited to assist endothelialization and served as the basis for preclinical and clinical studies as described below.

When cord blood EPCs and MSCs were injected subcutaneously into nude mice, they formed vascular structures that were stable for four weeks. The vascular network had endothelialized lumen and pericytes expressing smooth muscle actin. Engineered blood vessels made with HUVECs and human MSCs remained stable and functional for more than 130 days *in vivo*. These findings could guide future practices in tissue engineering and regenerative medicine, allowing physicians to form stable and long-lasting vasculature for engineered tissue.

Given the morbidity associated with limb ischemia, EPCs may be used for vascular therapy as an alternative to bypass approaches. In preclinical studies, introduction of BM-derived EPCs significantly improved collateral vessel formation and minimized limb ischemia. In patients suffering from peripheral arterial disease, injection of autologous whole bone marrow mononuclear cells into ischemic gastronemius muscle resulted in restoration of limb function. The improvement in muscle perfusion suggested that it was due to the presence of EPCs in the cell preparation. However, it remains to be determined whether the improvement was due in part to the introduction of myelomonocytic cells.

BM-derived MSCs were shown to contribute to myocardial regeneration and revascularization. In nude rats that underwent myocardial infarction, cytokine-mobilized EPCs homed to the infarcted tissue and contributed to neoangiogenesis. In similar studies, BM-derived MSCs were injected into the infarcted border and were shown to differentiate into myocardial cells and ECs. In most studies, direct introduction of these cells into an active angiogenic site, such as infarcted or ischemic myocardium, was essential for successful incorporation of the cells and improvement of cardiac function.

Acute myocardial infarction or chronic ischemic heart disease result in the loss of cardiomyocytes and vasculature. Several animal studies have shown that introduction of autologous bone marrow MSCs contributes to neoangiogenesis in the ischemic myocardium. In patients, whole autologous bone marrow mononuclear cells were delivered into the coronary arteries feeding the infarcted and ischemic tissue. In all of these studies, there was improved cardiac perfusion and left ventricular function, suggesting that delivery of autologous progenitor cells is feasible and safe, and may have a short-term therapeutic benefit. However, follow-up studies in animals and humans detected only a few BM-derived cells in the regenerated vascular network, suggesting that only a small portion of the cells may contribute to revascularization.

Despite the excitement generated by these initial observational clinical trials, it remains to be determined in double-blind placebo-controlled randomized clinical trials whether this cellular therapy approach will result in any long-standing cardiac benefits. Importantly, it remains unclear whether any long-term toxicity exists with this therapy. Such toxicity may result if myeloid cells are incorporated into regenerating myocardium and generate non-cardiac or fibrotic tissues. Therefore, progenitor cells that have been predifferentiated into EPCs should be used with caution and long-term monitoring.

17.5.2 Mesenchymal Stem Cells

In the case of MSCs, the lineage-committed cells can generate a variety of specialized mesenchymal tissues including bone, cartilage, muscle, marrow stroma, tendon, ligament, fat, and a variety of other connective tissues. As such, MSCs may have a dramatic impact on the overall health status of individuals by controlling the body's capacity to naturally remodel, repair, and upon demand rejuvenate various tissues. In human clinical research, initial efforts are focused on applications of MSC-based tissue repair using cell delivery approaches. The most logical application of MSCs is to regenerate non-union bone defects. A number of studies showed that MSCs from animals and humans, delivered in a porous, calcium phosphate vehicle, were able to regenerate bone tissue. Additionally, these cells may be beneficial for cartilage research. The cartilage is a tissue that cannot repair itself in adults.

MSCs have been applied in hyaluronan scaffolds for cartilage tissue repair with good results and are now in clinical trials. BM-derived MSCs have also been used for muscle repair and fuse with the host myotubes and form functional muscle fibers. Systemic delivery of BM-derived MSCs showed that they can home back to the bone marrow. This observation prompted clinical studies to use MSCs to restore the bone marrow in patients undergoing radiation and chemotherapy-mediated myeloablation.

Although a subset of MSCs was reported to differentiate into cardiomyocytes under specific conditions *in vitro*, it is still controversial whether MSCs can differentiate into cardiomyocytes. *In vivo* MSCs differentiating into cardiomyocytes have also been observed, but at an extremely low rate. On the other hand, like EPCs, MSCs can provide paracrine factors to support injured myocardium. This could be the major mechanism for the beneficial effects of these cells. So far, most clinical studies using bone marrow MSCs showed no or small (but possibly clinically important) improvement in cardiac function and the functional improvement was considered related to paracrine instead of mesenchymal cell differentiation.

17.5.3 The Use of Peripheral Blood Stem Cells for Gene Therapy

Gene and cell therapy have been proposed for regenerative medicine and tested in a number of clinical trials. Genetically modified MSCs offer a unique approach as cells with growth potential may represent a useful tool for tissue engineering and cell therapy. A detailed knowledge of vector delivery systems is critical for practical applications. One of the most popular vectors used for gene delivery to progenitor cells is replication-deficient adenovirus (Ad). Ad vectors offer two important advantages that make them ideal for gene therapy. First, they can efficiently infect non-dividing cells, which is important for MSCs that live primarily in the G0/G1 phase of the cell cycle. Second, the Ad vector can offer transient expression of the recombinant gene for a time period of approximately three weeks. However, Ad vectors have been shown to elicit an unwanted inflammatory response. Genetically modified stem cells have been explored in a number of studies to regenerate bone and cartilage or for neovascularization. The most common genes used in these studies are growth factors such as VEGF. VEGF, as mentioned above, is a potent angiogenic factor that supports the differentiation of MSCs along endothelial lineages. In order to enhance vascularization of engineered muscle tissue, we have transfected primary cultures of rat myoblasts with a plasmid encoding VEGF and green fluorescence protein (GFP). Cells expressing GFP were selected by a FACS and injected, mixed with gelatin, into the subcutaneous space of immune-deficient mice. Tissue volumes of VEGF-transfected cells increased during 21 days and tripled their size. In contrast, the volume of tissues containing cells that were transfected with control

plasmid gradually decreased and the tissues were minimally visible after 21 days. Immunohistochemical analysis of VEGF-expressing tissue with anti-von Willebrand factor revealed typical muscle formation and a developed vascular network. VEGF gene transfer to stem cells has been used by *in situ* neovascularization and angiogenesis in order to salvage ischemic limbs. Other studies looked at the combinations of growth factors to mimic the environment of vascular development. Both bFGF and angiopoietin-1 have been transfected with VEGF into progenitor cells to induce the development of mature blood vessels including the medial and outer adventitial layers. This approach also succeeded in reducing the VEGF-mediated permeability and fluid leakage of the new vessels. The future of stem cell-mediated gene therapy is dependent on the resolution of some key questions. The efficiency of gene transfer needs to be close to 100% to ensure that unmodified cells do not interfere with the regenerative process. The most feasible stem cell source needs to be used for successful clinical applications. Finally, the mode of cell delivery, systemic or local injection, needs to be decided. Regardless of the solution to each of these questions, stem cell-based therapies will benefit enormously from gene modification.

17.6 CONCLUSIONS AND FUTURE DIRECTIONS

The bone marrow is probably the source of peripheral blood stem and progenitor cells. Hemangioblasts are the embryonic precursors of HSCs, giving rise to committed hematopoietic progenitors. The bone marrow is also a source of other progenitor and stem cells, the MSCs, which can be expanded *in vitro* and have multilineage differentiation potentials. Numerous studies, described here, have shown that there is a constant exchange of cells from the bone marrow to peripheral blood. On the other hand, bone marrow transplantation studies have indicated that this process may be reversed and cells from peripheral blood may repopulate the bone marrow. Future success in applying adult peripheral blood-derived stem cells for clinical applications will depend on development of strategies to mobilize, isolate, expand, differentiate, and deliver these cells. For example, EPCs may be isolated from peripheral blood and used for therapeutic angiogenesis directly or after a period of *ex vivo* expansion. Understanding the signals involved in the recruitment of these cells to the regenerating tissues will play a crucial role in optimizing this technology for clinical use. The studies summarized here provide support for the presence of stem cells in peripheral blood and mechanisms by which they can be mobilized from bone marrow in order to increase their numbers in blood. Although various attempts have been made to use peripheral blood-derived stem cells in humans, and some encouraging results have been obtained, standard clinical use of these techniques must await further validation and long-term toxicity evaluations.

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CHAPTER 18

Multipotent Adult Progenitor Cells

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18.1 PLURIPOTENT STEM CELLS – EMBRYONIC STEM CELLS

ESCs are derived from the inner cell mass (ICM) of the blastocyst and are true pluripotent stem cells. Mouse ESCs express the cell surface antigen SSEA1 and human ESCs SSEA4, and both are characterized by the expression of a number of relative ESC-specific genes, including the transcription factors (TFs) *Oct4*, *Rex1*, *Nanog*, and *Sox2*. *Oct4* is expressed in the pre-gastrulation embryo, primordial germ cells, the ICM, and germ cells. While normal expression levels of *Oct4* maintain mouse ESC self-renewal, a decrease in expression to $<50\%$ leads to spontaneous trophectoderm differentiation, and an increase to levels $>200\%$ to primitive endoderm differentiation. *Oct4* promotes self-renewal by promoting transcription of genes such as *Oct4* and *Sox2*, and repressing genes such as *Hand1* and *Cdx2* that promote trophectoderm differentiation.

Initial studies have shown that Sall4, Epas1 (Hif-2α), *SF1*, and *RAR* activate the *Oct4* promoter, while *Tcf3* suppresses *Oct4* transcription. More recently, DNA methylation of regulatory enhancers by *Dnmt3a* and *Dnmt3b* were found to drive the *Oct4* expression. A number of orphan receptors were identified that can either be suppressive (for example, *GCNF* and *COUP-TFII*) or stimulatory (*Nr5a2*). The homeoprotein *Nanog* is found to be equally essential for early mouse development and ESC propagation. *Nanog* prevents ICM cells from differentiating into extra-embryonic endoderm by inhibiting genes such as *Gata4* and *Gata6* that promote primitive endoderm differentiation. Older studies suggested that *Nanog*−/− mice do not develop an epiblast, and *Nanog*−/− ESCs differentiate into mesoderm and endoderm. More recently it has been demonstrated that *Nanog*−/− cells are blocked in a transitional pre-pluripotent stage and eventually will develop into trophoblast rather than mesendoderm or will undergo apoptosis. Forced expression of *Nanog* in ESC results in leukocyte inhibitory factor (LIF)-independent proliferation, demonstrating its important role in maintaining ESC pluripotency.

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Intricate TF binding networks involving *Oct4*, *Sox2*, and *Nanog* are crucial for global transcriptional activation and repression in ESCs. Using ChiP on ChiP assays, unique and overlapping promoter binding sites have been identified for *Oct4*, *Sox2*, and *Nanog*, which serve as positive or negative regulators of transcription. These interactions are controlled by feed-forward loops, where initial regulators control other regulators with the option of converging and controlling downstream target genes. Others have used proteomics to identify *Nanog* partners. This technique has identified *Nanog*-bound genes such as *Oct4*, as well as other TFs including *Sall1* and *Sall4*.

18.2 POSTNATAL TISSUE-SPECIFIC STEM CELLS – ARE SOME MORE THAN MULTIPOTENT?

During gastrulation, the pluripotent cells in the ICM become restricted, first to a specific germ layer and then to a specific tissue. The latter persist throughout adult life, and are termed multipotent stem cells.

Even since the late 1990s, studies have suggested that classical adult stem cells, once thought to be multipotent, may actually be more pluripotent, as adult stem cells from a given tissue were reported to be able to become, under some circumstances, a cell of an unexpected tissue. Reports describing stem cell plasticity initially caused great excitement, as they challenged the concept that adult stem cells function solely to maintain the tissue of origin, suggesting that they might therefore provide a source of easily accessible cells not marred by ethical considerations, and that they could be used to treat a number of degenerative and genetic diseases. For instance, hematopoietic stem cells (HSCs) have been reported to differentiate into a variety of cell types of endoderm (lung epithelium, intestinal epithelium, kidney epithelium, endocrine pancreas, liver, bile ducts), ectoderm (epidermis and neural cells), as well as into mesoderm derivatives other than blood cells (skeletal and cardiac muscle, endothelium).

However, after the initial series of optimistic reports, a number of reports appeared that challenge the initial observation, or provide alternative explanations to the claim of greater potency of adult stem cells. For instance, there is evidence that stem cells, such as HSCs, may not only reside in the bone marrow (BM) but can also be present in other tissues.

A second explanation for the perceived plasticity of chiefly hematopoietic cells is fusion between the hematopoietic cells and certain host cells *in vivo*, a phenomenon known from hybridoma cell production and also shown to occur *in vitro* between hematopoietic cells or neurospheres and ESCs. Indeed, a number of studies described fusion between cells of hematopoietic origin and hepatocytes, cardiomyocytes, skeletal muscle cells, and Purkinje cells in the brain. In many instances, the nucleus of the donor cell becomes partially

reprogrammed with suppression of the hematopoietic program and activation of genes from which the donor cell fused. Others have presented relatively convincing evidence that not all apparent plasticity is due to cell fusion, including differentiation of hematopoietic cells to lung epithelial cells, and neuronal lineage cells into endothelial cells. However, the efficiency with which one stem cell appears to acquire the phenotype of a tissue cell different from the tissue of origin, whether via fusion or direct, is limited, and it remains to be determined whether this would have clinical relevance.

The two remaining possible explanations for the apparent ability of some adult stem cells to generate cells of a tissue lineage different from the tissue of origin are that stem cells with more pluripotent characteristics persist into adulthood, or that adult stem cells can be reprogrammed via a process of dedifferentiation and redifferentiation to another lineage, or via a process of transdifferentiation.

18.3 CAN PLURIPOTENCY BE ACQUIRED?

Mouse adult fibroblasts can be reprogrammed towards cells with all ESC characteristics – so-called induced pluripotent stem cells (iPSCs) – by the introduction of four transcription factors known to be expressed in ESCs (*Oct4*, *Sox2*, *Klf4*, and *c-Myc*), and selecting for cells that start to express endogenous *Nanog* or *Oct4*. Transfection with *Oct4*, *Sox2*, and *Klf4* drives somatic cells to a *Nanog*-pre-pluripotent stage, and acquisition of *Nanog* is mandatory to gain full reprogramming to pluripotent cells. This provides proof of the principle that adult somatic cells can be reprogrammed.

Since the initial description, many groups have created iPSCs from cell types and species other than mouse fibroblasts. Moreover, a number of similar protocols have been generated by replacing one or more of the initial transcription factors with fewer, or different transcription factors (*Nanog* and *Lin28*), nuclear orphan receptors (*Esrrb*, *Nr5a2*), or small molecules.

In 1993, spermatogonial stem cells were isolated for the first time from the mouse testis, representing only 0.03% of all germ cells. These spermatogonial stem cells can be transformed into ES-like cells easily *in vitro*, by growing them on feeder layers or by the addition of LIF to the culture. The four transcription genes used for reprogramming are already present at low levels in spermatogonial stem cells, but not *Nanog*, representing the pre-pluripotent status mentioned above. Upon transition to ES-like cells, *Nanog* and *Sox2* are highly upregulated while typical spermatogonial genes are downregulated.

Since 2001, a number of papers have reported that cells with greater potency can be isolated from other tissues than the testis. These include the isolation of SKPs (skin-derived progenitors), PMPs (pancreas-derived multipotent

precursors), and hFLMPCs (human fetal liver multipotent progenitor cells), which can differentiate into cells of two germ layers. We isolated stem cells with increased pluripotency from the BM of mouse and rat, termed multipotent adult progenitor cells (MAPCs). Since the initial description of MAPCs, a number of other cell populations isolated by culture of BM, umbilical cord blood, placental tissue, and amniotic fluid have been described which have the ability to differentiate into cells of the three germ layers. They have been named marrow-isolated adult multilineage inducible cells (MIAMI cells), human bone-marrow-derived stem cells (hBMSCs), unrestricted somatic stem cells (USSCs), fetal stem cells from somatic tissue (FSSCs), very small embryonic-like cells (VSELs), pre-mesenchymal stem cells (pre-MSCs), multipotent adult stem cells (MASCs), and amniotic fluid stem cells (AFSs). Although the phenotype differs somewhat between these different cell populations, they have in common that they can be expanded extensively *in vitro*, that some of them reportedly express stem cell specific genes such as *Oct4*, and that they can differentiate *in vitro* to cells with at least some features of mesoderm, endoderm, and ectoderm. However, not all studies show this at the single-cell level, and the proof of differentiation differs between publications. Moreover, few if any of the studies have shown that the more potent cells can also regenerate a tissue *in vivo*.

18.4 ISOLATION OF RODENT MAPCs

In 2001 and 2002, we described the isolation of MAPCs from the BM of human, mouse, and rat. Rodent MAPCs can be expanded *in vitro* without obvious senescence, and can at the single-cell level give rise to cells of mesoderm, endoderm, and ectoderm *in vitro*. We also demonstrated that a Rosa26 mouse-derived MAPC line contributed to many somatic tissues of the mouse when injected into the blastocyst.

Since the initial description of MAPC isolation, we have made changes to the culture method. MAPC isolation is now performed under hypoxic conditions: BM cells are plated at relatively high density on fibronectin-coated plates in 5% O_2 and 6% CO_2. After approximately one month, cells are passed through a Miltenyi column to remove $CD45^+$ cells and $Ter119^+$ cells, and cells subcloned at five cells/well. Clones are identified on the basis of their morphology and *Oct4* mRNA levels (q-RT-PCR), and expanded. This has led to the isolation of MAPCs which have significantly higher levels of Oct4. In addition, 90% of MAPCs thus isolated and maintained express Oct4 protein in the nucleus. The phenotype of mouse MAPC is B220, CD3, CD15, CD31, CD34, CD44, CD45, CD105, Thy1.1, Sca-1, E-cadherin, major histocompatibility complex (MHC) classes I and II negative, epithelial cell adhesion molecule (EpCAM) low, and c-Kit, VLA-6, and CD9 positive. For rat MAPC, the phenotype is CD44, CD45, MHC classes I and II negative, but CD31 positive.

To generate single-cell-derived populations of MAPC, we subclone established MAPC lines at 0.8 cells/well. Such subcloning is not usually possible at the initial subcloning step, but has 30% efficiency when cells initially subcloned at five cells/well are subsequently subcloned at 0.8 cells/well.

Transcriptome analysis demonstrates that rodent MAPCs differ significantly from MSCs, but also differ significantly from ESCs. Rodent MAPCs express a number of genes identified to be ESC-specific (ES cell-associated transcripts or ECATs), including *Oct4*, *Rex1*, and eight other genes, but they do not express Nanog and Sox2, as well as eight other ECATs. Of note, rMAPCs also express gene characteristics for primitive endoderm, such as *Sox7*, *Sox17*, *Gata4*, *Gata6*, *Foxa2*, and *Hnf1*β.

18.5 ISOLATION OF HUMAN MAPCs

Like rodent MAPCs, human MAPCs are isolated from BM and, like rodent MAPCs, they can be expanded extensively, although they do undergo eventual senescence. The cell surface is CD31, CD34, CD36, CD44, CD45, HLA class I, HLA-DR, c-Kit, Tie, VE-cadherin, VCAM, and ICAM-1 negative. Human MAPCs express very low levels of β2-microglobulin, AC133, Flk1, and Flt1, and high levels of CD13 and CD49b. Like rodent MAPCs, transcriptome studies have shown that human MAPCs differ from human MSCs and human ESCs; unlike rodent MAPCs, however, human MAPCs do not express significant levels of *Oct3a*.

18.5.1 Differentiation Ability of MAPCs *in Vitro*

Rodent and human MAPCs differentiate to mesenchymal cell types such as smooth muscle cells, osteoblasts, chondroblasts, and adipocytes, as well as to endothelial cells *in vitro* and *in vivo*.

Since the initial description of differentiation of MAPCs to hepatocyte-like cells, we have developed a differentiation protocol that induces a more robust acquisition of phenotypic and functional characteristics of hepatocytes from rodent MAPCs. However, the differentiation ability of human MAPCs using this new protocol is not enhanced compared with what we described in 2003. These culture conditions consist of initial induction of endoderm using Wnt3 and Activin-A; induction of hepatic endoderm using sequentially the mesodermal-derived cytokines BMP4 and FGF2 followed by FGF1, FGF4, and FGF8; and finally hepatocyte growth factor (HGF) and follistatin. This yields a mixed population of cells wherein a fraction expresses mature liver markers and has several functional characteristics of hepatocytes including albumin secretion, conversion of ammonia to urea, glycogen storage, bilirubin conjugation, and inducible cytochrome P450 activity. With minor adjustments, the

protocol can also be applied to induce differentiation of mouse and human ESCs towards functional hepatocyte-like cells.

18.5.2 Engraftment of MAPCs *in Vivo*

When mouse MAPCs were grafted intravenously, we found hematopoietic reconstitution. This study was further elaborated on in 2007, when two independent lines of MAPCs were grafted in sublethally irradiated NOD-SCID mice also treated with anti-natural killer (NK) antibodies: Tolar et al. demonstrated that engraftment of MAPCs that are MHC class-I-negative is inhibited by NK activity. We found multilineage hematopoietic reconstitution in 75% of animals, without evidence of fusion in the hematopoietic progeny. MAPC-derived KLS cells from primary recipients could rescue secondary C57/Bl6 mice from lethal irradiation and establish long-term hematopoiesis. MAPC-derived progeny cells that are CD45-negative can be found in multiple organs, although differentiation in a tissue-specific manner was not seen. In 2008, we demonstrated that both human and murine MAPCs improve both blood flow and function of ischemic limb in mice, via chiefly trophic effects, although some direct contribution to endothelial cells and skeletal muscle was observed. Likewise, when injected in the heart following left anterior descendant artery occlusion, we and others have shown that murine and swine MAPCs improve cardiac function in comparison with other cell populations, such as mouse embryonic fibroblasts (MEFs), and this again via trophic effect on cardiac cell survival and function, as well as angiogenesis.

Like MSCs, murine, rat, and human MAPCs have extensive immunomodulatory functions and can decrease T-cell-mediated immune reactions. In some of the studies this was found following systemic injection, whereas in other studies this was only observed by local injection.

18.5.3 Contribution of Rodent MAPCs to Chimeras

Although the initial MAPC line described in *Nature* contributed to chimeras, subsequent lines do not contribute in a significant manner. As the cells isolated under the new culture conditions have a primitive endoderm phenotype, for example Xen-P cells, and the latter have been shown to contribute to the visceral endoderm, we are currently evaluating the contribution of MAPCs to the yolk sac.

18.5.4 The Mechanisms Underlying the Greater Potency of MAPCs and Similar Adult Stem Cells with Greater Potency

One question that has not been answered is whether the cell populations described above (SKPs, PMPs, hFLMPCs, MAPCs, MIAMI cells, hBMSCs,

USSCs, FSSCs, AFS, MASCs, VSELs, and pre-MSCs) exist *in vivo* or are created in culture as the result of dedifferentiation. Of all the cells described, SKPs have been isolated directly from skin without the intervening culture step. SKPs can also be derived freshly, without the preceding culture, from fetal mice as well as from adult mice, where they appear to reside in a niche in the hair papillae and whisker follicles. SSEA1$^+$ pre-MSCs which express high levels of *Oct4* and can be expanded under MAPC conditions to generate cells capable of differentiating to the mesodermal, endodermal, and ectodermal lineage, and can contribute to hematopoiesis when grafted *in vivo*, can be isolated from mesenchymal cultures at passage 1. In contrast to MAPCs, the cells also expressed *Nanog* and Sox2. In addition, a homogeneous population of rare Sca-1+ Lin− , CD45− cells can be selected directly from the BM of mice and humans. These VSELs express *SSEA1*, *Oct4*, *Nanog*, and *Rex1*. The latter two studies suggest that rare cells exist in murine and human marrow with phenotypic features of MAPCs, MIAMI cells, hBMSCs, USSCs, AFS, or FSSCs. Whether the differentiation ability ascribed to MAPCs and similar cells is already present in the primary selected, uncultured BM cells, hence representing cells with greater potency persisting *in vivo* into postnatal life, or whether the differentiation ability is acquired once cells are culture-expanded *in vitro*, therefore representing dedifferentiation of a rare *Oct4*$^+$ cell, is not known.

The question as to whether MAPCs, and similar cells, exist as such is not only of academic importance; the answer may have profound biological implications as well as potential clinical applications. *In vitro*-generated cells have tremendous potential clinical usefulness, as long as the cells can be generated in an efficient and reliable manner. If MAPCs exist as such *in vivo*, it may one day be possible to manipulate their function *in vivo*, without the need for *in vitro* manipulation. Hence, future studies should aim to determine whether MAPCs and similar cells exist *in vivo*, and if so what their optimal method of isolation and *in vitro* expansion is; and whether they could be mobilized and/or activated *in vivo*. If the answer is 'no,' then it will be of the utmost importance to determine which cell population in a given tissue generates cells with greater potency *in vitro*, and develop strategies to select the precursor and induce with great efficiency the phenotype *in vitro*.

18.6 RECENT DEVELOPMENTS

Since 2010, new insights into human MAPC behavior and culture methods have been demonstrated. Human MAPCs were compared with human mesenchymal stem cells (hMSCs) and human mesoangioblasts (hMabs) and were found to be a distinct cell type based on surface marker expression and functional capacities. CD140b was found to be a marker which distinguishes between the three cell types, being highly expressed on hMSCs, expressed at

low levels on hMabs and not expressed on hMAPCs. Furthermore, hMAPCs showed a greater expansion capacity in comparison with hMSCs and hMabs. The differentiation capacity to osteogenic, chondrogenic, and smooth muscle lineage was similar in all three cell types, but only hMAPCs were capable of generating endothelial cells, while hMabs were the only cells that could generate skeletal myocytes. Transcriptome analysis confirmed the distinction between the three cell types. Interestingly, these differences could be overturned by changing culture conditions. When hMSCs or hMabs were grown under hMAPC conditions, both cell types acquired the ability to generate endothelial cells while hMabs also lost their ability to generate skeletal myocytes. On the other hand, hMAPCs lost their ability to generate endothelial cells when grown under hMSC conditions but not hMab conditions. Under hMab conditions no difference in functional capacities of hMAPCs was observed. Beside changes in functional capacities, the transcriptome was also altered according to the culture condition applied. These experiments indicate that the phenotype, transcriptome, and functional capacities are partially mediated by the applied cell culture conditions.

New insights into the immunological behavior of mouse MAPCs have also been published. Mouse MAPCs with high *Oct4* expression ($Oct4^{high}$ mMAPCs) were found to suppress local alloreactive T-cell expansion in lymph nodes *in vivo* but failed to suppress immunity in a systemic graft-versus-host disease (GVHD) animal model. Moreover, a bimodal modulatory effect was observed *in vitro* with immunostimulatory effect at low stimulator-to-effector (S:E) ratio (100:1 and 10:1) while a suppressive effect was found at S:E ratio 1:1. In similar experiments with mouse mesenchymal stem cells (mMSCs) and mouse embryonic stem cells (mESCs) only a suppressive effect was found, irrespective of the S:E ratio.

On the other hand, $Oct4^{neg}$ mMAPCs were also tested for their immunomodulatory capacities, as these cells more closely resemble hMAPCs. The immunostimulatory effect observed in $Oct4^{high}$ mMAPCs was no longer present in $Oct4^{neg}$ mMAPCs, as a suppressive effect was noted in all experiments irrespective of S:E ratio, similar to mMSCs. Also, *in vivo*, a local suppressive effect was found after both $Oct4^{neg}$ mMAPC and mMSC injection. Although the exact mechanisms are not fully understood, these experiments demonstrate that mMAPCs have immunosuppressive effects *in vivo* if delivered locally and that there might be a critical role for stem cell homing.

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CHAPTER 19

Mesenchymal Stem Cells

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19.1 THE DEFINITION OF MSCs

The precise definition of MSCs remains a matter of debate. Nevertheless, to date MSCs are widely defined as a plastic-adherent cell population that can be directed to differentiate *in vitro* into cells of osteogenic, chondrogenic, adipogenic, myogenic, and other lineages. As part of their stem cell nature, MSCs proliferate and give rise to daughter cells that have the same pattern of gene expression and phenotype and, therefore, maintain the 'stemness' of the original cells. Self-renewal and differentiation potential are two criteria that define MSCs as real stem cells, however, these characteristics have only been proved after *in vitro* manipulation, in bulk and at single-cell level, and there is no clear description of the characteristics displayed by unmanipulated MSCs *in vivo*.

In contrast to other stem cells such as hematopoietic stem cells (HSCs), which are identified by the expression of the CD34 surface marker, MSCs lack a unique marker. The CD105 surface antigen (endoglin) has been recently used to isolate hMSCs (human mesenchymal stem cells) from the bone marrow (BM), and such an approach enabled the characterization of freshly isolated hMSCs before culture. A distinct expression of certain surface antigens such as CD45 and CD31 was demonstrated in freshly isolated hMSCs, and the expression of these molecules was lower in culture-expanded hMSCs. These data suggest, again, the alterations that hMSCs may undergo during culture.

In several studies, cultured MSCs have been characterized either by using cell surface antigens or by examining the cells' differentiation potential. Lately, the Mesenchymal and Tissue Stem Cell Committee of the International

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Society for Cellular Therapy proposed the following minimal criteria to define human MSCs:

1. MSCs must be plastic-adherent when maintained in standard culture conditions and form CFU-Fs,
2. MSCs must express CD105, CD73, and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19, and HLA-DR surface molecules, and
3. MSCs must differentiate to osteoblasts, adipocytes, and chondroblasts *in vitro*.

19.2 THE STEM CELL NATURE OF MSCs

Stem cells are defined by their ability to self-renew and by their potential to undergo differentiation into functional cells under the right conditions. As detailed below, MSCs exhibit the potential to differentiate into the osteogenic, chondrogenic, adipogenic, tenogenic, myogenic, or stromal lineages.

The ongoing public discussion regarding whether MSCs are strictly stem cells requires a revision of the definition of stem cells, as MSCs apply to a wide cluster of non-hematopoietic stem-like cells isolated from mesenchymal tissues such as bone marrow, adipose, amniotic fluid, and blood vessels. The central question would be whether they might be differentiated into cells of other than a mesenchymal nature. Researchers have reported that MSCs from bone marrow and other tissues can be differentiated into epithelial, endothelial, and neural cells. As stated above, there is a consensus on specific MSC markers, but a unique marker of 'stemness' and multipotentiality has not yet been defined, since culture-expanded MSCs may lose some of these markers and acquire others, which are non-specific, but cells retain their multipotentiality. The molecular signature and *in vivo* distribution status of MSCs remain unknown and, as such, subject to investigation, even though *ex vivo*-expanded MSCs have been widely used in numerous studies.

In local models, direct injection of hMSCs into the brain tissue of rats resulted in the cells' long-term engraftment and subsequent migration along pathways similar to those used by neural stem cells. The results of these studies demonstrate the multilineage differentiation potential of BM-derived adult MSCs, and aid in defining them as suitable candidates for the regeneration of several mesenchymal tissues.

19.3 WHICH TISSUES CONTAIN MSCs?

The embryonic origin of MSCs is still unclear; however, some findings indicate a possible origin of MSCs in a supporting layer of the dorsal aorta in the aorto–gonadal–mesonephric region. Consistent with these findings, MSC-like

cells were found circulating within early human blood. In adults, MSCs appear to be 'resident' stem cells in many tissues, and they function in the normal turnover of these tissues. When tissue repair is required, these cells can be stimulated to proliferate and differentiate.

The most studied MSCs compose the stroma-supportive system of BM along with endothelial cells and adipocytes. An MSC population was also found in the BM of the craniofacial complex. However, many studies have demonstrated the presence of MSCs or MSC-like cells within other tissues such as adipose tissue (ASCs), dermal tissue, intervertebral disc, amniotic fluid, various dental tissues, human placenta, cord blood, and peripheral blood, although the latter finding is still controversial.

ASCs are quite similar to BM-derived MSCs morphologically and immunophenotypically, however, ASCs form more CFU-Fs when plated in culture. Adipose tissue is an attractive source of MSCs for regenerative medical purposes: it is relatively easy to obtain, can be collected with the use of local anesthesia, and is associated with minimal discomfort and risks.

19.4 MSC ISOLATION TECHNIQUES

Application of MSCs requires their isolation, and direction of their differentiation into the appropriate lineage. Since the 1980s, a density gradient has been used to separate mononuclear cells (MNCs) and red blood cells in the BM. The MNCs are then collected and seeded in medium containing 10% fetal bovine serum (FBS) at a density of $10–15 \times 10^5$ cells/cm^2 growth area. Adherent spindle-shaped cells appeared within 48 h after the initial seeding, and the estimated percentage of MNCs ranges from 0.001 to 0.01%.

Adipose-tissue-derived stem cells (ASCs) can be isolated also from adipose tissue after enzymatic treatment with collagenase. Then, a stromal vascular fraction (SVF) is obtained that parallels the MNC fraction in BM. This fraction is collected while the adipocyte-containing fraction is removed during the first steps of centrifugation due to its high content of fatty acids. Plastic-adherent cells within the SVF were shown to have a high potential for *in vitro* expansion and for differentiation into several mesenchymal lineages.

The major disadvantages of these methods are the presence of adherent cells of hematopoietic origin within the cultures during the first days and the need for *in vitro* culturing and expansion. The solution to these downsides will include isolation of cells based on intrinsic properties of MSCs avoiding culturing and the generation of immortalized cell lines.

Immunoisolation is a method to isolate non-cultured MSCs based on cell surface markers. Several studies employed the positive selection technique by immunoisolating MSCs with antibodies directed against the endoglin

(CD105), Stro-1, CD146, and MSC markers. Furthermore, immunodepletion is a 'negative selection' approach, in which the MSC population is enriched by washing out the cells labeled with antibodies, mostly directed against hematopoietic markers. Recently, more specific and pure populations were isolated utilizing a combination of immunoisolation and immunodepletion based on different surface markers.

Roda and collegues recently developed another technique for non-cultured MSC isolation that does not rely on surface marker, but on biophysical properties that cells acquire when in suspension under fluidic conditions. This was further described in a detailed protocol.

19.5 IMMUNOMODULATORY EFFECTS OF MSCs

Several studies have shown that MSCs escape immune recognition and inhibit immune responses. The modulation of the immune system was detected in both BM-MSCs and ASCs. This property of MSCs facilitates clinical use of MSCs in an allogeneic manner in diverse regenerative medicine approaches, for example liver transplantation.

A variety of suggested mechanisms explicate how MSCs prevent allogeneic rejection among different species, such as weak immunogenicity, interference in the maturation and function of dendritic cells (DCs), abolishment of T-cell proliferation, or interaction with natural killer (NK) cells in cell-to-cell contact or through the release of soluble secreted factors. Although there have been discrepancies, probably due to the different implemented experimental systems, the majority of the reports have indicated no or low expression of major histocompatibility complex (MHC) class II proteins. There is evidence for interference in the maturation of DCs: it was demonstrated that, although hMSCs are able to promote antigen-induced activation of purified T-cells, an addition of antigen-presenting cells (APCs) – monocytes or DCs – to cultures inhibited, in a contact-dependent manner, the T-cell responses. This inhibition could be partially overridden by the addition of factors that promote APC maturation. These data have been supported by findings of co-culture experiments, in which both MSCs and their supernatants interfered with the endocytosis of DCs and decreased their capacity to secrete (interleukin) IL-12 and activate alloreactive T-cells. Similar conclusions have been reported in co-cultures of hMSCs and DCs where decreased tumor necrosis factor secretion in mature type I DCs and increased secretion of IL-10 was observed.

Numerous groups support the direct interaction of MSCs and T-cells, either by cell contact or by the release of soluble factors. There is a distinction between T-cell stimulation in culture by mitogen and alloantigens. MSCs increased the levels of IL-2 and the IL-2-soluble receptor, as well as that of

IL-10 in mixed lymphocyte culture (MLCs). None of these factors are constitutively secreted by MSCs. When peripheral blood lymphocytes were stimulated with phytohemagglutinin (PHA), decreases in levels of IL-2 and the IL-2 soluble receptor were observed, whereas IL-10 levels were not affected. Moreover, the addition of a prostaglandin inhibitor, indomethacin, restored the inhibition induced by MSCs in PHA cultures, but did not influence MLCs. TGFβ1 and hematopoietic growth factor (HGF) were shown to be mediators of MSC effects on T-lymphocyte-suppressed proliferation by using neutralizing monoclonal antibodies. Cellular stimuli were effective as well as nonspecific mitogens, and T-cell inhibition is conducted by soluble factors, as shown by transwell experiments, in which cell-to-cell contact between MSCs and effector cells was avoided.

Alternatively, MSCs alter the phenotype of NK cells and suppress proliferation and cytokine secretion. Some of these effects were mediated by soluble factors including TGFβ1 and PGE-2. Others reported no involvement in T-cell inhibition by MSCs. The upregulation of PGE-2 in co-cultures has been observed as well, although the role of PGE-2 in the downregulation of MLCs diverged from that mentioned above.

Overall, the way by which MSCs avoid detection by the immune system is not thoroughly elucidated yet. Still, novel mechanisms might be revealed, as additional soluble factors and cells are actively under research.

19.6 SKELETAL TISSUE REGENERATION BY MSCs

19.6.1 Bone

Bone fractures and small defects usually regenerate and heal without the need for surgical intervention. Yet, in certain conditions, tissue loss is too extensive and complete spontaneous healing cannot be achieved. This is the case for non-union fractures and other critically-sized defects that might occur in long bones, the spinal column, or the craniofacial complex. In addition, certain procedures, such as spine fusion, require neo-formation of bone in sites where osteogenesis does not physiologically occur.

Numerous studies have attempted to demonstrate the feasibility of MSC-mediated bone regeneration. In general, MSCs can either be systemically administered using intravenous (i.v.) injection or directly implanted in the bone defect site. The systemic approach assumes that MSCs have the capability to migrate across the endothelium and home to injured tissues in a manner similar to the migration of leukocytes to sites of inflammation. This phenomenon has been shown in different experimental models, including injuries to heart, brain, liver, and lungs. Several studies have also shown that MSCs home to sites of bone fractures or to bones with impaired

development, as in several patients of osteogenesis imperfecta treated with allogeneic MSCs. Yet, although the systemic approach is attractive for clinical use, it is still unknown what percentage of the injected cells will eventually engraft at the injured tissue. It has been shown that, shortly after i.v. injection, the MSCs are entrapped in the lungs and are probably released to the circulation a few days later. Thus, the direct implantation approach aims at concentrating a high number of MSCs at the site of the injury without the risk of cell migration to other sites in the body.

Undifferentiated MSCs tend to form a non-specific connective tissue even in bone defects, therefore, it is essential to either induce osteogenic differentiation of the cells *in vitro* prior to implantation, or to seed them onto an osteoinductive and osteoconductive scaffold, which is usually composed of hydroxyapatite and β-tricalcium phosphate. The potential of MSC-loaded osteoinductive scaffolds to repair segmental defects in long bones has been shown in a number of animal models. Using a similar approach, spine fusion was achieved in large animals including rabbits, sheep, and rhesus monkeys. Following this solid experimental proof of principle, this tissue-engineering method was used to treat three human patients who suffered a bone loss of 4–7 cm in long bones. A good integration of the implants was evident two months post-surgery. The patients recovered function in 6–7 months after surgery (one half to one third of the time needed for recovery using 'conventional' bone grafts) and no special problems were recorded over a six-year follow-up period. Since then, several reports have described the use of this approach for bone regeneration in human patients in different sites, including the jaw, spine, and femoral head.

The downside of using hydroxyapatite scaffolds is their slow resorption rate *in vivo*. In fact, a large portion of these scaffolds does not resorb even after a few years, thus preventing complete bone regeneration. An alternative approach could be the combination of MSCs and an osteogenic factor such as bone morphogenetic protein (BMP)-2. BMP-2 can be incorporated into a scaffold during its preparation and then combined with MSCs. In this manner, BMP-2 is slowly released from the scaffold upon implantation, and its release is in correlation with the degradation rate of the scaffold itself. It is assumed that BMP-2 induces the osteogenic differentiation of the implanted MSCs and resident MSCs at the site of implantation. The shortcoming of this strategy lies in the short half-life of BMP. Thus, the effect of BMP-2 in this system could be limited.

A different approach, which combines MSCs and a continuous secretion of an osteogenic protein at the fracture site, is known as MSC-based gene therapy. This method requires the genetic modification of MSCs to overexpress a transgene encoding for an osteogenic gene. BMP-2 has been widely used

for this purpose, and also other members of the BMP family, such as BMP-4, BMP-6, and BMP-9. There are several advantages to this approach of tissue regeneration. First, the implanted MSCs secrete physiological quantities of the osteogenic factor, over a period of time. Second, MSCs tend to migrate to the fracture edges and induce an organized pattern of fracture repair, when compared with BMP-2 treatment, which induces the formation of scattered foci of ossification instead. Third, due to a continuous secretion of the osteogenic factor, an autocrine-paracrine effect is exerted inducing the osteogenic differentiation of the implanted MSCs and resident stem cells in the surrounding tissue. It is important to note that, when new bone generated by BMP-2-engineered MSCs was analyzed for its chemical, structural, and nanobiomechanical properties, it showed remarkably similar values to its natural counterpart.

19.6.2 Cartilage

Regeneration of damaged cartilage presents a great challenge for orthopedic medicine, because articular cartilage has very limited capacity for effective repair. Adult MSCs have the potential to proliferate and differentiate into chondrocytes, they can therefore be considered ideal candidates for cartilage tissue repair. Several attempts have been made to implant cells in cartilage defects. The first attempt involved the culture of autologous chondrocytes and their implantation in a cartilage defect in patients younger than 50 years of age who were believed to have healthy chondrocytes. It appeared, however, that chondrocytes could only achieve limited success in regenerating cartilage defects. It was also shown that chondrocytes loaded onto a polymeric carrier underwent apoptosis, which limited their therapeutic potential. These results prompted research into autologous pluripotent cells with chondrocyte-differentiating capacities. Evidence that MSCs can produce cartilage regeneration has been controversial. Findings of some studies indicate that MSCs fail to produce full regeneration over long time periods. MSCs have also been found to have limited success in forming long-lasting cartilage tissue. Other studies, in which sheep, pig, and rabbit models were used, have demonstrated the feasibility of using biodegradable scaffolds seeded with MSCs for articular cartilage repair.

Genetically modified MSCs have also been used in an attempt at cartilage formation, however, only a few genes have been shown to induce chondrogenic differentiation in these cells. When infected with adeno-TGFβ but not with adeno-IGF-1, MSCs differentiated into chondrocytes *in vitro*. A combination of IGF-1 and TGFβ or BMP-2 gene delivery to MSCs led to enhanced chondrogenesis *in vitro*, however, with the expression of collagen X, a marker of hypertrophic cartilage. Successful induction of MSC chondrogenic differentiation *in vivo* was achieved using the overexpression of Brachyury transcription

factor. Brachyury-expressing MSCs secreted collagen II, but not collagen X, *in vitro* and *in vivo*. Moreover, the implantation of these cells in ectopic sites *in vivo* has led to the formation of a chondrogenic tissue composed of proliferative chondrocytes. Interestingly, the engineered chondrogenic tissue generated *in vivo* was resistant to the destructive effect of rheumatoid arthritis synovial fibroblasts.

19.6.3 Tendon

Although of low occurrence, tendon and ligament lesions (especially rotator cuff, Achilles' tendon, and patellar tendon defects) are among the most common soft-tissue injuries. Repairing these defects is not a simple task, and indeed the available surgical treatments are not satisfactory. The *in vitro* differentiation of MSCs into tendon or ligament cells has only been shown in a few studies, either by application of exogenous forces to the scaffold on which the cells are grown or by the use of a specific scaffold made of hyaluronic acid, which induces ligament differentiation of hMSCs. There is no evidence that MSCs that have differentiated *in vitro* into tendon or ligament cells can indeed repair those tissues *in vivo*.

One possible treatment for *in vivo* tendon repair involves the implantation of non-differentiated MSCs that have been seeded onto various biodegradable scaffolds. So far there have been contradictory reports in the literature. It has been shown that the implantation of autologous MSCs in rabbit Achilles' tendon defects improves the physical properties of the damaged tendon when compared with tendons treated only with hydrogel, scaffold, or sutures, yet this effect could be detected only for a few weeks post-surgery. MSCs obtained from older animals are able to induce tendon repair in young ones. A recent publication found no added value for the implantation of MSCs in a rat rotator cuff tear. One adverse effect discovered in some of these studies was the formation of ectopic bone within tendons implanted with MSCs. Some have posited that there is no morphometric difference between tendons implanted with MSCs and ones implanted with collagen gel.

MSCs genetically modified to overexpress the Smad8 and BMP-2 cDNAs were shown to differentiate to tenocyte-like cells *in vitro* and *in vivo*. In addition, when implanted into a 3 mm defect in a rat's Achilles' tendon defect, complete regeneration was achieved, as demonstrated by double-quantum filtered magnetic resonance (MR) and histology. So far this has been the only report of genetically engineered MSCs used for tendon or ligament regeneration.

19.6.4 Intervertebral Disc

Regeneration of an intervertebral disc (IVD) poses great challenges for stem cell therapy due to the hostile environment in which implanted cells must

survive. The IVD is avascular and hypoxic, in the rabbit IVD, the nearest blood vessel can be 5–8 mm away from cells at the disc center. The disc cells (mainly nucleus pulposus (NP) cells) use anaerobic metabolism to generate energy. As a result, lactic acid (the main product of glycolysis) can accumulate, resulting in a low pH environment.

Studies have outlined two strategies for stem cell-mediated IVD regeneration. The first, which is indicated for early disc degeneration, is to regenerate only the NP. This could be achieved by direct injection of MSCs, similarly to what is done in discography procedures in the clinic today. Several works have shown that MSCs differentiate to NP-like cells when co-cultured with NP cells injected into a disc organ culture or cultured in specific scaffolds. It has been shown that low pH levels that exist in degenerated discs might have a significant effect on MSC proliferation and differentiation. Nevertheless, studies in rodents, canines, and rabbits showed that MSCs could survive in 'nucleotomized' discs for several weeks (up to 48), enhance extracellular matrix production, and increase disc height. A comprehensive biomechanical comparison between native and engineered tissues should be performed to evaluate the ability of this approach to generate functional NP tissue. In addition, care should be taken when choosing the right needle for stem cell injection, since its diameter might have an effect on the damage caused to the disc. Moreover, the injected cells might leak out via the entry site of the needle, as shown in a pig model.

A second strategy for IVD regeneration relates to the complete regeneration of the IVD, which could be relevant for a late-stage disease. This is a more challenging tissue-engineering goal, which will require the combination of designated scaffolds and inducing factors that will regenerate both the NP and the annulus fibrosus.

Interestingly, there is evidence showing the presence of resident MSCs in the degenerated IVD, which might imply that a potential therapy could include the activation of these cells in order to regenerate the disc.

19.7 NON-SKELETAL TISSUE REGENERATION BY MSCs

During the mid 1990s, the first two reports demonstrating non-skeletal differentiation potential of MSCs were presented. These reports were validated a few years later. Since then, MSCs have been used as regenerators of heart, skeletal muscle, nerve, liver, kidney, lungs, and pancreas.

The use of pluripotent stem cells to regenerate damaged heart tissue is being advocated as the new treatment for heart failure secondary to heart disease or

severe myocardial infarction. Promising results at the research stage have now led to the challenge of applying stem cell technology in the clinical setting.

Cardiomyocytes generated from MSCs were able to stay differentiated after being transplanted into the adult murine heart. Transplantation of MSCs improved cardiac function in animal models, possibly through induction of myogenesis and angiogenesis and inhibition of myocardial fibrosis by the cells' ability to supply angiogenic, anti-apoptotic, and mitogenic factors.

In order to achieve clinical application, studies were conducted in preclinical large animal models, with encouraging results. Methods for introducing the cells to the tissue and monitoring their homing, survival, and post-implantation effect were developed. New sources from which stem cells can be isolated for cardiac applications were identified and, in recent research, the effect of immunosuppressive drugs that will probably be used in implanted patients was tested on MSC activity. Finally, clinical studies have been undertaken and report excellent results: while the use/application of MSCs is now studied in small scale, mainly for safety and feasibility, the use of bone marrow mononuclear cells has been extensively studied and showed a significant reduction in subsequent cardiovascular events.

MSCs have been shown to promote neuron survival and limit the severity of neurological impairment in animal models of traumatic brain injury and stroke. Direct implantation of MSCs, either native or genetically engineered, into the spinal column has also been shown to promote functional recovery following spinal cord injury. Many preclinical animal studies have been conducted recently, with promising results that show MSC migration, differentiation, and regeneration effects in the brain. MSCs were able to reduce neuropathic pain, confer neuroprotection in a rat model for glaucoma, induce neuronal regeneration after neonatal ischemic brain injury, and treat depression, Parkinson's disease, and even epilepsy. The neuroprotective effects of MSCs are thought to result in part from their ability to replace diseased or damaged neurons via cellular differentiation as well as by induction of neurogenesis, angiogenesis, synapse formation, activation of endogenous restorative processes, and modulation of inflammatory response. Still, few clinical studies have been performed that have demonstrated the safety and regenerative effect of MSCs.

As the prevalence of diabetes increases, new treatment avenues are being sought and MSCs have been identified as prime candidates. Scientists have been able to obtain islet-like functional cells through differentiation of both animal and human MSCs from BM by modifying the cell culture environment. Lately, MSCs were also identified in pancreatic tissue cultures, in fact, a combined implantation of MSCs with islet graft improved the graft's function significantly. Moreover, MSC systemic and local administration was

found to be effective in different reports: reversion of hyperglycemia, reduction of albuminuria, and regeneration of beta-pancreatic islets on mouse and rat models of diabetes, amelioration of diabetic nephropathy in rats and mice, and even improvement of diabetic polyneuropathy in rats. Large animal models such as dogs and pigs confirm the feasibility of MSC-based therapy for diabetes. The therapeutic effect of MSCs in diabetes models may be due to their immunomodulatory capacity and paracrine activity, apart from their direct cell differentiation.

Schwartz and associates reported for the first time that multipotent adult progenitor cells (MAPCs) could differentiate into functional hepatocyte-like cells. Since then, many studies have demonstrated hepatic differentiation of MSCs and their applications in preclinical models and even in a phase-1 clinical trial.

In summary, even for non-skeletal tissue injuries and diseases, MSCs are seen as serious candidates that indeed possess the potential for future treatment of choice.

19.8 CONCLUSIONS

MSCs constitute a unique population of adult stem cells that hold great promise for various tissue-engineering applications. These cells can readily be isolated from various sites in the human body, especially from BM and adipose tissues. Established protocols exist for the induction of specific differentiation patterns of MSCs into different committed cells, most notably into osteoblasts, chondrocytes, and adipocytes. So far it has been demonstrated that the use of genetically modified MSCs, with overexpression of various therapeutic transgenes, is a powerful tool in the induction of differentiation and in the promotion of tissue regeneration *in vivo*. Novel technologies, which utilize electroporation-based systems, allow for the safe and efficient gene delivery into MSCs and bypass the need for using non-safe viral vectors. It has been shown that the ultrastructural, chemical, and nanobiomechanical properties of engineered bone derived from MSCs were similar to those of native origin. The conventional method of MSC isolation using plastic adherence has been shown to be costly and might reduce the stemness of the cells. Therefore, an attractive alternative has been developed that includes the immediate use of immuno-isolated, non-cultured MSCs for *in vivo* implantation. Future challenges require the identification of an optimal scaffold for MSC implantation *in vivo* and, finally, the development of a preservation method for future reuse of autologous cells. Non-invasive imaging will continue to play an important role in analyzing the power of MSCs to regenerate tissues in various defect models. Overcoming these hurdles will no doubt make MSCs the optimal tool for biological tissue replacement within this century.

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FOR FURTHER STUDY

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CHAPTER 20

Skeletal Muscle Stem Cells

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20.1 INTRODUCTION

Tissue-specific stem cells in adult animals have been described most widely in tissues with high turnover, such as blood, skin, or in tissues with a diverse cellular composition, such as the central nervous system (CNS). These tissues have a need for either frequent replenishment or for frequent remodeling; both are processes that lend themselves to the activity of a proliferative subpopulation of cells with the capacity to replace any cell type within that tissue. The classical characteristics used to define a cell as a stem cell derive from the best studied system, hematopoiesis. Accordingly, stem cells must be self-renewing, highly proliferative, and capable of differentiating into at least one other cell type. Unlike blood, skin, and CNS tissues, skeletal muscle fibers, the essence of muscle tissue, do not have an apparently rapid turnover. Because muscle tissue possesses a highly specialized cellular architecture and is prone to being damaged by physiologic use throughout life, its persistence and function necessitates the ability to regenerate.

Skeletal muscles are composed of bundles of muscle fibers (myofibers) that are large, terminally differentiated, multinucleate cells formed by the fusion of mononucleate MuSCs. Myofibers can generally be grouped into two different types based on function; fast or slow contracting, a distinction that depends largely on the composition of myosin heavy chain (MyHC) isoforms they express. As shown by interspecies grafting techniques or lineage tracing experiments during embryogenesis, MyHC-expressing mononucleate cells that produce muscle fibers in the limbs originate from the mesodermal somites. By contrast, the muscles of the craniofacial region originate from the sometomeres. To form the muscles of the limb, mononucleate myocytes migrate from the somites into the limb buds in two waves. In the mouse, primary muscle fibers are formed at embryonic day 13 (E13) and are followed by the formation of secondary muscle fibers at E16, which surround and align

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themselves in parallel with the primary fibers. In the limb, these two phases of myogenesis are accompanied by changes in fiber type: primary fibers are relatively large in diameter and they express slow MyHCs, whereas secondary fibers are smaller and express fast MyHCs when they are first formed. Eventually, the primary or secondary origin of the fibers cannot be distinguished, because at birth muscle consists of a mosaic of both fast and slow fibers of similar size. Even the MyHC composition within an individual fiber can differ, because myosins are encoded by distinct nuclei and maintained in nuclear domains. Myoblasts isolated from mice and cloned express all MyHCs irrespective of their muscle of origin, suggesting that regulation is imposed *in vivo*. The myogenic process is regulated by a well-known cascade of basic helix-loop-helix transcription factors, known as muscle regulatory factors (MRFs), expressed sequentially during myogenic development. Myf-5 and MyoD are expressed during the monocyte stage and then decline during differentiation and are followed by the expression of myogenin and MRF4. Once formed, myofibers express myosins, actins, and other proteins that comprise the contractile apparatus and the complex cell surface array of dystroglycans, integrins, and dystrophin. The constant threat of damage in adult animals to these structurally complex, postmitotic cells resulting from exercise, chemical agents, or genetic deficiencies suggests that the need for a regenerative pool of cells within the muscle is profound. This need is particularly apparent when a muscle's pool of regenerative cells is exhausted or becomes nonfunctional, as happens in muscular dystrophies such as Duchenne's, the aging process, or following high doses of γ-irradiation. When MuSCs are inadequate in number or function, the result is progressive muscle degeneration and atrophy.

20.2 THE ORIGINAL MUSCLE STEM CELL: THE SATELLITE CELL

The canonical MuSC in adult animals, designated 'satellite cell,' was anatomically defined in 1961 by transmission electron microscopy (TEM) studies of the peripheral region of muscle fibers in the tibialis anticus muscle of the frog. The discovery of the satellite cell heralded the birth of the field of muscle regeneration. Satellite cells have a high ratio of nucleus to cytoplasm, are intimately juxtaposed to muscle fibers, and resident in their own membrane-enclosed compartment, between the sarcolemma of the myofiber and the surrounding basal laminal membrane. So intimately are the satellite cells associated with the myofiber that they are impossible to discern from myonuclei within the fiber by conventional light microscopy. As a result, definitive identification requires laser scanning confocal microscopy or TEM. By grafting quail somites into chicken embryos, organisms with similar developmental time courses, but distinct nuclear morphology, it was shown that these cells in the adult muscle

are descended from the cells of the somites. Before the discovery of the satellite cell it was unclear whether there existed a mononucleate cell with the sole purpose of repairing damaged muscle fibers, or whether the nuclei of damaged fibers underwent a process whereby they replicated and ensheathed themselves in their own membrane, thereby proceeding to participate in their own repair. The former model is widely accepted, but it has yet to be demonstrated conclusively. Thus far, it has proven impossible to observe the same satellite cell in its characteristic anatomic position divide asymmetrically, renew itself, and then differentiate and give rise to a myonucleus in a myofiber. However, as described later, there are several lines of evidence strongly suggesting that the satellite cell is a MuSC that does just that.

The first evidence that satellite cells are derived from MuSC was provided by electron microscopy (EM) studies, which showed that after a single injection of [^{3}H] thymidine, only a small number of satellite cells, not myonuclei, had incorporated the radioactive label. These findings suggested that satellite cells were quiescent most of the time and were the only muscle-associated cells that could proliferate. A subsequent study showed that after transplantation of a [^{3}H]+thymidine labeled extensor digitorum longus (EDL) into the muscle bed of another animal, the myonuclei of the myofibers of the host animal had [^{3}H] thymidine labeled myonuclei. Apparently, the damage inflicted to the muscle during the transplantation procedure caused the satellite cells of the donor EDL to proliferate and differentiate, contributing donor myonuclei to the host myofibers. Taken together, these data suggest that satellite cells were stimulated to multiply and contribute to muscle in response to tissue damage.

The second line of evidence that satellite cells are MuSC derived came from experiments that showed that, like other populations of proliferative cells, satellite cells are sensitive to γ-irradiation, which renders them unable to meet the demands imposed by increased weight or exercise. When part of the tibialis anterior (TA) muscle is surgically removed, the demands on the neighboring EDL muscle are increased. In response to the additional weight and exercise, the EDL increases in mass because of a hypertrophic adaptive response. Both the gram weight and the average myofiber diameter of the overloaded EDL increased substantially when compared with the EDL of the contralateral leg in which the TA was not resected. Moreover, if one limb was treated with a high dose of γ-irradiation (25 Gy) before TA resection, the EDL could not adapt as well and was both reduced in mass and had significantly smaller myofiber diameters than nonirradiated controls. These results provided evidence that the proliferative activity of satellite cells was integral to the hypertrophic response, an observation confirmed by others.

A third line of investigation that implicated satellite cells as the primary regenerative cell of skeletal muscle derived from the characterization of animal

models with muscular dystrophy. One of the most commonly used mouse models of Duchenne's muscular dystrophy (DMD) is the mdx mutant, which carries a point mutation in the dystrophin gene that creates a translational block leading to a truncated protein. As described previously, dystrophin is a key protein in the membrane-bound dystrophin glycoprotein complex (DGC), which is thought to fortify the plasma membrane of the muscle against the intense shearing forces that are generated during daily exercise. The muscles of mdx mice have spontaneously revertant muscle fibers which express dystrophin resulting from a compensatory point mutation that corrects the translational block. Thus, as the mdx mice age, revertant fibers are found in clusters, in which each constituent myofiber harbors the same compensatory mutation. Interestingly, the compensatory mutations found in different bundles are unique to each bundle, suggesting that they are derived from the progeny of a single cell. This interpretation is supported by experiments in which limiting dilutions of β-galactosidase (β-gal) encoding retroviruses were used to demonstrate that a satellite cell infected with a single retrovirus has the ability to proliferate and migrate laterally. As a result, it can participate in regeneration of not only its own fiber but also of nearby fibers, leading to bundles of fibers regenerated by the progeny of the same satellite cell. Taken together, these data suggest that the bundles of revertant fibers in the mdx mouse are the result of fusion and differentiation of progeny derived from a single satellite cell or clone.

The fourth line of evidence that satellite cells are MuSCs derives from a mouse model used to study pathogenesis in dystroglycan-based muscular dystrophies, another constituent of the DGC. In these experiments satellite cells are labeled as they fuse with muscle fibers. A mouse was engineered with a transgene, in which Cre-recombinase was under control of the muscle creatine kinase promoter, which is only active in differentiated myofibers. This mouse was mated with transgenic mice, in which the gene encoding dystroglycan was flanked by LOX sites. The satellite cells expressed normal levels of dystroglycan until they fused with the existing muscle fibers at which time the dystroglycan transgene in the newly contributed myonuclei was excised by the Cre enzyme already present in the myofiber cytoplasm. In this mouse model, the Cre-induced LOX-recombination event did not occur immediately following fusion, and during the lag-time dystroglycan was transiently expressed in the muscle fiber. The result was that the usually strong onset of this type of dystrophy was delayed. Moreover, the transient presence of the satellite-cell-derived dystroglycan strongly suggests that satellite cells were fusing with the myofibers.

Fifth, MuSCs are typically isolated on collagen-coated or gelatin-coated plates from crude preparations of skeletal muscle. When isolated by this method they are usually termed myoblasts. These cells, if grown at clonal density, can differentiate into multinucleate myotubes when cultured in low-mitogen media, can fuse with existing myofibers when injected *in vivo*, and

are considered to be the highly proliferative descendants of satellite cells. However, the method by which they were isolated, their exact origin, and their relation to the anatomically defined satellite cell have been unclear. Myoblasts can be identified by scanning EM and can also be isolated from individual physically dissociated myofibers plated in tissue culture, a laboratory method capable of tracking satellite cell progeny. Mononucleate cells that migrated off the fibers formed colonies that differentiated into myotubes in culture. Unfortunately, the method used and the absence of specific markers made it difficult to confirm that the cells that migrated off the muscle fibers were initially in the appropriate anatomic position, underneath the basal lamina juxtaposed to the myofiber sarcolemma. As a result, the evidence that satellite cells beget myoblasts is strong but not conclusive.

Finally, there are physiologic and biochemical properties that implicate satellite cells as essential to muscle regeneration. In addition, in the fast fibers of the cat plantaris muscle, denervation and consequent immobilization caused muscle atrophy, leading both to a decrease in myonuclear number and fiber diameter compared with controls. Conversely, innervated plantaris muscle overloaded with increased weight exhibited augmentation of both myonuclear number and fiber diameter. These results are consistent with the interpretation that the satellite cells respond to exercise-induced muscle damage or stress. Similarly, myoblasts transduced with a nuclear localized β-gal expressing retrovirus, proliferated in tissue culture, and then injected into the TA and EDL muscles of an mdx host, exhibited dystrophin expression in single isolated myofiber in domains surrounding a β-gal-expressing donor nucleus, providing evidence that MuSC contribution restored dystrophin expression.

Taken together, these experiments strongly support the hypothesis that satellite cells are MuSCs, because they meet the criteria of self-renewal, proliferation, and the ability to give rise to more than one cell type (satellite cells, myoblasts, and muscle fibers). Like hematopoietic stem cells (HSCs), quiescent satellite cells can self-renew following isolation in culture or serial transfer in mice and are capable of robust proliferation as myoblasts *in vitro* and *in vivo*, and they are capable of differentiating into differentiated postmitotic myotubes in tissue culture and fusing with myofibers *in vivo*. These findings suggest that the canonical MuSC, the satellite cell, is capable of regeneration and maintenance of skeletal muscle.

20.3 FUNCTIONAL AND BIOCHEMICAL HETEROGENEITY AMONG MUSCLE STEM CELLS

During the study of MuSCs and their role in muscle regeneration, an apparent functional and biochemical heterogeneity within the population has become

apparent. Differences among MuSCs isolated at different ages from distinct myofiber types, fast and slow, and from muscles with distinctive embryonic origins, the limbs versus the masseter, are characterized by distinct physiologic and biochemical phenotypes. The purpose of this complexity remains unclear. Are the same cells observed at different time points or are there distinct subpopulations of MuSCs (e.g., one reserve population that gives rise to another that is poised to participate in the regeneration of specific muscles at specific stages of development)?

Several lines of evidence suggest that satellite cells are functionally heterogeneous. For example, a population of satellite cells was reported that underwent mitosis at least once in 32 hours, was committed to a myogenic fate, and readily differentiated, whereas another much smaller population did not enter mitosis but was thought to give rise to the first population following asymmetric divisions. Others have reported a radiation-resistant population of satellite cells that could be activated by an injection of the myotoxic snake venom, notexin. Notably, the notexin-responsive population was absent in the mdx mouse. Additional observations of the C2C12 mouse myoblast cell line and human primary myoblasts also suggested the existence of two distinct MuSC populations, one that gave rise to the other, which became quiescent instead of differentiating. In the case of C2C12 myoblasts, transcription factors were correlated with cells cultured in proliferative conditions (Myf-5$^+$ and MyoD$^+$) and in differentiation conditions (myogenin$^+$ and MRF4$^+$); however, even in differentiation media, a very small subpopulation of cells remained mononucleated but ceased to express any Myf-5 or MyoD. These mononucleate cells recapitulated the properties of their parental population when recultured in proliferative conditions and subsequently switched to conditions that favored differentiation. Taken together, these studies suggest functional heterogeneity among satellite cells and their derivatives.

Biochemical heterogeneity among MuSCs has also been documented. Single cell reverse transcriptase polymerase chain reaction (RT-PCR) was used to determine gene expression in quiescent and activated satellite cells by isolating single myofibers, and injecting each with toxin to cause necrosis of the fiber, so that the remaining associated viable cells were thought to be quiescent satellite cells that could be readily collected for analysis. The results showed that all quiescent satellite cells expressed the tyrosine kinase receptor cMet, whereas only 20% expressed the adhesion protein m-cadherin, and few expressed the transcription factors Myf-5 or MyoD. On the other hand, 'activated' satellite cells that crawled off these fibers proliferated and expressed all four of the MRF transcripts (Myf-5, MyoD, myogenin, and MRF4), and cMet and m-cadherin. In another study, the cell surface antigen, CD34, was shown to be expressed on quiescent satellite cells, whereas following activation an alternatively spliced isoform was transiently expressed. In contrast to the first study, the second

study found that Myf-5 and m-cadherin were detected on quiescent $CD34^+$ satellite cells. The authors speculated that CD34 plays a role in maintaining a quiescent state, and that $CD34^+$/Myf-5^+ satellite cells are the descendants of CD34 − /Myf-5− satellite cells. Taken together, the cell surface markers and transcription factors $cMet^+$/m-$cadherin^+$/$CD34^+$/Myf-5^+/$MyoD^+$ characterize most committed and activated satellite cells. By contrast the quiescent parent cells are far less well defined: $cMet^+$/m-cadherin ± /CD34 ± /Myf-5 ± /MyoD− (the transcription factor Pax7 has also been implicated as a marker of these cells). Moreover, not all of the markers are specific to satellite cells. For example, some cells of the blood are also known to express cMet receptor, and, therefore, it is only a good marker of satellite cells in the context of their anatomic position: membrane encircled and juxtaposed to a myofiber. The only direct evidence that the quiescent population gives rise to the activated and committed form of satellite cell is derived from the *in vitro* experiments conducted with the C2C12 cell line not primary cells. Clearly, lineage studies and more markers are needed to define unambiguously the heterogeneity of MuSCs and their etiology.

20.4 UNORTHODOX ORIGINS OF SKELETAL MUSCLE

The observed heterogeneity within the MuSC population and lack of conclusive evidence that satellite cells are the only MuSC raises the possibility that other cells associated with muscle tissue also give rise to MuSCs. During the past 30 years several reports have suggested that alternate sources of MuSC may include the thymus, dermis, vasculature, synovial membrane, and the bone marrow. This section focuses on evidence that MuSCs can derive from cells of the bone marrow, because the other tissue sources have already been extensively reviewed.

A number of investigators have demonstrated that following a marrow transplant, bone-marrow-derived cells (BMDCs) are present in diverse tissues in mice and humans, where they express characteristic tissue-specific proteins. These tissues include the heart, epithelium, liver, skeletal muscle, and brain. These combined results suggest that repair of tissues, including skeletal muscle, may derive from cells other than tissue-specific stem cells that derive from bone marrow.

As early as 1967 and 1983, the idea that muscle could derive from circulating cells was tested, but the experiments failed to detect such transitions because of technical limitations in the available methods of detection. The more recent studies benefited from sensitive genetic markers of individual bone marrow cells that could be detected by *in situ* hybridization, β-gal or green fluorescent protein (GFP) expression, as well as the use of sensitive instruments such as laser scanning confocal microscopes and flow cytometers. Using bone marrow from a transgenic mouse in which β-gal expression was under the control of

the muscle-specific promoter for myosin light chain 3F (MLC3F-nLacZ), marrow-derived cells were shown to contribute to muscle fibers damaged by cardiotoxin. Either following intramuscular injection of adherent or nonadherent bone marrow fractions into the tissue or bone marrow transplantation, donor-derived myonuclei were detected after two weeks. When bone marrow of wild-type mice was transplanted into lethally irradiated dystrophic mdx recipients, a somewhat greater frequency of dystrophin-expressing fibers was observed relative to nontransplanted mdx mice. These experiments suggested that cells of the bone marrow could respond to cues from damaged muscle and contribute to muscle regeneration. However, the frequency of the donor-derived fibers was low, and generally did not exceed 0.3% even after 10 months.

Occasionally mononuclear BMDCs were observed in regenerating muscle of transplant recipients that apparently were nonhematopoietic and were not incorporated into regenerating myofibers, suggesting that bone marrow cell contribution to mature muscle fibers might occur through an intermediate, mononucleate MuSC-like stage. This hypothesis was confirmed when, following whole bone marrow transplantation of GFP-labeled marrow into lethally irradiated wild-type hosts, GFP-labeled cells were observed on single muscle fibers isolated from transplant recipients in the appropriate anatomic position for satellite cells expressing the characteristic markers desmin, Myf-5, cMet receptor, and a newly defined marker α7-integrin. In one study, when isolated, these cells were heritably altered and were capable of self-renewal and proliferation as myoblast-like cells, and some fused to form myotubes in culture or following direct injection into host muscle. In these studies, two thirds of the endogenous satellite cells died at the radiation doses necessary for marrow transplant, resulting in a vacated niche to which the BMDCs could contribute. BMDC contribution to host myofibers was very modest, approximately 0.3%, on a par with previous reports. To contribute substantially to mature myofibers in the host mouse, a second insult, voluntary exercise-induced stress, led to a 4% contribution of BMDC GFP^+ satellite cells to myofibers, a 20-fold increase over non-exercised mice. These data demonstrated that cells of the bone marrow can give rise to MuSCs that persist long term, self-renew, and regenerate muscle in response to the same cues as endogenous satellite cells.

Taken together, these data provide strong evidence that cells within bone marrow can act as precursors to MuSCs, but their precise nature and origin remains to be determined. Possibilities include:

1. The existence of MuSCs within the marrow;
2. HSCs that give rise to both blood and other mesenchymal cell types such as muscle; and
3. A precursor that gives rise to HSCs, MuSCs, and other mesenchymal stem cells.

Although myogenic cells had previously been shown to give rise to other mesodermal cell types such as adipocytes, cartilage, and osteocytes in culture, the possibility that they could give rise to circulating mesodermal cells such as HSCs was not thought to occur. However, in some studies a side population (SP) of cells, was identified by fluorescence activated cell sorting (FACS) in bone marrow as a population that excludes Hoechst dye more than other marrow-derived cells and were capable of yielding both skeletal muscle and blood, whereas reports also appeared suggesting that SP cells isolated from muscle tissue (muscle-SP) could give rise to blood and muscle. Further studies have suggested that $CD45^+$ muscle-SP cells, a pan-hematopoietic marker, primarily give rise to blood, whereas CD45− muscle-SP cells give rise to muscle, suggesting that the former derived from circulating HSCs and the latter from MuSCs or a cell population with similar 'stem-like' properties within muscle. Analogous studies by others of $CD45^+$/$Sca\text{-}1^+$ muscle-SP showed that they did not express Myf-5 and gave rise only to blood colonies, whereas Sca-1 + /CD45− muscle-SP gave rise only to muscle *in vitro*. However, on injection into muscle *in vivo*, the Sca-1 + /CD45 + /Myf-5− muscle SP cells expressed Myf-5, and differentiation into muscle was observed. One could envision a model in which cells within the bone marrow give rise to vascular-associated $Sca\text{-}1^+$/ $CD45^+$ cells, which in turn give rise directly to MuSCs or a muscle-SP intermediate that persists and participates in muscle and possibly other mesenchymal tissue regeneration. Taken together, these results are provocative in that they suggest a closer relationship among adult mesodermal tissues, blood, and muscle than was previously recognized. However, a direct lineage from any single cell of the bone marrow to a BMDC-derived MuSC has yet to be demonstrated.

20.5 THE MUSCLE STEM CELL NICHE

Tissue-specific stem cells occupy niches, microenvironments that instruct and support stem cell self-renewal, proliferation, and differentiation, providing specific cellular neighbors, signaling molecules, and extracellular matrix components. From studies of muscle aging and muscular dystrophy it has been suggested that MuSCs are wedded to their niches. Satellite cells not only become dysfunctional because of exhaustion of their replicative capacity but also because of changes in their microenvironment imposed on them because of disease or aging. Complex mechanisms are likely to dictate how the milieu of factors in an MuSC niche might affect the recruitment and contribution of circulating cells to the MuSC population.

A portrait of the MuSC niche can be envisioned based on knowledge gained from the collective set of mutations that cause muscular dystrophy and from the signals that are released following muscle damage, both of which are known to affect satellite cell behavior. A number of dystrophies result from

defects in constituents of the DGC (e.g., dystrophin, α7-integrin, dysferlin, caveolin-3, sarcoglycans, dystroglycans) and the extracellular proteins that associate with the DGC (e.g., laminin α2). It is generally thought that the DGC fortifies the myofiber membrane against repetitive shearing forces encountered during normal exercise and mediates communication between the myofiber and the extracellular environment. When the DGC is disrupted, the muscle undergoes rapid cycles of degeneration and regeneration. Before and immediately following the onset of most dystrophies, satellite cells are capable of successfully regenerating muscle; however, as the disease progresses, the satellite cells become senescent and cease to participate in regeneration. This is certainly true for DMD, one of the most severe forms of human muscular dystrophy. The cells usurp their proliferative potential in repair of muscle in a manner similar to that which accompanies aging, only on a much shorter time-scale. Other factors that appear to characterize the MuSC niche are secreted factors, such as insulin-like growth factors (IGF)-1 and IGF-2, both of which help mediate muscle hypertrophy and are known to stimulate satellite cell proliferation and differentiation. By contrast, more transforming growth factor-β (TGF-β) is expressed in muscle of Duchenne patients and is correlated with an inhibition of satellite cell proliferation. The composition of molecules within the MuSC niche is likely to play a role in instructing MuSCs during muscle regeneration and during the aging process and also possibly in the recruitment and reprogramming of BMDCs en route to becoming MuSCs.

The process of bone-marrow-derived myogenesis is typically associated with a response to damage: genetic, chemical, or physical. It is known that high doses of γ-irradiation do not injure myofibers. Thus, it is intriguing that BMDCs still contribute to myofibers (albeit at a low frequency) in otherwise genetically normal, uninjured mice. Although all of the effects of γ-irradiation remain unknown, they include:

1. Damage of the regenerative satellite cells with proliferative potential, not the postmitotic myofibers;
2. A vacated niche leading to a regenerative deficit compared with normal muscle; and
3. A release of factors that could modify the vacated MuSC niche, leading to occupation of that niche by circulating stem cells that are instructed by the local environment to assume the role of the lost cells.

The end result is that BMDC are recruited to muscle, adopting a novel cell fate that allows them to participate in regeneration, thereby reducing the regenerative deficit in irradiated skeletal muscle.

Several lines of evidence suggest that γ-irradiation can also positively affect cell proliferation, in addition to the inhibitory effects described previously for satellite cells. After injection of a myogenic cell line into host muscles, tumors

were found to form more rapidly in γ-irradiated muscle than in nonirradiated muscle and the effect was dose-dependent, suggesting that the cells had a selective advantage once local proliferative cells were abated. Moreover, in contrast to damaging high-energy γ-irradiation, low energy laser irradiation (LELI) was shown to promote survival of both MuSCs and myofibers. These findings fit well with those of others who argue that when the environment of precancerous cells is changed by transient insults, such as irradiation, conditions are created that favor proliferation and the cells divide uncontrollably. Thus, modification of the niche or microenvironment of a cell, including growth factors, cytokines, and adhesion molecules, can influence its ability to promote proliferation and plasticity, as in the case of the conversion of bone marrow to MuSC-like cells and fibers as described previously. Following injury or stress to the muscle or its stem cells, the proportion of satellite cells derived from bone marrow found within the total MuSC population could increase and participate to a greater extent in regeneration, serving as a back-up reservoir to the tissue-specific stem cells. This cell source may prove advantageous in heritable muscular dystrophies in which the MuSC population is deficient or defective.

20.6 CONCLUSION

For more than 40 years the satellite cell has been considered the primary mediator of muscle regeneration in postnatal animals. This conclusion was first based on anatomic criteria using TEM, then by use of antibodies to specific proteins, and ultimately by the ability of the cells to contribute to the repair of locally damaged muscle fibers. However, evidence suggests that this interpretation may be an oversimplification, and that more than one source of cell may aid in muscle regeneration, or that a heretofore unrecognized lineage(s) may contribute to myofibers, either via the satellite cell pool or directly. First, both functional and biochemical heterogeneity has now been shown. Second, other myogenic populations have been implicated in muscle repair, for example muscle SP cells. Finally, cells that are not of myogenic origin appear to be capable of repairing muscle, such as meso-angioblasts, BMDCs, and possibly HSCs or their more multipotent precursors. It is unclear at present whether these cells originate from a similar cellular origin and represent different points in a single development pathway or whether there exist multiple distinct cell types that each have the capacity to participate in muscle regeneration. Finally, because cells from widely disparate nonmuscle origins have been observed to have myogenic potential, it is particularly important to understand their role in normal muscle regeneration and their relationship to the anatomically described satellite cell. Clearly, the interrelationships of these cells with their diverse markers must be further investigated and compared with one another and the classical satellite cell MuSC.

In conjunction with lineage tracing studies, a more global approach to defining a cell as a MuSC appears warranted, including consideration of the *functional* properties that are classically ascribed to satellite cells:

1. They are highly proliferative and capable of fusing with existing muscle fibers or forming muscle fibers *de novo*;
2. They possess the necessary molecular constituents to respond appropriately to the regenerative needs of a muscle environment;
3. Their absence or inability to function properly results in deleterious effects such as atrophy or myopathy;
4. They express key intracellular and cell surface regulatory proteins characteristic of muscle before their fusion with existing muscle fibers; and
5. In the absence of an experimentally manipulated environment their default fate is skeletal muscle.

Thus, the challenge for the next few years will be to understand the role played during skeletal muscle regeneration by circulating cells, vascular-associated cells, nonsatellite cells resident in muscle with myogenic potential, and cells from other nonmuscle tissues. Even if cells that originate from these sources do not play a significant role in normal muscle repair, their further study will illuminate the range of possible outcomes for those cells. Perhaps an understanding of the factors that govern their behavior and increase their potential to express muscle genes (nuclear reprogramming) will lead to information that can be used for developing new biomedical treatments of myopathies.

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FOR FURTHER STUDY

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CHAPTER 21

Stem Cells and the Regenerating Heart

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21.1 INTRODUCTION

What underlies the inability of the mammalian heart to rebuild itself in response to injury? Where are the stem cells of this vital organ, and, if there are none, how does it maintain its structural and functional integrity for decades? The impediment to adult mammalian cardiac regeneration has been attributed to its distinct embryonic history. The heart is the first fully differentiated structure to form and function during vertebrate development. The primitive heart tube, composed of contracting cardiomyocytes lined by a layer of endocardial cells, ensures the establishment of a circulatory system that is critical to support rapid rates of embryonic growth. The astonishing capacity of the embryonic heart to perform at such an early stage depends on its rapid assembly from the multipotent mesoderm, a cumulative process that depends on a series of sequential inductive interactions. The progressive acquisition of the cardiac phenotype by precursor cells starts early in the primitive streak stage, so that by the time a cardiac crescent is fully formed, there are coordinated contractions in the primitive heart tube. In contrast to fetal skeletal myocytes, which no longer proliferate once a functioning contractile apparatus has been elaborated, actively contracting fetal cardiomyocytes must continue to divide to provide for further growth of the embryonic heart. This phase of cardiomyocyte division ends soon after birth, when increase in myocardial mass is achieved largely through cellular hypertrophy, at which point the heart is considered fully formed.

The unusual capacity of embryonic and fetal cardiomyocytes to re-enter the cell cycle appears to be largely lost once the heart is developed. In contrast to mammalian skeletal muscle, which regenerates injured tissue through activation of quiescent myogenic precursor or multipotent adult stem cell populations, the heart does not appear to retain equivalent reserve cell populations

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to promote myofiber repair. The relative paucity of progenitor cells residing within the heart may impose severe limits on replacement of damaged myocardium. Thus, the prevailing assumption has been that the heart cannot regenerate as well as other organs because it does not maintain a sufficiently robust progenitor cell population.

21.2 RECRUITING CIRCULATING STEM CELL RESERVES

The relative scarcity of progenitor cells residing in the adult myocardium has prompted a search for a renewable source of circulating somatic progenitor cells that might home to the heart in response to damage. The existence of such cell populations has gained credibility from observations of sex mismatched cardiac human transplants, in which a female heart is transplanted into a male host. In these patients, the presence of the Y chromosome marks host-derived cells in the transplanted heart. Various numbers of Y chromosome-positive myocytes and coronary vessels in the transplanted heart's male cells could be found. Cell fusion of host cells with donor cardiac cells, as has been proven for other regenerating tissues, was ruled out in by the presence of a single X chromosome. The presence of differentiated host cells in the transplanted tissues proves the existence of migratory precursor cells that are induced to differentiate by the cardiac milieu. Although this phenomenon could be a response to organ transplantation, it may also reflect a normal homeostatic process for the maintenance of cardiac muscle and coronary vasculature.

The lack of information regarding the precise origin of donor cells in these human transplants has prompted animal experiments in which stem cells isolated from bone marrow were enriched for various surface markers. A stem cell-enriched side population (SP) can be isolated by the relative efflux of Hoechst dye 33342 through MDR1, a P-glycoprotein capable of extruding dyes, toxic substances, and drugs. The movement of bone marrow SP cells can be traced if they are isolated from donor mice expressing a genetic marker such as lacZ and used to reconstitute the bone marrow of lethally irradiated recipient mice. In such experiments, very few marked cells can normally be found in extrahematopoietic tissues of the reconstituted animals. However, in reconstituted mice that were subsequently subjected to coronary artery occlusion, lacZ marked cells could be found in vascular endothelium and cardiomyocytes of the border zone adjacent to the infarct. In other studies, cell populations expressing c-kit, the receptor for stem cell factor, were isolated from bone marrow and injected directly into the border zone of an experimentally induced infarct where they migrated into the damaged region, differentiated into cardiomyocytes and vascular cells and partially

replaced necrotic myocardium. To what extent these different subsets of marked bone marrow cells represent the same cell population as migratory Y chromosome-containing donor cells in the human transplant studies remains to be determined.

Other bone-marrow-derived candidates for cardiac regeneration include mesenchymal stem cells, distinct from hematopoietic stem cells, which have multilineage potential, normally generating multiple mesenchymal tissue types. When mesenchymal stem cells isolated from human donors were injected into the ventricular cavity of uninjured mice, they were subsequently found at low levels in the muscle wall, having acquired cardiomyocyte characteristics. Higher levels of mesenchymal stem cell engraftment into the myocardium were achieved by direct injection into the porcine ventricular wall after acute ischemic damage underscoring the importance of injury in the homing process. Numerous cell types have since shown promise in improving cardiac regeneration after acute myocardial infarction in patients, including bone marrow cells, endothelial progenitor cells, or skeletal myoblasts. Whatever their source, the newly formed cardiomyocytes must integrate precisely into the existing myocardial muscle to avoid life-threatening arrhythmia, a frequently cited complication of the therapeutic use of cells from noncardiac tissue when treating acute postinfarct ischemia.

Insufficient revascularization represents another major impediment to the reconstitution of ischemic myocardial tissue and the prevention of further scar tissue formation. Although angiogenesis within the infarcted area is an integral component of the remodeling process, the capillary network is normally unable to support the greater demands of the hypertrophied myocardium. Fortunately, adult bone marrow contains endothelial precursors that resemble embryonic angioblasts that, if sufficiently mobilized, could participate in revascularization of the ischemic tissue. In an exciting proof of concept, endothelial progenitor cells, isolated from human adult bone marrow on the basis of CD34 and c-kit expression, were injected intravenously into athymic rats in which myocardial infarction had been recently induced. The human endothelial precursors selectively migrated to ischemic myocardium, where they mediated new blood vessel formation in the infarct bed (vasculogenesis) and proliferation of vasculature (angiogenesis) from pre-existing mature host endothelium in the border zone. The treated animals displayed decreased apoptosis of hypertrophied myocytes in the peri-infarct region, long-term salvage and survival of viable myocardium, reduction in collagen deposition, and sustained improvement in cardiac function. Intracoronary infusion of endothelial progenitor cells in patients with acute myocardial infarction (AMI) significantly improves postinfarction left ventricular (LV) remodeling processes, regional contractile function of the infarcted segment, and coronary blood flow reserve in the infarct artery.

Although cardiomyocyte survival in these studies was undoubtedly enhanced by the revascularization of injured myocardium, it is also possible that endothelial precursors contribute directly to regenerating myocardial tissue. Indeed, human umbilical vein endothelial cells, or clonal cultures of endothelial cells isolated from embryonic mouse dorsal aorta, can be induced to express sarcomeric proteins after only five days of co-culture with neonatal rat cardiomyocytes. Additional evidence supporting this possibility comes from the conversion of adult human endothelial progenitor cells, derived from peripheral blood mononuclear cells, or $CD34^+$ hematopoietic progenitor cells into cardiomyocytes on co-culture with rat cardiomyocytes. In both studies, cell-cell contact or an extracellular matrix-associated signaling appeared to be critical, because conditioned media from cardiocyte cultures was not sufficient for conversion to a cardiomyocyte phenotype.

Whatever the provenance and potential of cardiac progenitors, it is clear that, at least in mammals, the relatively poor recruitment of circulating stem cells to the site of myocardial injury limits the body's ability to aid in the repair process. Numerous chemotactic signals associated with inflammation, including cytokines and adhesion molecules, are preferentially expressed by the infarct border zone, and may improve stem cell homing as well. Indeed, increasing evidence supports the notion that chemokines play a central role in directing angioblasts from the bone marrow to ischemic myocardium. The angioblast population of injured cardiac tissue can be experimentally increased either by inhibiting interactions between the bone-marrow-derived CXC chemokine SDF-1 and its receptor CXCR4 on angioblasts or by increasing expression of SDF-1 in the ischemic rat heart. The resulting sustained improvement in cardiac tissue involves both protection against apoptosis and induction of proliferation of endogenous cardiomyocytes, suggesting new therapeutic avenues in which enhancement of stem cell trafficking could be harnessed to amplify the endogenous homing process.

21.3 THE ELUSIVE CARDIAC STEM CELL

The adult mammalian heart has long been considered a postmitotic organ without an endogenous population of stem cells, which instead contains a relatively constant number of myocytes that cease to divide shortly after birth and remain constant into senescence. A longstanding view is that the inability of differentiated cardiac myocytes to re-enter the cell cycle may present the ultimate impediment to the heart's regenerative capacity. The terminal differentiation of cardiomyocytes is likely to be under the control of tumor suppressors, such as Rb, and cyclin-dependent kinase inhibitors. Because mice in which individual components of these cell cycle checkpoints have been systemically disrupted by gene knockout do not show a dramatic increase in

myocyte cell numbers, multiple mechanisms must exist to prevent adult myocytes from further proliferation.

The assumption that the myocardium is a terminally differentiated tissue has been subjected to intense scrutiny and considerable debate. The irreversible withdrawal of cardiomyocytes from the cell cycle soon after birth has been held responsible for the drastic effects of acute and chronic myocyte death in the surviving myocardium after infarction and for the failure to derive replicating mammalian myocyte cell cultures from the adult mammalian heart. The fact that cardiac myocytes are multinucleated and polyploid in many mammalian species has complicated interpretation of any observed DNA synthesis that might represent myocyte proliferation. Nevertheless, it has been argued that, in the face of massive cardiomyocyte apoptosis and necrosis, the diseased heart could not continue to function in the absence of new myocyte formation. Increases in myocyte number do not provide information about the origin of these new cells, however. Although most of the controversy surrounding mammalian heart regeneration has focused on the evidence for or against the replication of existing myocytes, cells capable of differentiating into a myocyte in the adult heart could originate through the commitment of precursor cells to the myocyte lineage, through replication of pre-existing myocytes, or by a combination of these two mechanisms.

Decades of frustrated searching for a resident cardiac stem cell population have yielded more encouraging results through the application of methods used to study stem cells in the adult hematopoietic compartment. SPs of cells resembling those isolated from the bone marrow have been found in adult rodent myocardium expressing the corresponding transport proteins. Cell surface proteins that mark stem cell populations in other tissues are also found on a subpopulation of undifferentiated precursor cells in the adult heart. These primitive cells can be detected by Sca-1, which is involved in cell signaling and cell adhesion. Sca-1 is not specific for stem cells because it is found on the surface of hematopoietic stem cells and other cell types. In certain instances, cells isolated from adult heart by virtue of their stem cell markers not only express appropriate markers but behave like cardiac progenitor cells *in vitro*, giving rise to clones that express biochemical markers of myocytes, smooth muscle, and endothelial cells.

Another hallmark of progenitor cells is the maintenance of chromosomal telomere integrity through the action of telomerase. In the heart, telomerase levels decrease after birth when most myocardial cells withdraw from the cell cycle. Although telomere shortening has been shown in a small percentage of adult rat cardiomyocytes, forced expression of telomerase prolongs cardiac myocyte cycling *in vivo* and confers protection from stress-induced telomere shortening and apoptosis in the adult heart. Notably, telomerase activity in

the adult myocardium is restricted to small interstitial cells that express Sca-1 but lack other markers of hematopoietic stem cells (c-kit, CD45, CD34) or endothelial progenitor cells (CD45, CD34, Flk-1, Flt-1). Purified cardiac Sca-1^+ cells home specifically to infarcted myocardium and activate the cardiogenic program, with a significant proportion fusing to existing cardiocytes. These studies suggest that, in the heart, telomere integrity is a key factor in maintaining a Sca-1^+ progenitor cell pool.

It remains to be seen how these cells relate to a population of rare small cycling cardiomyocytes that retain the capacity to proliferate in response to damage and are continuously renewed by the differentiation of stem-like cells as a normal function of cardiac homeostasis. The origins of cycling myocardial cells may be attributed to Lin − /c-kit+ cells isolated from the adult rat heart that retain stem cell characteristics. These cells are self-renewing, clonogenic, and multipotent *in vitro* and *in vivo* and give rise to myocytes and smooth muscle and endothelial vascular cells. When injected into an ischemic rat heart, a population of these cells or their clonal progeny reconstitute up to 70% of the injured myocardial wall. The regenerated myocardium contains small myocytes that present the anatomical, biochemical, and functional properties of young myocytes. These data support the notion that myocyte renewal in the adult mammalian heart occurs constantly, albeit at a very low rate. The possibility that endogenous cardiac stem cells can be mobilized to migrate from their niche within the healthy heart to support regeneration of diseased myocardium has exciting implications for therapeutic intervention.

21.4 EVOLVING CONCEPTS OF REGENERATION

The limited restorative capacity of the adult mammalian heart has been attributed to the loss of cardiomyocyte versatility soon after birth. The emerging concept of regeneration as an evolutionary variable is dramatically illustrated by the relatively robust proliferative capacity of the injured heart in other vertebrate species. The dramatic regeneration of urodele amphibian limb and lens extends to their efficient repair of injured myocardium. Newts repair their hearts in response to cardiac damage, leaving none of the dysfunctional scar tissue typical of the postinfarct mammalian myocardium. Unlike their mammalian counterparts, adult newt cardiomyocytes can readily proliferate after injury and contribute to the functional regeneration of the damaged heart. The recognition that cardiac tissue in certain vertebrates can undergo extensive repair has prompted the proposal that regeneration may be a primordial attribute that has been lost during mammalian evolution.

Studies of newt limb regeneration provide a mechanism whereby the rebuilding of damaged tissue could couple acute response to injury with local activation of plasticity in surrounding tissues and/or activation of stem cell pools.

A transient activity generated by thrombin, a critical component of the clotting cascade that ensures hemostasis and triggers other events of wound healing, has been linked to cell cycle re-entry in multinucleate myotubes during urodele limb regeneration. Selective activation of thrombin protease action in response to injury provides a feasible connection between damage control and initiation of regenerative growth. It remains to be seen whether such a linkage can be established in the regenerating myocardium.

The capacity of the newt heart to regenerate may not be a common attribute shared by all cardiomyocytes. Although longitudinal analyses of single cultured newt cardiomyocytes revealed that many cells enter into S phase in response to serum-activated pathways dependent on the phosphorylation of the Rb protein, the majority of these cells stably arrest at either entry to mitosis or during cytokinesis. Nevertheless, a significant cell subset progresses through mitosis and participates in successive cell divisions, providing a tractable model system to investigate the mechanisms underlying the ability of newt cardiomyocytes to maintain their remarkable proliferative potential.

Regeneration of the zebra fish heart offers a more genetically accessible model for dissecting the molecular basis of cardiac repair. After surgical removal of the ventricular apex and rapid clotting at the site of amputation, proliferating cardiac myofibers replace the clot and regenerate missing tissue, with minimal scarring. The requirement for cell cycle re-entry in this model is supported by the decreased regeneration and increased fibrosis in a temperature-sensitive mutant of a mitotic checkpoint kinase, mps1.

It is still formally possible that the activation of cardiac progenitor cells is largely responsible for the extraordinary capacity of the adult zebra fish to restore extensive portions of the heart. Regeneration has been traditionally assumed to involve the recapitulation of genetic pathways used during embryonic development. This scenario has been challenged by the finding that markers, such as Nkx2-5 or Tbx-5, which play a critical role in heart development, were not detected in regenerating zebrafish myocardium. Rather, an increase in cycling cells expressing myocardial markers was accompanied by activation of genes such as Msx transcription factors and Notch pathway components that are expressed during heart development. The novel profile of genes activated during zebrafish cardiac regeneration, distinct from that associated with cardiogenesis, is consistent with the emerging concept that differentiated myocytes can re-enter the cell cycle and proliferate in response to heart injury. This would provide evidence for true epimorphic regeneration in the vertebrate heart and argues for a clear distinction between mechanisms at work during regeneration versus development. If cardiac progenitor cells are indeed involved in this process, they may play a more instructive role in reforming damaged heart tissue.

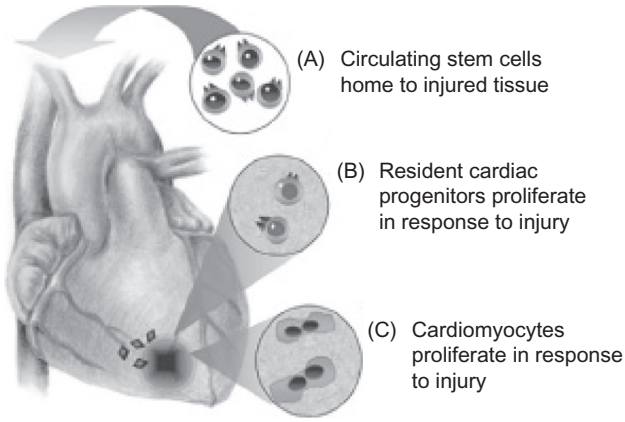

FIGURE 21.1 **Several modes of cardiac regeneration supported by recent studies.** (A) Circulating stem cells home to the infarcted area, guided by chemoattractive mechanisms, and participate in multiple functions including neoangiogenesis and myocyte renewal. (B) Injury stimulates the proliferation of resident cardiac progenitors, which resemble tissue stem cells in their phenotype and ability to participate in cardiac renewal. (C) A subset of competent cardiomyocytes re-enter the cell cycle to replace and rebuild missing tissue. Although these endogenous regenerative modes are not mutually exclusive, they provide different possibilities for therapeutic intervention.

The possibility that a process of limited cardiomyocyte proliferation may not be formally excluded in mammalian species is supported by the 'healer' phenotype of the MRL mouse strain, a well-characterized model of autoimmunity. Significant myocyte re-entry to S phase and myocardial replacement without scarring has been observed after cryogenic injury of MRL mouse heart tissue. The enhanced and heritable capacity of the MRL mouse to heal surgical wounds is a complex trait that maps to at least 20 genetic loci. Although the potential role of autoimmunity remains to be determined, MRL wound repair is potentially mediated by differences in the activity of matrix metalloproteinases and their inhibitors. The similarity of the MRL healer phenotype to newt regenerative processes has suggested a general mechanism whereby a regeneration blastema forms by local dedifferentiation of cells underlying the wound, followed by growth and then reversal to the differentiated cell type. Further study of the MRL mouse will be necessary to define molecular commonalities with the more regenerative vertebrates and to determine the potential role of cardiac stem cells in the healing capacity of this tantalizing mouse model.

The regenerative potential of the mammalian heart is a rapidly evolving concept. In the near future, cardiac repair is likely to be augmented through a number of avenues (Figure 21.1). The dramatic improvements that exogenously administered progenitor cells can effect in both animal and human myocardial repair underscore their therapeutic potential. Although resident

cardiac progenitor cell populations have now been identified, the insufficiencies of endogenous stem cells to alleviate acute and chronic damage to mammalian cardiac tissue remain to be overcome. Advances in our understanding have uncovered an unexpected dynamism in cardiac homeostasis and highlight the heterogeneous proliferative potential of resident cardiomyocytes. Enhancing the functional regeneration of this most obdurate of organs raises the exciting prospect that regenerative processes in other tissues of the adult mammalian soma might be similarly harnessed to fend off the ravages of aging and disease in a new paradigm of self-renewal.

FOR FURTHER STUDY

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CHAPTER 22

Cell Lineages and Stem Cells in the Embryonic Kidney

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22.1 THE ANATOMY OF KIDNEY DEVELOPMENT

For a detailed description of early renal development and nephron formation, the review by Kuure (2000) listed at the end of the chapter will be helpful. A brief summary of renal patterning and the origin of the renal progenitor cells must begin with gastrulation. In vertebrates, the process of gastrulation converts a single pluripotent sheet of embryonic tissue, the epiblast or embryonic ectoderm, into the three primary germ layers: the endoderm, the mesoderm, and the ectoderm (Figure 22.1A). In mammals, gastrulation is marked by a furrow called the primitive streak, which extends from the posterior pole of the epiblast. Extension of the primitive streak occurs by proliferation and migration of more lateral epiblast cells to the furrow, followed by invagination through the furrow and migration back laterally under the epiblast sheet. At the most anterior end of the primitive streak is the node or organizer, called Hensen's node in the chick, and the functional equivalent to the blastopore lip or Speeman's organizer in the amphibian embryo. The node is a signaling center that expresses a potent combination of secreted factors for establishing the body axes and left-right asymmetry. The node is positioned at the anterior pole of the primitive streak at approximately the midpoint of the epiblast. The more anterior epiblast generates much of the head and central nervous system and does not undergo gastrulation in the same manner. Once the streak reaches the node, it begins to regress back to the posterior pole. During this process of primitive streak regression, the notochord is formed along the midline of the embryo and just ventral to the neural plate. The notochord is a second critical signaling center for dorsal ventral patterning of both neural plate and paraxial mesoderm. The axial mesoderm refers to the most medial mesodermal cells, which in response to regression of the streak become segmented into somites, blocks of cells surrounded by a simple epithelium. At the stage of the first somite formation,

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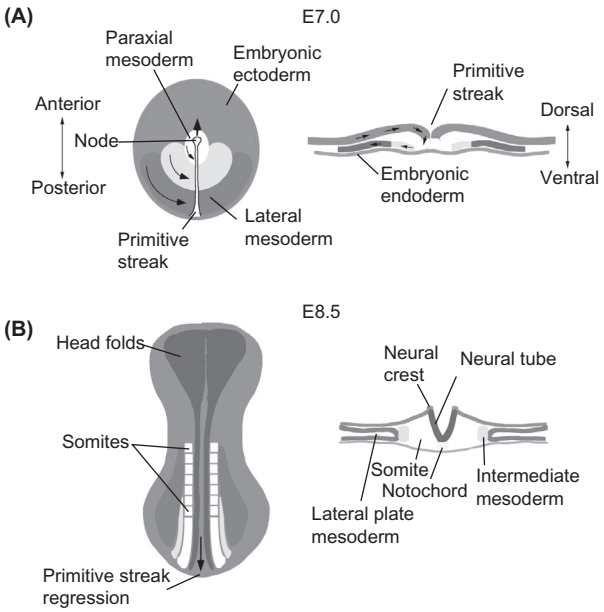

FIGURE 22.1 **The origin of the intermediate mesoderm.**

(A) At gastrulation, cells of the epiblast, or embryonic ectoderm, migrate through the primitive streak. In the mouse, the single sheet of embryonic ectoderm lines a cup-shaped egg cylinder. The left panel is a planar representation of a mouse epiblast looking down into the cup from above. The streak begins at the posterior and moves toward the anterior. Fate mapping studies indicate that lateral plate mesoderm originates from the posterior epiblast, whereas axial mesoderm is derived from more anterior epiblast cells. The intermediate mesoderm most likely originates from cells between these two regions. At the mid-streak stage, the expression of lim1 can already be detected before gastrulation in cells of the posterior epiblast. The schematic on the right is a cross section through the primitive streak, showing the formation of mesoderm as cells of the epiblast migrate toward the streak then invaginate and reverse direction underneath the epiblast sheet. (B) At the time of primitive streak regression, the notochord is formed along the ventral midline. Paraxial mesoderm begins to form segments, or somites, in an anterior to posterior direction. The lateral plate mesoderm, consists of two sheets called the somatopleure (dorsal) and the splanchnopleure (ventral). The region between the somite and the lateral plate is the intermediate mesoderm, where the first renal epithelial tube will form.

going medial to lateral, the notochord marks the midline, the somites abut the notochord on either side, and the unsegmented mesoderm is termed intermediate near the somite and lateral plate more distally (Figure 22.1B). It is this region of intermediate mesoderm within which the kidney will form that is the primary focus of this chapter.

The earliest morphologic indication of unique derivatives arising from the intermediate mesoderm is the formation of the pronephric duct, or primary

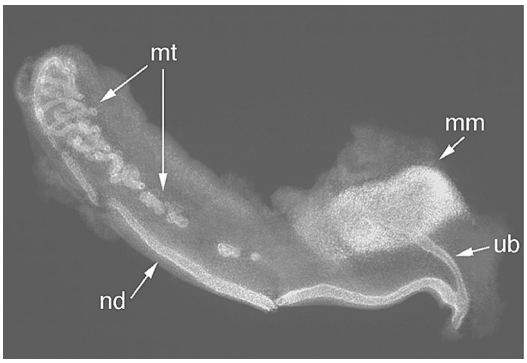

FIGURE 22.2 **Expression of Pax2 at the time of metanephric induction.**
One side of the intermediate mesoderm-derived nephric chord was microdissected from an E11.5 mouse embryo and stained with anti-Pax2 antibodies. The micrograph shows Pax2 in the nephric duct (nd) and the ureteric bud (ub) branching from the posterior duct. Pax2 is in mesonephric tubules (mt), even those more posterior that are not connected to the nephric duct. At the posterior end, Pax2 is in the metanephric mesenchyme cells (mm), surrounding the ureteric bud, that has not yet begun to form epithelia.

nephric duct. This single-cell-thick epithelial tube runs bilaterally beginning at around the 12th somite in birds and mammals. The nephric duct extends caudally until it reaches the cloaca. As it grows, it induces a linear array of epithelial tubules, which extend medioventrally and are thought to derive from periductal mesenchyme (Figure 22.2). The tubules are referred to as pronephric or mesonephric, depending on their position and degree of development, and represent an evolutionarily more primitive excretory system that forms transiently in mammals until it is replaced by the adult or metanephric kidney. Along the nephric duct, there is a graded evolution of renal tubule development, with the most anterior, or pronephric tubules, being very rudimentary, and the mesonephric tubules becoming well developed with glomeruli and convoluted proximal tubule-like structures. In contrast, the pronephros of the zebra fish larvae is a fully developed, functional filtration unit with a single midline glomerulus. Amphibian embryos such as *Xenopus laevis* have bilateral pronephric glomeruli and tubules that are functional until replaced by a mesonephric kidney in the tadpole. In fact, it is not altogether obvious in mammals where to draw the distinction between pronephric tubules and mesonephric tubules. Mature mesonephric tubules are characterized by a vascularized glomerulus at the proximal end of the tubule that empties into the nephric duct; the most anterior and posterior mesonephric tubules are more rudimentary, and the most posterior tubules are not connected to the duct at all.

The adult kidney, or metanephros, is formed at the caudal end of the nephric duct when an outgrowth, called the ureteric bud or metanephric diverticulum, extends into the surrounding metanephric mesenchyme. Outgrowth or budding of the epithelia requires signals emanating from the mesenchyme. Genetic and biochemical studies indicate that outgrowth of the ureteric bud is mediated by the transmembrane tyrosine kinase RET, which is expressed in the nephric duct, and the secreted neurotrophin glial derived neurotrophic factor (GDNF), which is expressed in the metanephric mesenchyme. Once the ureteric bud has invaded the metanephric mesenchyme, inductive signals emanating from the bud initiate the conversion of the metanephric mesenchyme to epithelium (Figure 22.3). The induced, condensing mesenchymal cells aggregate around the tips of the bud and will form a primitive polarized epithelium, the renal vesicle. Through a series of cleft formations, the renal vesicle forms first a comma then an S-shaped body, whose most distal end remains in contact with the ureteric bud epithelium and fuses to form a continuous epithelial tubule. This S-shaped tubule begins to express genes specific for glomerular podocyte cells at its most proximal end, markers for more distal tubules near the fusion with the ureteric bud epithelia, and proximal tubules markers in between. Endothelial cells begin to infiltrate the most proximal cleft of the S-shaped body as the vasculature of the glomerular tuft takes shape. At this stage, the glomerular epithelium consists of a visceral and parietal component, with the visceral cells becoming podocytes and the parietal cells the epithelia surrounding the urinary space. The capillary tuft consists of capillary endothelial cells and a specialized type of smooth muscle cell, termed the mesangial cell, whose origin remains unclear.

As these renal vesicles are generating much of the epithelia of the nephron, the ureteric bud epithelia continues to undergo branching morphogenesis in response to signals derived from the mesenchyme. Branching follows a stereotypical pattern and results in new mesenchymal aggregates induced at the tips of the branches, as new nephrons are sequentially induced. This repeated branching and induction results in the formation of nephrons along the radial axis of the kidney, with the oldest nephrons being more medullary and the younger nephrons located toward the periphery. However, not all cells of the mesenchyme become induced and convert to epithelia, some cells remain mesenchymal and migrate to the interstitium. These interstitial mesenchymal cells, or stromal cells, are essential for providing signals that maintain branching morphogenesis of the ureteric bud and survival of the mesenchyme.

From a stem cell perspective, defining the population of cells that generate the kidney depends in part on which stage one considers. At the time of metanephric mesenchyme induction, there are at least two primary cell

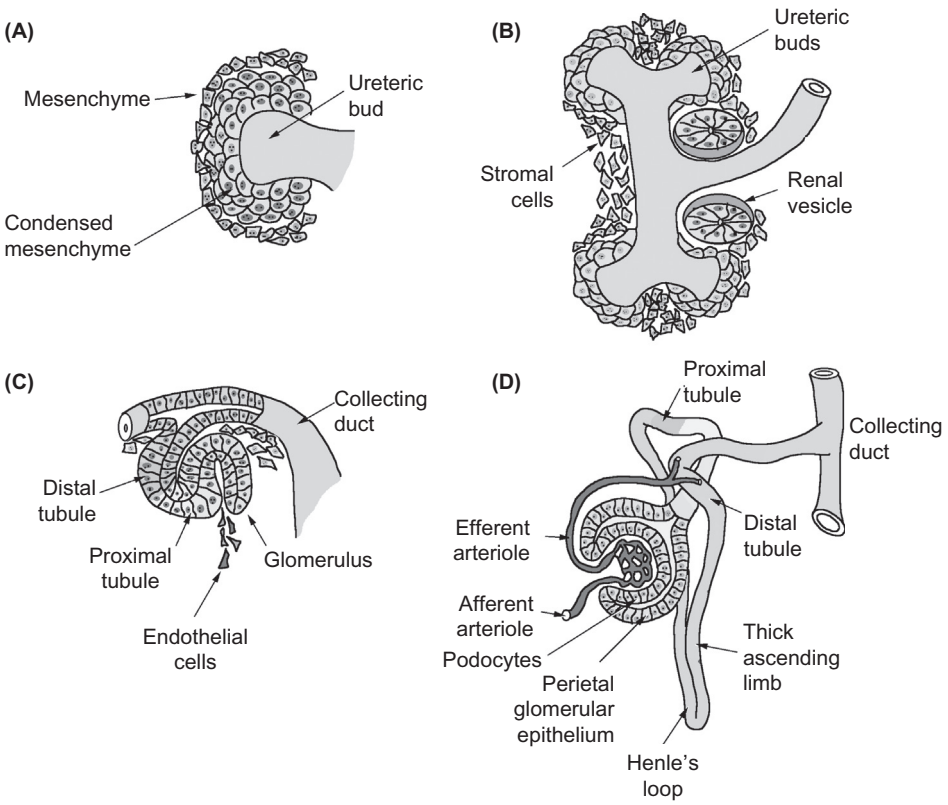

FIGURE 22.3 **The sequential conversion of metanephric mesenchyme to renal epithelia.** A schematic of the condensation and polarization of the metanephric mesenchyme at the tips of the ureteric bud epithelia is shown. (A) Epithelial precursors aggregate at the tips, whereas stromal cells remain peripheral. (B) The initial aggregates form a primitive sphere, the renal vesicle, as branching ureteric bud epithelia cells extend outward to induce new aggregates. Stromal cells begin to migrate into the interstitium. (C) At the S-shaped body stage, the mesenchymal derived structure is fused to the ureteric bud epithelial, which will make the collecting ducts and tubules. The proximal cleft of the S-shaped body is invaded by endothelial cells. Expression of glomerular, proximal tubule, and more distal tubule specific markers can be seen at this stage. (D) The architecture of the nephron is elaborated. Podocyte cells of the visceral glomerular epithelium contact the capillary tuft and the glomerular basement membrane is laid down. The proximal tubules become more convoluted and grow into the medullary zone to form the descending and ascending limbs of Henle's loop. The distal tubules and collecting ducts begin to express markers for more differentiated, specialized epithelia.

types: the mesenchyme and the ureteric bud epithelia. Although these cells are phenotypically distinguishable, they do express some common markers and share a common region of origin. As development progresses, it was thought that most of the epithelium of the nephron was derived

from the metanephric mesenchyme, whereas the branching ureteric bud epithelium generates the collecting ducts and the most distal tubules. This view has been challenged by cell lineage tracing methods *in vitro*, which indicate some plasticity at the tips of the ureteric bud epithelium such that the two populations may intermingle. Thus, at the time of induction, epithelial cells can convert to mesenchyme, just as the mesenchymal aggregates can convert to epithelia. Regardless of how the mesenchyme is induced, the cells are predetermined to make renal epithelia. Thus, their potential as renal stem cells has begun to be explored. To understand the origin of the metanephric mesenchyme, we begin with the patterning of the intermediate mesoderm.

22.2 GENES THAT CONTROL EARLY KIDNEY DEVELOPMENT

Genetic studies in the mouse have provided substantial new insights into the regulatory mechanisms underlying renal development. Although there are many genes that can affect the growth and patterning of the kidney, of particular importance with respect to potential renal stem cells are the genes that control formation of the nephric duct epithelia and proliferation and differentiation of the metanephric mesenchyme (Table 22.1).

Table 22.1 Genes that Regulate Early Kidney Cell Lineage

Gene	Expression	Mutant Phenotype
lim1	Lateral plate	No nephric duct, no kidneys
	Nephric duct	
Pax2	Inter, mesoderm	No mesonephric tubules
	Nephric duct	No metanephros
Pax2/Pax8	Inter, mesoderm	No nephric duct, no kidneys
WT1	Inter, mesoderm	Fewer mesonephric tubules
	Mesenchyme	Apoptos is of mesenchyme
Eya1	Met. mesenchyme	No induction of mesenchyme
wnt4	Mesenchymal aggregates	No polarization of aggregates
bmp7	Ureter bud and met.	Developmental arrest postinduction
	Mesenchyme	Some branching few nephrons
bf-2	Met. mesenchyme	Developmental arrest, few nephrons
	Interstitial stroma	Limited branching
pod-1	Stroma and podocytes	Poorly differentiated podocytes
pdgf(r)	S-shaped body	No vascularization of glomerular tuft

inter., intermediate; met., metanephric.

22.2.1 Genes that Determine the Nephrogenic Field

From a perspective of stem cells and potential therapy, the adult or metanephric kidney should be a primary focus. However, the early events controlling the specification of the renal cell lineages may be common among the pronephric and mesonephric regions. Indeed, many of the same genes expressed in the pronephric and mesonephric tubules are instrumental in early metanephric development. Furthermore, the earliest events that underlie regional specification have been studied in more amenable organisms, including fish and amphibians, in which pronephric development is less transient and of functional significance.

Although formation of the nephric duct is the earliest morphologic evidence of renal development, the expression of intermediate mesodermal-specific markers precedes nephric duct formation temporally and marks the intermediate mesoderm along much of the anterior–posterior (A–P) body axis. The earliest markers specific of intermediate mesoderm are two transcription factors of the Pax family (Figure 22.4): *Pax2* and *Pax8*, which appear to function redundantly in nephric duct formation and extension. The homeobox gene *lim1* is also expressed in the intermediate mesoderm but is initially expressed in the lateral plate mesoderm before becoming more restricted. Genetic studies implicate all three genes in some aspects of regionalization along the intermediate mesoderm. In the mouse, *Pax2* mutants begin nephric duct formation and extension but lack mesonephric tubules and the metanephros.

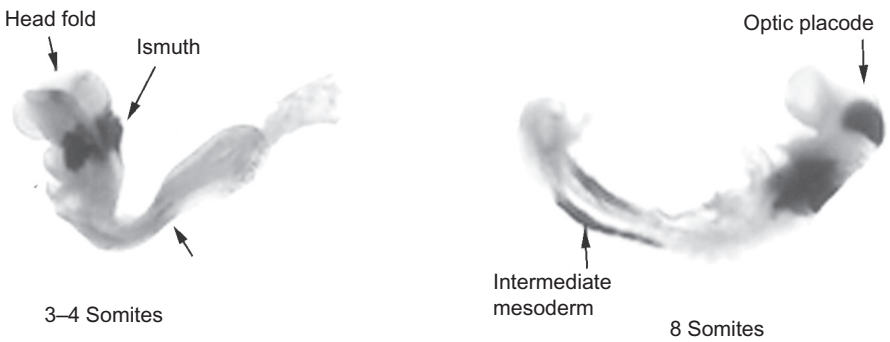

FIGURE 22.4 **The activation of Pax2 expression in the intermediate mesoderm.**
One of the earliest markers for the nephrogenic region Pax2 expression is activated around the four- to five-somite stage in the cells between the axial and lateral plate mesoderm. The embryos shown carry a Pax2 promoter driving the *LacZ* gene and expression is visualized by staining for beta-galactosidase activity. By the eight-somite stage, Pax2 marks the growing intermediate mesoderm even before the nephric duct is formed. Pax2 is also expressed in parts of the nervous system, especially the mid-brain–hindbrain junction, known as the rhombencephalic ismuth, and in the optic placode and cup.

Pax2/Pax8 double mutants have no evidence of nephric duct formation and do not express the *lim1* gene. Null mutants in *lim1* also lack the nephric duct and show reduced ability to differentiate into intermediate mesoderm-specific derivatives. The reduced expression of *Pax2* in *lim1* mutants could explain the inability of these cells to differentiate into urogenital epithelia. Although in *Pax2/Pax8* double null embryos, the lack of lim1 expression may also be an integral part of the phenotype. Because lim1 expression precedes Pax2 and Pax8 and is spread over a wider area in the pregastrulation and postgastrulation embryo and is Pax independent, it seems likely that maintenance and restriction of lim1 expression within the intermediate mesoderm requires activation of the *Pax2/8* genes at the five- to eight-somite stage. Are *Pax2/8* sufficient then to specify the renal progenitors? This question was addressed in the chick embryo using replication competent retroviruses expressing a Pax2b cDNA. Ectopic nephric ducts were generated within the general area of the intermediate mesoderm upon retrovirally driven Pax2b expression. These ectopic nephric ducts were not obtained with either lim1 or Pax8 alone. Strikingly, the ectopic nephric ducts paralleled the endogenous ducts and were not found in more paraxial or lateral plate mesoderm. This would suggest that Pax2's ability to induce duct formation does require some regional competence, perhaps only in the lim1-expressing domain.

If Pax2/8 and lim1 restriction in the intermediate mesoderm are the earliest events that distinguish the nephrogenic zone from surrounding paraxial and lateral plate mesoderm, the question then remains as to how these genes are activated. In the axial mesoderm, signals derived from the ventral notochord pattern the somites along the dorsal-ventral axis. Similar notochord derived signals could also pattern mesoderm along the mediolateral axis. However, this does not appear to be the case. In the chick embryo, the notochord is dispensable for activation of the *Pax2* gene in the intermediate mesoderm. Instead, signals derived from the somites, or paraxial mesoderm, are required for activation of Pax2. It remains to be determined what these somite-derived signals are, although it is worth noting that in the amphibian embryo a combination of retinoic acid and activin-A is able to activate early markers of the pronephric field including lim1.

In addition to somite-derived signals, additional signals may be required for the formation of epithelia within the predisposed intermediate mesoderm. Activation of Pax2/8 is found more anterior to and precedes formation of the nephric duct. Thus, not all Pax2 positive cells will make the primary nephric duct. In the chick embryo, the overlying ectoderm is necessary for formation of the nephric duct, which initiates adjacent to somites 10–12, significantly more posterior to the initial Pax2 positive domain. Removal of overlying ectodermal tissues, which express the secreted signaling molecule bone morphogenetic protein-4 (BMP4), blocks nephric duct formation and extension.

This block can be overcome by recombinant BMP4 protein, suggesting that this is indeed the essential ectodermally derived factor.

If Pax2/8 mark the entire nephric region, there must be additional factors that specify the position of elements along the A–P axis in the intermediate mesoderm. Such patterning genes could determine whether a mesonephric or metanephric kidney is formed within the Pax2 positive domain. Among the known A–P patterning genes are members of the HOX gene family. Indeed, mice that have deleted all genes of the Hox11 paralogous group have no metanephric kidneys, although it is not clear whether this is truly a shift in A–P patterning or a lack of induction. A–P patterning of the intermediate mesoderm may also depend on the FoxC family of transcription factors. *Foxc1* and *Foxc2* have similar expression domains in the presomitic and intermediate mesoderm, as early as E8.5. As nephric duct extension progresses, Foxc1 is expressed in a dorsoventral gradient, with the highest levels near the neural tube and lower levels in the BMP4 positive ventrolateral regions. In *Foxc1* homozygous null mutants the anterior boundary of the metanephric mesenchyme, as marked by GDNF expression, extends rostrally. This results in a broader ureteric bud forming along the A–P axis and eventual duplication of ureters. Similar defects are observed in compound heterozygotes of *Foxc1* and *Foxc2*, indicating some redundancy and gene dosage effects. Thus, *Foxc1* and *Foxc2* may set the anterior boundary of the metanephric mesenchyme, at the time of ureteric bud outgrowth, by suppressing genes at the transcriptional level.

22.2.2 Genes that Function at the Time of Metanephric Induction

Pax2 and Pax8 are coexpressed in the nephric duct, but the Pax2 expression domain is broader and encompasses the mesonephric tubules and the metanephric mesenchyme. Thus, Pax8 mutants have no obvious renal phenotype, Pax2 mutants have a nephric duct but no mesonephric tubules or metanephros, and Pax2/8 double mutants lack the nephric duct completely. Thus, either Pax2 or Pax8 is enough for duct formation, but Pax2 is clearly essential for conversion of the metanephric mesenchyme into epithelia.

The Pax2 phenotype is complex. Despite the presence of a nephric duct, there is no evidence of ureteric bud outgrowth. Ureteric bud outgrowth is controlled primarily by the receptor type tyrosine kinase RET, which is expressed on the nephric duct epithelia, the secreted signaling protein GDNF, which is expressed in the metanephric mesenchyme, and the GPi linked protein GFRα1, which is expressed in both tissues. Pax2 mutants have no ureteric buds because they do not express GDNF in the mesenchyme and fail to maintain high levels of RET expression in the nephric duct. Despite the

lack of bud, the metanephric mesenchyme is morphologically distinguishable in Pax2 mutants. Although lacking GDNF, it does express other markers of the mesenchyme, such as Six2. *In vitro* recombination experiments using Pax2 mutant mesenchyme, surgically isolated from E11 mouse embryos, and heterologous inducing tissues indicate that *Pax2* mutants are unable to respond to inductive signals. Thus, *Pax2* is necessary for specifying the region of intermediate mesoderm destined to undergo mesenchyme-to-epithelium conversion. In humans, the necessity of Pax2 function is further underscored because the loss of a single *Pax2* allele is associated with renal-coloboma syndrome, which is characterized by hypoplastic kidneys with vesicoureteral reflux.

A second essential gene for conversion of the metanephric mesenchyme to epithelia is *Eya1*, a vertebrate homologue of the *Drosophila eyes absent* gene. In humans, mutations in the *Eya1* gene are associated with branchio-oto-renal syndrome, a complex multifaceted phenotype. In mice homozygous for an *Eya1* mutation, kidney development is arrested at E11 because ureteric bud growth is inhibited and the mesenchyme remains uninduced, although Pax2 and WT1 expression appears normal. However, two other markers of the metanephric mesenchyme, Six2 and GDNF expression, are lost in the Eya1 mutants. The loss of GDNF expression most probably underlies the failure of ureteric bud growth. However, it is not clear if the mesenchyme is competent to respond to inductive signals if a wild-type inducer were to be used *in vitro*. The 'eyes absent' gene family is part of a conserved network that underlies cell specification in several other developing tissues. Eya proteins share a conserved domain but lack DNA binding activity. The Eya proteins interact directly with the Six family of DNA-binding proteins. Mammalian Six genes are homologues of the *Drosophila sina oculis* homeobox gene. This cooperative interaction between Six and Eya proteins is necessary for nuclear translocation and transcriptional activation of Six target genes.

The Wilms tumor suppressor gene, *WT1*, is another early marker of the metanephric mesenchyme and is essential for its survival. Wilms tumor is an embryonic kidney neoplasia that consists of undifferentiated mesenchymal cells, poorly organized epithelium, and surrounding stromal cells. Expression of WT1 is regulated spatially and temporally in a variety of tissues and is further complicated by the presence of at least four isoforms, generated by alternative splicing. In the developing kidney, WT1 can be found in the uninduced metanephric mesenchyme and in differentiating epithelium after induction. Early expression of WT1 may be mediated by Pax2. Initial expression levels are low in the metanephric mesenchyme, but become upregulated at the S-shaped body stage in the precursor cells of the glomerular epithelium, the podocytes. High WT1 levels persist in the adult podocytes. In the mouse, *WT1* null mutants have complete renal agenesis, because the metanephric mesenchyme undergoes apoptosis and the ureteric bud fails to

grow out of the nephric duct. The arrest of ureteric bud growth is most probably due to lack of signaling by the *WT1* mutant mesenchyme. As in *Pax2* mutants, the mesenchyme is unable to respond to inductive signals even if a heterologous inducer is used *in vitro*. Thus, it appears that *WT1* is required early in the mesenchyme to promote cell survival, enabling cells to respond to inductive signals and express ureteric bud growth promoting factors.

22.3 THE ESTABLISHMENT OF ADDITIONAL CELL LINEAGES

At the time of ureteric bud invasion, there appear to be at least two cell lineages established: the metanephric mesenchyme and the ureteric bud epithelia. As branching morphogenesis and induction of the mesenchyme progresses, additional cell lineages are evident. The early E11.5 mouse metanephros contains precursors for almost all cell types, including endothelial, stromal, epithelial, and mesangial cells. However, it is far from clear if these cell types share a common precursor, or if the metanephric mesenchyme is a mixed population of precursors. The latter point may well be true for the endothelial lineage. Although transplantation studies with lineage markers indicate that the vasculature can be derived from E11.5 metanephric kidneys, the Flk1 positive endothelial precursors have been observed closely associated with the ureteric bud epithelium, shortly after invasion, and are probably not derived from the metanephric mesenchyme. The lineages most likely to share a common origin within the metanephric mesenchyme are the stromal and epithelial lineages. The maintenance of these two lineages is essential for renal development, because the ratio of stroma to epithelia is a critical factor for the renewal of mesenchyme and the continued induction of new nephrons.

22.3.1 Epithelia versus Stroma

What are the early events in the induced mesenchyme that separates the stromal lineage from the epithelial lineage? In response to inductive signals, Pax2 positive cells aggregate at the tips of the ureteric bud. Activation of the *Wnt4* gene in these early aggregates appears critical to promote polarization. *Wnt* genes encode a family of secreted peptides that are known to function in the development of many tissues. Mice homozygous for a *Wnt4* mutation exhibit renal agenesis resulting from growth arrest shortly after branching of the ureteric bud. Although some mesenchymal aggregation has occurred, there is no evidence of cell differentiation into a polarized epithelial vesicle. Expression of *Pax2* is maintained but reduced. Thus, *Wnt4* may be a secondary inductive signal in the mesenchyme that propagates or maintains the primary induction response in the epithelial lineage. The transcription factor FoxD1/BF-2

is expressed in uninduced mesenchyme and becomes restricted to those cells not undergoing epithelial conversion after induction. FoxD1 expression is found along the periphery of the kidney and in the interstitial mesenchyme, or stroma. After induction, there is little overlap between FoxD1 and the Pax2 expression domain, prominent in the condensing pretubular aggregates. Clear lineage analysis is still lacking, although the expression patterns are consistent with the interpretation that mesenchyme cells may already be partitioned into a FoxD1 positive stromal precursor and a Pax2 positive epithelial precursor before or shortly after induction. Mouse mutants in FoxD1 exhibit severe developmental defects in the kidney that point to an essential role for FoxD1 in maintaining growth and structure. Early ureteric bud growth and branching are unaffected, as is the formation of the first mesenchymal aggregates. However, at later stages (E13–14) these mesenchymal aggregates fail to differentiate into comma- and S-shaped bodies at a rate similar to wild-type. Branching of the ureteric bud is greatly reduced at this stage, resulting in fewer new mesenchymal aggregates forming. The fate of the initial aggregates is not fixed, because some are able to form epithelium and most all express the appropriate early markers, such as Pax2, wnt4, and WT1. Nevertheless, it appears that the FoxD1 expressing stromal lineage is necessary to maintain growth of both ureteric bud epithelium and mesenchymal aggregates. Perhaps factors secreted from the stroma provide survival or proliferation cues for the epithelial precursors, in the absence of which the non-self-renewing population of mesenchyme is exhausted.

Some survival factors that act on the mesenchyme have already been identified. The secreted transforming growth factor beta (TGFβ) family member BMP7 and the fibroblast growth factor-2 (FGF2) in combination dramatically promote survival of uninduced metanephric mesenchyme *in vitro*. FGF2 is necessary to maintain the ability of the mesenchyme to respond to inductive signals *in vitro*. BMP7 alone inhibits apoptosis but is not sufficient to enable mesenchyme to undergo tubulogenesis at some later time. After induction, exogenously added FGF2 and BMP7 reduce the proportion of mesenchyme that undergoes tubulogenesis while increasing the population of FoxD1 positive stromal cells. At least after induction occurs, there is a delicate balance between a self-renewing population of stromal and epithelial progenitor cells, the proportion of which must be well regulated by both autocrine and paracrine factors. Whether this lineage decision has already been made in the uninduced mesenchyme remains to be determined.

The role of stroma in regulating renal development is further underscored by studies with retinoic acid receptors. It is well documented that vitamin A deficiency results in severe renal defects. In organ culture, retinoic acid stimulates expression of RET to dramatically increase the number of ureteric bud branch points, increasing the number of nephrons. However, it is the stromal cell

population that express the retinoic acid receptors (RARs), specifically RARα and RARβ2. Genetic studies with *RAR*α and *RAR*β*2* homozygous mutant mice indicate no significant renal defects when either gene is deleted. However, double homozygotes mutant for both *RAR*α and *RAR*β*2* exhibit severe growth retardation in the kidney. These defects are primarily due to decreased expression of the RET protein in the ureteric bud epithelia and limited branching morphogenesis. Surprisingly, overexpression of RET with a HoxB7/RET transgene can completely rescue the double RAR mutants. These studies suggest that stromal cells provide paracrine signals for maintaining RET expression in the ureteric bud epithelia and that retinoids are required for stromal proliferation. Reduced expression of stromal cell marker FoxD1, particularly in the interstitium of RAR double mutants, supports this hypothesis.

22.3.2 Cells of the Glomerular Tuft

The unique structure of the glomerulus is intricately linked to its ability to retain large macromolecules within the circulating bloodstream while allowing for rapid diffusion of ions and small molecules into the urinary space. The glomerulus consists of four major cell types: the endothelial cells of the microvasculature, the mesangial cells, the podocyte cells of the visceral epithelium, and the parietal epithelium. The development of the glomerular architecture and the origin of the individual cell types are just beginning to be understood.

The podocyte is a highly specialized epithelial cell whose function is integral to maintaining the filtration barrier in the glomerulus. The glomerular basement membrane separates the endothelial cells of the capillary tufts from the urinary space. The outside of the glomerular basement membrane, which faces the urinary space, is covered with podocyte cells and their interdigitated foot processes. At the basement membrane, these interdigitations meet to form a highly specialized cell-cell junction, called the slit diaphragm. The slit diaphragm has a specific pore size to enable small molecules to cross the filtration barrier into the urinary space, while retaining larger proteins in the blood stream. The podocytes are derived from condensing metanephric mesenchyme and can be visualized with specific markers at the S-shaped body stage. Although there are a number of genes expressed in the podocytes, there are only a few factors known to regulate podocyte differentiation. These include the *WT1* gene, which is required early for metanephric mesenchyme survival but whose levels increase in podocyte precursors at the S-shaped body stage. In the mouse, complete *WT1* null animals lack kidneys, but reduced gene dosage and expression of WT1 results in specific podocyte defects. Thus, the high levels of WT1 expression in podocytes appear to be required and make these precursor cells more sensitive to gene dosage. The basic helix-loop-helix protein Pod1 is expressed in epithelial precursor cells

and in more mature interstitial mesenchyme. At later developmental stages, Pod1 is restricted to the podocytes. In mice homozygous for a *Pod1* null allele, podocyte development appears arrested. Normal podocytes flatten and wrap their foot processes around the glomerular basement membrane. *Pod1* mutant podocytes remain more columnar and fail to fully develop foot processes. Because Pod1 is expressed in epithelial precursors and in the interstitium, it is unclear whether these podocyte effects are due to a general developmental arrest because of the stromal environment or a cell autonomous defect within the *Pod1* mutant podocyte precursor cells.

Within the glomerular tuft, the origin of the endothelium and the mesangium is less clear. At the S-shaped body stage, the glomerular cleft forms at the most proximal part of the S-shaped body, furthest from the ureteric bud epithelium. Vascularization of the developing kidney is first evident within this developing tuft. The origin of these invading endothelial cells has been studied in some detail. Under normal growth conditions, kidneys excized at the time of induction and cultured *in vitro* do not exhibit signs of vascularization, leading to the presumption that endothelial cells migrate to the kidney some time after induction. However, hypoxygenation or treatment with vascular endothelial growth factor (VEGF) promotes survival or differentiation of endothelial precursors in these same cultures, suggesting that endothelial precursors are already present and require growth differentiation stimuli. *In vivo* transplantation experiments using lacZ expressing donors or hosts also demonstrate that the E11.5 kidney rudiment has the potential to generate endothelial cells, although recruitment of endothelium is also observed from exogenous tissue depending on the environment. The data are consistent with the idea that cells within the E11.5 kidney have the ability to differentiate along the endothelial lineage. Are these endothelial cells generated from the metanephric mesenchyme? Using a lacZ knockin allele for the endothelial specific receptor Flk-1, presumptive angioblasts were observed to disperse along the periphery of the E12 kidney mesenchyme, with some positive cells invading the mesenchyme along the aspect of the growing ureteric bud. At later stages, Flk-1 positive angioblasts were localized to the nephrogenic zone, the developing glomerular cleft of the S-shaped bodies, and the more mature capillary loops, whereas VEGF localizes to the parietal and visceral glomerular epithelium. Injection of neutralizing VEGF antibodies into newborn mice, at a time when nephrogenesis is still ongoing, disturbs vascular growth and glomerular architecture. The data suggest that endothelial cells originate independently of the metanephric mesenchyme and invade the growing kidney from the periphery and along the ureteric bud.

The mesangial cells are located between the capillary loops of the glomerular tuft and have been referred to as specialized pericytes. The pericytes are found within the capillary basement membranes and have contractile

abilities, much like a smooth muscle cell. Whether the mesangial cell is derived from the endothelial or epithelial lineage remains unclear. However, genetic and chimeric analyses in the mouse have revealed a clear role for the platelet-derived growth factor receptor (PDGFR) and its ligand platelet-derived growth factor (PDGF). In mice deficient for either PDGF or PDGFR, a complete absence of mesangial cells results in glomerular defects, including the lack of microvasculature in the tuft. PDGF is expressed in the developing endothelial cells of the glomerular tuft, whereas the receptor is found in the presumptive mesangial cell precursors. Using ES cell chimeras of *Pdgfr* −/− and +/+ genotypes, only the wild-type cells could contribute to the mesangial lineage. This cell autonomous effect indicates that signaling from the developing vasculature promotes proliferation and/or migration of the mesangial precursor cells. Also, expression of PDGFR and smooth muscle actin supports a model in which mesangial cells are derived from smooth muscle of the afferent and efferent arterioles during glomerular maturation.

22.4 WHAT CONSTITUTES A RENAL STEM CELL?

The issue of renal stem cells is beginning to draw more attention as new information regarding development and lineage specification is unraveled. A prospective renal stem cell should be self-renewing and able to generate all of the cell types in the kidney. Whether such a cell exists remains to be demonstrated *in vivo*, although the *in vitro* data appear promising. In simplest terms, all of the cells in the kidney could be generated by a single stem cell population as outlined in Figure 22.5. Despite the potential, there are several issues outstanding. Even at the earliest stage of kidney development, there are already two identifiable cell types. The presence of early endothelial precursors at the time of induction would make a third distinct cell type. If the three earliest cell types can indeed be derived from the metanephric mesenchyme, a single stem cell may indeed exist and continue to proliferate as development progresses. At present, the data suggest that stroma and epithelia may share a common origin, whereas endothelial cells and their potential smooth muscle derivatives constitute a second lineage. However, even if there are three separate lineages already demarcated within the metanephric mesenchyme, the most relevant with respect to the repair of renal tissue is the epithelial lineage. Thus, if we consider the possibility of an epithelial stem cell, the following points would be among the criteria for selection:

1. The cells would most likely be a derivative of the intermediate mesoderm;
2. The cells would express a combination of markers specific for the metanephric mesenchyme; and
3. The cells should be able to contribute to all epithelial components of the nephron, *in vitro* and *in vivo*.

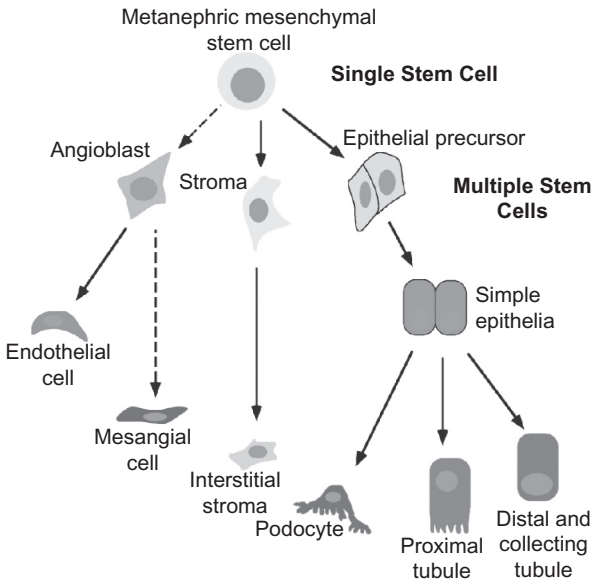

FIGURE 22.5 **The major cell lineages of the kidney.**
Whether the kidney arises from a single renal stem cell or from multiple independent lineages remains to be determined. However, the basic differentiation scheme is becoming clearer. The cell lineage relationships are outlined schematically, with dotted lines reflecting ambiguity in terms of direct lineages. The metanephric mesenchyme contains angioblasts and stromal and epithelial precursors. Whether angioblasts arise from mesenchymal cells or are a separate lineage that surrounds the mesenchyme is not entirely clear. Similarly, the origin of the mesangial cell is not well defined. Stromal and epithelial cells may share a common precursor, the metanephric mesenchyme, but segregate at the time of induction. The epithelial cell precursors, found in the aggregates at the ureteric bud tips, generate almost all of the epithelial cell types in the nephron.

Unlike ES cells, it seems improbable that cells from the intermediate mesoderm can be cultured indefinitely without additional transformation or immortalization taking place. Embryonic fibroblasts can be cultured from the mouse, but, in almost every case, a limited number of cell divisions occurs. The problem is apparent even *in vivo*, because the E11 metanephric mesenchyme is essentially quiescent and does not proliferate in the absence of induction. However, growth conditions that mimic induction might be able to allow the mesenchyme cells to proliferate while suppressing their differentiation into epithelium. Alternatively, it may be possible to differentiate ES cells into intermediate mesodermal cells using combinations of growth and patterning factors, as has been done for motor neuron differentiation.

If metanephric mesenchymal cells were able to proliferate *in vitro*, the markers they might express should include the following: Pax2, lim1, WT1, GDNF, Six2,

and FoxD1. Expression of these markers would indicate a mesenchymal cell that had not decided between the stromal and epithelial lineage. If cells express Pax2, WT1, and Wnt4, but not FoxD1, they could be epithelial stem cells. Such epithelial stem cells when injected into, or recombined with, an *in vitro* cultured metanephric kidney should be able to make all of the epithelial cells along the proximal–distal axis of the nephron. Such epithelial stem cells could prove significant in regenerating damaged tubules in acute and chronic renal injury.

At present, the complexity of the kidney still impedes progress in the area of tissue and cell-based therapies. Not only must the right cells be made, but they must be able to organize into a specialized three-dimensional tubular structure capable of fulfilling all of the physiologic demands put on the nephrons. Developmental biology can provide a framework for understanding how these cells arise and what factors promote their differentiation and growth. Although we may not be able to make a kidney from scratch, it seems within the realm of possibility to provide the injured adult kidney with cells or factors to facilitate its own regeneration. Given the high incidence and severity of acute and chronic renal insufficiency, such therapies would be most welcome indeed.

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FOR FURTHER STUDY

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CHAPTER 23

Adult Liver Stem Cells

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23.1 ORGANIZATION AND FUNCTIONS OF ADULT MAMMALIAN LIVER

To provide background for this chapter on liver stem cell biology, the normal anatomy and function of adult liver is briefly described.

The liver consists of several separate lobes and represents about 2% of human and 5% of mouse body weight. It is the only organ with two afferent blood supplies. The portal vein brings venous blood rich in nutrients and hormones from the splanchnic bed (intestines and pancreas), and the hepatic artery provides oxygenated blood. Venous drainage is into the vena cava. The bile secreted by hepatocytes is collected in a branched collecting system, the biliary tree, which drains into the duodenum. The hepatic artery, portal vein, and common bile duct enter the liver in the same location, the porta hepatis.

The main cell types resident in the liver are hepatocytes, bile duct epithelium, stellate cells (formerly called Ito cells), Kupffer cells, vascular endothelium, fibroblasts, and leukocytes. An adult mouse liver contains about 5×10^7 hepatocytes, and an adult human contains 8×10^9 hepatocytes. The microscopic structure of the liver is essential to the understanding of hepatic stem cell biology. The hepatic lobule (Figure 23.1) is the functional unit of the liver. The portal triad, consisting of a small portal vein, a hepatic artery branch, and a bile duct, is located on the perimeter. Arterial and portal venous blood enter here, mix, and flow past the hepatocytes toward the central vein in the middle of the lobule. Liver sinusoids are the vasculature connecting the portal triad vessels and the central vein. Contrary to other capillary beds, sinusoidal vessels have a fenestrated endothelium, thus permitting direct contact between the blood and the hepatocyte cell surface. In two-dimensional images, rows of hepatocytes oriented from portal to central form a hepatic plate. A channel formed by adjacent hepatocytes forms a

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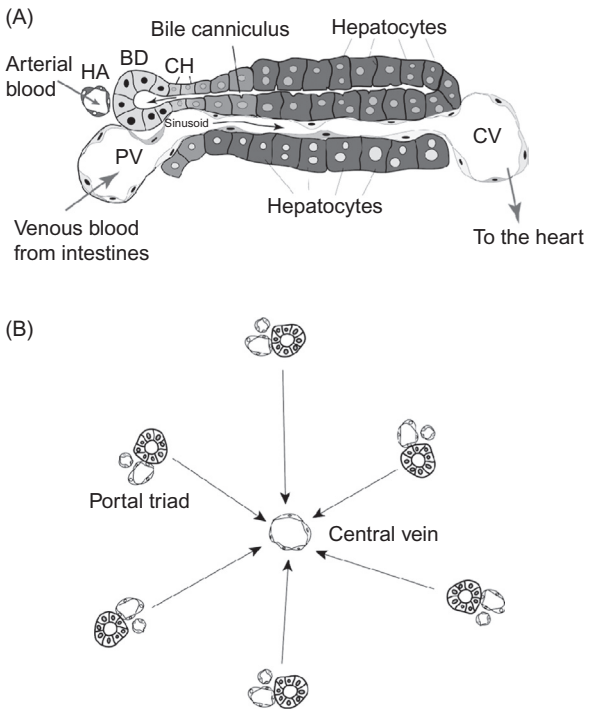

FIGURE 23.1 **Structure of the hepatic lobule.**
(A) The portal triad consists of bile ducts (BD), hepatic artery (HA), and portal vein (PV). Mixed blood from the hepatic artery and portal vein flows past hepatocytes through the sinusoids, covered with fenestrated endothelial cells to the central vein (CV). Bile produced by the hepatocytes is collected in the bile canaliculus and flows toward the bile duct. The canal of Hering (CH) is the junction between the hepatic plate and the bile ducts. This is the region where oval cell precursors reside. (B) Each hepatic lobule consists of one central vein and six surrounding portal triads.

bile canaliculus, which drains secreted bile toward the bile duct in the portal triad. Bile secreted by the hepatocytes is collected in bile ducts, lined by duct epithelial cells. The canal of Hering is the connection between the bile canaliculi (the interhepatocyte space into which bile is secreted) and the bile ducts at the interface between the lobule and the portal triad. Stellate cells represent about 5–10% of all hepatic cells. In addition to storing vitamin A, they are essential for the synthesis of extracellular matrix proteins and produce many hepatic growth factors that play a vital role in the biology of liver regeneration. Kupffer cells also represent about 5% of all liver cells and are tissue macrophages.

The liver is responsible for the intermediary metabolism of amino acids, lipids, and carbohydrates; the detoxification of xenobiotics; and the synthesis of serum proteins. In addition, the liver produces bile, important for the

intestinal absorption of nutrients as well as the elimination of cholesterol and copper. All of these functions are primarily executed by hepatocytes. The biochemical properties and pattern of gene expression is not uniform among all hepatocytes. The term metabolic zonation has been coined to indicate the different properties of periportal (adjacent to the portal triad) and pericentral (adjacent to the central vein) hepatocytes. For example, only pericentral hepatocytes express glutamine synthase and use ammonia to generate glutamine. In contrast, periportal hepatocytes express urea cycle enzymes and convert ammonia to urea.

23.2 LIVER STEM CELLS

The liver is known to have a very high capacity for regeneration. Mammals (including humans) can survive surgical removal of up to 75% of their total liver mass. The original number of cells is restored within one week, and the original tissue mass is returned within 2–3 weeks. Importantly, liver size is also controlled by prevention of organ overgrowth. Hepatic overgrowth can be induced by a variety of compounds, such as hepatocyte growth factor (HGF) or peroxisome proliferators, but the liver size rapidly returns to normal after removal of the growth-inducing signal. The role of liver stem cells in regeneration has been controversial, but many of the apparent inconsistencies can be reconciled by considering the different definitions used for these cells. Current evidence strongly suggests that different cell types and mechanisms are responsible for organ reconstitution depending on the type of liver injury. In addition, tissue replacement by endogenous cells (regeneration) must be distinguished from reconstitution by transplanted donor cells (repopulation). Thus, the definition of liver stem cells includes:

1. Cells responsible for normal tissue turnover,
2. Cells that give rise to regeneration after partial hepatectomy,
3. Cells responsible for progenitor-dependent regeneration,
4. Transplantable liver-repopulating cells, and
5. Cells that produce hepatocyte and bile duct epithelial phenotypes *in vitro*.

In the following sections, liver stem cells are described according to each of these definitions.

23.2.1 Cells Responsible for Normal Liver Tissue Turnover

The average life span of adult mammalian hepatocytes can be estimated to be between 200 and 300 days. The mechanism by which these cells are replaced has been of interest for some time. One of the main models regarding normal liver turnover was termed the *streaming liver*. According to this model, normal

liver turnover is similar to regeneration in the intestine, with young hepatocytes being born in the portal zone then migrating toward the central vein. The different patterns of gene expression in periportal and pericentral hepatocytes were explained by the maturation process during this migration and thus represented a typical lineage progression. However, recent work has provided strong evidence against the streaming liver hypothesis. First, it has been shown that the gene expression pattern in hepatocytes is crucially dependent on the direction of blood flow. If blood flow was reversed such that portal blood entered the lobule through the central vein and exited through the portal vein, the pattern inverted. Therefore, the lobular zonation is best explained by metabolite-induced gene regulation, not by lineage progression. Second, retroviral marking studies provide clear evidence against any hepatocyte migration during normal turnover. These results have been confirmed using the mosaic pattern of X-inactivation in female mice to analyze patterns of hepatocyte growth. Together, current evidence clearly indicates that normal liver turnover in adult animals is mediated primarily by *in situ* cell division of hepatocytes and bile duct epithelial cells themselves and not by stem cells.

23.2.2 Cells that Give Rise to Regeneration after Partial Hepatectomy

The process of liver regeneration after partial hepatectomy has been well studied and is the subject of several excellent reviews. During partial hepatectomy, specific lobes are removed intact without damage to the lobes left behind. The residual lobes grow to compensate for the mass of the resected lobes, although the removed lobes never grow back. The process is completed within one week. Again, as in normal liver turnover, there is no evidence for the involvement or requirement for stem cells in this process. Classic thymidine-labeling studies show that virtually all hepatocytes in the remaining liver divide once or twice to restore the original cell number within 3–4 days. The earliest labeled hepatocytes are seen 24 hours after partial hepatectomy, with the peak of thymidine incorporation occurring 24–48 hours depending on the species. Interestingly, there is zonal variation depending on how much tissue is removed. When only 15% of the liver is surgically removed, periportal (zone 1) hepatocytes divide preferentially, whereas cell division is seen equally in all three zones after 75% partial hepatectomy. Following the hepatocytes, the other hepatic cell types undergo a wave of mitosis, thereby restoring the original number of all liver cells within seven days.

Many positive and negative factors important for the initiation of regeneration after partial hepatectomy have been identified. The most important ones are HGF, interleukin-6, tumor necrosis factor-alpha, transforming growth factor-alpha (TGF-α), and epidermal growth factor. Nonpeptide hormones also have a significant role in the regenerative response after liver injury.

Triiodothyronine and norepinephrine can stimulate hepatocyte replication *in vivo*. It is unknown whether any of these factors are also important for progenitor-dependent liver regeneration or engraftment and expansion of liver stem cells (see the next section of this chapter).

Less knowledge exists about the mechanisms by which hepatocyte cell division and liver regeneration are stopped after the appropriate liver mass has been restored. In particular, the exogenous signals (endocrine, paracrine, or autocrine) involved in sensing the overall liver cell mass and negatively regulating its size are not known. Some evidence suggests that TGF-β1 may be important in termination of liver regeneration.

23.2.3 Cells Responsible for Progenitor-Dependent Regeneration

23.2.3.1 Oval Cells

Although neither cell replacement during normal tissue turnover nor cell replacement after injury by partial hepatectomy requires stem cells for organ regeneration, this is not true for all types of liver injury. In some types of damage to liver, small cells with a high nuclear:cytoplasmic ratio emerge in the portal zone, proliferate extensively, and migrate into the lobule. These small cells, which eventually become differentiated hepatocytes, are termed *oval cells* because of their morphology. Importantly, oval cells are not derived from hepatocytes; instead, they are the offspring of a cell in the canal of Hering (Figure 23.1). Oval cell proliferation therefore represents an example of progenitor-dependent liver regeneration, and the cell that produces oval cells can be considered a 'facultative liver stem cell.' In the rat, chronic liver injury by chemicals such as DL-ethionine, galactosamine, and azo dyes are examples of this type of liver damage (see Table 23.1). The toxic drugs are often combined with surgical partial hepatectomy. Many of the compounds that induce oval cell proliferation are DNA-damaging agents or carcinogens, and oval cells can therefore be considered precancerous. A common feature of progenitor-dependent liver regeneration is that the hepatocytes themselves cannot divide normally. Thus, progenitor-dependent regeneration may be used when parenchymal hepatocytes are severely damaged on a chronic basis, unable to regenerate efficiently, or both. Oval cells express markers of both bile duct epithelium (CK19) and hepatocytes (albumin). In addition, in the rat they express high levels of α-fetoprotein and are thus similar to fetal hepatoblasts in their gene expression profile. Furthermore, oval cells are bipotential *in vitro* and retain the ability to differentiate into both the bile duct epithelial and hepatocyte lineages. Because of their similarity to hepatoblasts and their bipotential capacity, oval cells have been considered early progenitors in analogy to committed hematopoietic progenitors. Thus, oval cell precursors located in the canal of Hering are likely candidates to be liver-repopulating stem cells.

Table 23.1 Induction of Progenitor-Dependent Liver Regeneration

Chemical Manipulation
Mouse
Dipin
3,5-Diethoxycarbonyl-1,4-Dihydrocollidine (DDC)
Phenobarbital + cocaine + p.H.[a]
Choline-deficient diet + DL-ethionine
Rat
2-Acetylaminofluorene (AAF)
Diethylnitrosamine (DEN)
Solt-Farber model (DEN + AAF + p.H)
Modified Solt-Farbermodel (AAF + p.H)
Choline-deficient diet + DL-ethionine
D-Galactosamine + p.H.
Lasiocarpine + p.H.
Retrorsine + p.H.

[a] *p.H. = partial hepatectomy.*

In the rat, several monoclonal antibodies have been raised against oval cells and used to study lineage progression. The cell surface marker OV6 has found application in a variety of studies. Generally, studies with these reagents have confirmed the similarity between oval cells and fetal hepatoblasts.

Until recently, it has been difficult to induce oval cell proliferation in the mouse and take advantage of the powerful genetics in this organism. Using transgenic mice, it would be possible to determine whether factors known to be important in liver regeneration after partial hepatectomy are also required for oval cell driven regeneration.

However, several protocols have been developed that result in progenitor-dependent hepatocyte regeneration in the mouse. One particularly useful regimen uses the chemical 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC). Mouse oval cells differ from their rat and human counterparts by not expressing α-fetoprotein. OV6 also does not react with murine oval cells, and to date only one oval cell-specific antibody, termed A6, has been developed for murine oval cells. Nonetheless, work on the genetics of oval cell proliferation is now possible. An example is the discovery, using transgenic mice, that TGF-β1 inhibits oval cell proliferation.

Table 23.1 lists conditions that result in oval cell proliferation in the rat and mouse. Oval cell proliferation has also been described in a variety of human

liver diseases, indicating that progenitor-dependent regeneration can be found in multiple organisms. Oval cells are found in disorders associated with chronic liver injury and are located at the edges of nodules in liver cirrhosis. In both rats and humans, OV6 is a useful marker for these cells.

Interestingly, rat oval cells induced by a classic carcinogen regimen express multiple genes typically associated with hematopoietic stem cells (HSCs). The first of these to be identified were stem cell factor (SCF) and its cognate receptor, the c-kit tyrosine kinase. Isolation of HSCs by fluorescence-activated cell sorting (FACS) uses c-kit antigen as an important positive marker. Both the *SCF* and the *c-kit* genes were expressed during the early stages of oval cell proliferation after partial hepatectomy in the 2-acetylaminofluorene (AAF)-partial hepatectomy model, but neither simple partial hepatectomy nor AAF administration alone induced a noticeable expression of the SCF-c-kit system. mRNA *in situ* hybridization revealed that the c-kit transcripts were restricted to oval cells, whereas the SCF transcripts were expressed in both oval and stellate cells.

In addition, later work found that the Thy-1 marker used for sorting of HSCs in mouse was also highly expressed on rat oval cells. Thy-1 staining clearly colocalized with classic oval cell markers such as α-fetoprotein.

The expression of HSC markers in oval cells is not unique to the rat. Human oval cells isolated from patients with chronic biliary diseases were found to express CD34 as well as the bile duct marker cytokeratin 19 (CK19). In addition, cells that are c-kit positive but are negative for hematopoietic markers have been identified in human pediatric liver disease. Very high levels of stem cell antigen-1 (Sca-1) were found to be expressed on murine oval cells induced by DDC. Therefore, multiple independent studies support the concept that hepatic oval cells, but not regenerating hepatocytes, can express genes found in HSCs. These findings have resulted in the hypothesis that oval cell precursors may be bone marrow derived (see later sections of this chapter).

23.2.3.2 Other Hepatocyte Progenitors

Oval cells are defined by their morphologic appearance in the rat, but there is variability in the marker genes expressed at different times after induction of oval cell proliferation. In addition, the different induction regimens vary the phenotype. Therefore, it is not clear whether all oval cells are equal or whether subclasses of oval cells exist. Another class of hepatocyte progenitors was described after treatment of rats with retrorsine and partial hepatectomy. Retrorsine blocks the division of mature hepatocytes, but it does not result in the emergence of classic oval cells that are α-fetoprotein and OV6 positive. Instead, foci of small, hepatocyte-like cells emerge and eventually result in

organ reconstitution. These small cells express both hepatocyte and bile duct markers. At this time, their origin (dedifferentiated hepatocytes, transitional cells in the canal of Hering, or bone marrow) is unknown.

23.2.4 Transplantable Liver-Repopulating Cells

One way to define stem cells is by their ability to repopulate the respective organ and restore its function. The HSC, for example, was defined by reconstitution of the blood lineages of lethally irradiated hosts. In the 1990s, similar repopulation assays were developed for the liver in several animal models. In liver repopulation, a small number of transplanted donor cells engrafts in the liver, the cells expand, and they replace > 50% of the liver mass. Thus, it has become possible to perform experiments analogous to those done in the hematopoietic system, including cell sorting, competitive repopulation, serial transplantation, and retroviral marking. Hepatic stem cells can now be defined by their ability to repopulate liver. It should be emphasized that liver repopulation refers to replacement of only the hepatocytes by transplanted cells. Efficient repopulation of the biliary system by transplanted cells has not yet been reported.

The main animal models for liver repopulation studies are summarized in Table 23.2. In all cases, liver repopulation by transplanted cells is based on a powerful selective advantage for the transplanted cells over the pre-existing host hepatocytes. In many models, this selection is achieved by genetic differences (transgene-knockout), but DNA damage has also been used successfully, particularly in the rat.

The animals described previously have been used to determine the nature of transplantable liver-repopulating cells and to determine whether undifferentiated stem cells are driving this process. The stem cell hypothesis was strengthened by the observation that liver-repopulating cells could be

Table 23.2 Animals Models for Liver Repopulation

Animal	Model	Selective Pressure
Mouse	Albumin-urokinase transgenic	Uruokinase-mediated hepatocyte injury
Mouse	Fah knockout	Accumulation of toxic tyrosine metabolite
Mouse	Albumin-HSVTK transgenic	HSVTK- mediated conversion of ganciclovir to toxin
Mouse	Mdr3 knockout	Bile acid accumulation
Mouse	Bcl2-transgenic donor cells	Fas ligand (Jo2)-induced apoptosis in hepatocytes not expressing Bcl2
Rat–mouse	Retrorsine conditioning	Host hepatocytes inactivated by retrors line (DNA damage)
Rat	Radiation conditioning	Host hepatocytes inactivated by X-rays (DNA damage)

serially transplanted for > 100 cell doublings without loss of functionality. Interestingly, the only donor-derived cells in this experiment were hepatocytes. No biliary epithelium or other cell types of donor origin were found, thus raising the possibility of a 'unipotential' stem cell.

23.2.4.1 Hepatocytes as Liver-Repopulating Cells

It is reasonable to hypothesize that adult liver cells are not homogeneous in their capacity for cell division and that subpopulations with high repopulation capacity might exist. In the hematopoietic system, repopulation experiments with purified fractions of total bone marrow were used to identify subpopulations with high reconstitution activity. Similar experiments have recently been performed with liver cells and indicate that, in contrast to the hematopoietic system, differentiated hepatocytes have a high capacity for liver repopulation.

In the *Fah*-mutant mouse model, size fractionation, retroviral marking, and competitive repopulation between naïve liver cells and those that had been serially transplanted were performed. All three experimental approaches indicated that large, binucleated hepatocytes representing ~70% of the population were primarily responsible for liver repopulation. Others confirmed this finding by transplanting hepatocytes that had been sorted based on their ploidy (DNA content). No differences in the repopulating ability of 2n, 4n, and 8n hepatocytes were found. Thus, no evidence for a rare stem cell responsible for liver repopulation was detected. Together, these experiments strongly suggest that fully differentiated hepatocytes, which constitute most liver cells, are efficient in liver repopulation and have a stem cell-like capacity for cell division.

23.2.4.2 Nonhepatocytes as Liver-Repopulating Cells

Despite the evidence that hepatocytes are serially transplantable liver-repopulating cells, other cell types also capable of repopulating the liver. This finding is analogous to the situation in liver regeneration, where hepatocytes as well as undifferentiated hepatocyte progenitors are capable of reconstituting the organ. In the following sections, liver repopulation by several nonhepatocyte cell types will be described:

1. Fetal hepatoblasts,
2. Oval cells,
3. Pancreatic liver progenitors, and
4. HSCs.

23.2.4.2a Liver Repopulation With Fetal Hepatoblasts

During embryonic development, the fetal liver bud contains hepatoblasts, cells that express α-fetoprotein as well as hepatocyte (albumin) and biliary

(CK19) markers. These cells therefore may represent fetal liver stem cells capable of hepatocyte repopulation and potentially capable of reconstitution of the biliary system.

Two reports on transplantation of hepatoblasts have been published. One study, using fetal rat liver cells in the retrorsine model, indicated at least three distinct subpopulations of hepatoblasts between embryonic days (E) 12 and 14. One population appeared to be bipotential on the basis of histochemical markers, and the other two had either a unipotent hepatocyte or biliary epithelial cell phenotype. After transplantation, the bipotential cells were able to proliferate in retrorsine-treated cell transplantation recipients, whereas the unipotent cells grew even in untreated rats. However, none of the fetal liver cell populations proliferated spontaneously. Partial hepatectomy or thyroid hormone treatment were required to augment proliferation of transplanted cells. Nonetheless, fetal liver cells proliferated more readily than adult cells. Finally, the transplanted fetal cells produced both hepatocyte cords and mature bile duct structures. It was not formally proven, however, that both of these cell lineages originated from a clonal precursor.

Together, these results indicated that transplanted fetal hepatoblasts proliferate more readily than adult hepatocytes and that some fetal liver cells may remain bipotential.

23.2.4.2b Liver Repopulation With Oval Cells

Oval cells are similar to fetal hepatoblasts in that they are bipotent. These cells have therefore been of interest in liver repopulation experiments. Transplantation of either hepatic- or pancreatic-derived oval cells has been reported in the rat. Upon transplantation, the oval cells proliferated modestly and differentiated into mature hepatocytes even under nonselective conditions. However, because no *in vivo* selection model was used, the true capacity for liver repopulation was not demonstrated in these experiments. More recently, however, repopulation studies have been performed with purified murine oval cells induced by DDC. These experiments clearly indicated that oval cells have extensive liver repopulation capacity and constitute a potential population for use in cell therapy.

23.2.4.2c Liver Repopulation With Pancreatic Progenitors

During embryogenesis, the main pancreatic cell types develop from a common endodermal precursor located in the ventral foregut, including ducts, ductules, acinar cells, and the endocrine α, β, and δ cells. Importantly, the main epithelial cells of the liver, hepatocytes, and bile duct epithelium are thought to arise from the same region of the foregut endoderm. This tight relationship between liver and pancreas in embryonic development has

raised the possibility that a common hepatopancreatic precursor-stem cell may continue to persist in adult life in both the liver and the pancreas. Indeed, several independent lines of evidence suggest that adult pancreas contains cells that can generate hepatocytes. The earliest description of pancreatic hepatocytes was in hamsters treated with a pancreatic carcinogen, *N*-nitroso-*bis*(2-oxopropyl)amine. Similarly, small, occasional clusters of hepatocyte-like cells were found in rats being treated with a peroxisome proliferator, Wy-14643. Interestingly, some of the same carcinogens can be used to produce hepatic oval cells, particularly when combined with partial hepatectomy. The best known example is the emergence of hepatocytes in copper-depleted rats after refeeding of copper. In this system, weanling rats are fed a copper-free diet for eight weeks, which leads to complete acinar atrophy, and then are refed copper. Within weeks, cells with multiple hepatocellular characteristics emerge from the remaining pancreatic ducts. This work has been interpreted to suggest the presence of a pancreatic liver stem cell. This notion is also supported by the appearance of hepatocellular markers in human pancreatic cancers. More recently, a specific cytokine has been identified as a candidate to drive this process. Transgenic mice in which the keratinocyte growth factor gene is driven by an insulin promoter consistently develop pancreatic hepatocytes. Thus, the existence of pancreatic liver precursors has been shown in several mammalian species and under multiple experimental conditions. There is also evidence for the reverse: that is, the existence of pancreatic precursors in the liver. Some cases of liver tumors, particularly cholangiocarcinomas, display expression of markers typical for pancreatic cell types. In one small series of analyzed liver tumors, 61% of cholangiocarcinomas expressed pancreatic-type amylase. Other tumors have been reported to produce lipase, another enzyme specific for pancreatic exocrine cells. Similarly, cultured rat oval cell lines derived from the liver could be differentiated *in vitro* into cells with regulated insulin secretion (i.e., properties similar to those of pancreatic β-cells). These cells were even able to rescue diabetic rats in transplantation experiments. Others have demonstrated the emergence of insulin-secreting cells *in vivo* in the livers of animals treated with vectors expressing developmental pancreatic transcription factors.

Therefore, we can hypothesize that both adult liver and adult pancreas continue to harbor a small population of primitive hepatopancreatic stem cells with the potential to produce the same differentiated progeny as during embryogenesis.

Transplantation experiments have been conducted to verify the pancreatic liver stem cell hypothesis. As mentioned previously, pancreatic oval cells induced by copper depletion were shown to generate morphologically normal hepatocytes *in vivo*. In addition, the Fah knockout model has been used

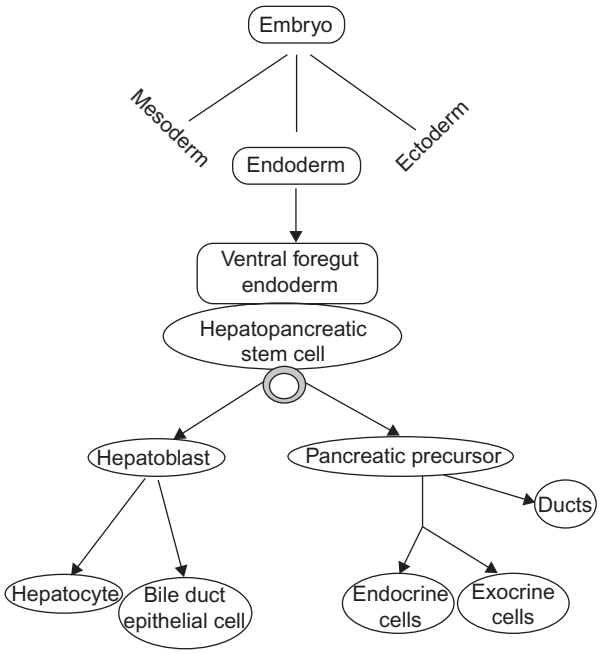

FIGURE 23.2 **Hypothetical embryonic lineage relationships between liver and pancreas.** The differentiation sequence from the totipotent endoderm to the liver and pancreatic lineages is depicted. The ventral foregut endoderm cell that produces both pancreas and liver during development may persist as a hepatopancreatic stem cell in adult life.

to demonstrate the existence of hepatocyte progenitors in adult pancreas. The pancreata of wild-type *Fah* mice were digested into single cells and then transplanted into *Fah*-mutant mice. Importantly, the cells used in these experiments were from normal adult pancreata, and no chemical treatments or oval cell induction regimens were used in the donor mice. Although only ~10% of pancreas cell-transplanted Fah-mutant mice survived long term and had extensive liver repopulation, > 50% of recipients had donor-derived Fah^+ hepatocyte nodules. Based on the number of hepatocyte nodules formed, it could be estimated that pancreatic liver precursors were rare (~1/5,000) in the donor cell population. Additional experiments showed that the pancreatic ducts did not generate hepatocytes in the liver repopulation assay. These findings are not inconsistent with the results obtained with the copper depletion model, because periductular, interstitial cells as well as the ducts themselves were considered candidates to be oval cell precursors.

A model for the lineage relationships between hepatic and pancreatic cells is shown in Figure 23.2. This model predicts the existence of an adult hepatopancreatic stem cell similar to the ventral foregut endodermal precursor that exists during embryonic development.

23.2.4.2d Liver Repopulation With Bone-Marrow-Derived Progenitors

The adult bone marrow of mammals contains cells with a variety of differentiation capacities. The HSC generating all blood cell lineages has been known for many years to reside in this compartment. However, bone marrow also contains mesenchymal stem cells capable of differentiating into chondrocytes, osteoblasts, and other connective tissue cell types. HSCs are nonadherent; mesenchymal stem cells adhere to plastic dishes in tissue culture and can be expanded there. A population of primitive, nonadherent cells characterized by their ability to pump the DNA-staining dye Hoechst can generate both muscle cells and hematopoiesis. Thus, adult bone marrow had been shown to produce a variety of tissue types of mesodermal origin. Bone marrow also contains epithelial precursors. Bone marrow and whole liver transplantation in genetically distinct animals were used to demonstrate that a portion of liver oval cells were donor derived after induction with AAF. Bone-marrow-derived (BM-derived) hepatocytes also exist in the mouse and oval cell induction is not required for this phenomenon. More recently, several reports demonstrated that donor-derived epithelial cells are also in human patients that have undergone a gender-mismatched bone marrow transplantation. In all these studies, the epithelial nature of the cells was demonstrated morphologically and by the expression of hepatocyte-specific markers.

23.2.4.2d.i HSCs as Hepatocyte Precursors Cell sorting experiments and the transplantation of purified HSCs of the c-kit^{high}Thy^{lo}Lin^{neg}Sca-1^{+} (KTLS) phenotype and at limiting dilution showed that the primitive HSC was the bone marrow cell responsible for the donor-derived hepatocytes in *Fah* knockout mice. This study also demonstrated extensive liver repopulation and a complete correction of the metabolic liver disease hereditary tyrosinemia, thus demonstrating conclusively that BM-derived hepatocytes function normally. The metabolic correction involved hepatocyte-specific parameters such as plasma amino acid levels, bilirubin conjugation, and excretion and serum transaminase levels.

In single-cell transplants, a single HSC not only could reconstitute the hematopoietic system of recipient mice but also could contribute to multiple tissues, including liver epithelium. Interestingly, in this experiment, donor cell markers were found only in rare hepatic ducts, not hepatocytes. It should also be noted that the bone marrow stem cell type used was not isolated using the KTLS phenotype; instead, it used a very different protocol. Therefore, it is not known whether the single-cell reconstitution with a KTLS stem cell would produce similar results.

It has been suggested that human HSCs, particularly those isolated from cord blood, can also produce hepatocyte-like cells in a xenotransplantation setting. Human CD34^{+} cord blood cells were engrafted into nonobese diabetic

severe combined immunodeficiency (NOD-SCID) mice after low dose irradiation using the standard procedure. If such animals were then exposed to liver injury – particularly carbon tetrachloride – human cells that produce albumin, a hepatocyte marker, can be found at low frequency (<1/1,000). This finding suggests that human HSCs may also be able to produce hepatocytes, especially after injury. This finding has been reported independently by multiple groups. It has been suggested that only a subpopulation of HSCs positive for the receptor for the complement molecule C1q [C1qR(p)] is capable of generating hepatocytes. However, because of the rarity of the human albumin-producing cells, it is unknown whether these cord-blood-derived cells express only some hepatocyte markers or whether they are true hepatocytes with all requisite functions.

23.2.4.2d.ii Multipotent Adult Progenitor Cells as Hepatocyte Progenitors Despite the evidence that KTLS HSCs are the primary cell type responsible for the emergence of BM-derived liver epithelial cells *in vivo*, they are not the only BM-derived cells that can differentiate toward the hepatocytic lineage. Multipotent adult progenitor cells (MAPCs) are a unique population of adult stem cells that can be isolated from the marrow of multiple mammalian species, including human, rat, and mouse. MAPCs are generated in culture by plating nonhematopoietic adherent cells and serial passaging. They are telomerase positive and grow stably in culture for many passages if kept at low density. These cells have properties similar to embryonic stem cells in that they can be differentiated toward many lineages *in vitro* under the appropriate conditions. A protocol was developed that permitted the efficient induction of hepatocyte gene expression in human and murine MAPCs. These conditions included culture on Matrigel and the use of fibroblast growth factor 4 (FGF-4) and HGF. Over two weeks, most MAPCs changed their phenotype and expressed multiple hepatocyte functions, including urea synthesis, albumin secretion, and phenobarbital-inducible cytochrome p450 induction.

MAPCs have also been transplanted *in vivo*. First, they were injected into blastocysts in a procedure similar to that done with embryonic stem cells. Embryos generated in this fashion had quantitative MAPC contribution to many tissues, including liver. When transplanted into adult mice, MAPC-derived cells were also found in liver and appeared to be hepatocytes. Although these MAPC-derived liver epithelial cells expressed hepatocyte markers, their ability to functionally correct liver disease has not yet been tested.

23.2.4.2d.iii Physiologic Significance of BM-Derived Hepatocytes Despite the unambiguous finding that fully functional BM-derived hepatocytes exist, there has been considerable controversy regarding their functional

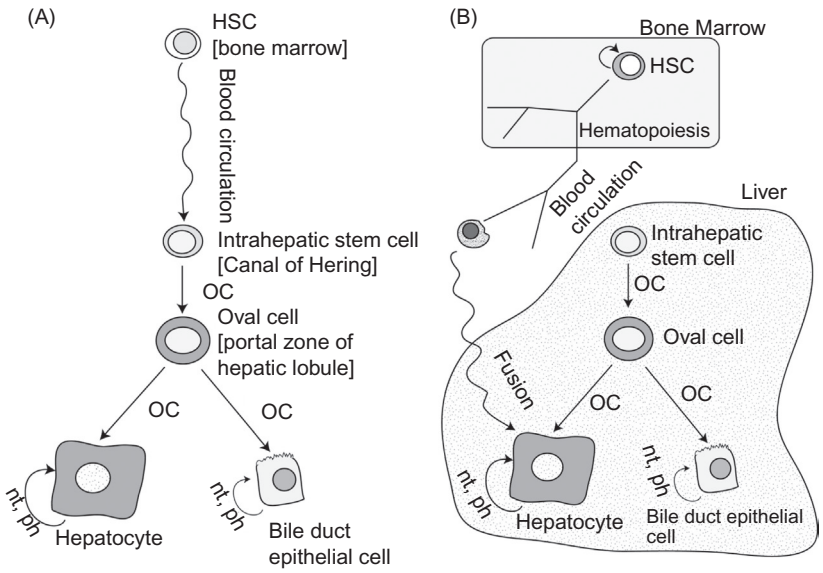

FIGURE 23.3 Models for the role of bone marrow resident stem cells in liver cell maintenance.

The different kinds of liver regeneration (see text) are indicated. (A) Transdifferentiation model. The anatomic location of the cells is given in square brackets. HSCs generate hepatocytes by trafficking to the liver through the blood circulation to then engraft and differentiate into epithelial cell precursors (intrahepatic stem cells). These cells can become activated during progenitor-dependent liver regeneration (oval cell response) and produce oval cells. Oval cells differentiate into hepatocytes or bile ducts. (B) Fusion model. There is no direct lineage relationship between HSCs and liver epithelial cells. Cell fusion among circulating progeny of the HSCs and hepatocytes are responsible for the emergence of BM-derived liver epithelial cells. Progenitor-dependent liver regeneration (oc) occurs by activation of intrahepatic liver stem cells, which are not derived from the bone marrow (nt: normal tissue turnover, ph: regeneration after partial hepatectomy, and oc: progenitor-dependent regeneration or oval cell response).

importance in liver injury. In one model, illustrated in Figure 23.3A, replenishment of liver cells from the bone marrow is an important injury response pathway, particularly in progenitor-dependent regeneration (oval cell response). A hepatocyte would be generated either by direct differentiation of an HSC or by indirect differentiation through an oval cell intermediate. The opposing model, illustrated in Figure. 23.3B, suggests that oval cell precursors reside only in the liver (tissue resident stem cell) and that the bone marrow contributes few liver epithelial cells, even during injury. To determine whether liver damage enhances the transition of HSCs to hepatocytes, the degree of cell replacement after a bone marrow transplant in mice has been measured not only in healthy animals but also in the context of preexisting hepatocyte injury or oval cell regeneration. Importantly, the frequency

of BM-derived hepatocytes was not higher in the acute liver injury seen in Fah knockout mice than in healthy control livers. In addition, no contribution from bone marrow precursors to the oval cell reaction induced by the chemical DDC was detected. Together, these results indicate that HSCs do not serve as epithelial cell precursors in all forms of hepatic injury. It remains to be determined whether HSCs play a significant role in any clinically relevant hepatic injury models.

23.2.4.2d.iv Mechanism for the Formation of Bone Marrow Stem-Cell-Derived Hepatocytes Three basic mechanisms for the repopulation of liver by BM-derived cells can be considered. First, bone marrow could theoretically harbor a specialized endodermal stem cell capable of producing hepatocytes and other epithelial cells. This cell would be analogous to the mesenchymal stem cell, which produces mesodermal derivatives such as muscle, cartilage, and fat. Second, hepatocytes and blood cells could be derived from the same stem cell by hierarchical differentiation (Figure 23.3A). Until recently, this has been the hypothesis favored by many in the field. Third, BM-derived hepatocytes could potentially be derived not from differentiation but from cell fusion (Figure 23.3B). This possibility was raised by the observation that hematopoietic cells could spontaneously fuse with embryonic stem cells *in vitro* and then produce multiple tissues in chimeric mouse embryos.

To address these hypotheses, transplantation experiments were designed in which genetic markers for both donor marrow and host hepatocytes were used in the *Fah* knockout model. The results from this study were unambiguous: Most BM-derived hepatocytes contained genetic information from both the donor and the host, indicating cell fusion. Cytogenetic analysis of female ∅ male gender-mismatched transplants indicated a high frequency of tetraploid XXXY and hexaploid XXXXYY karyotypes, as predicted for fusion. Importantly, similar experiments were independently performed by another laboratory and confirmed that fusion was the predominant mechanism for the derivation of hepatocytes from bone marrow stem cells in the *Fah* knockout model.

It remains to be seen whether functional hepatocytes can derive from bone marrow progenitors by a fusion-independent mechanism (i.e., proper differentiation). A study using xenotransplantation of human cord blood cells into NOD-SCID mice suggests that the observed cells producing human albumin did not derive by fusion.

23.2.5 Cells that Produce Hepatocyte and Bile Duct Epithelial Phenotypes *In Vitro*

Several *in vitro* models for hepatic stem cell growth and differentiation have been developed. Two general approaches to the *in vitro* study of liver stem cells can be distinguished. First, cell sorting can be used to isolate putative

liver stem cells by cell surface markers. These cells can then be cultured, and their growth and differentiation potential can be determined *in vitro*. Second, immortal cell lines can be derived from liver tissue by extensive *in vitro* manipulation and growth. To date, little work has been done on the prospective isolation of hepatic progenitors by cell sorting. In contrast, putative liver progenitor cell lines from several mammalian species have been isolated and propagated in tissue culture, including mouse, rat, pig, and human. These *in vitro* systems have scientific and potential medical uses. The medical purpose is to generate large numbers of hepatocytes *in vitro* for therapeutic transplantation. The scientific aims are to understand the factors that control the differentiation of these cells into hepatocytes and biliary duct epithelium. A common theme, applying to sorted primary cells as well as immortal progenitor cell lines, is that these cells can express markers typical for both pancreatic and hepatic cell fates.

In addition to the creation of hepatocyte progenitor cell lines, several investigators have devised strategies to conditionally immortalize differentiated hepatocytes for *in vitro* expansion and subsequent transplantation. To date, the only cell lines that have had documented therapeutic effects in animal models have been these cell lines or primary cultures derived from hepatocytes.

23.2.5.1 Prospective Isolation of Hepatocyte Progenitors by Cell Sorting

Fetal mouse liver cells were fractionated by FACS and analyzed for their proliferative as well as differentiation capacity. A multipotent population of cells was identified in E13.5 mouse liver. These cells were characterized as c-kit −, CD45 −, c-met +, Ter119 −, and CD49f + /low. *In vitro*, they proliferated extensively and could be differentiated into either hepatocytic or biliary lineages. However, the same cells could also express multiple pancreatic markers when cultured long term or transplanted into a pancreatic environment *in vivo*. The genes expressed *in vitro* included endocrine markers (preproinsulin and preproglucagon), pancreas-specific transcription factors (pdx-1 and pax6), and exocrine markers (amylase-2 and lipase). The same group has recently used a similar sorting approach in neonatal mouse pancreas (Suzuki et al., 2002b). The c-kit −, CD45 −, c-met +, Ter119 −, and CD49f + /low cells from the pancreas were able to be differentiated into hepatocytes *in vitro*.

23.2.5.1a Rat Cell Lines

23.2.5.1a.i WB-344 Cell Line This cell line was clonogenically derived from nonparenchymal rat liver cells and is probably the most intensely studied liver stem cell line. WB-344 cells are likely derived from canal of Hering cells. Although WB-344 cells can be cultured indefinitely *in vitro*, they retain

the ability to differentiate into morphologically normal hepatocytes after transplantation without forming tumors. To date, WB-344 cells are the only cells to fulfill this stringent criterion to represent a true liver stem cell line. Nonetheless, little is known about the molecular mechanisms regulating the stem cell-hepatocyte transition, and liver repopulation with this cell line has not yet been reported.

23.2.5.1a.ii Rat Oval Cell Lines Multiple laboratories have isolated oval cell lines from carcinogen-treated rats. Consistent with the proposed role of oval cells in the formation of hepatocarcinoma, these cell lines form tumors upon transplantation into immunodeficient recipients. The isolation, culture, and transplantation of these cells have been well reviewed.

23.2.5.1b Mouse Cell Lines

Although it is normally difficult to establish permanent cell lines from mouse liver, such lines can be established routinely from transgenic mice that overexpress a constitutively active form of the HGF receptor c-met. Two morphologically distinct types of cells emerge from such cultures. Both grow extensively in culture under certain media conditions but can be induced to differentiate with the appropriate signals. Clonal cell lines with the 'epithelial' morphology resemble hepatocytes and produce only hepatocyte-like offspring. In contrast, 'palmate' clones can produce two distinct lineages depending on the differentiation conditions used. Some general conditions that can cause the differentiation of palmate cells in either direction have been discovered. Acidic FGF or dimethyl sulfoxide (DMSO) induce hepatocytic differentiation, whereas culture in Matrigel induced the formation of bile duct-like structures. *In vivo* transplantation and differentiation of these cells have been reported.

HBC-3 cells are a novel bipotential cell line derived from day E9.5 mouse embryonic liver. This clonal cell line can be induced to differentiate into hepatocytes by DMSO or sodium butyrate.

23.2.5.1c PIG Cell Lines

An interesting cell line was established from cultured pig epiblast. PICM-19 cells are bipotential, similar to the murine cell lines reported previously. In contrast to other cell lines, however, PICM-19 cells were derived from a very early embryo prior to the formation of a liver premordium.

23.2.5.1d Human Cell Lines

Only a single human cell, AKN-1, that may have liver progenitor characteristics has been described.

23.2.5.1e Pancreas-Derived Cell Lines

Several laboratories have produced cell lines from the adult pancreas and used them in transdifferentiation studies. In one study, permanent cell lines with epithelial characteristics were established from both liver and pancreas under similar culture conditions. Extensive phenotypic characterization was performed; under the conditions of analysis, none of the cell lines expressed differentiated markers of either pancreas or liver. However, their morphological characteristics and expression profiles for aldolase, lactate dehydrogenase, and cytokeratins were indistinguishable, suggesting that primitive epithelial cells from both organs were similar.

Others established permanent 'duct' epithelial cell lines from the rat that expressed CK8, CK19, and carbonic anhydrase. These cells were then implanted in a collagen matrix into a normal rat – subcutaneously or into the peritoneal cavity. Upon transplantation, several hepatocyte-specific markers were expressed and pancreatic duct markers were silenced. This was particularly true for those cells transplanted into the mesenteric fat pad. The expression levels of albumin, transferrin, and aldolase B approached those seen in normal adult hepatocytes. Again, these data are consistent with the hypothesis of a common endodermal hepatopancreatic stem cell.

FOR FURTHER STUDY

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CHAPTER 24

Pancreatic Stem Cells

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24.1 INTRODUCTION

From a clinical perspective, the pancreas is an important focus of stem cell research, because it is an attractive target for cell replacement therapy. In type I diabetes, the insulin-producing β-cells that reside in the pancreatic islets of Langerhans are destroyed by autoimmune attack, and it is thought that self-renewing stem cells could provide an unlimited source of β-cells for transplantation. Such therapeutic efforts require the prospective isolation of stem cells with the potential to produce β-cells and the development of methods to direct their expansion and differentiation.

From a developmental biology perspective, the role of stem cells in the pancreas is a fascinating problem. New cells are produced during adulthood, but their origin is not clear. Much of the field is focused on the identification of β-cell progenitors and the characterization of their molecular requirements, but it is not known what role such cells play during pancreas maintenance and regeneration, or whether the adult pancreas contains a population of true stem cells. Regardless, information about the specification of the β-cell fate from undifferentiated progenitors will be important in directing the differentiation of stem cells *in vitro*. In this chapter, we review evidence for the existence and identity of pancreatic progenitor and stem cells and describe the criteria for experimental demonstration of such cells.

24.2 DEFINITION OF STEM CELLS AND OF PROGENITOR CELLS

The most rigorous definition of a stem cell is a cell that, upon proliferation, produces some progeny that have the same developmental potential

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(a process called self-renewal), as well as other progeny that have a more restricted developmental potential. Such cells may be present transiently during embryonic development, or persistently during the entire life of the organism, but their defining property is self-renewal. A progenitor cell, on the other hand, is any cell that generates another differentiated cell type.

Another important notion in stem cell biology, with potential relevance to the pancreas, is that of the facultative stem cell. These cells are thought to be functional, differentiated cells in an adult organ that can dedifferentiate in response to a specific signal (usually believed to be tissue damage), and then differentiate into another cell type. For example, the liver is thought to contain facultative stem cells that reside in the bile ducts and are capable of producing hepatocytes. Thus, the bile duct cells provide an effective reservoir of liver progenitors in case of tissue damage, although they may not meet the criteria of being stem cells at the single cell level.

How can we demonstrate that a particular cell is a stem cell? Formally, such a demonstration must be based on clonal analysis: a single cell has to be followed over time in order to show that it can produce more stem cells, as well as differentiated cells. The gold standard for the identification of stem cells was set in the hematopoietic system, where clonal analysis has been carried out *in vitro* (by subcloning individual colonies), as well as *in vivo* (by serial transplantation of single stem cells into lethally irradiated mice). By contrast, the identification of progenitor cells is an easier task. It requires demonstration, using some sort of lineage tracing experiment, that an undifferentiated cell population generates differentiated cells.

Even when stem cells cannot be identified or isolated in a particular organ, their existence may be inferred from kinetic studies of 5′-bromo-2′-deoxyuridine (BrdU) incorporation. Because stem cells are believed to be slowly dividing, the presence of label-retaining cells can point to the anatomical location of a stem cell niche. Such an analysis was carried out in self-renewing organ systems, such as skin and hair. In light of these definitions, in the next sections we discuss the evidence for stem cells and progenitors during pancreas development and adult life.

24.3 PROGENITOR CELLS DURING EMBRYONIC DEVELOPMENT OF THE PANCREAS

The adult pancreas contains three major cell types: exocrine cells, organized in acini, which secrete digestive enzymes; duct epithelial cells which flush these enzymes to the duodenum; and endocrine cells, organized in the islets of Langerhans, that secrete hormones to the blood. The islets, accounting for ~1% of the cells in the adult pancreas, contain four major cell types that

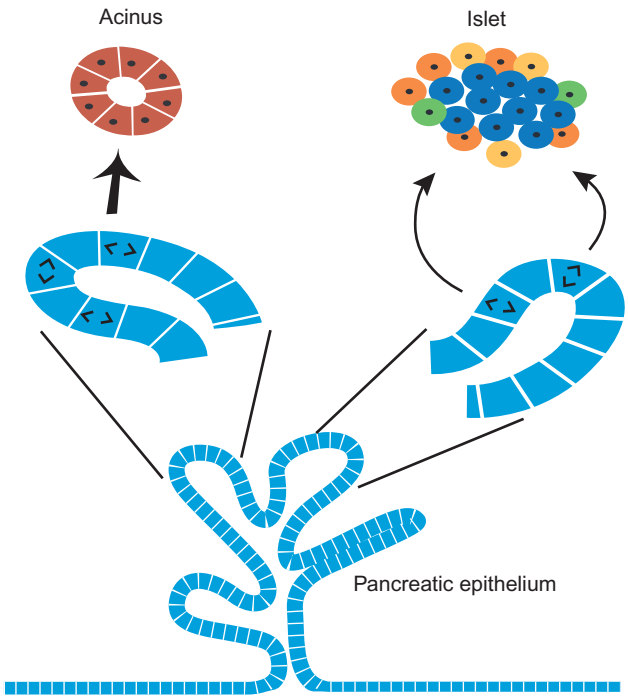

FIGURE 24.1 **Embryonic development of the pancreas.**
Pdx1$^+$ epithelial cells of the early pancreas (pale blue); acinar cells (red); and α-, β-, δ-, and pp-cells (indicated by orange, blue, yellow, and green in the islet). The proposed plane of division of endocrine and exocrine progenitors is indicated.

secrete different hormones: α-, β-, δ-, and pp-cells, secreting glucagon, insulin, somatostatin, and pancreatic polypeptide, respectively. In recent years, a detailed cellular and molecular understanding of pancreas development has emerged (Figure 24.1). Although many questions remain, one recurring theme is the essential role played by progenitor cells in organogenesis. Early patterning of the gut tube generates a sheet of epithelial cells that express the homeobox gene *pdx1* around embryonic day 9 (E9) in the mouse. These epithelial cells bud from the gut tube, proliferate, and branch to form a tubular structure around E12.5, from which cells differentiate and organize into the exocrine and endocrine tissues of the mature pancreas. Numerous experiments demonstrate that this process is based on the proliferation and stepwise differentiation of progenitor cells. As shown by lineage tracing experiments using tamoxifen-induced Cre recombinase, the early *pdx1*$^+$ cells (which by E10.5 also express the transcription factor p48/ptf1) produce all cell types in the adult pancreas. Later on, progenitors for duct, acinar, and endocrine lineages segregate, proliferate, and differentiate. Endocrine progenitors migrate from the tubular structure of the early pancreas, and coalesce

to form the islets of Langerhans just before birth. During that period, they undergo further restrictions in their potential, marked by the transient expression of several genes. Most notably, neurogenin3 expression marks progenitor cells for all endocrine lineages. Downregulation of progenitor markers and expression of the hormone genes mark the terminal differentiation of endocrine cells. A similar mechanism of gradual commitment, though less well defined, is thought to act in the developing exocrine pancreas.

How do pancreatic progenitors choose their fates? A longstanding hypothesis is that the axis of mitosis in epithelial cells of the early pancreas correlates with, or even determines, the identity of daughter cells (Figure 24.1). When the plane of division is perpendicular to the lumen, both daughter cells remain epithelial (symmetric division), and may lobulate to form exocrine acini. When the plane of division is parallel to the lumen, one daughter cell detaches from the lumen, and may become an endocrine progenitor cell (asymmetric division). This hypothesis, although untested, supports the popular view that adult pancreatic stem cells reside in the ducts. More recently, genetic evidence has suggested that cell-cell interactions mediated by the Notch pathway affect lateral specification of progenitor cells at multiple developmental junctions. Accordingly, mutations in Notch pathway genes can lead to accelerated, premature endocrine differentiation at the expense of the exocrine pancreas. These two models for specification may be compatible, as the Notch pathway is known to affect the plane of division in neural progenitors.

What is the relative contribution of progenitors and differentiated cells to proliferation of the embryonic pancreas? Although this question has not been addressed directly, the fate of hormone-expressing cells that appear early in pancreas development (E9.5–10.5) has been examined. These cells, many of which coexpress multiple endocrine hormones, were initially thought to replicate and generate the mature islets. However, lineage tracing and ablation experiments suggest that the multiple-hormone-expressing cells do not contribute to the adult endocrine pancreas. Rather, mature endocrine cells appear to be derived from midgestation progenitors.

Following this progenitor-based formative stage, rapid growth of the pancreas in late gestation and early postnatal life is thought to involve gradually less differentiation of progenitor cells, and more replication of fully differentiated cells (see later sections of this chapter).

Although the identity and importance of progenitors during pancreas development is clear, there is no indication for true self-renewal of cells during this period. The recent demonstration of heterogeneity among the pdxl$^+$ cells of the early pancreas suggests that lineage segregation occurs very early during pancreas organogenesis. Furthermore, no clonal analysis of the embryonic pancreas has been carried out *in vivo*. *In vitro* clonal analysis demonstrated

the common origin of the exocrine and endocrine pancreas, but not self-renewal. Therefore, it is fair to conclude that progenitor cells play a major role in pancreas formation. However, there is no evidence for self-renewing pancreatic stem cells during embryonic development. We now turn to the postnatal growth and maintenance of the pancreas, with special attention to β-cells. Do progenitor cells of the embryonic type persist in adult life in an active or latent form? Is there evidence for stem cells capable of generating new pancreatic cells in the adult?

24.4 PROGENITOR CELLS IN THE ADULT PANCREAS

When describing stem/progenitor cells in the adult pancreas, it is important to deal separately with two questions: first, what is the turnover rate of the different components of the pancreas during postnatal growth, throughout adult maintenance, and in response to injury? Second, are the new cells derived from stem/progenitor cells (neogenesis), or from replicating differentiated cells? It must be remembered that an impressive capacity for homeostatic maintenance, or even regeneration, does not indicate neogenesis. For example, dramatic regeneration of the injured liver can occur solely by proliferation of differentiated hepatocytes, without a requirement for stem cells.

24.4.1 Evidence from Cell Dynamics

After birth, the growth of the pancreas continues, but slows significantly around one month of age in the mouse and rat. However, even in old animals there is a measurable rate of cell birth in all pancreatic compartments. In the β-cell compartment, where most studies have been done, the replication rate falls from ~5% in four-week-old animals to ~0.1% in mice older than three months. Embryonic-type progenitors (based on expression patterns) are not seen in the normal adult animal (with the possible exception of rare islet cells generated from neurogenin3^{+} progenitors).

In spite of its low basal turnover rate, significant hyperplasia is seen in the adult pancreas under certain physiological and pathological conditions. For example, during pregnancy the β-cell mass doubles, a response attributed to a combination of cell hypertrophy and cell proliferation. More dramatically, several reports have documented an ability of the β-cell compartment to recover from genetically programmed, autoimmune, surgical, or chemical damage.

Can the new cells, in normal homeostasis or in a regeneration setting, be fully accounted for by the replication of differentiated cells? Empirically, for the β-cell compartment, can the number of BrdU+ pulse-labeled β-cells explain the accumulation of BrdU+ β-cells following continuous

administration of BrdU? If not, the presence of undifferentiated progenitors must be invoked. This type of analysis has, however, proven difficult because it requires reliable values for several elusive parameters: what is the duration of the S phase and the total cell cycle in a specific compartment? What is the death rate, and how long does it take for a dying cell to be cleared? Furthermore, cell number is not easily inferred from total cell area as obtained by immunostaining, because of cellular hypertrophy. Thus, a study of β-cell dynamics must include β-cell counting by fluorescence-activated cell sorting or by careful histological analysis across the whole pancreas, a criterion that is not always met.

The most comprehensive effort in this direction was carried out in studies of β-cell dynamics throughout the lifespan of the rat. A significant contribution of progenitor cells to the β-cell mass was observed in the first weeks after birth, followed by a shift to tissue maintenance and slower replication of β-cells. In addition, significant β-cell neogenesis was deduced in a similar study of chronic hyperglycemia in rats. These kinetic studies suggest that adult pancreatic progenitors exist. However, they do not help determine the molecular and anatomical origins of these cells.

Another argument for the existence of β-cell progenitors is the identification of single β-cells embedded in the adult exocrine tissue. These isolated cells are reportedly more frequent following insults, which led to the notion that they are newly generated from progenitors that reside in ducts or acini (see later sections of this chapter). The new β-cells are then believed to coalesce into islets in a mechanism resembling developmental islet morphogenesis. However, careful analysis is required to distinguish this interpretation from other potential explanations (e.g., disintegration of existing islets). In fact, genetic lineage tracing experiments suggest that the small clusters of β-cells are derived from β-cells, rather than from stem/progenitor cells.

A combination of BrdU pulse-chase experiments and genetic labeling of differentiated cells (for example, using an inducible Cre recombinase) may allow a direct comparison between the contribution of progenitors and the contribution of differentiated cells to pancreas growth and maintenance.

Many experiments neglect the kinetic aspects of neogenesis, and focus on histological identification of progenitors, based on expression markers in certain anatomical locations. However, without lineage tracing, these studies cannot demonstrate the fate of the putative progenitors, or determine their importance. In the next sections, we describe the most notable proposals regarding the identity of adult pancreatic stem-progenitor cells.

24.4.1.1 Ducts

It is widely believed that adult pancreatic stem-progenitor cells are located in the duct epithelium. Indeed, cells expressing β-cell markers (insulin, glut-2,

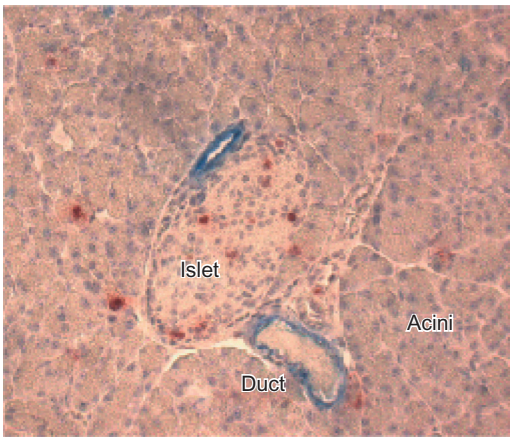

FIGURE 24.2 **Histology of the adult pancreas.**
Exocrine tissue (acini and ducts) and endocrine tissue (islet of Langerhans) are shown. Section from a four-week-old mouse pancreas, pulse labeled with BrdU (red). Ducts are stained blue. Note the close association between the ducts and the islet. Original magnification = 200×.

pax6, isl1, and HNF3b) can often be found embedded in or adjacent to adult ducts following an insult to the pancreas.

Moreover, duct cell replication (as assessed by BrdU incorporation) is increased after such insults. In addition, adult duct preparations are claimed to be capable of endocrine differentiation *in vitro* (see later sections of this chapter). Conceptually, the appearance of endocrine cells in or near ducts is interpreted as recapitulation of embryonic pancreas morphogenesis, where endocrine progenitors bud from the epithelium. However, adult ducts are not necessarily identical in their genetic program to the embryonic tubes that should be referred to as 'duct-like structures.' Indeed, lineage experiments indicate that the definitive ductal and endocrine lineages are separated as early as E12.5. With regard to endocrine cells seen budding from ducts, caution is required when suggesting dynamic interpretations to static histological snapshots (Figures 24.2 and 24.3).

The preceding description shows the importance of specific duct markers for the assessment of the ductal origins of endocrine cells. Although several duct markers have been found (such as carbonic anhydrase, cystic fibrosis transmembrane-conductance regulator, and cytokeratin-19), so far none has been translated to a useful lineage marker in transgenic mice.

In summary, it seems that ducts can elicit a proliferative response to tissue damage, and that endocrine markers are occasionally expressed in cells of the ductal epithelium. However, the fate of these cells and their relative contribution to the endocrine or exocrine pancreas has yet to be demonstrated.

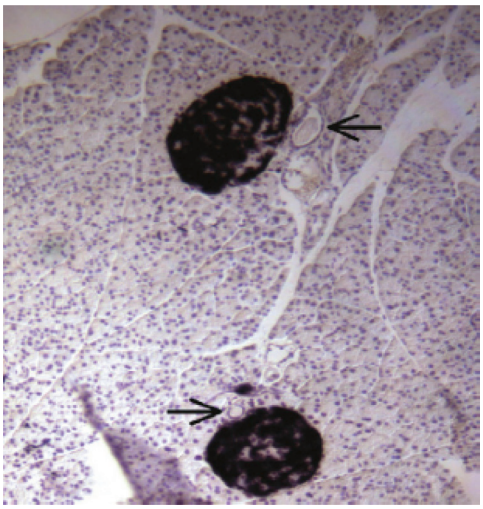

FIGURE 24.3 **Lineage labeling of the endocrine pancreas.**
Section from the pancreas of a four-week-old ngn3-cre; Z/AP double-transgenic mouse, stained for alkaline phosphatase activity (dark blue). In this mouse, cre-mediated recombination in *ngn3*$^{+}$ embryonic endocrine progenitor cells leads to heritable expression of the human placental alkaline phosphatase gene. Only islet cells are labeled, suggesting that ducts (arrows) and acini never expressed the *ngn3* gene. Original magnification = 100×.

24.4.2 Acini

Starting from the observation of isolated β-cells embedded in exocrine tissue, other investigators have proposed that acinar cells can transdifferentiate, with or without replication, into endocrine cells. This possibility is supported by *in vitro* studies showing that an acinar cell line can adopt endocrine features under certain conditions. However, as in the case of duct-embedded progenitors, the lineage of these cells was not followed *in vivo*; therefore, their origin and fate could not be determined. In this case, however, good lineage markers exist, so the possible acinar origin of endocrine cells can be directly tested. Preliminary experiments with an Elastase promoter-driven Cre recombinase have suggested that, under normal conditions, Elastase-expressing cells do not produce endocrine or duct cells. It remains to be tested, however, whether acinar cells contain 'facultative' endocrine progenitors activated upon injury.

24.4.2.1 Intra-islet Progenitors

Several groups have suggested the existence of intra-islet progenitor cells capable of proliferation and differentiation into β-cells. These reports are based on the expression of putative stem-progenitor cell markers in islets. One such proposal is that the expression of Nestin, a marker of neuronal progenitors, labels intra-islet endocrine progenitors with a potential for *in vitro*

differentiation into several fates. However, a recent lineage analysis using Cre recombinase driven by the Nestin promoter showed that pancreatic endocrine cells do not form by the differentiation of Nestin$^+$ cells. In addition, Nestin was recently shown to be expressed in the mesenchymal, but not in epithelial, cells of the embryonic pancreas, further questioning its relevance for pancreatic lineages.

Others have documented the coexpression of pancreatic hormones in islets following a diabetogenic insult. For example, It was proposed that, following streptozotocin treatment, somatostatin+ cells and glucagon+ cells generate β-cells by proliferation and differentiation through somatostatin$^+$pdxl$^+$, and glucagon$^+$/glut-2$^+$intermediate cell types. These proposals have yet to be confirmed by lineage analysis.

Finally, clinical transplantation of islets may provide important clues about islet cell dynamics. Islet grafts into the portal vein (as done with diabetic patients), or under the kidney capsule (as done routinely with rodents), survive for many months and provide glycemic control. Although it is clear that cells in the grafts die and proliferate, it is not known if new endocrine cells in the graft are derived only from replicating differentiated cells or also from progenitors. Since purified islets with minimal exocrine tissue are used for transplantations, any indication for the existence of progenitors in the graft would point to an intra-islet source.

24.4.2.2 Bone Marrow

Bone-marrow-derived cells have been found to contribute to the β-cell compartment in the absence of tissue damage other than irradiation. To facilitate the detection of marrow-derived β-cells, marrow was taken from transgenic mice expressing Cre recombinase under the insulin promoter, as well as enhanced green fluorescent protein (eGFP), preceded by a Cre-removable transcriptional stop signal. Thus, bone marrow cells of the donor mice should express eGFP only if they turn on the insulin promoter. By sorting eGFP$^+$ cells in pancreatic islets of recipient mice, up to 3% of all β-cells were identified as being of donor origin two months after marrow transplantation. The elegant design of the experiment was such that the fusion of marrow cells with recipient β-cells could be ruled out. These surprising results still await independent confirmation.

24.4.3 Other Evidence for Adult Pancreatic Progenitor Cells

It has been reported that cells in the adult pancreas can reconstitute a degenerating liver deficient in the enzyme fumarylacetoacetate hydrolase. This observation suggested the existence of progenitors in the pancreas capable of liver differentiation. However, more recent results indicate that liver

reconstitution by bone marrow cells represents complementation of defective hepatocytes by cell fusion, rather than transdifferentiation of marrow cells to liver. Thus, it is likely that liver reconstitution by pancreatic cells represents the 'fusibility' rather than the plasticity of these cells.

In summary, indirect evidence suggests that endocrine progenitor cells exist, or can appear, in the adult pancreas following injury. Careful lineage analysis has yet to demonstrate the existence, origin, fate, and importance of these cells to pancreas homeostasis. Similar to the situation in the embryo, there is no evidence for the existence of self-renewing stem cells in the adult pancreas.

Very recently, we have used a genetic lineage analysis system to study the dynamics of adult pancreatic β-cells. This analysis shows that adult β-cells are maintained largely by self-duplication, rather than stem cell differentiation during normal life in the mouse, as well as during regeneration from pancreatectomy. The results cast doubt on the idea that stem cells contribute significantly to the formation of new β-cells during adult life. We cannot rule out the existence of adult pancreatic stem cells; however, it seems that the contribution of such putative cells to new β-cells is minimal. In addition, facultative pancreatic stem cells might exist and be recruited following other types of injury. The same system can now be used for testing claims about stem cell differentiation into β-cells, *in vitro* and vitro.

24.5 FORCING OTHER TISSUES TO ADOPT A PANCREATIC PHENOTYPE

From a therapeutic perspective, artificial induction of a β-cell fate in other cell types is as useful as finding and expanding endogenous pancreatic progenitors. Moreover, nonpancreatic cells, if indeed competent to adopt a β-cell program, may be more accessible for expansion and *in vitro* manipulation. Efforts in this direction have focused on tissues thought to be capable of pancreatic differentiation because of a common lineage origin or a similar genetic program. Here, we explain the pancreatic connection of the most popular starting tissues for such experiments.

24.5.1 Liver to Pancreas

Several observations led to the notion that the adult liver may be a close relative of the pancreas in terms of the steps required for switching fates. First, both organs are derived from the endoderm of the embryonic gut tube. Moreover, studies by Zaret and colleagues suggest that a bipotential liver-pancreas progenitor cell population exists by E8 in the ventral foregut endoderm. Using embryo tissue explantation experiments, they have shown that fibroblast growth factor signaling from the cardiac mesoderm can divert these progenitors

from a default pancreatic fate to a liver fate. However, lineage or clonal analysis was not performed to formally demonstrate that bipotential cells exist.

Second, transdifferentiation of pancreas to liver, in which clusters of hepatocytes emerge in the pancreas, occurs spontaneously in humans and can be induced experimentally in rats. In cultured pancreatic cells, liver transdifferentiation was demonstrated by activation of a single transcription factor (C/EBP-b) without a requirement for cell division. These observations suggest that adult liver and pancreas cells may retain a degree of plasticity that allows transdifferentiation to occur. Alternatively, a rare stem-progenitor cell population in these organs, yet to be identified, may be responsible for the metaplastic phenomenon.

Notably, no evidence has been found for deep similarities in the genetic program of the adult pancreas and liver. Based on these observations, several groups have tried experimentally to convert liver to pancreas by enforced expression of key pancreatic genes. One candidate for imposing a pancreatic fate is Pdxl, the most upstream component of the pancreatic program. Indeed, adenoviral-mediated transfer of Pdxl to adult mice was reported to induce low-level expression of insulin or exocrine pancreas markers in the liver.

A conceptual problem with simple overexpression of Pdxl is the need for specific transcriptional cofactors to be present. For example, Pdxl is known to require the Pbx and Meis proteins for proper function. With the hope of bypassing the need for Pdxl cofactors absent from the liver, a Pdxl-VP16 fusion gene was recently constructed. Transgenic expression of Pdxl-VP16 (but not unmodified pdxl) in livers of *Xenopus* tadpoles led to significant expression of pancreatic endocrine and exocrine genes in the liver, concomitant with downregulation of liver genes. The use of a liver-specific promoter (from the transthyretin gene), and the finding that the same construct induces a pancreatic phenotype in a hepatocyte cell line, suggests that the responding cells are differentiated hepatocytes rather than uncommitted progenitors or stem cells.

In summary, it seems that ectopic expression of pancreatic transcription factors in the embryonic and perhaps the adult liver can lead to activation of a pancreatic program. The available data suggest that the mechanism is direct hepatocyte transdifferentiation, rather than activation of a common liver-pancreas progenitor. In addition to the therapeutic potential of the approach, analysis of the molecular details may provide significant insights into the problems of competence and potential.

24.5.2 Neurons to Pancreas

Although arising from different lineages during embryogenesis, the endocrine pancreas and the nervous system share a significant portion of their

developmental programs. For example, most transcription factors participating in the stepwise differentiation of the endocrine pancreas are also expressed in the developing brain. Some examples are isl1, Hb9, neurogenin3, NeuroD, Nkx2.2, Nkx6.1, pax6, and brn4. Notable exceptions are the absence of Pdxl, as well as insulin, expression in the brain. However, the insulin promoter does contain neuronal regulatory elements that are repressed in the context of the endogenous insulin locus, but give rise to neural expression when driving transgenes. The significance of this intriguing similarity is not fully understood. One possibility is that it reflects a common ancestral lineage for brain and endocrine pancreas, which diverged during evolution as the endocrine program came under Pdxl control. This idea is supported by the finding that insulin and glucagon are expressed in specific neurons in the fly (which lacks a pancreas). Thus, neuronal progenitors are the closest known relatives of pancreatic progenitors in terms of their genetic program. This suggests that neuronal progenitors (easily derived from embryonic stem (ES) cells) may be only a few steps from adopting a pancreatic fate. However, attempts to induce ES-derived neural progenitors to a pancreatic fate have so far failed.

24.5.3 Gut to Pancreas

Several studies explored the potential of the nonpancreatic segments of the digestive tract to adopt a pancreatic fate. Ectopic expression of Pdxl in the embryonic gut outside the normal expression domain of Pdxl led to a partial adoption of a pancreatic fate, judged by gene expression and cell budding out of the epithelium. Similarly, ectopic expression of neurogenin3 in the embryonic chick or mouse led to the appearance of endocrine cells expressing glucagon and somatostatin (but not insulin). Inactivation of ptfla-p48, a transcription factor normally expressed in pancreatic progenitors, led to a cell-autonomous switch in the reverse direction, namely, the conversion of pancreas to duodenal epithelium. These results suggest that, even after the onset of segment-specific genetic programs, the embryonic gut epithelium remains plastic. It is not known if such plasticity is retained in the adult intestine.

24.6 *IN VITRO* STUDIES

Numerous attempts have been made to generate β-cells *in vitro* from dissociated pancreatic tissue using the available information on the identity of putative pancreatic stem-progenitors. Accordingly, pancreatic duct preparations were cultured, and the appearance of differentiated cell types was monitored. Some groups have reported on the generation of endocrine cells from duct cultures, and some even claimed to correct hyperglycemia in animals by grafting the *in vitro*-generated islet-like clusters. Others have used

islet preparations as a starting material. These studies, however, share several methodological problems that complicate their interpretation. First, the number of endocrine cells at the beginning and at the end of the culture period was not always measured carefully. Second, all studies were carried out on enriched rather than pure cell populations, thus failing to rule out the presence of contaminating cells from unintended compartments. Third, clonal analysis was not performed. Fourth, many of these studies used insulin immunostaining as a marker for β-cell differentiation, and supplied large amounts of exogenous insulin in the culture medium. Therefore, they may have scored for insulin uptake rather than *de novo* synthesis.

Perhaps a more promising approach to the problem is the use of embryonic pancreas as a starting material, since it is clear that it contains progenitor cells that can proliferate and differentiate. This, however, has also proved a difficult task. Much of the difficulty may result from the lack of proper culture conditions that mimic the embryonic environment.

24.7 SUMMARY

The search for pancreatic stem or progenitor cells may provide insights into the basic mechanisms of organ homeostasis, as well as a promising therapeutic approach for diabetes. It is clear that pancreatic progenitor cells exist and play a major role during embryonic development, although much has to be learned about the mechanisms and molecules controlling them. There is no evidence, however, for pancreatic stem cells capable of self-renewal.

As for the adult pancreas, no conclusive evidence shows the importance or mere existence of pancreatic progenitor or stem cells in the adult organism. On the contrary, it appears that, at least for pancreatic beta cells, the major mechanism for tissue maintenance is self-duplication rather than stem cell differentiation. It is possible, however, that under certain stresses progenitor cells appear and may contribute to the maintenance of the pancreas. Most evidence points to the pancreatic ducts as the likely niche of such 'facultative' progenitors. Development of culture conditions that support the growth and differentiation of dissociated pancreatic tissue, as well as *in vivo* lineage analysis systems, are the tools most needed for tackling this problem.

Other cell types may retain a degree of plasticity that allows for pancreatic differentiation under artificial conditions. Embryonic stem cells are the only cells available *in vitro* that can definitely differentiate into pancreatic cell types; however, this potential has yet to be demonstrated *in vivo*. Theoretical considerations and some experimental evidence suggest that neuronal progenitors, liver cells, and intestinal epithelial cells may also be forced to adopt a pancreatic fate.

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CHAPTER 25

Stem Cells in the Gastrointestinal Tract

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25.1 INTRODUCTION

There has been a tremendous increase in interest in stem cell biology and its potential applications in recent years. Although this has been galvanized by the exploitation of research in embryonic stem cells, it is interesting to note that what might be called the 'intestinal stem cell community,' albeit small, has been working productively for some 40 to 50 years. With stem cells now claiming considerable attention, in retrospect, many of the basic tenets that govern our understanding of organ-specific stem cells have come from studies in the gastrointestinal tract and the hematopoietic system.

In the gastrointestinal tract, there is a large body of evidence that multipotent stem cells are found in specific zones, or niches, within gastric glands and intestinal crypts, composed of and maintained by myofibroblasts in the adjacent lamina propria. In this chapter, we review evidence that these multipotent stem cells generate all gastrointestinal epithelial cell lineages through committed precursor cells housed in the proliferative compartments of intestinal crypts and gastric glands, a concept that has had a long and difficult gestation. Notwithstanding their obvious significance, the gastrointestinal stem cells remain elusive and unidentified, mainly because of a lack of accepted morphological and functional markers at the single-cell level. We also explore concepts of stem cell number, location, and fate, and we touch on the ability of gastrointestinal stem cells to regenerate cell lineages of whole intestinal crypts and villi after damage.

The luminal gut shows regional specializations of function – the stomach primarily for absorption and the intestinal mucosa for both absorption and secretion. This is reflected by variation in the adult cell lineages native to each tissue, and it is thereby consistent that stem cell fate within each tissue is also different. We are beginning to understand the mechanisms that

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govern such variation. Controversial findings regarding stem cell plasticity in the gastrointestinal tract are examined in this chapter. Because of their longevity, putatively the same as that of the organism itself, stem cells are often viewed as the target cells for carcinogens and the cells of origin for spontaneous tumors. Recent thought on the location of stem cells in the colon has sparked debate concerning the possible pathways of morphological progression of transformed stem cells. This includes a *top-down* proliferation of mutated stem cells located within intercryptal zones on the mucosal surface, which spread downward into the adjacent crypts. Contrasting to this is a *bottom-up* theory of the upward proliferation of mutated stem cells in the crypt base to produce dysplastic crypts that replicate and expand by crypt fission. This brings other facets of gut biology into sharp focus: the mechanisms of crypt reproduction; the clonal architecture of normal and dysplastic gastric glands, intestinal crypts, and their derivative tumors; and the role that stem cells take in these events. Here, we propose that the stem cells accumulate the multiple genetic events leading to tumorigenesis, and we explore the manner by which such mutated clones spread in gastrointestinal epithelia. Many of these concepts are being explored at the level of molecular regulatory pathways, including the signaling pathways of Wnt and transforming growth factor β (TGFβ).

25.2 GASTROINTESTINAL MUCOSA CONTAINS MULTIPLE LINEAGES

In the small intestine, the epithelial lining forms numerous crypts and larger, finger-shaped projections called villi. In the colon there are many crypts, which vary in size throughout the colon; the shortest is in the ascending colon. Overall, four main epithelial cell lineages exist in the intestinal epithelium: the columnar cells, the mucin-secreting cells, the endocrine cells, and Paneth cells in the small intestine. Other less common cell lineages are also present, such as the caveolated cells and membranous or microfold cells. Columnar cells, with apical microvilli, are the most abundant epithelial cells, termed enterocytes in the small intestine and colonocytes in the large intestine. 'Goblet' cells containing mucin granules – and thus producing swollen, goblet-shaped cells – are found throughout the colonic epithelium, secreting mucus into the intestinal lumen. Endocrine, 'neuroendocrine,' or 'enteroendocrine' cells form an abundant cell population distributed throughout the intestinal epithelium; these cells secrete peptide hormones in an endocrine or paracrine manner from their contained dense core of neurosecretory granules. Paneth cells are located almost exclusively at the crypt base of the small intestine and ascending colon, contain large apical secretory granules, and express several proteins – including lysozyme, tumor necrosis

factor, and the antibacterial cryptins (small molecular weight peptides related to defensins).

In the stomach, the epithelial lining forms long, tubular glands divided into the foveolus, isthmus, neck, and base regions. Gastric foveolar or surface mucus cells are located on the mucosal surface and in the foveola. They contain tightly packed mucous granules in the supranuclear cytoplasm and do not possess a theca. The mucus neck cells are situated within the neck and isthmus of the gastric glands and contain apical secretory mucin granules. The peptic-chief or zymogenic cells are located in the base of the glands in the fundic and body regions; they secrete pepsinogen from oval zymogenic granules. The parietal or oxyntic acid-secreting cells are located in the body of the stomach in the base of the glands. These cells have many surface infoldings, or canaliculi, which form a network reaching almost to the base of the gland. Endocrine cell families include the enterochromaffin-like cells in the fundus or body that produce histamine; the gastrin-producing cells are a major component of the antral mucosa.

The intestinal crypts and gastric glands are enclosed within a fenestrated sheath of intestinal subepithelial myofibroblasts (ISEMFs). These cells exist as a syncytium that extends throughout the lamina propria and merges with the pericytes of the blood vessels. The ISEMFs are closely applied to the intestinal epithelium and play a vital role in epithelial-mesenchymal interactions. ISEMFs secrete hepatocyte growth factor (HGF), TGFβ, and keratinocyte growth factor (KGF), but the receptors for these growth factors are located on the epithelial cells. Thus, the ISEMFs are essential for the regulation of epithelial cell differentiation through the secretion of these and possibly other growth factors. Platelet-derived growth factor-α (PDGF-A), expressed in the intestinal epithelium, acts by paracrine signaling through its mesenchymal receptor, PDGFR-α, to regulate epithelial-mesenchymal interactions during development. Typically, ISEMFs are α-smooth muscle actin-positive (αSMA +) and desmin-negative, but some myofibroblasts also express myosin-heavy chains. ISEMFs undergo proliferation despite this MyoD expression – unlike the skeletal muscle myoblast, which decycles once MyoD is expressed. It has been proposed that these cells form a renewing population, migrating upwards as they accompany the epithelial escalator. Although they appear to proliferate and migrate, they move relatively slowly and then move off into the lamina propria to become polyploid.

A second myofibroblast population in the intestine is the interstitial cells of Cajal. These cells are located close to neurons in the muscular layers; they act as pacemakers for gastrointestinal smooth muscle activity, propagate electrical events, and modulate neurotransmission. They are said to be αSMA+ and desmin+ and to immunostain for *c-kit* and CD45.

25.3 EPITHELIAL CELL LINEAGES ORIGINATE FROM A COMMON PRECURSOR CELL

Little is known of the location and fate of the stem cells within the gastrointestinal tract because of the lack of distinctive and accepted stem cells markers, although they are usually said to appear undifferentiated and can be identified operationally by their ability to repopulate crypts and glands after damage.

The unitarian hypothesis states that all the differentiated cell lineages within the gastrointestinal epithelium emanate from a common stem cell origin. Although widely propounded, until fairly recently little definitive evidence existed to underpin this hypothesis. Moreover, it has been proposed that gastrointestinal endocrine cells derive from migrating neuroendocrine stem cells in the neural crest, a concept that still has its adherents. Although studies of quail neural crest cells transplanted into chick embryos, or experiments where the neural crest is eradicated, show gut endocrine cells to be of endodermal origin, it has been suggested that the endoderm is colonized by 'neuroendocrine-programmed stem cells' from the primitive epiblast, which generate gut endocrine cells. This hypothesis was not ruled out by chick-quail chimera experiments; therefore, other models must be used to ascertain the gut endocrine cell origins, such as the chimeric mouse studies described later.

Several lines of evidence suggest that stem cells reside in the base of the crypts of Lieberkuhn in the small intestine, just superior to the Paneth cells (approximately the fourth or fifth cell position in mice). In the large intestine, they are presumed to be located in the midcrypt of the ascending colon and in the crypt base of the descending colon. However, within the gastric glands, migration of cells is bidirectional from the neck-isthmus region to form the simple mucous epithelium of the foveolus or pit, and cells migrate downward to form parietal cells and chief cells. Therefore, the stem cells are believed to be within the neck-isthmus region of the gastric gland. The unitarian hypothesis is now supported by a considerable body of research.

25.4 SINGLE INTESTINAL STEM CELLS REGENERATE WHOLE CRYPTS CONTAINING ALL EPITHELIAL LINEAGES

The ability of intestinal stem cells to regenerate epithelial cell populations of entire intestinal crypts and villi following cytotoxic treatment has been demonstrated using the crypt microcolony assay. Four days after irradiation, sterilized crypts undergo apoptosis and disappear, but they can be identified by remaining radio-resistant Paneth cells at the crypt base. At higher radiation

dose levels, only single cells survive in each crypt, since a unit increase in radiation leads to unit reduction in crypt survival. Survival of one or more clonogenic cells in a crypt after radiation ensures crypt persistence, and there is regeneration of all epithelial cell populations of that crypt and, in the small intestine, of the overlying villi. Therefore, following cytotoxic damage, a single surviving stem cell can produce all cell types of the intestinal epithelium to reproduce a crypt.

25.5 MOUSE AGGREGATION CHIMERAS SHOW THAT INTESTINAL CRYPTS ARE CLONAL POPULATIONS

Mouse embryo aggregation chimeras are readily made, wherein the two populations can be readily distinguished. The lectin *Dolichos biflorus* agglutinin (DBA) binds to sites on the B6-derived but not on the SWR-derived cells in C57BL/6J Lac (B6) × SWR mouse embryo aggregation chimeras, and it can be used to distinguish the two parental strains in gut epithelia. The intestinal crypts in each chimera studied were either positive or negative for DBA, and there were no mixed crypts in the tens of thousands studied (Figure 25.1A). Therefore, each crypt forms a clonal population. This is the case for Paneth, mucous, and columnar cells, although it was not possible to detect the markers in endocrine cells because of their inability to bind the lectin on their surface. In neonatal C57BL/6J Lac (B6) × SWR chimaeras, there were mixed (i.e., polyclonal) crypts for the first two weeks after birth, suggesting that multiple stem cells exist during development (Figure 25.1B through 25.1D). However, all crypts ultimately become derived from a single stem cell between birth and postnatal day 14, so-called monoclonal conversion. This apparent cleansing or 'purification' of crypts could be caused by the stochastic loss of one stem cell lineage or by the segregation of lineages because of an extremely active replication of crypts by fission, which occurs at this developmental period. To exclude the possibility that crypts from distinct strains segregate differentially during organogenesis, These findings were confirmed in mice bearing an X-linked defective gene for glucose-6-phosphate dehydrogenase (*G6PD*) (Figure 25.1E and later sections of this chapter).

In the stomach, the situation is similar although more complex. Epithelial cell lineages in the antral gastric mucosa of the mouse stomach, including the endocrine cells, derive from a common stem cell. Identification of the Y-chromosome by *in situ* hybridization in XX–XY chimeric mice showed that gastric glands were also clonal populations (Figure 25.1F). These findings were confirmed in CH3 × BALB/c chimeric mice, where each gastric gland was composed of either CH3 or BALB/c cells; there were no mixed glands. Thus, we might advance the general hypothesis that gastric glands in the

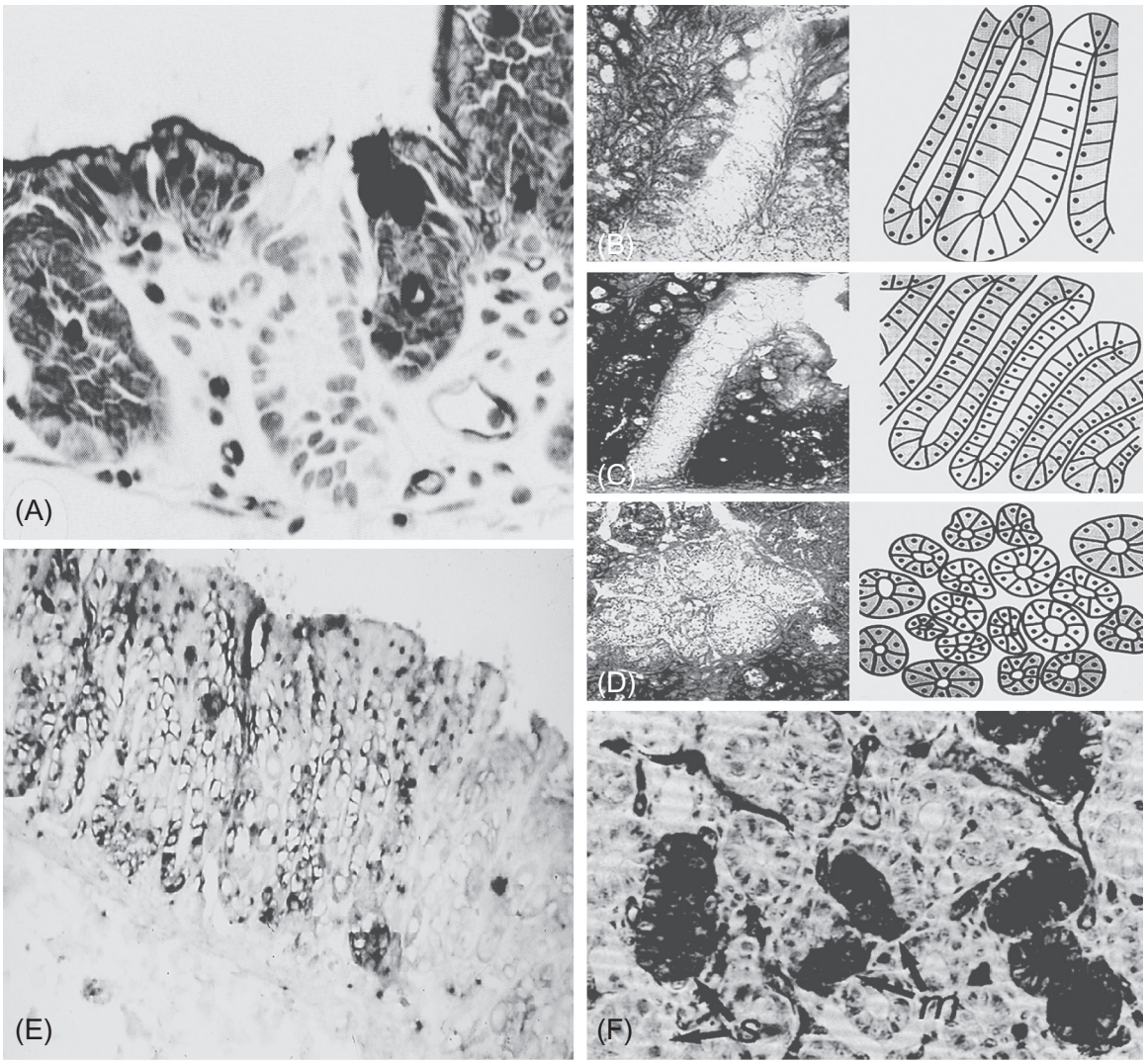

FIGURE 25.1 **Gastrointestinal clonality studies. Mouse embryo aggregation chimeras, XX–XY chimeric mice, and X-inactivation mice.**

(A) DBA staining in the small intestine of ENU-treated (12 weeks) C57BL/6J-SWR F1 chimeras showing entire negative and positive (black) crypts. (B–D) G6PD histochemistry in frozen sections of colonic mucosa in an ENU-treated C3H mouse: (B) partially negative crypt, (C) completely negative crypt, and (D) cross section of an eight-crypt patch at 21 weeks. (E) Y-spot pattern in the gastric mucosa and underlying tissues of an XX–XY chimera. (F) Cross section through crypts in neonatal duodenum of B6-SWR chimera stained with DBA (B6 = black staining, SWR = unstained). A balanced contribution to mixed crypts (m), and monoclonal crypts (s) is seen. *Panel A reproduced with permission from Winton DJ, Ponder BA. 1990. Proc Biol Sci 241: 13–8; panel B with permission from Park HS, Goodlad RA, Wright NA. 1995. Am J Pathol 147: 1416–27; panel E courtesy of Thompson EM, and panel F with permission from Schmidt GH, Winton DJ, Ponder BA. 1988. Development 103: 785–90.*

mouse, in addition to the intestinal crypts, are clonally derived. Additionally, by combining immunohistochemistry for gastrin, an endocrine cell marker, with *in situ* hybridization to detect the Y-chromosome, the male regions of the gastric glands were shown to be almost exclusively Y-chromosome positive with gastrin-positive endocrine cells, whereas the female areas in the chimeric stomach were gastrin positive and Y-chromosome negative (Figure 25.1D). These results finally negate the Pearse concept that gut endocrine cells originate from a separate stem cell pool.

25.6 SOMATIC MUTATIONS IN STEM CELLS REVEAL STEM CELL HIERARCHY AND CLONAL SUCCESSION

Somatic mutations at certain loci allow us to study stem cell hierarchy and clonal succession within the gastrointestinal tract. Mutations in the *DLb-1* on chromosome 11 are one good example of this; C57BL/6J × SWR F1 chimeric mice show heterozygous expression of a binding site on intestinal epithelial cells for the DBA lectin. This binding site can be abolished when the *Dlb-1* locus becomes mutated either spontaneously or by the chemical mutagen ethyl nitrosourea (ENU). After ENU treatment, crypts emerge that initially are partially and then are entirely negative for DBA staining. Perhaps the simplest explanation for this phenomenon is that a mutation occurs at the *Dlb-1* locus in a stem cell within the small intestinal crypt. This mutated cell could expand stochastically to produce a clone of cells that cannot bind DBA and remain unstained (Figure 25.1E). If this is the case, then a single stem cell can generate all the epithelial lineages within an intestinal crypt of the small intestine.

A 'knock-in' strategy at the *Dlb-1* locus can also be used to explain the preceding findings. If SWR mice do not express a DBA-binding site on their intestinal epithelial cells but can be induced to bind DBA by ENU treatment, wholly DBA+ or DBA− intestinal crypts would result. From the use of this model, it was proposed that 'committed epithelial progenitor' cells exist in mouse intestinal crypts, by visualizing the morphology, location, and longevity of mutant clones in crypts and villi of the mouse small intestine. These transitory committed progenitor cells – the columnar cell progenitors (Co) and the mucus cell progenitors (Mo) – evolve from pluripotential stem cells and then differentiate further into adult intestinal epithelial cell types.

Not much is known about the mechanisms that regulate the proliferation of these progenitor cells, but administration of proglucagon-derived, glucagon-like peptide 2 (GLP-2) to SWR mice was found to induce intestinal epithelial growth and repair specifically by stimulating the columnar cell progenitors,

resulting in increased crypt and villus size in the normal small intestine. The receptor for GLP-2 (GLP2R) was shown to be located on enteric neurons and not on the gut enteroendocrine cells and in the brain as previously thought. GLP-2 activation of enteric neurons produces a rapid induction in *c-fos* expression, which signals growth of columnar epithelial cell progenitors and stem cells that generate adult columnar cell types. There is no stimulatory effect on the mucus cell lineage; instead, it is stimulated by KGF. Thus, the committed progenitor cells are involved in regeneration of damaged epithelia, possibly through a neural regulatory pathway (Figure 25.2B).

Mice treated with the colon carcinogen dimethylhydrazine (DMH) or ENU also develop crypts that initially are partially and later are wholly negative for G6PD. The partially negative crypts could conceivably result from the mutation of a cell in the dividing transit population of the crypt that lacks stem cell properties. This is supported by the observation that these partially negative crypts are transient and decrease in frequency parallel to an

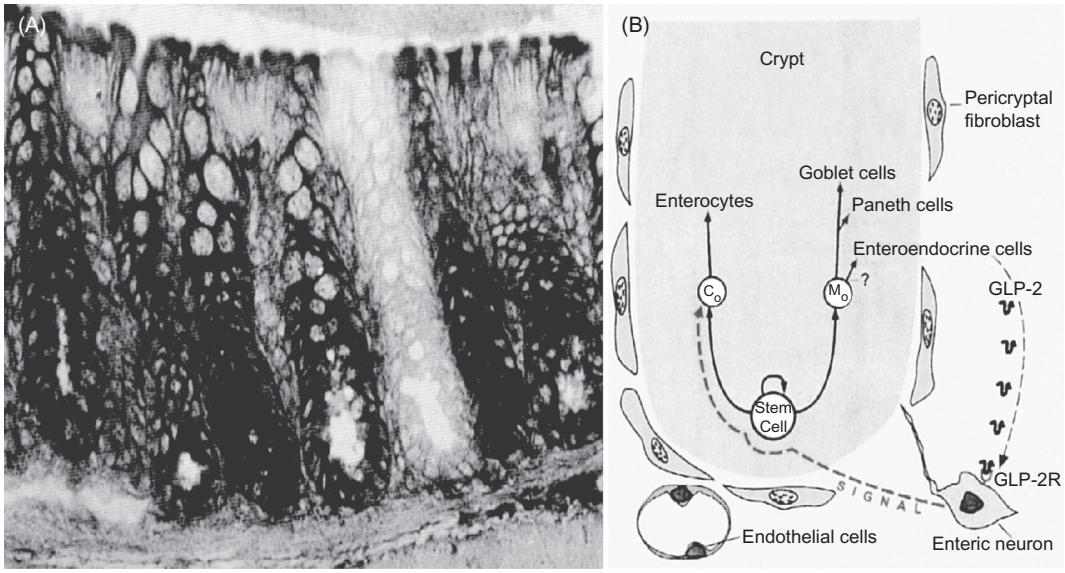

FIGURE 25.2 **Stem cell niche hypothesis.**

(A) G6PD-stained frozen section of colonic mucosa from a CH3 mouse 14 days after a single dose of DMH, showing a single, wholly mutated crypt in which all cells have been replaced by a mutant phenotype. (B) Diagrammatic representation of the stem cell niche. An active, multipotent stem cell produces a daughter cell (C_0) that generates enterocytic lineages and another that generates goblet, Paneth, and enteroendocrine cells (although it is not known whether all derive from M_0). GLP-2, produced by a subset of enteroendocrine cells, stimulates proliferation of the C_0 daughter through an interaction with enteric nervous system neurons that express the GLP-2 receptor (GLP-2R). *Panel A reproduced with permission from Williams ED, Lowes AP, Williams D, Williams GT. 1992. Am J Pathol 141: 773–6; and panel B reproduced with permission from Mills JC, Gordon JI. 2001. Proc Natl Acad Sci USA 98: 12334–6.*

increase in wholly negative crypts. Conversely, such partially negative crypts could become wholly negative by stochastic expansion of a mutant stem cell. Wholly negative crypts would then be a clonal population derived from this mutant stem cell.

After administration of a mutagen, in both the *Dlb-1* and the G6PD models, the time taken for the decrease in partially mutated crypts and the emergence of entirely negative crypts to reach a plateau is approximately four weeks in the small intestine and up to 12 weeks in the large intestine. This difference is intriguing, and was initially thought to be because of cell cycle time differences between the colon and the small intestine. However, a favored explanation can be found in the *stem cell niche hypothesis.* This hypothesis suggests that multiple stem cells occupy a crypt with random cell loss after stem cell division. The numbers of stem cells may be larger in the small intestine than in the large intestine, causing the difference in time taken for phenotypic changes following mutagen treatment as the mutant stem cell expands stochastically (Figure 25.2A). An alternative hypothesis might lie in crypt fission: the rate of fission at the time of mutagen administration was higher in the colon than in the small intestine. During crypt fission, when crypts divide longitudinally, selective segregation of the two cell populations could occur, 'cleansing' the partially mutated crypts by segregating the mutated and non-mutated cells and by duplicating the wholly negative crypts to create monoclonal crypts.

25.7 HUMAN INTESTINAL CRYPTS CONTAIN MULTIPLE EPITHELIAL CELL LINEAGES DERIVED FROM A SINGLE STEM CELL

In most of the human population, the colonic goblet cells secrete O-acetylated mucin. However, approximately 9% of the human Caucasian population has a homozygous genetic mutation in the enzyme O-acetyl transferase (*OAT*~ − /~ −) and in goblet cells secreting this non-O-acetylated sialic acid; these are then positive when stained with mild periodic acid-Schiff (mPAS) stain. In heterozygotes, which comprise approximately 42% of the population (*OAT*~ − /*OAT* +), O-acetylation proceeds, and crypts thereby stain negative for mPAS. Loss of the remaining active *OAT* gene converts the genotype to *OAT* − /*OAT* −, resulting in occasional, apparently randomly located positive mPAS-stained crypts with uniform staining of goblet cells from base to luminal surface, an effect that increases with age. This could be because of a somatic mutation or nondisjunction in a single crypt stem cell and subsequent colonization of the crypt by the mutated stem cell. The frequency of these events is racially determined and increases after irradiation. This has been interpreted as indicating an increased rate of stem cell

mutation. Interestingly, there is no increase in the rate of apparent stem cell mutation in hereditary nonpolyposis carcinoma patients, nor in the background mucosa of left- and right-sided carcinomas. However, just as in the mouse stem cell mutation models, when patients are followed by the mPAS method with time after irradiation, initially there is partial crypt staining, and then whole crypts appear where the goblet cells are mPAS +. A *clonal stabilization time* (defined as the period required for the emergence of most of such wholly stained crypts) in humans is approximately one year, a process we referred to previously as monoclonal crypt conversion.

These results have implications for the origins of goblet cell lineages in the gut: they indicate that they arise from crypt stem cells; however, they say little about the other cell lineages.

Perhaps the best evidence for the clonality of human intestinal crypts, and the stem cell derivation of all contained epithelial cell lineages, comes from studies of the colon of a rare XO–XY patient who received a prophylactic colectomy for familial adenomatous polyposis (FAP). Nonisotopic *in situ* hybridization (NISH) using Y-chromosome-specific probes showed the patient's normal intestinal crypts to be composed almost entirely of either Y-chromosome positive or Y-chromosome negative cells, with about 20% of crypts being XO. Immunostaining for neuroendocrine specific markers and Y-chromosome NISH used in combination showed that crypt neuroendocrine cells shared the genotype of other crypt cells. In the small intestine, the villus epithelium was a mixture of XO and XY cells, in keeping with the belief that the villi derive from stem cells of more than one crypt. Of the 12,614 crypts examined, only four were composed of XO and XY cells, which could be explained by nondisjunction with a loss of the Y-chromosome in a crypt stem cell. Importantly, there were no mixed crypts at patch boundaries. These observations agree with previous findings using chimeric mice that intestinal crypt epithelial cells, including neuroendocrine cells, are monoclonal and derive from a single multipotent stem cell. Consequently, the hypothesis that enteroendocrine cells and other differentiated cell types within the colorectal epithelium share a common cell of origin (the unitarian hypothesis) appears to apply to both mice and humans. These observations have been confirmed in Sardinian women heterozygous for a defective *G6PD* gene.

It has been proposed that insight into stem cell organization can be gained from the study of the methylation pattern of nonexpressed genes in the colon. In the normal human colon, methylation patterns are somatically inherited endogenous sequences that randomly change and increase in occurrence with aging. Investigation of methylation patterns is a possible alternative to histological markers to investigate crypt histories and allow fate mapping. Examination of methylation tags of three neutral loci in cells from

normal human colon showed variation in sequences between crypts and mosaic methylation patterns within single crypts. Multiple unique sites were present in morphologically identical crypts; for example, one patient had no identical methylation sequences of one gene within any of the crypts studied, even though all sequences were related. This indicates that some normal human colonic crypts are quasi-clonal with multiple stem cells per crypt. Differences in methylation tags can highlight relationships among cells in a crypt where less closely related cells show greater sequence alterations and where closely related cells have similar methylation patterns. Sequence differences suggest that crypts are maintained by stem cells, which are randomly lost and replaced in a stochastic manner, eventually leading to a 'bottleneck' effect in which all cells within a crypt are closely related to a single stem cell descendant. This reduction to the most recent common crypt progenitor is predicted to occur several times during life, superficially resembling the clonal succession of tumor progression.

In situ analyses of glandular clonality in the human stomach have been more problematic. X-chromosome-linked inactivation was used to study fundic and pyloric glands in human female stomachs. Studies using polymorphisms on X-linked genes, such as the androgen receptor (HUMARA) to distinguish between the two X-chromosomes revealed that, although pyloric glands appear homotypic and thus monoclonal, about half of the fundic glands studied were heterotypic at the HUMARA locus and were consequently polyclonal. This finding suggests that a more complex situation occurs in humans than the studies of gastric gland clonality in chimeric mice indicate. However, we have seen that some glands in the mouse remain polyclonal throughout life.

25.8 BONE MARROW STEM CELLS CONTRIBUTE TO GUT REPOPULATION AFTER DAMAGE

The hematopoietic bone marrow stem cell is of mesodermal origin, and its functionality and cell surface markers have been well characterized. When transplanted into lethally irradiated animals and humans, as in clinical bone marrow transplantation, it has been long considered that the hematopoietic stem cell colonizes host tissues to form only new erythroid, granulocyte-macrophage, megakaryocyte, and lymphoid lineages. Although earlier studies suggest that vascular endothelium could derive from transplanted donor marrow, more recent studies not only have confirmed these earlier proposals concerning endothelial cells but also have indicated that adult bone marrow stem cells possess a considerable degree of plasticity and can differentiate into different cell types, including hepatocytes, biliary epithelial cells, skeletal muscle fibers, cardiomyocytes, central nervous system cells, and renal

tubular epithelial cells. These pathways can be bidirectional, as muscle and neuronal stem cells can also apparently form bone marrow. Furthermore, it appears that selection pressure induced by target organ damage can intensify the efficacy of this process, as bone marrow stem cells differentiate into cardiomyocytes, endothelial cells, and smooth muscle cells in mice, with ischemic cell death following myocardial infarction and coronary artery occlusion. Bone marrow stem cells have also been shown to differentiate into pancreatic β-cells, and possibly more persuasively, fully differentiated cells can transdifferentiate into other adult cell types without undergoing cell division; for example, exocrine pancreatic cells can differentiate into hepatocytes *in vitro*. Furthermore, isolated potential hepatic stem cells from fetal mouse livers, which differentiate into hepatocytes and cholangiocytes when transplanted into recipient animals, can form pancreatic ductal acinar cells and intestinal epithelial cells when transplanted directly into the pancreas or duodenal wall. Thus, the conventional view that bone marrow stem cells generate cell types of a single lineage (i.e., all formed elements in the peripheral blood) has been rectified in favor of the findings that adult bone marrow stem cells are highly plastic and can differentiate into many cell types within various organs.

These observations have raised the possibility of regeneration of a failing organ by transplanting an individual's own bone marrow stem cells to colonize and repopulate the diseased tissue, thus avoiding the allograft reaction. In apparent proof of principle, fumarylacetoacetate hydrolase (FAH)-deficient mice, which resemble type 1 tyrosinemia in humans, are rescued from liver failure by transplantation of purified hematopoietic stem cells that become morphologically normal hepatocytes, express the FAH enzyme, and therefore are functionally normal.

There are now several reports that bone marrow cells can repopulate both epithelial and mesenchymal lineages in the gut. Colons and small intestines of female mice that received a bone marrow transplant from male mice donors and gastrointestinal biopsies from female patients with graft-versus-host disease following bone marrow transplant from male donors were analyzed. Bone marrow cells frequently engrafted into the mouse small intestine and colon and differentiated to form ISEMFs within the lamina propria. *In situ* hybridization confirmed the presence of Y-chromosomes in these cells; their positive immunostaining for αSMA and negativity for desmin, the mouse macrophage marker F4/80, and the hematopoietic precursor marker CD34 determined their phenotype as pericryptal myofibroblasts in the lamina propria derived from transplanted bone marrow. This engraftment and transdifferentiation occurred as early as one week after bone marrow transplantation; almost 60% of ISEMFs were bone-marrow-derived six

weeks after the transplantation, indicating that transplanted bone marrow cells are capable of a sustained turnover of the ISEMF cells in the lamina propria. Y-chromosome-positive ISEMFs were also seen in the human intestinal biopsy material. Lethally irradiated female mice given a male bone marrow transplant and a subsequent foreign-body peritoneal implant formed granulation tissue capsules containing myofibroblast cells derived from the hematopoietic stem cells of the transplanted bone marrow. This suggests that myofibroblasts may generally derive from bone marrow cells.

There are a growing number of reports that bone marrow cells can repopulate gastrointestinal epithelial cells in animals and man. Bone-marrow-derived epithelial cells were found in the lung, gastrointestinal tract, and skin 11 months after transplantation of a single hematopoietic bone marrow stem cell in the mouse. In the gastrointestinal tract, engrafted cells were present as columnar epithelial cells in the esophageal lining, a small intestinal villus, colonic crypts, and gastric foveola. No apparent engraftment into the pericryptal myofibroblast sheath was reported, and as only a single hematopoietic stem cell was transplanted, it is possible that the ISEMFs derive from mesenchymal stem cells within transplanted whole bone marrow. It is, however, generally believed that stromal cell populations do not survive following bone marrow transplantation – although if an empty niche exists, as after irradiation in the gut, engraftment might occur. Local application of bone marrow stem cells, either directly injected into the stomach and duodenum or applied to the mucosa after the induction of experimental colitis, can also apparently lead to epithelial transdifferentiation.

In biopsies from female patients who had undergone sex-mismatched hematopoietic bone marrow transplantation, *in situ* hybridization for a Y-chromosome-specific probe with immunohistochemical staining for cytokeratins demonstrated mucosal cells of donor origin in the gastric cardia. Moreover, four long-term bone marrow transplant survivors with multiple engraftment of esophageal, gastric, small intestinal, and colonic epithelial cells by donor bone marrow cells up to eight years after transplantation have been observed, emphasizing the long-term nature of this transdifferentiation.

It would be impossible to finish even such a short section on this topic without mentioning the mechanisms and significance of such phenomena. At first considered to be caused by transdifferentiation or lack of lineage fidelity – commonly seen in invertebrates, during gastrulation, or during organogenesis – it is becoming clear that such changes are neither simple nor readily reproducible. Some labs have been unable to reproduce earlier findings, and there are claims that adult tissues are contaminated with bone marrow precursors. Finally, the fusion of a transplanted bone marrow cell with an indigenous

adult cell has been proposed as the mechanism by which bone marrow stem cells acquire the phenotype of target cell lineages. Initially, cell fusion was seen as a rare event, occurring only *in vitro* and under circumstances of extreme selection. In the FAH model described previously, cell fusion was recently shown to be common, and it cannot be ruled out as the main mechanism by which bone marrow stem cells transform to functional hepatocytes. However, the genetic mechanisms whereby gene expression is switched off in the recipient cells – followed by clonal expansion to repopulate large areas of the liver, for example – are as yet unclear. We should also recall that several tissues in the mouse are polyploid, such as the liver and the acinar cells of the exocrine pancreas. Other studies in which mixed-sex bone marrow transplants have been used to show plasticity have not reported evidence of cell fusion in animals or man. For example, bone marrow-engrafted cells in the human stomach, intestine, buccal mucosa, and pancreatic islet cells showed a normal complement of X- and Y-chromosomes. Whatever the mechanism, it is clear that the most important criterion for altered lineage commitment, that of function, has only been fulfilled in a few models, such as the FAH model and possibly postinfarction cardiomyocyte engraftment. The future in this field will prove interesting.

In inflammatory bowel diseases such as Crohn's disease, intestinal myofibroblasts are activated to proliferate and synthesize an extracellular matrix, and excessive collagen deposition causes fibrosis and postinflammatory scarring in the lamina propria and muscularis layers of the gut wall. Intestinal inflammation is believed to be mediated by luminal bacteria and bacterial wall polymers, and tumor necrosis factor (TNF) plays a key role in the pathogenesis of intestinal inflammatory disease, since mice with targeted deletion of the AU-rich elements in TNF develop chronic ileitis resembling a Crohn's disease-like phenotype (Figure 25.3A–D). Moreover, a single dose of anti-TNF antibody to Crohn's disease patients can dramatically alleviate inflammation. Other cytokines, including interleukin-10 and TGFβ are implicated in the development of fibrosis in inflammatory bowel disease.

It has previously been concluded that fibrotic reactions, which occur in inflammatory bowel disease, and other diseases are caused by the local proliferation of myofibroblasts and fibroblasts. Our data showing that transplanted bone marrow contributes to the intestinal subepithelial myofibroblast population implicates that extraintestinal cells may have a role in fibrosis. In this respect, we have shown that cells with a fibroblast-fibrocyte phenotype can derive from transplanted bone marrow and contribute to fibrotic reactions in and around the intestinal wall (Figure 25.3F). The concept that cells of the lamina propria, other than the lymphoid and myeloid lineages, exist in equilibrium with bone marrow precursors is an interesting one and could provide an opportunity for therapeutic delivery of cytokines to the intestine to

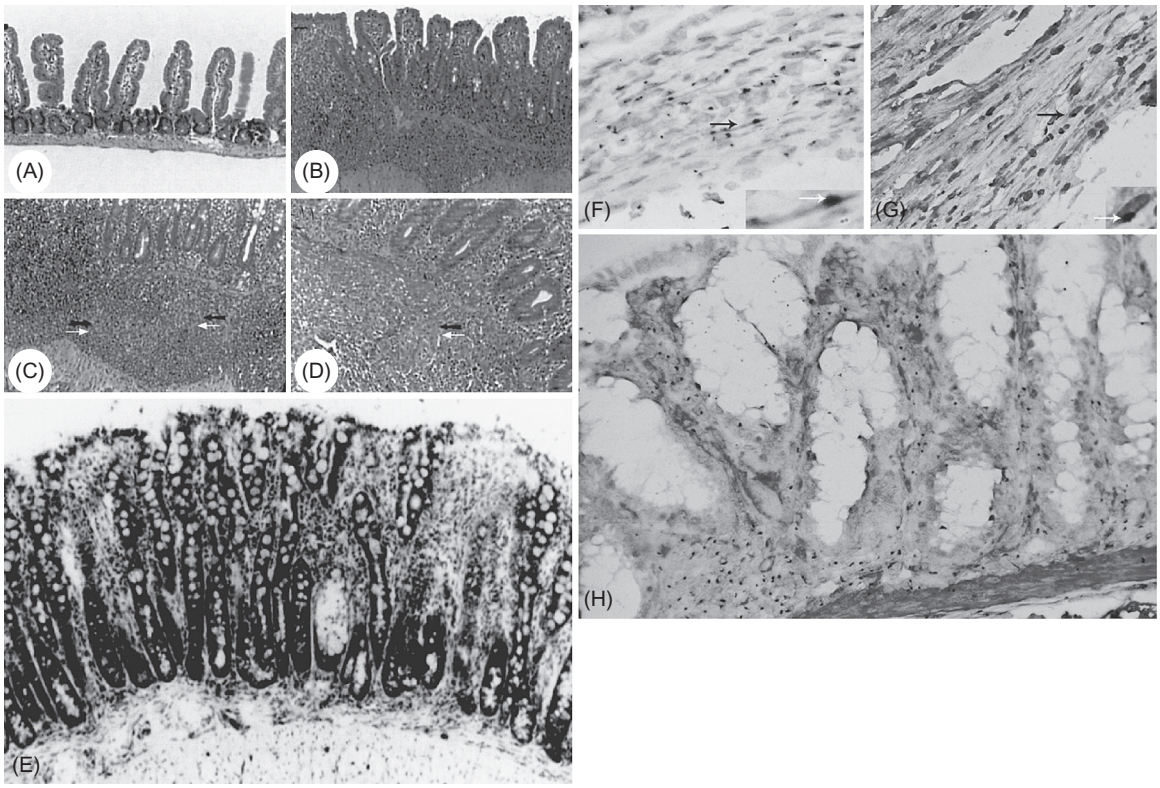

FIGURE 25.3 **Murine models of fibrosis. Inflammatory bowel disease in TNFΔARE and SAMP/Yit mouse ilea resembling human Crohn's disease.**

(A) Normal ileal morphology in an 8-week control mouse. (B) TNFΔARE homozygous knockout at seven weeks with blunt, distorted villi and increased inflammatory infiltrate. (C) TNFΔARE heterozygote at 16 weeks with villus blunting and chronic inflammation. An ill-defined noncaseating granuloma is in the submucosa (arrows). (D) Ileocecal region from a human patient with Crohn's disease demonstrating similar location and composition of granuloma compared with mouse model (arrow). (E) SAMP/Yit mouse at 20 weeks showing severe mucosal inflammation, crypt hyperplasia, elongation, and villous atrophy. (F) A fibrotic reaction in the serosal tissues of a paracetamol-treated male-female radiation chimeric mouse, showing numerous spindle-shaped cells with Y-chromosomes revealing their bone marrow origin, which are also (G) vimentin-positive, confirming their fibroblast lineage. (H) Massive expansion of myofibroblasts of bone marrow origin in a male-female chimeric mouse with colitis induced by TNBS. *Panel D reproduced with permission from Kontoyiannis D, Pasparakis M, Pizarro TT, Cominelli F, Kollias G. 1999. Immunity 10: 387–98; panel E with permission from Matsumoto S, Okabe Y, Setoyama H, Takayama K, Ohtsuka J, et al. 1998. Gut 43: 71–8; panels F–G courtesy of N. Direkze; and panel H courtesy of M. Brittan.*

prevent the development of fibrosis or even treat it. Further data show that many of the myofibroblasts and fibroblasts recruited to, or expanding in, the lamina propria and submucosa of mice with induced 2,4,6-trinitrobenzene sulfonic acid (TNBS) colitis are bone marrow derived (Figure 25.3G).

25.9 GASTROINTESTINAL STEM CELLS OCCUPY A NICHE MAINTAINED BY ISEMFS IN THE LAMINA PROPRIA

Stem cells within many tissues are thought to reside within a niche formed by a group of surrounding cells and their extracellular matrices, which provide an optimal microenvironment for the stem cells to function. The identification of a niche within any tissue involves knowledge of the location of the stem cells; as we have seen, this has proved problematical in the gastrointestinal tract. To prove that a niche is present, the stem cells must be removed and subsequently replaced while the niche persists, providing support to the remaining exogenous cells. Although this has been accomplished in *Drosophila*, such manipulations have not yet been possible in mammals. In this context, the survival of a single epithelial cell following cytotoxic damage to intestinal crypts in the microcolony assay is interesting, as many of the intestinal subepithelial myofibroblasts are also lost after irradiation, although sufficient numbers may remain or be replaced by local proliferation or migration from the bone marrow to provide a supportive niche for the surviving stem cell or cells. The ISEMFs surround the base of the crypt and the neck-isthmus of the gastric gland, a commonly proposed location for the intestinal and gastric stem cell niches, respectively. It is proposed that ISEMFs influence epithelial cell proliferation and regeneration through epithelial-mesenchymal cross talk and that they ultimately determine epithelial cell fate.

There has been a long quest for markers of stem cells in the intestine. The neural RNA-binding protein marker Musashi-1 (Msi-1) is a mammalian homolog of a *Drosophila* protein evidently required for asymmetrical division of sensory neural precursors. In the mouse, Msi-1 is expressed in neural stem cells and has recently been proposed as the first intestinal stem cell marker, because of its expression in developing intestinal crypts and specifically within the stem cell region of adult small intestinal crypts. This is further substantiated by its expanded expression throughout the entire clonogenic region in the small intestine after irradiation.

The regulatory mechanisms of stem cell division within the niche to produce, on average, one stem cell and one cell committed to differentiation are as yet unknown, although there is no shortage of potential models. In the stem cell zone hypothesis, the bottom few cell positions of the small intestinal crypt are occupied by a mixture of cell types: Paneth, goblet, and endocrine. The migration vector is toward the bottom of the crypt. Above cell position 5, cells migrate upward, although only the cells that divide in the stem cell zone beneath are stem cells. Other models envisage the stem cells occupying a ring

immediately above the Paneth cells, although there is little experimental basis for such an assertion since 'undifferentiated' cells of similar appearance are seen among the Paneth cells in thin sections. Moreover, there is no difference in the expression of Msi-1 and Hes1 – a transcriptional factor regulated by the Notch signaling pathway also required for neural stem cell renewal and neuronal lineage commitment – in undifferentiated cells located in either the stem cell zone or immediately above the Paneth cells. This suggests that both populations may have the same potential as putative stem cells.

The number of stem cells in a crypt or a gland is presently unknown. Initially, all proliferating cells were believed to be stem cells. Although clonal regeneration experiments using the microcolony assay indicated that intestinal crypts contained a multiplicity of stem cells, it was clear that this was less than the proliferative cellularity. Proposed stem cell numbers have varied from a single stem cell to 16 or more. Others have proposed that the number of stem cells per crypt varies throughout the crypt cycle, with the attainment of a *threshold number* of stem cells per crypt being the signal for fission to occur. There is little experimental evidence to support these proposals. Although all cells in a crypt are initially derived from a single cell, as shown by the chimeric and X-inactivation experiments discussed previously, mutagenesis studies such as those shown in Figure 25.1E and Figure 25.2A argue strongly for more than one stem cell per crypt, with stochastic clonal expansion of a mutant clone. A three stem cell colonic crypt has been suggested on this basis.

In organisms such as *Drosophila* and *Caenorhabditis elegans,* stem cell divisions are known to be asymmetric. We have no such firm concept for the mammalian gut, although there is some evidence to support the proposal. By labeling DNA template strands in intestinal stem cells with tritiated thymidine during development or tissue regeneration, and by labeling newly synthesized daughter strands with bromodeoxyuridine, segregation of the two markers can be studied. The template DNA strand labeled with tritiated thymidine is retained, but the newly synthesized strands labeled with bromodeoxyuridine become lost after the second division of the stem cell. This indicates not only that asymmetric stem cell divisions occur, but also that by discarding the newly synthesized DNA, which is prone to mutation, into the daughter cell destined to differentiate, a mechanism of stem cell genome protection is afforded.

When a stem cell divides, the possible outcomes are that two stem cells (P) are produced, that two daughter cells destined to differentiate (Q) cells are produced, or that there could be an asymmetric division resulting in one P and one Q cell. These are sometimes called p, q, and r divisions

or p and q divisions. If $p = 1$ and $q = 0$, then regardless of the number of stem cells per crypt, the cells are immortal and there will be no drift in the niche with time. Such a situation is called 'deterministic.' However, if $p < 1$ and $Q > 0$ (i.e., a stochastic model), there will be eventual extinction of some stem cell lines and a drift toward a common stem cell from which all other cells derive. We previously described the variation in methylation patterns or 'tags' that occur in human colonic crypts and explained that crypts apparently show several unique tags. The variance of these unique tags was compared with those expected using a variety of models, including no drift with aging (the deterministic model), drift with immortal stem cells with divergence (the numbers of unique tags are proportional to the stem cell number), drift with one stem cell per crypt, and a stem cell niche with more recent divergence (with loss of stem cells occurring proportional to the time since divergence). Multiple unique tags were found in some crypts, and the number of unique tags increased with the number of markers counted, which favors random tag drift and multiple stem cells per crypt. The variances were consistent with drift in immortal stem cells, where N (the number of stem cells) = 2, but favored a model where $0.75 < P < 0.95$ and $N < 512$. Thus, the data supported a stochastic model with multiple stem cells per crypt. However, as in many such attempts, there are several major assumptions necessary, such as a constant stem cell number. It is clear that variation in both P and N occur in this model. However, this analysis is consistent with data that the time taken for monoclonal conversion, or the 'clonal stabilization time,' of OAT +/− individuals to convert to OAT −/− cells following irradiation was found to be about one year. Assuming 64 stem cells per niche and $P = 0.95$, the mean time for conversion should be some 220 days. The same assumptions suggest a bottleneck, where all stem cells are related to the most recent common ancestral cell, occurring every 8.2 years.

25.10 MULTIPLE MOLECULES REGULATE GASTROINTESTINAL DEVELOPMENT, PROLIFERATION, AND DIFFERENTIATION

Although the molecular mechanisms by which pluripotent stem cells of the gastrointestinal tract produce differentiated cell types are not clearly understood, an increasing number of genes and growth factors have been identified that regulate development, proliferation, and differentiation as well as development of tumors. These are expressed by intestinal mesenchymal and epithelial cells and include members of the fibroblast growth factor family, epidermal growth factor family, TGFβ, insulin-like growth factors 1 and 2, HGF-scatterfactor, Sonic and Indian hedgehog, and PDGF-α, among others.

25.11 WNT/β-CATENIN SIGNALING PATHWAY CONTROLS INTESTINAL STEM CELL FUNCTION

The Wnt family of signaling proteins is critical during embryonic development and organogenesis in many species. There are 16 known mammalian *Wnt* genes, which bind to receptors of the frizzled (*Fz*) family, eight of which have been identified in mammals. The multifunctional protein β-catenin normally interacts with a glycogen synthase kinase 3-β (GSK3-β), axin, and adenomatous polyposis coli (APC) tumor suppressor protein complex. Subsequent serine phosphorylation of cytosolic β-catenin by GSK3-β leads to its ubiquitination and to its proteasomal degradation, thereby maintaining low levels of cytosolic and nuclear β-catenin. Wnt ligand binding to its *Fz* receptor activates the cytoplasmic phosphoprotein *dishevelled*, which in turn initiates a signaling cascade resulting in increased cytosolic levels of β-catenin. β-Catenin then translocates to the cell nucleus, where it forms a transcriptional activator by combining with members of the T-cell factor/lymphocyte enhancer factor (Tcf/LEF) DNA-binding protein family. This activates specific genes, resulting in the proliferation of target cells, for example, in embryonic development (Figure 25.4). In addition to its role in normal embryonic development, the Wnt/β-catenin pathway plays a key role in malignant transformation. Mutations of the *APC* tumor suppression gene are present in up to 80% of human sporadic colorectal tumors. This mutation prevents normal β-catenin turnover by the GSK3β/axin/APC complex. This results in increased nuclear β-catenin/Tcf/LEF gene transcription and a subsequent increase in β-catenin-induced Tcf/LEF transcription. One of the main functions of *APC* appears to be the destabilisation of β-catenin. Free β-catenin is one of the earliest events, or perhaps even the initiating event, in tumorigenesis in the murine small intestine and in the human colon. Many genes, including *c-myc*, *cyclin D1*, *CD44*, *c-Jun*, *Fra-1*, and urokinase-type plasminogen receptor have been identified as targets of the β-catenin/Tcf/LEF nuclear complex, although the precise mechanisms that lead to carcinogenesis are not entirely understood.

The Tcf/LEF family of transcription factors has four members; Tcf-1, LEF1, Tcf-3, and Tcf-4. Tcf-4 is expressed in high levels in the developing intestine from embryonic day (E) 13.5 and in the epithelium of adult small intestine, colon, and colon carcinomas. When there is loss of function of APC or mutations in β-catenin, increased β-catenin/Tcf-4 complexes are formed that lead to uncontrolled transcription of target genes. Mice with targeted disruption of the *Tcf-4* gene have no proliferating cells within their small intestinal crypts and lack a functional stem cell compartment. This suggests that *Tcf-4* is responsible for establishing stem cell populations within intestinal crypts; this in turn is

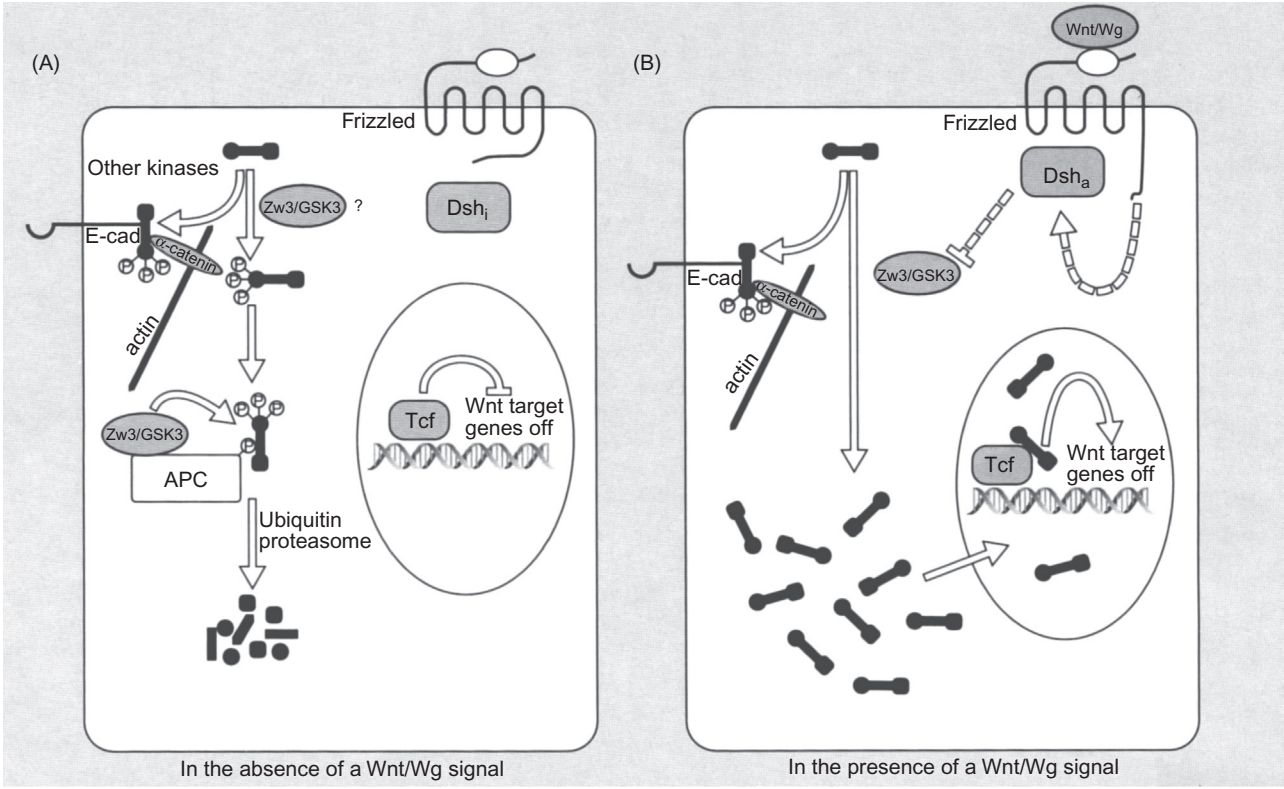

FIGURE 25.4 **Wnt signaling pathway.**

(A) In the absence of Wnt signaling, *dishevelled* is inactive (*Dsh*$_i$), and *Drosophila* zeste-*white* 3 or its mammalian homolog glycogen synthase kinase 3 (Zw3/GSK3) is active. β-Catenin (black dumbbell), through association with the APC–Zw3/GSK3 complex, undergoes phosphorylation and degradation by the ubiquitin-proteasome pathway. Meanwhile, T-cell factor (TCF) is bound to its DNA-binding site in the nucleus, where it represses the expression of genes such as *Siamois* in *Xenopus*. (B) In the presence of a Wnt signal, *dishevelled* is activated (*Dsh*$_a$), leading to inactivation of Zw3/GSK3 by an unknown mechanism. β-Catenin fails to be phosphorylated and is no longer targeted into the ubiquitin-proteasome pathway; instead, it accumulates in the cytoplasm and enters the nucleus by an unknown pathway, where it interacts with TCF to alleviate repression of the downstream genes and provide a transcriptional activation domain. *Reproduced with permission from Willert K, Nusse R. 1998. Curr Opin Genet Dev 8: 95–102.*

thought to be activated by a *Wnt* signal from the underlying mesenchymal cells in the stem cell niche. Chimeric ROSA26 mice expressing a fusion protein containing the high mobility group box domain of Lef-1 linked to the transactivation domain of β-catenin (B6Rosa26< > 129/Sv(Lef-1/β-cat) display increased intestinal epithelial apoptosis. This occurs specifically in 129/Sv cells throughout crypt morphogenesis, unrelated to enhanced cell proliferation. On completion of crypt formation and in adult mice, there is complete loss of all 129/Sv cells. Stem cell selection appears to be biased toward the unmanipulated ROSA26 cells in these chimeras, suggesting that 'adequate threshold' levels of β-catenin during development permit sustained proliferation and selection of cells, establishing a stem cell hierarchy. Increased β-catenin expression appears to induce an apoptotic response, and thus the stem cell niche is unaffected by increased Lef-1/β-catenin during intestinal crypt development.

25.12 TRANSCRIPTION FACTORS DEFINE REGIONAL GUT SPECIFICATION AND INTESTINAL STEM CELL FATE

25.12.1 Hox Genes Define Regional Gut Specification

Mammalian homeobox genes *Cdx-1* and *Cdx-2* display specific regional expression in developing and mature colon and small intestine. During embryogenesis, *Cdx-1* localizes to the proliferating cells of the crypts and maintains this expression during adulthood. The Tcf-4 knockout mouse does not express *Cdx-1* in the small intestinal epithelium, thus the Wnt/β-catenin complex appears to induce *Cdx-1* transcription in association with Tcf-4 during the development of intestinal crypts. Mice heterozygous for a *Cdx-2* mutation develop colonic polyps composed of squamous, body, and antral gastric mucosa with small intestinal tissue. Proliferation of *Cdx-2*-colonic cells with low *Cdx-2* levels can produce clones of cells phenotypically similar to epithelial cells of the stomach or small intestine. This could indicate a possible homeotic shift in stem cell phenotype. Region-specific genes such as *Cdx-1*, *Cdx-2*, and *Tcf-4* appear to define the morphological features of differential regions of the intestinal epithelium and regulate the proliferation and differentiation of the stem cells.

25.12.2 Forkhead Family is Essential for Intestinal Proliferation

The winged helix-forkhead family of transcription factors are essential for proper development of the ectodermal and endodermal regions of the gut. There are nine murine forkhead family members, which generate the forkhead box (Fox) proteins, three homologs of the rat hepatic nuclear factor 3

gene (*HNF3*α, -β, and -γ), and six genes referred to as forkhead homologs (*fkh-1* through *fkh-6*). *Fkh-6* is expressed in gastrointestinal mesenchymal cells now reclassified as *Foxl1*. *Foxl1* knockout mice have a dramatically altered gastrointestinal epithelium with branched and elongated glands in the stomach, elongated villi, hyperproliferative crypts, and goblet cell hyperplasia because of increased epithelial cell proliferation. They show upregulated levels of heparin sulfate proteoglycans (HSPGs), which increase *Wnt*-binding efficacy to the *Fz* receptors on gastrointestinal epithelial cells. This results in overactivation of the Wnt/β-catenin pathway and increased nuclear β-catenin. The resultant β-catenin/Tcf/LEF complex activates target genes such as *cyclin D1* and *c-myc*, which increase epithelial cell proliferation. Therefore, *Foxl1* regulates the Wnt/β-catenin pathway in association with an increase in HSPGs, demonstrating epithelial cell regulation by mesenchymal factors during embryogenesis in the gastrointestinal tract. As *c-myc* is a known proto-oncogene, a mutation of *Foxl1* and the resultant increase in epithelial cell proliferation through inappropriate *c-myc* activation may lead to the development of colorectal cancers.

25.12.3 E2F Transcription Family is Essential for Development of the Crypt Proliferative Zone

The E2F family of transcription factors regulates cell proliferation, allowing transit from the G_1 to the S phase. E2F4 is expressed in the proliferative regions of embryonic intestine and in the adult small intestine and colon. In E2F4-knockout mice, intestinal crypts fail to develop, and the lamina propria appears thickened. E2F4 is thus essential for the development of the proliferative compartment of the intestinal epithelium, although the molecular pathways that influence E2F4 during development are unknown.

25.12.3.1 Multiple Molecules Define Stem Cell Fate and Cell Position in the Villus–Crypt Axis

The colonic crypt and the villus–crypt axis offer a system in which the fate of stem cell progeny is defined. Their position within this axis can readily be determined. The goblet cell number normally remains relatively constant, and Paneth cells derive positional information and use it to remain in the crypt base. In mice, deletion of the *Math1* gene, a basic helix-loop-helix transcription factor and downstream component of the *Notch* signaling pathway, depletes goblet, Paneth, and enteroendocrine cell lineages in the small intestine. This indicates that Math1 is essential for stem cell commitment to one of three epithelial adult cell types. Math1 progenitors merely become enterocytes. High levels of Notch switch on the Hes1 transcriptional repressor. This in turn blocks expression of Math1, so that cells remain progenitors and ultimately become enterocytes. Conversely, low Notch expression

increases levels of its ligand Delta, which induces Math1 expression by blocking Hes1, causing cells to become goblet cells, Paneth cells, or enteroendocrine cells. Hes1-null mice have elevated Math1 expression, with more enteroendocrine and goblet cells and fewer enterocytes. This supports the evidence that Math1 regulates the determination of cell fate through a Notch–Delta signaling pathway (Figure 25.5).

Recent studies show that β-catenin and Tcf inversely control the expression of the EphB2/EphB3 receptors and their ligand ephrin-B1 in colorectal cancer

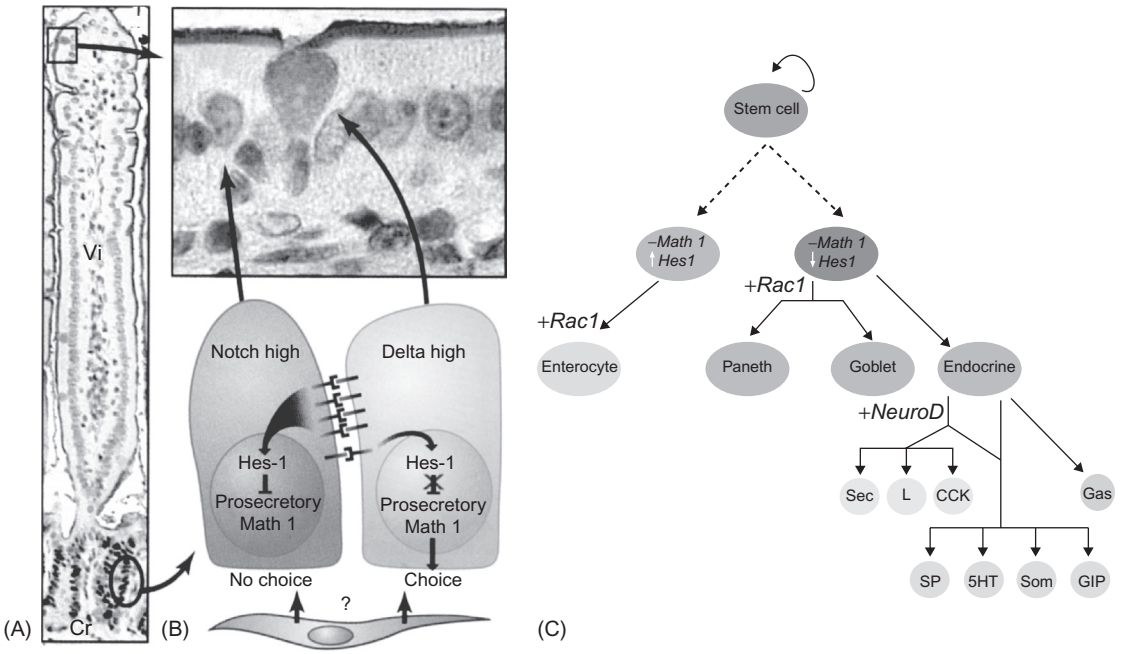

FIGURE 25.5 **Math1 signaling pathway.**

(A) Low-power section of adult murine small intestine. Precursor cells are stained for cyclin proliferating cell nuclear antigen, enterocytes express intestinal alkaline phosphatase, and goblet cells secrete mucins. Inset shows high-power image of small intestinal enterocytes and goblet cells. (B) Math1, a component of the Notch signaling pathway, influences intestinal epithelial cell fate decisions. In crypt progenitor stem cells that express high levels of Notch, the Hes1 transcription factor is switched on, and the expression of Math1 and of other 'prosecretory' genes is blocked. The result is that the precursor cells become enterocytes. In cells expressing low amounts of Notch, levels of Delta are high, production of Hes1 is blocked, and Math1 expression is induced. Production of the Math1 helix-loop-helix transcription factor allows precursor cells to make a choice: whether to become goblet cells, Paneth cells, or enteroendocrine cells (C). Math1 is essential for secretory cells. Whether Math1-expressing cells descend directly from stem cells or an intermediate progenitor remains unknown. (Vi: villus, Cr: crypt, Sec: secretin, L: glucagons-peptide YY, CCK: cholecystokinin, SP: substance P, 5HT: serotonin, Som: somatostatin, GIP: gastric inhibitory peptide, and Gas: gastrin). *Panels A and B reproduced with permission from van Den Brink et al. (2001), and panel C reproduced with permission Yang Q, Bermingham NA, Finegold MJ, Zoghbi HY. 2001. Science 294: 2155–8.*

and along the crypt–villus axis. When *EphB2* and *EphB3* genes are disrupted, cell positioning within the crypt is also disrupted. For example Paneth cells do not migrate downward to their normal position at the bottom of the crypt but scatter along crypt and villus. This indicates that β-catenin and Tcf contribute to the sorting of cell populations through the EphB/ephrin-B system.

In the future, it is clear that functional genomics will have an increasing role to play in the study and identification of intestinal stem cells. A consolidated population of stem cells was isolated by laser-capture microdissection from germ-free transgenic mice lacking Paneth cells. There were no fewer than 163 transcripts enriched in these stem cells compared with normal crypt base epithelium, which contains a predominance of Paneth cells. The profile showed prominent representation of genes involved in c-myc signaling, as well as in the processing, localization, and translation of mRNAs. Similar studies in the mouse stomach showed that growth factor response pathways are prominent in gastric stem cells, examples including insulin-like growth factor. A considerable fraction of stem cell transcripts encode products required for mRNA processing and cytoplasmic localization. These include numerous homologs of *Drosophila* genes needed for axis formation during oogenesis.

25.13 GASTROINTESTINAL NEOPLASMS ORIGINATE IN STEM CELL POPULATIONS

We can use the development of colorectal carcinoma as a paradigm. The concept of the adenoma-carcinoma sequence, whereby adenomas develop into carcinomas, is now widely accepted, and most colorectal carcinomas are believed to originate in adenomas. The initial genetic change in the development of most colorectal adenomas is thought to be at the *APC* locus, and the molecular events associated with these stages are clear: a second hit in the *APC* gene is sufficient to give microadenoma development, at least in FAP. There are basically two models for adenoma morphogenesis, both of which closely involve basic concepts of stem cell biology in the colon: in the first, mutant cells appear in the *intracryptal zone* between crypt orifices, and as the clone expands, the cells migrate laterally and downward, displacing the normal epithelium of adjacent crypts. A modification of this proposal is that a mutant cell in the crypt base, classically the site of the stem cell compartment, migrates to the crypt apex where it expands. These proposals are based on findings in some early non-FAP adenomas, where dysplastic cells were seen exclusively at the orifices and on the luminal surface of colonic crypts; measurement of loss of heterozygosity (LOH) for *APC* and nucleotide sequence analysis of the mutation cluster region of the *APC* gene carried out on microdissected, well-oriented histological sections of these adenomas showed that half the sample had LOH in the upper portion of the crypts, most with

truncating *APC* mutations. Only these superficial cells showed prominent proliferative activity, with nuclear localization of β-catenin indicating an APC mutation only in these apical cells. Earlier morphological studies have drawn attention to the same appearances. This top-down morphogenesis has wide implications for concepts of stem cell biology in the gut. It is clear that most evidence indicates that crypt stem cells are found at the origin of the cell flux, near the crypt base. These proposals, however, either re-establish the stem cell compartment in the intracryptal zone or make the intracryptal zone a favored locus where stem cells, having acquired a second hit, clonally expand.

An alternative hypothesis proposes that the earliest lesion is the *unicryptal* or *monocryptal adenoma*, where the dysplastic epithelium occupies an entire single crypt. These lesions are common in FAP, and although they are rare in non-FAP patients, they have been described. Here, a stem cell acquires the second hit, then it expands, stochastically or more likely because of a selective advantage, to colonize the whole crypt. Such monocryptal lesions thus should be clonal. Similar crypt-restricted expansion of mutated stem cells has been well documented in mice after ENU treatment and in humans heterozygous for the *OAT* gene, where after LOH, initially half then the whole crypt is colonized by the progeny of the mutant stem cell. Interestingly, OAT + /OAT− individuals with FAP show increased rates of stem cell mutation with clustering of mutated crypts. Thus, in sharp contrast the mutated clone expands not by lateral migration but by *crypt fission* in which the crypt divides, usually symmetrically at the base. Several studies have shown that fission of adenomatous crypts is the main mode of adenoma progression – predominantly in FAP, where such events are readily evaluated, but also in sporadic adenomas. The nonadenomatous mucosa in FAP, with only one *APC* mutation, shows a large increase in the incidence of crypts in fission. Aberrant crypt foci, thought to be precursors of adenomas, grow by crypt fission, as do hyperplastic polyps. This concept does not exclude the possibility that the clone later expands by lateral migration and downward spread into adjacent crypts, but, with the initial lesion the monocryptal adenoma, this model of morphogenesis is conceptually very different.

Work supporting the bottom-up spread of colorectal adenomas looked at a number of small (<3 mm) tubular adenomas. Here, nuclear accumulation of β-catenin was seen, indicating loss of function of one of the genes in the *Wnt* pathway, most likely *APC*, with subsequent translocation of β-catenin to the nucleus. Serial sections showed that the β-catenin nuclear staining extended to the bottom of the crypts and was present in crypts in the process of crypt fission. β-Catenin expression was particularly marked in the nuclei of buds. At the surface, there was a sharp cut-off between the adenomatous cells in the crypt that showed nuclear β-catenin and those surface cells that did not. The adjacent crypts were filled with dysplastic cells containing nuclear β-catenin,

which were not confined to the upper portions of the crypts. In larger adenomas, there was unequivocal evidence of surface cells growing down and replacing the epithelium of normal-looking crypts. Crypt fission was rare in normal and noninvolved mucosa and usually began with basal bifurcation at the base of the gland, whereas in adenomas, fission was commonly asymmetrical with budding from the superficial and mid-crypt. Multiple fission events were frequently observed in adenomas. The *crypt fission index* (the proportion of crypts in fission) in adenomas was significantly greater than that in noninvolved mucosa.

What are the implications of these considerations upon our concepts of the single (stem) cell origin or clonality in colorectal adenomas? We have seen that crypts are clonal units; thus, these lesions would be polyclonal because of the mixture of clonal crypts and clonal adenoma – though in this instance, they have different clonal derivation. A study of both sporadic and FAP adenomas, using X-linked restriction fragment length polymorphisms, showed that such lesions were apparently monoclonal in origin. On the other hand, the X-linked patch in the colon is large – and can be in excess of 450 crypts in diameter (see Figure 25.6). So, unless an adenoma grows over a patch boundary and involves crypts on either side of that boundary, X-inactivation analysis will always show that such lesions are monoclonal.

On the 'bottom-up' proposals, expansion of a clonal monocryptal adenoma by crypt fission would inevitably lead to a monoclonal microadenoma and thus adenoma. However, as mentioned before, studies on the XO–XY individual, who has a mean patch size of 1.48 crypts, indicated that some 76% of adenomas were polyclonal, supported by observations in Rosa26-Min chimeric mice. This could be explained by the transformation of noninvolved crypts by transformed stem cells.

So what is happening to the stem cells in the development of these early, monocryptal lesions? Adenomatous crypts in FAP contain two stem cell lines, *APC* −/+ and *APC* −/−, and interpreted this to mean that the *APC* −/− cells were expanding stochastically within the same crypt. There is good evidence that such monoclonal conversion occurs as a stochastic process. It has previously been shown that a quantitative analysis of age-related methylation suggests that crypts are maintained by niches containing multiple stem cells. Random stem cell loss with replacement suggests that all niche lineages except one will become extinct. It is fairly clear that clonal succession is related to tumor progression. The situation in a normal individual, where stem cells are wild type (APC +/+) and one APC allele is lost in a single stem cell. This APC +/− stem cell, unless it possesses a growth advantage, could be lost by the stochastic ejection from the niche – indeed, it could be argued that this will happen in the overriding majority of instances, but the

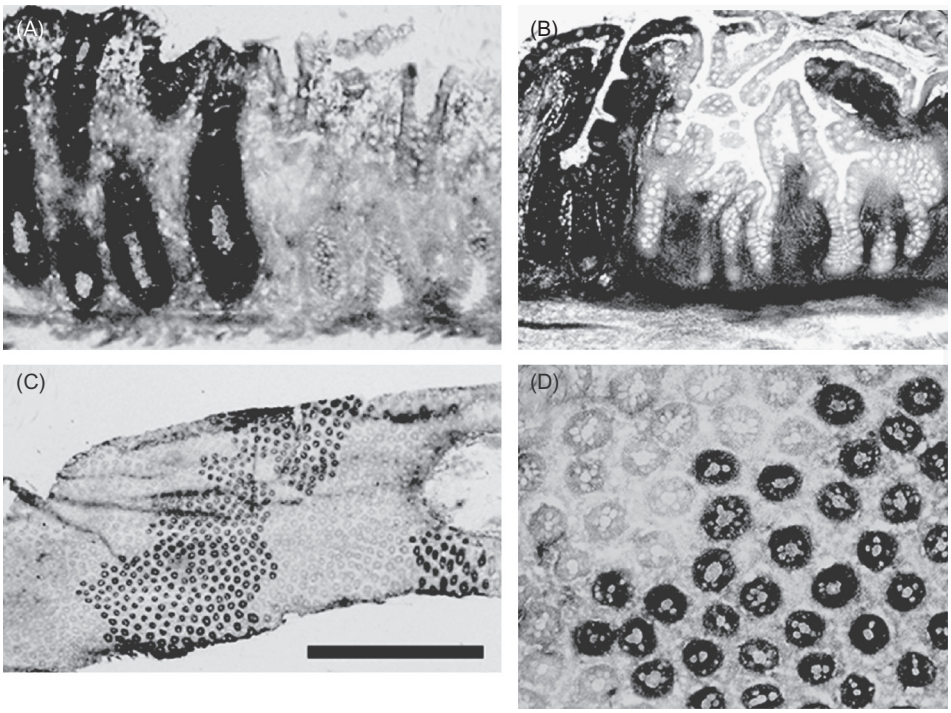

FIGURE 25.6 **Visualization of X-inactivation patches directly by enzyme histochemistry in surgical resections from Sardinian females heterozygous for the G6PD Mediterranean mutation (563 C 3 T), previously shown to have reduced G6PD enzyme activity.**
Heterozygosity for the G6PD Mediterranean mutation was confirmed by polymerase chain reaction analysis of genomic DNA followed by MBOII restriction endonuclease. (A) G6PD staining in longitudinal sections of colonic crypts. In individual crypts, all epithelial cells show a similar staining pattern. (B) Longitudinal sections of crypts and villi in small intestinal mucosa stained for G6PD activity. Epithelial cells in individual crypts show a similar staining pattern, but the villous epithelium shows areas of positive and negative staining, confirming its polyclonal derivation. (C) G6PD staining in colonic patches in low power. (D) High-power view of large patches of crypts with irregular patch borders. Bar indicates 2 mm. *Reproduced with permission from Novelli M, Cossu A, Oukrif D, Quaglia A, Lakhani S, et al. 2003. Proc Natl Acad Sci USA 100: 3311–4.*

APC +/− cell could survive and the niche will be populated by progeny of this APC +/− cell, which will resemble an FAP crypt. A further hit in this crypt will lead to the formation of the monocryptal adenoma on the model proposed previously.

Others have also concluded that migrating crypt epithelial cells in the upper part of the crypt are the primary targets for transformation by *APC* mutation, and this has received some experimental support. At the bottom of the crypt, progenitor cells accumulate nuclear β-catenin and express *Tcf* target genes as a result of *Wnt* stimulation from surrounding basal pericryptal

myofibroblasts. In normal crypts, cells that reach the mid-crypt region down-regulate β-catenin/*Tcf*, resulting in cell cycle arrest and differentiation. Cells that bear a β-catenin or an *APC* mutation do not respond to signals controlling β-catenin/*Tcf* activity, and these cells continue to behave as crypt progenitor cells in the surface epithelium, generating microadenomas. Computer modeling has yielded data to suggest that an expansion in the crypt stem cell population explained the putative proliferative abnormality in FAP, namely an upwards shift in the proliferative compartment toward the top of the crypt. Simulation of labeling index distribution curves from FAP crypts using a single mechanistic design was able to fit the data from both control and FAP crypts, indicating that the proliferative abnormality does not alter the rate of cell cycle proliferation, differentiation, or apoptosis of proliferating crypt cells. Instead, it suggests an expansion in the crypt stem cell population sufficient to explain the observed proliferative abnormality in FAP. Thus, β-catenin signaling in the colonic crypt controls the number of stem cells. The stem cell population is expanded in FAP crypts because of a germ line APC mutation activating Tcf-4. Any increase in the size of the stem cell population might be expected to result in an increase in the rate of crypt fission.

Crypt fission is therefore an essential event in the expansion of mutated clones in adenomas. Although the morphology of this process is distinct, the molecular mechanisms that govern it are far from clear. We further conclude that the initial event in the genesis of colorectal adenomas, of both sporadic and FAP adenomas, is the monocryptal adenoma; initial growth occurs through crypt fission, and spread into adjacent crypt territories is a later, secondary event.

25.14 SUMMARY

The cells of the gastrointestinal tract undergo constant renewal and respond to damage by regeneration and repopulation. Each region of the gastrointestinal tract is morphologically distinct, with its own repertoire of cell types. Although the stem cells are the most important cells of the gastrointestinal tract, responsible for the production of every other cell type in the gastrointestinal mucosa, they have not yet been closely characterized. There is, initially at any rate, a single stem cell in every intestinal crypt or gastric gland that indirectly generates a clone containing further stem cells, transit amplifying and differentiated cells, through the production of committed progenitor cells. This cell also produces new crypts by crypt fission, repairs entire crypts and villi when damaged, and generates gastrointestinal tumors. The stem cell or cells occupy a niche, formed by mesenchymal cells such as the ISEMFs, and extracellular matrix molecules, which regulate epithelial stem cells through mesenchymal-epithelial crosstalk. The molecular events that regulate the

development of the gastrointestinal tract and epithelial cell turnover in the normal tissue and in formation of carcinomas are beginning to be identified. It is clear that the Wnt/β-catenin signaling pathway and downstream molecules such as APC, Tcf-4, Fkh-6, Cdx-1, and Cdx-2 are vital for normal gastrointestinal stem cell function. We are beginning to identify molecular pathways that determine further proliferation of committed progenitor cells into specific epithelial cell lineages: The Notch–Delta signaling pathways involving Hes1 and Math1 transcription factors regulate differentiation of goblet, Paneth, and enteroendocrine cells in the small intestine. Factors secreted and expressed by the mesenchymal cells (KGF, HGF, etc.) that regulate gastrointestinal mucosal development and epithelial proliferation are rapidly becoming identified. Epithelial and intestinal subepithelial myofibroblast lineages are apparently derived from bone marrow, presenting the possibility of delivering therapeutic genes to the damaged lamina propria – for example, in diseases that cause fibrosis, such as Crohn's disease – and even repopulating the damaged gut. Finally, we conclude that the colonic stem cell is pivotal in understanding mechanisms of tumorigenesis in the colon. The isolation and characterization of gastrointestinal stem cells is a priority in gut biology.

FOR FURTHER STUDY

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CHAPTER 26

Induced Pluripotent Stem Cells

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26.1 GENERATION OF iPS CELLS

26.1.1 Reprogramming Factors

iPS cells are established by the forced expression of several transgenes. The classic mixture is Oct3/4, Sox2, Klf4, and c-Myc. This mixture can reprogram somatic cells of the mouse, human, rat, monkey, and dog. All of these factors have transcriptional activity, and Oct3/4, Sox2, and Klf4 regulate many ES cell-specific genes in combination. These factors also regulate their own expression. There are families of genes for Oct3/4, Sox2, and Klf4, and some of them can induce iPS cells. For example, Sox2 can be replaced with Sox1, Sox3, Sox7, Sox15, Sox17, or Sox18, and Klf4 with Klf2. Comparing the target genes among reprogramming factors and the family genes might be useful for understanding the molecular mechanisms underlying iPS cell formation. Other combinations, such as Oct3/4, Sox2, Nanog, and Lin28, have been reported for the generation of human iPS cells. Nanog is one of the most important transcription factors for stabilizing the pluripotent state in mouse ES cells. It also makes a transcriptional circuit with Oct3/4, Sox2, and Klf4. Oct3/4, Sox2, and Nanog bind and upregulate ES cell-specific genes such as STAT3 and ZIC3 with RNA polymerase II. On the other hand, they also localize to developmental regulator genes, such as PAX6 and ATBF1, with SUZ12, where they work as suppressors. The forced expression of some core components of ES cells would induce ES cell-like transcription networks in somatic cells and change their state. c-Myc is associated with many aspects of reprogramming, but its precise function is unclear. The process of iPS induction is thought to have some stochastic events dependent on cell proliferation, such as passive DNA demethylation. The expression of c-Myc blocks cell senescence, accelerates proliferation of fibroblasts, and leads to enhancement of iPS induction. c-Myc binds to more than 4,000 sites of the

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genome; therefore, it could loosen tightly packed chromosomes in somatic cells and increase the accessibility of other transcription factors to the genome during iPS induction. Overexpression of c-Myc itself also shifts the gene expression profile of mouse embryonic fibroblasts (MEFs) towards pluripotent cells. LIN28 is an RNA-binding protein and negatively regulates Let7 microRNA (miRNA) families. Let7 promotes differentiation of breast cancer cells and inhibits their proliferation. Therefore, LIN28 seems to indirectly enhance reprogramming efficiency through Let7 families. A combination of extra factors used in the induction can improve the reprogramming efficiency and quality. The addition of transcription factors, such as ESRRB26, UTF127, and SALL428, increased the efficiency. All of these factors are expressed in ES cells, and are involved in the formation of an ES-like transcriptional network. Tbx3 significantly improves the quality and the germ-line competency of mouse iPS cells. Some variations of inducing factors have been reported for iPS generation. The factor(s) in the reprogramming cocktail can be reduced if the somatic cells have sufficient endogenous expression of either of the reprogramming factor(s). For example, neural precursor cells express endogenous SOX2, KLF4, and c-MYC, and they only need OCT3/4 transgenes for iPS cell induction.

The acceleration of cell proliferation and the inhibition of senescence by the suppression of the p53 and p21 pathways can also dramatically increase the efficiency. An increase in the number of cells under induction results in high iPS colony formation because the reprogramming process includes stochastic events. Suppression of p53 increases reprogramming efficiency predominantly through acceleration of cell division. On the other hand, the addition of Nanog to the reprogramming factor upregulated the net reprogramming efficiency, in a cell-division-independent manner. However, the suppression of the p53 and p21 pathways increases the genomic instability of iPS cells. Therefore, the permanent suppression of the pathway should be avoided because it would lower the quality of iPS cells. The transient suppression of inhibitors or siRNAs could be useful for the enhancement of reprogramming.

iPS cell induction takes at least one week in the mouse and two weeks in humans. On the other hand, reprogramming by fusion of ES cells occurs very rapidly. The activation of endogenous Oct3/4 promoter of somatic cell nuclei is observed within two days. Although transgene expression in iPS cells requires a few days after vector transduction, the reprogramming of iPS cells seems to take much more time than that of cell fusion. ES cells must have other factor(s) that facilitate the reprogramming. Reprogramming events occur naturally *in vivo* during early developmental stages. The fertilized eggs erase almost all epigenetic status except imprinting before blastocyst formation, and they rebuild it as differentiation proceeds. The eggs also have high reprogramming activity, since they can produce a cloned animal after enucleation and fusion with somatic cells. Although the mechanism remains elusive, cloning might provide

helpful hints for improving the generation of iPS cells. However, cloned mice have some abnormalities, such as a large placenta and a tendency to gain excess weight. There may be some limitation in the artificial reprogramming that must be considered.

26.1.2 Transduction Methods

iPS cells were originally established by the delivery of transgenes by MMLV (Moloney murine leukemia virus)-based retroviral vectors. A retrovirus can robustly infect mouse fibroblasts and introduce its RNA genome into the host genome by reverse transcriptase. Therefore, the iPS cells integrate numerous transgenes, which thereby enable constant transgene expression. The inactivation of the retroviral promoter by DNA methylation is observed in ES cells as well as in iPS cells. Therefore, the expression of retroviral transgenes is gradually suppressed during the reprogramming process, and the silencing is complete when the cells become iPS cells. This automatic silencing mechanism is thought to provide effective reprogramming in somatic cells. However, the exogenous sequences remain in the genome of iPS cells and the alteration of genomic organization could induce some abnormalities. In particular, c-Myc, one of the reprogramming factors, is a proto-oncogene, and its reactivation could give rise to transgene-derived tumor formation. There have been improvements in the transduction methods for making safe iPS cells. Elimination of the c-Myc transgene for iPS cell induction is one important approach. Human and mouse iPS cells can be established from fibroblasts with only Oct3/4, Sox2, and Klf4, although the efficiency is significantly lower. Mouse iPS cells without c-Myc do not show enhanced tumor formation during the observation period (six months) in comparison to control mice. Another approach is to reduce the number of integration sites by attaching the reprogramming factors with internal ribosome entry sequences (IRES) or 2A self-cleavage peptide and putting them into a single vector. This reprogramming cassette was used with a lentivirus system containing a loxP sequence and produced iPS cells with only single insertions. The expression of Cre recombinase successfully cuts out the cassette, although a truncated long terminal repeat (LTR) remains in the iPS genome. The elimination of transgenes from the genome avoids the leaky expression of reprogramming factors, and improves the gene expression profile and the differentiation potential of iPS cells. A transposon system has also been used for iPS induction. A plasmid-based transposon vector with a reprogramming cassette can integrate into host genome with transposase. The re-expression of the transposase after establishment of iPS cells recognizes the terminal repeat of integrated transposon vector, and excises it from the genome. The excision of the transposon does not leave a footprint in most cases, so it maintains the original endogenous sequences. Non-integration methods were also reported with viral vectors

(adenovirus and sendaivirus), DNA vectors (plasmid, episomal plasmid, and minicircle vector), and direct protein delivery. Although the induction efficiency of iPS cells with these methods is still low, they could become future standard methods.

26.1.2.1 Culture Conditions and Cell Signaling

Culture conditions and cell signaling have a great influence on iPS generation. iPS cells are cultured in medium optimized for ES cells. Leukemia inhibitory factor and basic fibroblast growth factor are important for mouse and human ES cell maintenance, respectively. However, the roles of these cytokines in the induction process are still unclear. Wnt signaling supports the self-renewal of ES cells. The Wnt3a signal is mediated by glycogen synthase kinase (GSK) 3-β. Without the Wnt signal, GSK3-β inactivates target genes, such as β-catenin and c-Myc, by phosphorylation and proteasome-mediated degradation. Hence, the inhibition of GSK3-β with a chemical drug, such as CHIR99021, results in activation of Wnt signaling. Addition of Wnt3a or CHIR99021 enhances the reprogramming efficiency. Kenpaullone is an inhibitor whose targets are GSK3-β as well as cyclin-dependent kinase (CDK)s and can replace Kruppel-like factor 4 (Klf4) in reprogramming induction from MEF with Oct3/4, Sox2, and c-Myc. Although more specific GSK3-β inhibitors, such as CHIR99021, or CDK inhibitor, purvalanol A, were unable to generate mouse iPS cells with the same combination of transcription factors and Kenpaullone itself did not increase endogenous Klf4 expression, the function of Kenpaullone is still elusive. Importantly, Li et al. found that the combination of CHIR99021 and Parnate, an inhibitor of lysine-specific demethylase 1, can generate iPS cells from human primary keratinocytes with only Oct3/4 and Klf4. The addition of vitamin C enhances iPS cell generation from both mouse and human somatic cells. Vitamin C works at least in part by alleviating cell senescence.

O_2 tension is also an important factor for stem cell maintenance and differentiation. For instance, low O_2 tension promotes the survival of neural crest cells and hematopoietic stem cells, and prevents differentiation of human ES cells. Up to fourfold enhancement of the reprogramming efficiency is observed when the iPS induction is performed in hypoxic conditions (5% O_2), in both mouse and human fibroblasts.

26.1.3 Cell Source

iPS cells were first established from primary mouse fibroblast culture. Their origin was thought to be some tissue stem cells included in the culture since the efficiency of iPS cell induction was very low (less than 0.1%). Mouse iPS cells can be established from mouse hepatocytes and stomach epithelial cells and linage tracing experiments showed that most hepatocyte-derived iPS cells were from albumin-positive cells. Mouse iPS cells were also established

from pancreatic islet β cells. Therefore, the origin of iPS cells is not only tissue stem cells but also differentiated somatic cells. Human iPS cells have been established from various tissues, including fibroblasts (adult and embryo), adult keratinocyte, adipose tissue, peripheral blood, cord blood, amniotic fluid-derived cells, and neural precursor cells. Hence, all somatic cells are thought to have the ability to yield iPS cells, although they show differential efficiency. However, it is unclear whether iPS cells from different cell sources have the same potential. Mouse iPS cells derived by the current reprogramming method from different tissues apparently have divergent characteristics. Miura et al. compared neural differentiation potential and safety of mouse iPS cells derived from MEF, tail-tip fibroblasts (TTFs), and hepatocytes. Most iPS clones form a neural sphere under *in vitro*-directed differentiation conditions. The neural sphere contains neural precursor cells that can produce three neuronal cell types; neuron, astrocyte, and oligodendrocyte. The neurosphere from ES cells could contribute the neural tissues when transplanted into the mouse brain. However, the neurospheres prepared from TTF-derived iPS cells tended to form teratomas after transplantation into mouse brain. Teratoma formation has been reported in the transplantation of neurospheres formed from ES cells containing undifferentiated cells that remained after the differentiation process. The population of undifferentiated cells is rare in neurospheres from MEF-derived iPS cells and ES cells, but is obvious in those from TTF-derived iPS cells (up to 20%). The study revealed that the existence of undifferentiated cells varies depending on the cell source. Accessibility to a cell source is another important point in the selection of tissues, especially for induction of human iPS cells. Human iPS cells can be established from neural precursor cells with only OCT3/4 transgenes; however, constant acquisition of the neural tissue is difficult.

26.2 MOLECULAR MECHANISMS IN iPS CELL INDUCTION

26.2.1 Epigenetics

The generation of iPS cells includes epigenetic alterations. DNA methylation status and histone modifications of promoter regions including Nanog, Oct3/4, Sox2, and Fbxo15 achieve an ES-like state after reprogramming. The addition of a histone deacetylase (HDAC) inhibitor, valproic acid (VPA), improves the reprogramming efficiency in both mouse and human fibroblasts. Other HDAC inhibitors, such as suberoylanilide hydroxamic acid and trichostatin A, also work in mouse fibroblasts. Inhibitors of DNA methyltransferase, such as 5′-azacytidine and RG108, and BIX-01294 for G9a histone methyltransferase increased reprogramming efficiency. These results supported the hypothesis that the process of iPS generation involves epigenetic changes. Some of the

inhibitors could abolish the use of one or two reprogramming factor(s). For example, VPA treatment of human fibroblasts enables reprogramming with only two factors, Oct4 and Sox2, and eliminates the oncogenic c-Myc or Klf4. However, it is doubtful whether these drugs fill in the exact function of reprogramming genes; rather, they seemed to enhance the induction efficiency that allows the reduction of reprogramming factor(s).

iPS induction requires the establishment of an ES-like transcription factor circuit in somatic cells. In fact, iPS cells have the same expression profile as ES cells; however, they have differences in epigenetic modifications, especially in genes not involved in pluripotency. Cell differentiation is a process of limitation of the differentiation potential by epigenetic modification. Each type of somatic cell has its specific epigenetics by which cells are able to stabilize their state. The forced expression of reprogramming factors can affect several downstream genes in somatic cells and alter their epigenetic modifications. However, it is difficult to think that the factors control all genes throughout the genome. In fact, genome-wide analysis showed similar DNA methylation patterns of iPS and ES cells, but they also detected differentially methylated regions between iPS and ES cells. The uncontrolled genes would keep their epigenetic profiles even in iPS cells. This could influence the differentiation potential of iPS cells. For example, the methylation status of the enhancer binding site in the astrocyte gene, GFAP, controls the differentiation fate of neuronal precursor by changing the binding activity for an enhancer, STAT3. Without such methylation they instead tended to become astrocytes, whereas in the presence of methylation they tended to demonstrate neuronal differentiation.

26.2.2 MicroRNAs

miRNAs are small single-stranded RNAs (around 22 nt) that directly interact with target mRNAs through complementary base-pairing and inhibit the expression of the target genes. miRNAs also work at the transcriptional level. miRNAs are generated as long RNA sequences and are digested to the short mature form by Dicer. miRNAs are involved in many features of cell properties, such as proliferation, apoptosis, and differentiation, by fine-tuning gene expression. ES cells have the characteristic expression of miRNAs, and iPS cells also showed a similar expression profile. Over 70% of mRNAs in mouse ES cells are the miR-290 cluster, which contributes to the ES cell-specific rapid cell cycle progression. The cluster includes miR-291-3p, miR-292-3p, miR-293, miR-294, and miR-295. miR-291-3p, miR-294, or miR-295 increases the reprogramming efficiency from MEF with Oct4, Sox2, and Klf4. They appear to be downstream targets of c-Myc, because the miRNAs did not enhance reprogramming efficiency in the presence of c-Myc transgene, and c-Myc binds the promoter region of the cluster. The three miRNAs share a conserved seed sequence, which mainly specifies target genes, suggesting they work through common

targets. LIN28 is a negative regulator of Let7 miRNA families. Lin28 induced the uridylation of immature let7 RNA by a non-canonical poly (A) polymerase, TUTase4, and this leads to degradation of the RNA. Lin28 gradually decreases during ES cell differentiation, and mature let7 family miRNAs accumulate with inverse correlation. The addition of Lin28 enhances the reprogramming efficiency from both human and mouse fibroblasts. A detailed analysis showed that Lin28 accelerates the reprogramming efficiency in a cell cycle-dependent manner. This is consistent with the concept that the targets of mature let7 include oncogenic genes, such as K-Ras and c-Myc. Lin28 facilitates the expression of Oct4 at the post-transcriptional level by direct binding to its mRNA.

26.3 RECAPITULATION OF DISEASE ONTOLOGY AND DRUG SCREENING

Patient-derived iPS cells are useful in understanding disease ontology. The iPS cells have the same genomic information as the patient. Many iPS cells have been established from somatic cells obtained from patients with adenosine deaminase deficiency-related severe combined immunodeficiency, Duchenne and Becker muscular dystrophy, and amyotrophic lateral sclerosis. Ebert et al. established iPS cells from skin fibroblast of a spinal muscular atrophy (SMA) patient. SMA is an autosomal recessive genetic disorder that is characterized by degeneration of motor neurons following progressive muscular atrophy. The most common cause of SMA is a mutation of the survival motor neuron 1 (SMN1) gene, and it significantly reduces the level of protein expression. The motor neurons generated from the patient's iPS cells can recapitulate the disease ontology, as they show reduced survival motor neuron (SMN) expression in comparison to those derived from the child's unaffected mother. Treatment with VPA or tobramycin increases SMN expression. Importantly, the same treatment also worked in the motor neurons prepared from the patient's iPS cells. The results indicate that iPS cells could provide a useful screening system for the identification of a specific, effective drug from thousands of candidate compounds.

Such patient-derived iPS cells could also be used to find developing drugs that would be harmful to the human body. Some compounds work on target tissue, but have severe side effects. Long QT syndrome is an inborn heart defect that shows characteristic prolongation of the QT interval on electrocardiogram, increases the risk of irregular heartbeat, and threatens life. It occurs only after drug administration in some individuals. Cardiomyocytes established from patients with long QT syndrome via iPS cells could therefore be used to identify any possible toxic side effect of candidate compounds before starting clinical trials.

Most diseases do not have a simple cause; they are the total sum of genetic/epigenetic issues, environment, aging, etc., in a complicated relationship between several cell types in the body. It is therefore necessary to establish a way to recapitulate late-onset disease and environmental effects *in vitro* or in an animal model.

26.4 iPS CELL BANKING

It will require time to establish a clinical grade of useful cells from a patient's own somatic cells. The applicability and safety of each cell type must be assessed. The clinical applications of iPS cells must also be considered from an economic point of view. Complete tailor-made iPS cell therapy would cost too much to apply to a large number of people. Therefore, a banking system should be established for iPS cells. iPS cells having various human leukocyte antigen (HLA) haplotypes should be collected to avoid immune rejection. Experience with organ transplantation has revealed that the HLA class I molecules, HLA-A and HLA-B, and the class II molecule, HLA-DR, are the most important HLA molecules to match. Therefore, the HLA matching of these loci reduces the incidence of acute rejection and improves transplant survival. Estimations of stem cell bank size have been calculated in Japanese and UK populations. The random establishment of 170 lines of iPS cells would provide donor lines for 80% or more of patients with a single mismatch at one of three HLA loci (HLA-A, -B, and -DR) among the Japanese. A comparable bank of 150 lines could provide an acceptable or better match for 84.9% of the UK population. Importantly, a bank size of only 50 lines could provide a three-locus match in 90.7% of the Japanese population, if iPS cells are established from HLA homozygous cells. Screening an HLA-type database of 24,000 individuals would be required to identify at least one homozygote for each of 50 different HLA haplotypes. This could be possible if the iPS banks cooperate with other banks, in the same manner as do cord blood banks and bone marrow banks.

26.5 SAFETY CONCERNS FOR MEDICAL APPLICATION

Safety is extremely important for the clinical application of iPS cells. Each culture of iPS cells would have different properties in terms of differentiation and safety. Human iPS cells can be generated from several cell types with different combinations of reprogramming factors by various transduction methods, as described above. As yet, no one knows the best way to obtain fully reprogrammed, safe iPS cells. Assays of chimeric mice have revealed that genomic integration of c-Myc transgene is associated with a high risk of tumor formation and should be avoided. The integration of Oct3/4, Sox2,

and Klf4 seems to have no/little effect on tumorigenesis. However, the overexpression of Oct3/4 and Klf4 causes tumor formation, and various human tumors express OCT3/4, SOX2, and KLF4. Furthermore, retroviral insertion into the genome may itself disturb endogenous gene structure and increase tumor risks. However, there are between one and 40 genomic integration sites of retro- and lentivirus in iPS cells, and PCR-based analysis can detect them all. Therefore, it is possible to estimate the risk beforehand. Non-integration methods have been established, but they have low induction efficiency of iPS cells, which suggests they yield reprogrammed iPS cells of lower quality than integration methods. This might be improved by using better combinations of reprogramming factors and choosing a better cell source. The retroviral induction method might be selected after a careful risk assessment if it induces better reprogramming than other transient or non-integration methods.

Residual undifferentiated cells are a common problem when using stem cells for cell transplantation therapy. As described above, most mouse iPS cells can differentiate into neurospheres; however, a small portion of cells remains in an undifferentiated state in the sphere, thereby giving rise to tumor formation when transplanted. An effective protocol to eliminate undifferentiated cells should be established, such as improvement of the differentiation protocol and sorting by flow cytometry.

26.6 MEDICAL APPLICATION

Mouse iPS cells have been applied to the treatment of a humanized sickle cell anemia mouse model. Homozygous mice for mutant human β-globin genes show characteristic symptoms, including severe anemia due to erythrocyte sickling, splenic infarcts, urine concentration defects, and poor health. The iPS cells established from the mouse have the same genomic mutation. The mutation was corrected by homologous recombination with a non-mutated construct. The rescued iPS cells were differentiated into hematopoietic progenitors and transplanted into a 'patient' mouse. The study provided proof of principle for application of iPS cells in combination with gene repair for cell therapy. Efficient gene correction methods have been established in human pluripotent stem cells. Homologous recombination occurs in human ES cells with helper-dependent adenoviral vectors. Homologous recombination was also performed using zinc-finger nuclease-mediated genome editing in both human ES and iPS cells. Human disease-corrected iPS cells have been established from Fanconi anemia patients by lentiviral delivery of a normal gene. Duchenne muscular dystrophy is caused by defect of the Dystrophine gene, which has an extremely large size of 2.4 Mbp. iPS cells from a Duchenne muscular dystrophy (DMD) patient were transferred and corrected with the

human-artificial-chromosome-encoding Dystrophine gene. These techniques could supply patient-specific but gene-corrected iPS cells.

26.7 DIRECT FATE SWITCH

The establishment of iPS cells introduced a new paradigm: that forced expression of master genes can alter the cell state. This contributes to the study of direct reprogramming from one somatic cell type into another cell type without the mediation of stem cells. One group screened more than 1,100 transcription factors and chose nine candidates for β-cell induction. Combinations of these genes were inserted by adenovirus vectors into the pancreata of mice. Insulin-positive cells developed in one month with the mixture of Ngn3, Pdx1, and Mafa. The cells were derived from pancreatic exocrine cells and closely resembled normal β-cells. These cells could ameliorate hyperglycemia by remodeling the local vasculature and secreting insulin in mice rendered diabetic by streptozotocin injection. Another example is the conversion of mouse fibroblasts into neurons by induction of three transcription factors, Ascl1, Brn2, and Myt1l. The induced neuronal (iN) cells expressed several neuronal markers, generated action potentials, and formed functional synapses. The iN cells are useful for neurological disease modeling and regenerative medicine. Although further study is required, these approaches could therefore form an alternative method for making specific differentiated cells from a patient's somatic cells or iPS cells.

26.8 CONCLUSION

iPS cells have tremendous potential to supply patient-specific pluripotent stem cells for use in the study of disease pathogenesis, drug discovery, toxicology, and cell transplantation therapy. Several lines of evidence support the finding that iPS cells are very similar, but not identical, to ES cells. However, there is insufficient data to definitively determine whether or not this difference is critical. Mouse and rat iPS cells can contribute to chimeric animals after injection into blastocysts. Direct and detailed comparison between iPS cells and ES cells is required. The establishment of iPS cells would also apply not only to the medical field but also to the elucidation of the control mechanisms of stem cells and the development of efficient differentiation protocols. Studies of disease pathogenesis and drug discovery have already been launched, and the results could provide relief to countless people throughout the world. The application of iPS cells to human disease will take time. In addition, both the research and medical application of human iPS cells will also be subject to a wide range of laws and research ethical policies.

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CHAPTER 27

Embryonic Stem Cells: Derivation and Properties

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27.1 DERIVATION OF EMBRYONIC STEM CELLS

27.1.1 Embryonic Carcinoma Cells

Teratocarcinoma is a form of malignant germ cell tumor that occurs in both animals and humans. These tumors comprise an undifferentiated embryonal carcinoma (EC) component and differentiated derivatives that can include all three germ layers. Although teratocarcinomas had been known as medical curiosities for centuries, it was the discovery that male mice of strain 129 had a high incidence of testicular teratocarcinomas that made these tumors more routinely amenable to experimental analysis. Because their growth is sustained by a persistent EC cell component, teratocarcinomas can be serially transplanted between mice. In 1964, it was demonstrated that a single EC cell was capable of both self-renewal and multilineage differentiation, and this formal demonstration of a pluripotent stem cell provided the intellectual framework for both mouse and human ES cells.

The first mouse EC cell lines were established in the early 1970s. EC cells exhibit similar antigen and protein expression to the cells present in the inner cell mass (ICM) and this observation led to the notion that EC cells are the counterpart of pluripotent cells present in the ICM. When injected into mouse blastocysts, some EC cell lines are able to contribute to various somatic cell types, but most EC cell lines have limited developmental potential and contribute poorly to chimeric mice, probably reflecting genetic changes acquired during teratocarcinoma formation. Mutations that confer growth advantages to EC cells are likely to accumulate during tumorigenesis, and EC cells in chimeras can result in tumor formation. As a result, there are limitations in the application of EC cells to both regenerative medicine and to research in basic developmental biology.

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Following fertilization, as the one cell embryo migrates down the oviduct, it undergoes a series of cleavage divisions resulting in a morula. During blastocyst formation, the outer cell layer of the morula delaminates from the rest of the embryo to form the trophectoderm. The ICM of the blastocyst gives rise to all the fetal tissues (ectoderm, mesoderm, and endoderm) and some extraembryonic tissues, and the trophectoderm gives rise to the trophoblast. Although the early ICM can contribute to the trophoblast, the late ICM does not, suggesting there is some restriction in developmental potential at this stage. In normal embryos, the pluripotent cells of the embryo have a transient existence, as these cells quickly give rise to other non-pluripotent cells through the normal developmental program. Thus, the pluripotent cells of the intact embryo really function *in vivo* as precursor cells and not as stem cells. However, if early mouse embryos are transferred to extrauterine sites, such as the kidney or testis capsules of adult mice, they can develop into teratocarcinomas that include pluripotent stem (EC) cells. These ectopic transplantation experiments result in teratocarcinomas at high frequencies, even in strains that do not spontaneously have elevated incidence of germ cell tumors, suggesting that this process is not the result of rare neoplastic transformation events. These key transplantation experiments led to the search for culture conditions that would allow the *in vitro* derivation of pluripotent stem cells directly from the embryo, without the intermediate need to form teratocarcinomas *in vivo*.

27.1.2 Derivation of Embryonic Stem Cells

In 1981, pluripotent embryonic stem (ES) cell lines were derived directly from the ICM of mouse blastocysts using culture conditions previously developed for mouse EC cells. ES cell cultures derived from a single cell could differentiate into a wide variety of cell types, or could form teratocarcinomas when injected into mice. Unlike EC cells, however, these karyotypically normal cells contributed at a high frequency to a variety of tissues in chimeras, including germ cells, and thus provided a practical way to introduce modifications to the mouse germ line.

The efficiency in mouse ES cell derivation is influenced by genetic background. For example, ES cells can be easily derived from the inbred 129/ter-Sv strain, but less efficiently from C57BL/6 and other mouse strains, and these strain differences somewhat correspond with the propensity of mice of different strains to develop teratocarcinomas. These observations suggested that genetic and/or epigenetic components play an important role in the derivation of mouse ES cells. On the other hand, the efficiency of teratocarcinoma formation induced through extrauterine mouse embryo transplantations appears to be somewhat less strain dependent. This indicates that the difference in the efficiency of ES cell derivation from different mouse strains

might be due to suboptimal culture conditions. Indeed, mouse ES cells can be derived from some non-permissive strains using modified protocols; e.g., dual inhibition of differentiation-inducing signaling from mitogen-activated protein kinase and glycogen synthase kinase-3 (GSK3) enabled the efficient derivation of germ line-competent ES cells from non-obese diabetic mice.

ES cell lines are generally derived from the culture of the ICM, but this does not mean that ES cells are the *in vitro* equivalent to ICM cells, or even that ICM cells are the immediate precursor to ES cells. It is possible that, during culture, ICM cells give rise to other cells that serve as the immediate precursors. Some experiments suggest that ES cells more closely resemble cells from the primitive ectoderm, the cell layer derived from the ICM after delamination of the primitive endoderm. Isolated primitive ectoderm from the mouse gives rise to ES cell lines at a high frequency and allows the isolation of ES cell lines from mouse strains that had previously been refractory to ES cell isolation. Indeed, single primitive ectoderm cells can give rise to ES cell lines at a reasonable frequency, something not possible with early ICM cells. Although these experiments do suggest that ES cells are more closely related to primitive ectoderm than to ICM, they do not reveal whether ES cells more closely resemble primitive ectoderm or another cell type (for example, very early germ cells) derived from it *in vitro*. As no pluripotent cell in the intact embryo undergoes long-term self-renewal, ES cells are in some ways tissue culture artifacts. It is surprising that even more than 20 years after their derivation, the origin of these cells is not completely understood. Given the dramatic improvement in molecular techniques since the initial derivation in the 1980s, there is considerable value in re-examining the origin of ES cells to better understand the control of their proliferative pluripotent state.

In addition to their derivation from the ICM and isolated primitive ectoderm, mouse ES cells have also been derived from morula-stage embryos and even from individual blastomeres. Again, although the ES cell lines were derived from morula, there may well be a progression of intermediate states during the derivation process. The frequencies of success were lower when starting with morula or blastomeres, but these results do suggest that it might be possible to derive human ES cells without the destruction of an embryo. Such cell lines could prove useful to the child resulting from the transfer of a biopsied embryo, as they would be genetically matched to that child.

27.1.3 Derivation of Human Embryonic Stem Cells

In 1978 the first baby was born from an embryo fertilized *in vitro* and, without this event, the derivation of human ES cells would not have been possible. Although there were attempts to derive human ES cells as early as the 1980s, species-specific differences and suboptimal human embryo culture

media delayed their successful isolation until 1998. For example, the culture of isolated ICMs from human blastocysts was reported, but stable undifferentiated cell lines were not produced in medium supplemented with leukemia inhibitory factor (LIF) in the presence of feeder layers, conditions that allow the isolation of mouse ES cells. In the mid-1990s, ES cell lines were derived from two nonhuman primates: the rhesus monkey and the common marmoset. Experience with these ES cell lines and concomitant improvements in culture conditions for human in vitro fertilization (IVF) embryos resulted in the successful derivation of human ES cell lines. These human ES cells had normal karyotypes and, even after prolonged undifferentiated proliferation, maintained the developmental potential to contribute to advanced derivatives of all three germ layers.

To date, more than 120 human ES cell lines have been established worldwide. Although most were derived from isolated ICMs, some were derived from morulae or later blastocyst stage embryos. It is not yet known whether ES cells derived from these different developmental stages have any consistent differences or whether they are developmentally equivalent. Human ES cell lines have also been derived from embryos carrying various disease-associated genetic changes, which provide new *in vitro* models of disease.

27.2 CULTURE OF EMBRYONIC STEM CELLS

27.2.1 Culture of Mouse Embryonic Stem Cells

Mitotically inactivated feeder layers were first used to support difficult-to-culture epithelial cells, and were later successfully adapted for the culture of mouse EC cells and mouse ES cells. A medium that is 'conditioned' by co-culture with fibroblasts sustains EC cells. Fractionation of conditioned medium led to the identification of a cytokine, LIF, which sustains ES cells. LIF and its related cytokines act via the gp130 receptor. Binding of LIF induces dimerization of LIF/gp130 receptors, which in turn activates the latent transcription factor STAT3 and ERK mitogen-activated protein kinase (MAPK) cascade. STAT3 activation is sufficient for LIF-mediated self-renewal of mouse ES cells in the presence of serum. In contrast, suppression of the ERK pathway promotes ES cell proliferation. In serum-free medium, LIF alone is insufficient to prevent mouse ES cell differentiation but, in combination with BMP (bone morphogenetic protein, a member of the TGFβ superfamily), mouse ES cells are sustained. BMPs induce expression of Id (inhibitor of differentiation) proteins and inhibit the ERK and p38 MAPK pathways, thus attenuating the pro-differentiation activation of ERK MAPK pathway by LIF. These earlier works suggest the dependence on the extrinsic stimuli for the self-renewal of mouse ES cells, which was brought into question by recent studies. Inhibition of

the ERK cascade (e.g., SU5402 and PD184352 or PD0325901) and GSK3 (CHIR99021) was sufficient to support the derivation, proliferation, and pluripotency of mouse ES cells; i.e., mouse ES cells do not rely on the extrinsic signals for self-renewal. Indeed, such conditions enabled the efficient derivation of ES cells not only from previously non-permissive mouse strains, but also from refractory species.

27.2.2 Culture of Human Embryonic Stem Cells

Mitotically inactivated fibroblast feeder layers and serum-containing medium were used in the initial derivation of human ES cells, essentially the same conditions used for the derivation of mouse ES cells prior to the identification of LIF. However, it now appears largely to be a lucky coincidence that fibroblast feeder layers support both mouse and human ES cells, as the specific factors identified to date that sustain mouse ES cells do not support human ES cells. LIF and its related cytokines fail to support human or non-human primate ES cells in serum-containing media that supports mouse ES cells, and BMPs, when added to human ES cells, cause rapid differentiation in conditions that would otherwise support their self-renewal. Indeed, the LIF/STAT3 pathway has yet to be shown to have any relevance to the self-renewal of human ES cells.

In contrast to mouse embryonic stem (ES) cells, FGF signaling appears to be of central importance in the self-renewal of human ES cells. Basic FGF (bFGF or FGF2) allows the clonal growth of human ES cells on fibroblasts in the presence of a commercially available serum replacement. At higher concentrations, bFGF allows feeder-independent growth of human ES cells cultured in the same serum replacement. The mechanism through which these high concentrations of bFGF exert their functions is incompletely known, although one of the effects is the suppression of BMP signaling. Serum and the serum replacement currently used have significant BMP-like activity, which is sufficient to induce differentiation of human ES cells, and conditioning this medium on fibroblasts reduces this activity. At moderate concentrations of bFGF (40 ng/ml), the addition of noggin or other inhibitors of BMP signaling significantly decreases background differentiation of human ES cells. At higher concentrations (100 ng/ml), bFGF itself suppresses BMP signaling in human ES cells to levels comparable to those observed in fibroblast-conditioned medium, and the addition of noggin is no longer needed for feeder-independent growth. As more defined culture conditions are developed for human ES cells that lack serum products containing BMP activity, it is not yet clear how important the suppression of the BMP pathway will be, unless there is significant production of BMPs by the ES cells themselves. Also, the effects of BMP signaling could change depending on context. Even in mouse

ES cells, BMPs are inducers of differentiation unless they are presented in combination with LIF, and it is entirely possible that, in a different signaling context, the effects of BMPs on human ES cells could change.

Suppression of BMP activity by itself is insufficient to maintain human ES cells; thus, bFGF must be serving other signaling functions. Human ES cells themselves produce FGFs, and, in high-density cultures either on fibroblasts or in fibroblast-conditioned medium, it is not necessary to add FGFs. However, chemical inhibitors of FGF receptor-mediated phosphorylation cause differentiation of human ES cells under these standard culture conditions. The required downstream events are not yet well worked out, but some evidence implicates activation of the ERK pathway.

Although FGF signaling appears to have a central role in the self-renewal of human ES cells, other pathways have also been implicated. When combined with low to moderate levels of FGFs, TGFβ/Activin/Nodal signaling has a positive effect on the undifferentiated proliferation of human ES cells, and inhibition of this pathway leads to differentiation. However, one of the effects of inhibiting the TGFβ/Activin/Nodal pathway is a stimulation of the BMP pathway, which in itself would be sufficient to induce differentiation. Thus, it is not yet clear whether TGFβ/Activin/Nodal signaling has a role in human ES cell self-renewal independent of its effects on BMP signaling. Further studies directly inhibiting the BMP pathway in the context of inhibition or stimulation of the TGFβ/Activin/Nodal are needed to resolve this issue.

The molecular components of the Wnt pathway are well represented in human ES cells. In short-term cultures, activation of Wnt signaling by a pharmacological GSK-3-specfic inhibitor (BIO) has been reported to have a positive effect on human ES cell self-renewal, but, in a different study, inhibition of Wnt signaling or stimulation of Wnt signaling by the addition of recombinant Wnt proteins showed no effect on the maintenance of human ES cells. It is possible that the positive observed effect of BIO on human ES cells is mediated through other pathways.

For human ES cells to be used in a clinical setting, it would be useful for these cells to be derived and maintained in conditions that are free of animal products. For example, human ES cells derived with mouse embryonic fibroblasts were shown to be contaminated with immunogenic non-human sialic acid, which would cause an immune reaction if the cells were used in human patients. Towards this goal, protein matrices including laminin and fibronectin, and different types of human feeder cells, were developed to sustain human ES cells. New human ES cell lines have been derived in the absence of feeder cells, but in the presence of a mouse-derived matrix and a bovine-derived serum replacement product. Existing human ES cell lines have been grown in defined serum-free medium that included sphingosine-1-phosphate (S1P) and

platelet-derived growth factor (PDGF), but this medium does not eliminate the need for feeder layers. Existing human ES cell lines have also been adapted to feeder-free conditions in which none of the protein components are animal derived, but it is not yet known whether these specific conditions will allow derivation of new lines. Recent improvements in human ES cell culture have enabled the commercial development of completely defined, feeder-free culture conditions such as mTeSR1 and STEMPRO® hESC SFM. Such conditions allow the derivation of new cell lines that will be more directly applicable to therapeutic purposes.

During extended culture, genetic changes can accumulate in human ES cells. The status of imprinted genes appears to be relatively stable in human ES cells, but can also change. Such genetic and epigenetic alterations present a challenge that must be appropriately managed if human ES cells are to be used in cell replacement therapy. The rates at which these changes accumulate in culture likely depend on the culture system used and the particular selective pressures applied. For example, in all current culture conditions, the cloning efficiency of human ES cells is poor: typically 1% or less. If cells are dispersed into a suspension of single cells, there is a tremendous selective pressure for cells that clone at a higher efficiency, and indeed such an increase in cloning efficiency is observed in karyotypically abnormal cells. Enzymatic methods of passaging ES cells can allow long-term passage without karyotypic changes if the clump size is carefully controlled, but, if such methods are used to disperse cells to single cell suspensions or small clumps, karyotypic changes are more frequent. This is a likely explanation for why mechanical splitting of individual colonies allows such long-term karyotypic stability. Understanding the rates at which genetic changes occur and the selective pressures that allow them to overgrow a culture in different culture conditions will be critical to the large-scale expansion and clinical use of human ES cells. For example, ROCK inhibitors could significantly improve the survival of dissociated human ES cells. Inclusion of these small molecules could potentially minimize the selection pressure and facilitate the development of large-scale human ES cell culture.

27.3 DEVELOPMENTAL POTENTIAL OF EMBRYONIC STEM CELLS

27.3.1 Differentiation of Embryonic Stem Cells

Since ES cells have the ability to differentiate into clinically relevant cell types, such as dopamine neurons, cardiomyocytes, and β-cells, there is tremendous interest in using these cells both in basic biological research and in transplantation medicine. Both uses demand a great deal of control over lineage allocation and expansion. There are several experimental approaches to demonstrating the developmental potential of embryonic stem cells and to

directing their differentiation to specific lineages. These approaches range in complexity and experimental control; from allowing the ES cells to respond to normal developmental cues in a chimera within an intact embryo, to the addition of defined growth factors to a monolayer culture.

Mouse ES cells reintroduced into blastocysts participate in normal embryogenesis, even after prolonged culture and extensive manipulation *in vitro*. In such chimeras, the progeny of ES cells contribute to both somatic tissues and germ cells. When ES cells are introduced into tetraploid blastocysts, mice entirely derived from ES cells can be produced, as the tetraploid component is outcompeted in the ICM-derived somatic tissues. Although mice entirely derived from ES cells can be generated, signals from the ICM of the blastocyst are likely necessary for mouse ES cells to contribute to offspring, as fetal development has not been reported when the ICM is completely replaced with ES cells.

ES cells injected into syngeneic or immunocompromised adult mice form teratomas that contain differentiated derivatives of all three germ layers (ectoderm, mesoderm, and endoderm). This property is similar to both early embryos and EC cells, and is an approach now routinely used to demonstrate the pluripotency of human ES cells. Very complex structures resembling the neural tube, gut, teeth, and hair form in these teratomas in a very consistent temporal pattern, and these teratomas do offer an experimental model to study the development of these structures in human material, but the environment of differentiation is complex and difficult to manipulate.

Aggregates of EC cells or ES cells cultured in conditions that prevent their attachment form cystic 'embryoid bodies' that recapitulate some of the events of early development. Differentiated derivatives of all three germ layers form in these structures, and for ES cells the temporal events occurring mimic *in vivo* embryogenesis. The formation of embryoid bodies has been used, for example, to produce neural cells, cardiomyocyte, hematopoietic precursors, β-like cells, hepatocytes, and germ cells. The formation of a three-dimensional structure in embryoid bodies (EBs) is useful to promote certain developmental events, but the complicated cell-cell interaction makes it difficult to elucidate the essential signaling pathways involved.

A somewhat more controlled method to differentiate ES cells is to co-culture them with differentiated cells that induce their differentiation to specific lineages. For example, MS5, S2, and PA6 stromal cells have been used to derive dopamine neurons from human ES cells; bone marrow stromal cell lines S17 and OP9 support efficient hematopoietic differentiation. The inducing activity provided by such stromal cells, while efficient in directing ES cell differentiation, contains many unknown factors, and such activity can change both between and within cell lines as a function of culture conditions.

An even more controlled method is differentiation in monolayers on defined matrices in the presence of specific growth factors. Both mouse and human ES cells differentiate into neuroectodermal precursors in monolayer culture, and human ES cells can be efficiently induced to differentiate into trophoblasts with addition of BMPs. This method eliminates many unknown factors provided by either EBs or stromal cells, thus allowing precise analysis of specific factors on the differentiation of ES cells into lineages of choice. With improved understanding of regulatory events governing germ layer and cell lineage specifications, more cell types will likely be derived from ES cells in increasingly defined conditions.

27.3.2 Molecular Control of Pluripotency

We remain remarkably ignorant about why one cell is pluripotent and another is not, although some of the key players important to maintaining this remarkable state have been identified. Oct4, a member of the POU family of transcription factors, is essential for both the derivation and maintenance of ES cells. The expression of Oct4 in the mouse is restricted to early embryos and germ cells, and homozygous deletion of this gene causes a failure in the formation of the ICM. For mouse ES cells to remain undifferentiated, the expression of Oct4 must be maintained within a critical range. Overexpression of this protein causes differentiation into endoderm and mesoderm, while decreased expression leads to differentiation into trophoblast. The expression of Oct4 is also a hallmark of human ES cells, and its downregulation also leads to differentiation and expression of trophoblast markers.

Another transcription factor important for the pluripotency of ES cells is Nanog. Similar to Oct4, the expression of Nanog decreases rapidly as ES cells differentiate. However, unlike Oct4, overexpression of this protein in mouse ES cells allows their self-renewal to be independent of LIF/STAT3, although Nanog appears not to be a direct downstream target of the LIF/STAT3 pathway. Moreover, increased Nanog expression stimulates the activation of pluripotent genes from the somatic genome in cell–cell fusion models. In human ES cells, the expression of Nanog was directly activated by the transforming growth factor beta (TGFβ)/activin-mediated small 'mothers against' decapentaplegic (SMAD) signaling, and its overexpression enabled feeder-free growth. In both mouse and human ES cells, reduced expression of Nanog causes differentiation into extraembryonic lineages. Interestingly, although they are prone to differentiating, mouse ES cells can self-renew indefinitely and contribute to multilineages in chimeras in the absence of Nanog. The function of Nanog in ES cells, thus, is more likely involved in the stabilization of the pluripotent state, while dispensable for its establishment.

The expression of genes enriched in ES cells has been extensively studied by several groups (see, e.g., Rao and Stice, 2004, and references therein), and includes, for example, transcription factors Sox2 and *foxd3*, RNA-binding protein Esg-1 (Dppa5), and *de novo* DNA methyltransferase 3b. Deletion of some of them in mice does demonstrate a critical function in early development (Table 27.1). ES cells also express high levels of genes involved in protein synthesis and mRNA processing, and non-coding RNAs unique to ES cells.

Table 27.1 Some Genes Known to be Enriched in ES Cells and a Description of Features and Functions that have been Characterized to date

Genes	Protein Features and Functions
Sox2	HMG-box transcription factor; interacts with Oct4 to regulate transcription: Sox2 –/– mouse embryos died shortly after implantation with loss of epiblast at ~E6.0
FOXD3	Forkhead family transcription factor; FoxD3 –/– mouse embryos died shortly after implantation with loss of epiblast (~E6.5); no FoxD3 –/– ES cells can he established
Rex 1 (Zfp-42)	Zinc-finger transcription factor; direct target of Oct4:Rex-1 –/– EC cells failed to differentiate into primitive and visceral endoderm
Gbx2 (Stra7)	Homeobox containing transcription factor; Gbx –/– embryos displayed defects in neural crest cell patterning and pharyngeal arch artery
Sall1	Potent zinc-finger transcription repressor; heterozygous mutations in humans cause Townes–Brocks syndrome; Sall1 –/– mice died perinatally
Sall2	Homolog of Sall1; Sall –/– mice showed no phenotype
Hoxa11	Transcription factor; Hoxa11 –/– mice showed defects in male and female fertility
UTF1	Transcriptional coactivator; stimulate ES cell proliferation
TERT	Reverse transcriptase (catalytic component of telomerase)
TERF1	Telomere repeat-binding factor 1; TERF1 –/– mouse embryos died at E5–6 with severe growth defect in ICM
TERF2	Telomere repeat-binding factor *2*
DNMT3b	*De novo* DNA meyhyltransferase; required for methylation of centrimeric minor satellite repeats; DNMT3β –/– embryos died before birth
DNMT3a	*De novo* DNA methyltransferase; DNMT3a –/– mice died at the age of 4 weeks
Dppa2	Putative DNA binding motif SAP
Dppa3 (PGC7, Stella)	Putative DNA binding motif SAP
Dppa4 (FLJ10713)	Putative DNA binding motif SAP
Dppa5 (Ph34, Esg-1)	Similar to KH RNA-binding motif
ECAT11 (FLJ10884)	Conserved transposase *22* domain

A surprisingly high percentage of genes enriched in ES cells have unknown functions.

A recent genome-wide location analysis of human ES cells showed that Oct4 and Nanog, along with Sox2, co-occupy the promoters of a high number of genes, many of which are transcription factors such as Oct4, Nanog, and Sox2. These three proteins, in addition to regulating their own transcription as previously shown, could also activate or repress the expression of many other genes. These genome-wide approaches hold great promise in elucidating the networks that control the pluripotent state.

27.4 CONCLUSION

Progress in developmental biology has been dramatic over the last few decades, and one of the legacies of the derivation of human ES cells is that they provide a compelling link between that progress and the understanding and treatment of human disease. The derivation of mouse ES cells in 1981 and subsequent development of homologous recombination revolutionized mammalian developmental biology, as it allowed the very specific modification of the mouse genome to test gene function. Yet, although the use of mouse ES cells as an *in vitro* model of differentiation was established soon after their initial derivation, it was only after the derivation of human ES cells in 1998, and their potential use in transplantation medicine was immediately appreciated, that there was an explosion of interest in the *in vitro*, lineage-specific differentiation of ES cells. Significant progress has been made in lineage-specific differentiation of human ES cells, and progress in this area is accelerating as new groups are now rapidly entering this field. An understanding of the basic mechanism controlling germ layer and lineage specification is rapidly unfolding through the interplay of knockout mice, *in vitro* differentiation of ES cells, and conserved mechanisms identified in other model organisms.

The basic biology of pluripotency is another area of research that the isolation of human ES cells rekindled. Even though significant differences exist between mouse and human ES cells, they share many key genes involved in pluripotency, such as Oct4 and Nanog. Global gene expression analysis of mouse and human ES cells has revealed the existence of many novel genes unique to ES cells, but the challenge remains in identifying functions of those genes and understanding how the proliferative, pluripotent state is established and maintained. Indeed, although certain genes have been identified that are required to maintain the pluripotent state, it remains a central problem in biology to understand why one cell can form anything in the body and another cannot. Such a basic understanding has implications for regenerative medicine that go far beyond the use of ES cells in transplantation, and

may lead to methods of causing tissues to regenerate that fail to do so naturally. The derivation of ES cell-like-induced pluripotent stem cells from differentiated somatic cells with a small set of transgenes is a first groundbreaking step in this direction.

FOR FURTHER STUDY

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CHAPTER 28

Isolation and Maintenance of Murine Embryonic Stem Cells

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28.1 INTRODUCTION

I first discuss the growth and maintenance of cultures of mouse embryonic stem (ES) cells, because their isolation depends on the secure ability to maintain them. I would recommend that an established ES cell line be used to optimize growth conditions before attempts are made to isolate new ones.

ES cells grow well in culture, and are not particularly fastidious about the media, but it must be remembered that ES cell cultures are essentially primary cultures. Therefore, it is necessary to use conditions of tissue culture that maintain their primary properties, and do not select variants. Any growth in nonoptimal conditions will lead to selective pressure, and the appearance of better-growing but worse-differentiating strains. In some cases, these may be recognized as chromosomal variants, but in others there is no gross karyotypic change identifying the altered cell strain. They will certainly, however, be less able to differentiate and will produce abnormal chimeras.

28.2 MAINTENANCE OF EMBRYONIC STEM CELLS

There are major requirements for ES cell culture in the context of maintaining the totipotency necessary for genetic engineering and gene-targeting projects:

- The cells are maintained in their undifferentiated state.
- The cells maintain their normal capability and range of differentiation.
- The cells retain a normal karyotype, a prerequisite for germ-line transmission.

To maintain the stem cell state (suppression of differentiation), the cells need to be cultured with mitotically inactivated feeder cells, or in the presence of leukemia inhibitory factor (LIF). Each method has advantages and disadvantages. Feeders provide a more robust approach, for the supplementation of media by growth factors will lead to a more pulsatile addition of the factors and will

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depend more on meticulous attention. Conversely, sufficient provision of feeders requires setting them up and inactivating at each passage. Growth in the presence of LIF may seem less troublesome, and makes certain types of experiments much easier, but it can have its disadvantages. Some ES cell lines seemingly grow better on feeders than in the presence of LIF, whereas others can be switched between the two conditions with little difficulty.

By far the best practical method for maintaining totipotent cells in tissue culture remains the use of fibroblast feeders (STO or primary embryo fibroblast). For any serious long-term investment in ES cells, it is wise to expand and freeze ES cells over several passages, checking the karyotype (and germ-line transmission) at each passage. Use should only be made of ES cells with proven karyotype and germ-line transmission properties for critical experiments.

ES cell cultures are essentially primary cultures, and it is necessary therefore to use conditions of tissue culture that maintain their primary properties, and do not select variants. It appears that extremes of growth conditions, either by plating the cells too sparsely or by allowing the cultures to become too dense and exhausting their medium, will encourage loss of the desired normal characteristics of ES cells. Either situation sets up conditions in which selection for abnormal (fast-growing, aneuploid) cells can occur. Healthy cultures of ES cells grow with a population doubling time of 15 to 20 hours. In practice, therefore, it is necessary to subculture ES cells about every three days, and to renew the medium regularly.

In as much as ES cells are not particularly fastidious about the medium in which they are grown, the optimum formulation I have used is Dulbecco's modified Eagles medium (DMEM), high-glucose, low-pyruvate formulation mixed 1:1 with Ham's F12M medium. This peculiar mix is a compromise between the high-yielding DMEM, originally designed for maximal growth of tissue culture cell lines for virus production, and the finely balanced Ham's F12, originally developed for the clonal growth of cells. I originally supplemented the DMEM with nonessential amino acids (NEAA) and a mixture of nucleosides (adenosine, guanosine, cytidine, and uridine) to a final concentration of 30 mM, and thymidine to a final concentration of 10 mM. This supplement has not been tested properly when used in a DMEM/F12 mix, but it nevertheless may have an advantageous effect on the primary cell isolation. ES cells grow happily in DMEM alone, but they grow better in DMEM plus NEAA, and better still in DMEM/F12. The only disadvantage of the latter is that it becomes acidified faster, and the medium needs to be changed more frequently. These media may be prepared conveniently 'in house' from premixed powder. Alternatively, 10× concentrates may be used, or a medium may be bought ready to use at 1× concentration. The water used must be of the highest purity, and glassware cleanliness is critical. These latter points have proved to be the most important in establishing new ES cell lines. I suspect that the most damaging contaminants

are detergent residues, and that their effects are not necessarily seen in bulk passage cultures. The most useful tool for optimization of media, sera, and other conditions is the cloning efficiency test (explained later in this chapter), which should also be used for any troubleshooting.

28.3 MEDIA

The medium recommended for ES cells is as follows:

- DMEM/F12 (or DMEM)
- Glutamine (stored frozen as 100 × concentrate of 200 mM)
- β-Mercaptoethanol to a final concentration of 10^{-4} M, either 4 ml neat in 500 ml or from a 10^{-2} M (100 ×) concentrate. (Prepared by adding 72 ml to 100 ml phosphate-buffered saline (PBS))
- 10% calf serum and 10% fetal calf serum
- NEAA from 100 × concentrate
- Nucleosides from 100 × concentrate (if required)
- Antibiotics (if desired) – use only penicillin, streptomycin, kanamycin, or gentamicin. Do not use antimycotics.

28.4 SERA

Both fetal calf serum (FCS) and newborn calf serum are used. It is a mistake to imagine that the higher price of fetal calf serum makes it better. Some newborn sera are excellent, and some fetal calf sera are extremely toxic to ES cells. Empirical selection is imperative. All sera should be carefully batch-tested using a cloning efficiency test (explained later in this chapter) and should be ordered in a sufficient lot for the experiments planned. Look both for lack of toxicity (aim for a cloning efficiency of 20% or more) and for growth promotion (the size of colonies). Aim to buy new serum batches that equal or exceed the quality of the presently used (control) sera. Be cautious with sera that show toxicity at high (e.g., 40%) concentrations; they can be excellent at lower levels, but other sera can be just as good without extra toxicity.

28.5 COLONY-FORMING ASSAY FOR TESTING CULTURE CONDITIONS

The colony-forming assay described should be used to test all the components of the media, as well as some culture procedures (e.g., viability of cells after different regimens of dissociation):

- Set up experimental media in six-well cluster dishes (2 ml/well) at twice the intended final concentration of additives, and equilibrate in the

incubator at 37°C, 5% CO_2, in air before use. If feeder cells are used, these need to be added either at this stage, or with the sample cells. Use duplicate wells for each condition and appropriate controls (e.g., a known 'good' serum batch). For FCS batch testing, use growth media supplemented with the FCS batch at 5%, 10%, and 20%. All other components (e.g., mercaptoethanol concentrations and batches of media) may be tested in the same way.

- Disaggregate ES cell stock culture on day 2 after plating (i.e., semiconfluent), ensuring as close to a single cell suspension as possible. In a test of sera, remember, if using trypsin, to inactivate by a wash in serum-containing medium, before redispensing into serum-free medium. Count and resuspend in growth medium at a density of 10^3 cells/ml.
- Add 2 ml cell suspension to each well (making 4 ml total volume 1 × concentration of additives), and return to the incubator.
- Incubate for 6 to 8 days at 37°C, 5% CO_2 in air.
- Fix, and then stain, plates with Giemsa. ES cell colonies can be identified by characteristic morphology and dark staining properties. Differentiated colonies are paler in color. (It is useful to check sample colony appearance before fixing and staining by inverted phase contrast.) Count the total number of colonies (the plating index), and the proportion of ES cell colonies. In tests of LIF or feeders, the maintenance of undifferentiated colonies is important.

I still recommend the use of inactivated feeder layers for the maintenance of ES cells. I have found that the attraction and convenience of feeder-free methods are outweighed by the frequency with which stocks deteriorate under these conditions, and would therefore prefer that an important seed stock be maintained with feeders. It is possible to use a belt-and-braces policy – to use feeders and added LIF. For feeders, use either STO cells or primary mouse embryo fibroblasts.

STO cells are routinely grown in DMEM supplemented with 10% newborn calf serum. Remember that STO cells are effectively 3T3 cells, and should be passaged promptly when they reach confluence to prevent the accumulation of noncontact-inhibited cells in the population. Passage STOs at 5×10^3 cells per cm^2 and expect a harvest of up to 20 times this amount. If a confluent 10 cm Petri dish yields 10^7 cells, then the cells are losing their contact inhibition, and will no longer produce a good feeder layer. Either replace them with a new batch, or (and effectively) clone them out by seeding at 100 cells per dish and select a flat clone to establish a new stock. Some workers prefer to use primary embryo fibroblasts to prepare feeder layers rather than STO cells, because they feel that better ES cell growth can be obtained. This may, however, reflect abused STO cells rather than an intrinsic superiority of primary embryo fibroblasts.

Feeders are prepared in the following manner:

- Remove the medium from a confluent 10 cm dish of STO cells, and replace it with DMEM/10% NCS plus 10 mg/ml of mitomycin C. (Stock mitomycin C is made at 2 mg/ml with PBS, and can be stored at 4 °C for 2 weeks.)
- Incubate plates for 2 to 3 hours. Avoid longer exposure to mitomycin C.
- Remove mitomycin C from the STO cells, and wash each plate three times with 10 ml PBS.
- Trypsinize, resuspend, and pellet the cells by centrifugation. This is an important washing step.
- Resuspend in growth medium, and seed onto gelatinized tissue culture plates at 5×10^4 cells per cm^2. These can be kept for later use (up to a week), or the inactivated cells can be stored for later use in suspension in a serum-containing medium at 4 °C for a similar time.

28.6 EMBRYONIC STEM CELL PASSAGE CULTURE

Passage and maintenance of the ES cells is straightforward; bear in mind the following. The cells should not be allowed to become so confluent that the medium becomes excessively acidified. Given the chance, they will continue dividing until they start to kill themselves by overcrowding and exhaustion of the medium; avoid this. Expect to harvest about 3×10^5 cells per square centimeter, and plate at $2 \times 10^4/cm^2$. Feed regularly and passage every three days. It is important to seed the cells as a suspension of single cells with few, if any, aggregates. Such aggregates will initiate differentiation, and can severely complicate the ES culture for many purposes. Cells are best disaggregated by careful washing with PBS (remember to have 20% serum in the medium, and this needs to be fully removed) followed by trypsin/EGTA (0.125% trypsin with 10^{-4} M EGTA in PBS – an improvement on trypsin/EDTA). Incubation at room temperature is sufficient. (It is also possible to disaggregate ES cells by a prolonged incubation in PBS/EGTA, but their viability is reduced.) Immediately after the trypsin incubation, add growth medium and pipette up and down a few times to generate single cells. Because it is important that the cells are well disaggregated, it is good to check by counting in a hemocytometer. A mainly single cell suspension with round, phase-bright cells should be seen. Dead cells or clumps are obvious; ragged and larger cells are feeders.

28.7 ISOLATION OF NEW EMBRYONIC STEM CELL LINES

Contrary to some well-expressed opinions, it is clearly possible to isolate ES cells from a variety of mouse strains, and not just from strain 129. I append a prescriptive description of ES cell isolation (given later in this chapter), but

first it is useful to consider the background, and to point out that isolation is possible from the cleavage to early postimplantation stages and using different procedures.

Embryonic development starting from a single zygote proceeds through extensive cell proliferation and progressive cellular differentiation. At early stages some cells have wide prospective fates, but these are not necessarily self-renewing populations. Pluripotent stem cells were first identified experimentally in the mouse as the stem cell of teratocarcinomas when tumors were passaged by transferring single cells isolated from embryoid bodies. These testicular teratocarcinomas in mice arise spontaneously in specific genetic strains, and were extensively studied. The stem cells of these tumors arise from the primordial germ cells during gonad formation, and one question is whether this represents a parthenogenetic activation. This idea – that the teratocarcinoma stem cells might be embryo related rather than germ cell related – was tested directly by transplanting early embryos to adult testes or kidneys. Passageable, progressively growing teratocarcinomas were formed after transplantation of 1–3.5 day preimplantation embryos. From some of these tumors, clonal *in vitro* differentiating embryonal carcinoma cell lines were isolated and characterized. These were the direct forerunners of ES cells and their isolation. They demonstrated their pluripotentiality by forming well-differentiated teratocarcinomas on reinjection into mice. These also differentiated extremely well *in vitro*, via an embryo-like route. A series of observations of the properties of these and similar embryonal carcinoma (EC) cells started to provide convincing evidence of their homology with the pluripotential cells in the normal embryo, which were able to generate teratocarcinomas under conditions of ectopic transplantation. There seemed to be every reason to suppose that direct isolation into tissue culture should be possible.

Teratocarcinomas can also be formed from postimplantation mouse embryos. The embryonic part of the postimplantation mouse embryo undergoes gastrulation and forms an embryonic mesoderm by invagination from the ectoderm; this lies between the ectoderm and an embryonic endoderm. Skreb and his colleagues isolated these three layers of the rat embryo by microdissection, and tested their developmental potency by ectopic transplantation. Only the embryonic ectoderm produced teratomas with multiple tissue types and was hence pluripotent, but these were not progressively growing teratocarcinomas. When these experiments were repeated with gastrulating mouse embryos, it was discovered that, in contrast to the rat, transplantable teratocarcinomas were formed. At least, therefore, in the mouse, pluripotent stem cells could be recovered until about the end of gastrulation. Direct isolation of ES cells, however, from such a late stage has not been reported. Taking these comparisons and molecular data into consideration, we have argued that mouse

ES cells are – regardless of the route of isolation – homologous to the early postimplantational epiblast, rather than the inner cell mass (ICM), as is often erroneously stated.

The culture conditions – feeder cells and media – had been refined by culturing both mouse and human teratocarcinoma EC cells. By using implantationally delayed blastocysts as the embryo source, cultures of pluripotent cells were successfully established directly, without an *in vivo* tumor step. Subsequent studies showed isolation of such cell lines from both inbred and outbred strains, from normal 3.5-day blastocysts, and those that had been implantationally delayed. Independent work showed that isolated 3.5-day ICMs may also be used. These cells, which have become known as ES cells, share the properties of indefinite proliferative capacity, embryonic phenotype, and differentiative capacity with their forerunners – the EC cells; in addition, being primarily derived, they may be kept entirely karyotypically normal.

ES cell cultures may be established from disaggregated 16–21 cell morulae with an apparent immediate growth of the colonies from some of the explanted single cells. Up to four separate ES cell colonies were founded from a single embryo.

The latest stage of isolation into cultures of ES cells from used 4.5-day-old hatched, peri-implantational embryos flushed from the uterus. Epiblast cells were microdissected from both the primary endoderm and the trophectoderm. Both whole epiblasts and those that had been disaggregated into single cells readily generated ES cultures – if they were not left in contact with the endoderm. This work also reconfirmed the benefit of using delayed blastocysts, showing that the isolated epiblasts of these were the most efficient source of ES cell cultures.

28.8 METHOD FOR DERIVING EMBRYONIC STEM CELLS

I would still recommend the original method of explantation of implantationally delayed blastocysts as the most effective method. It works well, and involves no microdissection or immunosurgery.

First, it is essential to pay attention to the optimization of the culture conditions, as described previously. If things do not work, suspect the purity of the media and particularly any possible contamination by detergent residues. Seemingly stupid problems, such as aliquoting the medium into small batches and using untested vessels (e.g., bijou bottles), can arise. Suspect specific items that have not been tested in the ES cell cultures – for instance, the

cleanliness of the glass Pasteur pipettes from which micropipettes are pulled for manipulation of the embryos and growth of ES cell colonies.

Delayed blastocysts:

- Mate mice by caging together overnight and observing mating plugs in the morning. Separate the plugged females.
- On day 2 (counting the day of plug as day 0), it is necessary to remove estrogen activity but preserve progesterone. This was originally done by ovariectomy followed by injection of Depo-Provera, but we have introduced the less invasive, simpler, and considerably improved option of using the antiestrogenic effects of tamoxifen. The mice are treated with 10 mg tamoxifen and 1 mg Depo-Provera. Dissolve the tamoxifen in ethanol to make a 100 × stock, and dilute this in sesame oil before injecting a dose of 10 mg intraperitoneally. At the same time, administer a dose of 1 mg Depo-Provera subcutaneously. Kill the animals, and recover the delayed blastocysts by flushing from the uterus between day 6 and day 8.
- Prepare feeder layers preincubated in an ES cell medium. (Although it is routine to pretreat plastic Petri dishes with gelatin as an aid to ES cell culture, it is probably more important here than elsewhere.) I find that 1.6 cm four-well cluster dishes are convenient, but possibly better are 3 cm Petri dishes, each enclosed in a 10 cm plastic Petri dish (this aids handling and helps to minimize evaporation from the small volumes of medium).
- The embryos will enter diapause, and may be recovered by flushing from the uterus 6–12 days later. They will be large, slightly ragged, hatched blastocysts, usually with a visible ICM.
- Using a drawn-out Pasteur pipette, recover the blastocysts and place them on preincubated feeder layers in full ES cell medium.
- Incubate for about four days, observing daily.
- When the blastocyst has attached and spread out, with a visibly growing ICM derivative (but before this becomes encapsulated in a thick endodermal layer – if you see this happening, you have left it too long), carefully remove the medium and carefully wash twice in PBS with 10^{-4} M EGTA.
- Wash with PBS/EGTA containing 0.125% trypsin and aspirate, leaving a thin wetting layer of the trypsin solution. Very briefly incubate at 37 °C.
- Have a drawn-out Pasteur pipette with a tip aperture of 20–50 microns ready and filled with full ES cell medium. Observe under a dissecting microscope. When the cells are loosening, but before everything swims off the dish, carefully flood the medium over the ICM area from the drawn-out pipette and then suck it up.

- This should neatly disaggregate it into single cells and small (two- to four-cell) clumps – do not aim to make a complete single cell suspension at this stage. Blow these cells out beneath the surface of the medium of a preincubated feeder dish or well in the ES cell medium.
- Incubate this dish for 7–10 days, observing carefully. Do not be tempted to feed daily, but if it seems necessary, replace about half the medium after 5 days. If many small ES cell colonies are becoming established, let them grow to a reasonable size before passaging 1:1 onto new feeders; thereafter, you should be able to grow it in the normal manner. (The size of the colonies depends on the size of the cell aggregate that founded them – if they have come from a single cell, they can be left for 10 days before passage.) If few ES cell colonies appear, it is useful to repeat the trypsinization and passage by drawn-out Pasteur pipette on each one, before trusting a bulk passage.

With 129 mice and similar strains, and if all is going well, you should be able to establish new ES cell lines from up to half of the explanted blastocysts. It is useful to make a freeze stock at the earliest opportunity. There are usually plentiful cells by passage 4–6.

28.9 SUMMARY

Although it is relatively easy to create new cell lines, their full validation – stability in culture, karyotype, chimera-forming ability, and germ-line potential – takes a lot of time and work. It is, therefore, important to have sufficient characterized stocks of particular passage and to freeze batches to allow useful repeatable studies to be undertaken.

FOR FURTHER STUDY

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CHAPTER 29

Approaches for Derivation and Maintenance of Human Embryonic Stem Cells: Detailed Procedures and Alternatives

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29.1 INTRODUCTION

Since the generation of human embryonic stem (hES) cells in 1998, significant progress has been made in this field, as well as in the generation of induced pluripotent (iPS) cells. In spite of the growing number of hES and iPS cell lines, their number and quality are still limited, which is a testament to the difficulties encountered at various stages of their derivation and maintenance. While iPS cells hold great promise for regenerative medicine, there are a lot of safety issues at this stage which need to be resolved, and hES cells remain the gold standard for research leading to therapeutic application of pluripotent cells' derivatives. It is worth mentioning that there are more similarities than differences in maintenance of hES and iPS cells, which makes the techniques described in this chapter applicable to culture and differentiation of iPS cells.

The current techniques used for the maintenance of hES cells require scrupulous attention to the details of cell culture systems including: quality control of the reagents, monitoring of growth conditions and an ongoing assessment of the pluripotent status of the cells in order to promote consistency in the cell culture system. Distributors of the available hES cell lines frequently recommend attending special training courses before working with the hES cell lines they provide.

The authors have experience in derivation and culture of over 30 hES cell lines, including hES cells derived without feeder cells and lines established from single blastomeres. All our lines have been adapted to trypsinization, which makes passaging of the cells less labor intensive and generates cells in sufficient numbers to permit experimentation. All of these lines can be successfully frozen and thawed using very simple procedures with a recovery rate of 10–30% or higher.

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Techniques for deriving and maintaining pluripotent ES cells in culture have been described by a variety of labs, and there are similarities and vital differences. For the derivation and maintenance of hES cell lines in our lab we adapted previously published methods and developed an approach which has consistently produced new cell lines, and has been successfully taught to many researchers around the world.

The goal of this chapter is to describe aspects of derivation and maintenance of hES cells that we found to be critical for generating hES cell lines. We respond to questions received from other researchers with regards to: equipment, preparation and quality control of media and reagents, cell passaging techniques, and other aspects of hES cells' morphology and behavior. This updated chapter also includes our experience with good manufacturing practices (GMP)-compliant production of hES cells and their derivative, retinal pigment epithelium, which received Food and Drug Administration (FDA) approval for phase I clinical trials, i.e., quality control, safety issues, large-scale production, etc.

29.2 SETTING UP THE LAB

29.2.1 Equipment

Initial steps in the derivation process are conducted under a dissecting microscope. We make an effort to keep the embryos and the dishes containing the early mechanically passaged dispersions at 37°C. Dishes brought out of the incubators are set on 37°C slide warmers or viewed using microscopes fitted with heated stages. The mechanical dispersions necessitate having the dishes open for extended periods and, since the cultures are vulnerable to contamination at this time, we have the dissecting microscope within a bench-top laminar flow hood. A high quality stereo microscope with a wide range zoom is essential for the mechanical dispersion of colonies, including the inner cell mass (ICM) outgrowth; it permits an overall assessment of each plate and evaluation of the morphology of each colony when doing the mechanical passaging.

The equipment that is used in our lab is as follows:

Stereomicroscope for microdissection: Nikon SMZ-1500 with the magnification range 10–100× : works well with its easy zoom and the positioning mirror that regulates the depth and contrast of the image. A whole 35 mm dish can be scanned for colony morphology, and the zoom permits selection with precision of the parts of the colony that are the best for dissection.
Inverted microscope: A Nikon TE 300, or any similar microscope, set up with phase contrast and objectives 4×, 10×, 20×, 40×, and Hoffman Modulation Optics (HMC) 20×, 40×. Recommended for fine observation

of hES cells, some of their derivatives, and is highly desirable for embryo evaluation.
Heated microscope stage for both stereo and inverted microscope: Nikon. Slide warmer keeps plates at 37°C during observation and mechanical dispersions.
Bench-top laminar flow hood with an high efficiency particulate air (HEPA) filter. Vertical hoods by Terra Universal (Anaheim, CA) are a good inexpensive choice. We found these vertical hoods were tall enough to accommodate a dissecting microscope and were very convenient and reliable. Horizontal models sometimes produce too much vibration. Mid-Atlantic Diagnostics (Mount Laurel, NJ) manufactures workstations that include a biosafety cabinet and a built-in dissecting microscope; these models are more expensive but are compliant with the clean room requirements, allowing to keep everything warm while moving around, as the whole working area could be heated, and CO_2 circulation could be used to keep the pH of the plates stable while they are in use.
Reliable tissue culture incubator. All parameters (CO_2 concentration, humidity, and temperature) need to be checked daily with external monitoring equipment.

External monitoring equipment:

Surface thermometer
Mercury liquid-immersed thermometer
Hygrometer
CO_2 monitor and gas calibration kit (GD 444 SR-B by CEA Instruments, Emerson, NJ)

More sophisticated monitor/alarm systems (which would call the persons on the list if the monitored parameters fall out of the specified range) are a good (but rather expensive) alternative when immediate action would be needed to save an experiment or a manufactured lot.

29.2.2 Quality Assurance of Equipment

Consistency in growth conditions is very important for the development of the embryos and growth of hES cells. The checklist of parameters which are monitored daily includes the percent CO_2 (5.0%), temperature (37°C), and humidity of the incubators (>90%). A checklist with daily readings is very helpful for timely recalibration if any undesirable trend is noticed. Warming stages are constantly monitored with surface thermometers.

The incubators are checked prior to any new derivation round by growing mouse embryos from the two-cell stage to blastocyst; a passing score requires 50% to go to blastocyst. Ideally, until the newly derived lines can be frozen,

the cultures should be split between two incubators to prevent the loss of a line, in the case of equipment failure.

If hES cells need to be derived and maintained under the conditions of current Good Manufacturing Practices (cGMP) or current Good Laboratory Practices (cGLP), cGMP/cGLP protocols for equipment and reagents validation would have to be followed.

29.2.3 Sterility

There are certain aspects of hES cell lines derivation which put the associated work under more stringent sterility requirements than any typical cell culture lab. Among these is the nature of human embryos, each being unique and very valuable. Due to the labor intensive nature of derivation and expansion, the new lines usually require a team effort until they are safely frozen. Experiments may require long periods in culture, such as in the course of cell differentiation into desired derivatives that often takes many weeks. In addition, no antibiotics in cell culture media can be used during cGMP-compliant manufacturing.

The reagents should either arrive sterile from the manufacturer or be filter sterilized in the lab. Most of the cell culture supplies can be bought sterile. However, everything that is in-house sterilized by autoclaving, gas, or dry heat needs to be quality controlled. We use biological indicators (spore strips from Steris, Mentor, OH) – these are used with every load in an exact (size, shape) container as the materials autoclaved. After sterilization, the spore strips need to be incubated side by side with a positive control which should change color showing the bacterial growth while the test strip should remain negative; the materials are only released after passing this test.

If it comes to the worst, a triple-action drug Normocin (active against gram-negative and gram-positive bacteria, fungi, and micoplasma) appears to be well tolerated by hES cells without significant changes in their pluripotency or growth rate and often permits the rescue of contaminated cultures.

When the process is cGMP/cGLP compliant, the following needs to be in place: Standard Operating Procedures (SOP) and batch records must be kept for each step of manufacturing, i.e., gowning, environmental monitoring, cleaning, raw materials, preparation of reagents, every procedure used in manufacturing, etc. All personnel need to undergo training and certification.

29.3 PREPARING AND SCREENING REAGENTS

Many novel hES cell culture products have become available in the recent years. Some alternative media recipes may work better if differentiation towards certain cell types is desirable. Animal product-free culture systems are also a good alternative. We have seen the cells which can look very ‘proper’ under new conditions for 1–2 passages but spontaneously differentiate at

the next passage, or produce very low yield of a desired derivative; therefore, for any change in media and/or culture procedures we test for the following: passing hES cells for 3–4 passages to ensure they look the same or better and the yields are high, staining for alkaline phosphatase and markers of pluripotency, differentiation into desirable derivatives, karyotype.

29.3.1 Media Components

KO-DMEM (Invitrogen cat. # 10829)
DMEM high glucose (Invitrogen cat. # 11960–044)
Knockout Serum Replacement™ (KSR, Invitrogen cat. # 10828). Each lot needs to be tested; but as a guide, we found the lots with osmolarity higher than 470 mOsm/kg and endotoxicity lower than 0.9 EU/ml were the best. Upon thawing, make single-use aliquots and freeze.
Plasmanate (Talecric, Research Triangle Park, NC). Each lot needs to be tested.
Fetal bovine serum (FBS) (Hyclone cat. # SH30070.02). Each lot needs to be tested. Heat-inactivate at 55°C for 30 minutes, if desired, and freeze in aliquots.
Beta-mercaptoethanol, 1000× solution (Invitrogen cat. # 21985–023)
Non-essential amino acids (NEAA), 100× solution (Invitrogen cat. # 11140050)
Penicillin/Streptomycin, 100× solution (Invitrogen cat. # 15070–063).
Glutamax-I, 100× solution, a stable dipeptide of L-glutamine and L-alanyl, a glutamine substitute (Invitrogen cat. # 35050–061)
Penicillin/Streptomycin and Glutamax-I are kept in frozen single-use aliquots.
bFGF (Invitrogen cat. # 13256–029). Add 1.25 ml of protein-containing medium (we use basal medium, supplemented with Plasmanate and Serum Replacement, as described below) to a vial containing 10 µg of bFGF. This makes 8 µg/ml stock solution. Increasing final bFGF concentration to 8–20 ng/ml can be beneficial for the cells, especially at early stages of derivation, after thaw or when the cells are grown at low density. Make 120 µl aliquots and freeze.
Human leukocyte inhibitory factor (LIF) (Millipore cat. # LIF1010)
0.05% trypsin/0.53 mM EDTA (Invitrogen cat. #25300–054)
Gelatin from porcine skin (Sigma, cat. # G1880)
Phosphate-buffered saline (PBS), Ca^{2+}, Mg^{2+}-free (Invitrogen cat. # 14190–144)
Normocin, antibiotic active against gram-positive/gram-negative bacteria, also has anti-mycoplasma and anti-fungi activity (Invivogen, San Diego, CA; cat. # ant-nr-2; comes as a 500× solution).
Use of antibiotics is optional and is not allowed under GMP regulations, as it could conceal potential infection and/or lead to drug resistance.

29.3.2 Media Recipes

Bottles of media that are opened frequently rapidly become alkaline; we suggest making smaller quantities that will last approximately a week. An expiration date should be assigned to each prepared lot of reagents under GMP.

Primary Mouse Embryo Fibroblast (PMEF) growth medium:

To a 500 ml bottle of high glucose DMEM add:
6 ml penicillin/streptomycin
6 ml Glutamax-1
50 ml FBS

hESC basal medium (bM):

To a 500 ml bottle of KO-DMEM add:
6 ml penicillin/streptomycin
6 ml Glutamax-1
6 ml NEAA
0.6 ml beta-mercaptoethanol. ATTENTION! This volume pertains to the 1000× diluted solution (see the list of reagents) NOT to 100% beta-mercaptoethanol!

hESC derivation medium:

Use this medium at early stages of ICM outgrowth. It has higher LIF and bFGF concentration and contains FBS. Can switch to hESC growth medium when a steady growth of colonies has been reached (usually, passage 2–4).

To 100 ml of basal medium (bM) add:

5 ml Plasmanate
5 ml KSR
5 ml FBS
240 µl of human LIF (final concentration 20 ng/ml)
120 µl of bFGF stock solution (final concentration 8 ng/ml) or more (up to 20 ng/ml)
Sterilize by 0.22 µm filtration
Alternatively, two media can be prepared: one containing Serum Replacement and Plasmanate only, the other containing only FBS (10–15%). Mix them 1:1 or 2:1 for the earliest stages of derivation: FBS may help to stimulate the hES cell colony growth, but reduce its content if any undesirable spontaneous differentiation is observed.
We also supplement derivation medium with hES cell-conditioned medium. To produce it, derivation medium without FBS is added to an almost confluent culture of hES cells of very good morphology (see below) incubated overnight, collected, filtered through 0.22 µm filters and used at 25–30%.

hESC growth medium (hESCM):

To 200 ml of basal medium add:
20 ml Plasmanate
20 ml KSR
240 µl of human LIF for 10 ng/ml or 480 µl for 20 ng/ml
120 µl of bFGF stock solution (final concentration 4 ng/ml) for 1 × bFGF or more, if a higher concentration is desired
Sterilize by 0.22 µm filtration

Gelatin:

Dissolve 0.5 g of gelatin in 500 ml of warm (50–60 °C) Milli-Q water. Cool down to room temperature, sterilize by 0.22 µm filtration. Makes 0.1% solution. Treat dishes by flooding plates and leaving solution at room temperature for 30 minutes, aspirate solution prior to use.

Mitomycin C:

Add 2 ml of sterile Milli-Q water to a vial (2 mg) of lyophilized Mitomycin C (Sigma, cat. # M0503); makes 1 mg/ml stock solution. The solution is light sensitive and is good for one week at 4° C.

29.3.3 Screening Media Components

It is important to be consistent with the screening, aliquoting, and storage of the components of the media. Various lots of KSR, Plasmanate and FBS should be screened preferably on hES cells. The screening of Serum Replacement lots should be done prior to the first lot running out so an evaluation of its qualities can be compared side by side to the previous lot. Some newly derived hES cells will completely die out with a change in lots at the initial stages.

29.3.3.1 Screening of FBS, Plasmanate, or KSR

This test is based on a published procedure for screening FBS lots for mouse ES cell work (Robertson, 1987, p. 74). This approach can be used for screening any combination of reagents or finding the best concentrations for media supplements. The quality of the reagents is assessed by counting the number of colonies, evaluating the morphology of the hES cells and by staining for alkaline phosphatase activity as detected with a Vector Red Kit or Vector Blue (Vector Laboratories, Burlingame, CA).

1. Prepare 12-well plates with PMEFs. For each lot tested, you will need at least 12 wells to vary the concentration of the component being tested, from the working concentration to high enough concentrations to evaluate toxicity: 8%, 10%, 20%, 30%. Each concentration is done in triplicate and compared to known media components at working concentrations.

2. Split hES cells onto 6- or 12-well plates (24-well plates often have higher cell density in the middle, making it difficult to evaluate) with a ratio of 1:6–1:10. The difference in reagents will be more noticeable when the cells are started at low density; however, some cell lines grow very slowly and differentiate when they are kept at a low density, therefore adjust splitting ratio to the specific hES cell line. Resuspend the cells in a small volume of basal medium and add equal volumes of cell suspension to each well of the test plate; pipette up and down in each well or slowly move the plate in perpendicular directions for even distribution of the cells. Do not rotate, as doing so will move most of the freshly added cells to the center.
3. Change medium daily and evaluate colony morphology under the microscope. Human ES cells grow in flat, tightly packed colonies with sharp refractory borders. The colonies appear deep red when stained for alkaline phosphatase activity. In differentiating colonies, the cells are more loosely packed with diffuse borders and are pinker in comparison. Usually, the difference between the conditions being tested becomes more obvious as colonies grow bigger; however, as the colonies grow larger they can begin to touch each other, and such colonies have a tendency to differentiate. Staining one of the triplicate wells for alkaline phosphatase activity prior to seeing signs of differentiation is advisable. Continue with the other wells in each set for another day or two before staining (see Figure 29.1 for a sample test).

This test can also be used to compare the performance of different media formulations or new products offered commercially; 8% KSR/8% Plasmanate medium can be used as a control.

Adequate record keeping for all commercial and in-house prepared lots of reagents is essential for troubleshooting, should the performance of hES cells begin to fail.

29.3.4 Preparing Primary Mouse Embryo Fibroblast (PMEF) Feeders

We grow all of our hES cells on PMEF feeders that have been Mitomycin C-treated to generate stable monolayers. The PMEFs are made by standard procedures using 12.5 dpc ICR mouse embryos (Klimanskaya et al., 2007, p. 77). The 12.5 dpc embryos are eviscerated but the heads are left on during tissue disruption in trypsin; plating density is 1.5 embryos per 150 mm plate. PMEFs are expanded once after the initial plating (1:5 split) and then frozen (P1). The growth rate of PMEFs and their performance as feeders decreases as they go through multiple passages, therefore thawed PMEFs are only passaged once (P2) for expansion purposes prior to Mitomycin C treatment at which point a new vial of PMEFs would be thawed.

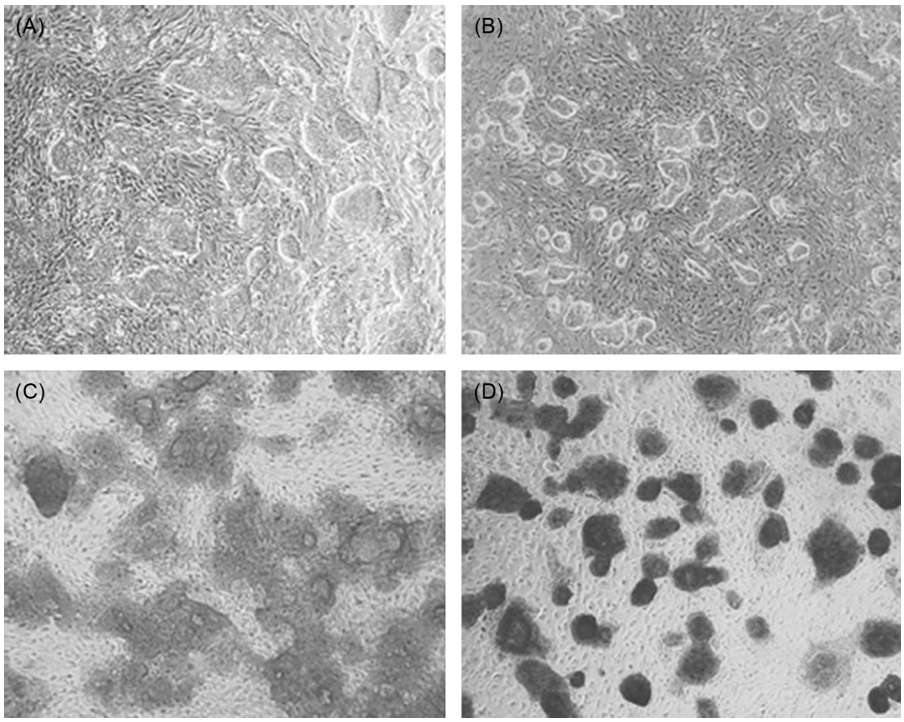

FIGURE 29.1
Media testing.
Comparison of 16% Serum Replacement (A, C), with 8% Serum Replacement; 8% Plasmanate (B, D). Quality of the media supplements is assessed by the morphology of the colonies in bright field (A, B), as described in the text, followed by staining for alkaline phosphatase activity (C, D). Note that although the morphology of the colonies in (A) and (B) is comparable, the activity of alkaline phosphatase is higher in medium with both Serum Replacement and Plasmanate (D). Magnification ×40.

If hES cell derivatives are intended for use in patients, it is important to use pathogen-free mouse embryo fibroblasts (MEF) (tests for viruses can be subcontracted). FBS needs to come from sources that guarantee the absence of spongiform or other transmittable bovine pathogens.

29.3.4.1 Mitomycin C Treatment and Plating

Mitomycin C is added to the medium of a confluent plate of PMEFs at a concentration of 10 μg/ml and incubated at 37°C for 3 hours. The cells are harvested by trypsinization and plated in PMEF medium onto gelatin-coated plates. In serum-free hESC growth media the PMEFs may appear less confluent due to the spindle-like form the cells take on, to ensure a confluent monolayer we recommend a plating density of 50–60 thousand cells/cm^2 (see Figure 29.1). We replace the PMEF medium with hESC growth medium the day after plating and use plates of PMEFs no longer than 3–4 days after Mitomycin C treatment. We have noticed that some lots of Mitomycin C may be inconsistent, the reagent forms an insoluble precipitate, and the characteristic purple color of the solution lightens dramatically. Reduced concentration of Mitomycin C causes proliferation of 'treated' PMEFs, which results in their detachment in a few days and/or formation of gaps in the monolayer

where the drops of medium hit the plate. Correctly inactivated PMEFs do not form such gaps and can remain intact on the plate for up to 3–4 weeks; such old PMEFs are not good for hES cell culture but are useful for preparation of extracellular matrix plates for feeder-free culture.

29.4 MECHANICAL PASSAGING OF hES CELL COLONIES

Many established hES cell lines are passaged with collagenase or by dispase in conjunction with mechanical dispersion. Mechanical dispersion can provide colonies of 'perfect' morphology, as it permits one to selectively pick undifferentiated colonies or even undifferentiated parts from differentiated and overgrown cultures, but it is time-consuming and does not yield large numbers of cells, thus limiting expansion of the hES cell lines and experimental design. Nevertheless, this procedure is invaluable at early stages of derivation or as a means of producing more homogeneously undifferentiated plates of cells for expansion or for adaptation to trypsin. It is also a tool for a 'rescue operation' in critical situations when the success of salvaging a few colonies means saving a new hES cell line. When hES cell lines recover from freezing with low rate (some providers can only ensure the viability of 1% or less) or show aneuploidy (less than 100%), they can be 're-derived' using mechanical passaging.

In new derivations or established cultures it is better to disperse colonies before they grow in contact with each other and prior to signs of differentiation such as becoming multilayered Figure 29.2. The dispersed colonies may be left in the same well or moved to a new well. If there are only a few colony pieces (1–5) they should be placed near to each other but with enough space to permit growth. When the cells are grown on a 35 mm dish, it is usually sufficient to disperse 50–100 average-sized colonies to populate a new 35 mm plate. In 1–2 days it may be necessary to disperse some of the larger colonies, leaving the pieces in the same well. Usually, mechanical passaging needs to be done every 5–6 days, but several larger colonies may need to be dispersed daily.

29.4.1 Materials Needed

29.4.1.1 Flame-Pulled Thin Capillaries

We use an alcohol or gas burner to pull capillaries from glass Pasteur pipettes (pre-sterilized). The capillaries are broken by hand into angled tips (the shape of a hypodermic needle). The diameter of the capillaries may vary, but the best results are achieved when it is about 10–100 ES cells diameter; this is how large the colony pieces are going to be. The choice of a diameter depends on the operation: for instance, to do initial dispersion of an ICM

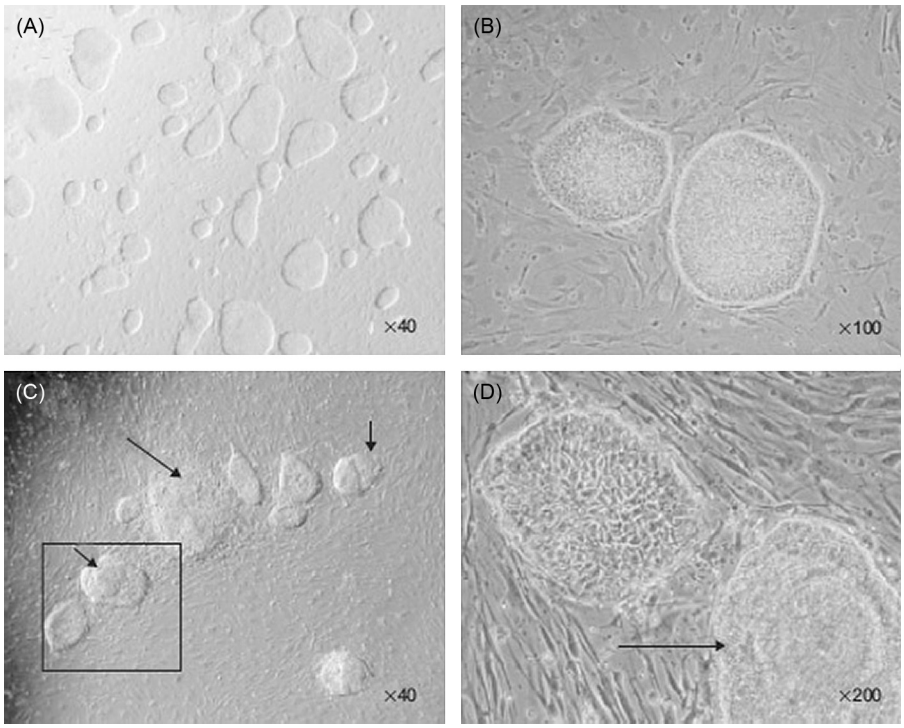

FIGURE 29.2 **Morphology of mechanically dispersed hES cells.**
Colonies on panels (A, B) show no signs of differentiation. Note that the cells are small, tightly packed, and the colonies have sharp borders. In (C) long arrow points to a very differentiated colony that should not be dispersed, short arrows show partially differentiated colonies which can be dispersed and may produce undifferentiated colonies at the next passage. (D) Higher magnification of the framed colonies on (C), shifted 90°. The arrow points to a partially differentiated colony, which has become multilayered in the middle. The other colony is undifferentiated and is similar to colonies shown on (A, B).

outgrowth or to target undifferentiated parts of a colony, a diameter of 10–30 cells would be used, and to quickly harvest a large number of colonies from a good quality culture, 50–100 cell capillary diameter would work the best. If immunosurgery is performed, a very narrow capillary of the ICM size is needed to strip off the throphoblast.

29.4.1.2 Mouth-Controlled Suction Device

Similar to a mouth pipette used for embryo transfer, this device consists of a mouthpiece (Meditech International, cat. # 15601P), rubber tubing, and a 0.22 µm syringe filter with a rubber tubing adapter for the capillary. It provides high precision in all manipulations for colony dispersions.

Alternatively, a 'Stem Cell Cutting Tool' (available from Vitrolife, Sweden) for picking colonies can be used instead of a mouth-controlled device and glass capillaries.

29.4.1.3 Preparation of PMEF Plate Receiving Mechanically Dispersed Colonies

1. When dispersing the initial ICM outgrowth, change the medium on a four-well plate of Mitomycin C-treated PMEFs to hESC derivation medium the night before the derivation day to let it get conditioned by the PMEFs, final volume of 250 µl. Alternatively, instead of conditioning it overnight, the derivation medium can be supplemented with 30% of hESC-conditioned medium. To collect hESC-conditioned medium, add medium to a sub-confluent culture of hES cells with good morphology (see Figure 29.2A for an example of colony density and morphology), leave for 24 hours, collect medium, filter and store for 2–3 days at 4 °C.

At early derivation stages when the colony growth is slow, change 2/3 of the medium every 2–3 days to keep it conditioned at all times. As more colonies appear change 2/3 of medium daily and increase volume to 500 µl/well of a four-well plate.

2. For established cultures, remove 1/3 of medium from growing culture, put on new PMEF dish and add 2/3 volume of fresh medium. Change 2/3 of medium daily.

29.4.1.4 Mechanical Dispersion

The procedure is similar to vacuuming. Gentle dispersion of the colonies is achieved by simultaneously cutting off the pieces with the angled end of the capillary, very lightly moving them off and sucking them in. With the opening of the capillary positioned horizontal to the bottom of the dish, begin moving from the sides in towards the colony center, chopping off and gently sucking in each piece. The light suction helps to detach the colony parts and is applied at all times, as you move from the periphery of the colony, collecting the colony parts. If the whole colony is coming away from the monolayer in one piece, it is probably differentiated and should be discarded.

When the desired number of colonies is dispersed, blow out the pieces into the same plate (like for the first ICM dispersion) or into a freshly prepared plate. To avoid having all the colonies stuck to each other in the center of the plate move the plate gently from side to side, do not swirl. (Figure 29.2 shows examples of mechanically passaged cultures, pointing out parts of colonies that have differentiated and should be avoided when passaging the culture.)

29.5 DERIVATION OF hES CELLS

It is clear there are many factors which are not fully understood that influence whether an isolated ICM will produce an hES cell line. Some of the factors to consider are: at what stage the embryo was frozen and by what procedure, the length of time the embryo must be in culture to generate a blastocyst, the culture conditions and the quality of both the ICM and the trophectoderm. The determination of when an embryo is ready for immunosurgery must be determined empirically, usually occurring between day 5 and day 7. Any embryo that has undergone cavitation and has relatively intact trophectoderm is a candidate for immunosurgery

29.5.1 Immunosurgery

The process of immunosurgery was performed essentially as described in Solter and Knowles 1975). It involves removing the zona pellucida with Acid Tyrode's solution, incubating the embryo in an antibody that binds to the trophectoderm and preferably not to the ICM cells (especially important for the embryos with non-intact trophectoderm), and then lysing the trophectoderm cells with complement. The dead cells that surround the ICM are removed by passing the ICM through a narrow capillary. The isolated ICM is put on a prepared PMEF (in microdrops or four-well plates) for further growth and dispersion.

29.5.2 Materials Needed

Acid Tyrode's (Specialty Media, cat. # MR-004.D)

Rabbit anti-human red blood cell (RBC) antibody (purified IgG fraction, Inter-Cell Technologies, Hopewell, NJ, cat. # AG 28840)
Aliquoted and stored at −80°C, freshly diluted 1:10 in hES C derivation medium
Complement (Sigma, cat. # S1639)
Aliquoted and stored at −80°C, freshly diluted 1:10 in derivation medium
Capillaries for embryo transfer
Thinly drawn capillaries (approximately the diameter of the ICM) for the trophectoderm removal

29.5.2.1 Prepared Mitomycin C-Treated PMEF Plates

For the initial ICM outgrowth, change the medium on a four-well plate of Mitomycin C-treated PMEFs to hESC derivation medium the night before the immunosurgery to let it become conditioned by the PMEFs, final volume of 250 µl.

Alternatively, instead of conditioning it overnight, the derivation medium can be supplemented with 30% of hESC-conditioned medium. To collect hESC-conditioned medium, add medium to a near-confluent culture of hES cells with good morphology (see Figure 29.2A for an example of colony density and morphology), leave for 24 hours, collect medium, filter, and store for 2–3 days at 4°C.

Microdrops under oil can also be used to minimize the volume of the medium conditioned by the colony growing from an ICM. To prepare a plate with microdrops, first, make gelatin drops on a 60 mm tissue culture dish, incubate for 30 min in a cell culture incubator. Aspirate each drop and immediately add to 'footprints' PMEF suspension, 1 million cells/ml. Overlay with embryo quality light mineral oil. Change the medium the next day to derivation medium by carefully aspirating the medium from each drop under the dissecting microscope and replacing with growth medium (P200 pipette can be used); repeat once or twice to get rid of all PMEF medium.

29.5.3 Immunosurgery Procedure

1. Each embryo is processed separately. A dish is prepared with a series of 30 µl microdrops, three for each step: Acid Tyrode's, anti-human RBC antibody, complement, and three drops of derivation medium for each of the washes between the steps. The drops are covered with embryo-tested mineral oil and are equilibrated in the CO_2 incubator for 60 minutes.
2. Under the dissecting microscope, transfer the embryo into the first Acid Tyrode's drop for a quick (1–2 seconds) wash and then move into the second. Watch the embryo closely, and as soon as the zona pellucida thins and is nearly digested, move the embryo into the next series of hES cell media drops. Move the embryos through the first antibody drop into the second and third drops and put the dish into the incubator for 30 minutes.
3. Transfer the embryos through three drops of derivation medium and through three drops of the complement solution as described above; incubate in the last drop of complement in the CO_2 incubator for 15 minutes and check for any 'bubbling' trophoblast cells. If no cells show signs of lysis or if only a few cells are 'bubbling,' continue the incubation and recheck in 5 minutes. The embryo should be transferred to the drop of derivation medium as soon as all trophoblast cells are lysed or if no new 'bubbling' cells appear after rechecking; the total incubation in complements should not exceed 30 minutes.
4. Gently pass the embryo through the opening of a thinly drawn capillary (about the diameter of the ICM): the lysed trophectoderm cells should detach after 1–2 passes.

5. Wash the ICM in the drops with derivation medium and place into the prepared well of a four-well plate. The ICM should attach within 24 hours.

29.5.4 ICM Dispersion

At early stages of derivation, we recommend doing the first dispersion as soon as at least 2–3 colony pieces can be obtained from the initial outgrowth (see Figure 29.3). The dispersed colonies may be left in the same well or

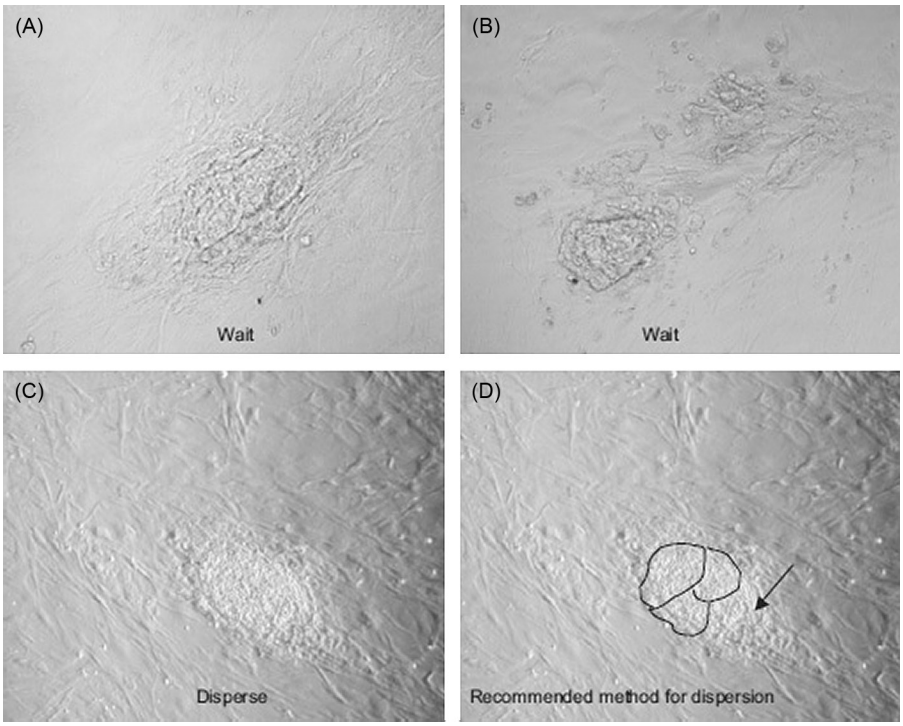

FIGURE 29.3 **Initial ICM outgrowth.**

The initial outgrowth of the ICM rarely shows typical ES colony morphology and often includes many differentiated-looking cells. When no obvious ES cell-like colonies large enough for dispersion can be located, more time is required before the first colony dispersion can be done. (A, B) The initial ICM outgrowth of two future hES lines. At this stage, when the dispersion is attempted, the outgrowth and the PMEF monolayer come up together, so it is better to wait longer before dispersing. (C) The ICM is ready for dispersion, when a colony of ES-like cells is formed which is large enough for dispersion into several pieces, leaving 20–50% of the outgrowth on the original place for future regrowth. (D) Dotted lines transcribe the number of pieces recommended for the dispersion of this colony. A narrow capillary is used and a small part of colony is left untouched (arrow). Magnification ×100.

moved to a new well. If there are only a few colony pieces (1–5) they should be placed close to each other but with enough space to permit growth. It is better to disperse colonies before they grow in contact with each other and prior to signs of differentiation such as becoming multilayered.

When the colony growth is slow, change 2/3 of the medium every 2–3 days to keep it conditioned at all times. As more colonies appear change 2/3 of medium daily and increase volume to 500 µl/well of a four-well plate.

Even when an original colony looks differentiated or comes off as a single piece, when replated it usually gives an outgrowth of hES cells. When doing the initial dispersion, a part of the original colony should be left untouched as a backup, especially if the picked pieces are transferred into a new well. Expect it to grow back in 1–2 days, and the new outgrowth can be picked and recombined with previously picked cells. Multiple harvests can be obtained from the initial outgrowth. It is critical at this stage to expand the number of colonies slowly and steadily. See Figure 29.4 for an example of the length of time between dispersions and appearance of the cultures during the process of derivation. In this case no immunosurgery was done because the trophectoderm was not sufficiently intact.

29.6 MAINTENANCE OF ESTABLISHED hES CELL CULTURES

Usually, once a steady growth of colonies is reached, use of the hESC derivation medium is discontinued and the cultures are maintained in hESC growth medium. For established cultures, replace 2/3 of the medium volume with fresh medium daily; do not allow the medium to turn yellow. Cultures should be expanded gradually by the progression from relatively sparsely populated four-well dishes to confluent four wells to 35 mm dishes. Throughout the process the cultures should be observed daily, differentiated colonies removed and undifferentiated colonies dispersed as necessary (see Figure 29.5 for examples of approaches to be used for dispersion of colonies with different morphologies).

By the time the cells are growing on a 35 mm dish, it is usually sufficient to disperse 50–100 average-sized colonies to populate a new well. In 1–2 days it may be necessary to disperse some of the larger colonies, leaving the pieces in the same well. Usually, mechanical passaging needs to be done every 5–6 days, but several larger colonies may need to be dispersed daily.

29.6.1 Adaptation of hES Cells to Trypsin

Our experience with trypsinization of over 30 hES cell lines demonstrates that after the initial adaptation of the lines to trypsin, this procedure can be

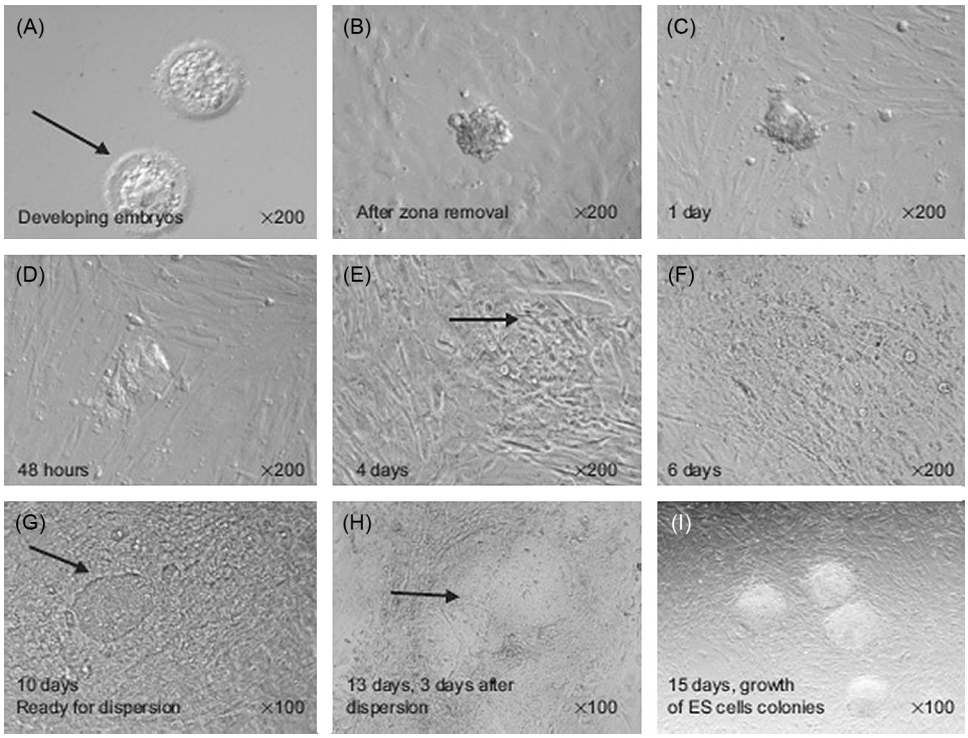

FIGURE 29.4 **Early stages of hES cell derivation.**

A blastocyst of poor quality, grade 3, arrow in (A) underwent the removal of the zona pellucida (B) and next day after being plated on the PMEF monolayer has attached (C). In 48 hours after plating the ICM appears smaller, possibly due to some cell death (D), and in 4 days after plating an outgrowing group of cells is visible with some small cells in the middle (E, arrow) which become less visible 2 days later (F). Ten days after plating (G): a small colony of ES-like cells (arrow) has formed within the large group of differentiated-looking cells and is now large enough for dispersion. Original ICM outgrowth (H) 2 days after it was dispersed; note regrowth of small ES-like cells in the cleared area (arrow) which are ready for another dispersion. Formation of ES cell colonies from the recombined first and second dispersion (I).

robust and yield large quantities of hES cells which exhibit all the properties of pluripotential cells. Trypsinized cells retain undifferentiated colony morphology, express characteristic molecular markers, i.e., Oct-4, alkaline phosphatase, SSEA-3, SSEA-4, TRA-1–60, TRA-1–81, differentiate into three germ layers *in vitro* and into teratomas *in vivo*, and maintain normal karyotypes.

Newly derived hES cells may be successfully passaged with trypsin as early as passage 2–3 from a four-well plate. However, trypsinization is not always successful and several attempts may be necessary before the cells are adapted to trypsin. We recommend always keeping a backup well of mechanically passaged cells. It has been shown that aneuploidy can develop in long-term

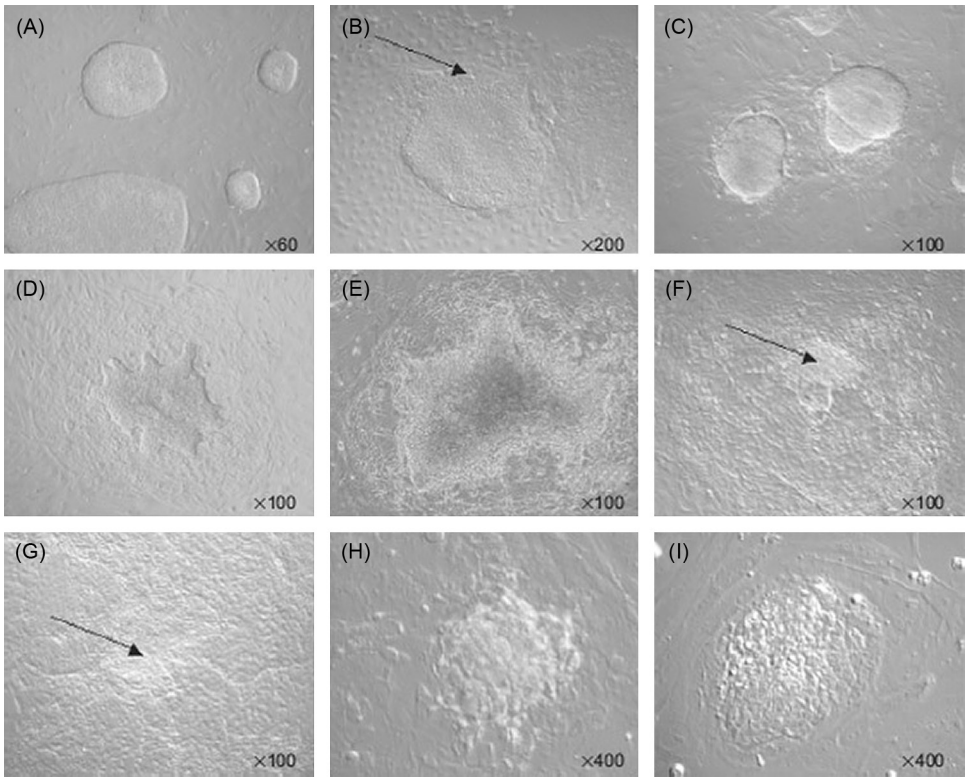

FIGURE 29.5 Evaluation of hES cell colony morphology.
Various types of morphology of hES cell colonies encountered at early stages of derivation when mechanical dispersion can be the tool of choice to selectively pick undifferentiated colony parts. (A) All colonies are undifferentiated and can be mechanically passaged. (B) Colony which has a few signs of differentiation (arrow) and is surrounded by differentiated cells; undifferentiated part is easily separated from surrounding differentiated cells. (C) In partially differentiated, multilayered colonies the centers are thickened and yellowish in color, can be mechanically dispersed into several small pieces but may result in both differentiated and undifferentiated colonies. (D, E, F) All these colonies are more extensively differentiated; a thin layer of differentiated cells covers them like a veil. They can be cut into pieces through the top cell layer and passaged, and may yield undifferentiated colonies. (G, H, I) These colonies are badly differentiated, the arrow on (G) shows a group of undifferentiated cells within the large differentiated area. If this one is to be saved, one should wait for a few days for this group to increase in size.

cultures, passaged enzymatically, therefore we recommend periodical karyotyping, and if aneuploidy is apparent, an earlier frozen stock should be expanded, or the line can be re-established by mechanical passaging if only a fraction of the cells appear aneuploid.

The safest approach is to begin with a sub-confluent 35 mm plate of colonies with good morphology. Mechanically pick 50–100 colonies and transfer into

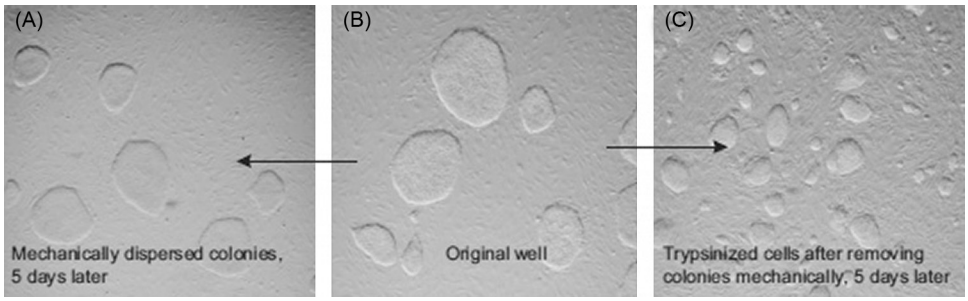

FIGURE 29.6 **Adaptation of mechanically passaged hES cells to trypsin.**
After 5 days of growth, colonies were mechanically dispersed and transferred to a fresh plate of PMEFs (A). Morphologically they are very similar to the original plate (B). The remaining colonies in the original plate, which were passaged with trypsin and plated onto the same diameter plate (C), show actively growing colonies and will probably be ready for passaging in 1–2 days.

a new well as a backup. Differentiated colonies may be removed mechanically prior to trypsinization. Trypsinize the remaining colonies in the original well and plate into the same diameter well. The cells should be ready for the next split in 5–7 days (Figure 29.6). For the second trypsinization, split 1:3. After this step the cells can usually be trypsinized routinely without problems, but always a mechanical backup should be maintained until the cells are frozen and the test vials are successfully thawed.

29.6.1.1 Trypsinization

Generally, hES cells recover from trypsinization better when not dispersed to single cells but remain as small cell clumps of approximately 5–20 cells. The procedure works best when hES cell colonies are dispersed by a combination of enzymatic digestion and pipetting; we do the pipetting before the PMEF monolayer and the colonies turn into a single-cell suspension. The time in trypsin required for the cells to detach varies depending on the hES cell density, degree of differentiation, age of the culture, temperature of trypsin, etc. Therefore, instead of providing a fixed incubation time in trypsin, we recommend checking the appearance of the hES culture under the microscope and empirically working out the best incubation time for each plate (see Figure 29.7).

1. Warm trypsin in a 37 °C water bath; keep warm until ready for procedure.
2. Rinse the cells with PBS, Ca^{2+}, Mg^{2+} free, 2 times (1–2 ml per 35 mm dish).
3. Add 1 ml of trypsin to each 35 mm dish. Incubate in the hood at room temperature for several minutes, usually 2–5, frequently checking the cells under the microscope. The cells are ready for mechanical dispersion when the PMEFs begin to shrink; the colonies should round up but remain attached. Some cells may begin to detach and float (Figure 29.7A,B).
4. Prepare a centrifuge tube with 10 ml of warm PMEF medium.

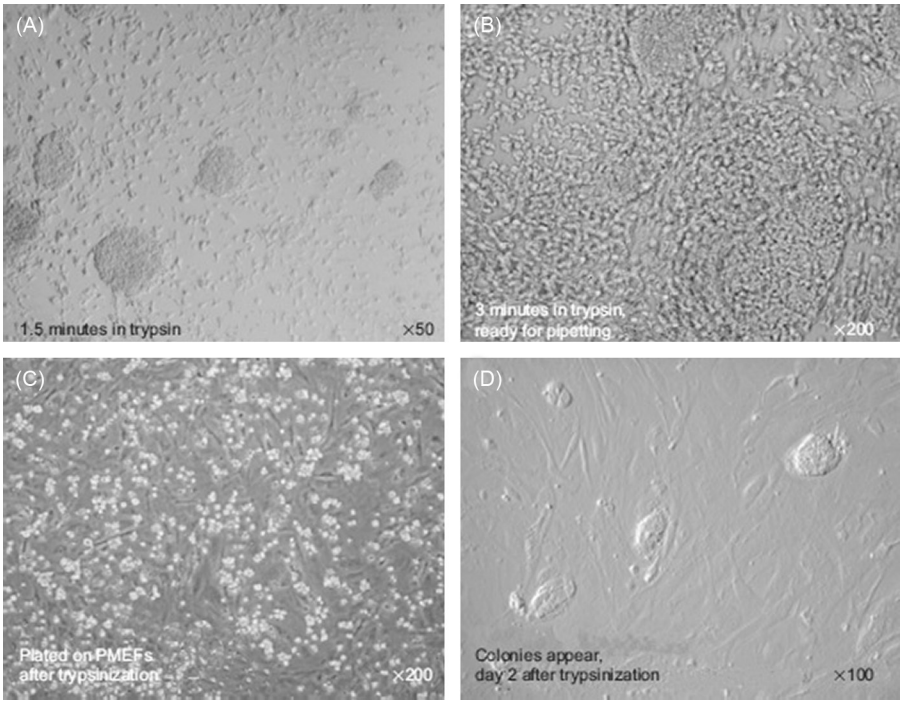

FIGURE 29.7 **Passaging by trypsinization.**

(A) 1.5 minutes in trypsin, the PMEFs look shrunken and the hES colonies have loosened a little. (B) 3 minutes in trypsin at higher magnification: the colonies compacted; this is a good time to begin pipetting. Please note that depending on the density of the colonies, days after passaging and the degree of differentiation the time required to reach this stage may vary and needs to be worked out empirically. (C) Suspension of hES cells after replating onto fresh PMEFs. Note the majority of the cells are in small cell aggregates. (D) Small colonies begin to appear on day 2 after plating (at 1:3 ratio). The time when the first colonies are seen may vary depending on the splitting ratio and the size of the cell aggregates.

Note: It is necessary to use PMEF medium to inactivate the trypsin because our hESC medium is serum free.

Tilt the plate and begin to gently pipette the trypsin solution up and down with a 1 ml pipetteman (Gilson type), pouring it over the cell monolayer at an angle. Properly digested cells should detach easily, leaving visible clear gaps in the monolayer, where the trypsin solution was poured. If no such gaps appear, leave it for another 1–2 minutes and test again. Expect the monolayer to detach after several repetitions. Usually, on cell cultures less than 5 days old you should be able to completely disperse the monolayer, but if the culture is older or very dense, there may be some undigested material

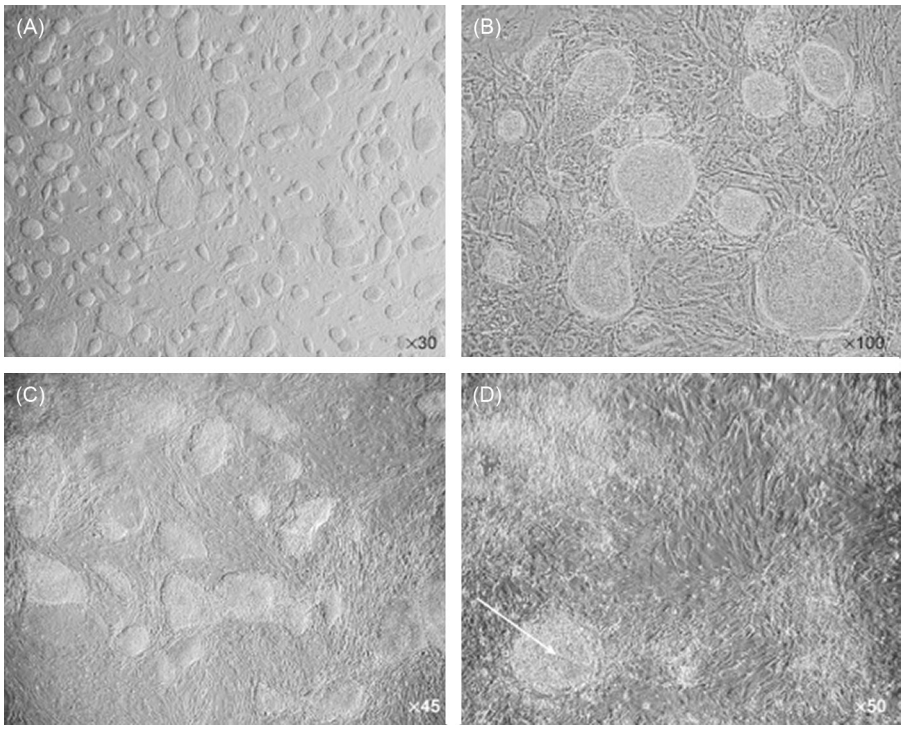

FIGURE 29.8
Evaluation of hES cell culture in preparation for the next trypsinization.
(A, B) Mostly undifferentiated hES cells ready for trypsinization. (C) The colonies are a little overgrown and show signs of differentiation but still can be safely passaged with trypsin. (D) Badly differentiated hES cells, an arrow points to a colony, which can still be rescued by mechanically picking colony pieces and passaging.

which can be discarded. Usually, it takes 5–10 pipetting strokes to break the colonies into small clumps of cells (see Figure 29.7C for approximate clump size). Extensive pipetting should be avoided. Using a cell culture pipette in place of an automatic pipettor will result in larger cell clumps and thus may require a longer time in trypsin.

5. Transfer the trypsinized cell suspension into the prepared centrifuge tube; centrifuge 5 minutes at 160 g.
6. Aspirate the medium and resuspend the pellet in hESC medium again, avoiding extensive pipetting to preserve small cell aggregates and replate at the desired ratio. The colonies should become visible in 1–2 days, depending on the splitting ratio and the clump size (Figure 29.7D).

Although hES cultures passaged with trypsin can be maintained in an undifferentiated state, if conditions are unfavorable due to changes in media/reagents quality, too high or low splitting ratio, or problems with PMEF quality, the cultures can have a degree of differentiation which should be evaluated prior to the next trypsinization (see Figure 29.8).

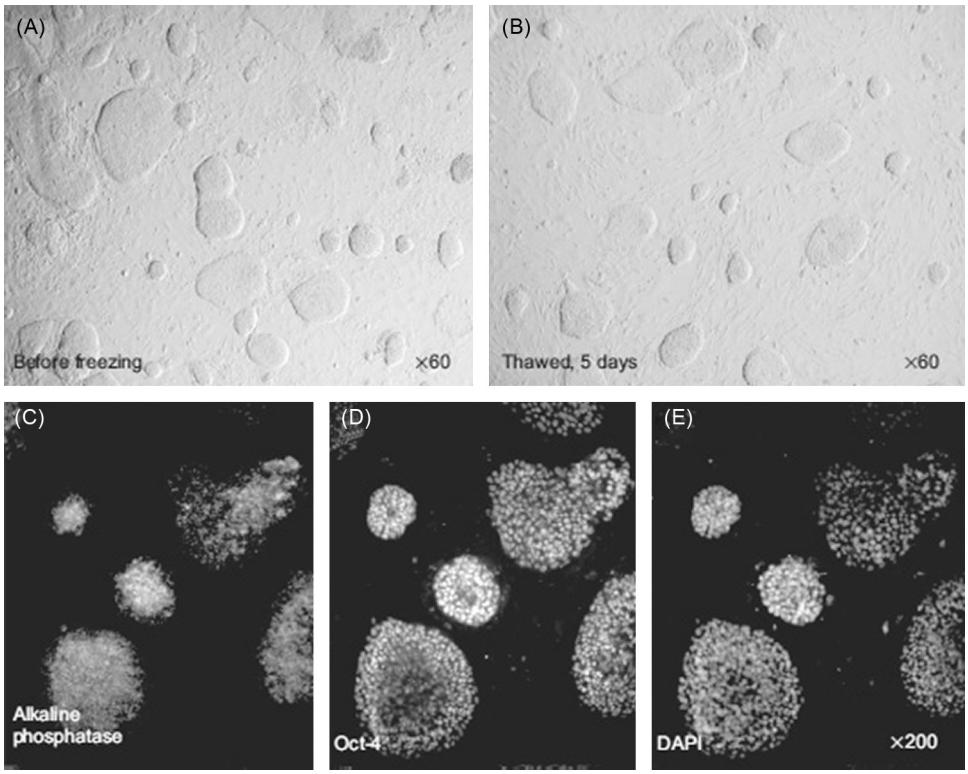

FIGURE 29.9 **Freezing and thawing of hES cells.**
The top panel shows the approximate density and morphology of the colonies of hES cells at freezing (A). When thawed onto the same diameter plate, the colonies should be ready for the next split in 5–7 days (B). Thawed hES cells show high expression of Oct-4 and alkaline phosphatase (C, D).

29.7 FREEZING hES CELLS

Many of the established hES cells have low recovery rates upon thawing, as low as 0.1–1%. This may be due to the method of passaging the cells. Mechanical picking or using collagenase dispersion usually results in large cell aggregates which presumably do not get cryopreserved as effectively as smaller clumps. Trypsinized cells in our lab have a recovery rate of about 10–20% or higher and do not require more complicated procedures like vitrification (Figure 29.9).

29.7.1 Freezing Medium

The best recovery rate was observed in freezing medium consisting of 90% FCS, 10% DMSO. However, Oct-4 expression in the thawed cells was lower

than in cells frozen in hESC growth medium with 10% DMSO. Nevertheless, by the next passage, the expression and distribution of Oct-4 and other markers of undifferentiated cells were indistinguishable between these two freezing conditions. We routinely use the 90% FBS, 10% DMSO medium.

29.7.2 Freezing Protocol

Select a high quality confluent culture with good morphology for freezing. We also recommend taking a picture of a sample field and staining for molecular markers characteristic of undifferentiated hES cells for future reference.

29.7.2.1 Materials Needed

Freezing medium: 90% FBS, 10% DMSO
Cryovials, labeled with the line, passage number, date
Cryovial rack (rack with ice reservoir by Corning)
Styrofoam rack from packaging for 15 ml centrifuge tubes
−80°C freezer

1. Trypsinize the cells; centrifuge in PMEF medium (see above).
2. Resuspend the pellet in cold freezing medium. We recommend freezing one confluent 35 mm plate per vial in 0.5 ml of freezing medium. Work quickly and keep the cells on ice after addition of the freezing medium.
3. Aliquot cell suspension into pre-chilled freezing vials and sandwich the vials between two Styrofoam racks; tape to prevent the two racks from separating and transfer to a −80°C freezer overnight. Transfer cryovials to liquid nitrogen for long-term storage.

29.8 THAWING hES CELLS

Thawing hES cells is a relatively simple procedure. The main rule to follow is to do everything quickly.

29.8.1 Preparation

1. Prepare Mitomycin C-treated PMEFs a day before thaw.
2. Make thawing medium. We use 70% hESC growth medium, supplemented with 20 ng/ml hLIF and 8 ng/ml bFGF with 30% hESC or PMEF-conditioned medium.
3. Change the medium on the PMEF plate to the thawing medium; equilibrate in the CO_2 incubator for 1 hour. For 35 mm plates use 1.5 ml, for four-well plates use 0.5 ml medium per well.
4. Prepare a 50 ml conical tube with 10–15 ml of warm hESC growth medium.

29.8.2 Thawing

1. Thaw the vial(s) in a 37 °C water bath, constantly agitating, while ensuring that the neck of the vial is above the water level. Check the content of the vial in about 40 seconds and then at 10-second intervals until only a small piece of ice remains.
2. Quickly spray the vials with 70% isopropanol, then using a 1 ml pipetteman add warm hESC medium to the contents of the vial dropwise with gentle agitation. Do it quickly but very gently. Immediately transfer the contents into the prepared 50 ml tube with warm hESC medium centrifuged at 160 g for 5 minutes.
3. Remove the medium completely, not touching the pellet.
4. Add 0.5 ml hESC thawing medium; gently resuspend the cells using a 1 ml pipetteman (2–4 repetitions), and transfer to prepared PMEF plates with equilibrated hESC thawing medium. Spread the cells evenly throughout the well by moving the plate several times in two directions, at 90 degrees to each other; avoid swirling.
5. Check the cells the next day; if there are many dead cells or the medium has changed color, change 2/3 of the medium, otherwise, do not change it for another day.
6. The colonies usually begin to appear in 3–4 days and can be ready for splitting in 5–10 days (Figure 29.7).

29.8.3 Challenging Situations

There are certain situations presenting an extra challenge, such as derivation of hES cells, especially feeder-free cultures, or isolating hES from a single blastomere, or from low quality embryos, etc. Success often depends on a combination of several factors, each of which, taken separately, may not necessarily provide a large improvement in success rate – but together they do. We consider the following factors to be critical:

- If feeder cells are used, they should be of excellent quality (tested prior to derivation), ideally treated with Mitomycin C no longer than 1 day prior to plating cells or embryo/ICM for outgrowth.
- Quality of Knockout Serum Replacement and Plasmanate (testing of each lot is needed).
- Concentration of bFGF (we use up to 20 ng/ml).
- Small initial volume of medium for outgrowth (20 µl drops under mineral oil works very well).
- Prompt response to any changes in the cells/outgrowth appearance – we check it at least daily and decide if it needs to be dispersed, or fresh/conditioned medium needs to be added, etc.

- If differentiation is observed, undifferentiated part of the colony should be separated from the differentiated cells. Based on the morphology, differentiated cells may be the easiest to remove, or undifferentiated parts can be moved to a fresh plate.
- When the first dispersion is done, we always leave a part of the colony intact – sometimes this would be the only surviving group of cells, as the replated colony pieces may not grow.
- For the first several passages, we keep all plates from previous passages, always leaving a part of the colony behind when mechanically passaging the cells, as it is possible to harvest from the same plate several times.

When a new line has gone through several passages and is growing on several plates, it is important to freeze down cells, even before they are adapted to trypsin. Freezing can be done by trypsinizing even one well of a four-well plate – although the recovery will probably not be the greatest, it should be possible to re-establish a line in a critical situation by mechanical passaging.

29.9 hES CELL QUALITY CONTROL

Although morphology of hES cells is often used for evaluating the quality of the culture and its readiness for passaging or freezing, this criteria alone cannot be used for assessment of the ES cells' pluripotency. Staining for the expression of Oct-4 or alkaline phosphatase even in colonies of 'perfect' morphology can result in one or both of these markers appearing in the cells only at the periphery of the colony. It is important, therefore, to regularly assess the cells by analyzing the expression of markers of pluripotent cells. We look at Oct-4, Nanog, SSEA-3, SSEA-4, TRA 1–60, and TRA 1–81 by immunostaining or perform an enzyme assay for alkaline phosphatase. The procedures for such assays and available antibodies are described. Routine karyotyping performed either in house or commercially avoids accumulation of aneuploidy in hES cultures, which can be especially important when establishing a master cell bank for manufacturing of differentiated derivatives. If aneuploidy is detected, a vial of an earlier passage should be thawed and tested. However, if the aneuploidy is present only in a subset of cells, it is often possible to 're-derive' a cell line by mechanical passaging, paying close attention to colony morphology. Testing for differentiation potential allows another important parameter to be assessed. In our laboratory we routinely perform differentiation of hES cells into retinal pigment epithelium and hemangioblast as a part of other ongoing projects, and if abnormal cell behavior such as low differentiation rate is observed in differentiation assays, the cultures are discarded and fresh stock is thawed. Other differentiation models for hES cells could be used for such routine assessment of the quality of the culture as determined by the research goals.

FOR FURTHER STUDY

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CHAPTER 30

Derivation and Differentiation of Human Embryonic Germ Cells

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30.1 INTRODUCTION

Embryonic germ (EG) cells are pluripotent stem cells derived from primordial germ cells (PGCs) that arise in the late embryonic and early fetal period of development. EG cells have been derived from several species, including the mouse, pig, chicken, and human. Mouse, pig, and chicken EG cells have been demonstrated to contribute to experimentally produced chimeric animals, including germ-line transmission in the latter two species. Furthermore, germ-line transmission of mouse and chicken EG cell derivatives have been demonstrated. Mouse and human EG cells can be differentiated *in vitro* to form embryoid bodies (EBs). Like EBs generated from embryonic stem (ES) cells, EG-derived EBs contain differentiated cells representing all three germ layers, as well as mixed-cell populations of less differentiated progenitors and precursors. These human EB-derived (EBD) cells are capable of considerable cell proliferation and express a variety of lineage-specific markers. Human EBD cell cultures have a normal and stable karyotype and normal patterns of genomic imprinting, including X-inactivation. Transplantation studies have demonstrated that human EBD cells can engraft into a variety of rodent tissues and can participate in the recovery of rats following motor neuron injury.

Pluripotent stem cells have been derived from two embryonic sources. ES cells were first derived from the inner cell mass of mouse preimplantation embryos, and EG cells were initially derived from mouse PGCs. Subsequently, EG cells have been derived from chicken, pig, and human PGCs. Pig, chicken, and mouse EG cells have been demonstrated to contribute to experimentally produced chimeric animals, including germ-line transmission in the latter two species.

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30.1.1 Primordial Germ Cells

PGCs are the sole means of genetic transmission between parent and offspring, as they generate eggs and sperm. In many species, such as *C. elegans*, germ cells are segregated very early in development, during the first embryonic cleavages, and are marked by deposition of ribonucleoprotein P-granules. In mammals, the process occurs later in development, and seems to be directed more by extrinsic factors than by preprogrammed intrinsic differences. For example, in mice, cells that generate PGCs are located close to extraembryonic ectoderm during gastrulation. Rather than having a previously determined fate, cells in this location receive external signals to further differentiate into PGCs, as demonstrated by the observation that transplantation of cells from other parts of the epiblast to this region can take on a PGC fate. Several components of this signaling process have been identified. Initially, bone morphogenetic protein 4 (BMP4) and BMP8b are produced by extraembryonic ectoderm program cells from the epiblast to become extraembryonic mesoderm precursors or PGCs. Cells destined to become PGCs express higher levels of membrane protein fragilis than nuclear protein stella.

In the mouse, PGCs are visible as alkaline phosphatase (AP) positive cells at the base of the allantois at 7.5 to 8.0 days postcoitus (dpc). They begin to associate with the endoderm that is invaginating to form the hindgut at 8.5 dpc. By 10.5 dpc, PGCs are associated with dorsal mesenteries and are translocated to the genital ridges. The migration of PGCs is caused by both cellular migration and association with moving tissues. Throughout this migration, PGCs expand from approximately 130 cells at 8.5 dpc to more than 25,000 at 13.5 dpc. Once they arrive at the genital ridge, PGCs continue proliferating until they enter prophase of the first meiotic division. In males, entry into meiosis is inhibited by signals from the developing testis, blocking PGCs at G0 until after birth. In the absence of inhibitory signals, female PGCs undergo oogenesis. Although not as thoroughly studied, much is known regarding the migratory path of human PGCs, including their association with gut endoderm and migration into developing genital ridges.

PGCs do not survive well under standard tissue culture conditions, and are not pluripotent stem cells *in vivo* or *in vitro*. Early attempts to use various growth factors and feeder layers succeeded in prolonging their survival, but proliferation was limited. The combination of leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF), and c-kit ligand (KL, also known as stem cell factor, mast cell factor, or steel factor) proved to result in an immortal cell population, especially if the KL was presented in the transmembrane form by a layer of 'feeder' cells (see the section 'Feeder Layer'). Instead of simply encouraging PGC proliferation, these factors cause the normally solitary PGCs to congregate and proliferate as multicellular colonies, known as EG cells, and to gain pluripotency. Mouse EG cell lines have been

derived from PGCs prior to migration around 8.0–8.5 dpc, during migration at 9.5 dpc, and after entry into the genital ridges between 11.5 and 12.5 dpc.

The roles played by KL and the tyrosine kinase receptor for KL, c-Kit, in the *in vitro* derivation of EG cells from PGCs have parallels *in vivo*. c-Kit is expressed in PGCs, and KL is expressed along the PGC migratory pathway and in the genital ridges. The roles of KL and c-Kit in PGC survival were originally characterized through several mutations at their respective loci, *Sl* and *W*, which resulted in subfertile or sterile mice. PGCs are formed in homozygous mutant embryos of *W* and *Sl*, but mitosis is severely impaired, and the few PGCs that reach the gonad do not survive. KL is produced as a membrane-bound growth factor that can undergo proteolytic cleavage to generate a soluble form. Mice lacking the membrane-bound KL, but not the soluble form, maintain low PGC numbers and are sterile, suggesting that the membrane-bound form but not the soluble form is essential for PGC survival. The mechanism involved in KL-induced PGC survival has been shown to involve suppression of apoptosis. The c-kit receptor has also been shown to be involved in the adhesion of mouse PGCs to somatic cells *in vitro*. Other recent studies attempting to identify signaling pathways activated by KL binding to its receptor in mouse PGCs have shown activation of AKT kinase and telomerase.

In contrast to the embryologically early and relatively undifferentiated epiblast, PGCs arise late and have a specialized role during normal development. In this regard, it is somewhat surprising that exposure to three cytokines can convert PGCs into pluripotent stem cells *in vitro*. It is possible that the flexibility provided by extrinsic signaling during PGC specification, rather than intrinsic preprogramming, allows for this conversion.

30.1.2 Comparison to Embryonic Stem Cells

Both mouse ES and EG cells are pluripotent and demonstrate germ-line transmission in experimentally produced chimeras. Mouse ES and EG cells share several morphological characteristics, such as high levels of intracellular AP and presentation of specific cell-surface glycolipids and glycoproteins. These properties are characteristic of, but not specific to, pluripotent stem cells. Other important characteristics include growth as multicellular colonies, normal and stable karyotypes, the ability to be continuously passaged, and the capability to differentiate into cells derived from all three EG layers: endoderm, ectoderm, and mesoderm.

30.2 HUMAN EMBRYONIC GERM CELL DERIVATION

Although many combinations of cytokines and feeder layers have been evaluated, the standard practice for derivation of human EG cells remains similar to

techniques developed for the mouse. As of 2003, approximately 140 human EG cultures have been derived in our laboratory using this general technique.

30.2.1 Initial Disaggregation and Plating

Gonadal ridges and mesenteries of week 5–9 post-fertilization human embryos (obtained as a result of therapeutic termination of pregnancies) are collected in 1 ml ice-cold growth media and rapidly transported to a sterile work space. The tissues are then soaked in calcium-magnesium-free Dulbecco's phosphate-buffered saline (DPBS) for 5 minutes, and then transferred to 0.1 ml trypsin-ethylenediaminetetraacetic acid (EDTA) solution. The concentration of trypsin and EDTA is varied, such that at the earliest developmental stages, a gentler 0.05% trypsin–0.5 mM EDTA is used, and at later developmental stages, a stronger 0.25% trypsin–0.5 mM EDTA solution is used. The tissue is mechanically disaggregated thoroughly using a fine forceps and iris scissors. This process is carried out for 5 to 10 minutes at room temperature and the result is then incubated at 37°C for 5 to 10 minutes. This disaggregation process often results in a single-cell suspension and large pieces of undigested tissue. To stop the digestion, serum-containing growth media is added. The digested tissue is transferred to wells of a 96-well tissue culture plate that has been previously prepared with feeder layer (see the sections 'Plating an STO feeder layer prior to inactivation' and 'Plating an STO feeder later after inactivation'). Usually, the initial plating occupies four to 10 wells of the 96-well plate. The plate is incubated at 37°C in 5% CO_2 with 95% humidity for seven days. Approximately 90% of the growth media is removed each day, and the plate is replenished with fresh growth media.

30.2.2 Subsequent Passage of Embryonic Germ Cell Cultures

In the first seven days of derivation (passage 0), most human EG cultures do not produce visible EG colonies. Staining for AP activity demonstrates the presence of solitary PGCs, with either stationary or migratory morphology (Figure 30.1 A,B). Often, colonies of cells that do not stain AP^+ are seen (Figure 30.1 D,E), as are small clumps of tissue remaining from the initial disaggregation (Figure 30.1 C). After seven days, the media is removed, and the wells are rinsed twice with calcium-magnesium-free DPBS for a total of five minutes. Then, 40 ml of freshly thawed trypsin solution is added to each well, and the plate is incubated on a heated platform or in a tissue culture incubator for five minutes at 37°C. As previously described, the trypsin solution is 0.05% trypsin–0.5 mM EDTA, 0.25% trypsin–0.5 mM EDTA, or a mixture of these two solutions. The important point at this stage is to facilitate the complete disaggregation of the STO cell feeder layer (see the section 'Feeder layer'), which can be a significant challenge. After the incubation in

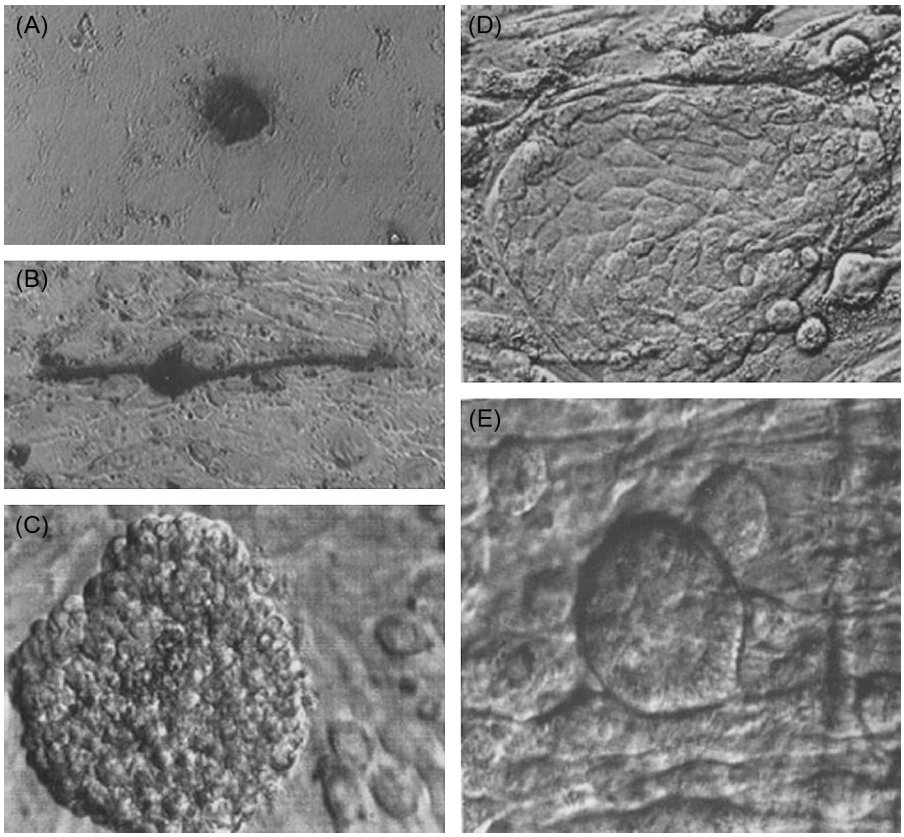

FIGURE 30.1 **Cell morphologies seen in early-passage human embryonic germ culture.** (A, B) Alkaline phosphatase-positive (AP^+) stationary and migrating primordial germ cells. (C) Multicellular piece of undisaggregated gonadal tissue. (D, E) Flat and round cell colonies that do not lead to human EG cells.

trypsin, the edge of the 96-well culture plate is hit firmly against a solid surface until the STO cells have completely lifted off the growth surface. This process can be aided by scraping the well and gently triturating. After the STO cells have been loosened, fresh growth media is added to each well, and the contents are triturated. This phase is critical to successful disaggregation of STO feeder layer and EG cells.

All subsequent passages are done as described. After 14 to 21 days (during passage 1 or 2), large and recognizable EG colonies will arise in some of the wells (Figure 30.2). At this point, wells that do not have EG colonies are discarded.

Several common problems occur during the passage of human EG cells. One observation is that the STO feeder layer will sometimes not fully disaggregate.

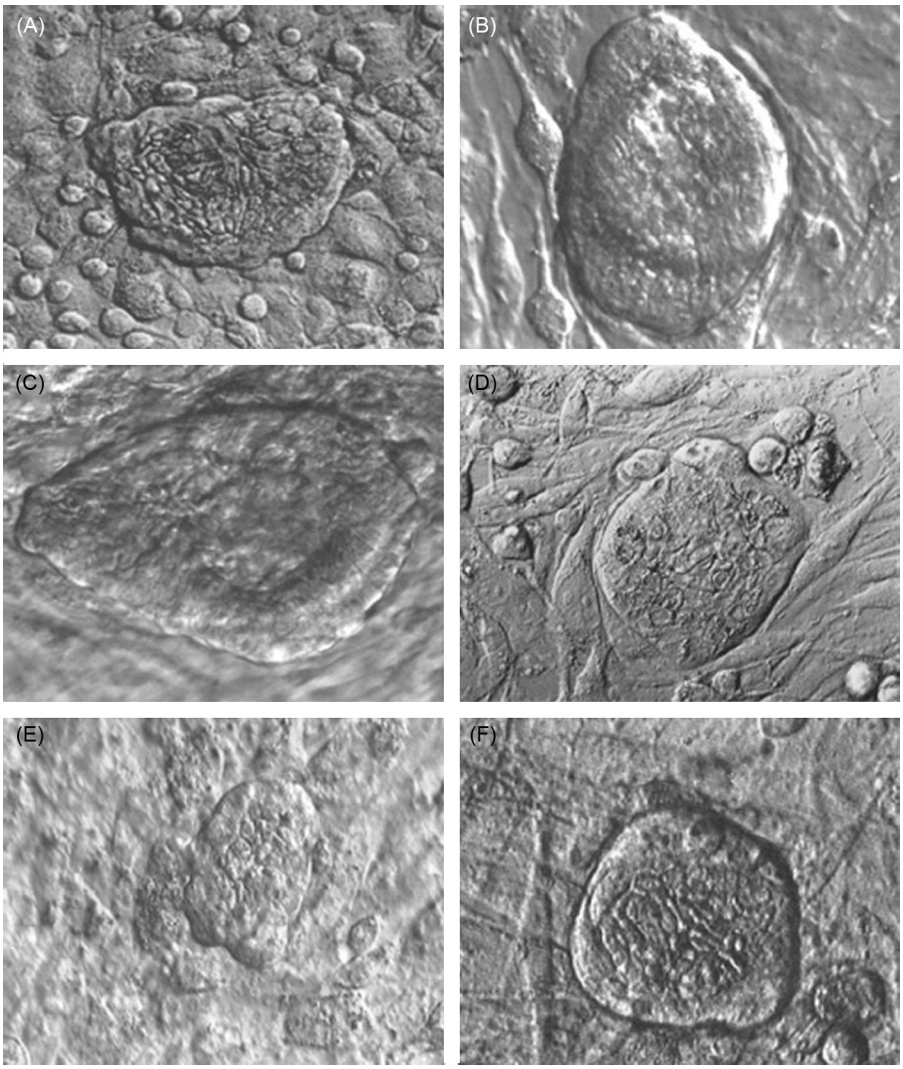

FIGURE 30.2 Human EG colonies growing on an STO cell feeder layer.

This can be observed by the presence of large cell aggregates immediately following disaggregation. If this occurs with regularity, it is a sign that the trypsinization method is insufficient, or that the STO cells have become less contact inhibited and have overgrown during the seven days of culture. Another common occurrence is that the EG colonies do not fully disaggregate. The consequences of poor disaggregation are that the large pieces differentiate or die, and fewer EG cells are available for continued culture

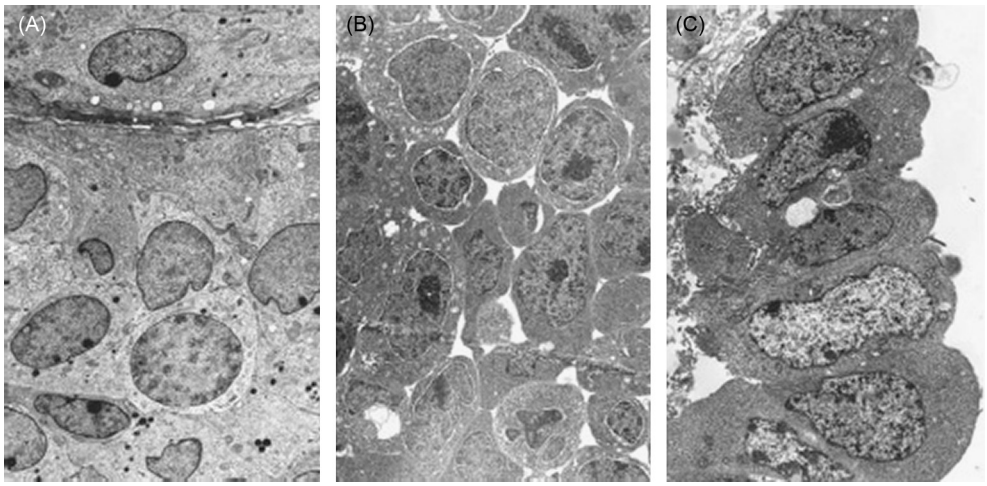

FIGURE 30.3 **Electron micrograph of EG and ES colonies.**
(A) Human EG colony; (B) mouse EG colony; and (C) mouse ES colony.

expansion. Although much effort has been expended in finding a solution to this problem, it remains the most difficult aspect of and challenging hurdle to human EG cell biology.

To gain some insight into this problem, a series of electron microscopic images were taken to compare the cell-cell interactions found in mouse ES, mouse EG, and human EG cell colonies. It is evident from these images that cells within the human EG colonies adhere more completely to each other than cells within mouse ES and EG colonies (Figure 30.3). It is possible that this tight association within the colony limits the access of disaggregation reagents. At this time, neither the nature of the cell-cell interactions nor an effective solution to this problem is evident.

Because of incomplete disaggregation and other intrinsic or extrinsic signals, many human EG colonies (10–30% per passage) differentiate to form three-dimensional structures termed EBs (see the section 'EB formation and analysis') or flatten into structures that are AP− and do not continue to proliferate (Figure 30.4). EG colonies that are more fully disaggregated go on to produce new EG colonies, and under the best circumstances, EG cultures can be expanded continuously for many months, and have routinely exceeded 20 passages. Inevitably, as large EG colonies are removed from the culture as a result of EB formation, the cultures become sparse, and are discontinued for practical considerations. Efforts employing standard dimethyl sulfoxide (DMSO) cryopreservation techniques have so far been unsuccessful.

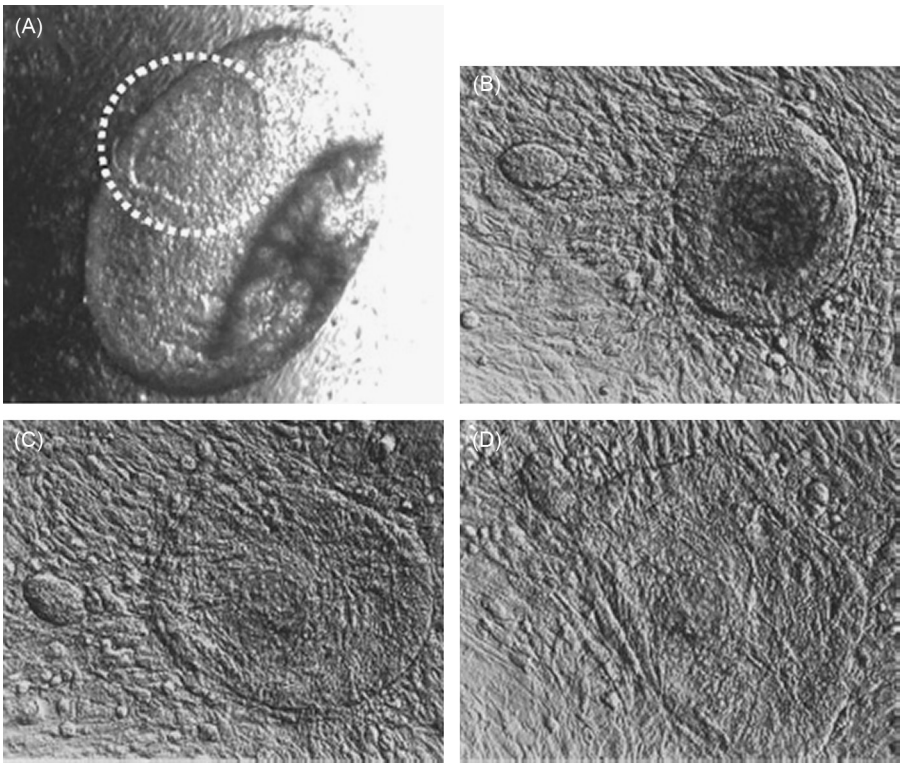

FIGURE 30.4 **Differentiating human EG colonies.**
(A) Stereomicrograph of an EG colony with attached embryoid body (EB). The EG colony is circled. The diameter of the EB is approximately 0.5 mm. (B–D) Time-lapse study of EG cell flattening. (B) Four days after disaggregation, the small EG colony on the left results from more complete disaggregation. The large colony on the right is composed of a dark residual colony surrounded by new EG cell growth. (C) Five days after disaggregation, the large EG colony has begun to flatten. (D) Seven days later, the large EG colony has completely flattened and did not survive as a recognizable EG cell colony after disaggregation and replating. In a separate experiment, EBs and these flattened structures are shown to be largely AP^{-}.

30.2.3 Feeder Layer

Unlike ES cells, EG cell derivation is highly dependent on a specific type of feeder layer. Mouse STO fibroblasts are a spontaneously transformed cell line from the Sandoz inbred mouse. They are thioguanine and ouabain resistant; features of historical interest, but not used in this context. STO cells have been used to derive mouse embryonal carcinoma (EC), ES, and EG cells, and to date they are the only cell type capable of generating human EG cells. The factor or factors provided exclusively by this cell type are not fully understood. However, the transmembrane form of KL is present on STO fibroblasts, but not on most other cell types evaluated in our laboratory.

Although STO is a clonal cell line, individual isolates vary greatly in their ability to support human EG derivation. This is further complicated by the known phenotypic variation of STO cells in continuous culture. Given the very limited supply of human tissue, it is prudent to screen STO cells for suitability prior to use. The most reliable screening method is to produce a number of clonal STO lines (by limiting dilution or cloning cylinder), and to evaluate them for their ability to support the derivation of mouse EG cells. The growth of existing mouse EG lines is not a sufficient method, as most mouse EG lines become feeder layer independent after derivation. Derivation of mouse EG cells is not a simple undertaking. In an effort to screen STO cells more rapidly, and to investigate the role of transmembrane KL, we have begun to screen STO cell lines by using an immunocytochemical method to detect KL. Once a supportive STO fibroblast line is identified, it should be immediately cryopreserved in several low-passage aliquots. One of these aliquots can then be expanded to provide many medium passage aliquots, which are thawed and used with limited further expansion. Continuous passage of STO fibroblasts without frequent screening should be avoided.

30.2.3.1 Plating an STO Feeder Layer Prior to Inactivation

Two different methods can be used to prepare an STO feeder layer: plate-then-irradiate and irradiate-then-plate. Most human EG cultures are derived using the former method. This is practical when a large g-irradiator is available. STO cells are passaged for short periods (not continuously) in PGC growth media without LIF, bFGF, or forskolin, and are disaggregated using 0.05% trypsin–EDTA solution. One day prior to use, a 96-well culture dish is coated with 0.1% gelatin for 30 minutes. The gelatin is withdrawn, and 5×10^4 STO cells are plated per well in PGC growth media without the LIF, bFGF, or forskolin. Similar cell densities ($\sim 1.5 \times 10^5$ cells/cm^2) can be achieved in other well configurations. The cells are grown overnight and then exposed to 5000 rads (1 rad $\times$ 0.01 Gy) g-radiation or X-ray. The cells are then returned to the tissue culture incubator until required. Prior to use, the growth media is removed, 0.1 ml PGC growth medium with added factors is added to each well (or half of the required well volume), and the dish is returned to the tissue culture incubator.

30.2.3.2 Plating an STO Feeder Layer after Inactivation

This method of STO cell preparation is used when a large g-radiation unit is not available, when large amounts of cells are required, or if better control of STO cell density is required. STO cells are grown as described previously, trypsinized, counted, and resuspended in PGC growth media without added factors. The cells are placed into one or more 50 ml conical tubes, and placed into the g-irradiator or X-ray device. Cells are exposed to 5000 rads g-radiation or X-ray. Following exposure, cells are adjusted to a convenient

concentration with PGC growth media without added factors, counted, and plated into tissue culture dishes that have been previously coated with 0.1% gelatin for 30 minutes. Cells are allowed to adhere overnight; then the media is replaced with half the final volume of PGC growth media, including factors prior to use.

30.2.4 Primordial Germ Cell Growth Media Components

Human EG cells are derived and maintained in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 15% fetal bovine serum (FBS, Hyclone), 0.1 mM nonessential amino acids (Gibco BRL), 0.1 mM 2-mercaptoethanol (Sigma), 2 mM glutamine (Gibco BRL), 1 mM sodium pyruvate (Gibco BRL), 100 μg/ml penicillin (Gibco BRL), 100 mg/ml streptomycin (Gibco BRL), 1000 μg/ml human recombinant LIF (hrLIF, Chemicon), 1–2 ng/ml human recombinant bFGF (hrbFGF, R&D systems), and 10 mM forskolin (Sigma) prepared in DMSO.

30.2.5 Evaluation of Embryonic Germ Cell Cultures

Of 150 human PGC cultures initiated, 142 (~95%) demonstrated morphological, biochemical, and/or immuno-cytochemical characteristics consistent with previously characterized pluripotent stem cell lines. The easiest and most consistent method for evaluating EG cultures is to note the presence of tightly compacted multicellular colonies resembling early-passage mouse ES and EG cell colonies (Figure 30.2), rather than the flattened and more loosely associated colonies seen in human EC and rhesus ES cells. Under the best circumstances, the number of colonies should double or triple over a one-week passage. The trend is usually to start with small colonies, and to end with larger colonies that result from incomplete colony disaggregation (see the earlier section 'Subsequent passage of EG cultures').

High levels of AP activity are associated with human EG cells. Under standard culture conditions, human EG colonies are >70–90% AP^+. As colonies differentiate, one can observe a lower staining percentage and weaker staining, sometimes restricted to the periphery of the colony.

Human EG cells have been further characterized by a bank of five monoclonal antibodies: SSEA-1; SSEA-3; SSEA-4; TRA-1-60; and TRA-1-81. Colonies stain strongly for four of the five antibodies. The antibody recognizing SSEA-3 antigen stains the cells inconsistently and weakly. As with the results of AP staining, the percentage of cells within a colony that stain positive is variable.

The histological profile of human EG cells (AP^+, SSEA-1^+, SSEA-3^+, SSEA-4^+, TRA-1-60^+, and TRA-1-81^+) differs from undifferentiated human EC and rhesus ES cells, which are SSEA-1^-. The fact that differentiation of the human EC line NTERA2 leads to increased expression of SSEA-1 may suggest that this is

indicative of differentiation in the human EG cultures. However, NTERA2 differentiation is accompanied by the loss of the other markers, which are not observed in these cultures.

Karyotypic analyses carried out at passage 8–10 (60–70 days in culture) indicated apparently normal human chromosomes at the 300-band level of resolution. Both XX and XY cultures have been derived. Other markers of pluripotency, such as mRNA expression of the human ortholog of mouse Oct3/4 and telomerase enzyme activity, have been examined. Results differ greatly, depending on the status of the EG colony, culture, or both. In general, relatively undifferentiated EG colonies are OCT4 mRNA$^+$ as detected by reverse transcriptase polymerase chain reaction (RTPCR) and have detectable levels of telomerase.

30.2.5.1 Alkaline Phosphatase and Immunocytochemical Staining

Cells are fixed for detection of AP activity in 66% acetone–3% formaldehyde and then stained with naphthol/ FRV-alkaline AP substrate (Sigma). For immunocytochemistry, cells are fixed in 3% paraformaldehyde in DPBS. Cell-surface glycolipid- and glycoprotein-specific monoclonal antibodies are used at 1:15 to 1:50 dilution. MC480 (SSEA-1), MC631 (SSEA-3), and MC813-70 (SSEA-4) antibodies were supplied by the Developmental Studies Hybridoma Bank (University of Iowa). TRA-1-60 and TRA-1-81 were a gift from Dr. Peter Andrews (University of Sheffield, UK). Antibodies are detected by using biotinylated antimouse secondary antibody, streptavidin-conjugated horseradish peroxidase, and AEC chromogen (BioGenex).

30.2.5.2 Embryoid Body Formation and Analysis

EBs form spontaneously in human EG cultures. Although this represents a loss of pluripotent EG cells from the culture, EBs provide evidence for the pluripotent status of the culture, and provide cellular material for subsequent culture and experimentation (see the section 'EBD cells'). Initially, EBs provided the only direct evidence that human EG cultures were pluripotent, as all attempts to form teratomas in mice from human EG cells failed. To this day, there is no evidence of teratoma formation from human EG cells or their derivatives.

30.2.5.3 Embryoid Body Embedding and Immunohistochemistry

The constituent cells of EBs can be identified most reliably by embedding them in paraffin and staining sections with a bank of well-characterized antibodies. This process avoids the significant problem of antibody trapping that complicates analyses of large three-dimensional structures when direct staining is attempted. EBs are collected from cultures and placed into a small drop

of molten 2% low melting point agarose (FMC), prepared in DPBS, and cooled to 42°C. Solidified agarose-containing EBs are then fixed in 3% paraformaldehyde in DPBS and embedded in paraffin. Individual 6 mm sections are placed on microscope slides (ProbeOn Plus, Fisher Scientific). Routinely, immunohistochemical analysis is carried out by using a BioTek-Tech Mate 1000 automated stainer (Ventana-BioTek Solutions). Manual staining is also carried out using standard immunohistochemical methods; however, antigen retrieval is required for some of the antibodies employed. Cryosections of EBs generally produce less satisfactory cell morphology and have not been used extensively.

Antibodies used on paraffin sections include HHF35 (muscle specific actin, Dako), M760 (desmin, Dako), CD34 (Immunotech), Z311, (S-100, Dako), sm311 (panneurofilament, Sternberger Monoclonals), A008 (a-1-fetoprotein), CKERAE1/AE3 (pancytokeratin, Boehringer Mannheim), OV-TL 12/30 (cytokeratin 7, Dako), and K_S20.8 (cyto-keratin 20, Dako). Primary antibodies are detected by using biotinylated antirabbit or antimouse secondary antibody, streptavidin-conjugated horseradish peroxidase, and DAB chromogen (Ventana-BioTek Solutions). Slides are counterstained with hematoxylin. Using these and other antibodies it can be demonstrated that, when human EG cells differentiate, they form EBs comprised of endodermal, ectodermal, and mesodermal derivatives.

30.3 EMBRYOID BODY-DERIVED CELLS

Although a compelling demonstration of the potential of human EG cells, the limited growth characteristics of differentiated cells within EBs, and the difficulties associated with their isolation, make extensive experimental manipulation difficult and limit their use in future cellular transplantation therapies. At least two possibilities exist to explain the presence of the observed differentiated cell types. These cells could be generated directly from the pluripotent EG cell, or they could proceed through a series of precursor or progenitor cell types, prior to the acquisition of a mature phenotype. It seems unlikely that cells within EBs would bypass normal pathways of differentiation, so efforts have been made to isolate and expand these populations. The hypothesis was that progenitor-precursor cells would have desirable proliferation characteristics, and could be recognized by the expression of molecules known to mark progenitor-precursor populations, as well as by the simultaneous expression of markers normally considered part of a mature cellular expression repertoire. Simultaneous expression of neuronal and glial markers by neural progenitors and the expression of a variety of lineage-affiliated transcription factors and cytokine receptors by multipotent hematopoietic progenitors provide some basis for this hypothesis. In this model of

differentiation, multi-lineage gene expression by precursor or progenitor cells defines a ground state from which cell-extrinsic and -intrinsic signals work to continuously define a differentiated expression pattern and phenotype, resulting in the developmental plasticity observed after the differentiation of bone marrow and central nervous system stem cells.

The method used to isolate cell populations from EBs is conceptually similar to microbiological selective media experiments. EBs are disaggregated and plated into several different cellular growth environments. These environments consist of combinations of a growth media and a matrix. Although many combinations were evaluated, most EBD cell cultures have been derived from one of six environments, formed by combinations of two growth media and three plating surfaces. The growth media are an RPMI 1640 media supplemented with 15% FBS, and a low (5%) FBS media supplemented with bFGF, epidermal growth factor (EGF), insulin-like growth factor 1 (IGF1), and vascular endothelial growth factor (VEGF). The plating surfaces are bovine type I collagen, human extracellular matrix extract, and tissue culture-treated plastic. These are not intended to be highly selective environments. Instead, they favor several basic themes: cells thriving in high serum and elevated glucose (10 mM) conditions versus cells proliferating in low glucose (5 mM) under the control of four mitogens. Surfaces included binding to type I collagen, a biomatrix often thought to favor undifferentiated proliferation versus human extracellular matrix, a more complex mixture of laminin, collagen, and fibronectin. The initial assay is to determine conditions that favor extensive cell proliferation, with the hypothesis that this condition will favor undifferentiated cell populations, or at least provide numerical disadvantage to the terminally differentiated constituents of the EB.

30.3.1 Embryoid Body-Derived Growth and Expression Characteristics

Cell populations capable of long-term and robust proliferation can be isolated in this way from human EG-derived EBs. Embryoid body-derived is the generic term used to describe cells derived in this way *and* capable of extensive further proliferation. In general, the type I collagen and human extracellular matrices combined with the low serum media provide the most rapid and extensive cell proliferation. EBD cell lines and cultures are routinely maintained in the environment in which they were derived. The EBD naming convention aids this process. The first two letters of the name refer to the EG culture from which it was derived. The second letter indicates the growth media (E for EGM2MV and R for RPMI1640), and the last letter indicates the matrix (C for collagen, E for human extracellular matrix extract, and P for plastic). For example, the embryoid body-derived (EBD) culture SDEC was derived from embryonic germ (EG) culture SD in EGM2MV medium on type I collagen.

To distinguish EBD cells from a simple population of cells, rapidly proliferating but presumably uninteresting in terms of molecules expressed, it is important to establish a robust expression profile assay. The assay should use redundant measures when possible and must combine breadth, sensitivity, specificity, and speed. A series of 24 RTPCRs detecting products from five cell lineages (neuronal, glial, muscle, hematopoietic–vascular endothelia, and endoderm) combined with immunocytochemical staining provides a rapid measure of cell expression. Inevitably, molecular markers are not as definitively specific as desired, so multiple markers for each lineage are advisable.

Using mRNA and antibody expression profiling, we can demonstrate that most rapidly proliferating EBD cell cultures simultaneously express a wide array of mRNA and protein markers normally associated with distinct developmental lineages. This is not a surprising property considering that EBD cells are, at least during the derivation stage, a mixed-cell population. More remarkable is the finding that most (11 of 13) EBD cell lines isolated by dilution cloning also exhibit a broad multi-lineage gene expression profile. It can also be demonstrated that the expression profile for a given EBD culture remains stable throughout the lifespan of the culture. This normally exceeds 70 population doublings, but is not unlimited since EBD cells are not immortal.

More than 100 EBD cell cultures and clonally isolated cell lines have been derived and characterized as described here. Most of these cultures share the properties of rapid and robust proliferation, and broad multi-lineage gene expression. Less than 10% of the EBD cultures derived have a narrow expression profile, with one extreme case expressing only nestin, vimentin, and a-1-fetoprotein mRNA. Other general trends in EBD expression are that many cultures appear to be neurally biased, with strong expression of neuronal, glial, and neural progenitor markers, and relatively weak in expression of muscle markers.

Other general characteristics of EBD cells are the relative ease with which they can be genetically manipulated, using lipofection and electroporation, as well as retroviral, adenoviral, and lentiviral vectors. Adenoviral and lentiviral vectors are capable of nearly 100% transduction efficiency. These techniques have been used to generate EBD lines that constitutively and tissue-specifically express enhanced GFP and contain many different genetic selection vectors. EBD cells can be immortalized by retroviral-based expression of the telomerase RNA subunit (pBABE). Interestingly, after several hundred population doublings, these lines often become genetically unstable, generating at least two rearrangements: [47,XX,-1, + del (1)(q12), + i (1)(q10)] and [46,XX, del(4)(p14)]. In addition, these EBD lines tend to have narrow expression profiles.

The imprinting pattern of several EBD cultures has been examined. In one study, expression levels of four imprinted genes (*TSSC5*, *H19*, *SNRPN*, and *IGF2*) were determined in five EBD cultures. Three of these genes (*TSSC5*, *H19*, and *SNRPN*) had normal monoallelic expression levels, and IGF2 had a partially relaxed imprinting pattern comparable to levels found in normal somatic cells. This study also determined that the imprinting control region that regulates H19 and IGF2 imprinting had a normal pattern of DNA methylation. A second study determined that two XX EBD cultures had a normal pattern of X-inactivation.

The proliferation and expression characteristics of EBD cells suggest that they may be useful in the study of human cell differentiation, and as a resource for cellular transplantation therapies. One important property in this regard is that no tumor of human origin has arisen in any animal receiving EBD cells, although hundreds of mice, rats, and African green monkeys have received EBD transplants in a variety of anatomical locations, often consisting of more than one million cells injected. This is in contrast to the infrequent, yet significant, number of teratocarcinomas that have arisen following transplantation of cells produced through neural and hematopoietic differentiation of mouse ES cells.

30.3.2 Embryoid Body-Derived Cell Transplantation

Transplantation of EBD cells into animal models of human disease constitutes an active and promising research avenue. Studies with EBD cells and many other cell types have suggested that tissue injury can be highly instructive to transplanted cells. This provides a powerful method to test the potential of cells to differentiate, without an initial understanding of the underlying mechanisms. These studies also suggest possibilities for the eventual treatment of patients suffering from these diseases.

One example of EBD cell transplantation is the use of an EBD culture named SDEC. This culture was initially selected for further study because of its strong neural expression bias. SDEC cells were introduced into the cerebrospinal fluid of normal rats and rats exposed to the neuroadapted Sindbis virus. This virus specifically targets spinal cord motor neurons, and infection results in permanent hind limb paralysis. SDEC cells transplanted into virally injured rats engrafted extensively the length of spinal cord and migrated into the cord parenchyma. Substantial engraftment was not observed in uninjured animals receiving SDEC cells. Engrafted SDEC cells took on expression characteristics of mature neurons and astrocytes. Remarkably, albeit at low frequency, engrafted SDEC cells became immunoreactive to choline acetyltransferase, and sent axons into the sciatic nerve. Even more remarkably, after 12 and 24 weeks, paralyzed animals receiving SDEC cells partially recovered their hind limb function. In this experiment, the frequency and total number of

neurons generated from SDEC cells was not sufficient to easily explain the significant recovery of function, which is not surprising since the transplanted SDEC cells are a mixed-cell population rather than a line or culture grown or differentiated to promote neural outcome. The mechanism proposed to explain the functional recovery involves EBD cells protecting host motor neurons from death, and facilitating host motor neuron reafferentation, possibly through the secretion of transforming growth factor-a and brain-derived neurotrophic factor.

This example illustrates several important points. The engraftment (and possibly one or more steps in cellular differentiation) of SDEC cells was promoted by an injury signal following viral infection. Once engrafted, cells within the SDEC population were capable of differentiation *in vivo* into mature astrocytes and neurons, some of which sent processes along the correct pathway to the sciatic nerve and were then capable of retrograde transport. It is difficult to see how this elaborate and spatially precise differentiation could be carried out *ex vivo* and then introduced into an animal or patient. The use of a mixed-cell population may have allowed the variety of cellular responses that ultimately resulted in functional recovery. Some cells within the population were capable of forming new neural cells, and others took on supportive and protective roles. Although, in this experimental model, the multiple roles carried out by use of a mixed-cell population may have resulted in functional recovery, future experiments will need to focus on isolation of subpopulations to increase the efficiency of differentiation into required cell types, and to address issues of safety. Lastly, the rats in all treatment groups received immunosuppressive drugs to discourage rejection of the human EBD cells. In the near term, this will likely be a feature of all EBD-based cellular transplantation experiments and therapies.

30.3.3 Embryoid Body-Derived Derivation, Growth, and Cryopreservation Methods

EBs formed in the presence of PGC growth media are harvested in groups of 10 or more and are dissociated by digestion in 1 mg/ml Collagenase/Dispase (Roche) for 30 minutes to 1 hour at 37°C. Cells are then spun at 1000 rpm for 5 minutes and resuspended in various growth media–matrix environments. These include RPMI growth media (RPMI 1640 (LTI), 15% FCS, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 100 μ/ml penicillin, and 100 mg/ml streptomycin) and EGM2MV media (Clonetics) (5% FCS, hydrocortisone, hbFGF, hVEGF, R^3-IGF1, ascorbic acid, hEGF, heparin, gentamycin, and amphotericin B). Matrices are bovine collagen I (Collaborative Biomedical, 10 mg/cm^2), human extracellular matrix (Collaborative Biomedical, 5 mg/cm^2), and tissue culture plastic. EBD cells are cultured at 37°C, 5% CO_2, 95% humidity, and are routinely passaged 1:10 to 1:40 using

0.025% trypsin–0.01% EDTA (Clonetics) for 5 minutes at 37°C. Low serum cultures are treated with trypsin inhibitor (Clonetics) and then spun down and resuspended in growth media. EBD cells are cryopreserved in the presence of 50% FCS, 10% DMSO, in a controlled rate freezing vessel and stored in liquid nitrogen.

FOR FURTHER STUDY

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CHAPTER 31

Genomic Reprogramming

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31.1 INTRODUCTION

From the earliest speculation about genomic reprogramming through many subsequent experiments, it has become clear that the maternally inherited factors contained within the oocyte also have the extraordinary property to restore totipotency to a differentiated somatic nucleus when transplanted into it. The components within the oocyte must have the property to alter the somatic nucleus so that it can recapitulate the entire developmental program, and thus give rise to an exact genetic copy or clone of the individual who donated the transplanted nucleus. This transformation of differentiated cell to a totipotent state is probably the most widely understood meaning of genomic reprogramming. However, it is important to note that extensive epigenetic reprogramming of the genome also occurs in the germ line and during early development, which is essential for generating the totipotent zygote, and for creating the pluripotent epiblast cells from which both germ cells and somatic cells are subsequently derived.

Specification of diverse cell types from pluripotent cells is determined by the expression of a precise set of genes, while the rest are repressed. These newly acquired cell fates are propagated by heritable epigenetic mechanisms through modifications of chromatin and by DNA methylation. These epigenetic modifications, although they are heritable, are also reversible and can be erased, which is why it is possible to change the phenotypic characteristics of cells and restore totipotency to somatic nuclei under specific conditions (see Figure 31.1). To understand the mechanisms of reprogramming, it is important to know the nature of chromatin modifications and the mechanisms that can reverse or erase the existing modifications, and also how new modifications are imposed. Because these reprogramming factors normally play a significant role during early development, it is important to determine

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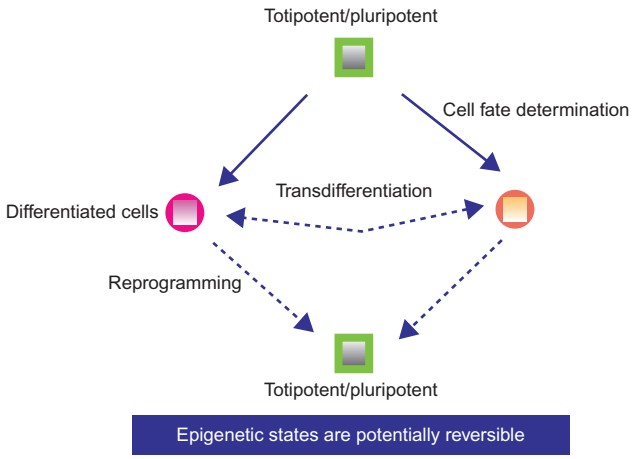

FIGURE 31.1 Genomic reprogramming involves heritable but reversible epigenetic modifications.

their role in this context, and how these factors act on somatic nuclei during restoration of totipotency or pluripotency.

31.2 GENOMIC REPROGRAMMING IN GERM CELLS

Germ cells provide the enduring link between generations, and for this reason this lineage exhibits many unique properties, including the extensive epigenetic reprogramming of the genome prior to gametogenesis. This reprogramming is crucial for generating viable and functional gametes, which in turn generate a totipotent zygote. Primordial germ cells (PGCs) are among the first cells to undergo specification from pluripotent epiblast cells, when the distinction between germ cells and soma is established. PGCs as precursors of sperm and oocyte are highly specialized cells, and are the only cells that can undergo meiosis. However, PGCs retain expression of some markers of pluripotency, such as *Oct4*. It is also possible to derive pluripotent embryonic germ cells (EG) from PGCs. In this context, it is interesting to determine both how PGC specification occurs, and how these cells undergo dedifferentiation to pluripotent EG cells, which may provide some insights into genomic reprogramming.

31.2.1 Stem Cell Model for the Specification of Germ Cells in Mammals

There are two key mechanisms for the specification of germ cells. The first involves inheritance of preformed germ plasm, which is found in *Drosophila*

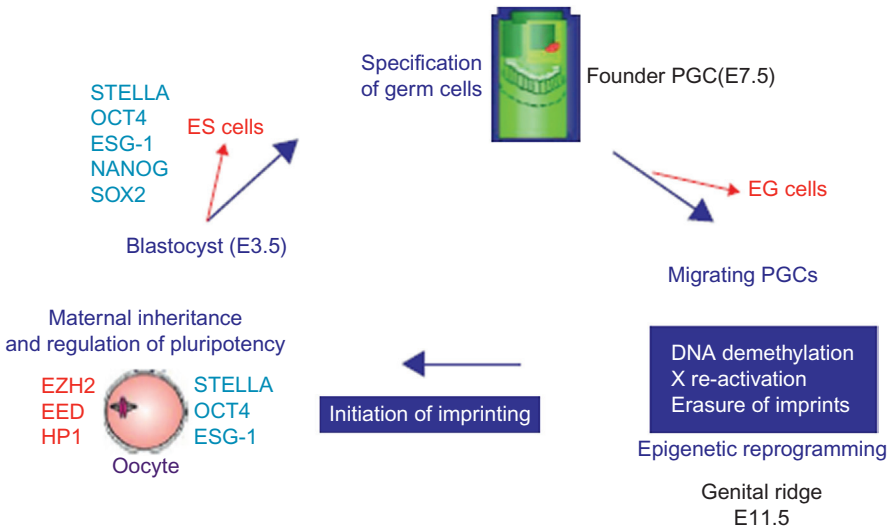

FIGURE 31.2 **Genomic reprogramming in the oocyte, early embryos, and the germ line.**
The origins of pluripotent embryonic stem cells (ES) and embryonic germ cells (EG) are indicated.

and *C. elegans*. In mammals, germ cell specification occurs according to the stem cell model, where germ cells are derived from pluripotent epiblast cells in response to signaling molecules from the extraembryonic ectoderm. BMP4 and BMP8b are among the key signaling molecules in conferring germ cell competence on pluripotent epiblast cells in mice, starting at E6.5 (Figure 31.2), an event that is detected by the expression of *fragilis*, a transmembrane protein. These germ cell competent cells are initially destined for a mesoderm somatic cell fate, as they show expression of *Brachyury* and some region-specific *Hox* genes as they migrate toward the posterior proximal region. However, at around E7.25, cells that ultimately acquire a germ cell fate switch off the somatic program through repression of a number of genes, which continue to be expressed in the neighboring somatic cells. Cells that acquire germ cell fate continue to show expression of markers of pluripotency, including *Oct4*. A unique marker of germ cells at this time is Stella, which is first detected in the 45–50 founder germ cells at E7.5. Thus, diversification between germ cell and somatic cell fate among neighboring cells occurs over approximately 6–10 hours between E7.25 and E7.5, with the repression of somatic cell programming being one of the major events during germ cell specification.

31.2.1.1 From Stem Cells to Germ Cells

Recent studies show that it is also possible to derive PGCs and gametes from pluripotent stem cells. In one study, embryonic stem (ES) cells with the

gcOct4-GFP reporter, which drives expression specifically in germ cells, were generated. The ES cells containing the reporter were allowed to undergo differentiation when cells with GFP were detected. These cells expressed a variety of germ cell-specific markers. Further cultures following cell sorting eventually produced oocyte-like cells that underwent development to form blastocyst-like structures. The latter shows that mouse ES cells can, under these conditions, differentiate into oocytes and, subsequently, blastocysts. With further detailed characterization of germ cells and gametes, such an *in vitro* system may be useful for investigations concerning specific aspects of germ cell development.

Another study has similarly been carried out to generate spermatogenic cells from pluripotent ES cells. In this case, the endogenous mouse homolog of *Vasa*, *Mvh* was used to knock in the reporters, *LacZ* and *GFP*. In this study, germ cells were generated in embryoid bodies, which were detected through the expression of MVH-GFP. This process was greatly enhanced by the exposure of ES cells to BMP4. These MVH-GFP cells were aggregated with E12.5–E13.5 male gonadal cells when germ cells within these aggregates developed into elongated spermatids.

These studies show that it may be possible to generate an efficient *in vitro* system to derive germ cells from pluripotent ES cells. Such a system would be useful to study the mechanism of PGC specification *in vitro*, as well as other aspects, including the formation of gametes, and aspects of epigenetic reprogramming of the genome. Derivation of germ cells from human ES cells would be particularly useful for studies on this lineage. Furthermore, derivation of human oocytes from ES cells would greatly add to this scarce resource, thus providing opportunities for fundamental studies on somatic cell reprogramming, and for the subsequent derivation of stem cell lines from human somatic cells for investigations of specific mutations and diseases.

31.2.1.2 From Germ Cells to Stem Cells

Embryonal carcinoma cells (EC) that are derived from PGCs *in vivo* were the first pluripotent stem cells to be identified, and several loci have been identified that have a critical role in this process. At the same time, derivation of pluripotent cells from PGCs has also been achieved *in vitro*. This conversion of germ cells into EG cells occurs in the presence of leukemia inhibitory factor (LIF), basic fibroblast growth factor (FGF2), and the Kit ligand (KL). The precise mechanism for conversion of the highly specialized germ cells into pluripotent stem cells is largely unknown. Further investigations would provide insights into dedifferentiation of cells, and on the mechanism of genomic reprogramming.

31.2.2 Epigenetic Reprogramming in Germ Cells

One of the properties of germ cells, of particular interest, is the epigenetic reprogramming of the genome. This event occurs when PGCs enter into the

developing gonads (Figure 31.2), when there is extensive erasure of epigenetic modifications, including erasure of genomic imprints and reactivation of the inactive X-chromosome. New parental imprints are initiated later during gametogenesis, particularly oogenesis, and these modifications that are heritable after fertilization dictate parent-of-origin-dependent gene expression.

As germ cells proliferate after the formation of the founder population at E7.5, they start to migrate to the developing gonads. At this stage, germ cells, as well as somatic cells, contain epigenetic marks associated with imprinted genes. During their migration, female germ cells also show inactivation of one X-chromosome. Upon the entry of germ cells into developing gonads at E10.5–E11.5, a major epigenetic reprogramming event occurs, which includes reactivation of the inactive X-chromosome, and the erasure of epigenetic marks associated with imprinted genes. Indeed, there appears to be genome-wide DNA demethylation of the genome at this time. This genomic reprogramming event occurs relatively rapidly, and it is completed by E12.5.

The mechanism involved in the erasure of epigenetic modifications in the germ line may provide insights into the erasure of epigenetic modifications from somatic nuclei that occur after transplantation into oocytes, which restores totipotency. If this is the case, similar factors may be transcribed during oocyte maturation at the germinal vesicle stage and translated, to be stored in oocytes as maternally inherited factors. The onset of genomic reprogramming in PGCs in the gonad may be triggered by a signal from somatic cells, or in response to a developmental timer, such as the number of cell divisions in PGCs since the establishment of the founder population of PGCs. Because the gonads at E11.5 are bipotent, it is possible that the signal from somatic cells, if it exists, should be the same in both male and female embryos. In this case, it would be of interest to discover the nature of the signal and determine how such an external cue can trigger extensive genomic reprogramming. However, there is also support for the alternative developmental timer model, since EG cells show erasure of imprints even when they are derived from PGCs where this process has not yet commenced. It is possible that erasure is initiated when germ cells complete a critical number of cell cycles, although, as these cells are cultured in a complex medium, a role for an environmental cue cannot be entirely discounted. Whatever the case may be, it is important to note that EG cells themselves have the property to induce erasure of epigenetic modifications from somatic nuclei (see below).

31.2.2.1 Genomic Reprogramming in Oocytes

Resumption of oocyte growth is accompanied by further epigenetic reprogramming events, particularly the initiation of genomic imprints. The majority of the epigenetic marks associated with imprinting are introduced during oocyte growth, although some genes acquire paternal-specific imprints in

the male germ line. These epigenetic marks are eventually detected as DNA methylation of specific *cis*-control elements. Some of the marks, for example, in the *Igf2r* locus, ensure that the gene will be active only when maternally inherited, while others such as *Peg3* will be silent in the female genome. *Dnmt3l* is a key gene involved in the initiation of parental imprints, which acts together with the *de novo* DNA methylase enzyme, Dnmt3a. A mutation in the *Dnmt3l* gene does not disrupt development or maturation of the oocyte, except that these oocytes do not carry appropriate maternal imprints or epigenetic marks. Following their fertilization, the resulting embryos are unable to develop normally, and they die shortly after implantation. Other genes, such as *H19*, undergo DNA methylation in the paternal germ line, and this gene is repressed in the paternal genome. This topic is discussed comprehensively elsewhere.

31.2.3 Maternal Inheritance and Reprogramming of Parental Genomes

As in other organisms, mouse oocytes contain a number of maternally inherited proteins and messages (Figure 31.2). In mammals, maternally inherited factors are essential for totipotency and pluripotency, such as Oct4, Esg1, and Stella, although there is no Nanog. Maternal inheritance of Stella is apparently necessary for normal preimplantation development. The oocytes also contain epigenetic modifiers, including the *Polycomb* group proteins, Ezh2 and eed, as well as the heterochromatin factor, HP1. These factors are essential for regulating early development, and for generating the pluripotent epiblast and trophectoderm cells of blastocysts. The oocyte is also likely to inherit some key chromatin remodeling factors.

In mammals, the parental genomes exhibit epigenetic asymmetry in the zygote as a result of imprinting, which confers functional differences between parental genomes. At fertilization, the maternal genome apparently has high methylated lysine 9 histone H3 (H3meK9). Immediately after fertilization, the hetrochromatin protein HP1b binds preferentially to the maternal genome. The Polycomb proteins, Ezh2 and eed, also bind preferentially to the maternal genome. While this takes place, the paternal genome that has relatively low levels of H3meK9 shows genome-wide DNA demethylation, thus enhancing the epigenetic differences between the parental genomes. Ezh2 has the conserved suvar E2 trithorax (SET) domain with histone methylase activity for methylation of histone H3-lysine 27/lysine 9 (H3meK27/9). The maternal inheritance of Ezh2 *per se* is apparently important, since depletion of this factor from oocytes results in development of very small neonates, presumably because of an effect on placental development. This seems likely because the neonates eventually grow and acquire normal size, indicating a placental functional deficiency during development. Whether this is due to an

effect on imprinted genes remains to be determined. These experiments show that factors present within the oocyte have the potential to exert a variety of epigenetic effects on development. Somatic nuclei transplanted into oocytes would be affected by the activities of these factors during reprogramming, but the variable expression of genes associated with totipotency and of imprinted genes argues that appropriate epigenetic reprogramming of the genome may not be accomplished in every case (see below).

Factors involved in chromatin remodeling are also likely to be important for early development and genomic reprogramming, as they regulate accessibility to DNA. The SWI/SNF-like complexes consist of at least two ATPase subunits, BRG1 and BRM. *Brg1* is important during preimplantation development, as loss of function is lethal during this phase. It is also known that mutation in *ATRX*, a member of the SNF2 helicase/ATPase family, has an effect on DNA methylation of highly repeated sequences. Mutation in *Lsh* similarly results in substantial demethylation of the genome. Lsh is related to the SNF2 subfamily; most members of the SNF2 family of proteins appear to have the capacity to alter chromatin structure. The activity of the nucleosome-dependent ATPase, ISWI, may be used in chromatin remodeling in nuclear reprogramming of somatic nuclei; if so, it is likely to be present in the oocyte, and would have a role in the zygote. One of the earliest changes observed following fertilization (or indeed after transplantation of the somatic nucleus) is the apparent increase in the size of the nucleus. This morphological change may be in response to chromatin remodeling factors belonging to the ISWI complexes. This activity may be necessary for the initial unwinding of the chromosomes, to facilitate epigenetic modifications of the chromatin.

31.2.4 Reprogramming During Early Development

Epigenetic reprogramming of the embryonic genome continues throughout preimplantation development, as judged by the continual changes in histone modifications and a decline in the genomic levels of DNA methylation. During preimplantation development, both pluripotent epiblast and differentiated trophectoderm cells are formed. There are differences in the epigenetic reprogramming in these two tissues. For example, there is preferential paternal X-inactivation in the trophectoderm, a process in which the *Polycomb* group proteins, Ezh2/eed complex, have a significant role. The cells of the late morula, which are positive for the expression of *Nanog*, and destined to form the inner cell mass, cease to show Ezh2/eed accumulation at Xi, as seen with the paternal X-chromosome in the trophectoderm. Ezh2 is also detected in the inner cell mass, which may account for the presence of the overall H3meK27 staining of epiblast cells, which are positive for Oct4 expression.

It appears that histone modifications, such as H3meK27, may have a role in the maintenance of epigenetic plasticity of the pluripotent epiblast cells, since

the loss of function of Ezh2 is early-embryonic-lethal, and it is not possible to derive pluripotent ES cells from blastocyst that are null for Ezh2. These experiments show the importance of appropriate epigenetic reprogramming of the genome for early development, and for generating pluripotent epiblast cells that are the precursors of both somatic and germ cell lineages. As we learn more about nuclear reprogramming events which occur normally in germ cells, oocytes, and early development, these studies are likely to be used to identify key candidates for genomic reprogramming.

31.3 REPROGRAMMING SOMATIC NUCLEI

31.3.1 Nuclear Transplantation

Epigenetic reprogramming of somatic nuclei transplanted into oocytes must require the erasure and initiation of appropriate epigenetic modifications compatible for development. This subject has already been reviewed extensively elsewhere. At least some of the key reprogramming events may be faulty, to account for the very low success rate, since somatic nuclei undergo variable reprogramming resulting in a wide variety of phenotypes. The effects of aberrant reprogramming are apparent, particularly soon after implantation and during postimplantation development. Both the embryo and extraembryonic tissue seem to be affected. Some epigenetic marks associated with imprinted genes are erased, resulting in the aberrant expression of these genes. It seems likely, therefore, that a large number of genes fail to show appropriate temporal and spatial patterns of expression. Further studies on the mechanisms of genomic reprogramming during normal development and following nuclear transplantation are necessary to assess the reasons for faulty reprogramming of somatic nuclei.

31.3.2 Reprogramming in Embryonic Stem–Somatic and Embryonic Germ–Somatic Cell Hybrids

Somatic nuclear reprogramming has also been demonstrated in hybrid cells between pluripotent ES/EG and somatic cells, which also restores pluripotency in somatic nuclei. These studies indicate that not only the oocytes, but also pluripotent ES/EG cells, must contain appropriate factors to reprogram the somatic nucleus. Reprogramming of somatic nuclei in ES/G–somatic cell hybrids is, however, relatively less complex compared to its transplantation into oocytes. This is because the somatic nucleus in the oocyte has to be reprogrammed to recapitulate the entire program of early development to the blastocyst stage. It is important to note that this donor somatic nucleus has to be reprogrammed to generate pluripotent epiblast cells, as well as the highly differentiated trophectoderm cells. The latter should be viewed as a transdifferentiation event, because somatic nuclei of diverse origin must direct

differentiation of highly specialized trophectoderm cells after only a few cleavage divisions. Indeed, in some respects, this transdifferentiation event is more striking as a reprogramming event. By comparison, reprogramming of somatic nuclei in ES/EG–somatic cell hybrids is less complex, as there is restoration of pluripotency without the necessity to recapitulate early events of development.

Although EG and ES cells on the whole have similar effects on somatic nuclei, there is at least one critical difference between them. Using EG–thymocyte hybrid cells, it was shown that the somatic nucleus underwent extensive reprogramming, resulting in the erasure of DNA methylation associated with imprinted genes, and the inactive X-chromosome was reactivated. The somatic nucleus also acquired pluripotency, as judged by the activation of the *Oct4* gene, and the hybrid cells could differentiate into all three germ layers in chimeras. This study shows that EG cells, apart from conferring pluripotency to the somatic nucleus, retained a key property found only in germ cells, which is the ability to erase parental imprints and, indeed, induce genome-wide DNA demethylation. Experiments using ES–thymocyte hybrid cells gave similar results, including the restoration of pluripotency to somatic nuclei, as shown by the activation of the Oct4-GFP reporter gene, and for the ability of these cells to differentiate into a variety of cell types. Unlike EG cells, however, ES cells do not cause erasure of imprints from somatic nuclei. Furthermore, in ES–EG hybrids, EG cells can induce erasure of imprints from ES cells, which shows that EG cells have dominant activity for the erasure of imprints and DNA demethylation. However, from these studies it is clear that DNA demethylation activity, at least for the erasure of imprints present in EG cells, is not essential for restoring pluripotency to somatic cells. It is possible to use this system to design cell-based assays in search of key reprogramming factors.

The ability of ES/EG cells to restore pluripotency in somatic nuclei is significant because it also opens up possibilities to identify the molecules involved in reprogramming somatic nuclei. Such studies are difficult with mammalian oocytes, partly because they are small compared to amphibian oocytes, and it is difficult to collect large numbers of them. More importantly, as discussed earlier, oocytes are complex cells containing factors essential for pluripotency, as well as for the early development and differentiation of trophectoderm cells. By contrast, pluripotent ES/EG cells are relatively less complex, and, more importantly, they can be grown indefinitely *in vitro*. Thus, they can provide a considerable source of material for analysis. For example, it is possible to use nuclear extracts from ES/EG cells to examine reprogramming of somatic nuclei, as described in one experimental approach. The availability of relatively large amounts of nuclear extracts from ES/EG cells also makes it possible to undertake biochemical studies to identify the key reprogramming factors.

31.4 CONCLUSIONS

The evidence from studies on early mammalian development shows that there is dynamic and extensive reprogramming of the genome in the oocyte, zygote, and germ cells. Pluripotent stem cells also appear to show considerable potential for genomic reprogramming, and while there are differences between ES and EG cells, both of them can restore pluripotency to somatic nuclei. There is maternal inheritance of factors for pluripotency, epigenetic modifications, and chromatin remodeling in the oocyte. Reprogramming in the oocyte is relatively complex, since the parental pronuclei exhibit epigenetic asymmetry in the zygote. The paternal genome becomes rapidly demethylated after fertilization, but the maternal genome does not, at least in part, because of the differential histone modifications, such as the preferential H3meK9 of the maternal genome, but not of the paternal genome, and the preferential binding of HP1b and Ezh2/eed proteins to it. It is possible that epigenetic reprogramming of somatic nuclei may be affected by their original epigenetic state.

The phenomenon of reprogramming somatic nuclei is now well established in mammals, both by nuclear transplantation studies and in heterokaryons, but the mechanisms and the key molecules involved are yet unknown. It is reasonable to assume that some of the factors involved in reprogramming the genome in the germ line are also present in the oocyte. It is also possible that some of the basic reprogramming factors present in pluripotent stem cells are also present in the oocyte.

A likely sequence of events for converting a somatic nucleus to a pluripotent nucleus may first require chromatin remodeling activity. Many of these complexes are known to exist in mammals, but at this stage it is not known precisely which are important for reprogramming of somatic nuclei. This may be followed by changes in histone modifications compatible with pluripotency. What these changes are has yet to be fully determined, together with the identity of the histone modifiers.

As ES/EG cells apparently have the capacity for reprogramming somatic nuclei to pluripotency, they may be used for identifying key molecules necessary for genomic reprogramming through cell-based assays, combined with appropriate biochemical and cellular analyses.

FOR FURTHER STUDY

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CHAPTER 32

Neural Stem Cells – Therapeutic Applications in Neurodegenerative Diseases

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32.1 INTRODUCTION

In this chapter, we review some of the work that has been performed in animal models of central nervous system (CNS) diseases, where transplanted neural stem cells (NSCs) have mediated a therapeutic effect. These disorders include rodent models of genetic and acquired (e.g., traumatic and ischemic) neurodegeneration, inheritable metabolic disorders, age-related degeneration, and neoplasms. These conditions often show widespread neural cell loss, dysfunction, or both. The disseminated nature of the pathology of these diseases is not readily treated by conventional transplantation approaches in which a solid tissue graft or a limited number of nonmotile cells are delivered to a restricted area. Similarly, most gene therapy approaches tend to fall short because of their limited 'sphere of influence' following injection of the vector into the CNS parenchyma. The use of bone marrow transplantation, even with hematopoietic stem cells, is typically inadequate because the cells do not efficiently breach the blood-brain barrier. Cells from non-neural organs, if they transdifferentiate into neural cells at all (a controversial prospect), do so far too inefficiently to be therapeutically reliable for cell replacement in these conditions.

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NSCs, on the other hand, circumvent many of these obstacles. Because they differentiate robustly into neural cells, integrate seamlessly into neural parenchyma as multiple neural cell types (both neuronal and glial), respond to normal developmental and regeneration cues, and migrate (even long distances) to multiple, disseminated areas of neuropathology, they appear to be ideally suited for the molecular and cellular therapies required by extensive, diffuse (even 'global') degenerative processes. Examples of such widespread neurodegenerative conditions include myelin disorders, storage diseases, motor neuron degeneration, dementing conditions such as Alzheimer's disease, and ischemic and traumatic pathologies such as stroke. Some diseases appear to be restricted in their involvement – Parkinson's disease localized to the mesostriatum, Huntington's to the caudate, spinal cord contusion to a few spinal segments, and cerebellar degeneration to the hindbrain. However, even these disorders require that cell replacement be distributed evenly over relatively large terrain, that multiple neural cell types be replaced even in a given region, or both. Again, these needs are best accomplished by a migratory, responsive, multipotent neural progenitor even if transplantation is directed toward a more circumscribed CNS region.

32.2 DEFINITION OF NEURAL STEM CELLS

NSCs are the most primordial cells of the nervous system. They generate the array of specialized cells throughout the CNS (and probably the peripheral, autonomic, and enteric nervous systems as well). NSCs are operationally defined by their ability to:

1. Differentiate into cells of all neural lineages (i.e., neurons, ideally of multiple subtypes; oligodendroglia, and astroglia) in multiple regional and developmental contexts;
2. Self-renew (i.e., to generate new NSCs with similar potential); and
3. Populate developing and/or degenerating CNS (and possibly other neural) regions.

To affirm that a single cell possesses these capabilities, clonal populations (i.e., the affirmed progeny of a single cell) must be examined. Although several antigenic markers have been proffered as distinguishing NSCs from other neural and non-neural progenitors – for example nestin (an intermediate filament consistent with an immature neuroectodermal lineage), musashi 1 (an RNA-binding protein), AC133 (a cell surface marker), Hoechst dye exclusion (the 'side population' after flow cytometric analysis) – none has proven sufficiently specific or sensitive to supplant the previously mentioned operational definitions. The presence of a panel of immunologic markers – not just one marker – will support but not clinch this assessment. Similarly, the ability of a cell to form a cytosphere (a floating cluster of cells *in vitro*) is a characteristic

of any actively propagating cell of any lineage when maintained in serum-free medium without an adherent substrate. Hence, it cannot be used by itself to define an NSC; it simply affirms that the cell is mitotic. Several studies were performed to determine whether NSCs share with embryonic stem (ES) cells and/or stem cells from other somatic organs the expression of certain 'universal stemness genes.' Some studies find a degree of overlap, and others find none – perhaps reflecting differences in the cellular populations being analyzed. Hence, the answer remains uncertain. It is likely that if any genes are held in common they will be those integral to self-renewal and hence involved in maintaining cells within the cell cycle.

Neural cells with stem cell properties have been isolated from the embryonic, neonatal, and adult rodent nervous system and propagated *in vitro* by a variety of equally effective and safe means – both epigenetic and genetic. When mitotic, a stem cell retains its greatest degree of multipotency. A rapid cascade of commitment steps and a progressive narrowing of potential, often under the instruction of pertinent external environmental cues, accompany its exit from the cell cycle. The extent to which the commitment of an NSC is preprogrammed within the cell (i.e., cell autonomous) versus the extent to which it is directed by external signals from the milieu (i.e., cell nonautonomous) remains to be determined and is controversial. Epigenetic means for inducing cells to enter and remain within the cell cycle have typically entailed adding mitogens – such as epidermal growth factor or basic fibroblast growth factor – to serum-free culture medium. In addition, or as an alternative, certain cell cycle-associated genes – including those that help mediate the action of mitogens – can be transduced into a stem cell to maintain its ability to self-renew, for blunt senescence, to hold commitment in abeyance, to preserve multipotency, or all of these. One such gene is *myc*, the overexpression of which will maintain stem-like behavior in an NSC. The gene does not preclude responsiveness of the NSC to normal environmental and growth control cues and is downregulated following transplantation *in vivo*.

It remains to be determined whether prolonged passaging of NSCs *in vitro* (whether by mitogens or by other techniques) changes these cells so that they no longer reflect the capacities and potential of their *in vivo* predecessors and counterparts. However, it is clear that maintaining NSCs in a proliferative state in culture does *not* subvert their ability to respond to normal developmental cues *in vivo* following transplantation, including the ability to withdraw from the cell cycle, to interact faithfully with host cells, and to differentiate.

With the earliest recognition that neural cells with stem cell properties, propagated in culture, could be reimplanted into the mammalian brain, where they could reintegrate appropriately and stably express foreign genes,

translational neurobiologists began to speculate about how such a phenomenon might be harnessed for therapeutic advantage as well as for understanding developmental mechanisms. These, and the studies they spawned, provided hope that the use of NSCs might circumvent some limitations of available graft material and gene transfer vehicles and make feasible a variety of new therapeutic strategies.

32.3 THERAPEUTIC POTENTIAL OF NEURAL STEM CELLS

The clinical potential of the NSC is rooted in its inherent biologic properties. The ability of NSCs and their progeny to develop into integral cytoarchitectural components of many regions throughout the host brain as neurons, oligodendrocytes, astrocytes, or even immature neural progenitors makes them capable of replacing a range of missing or dysfunctional neural cells. Although the field of neural repair has tended to place emphasis on the replacement of missing neurons in neurodegenerative diseases or oligodendrocytes in demyelinating diseases, it is becoming increasingly evident that simultaneously replacing a diseased cell's 'neighboring cells' – typically astrocytes – may be of equal importance because of the indispensable trophic, guidance, and detoxification role such 'chaperone' cells may play. Fortuitously, part of the biological repertoire of an NSC is to generate the variety of cells that constitute the 'fabric' of a given neural region. NSCs in particular differentiation states will spontaneously produce a variety of neurotrophic factors that may serve trophic, protective, or both functions – for example, glial-cell-line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and neurotrophin-3 (NT-3). NSCs also inherently express most of the 'housekeeping' enzymes and factors necessary for any cell to maintain normal metabolism.

NSCs may also be readily engineered *ex vivo* to express a variety of molecules that either are not produced by NSCs or are not produced in therapeutically adequate quantities. The NSCs are amenable to various types of viral vector transduction as well as to other gene transfer strategies, such as lipofection, electroporation, and calcium phosphate precipitation. Following transplantation, such engrafted NSCs may then be used as cellular vectors for the *in vivo* expression of exogenous genes of developmental and/or therapeutic relevance. Such gene products can be delivered either to circumscribed regions or, if necessary, to more widely disseminated areas throughout the host CNS. Because they display significant migratory capacity as well as an ability to integrate widely throughout the brain when implanted into germinal zones, NSCs may help to reconstitute enzyme and cellular deficiencies in a global fashion (Figure 32.1).

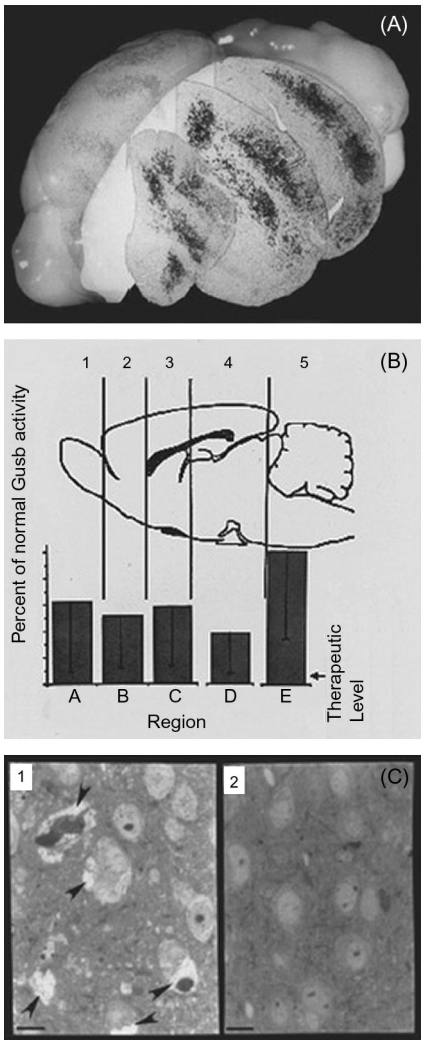

FIGURE 32.1 Widespread engraftment of NSCs expressing GUSB throughout the brain of the MPS VII mouse.
(A) Brain of a mature MPS VII mouse after receiving a neonatal intraventricular transplant of murine NSCs expressing GUSB. Donor NSC-derived cells, identified by their X-gal histochemical reaction for expression of the LacZ marker gene, have engrafted throughout the recipient mutant brain. Coronal sections – placed at their appropriate level by computer – show these cells to span the rostral-caudal expanse of the brain. (B) Distribution of GUSB enzymatic activity throughout brains of MPS VII NSC transplant recipients. Serial sections were collected from throughout the brains of transplant recipients and assayed for GUSB activity. Sections were pooled to reflect the activity within the demarcated regions. The regions were defined by anatomical landmarks in the anterior-to-posterior plane to permit comparison among animals. The mean levels of GUSB activity for each region (n = 17) are presented as the percentage of average normal levels for each region. Untreated MPS VII mice show no GUSB activity biochemically or histochemically. Enzyme activity of 2% of normal is corrective based on data from liver and spleen. (C) Decreased lysosomal storage in a treated MPS VII mouse brain at eight months. (1) Extensive vacuolation representing distended lysosomes (arrowheads) in both neurons and glia in the neocortex of an 8-month-old, untransplanted control MPS VII mouse. (2) Decrease in lysosomal storage in the cortex of an MPS VII mouse treated at birth from a region analogous to the untreated control section in panel 1. The other regions of this animal's brain showed a similar decrease in storage compared with untreated, age-matched mutants in regions where GUSB was expressed. Scale bars = 21 μm.
Adapted from Snyder EY et al. 1995. Nature 374: 367–70.

The ability of true NSCs to adjust to their regions of engraftment probably obviates the need for abstracting stem cells from specific CNS regions. Furthermore, NSCs appear to possess a tropism for degenerating CNS regions (Figure 32.2). NSCs may be attracted – across long distances – to regions of neurodegeneration in brains of all ages, including old age. Under some circumstances, an environment in which a particular type of neural cell has degenerated creates a milieu that directs the differentiation of an NSC toward maintaining homeostasis, including replenishment of a specific deficient neural cell type. Through mechanisms yet to be determined, it appears that some

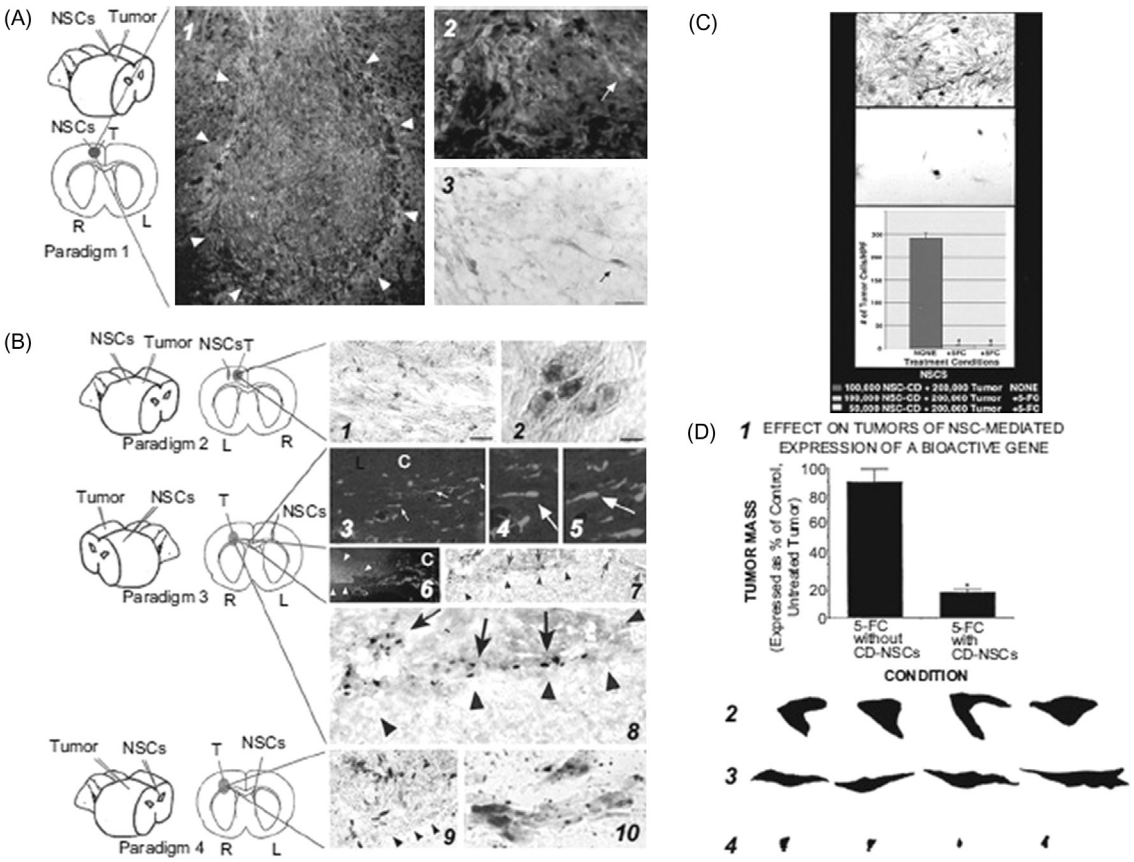

FIGURE 32.2 **Neural stem cells display extensive tropism for pathology in the adult brain and can express bioactive genes within such pathological situations: evidence from intracranial gliomas.**

(A) NSCs migrate extensively throughout a brain tumor mass *in vivo* and 'trail' advancing tumor cells. Paradigm (1), in which NSCs are implanted directly into an established experimental intracranial glioblastoma, is illustrated schematically. (1) The virulent and aggressively invasive CNS-1 glioblastoma cell line, used to create the tumor, has been labeled *ex vivo* by transduction with GFP cDNA. The NSCs stably express *lacZ* and produce βgal. This panel, processed for double immunofluorescence using an anti-βgal antibody (NSCs) and an anti-GFP antibody (glioblastoma cells), shows a section of brain (under low power) from an adult nude mouse sacrificed 10 days after NSC injection into the glioblastoma; arrowheads mark where the tumor mass interfaces with normal tissue. Donor βgal+ NSCs can be seen extensively distributed throughout the mass, interspersed among the tumor cells. This degree of interspersion by NSCs after injection occurs within 48 hours. Interestingly, although NSCs have extensively migrated and distributed themselves within the mass, they largely stop at the junction between tumor and normal tissue except where a tumor cell is infiltrating normal tissue; then, NSCs appear to follow the invading tumor cell into surrounding tissue. (1 and 2) Detail of the trailing of individual glioblastoma cells migrating from the main tumor bed (2). High-power view, under fluorescence microscopy, of single migrating infiltrating GFP+ tumor cells in apposition to βgal+ NSCs (white arrow). (3) Costaining with Xgal (the arrow points to the lacZ-expressing NSCs) and with neutral red (for the elongated glioblastoma cells). The NSC is in direct juxtaposition to a single migrating, invading the neutral red$^+$, spindle-shaped tumor cell (arrow), with the NSC 'piggybacking' the glioma cell. Scale bar = 60 μm. (B) NSCs implanted at various intracranial sites far from main tumor bed migrate through normal adult tissue toward glioblastoma cells. (1 and 2) Same hemisphere: A section through the tumor from an

neurodegenerative processes (e.g., those associated with apoptosis) elaborate neurogenic signals that recapitulate developmental cues to which NSCs can respond (Figure 32.3).

It would seem that NSC-mediated cell replacement would only be feasible in 'cell autonomous' disease states – that is, diseases in which the pathology is restricted to a particular cell whose life span is short-circuited but within an extracellular milieu otherwise normal. Conversely, a 'cell nonautonomous' condition, where normal cells die because of an inhospitable microenvironment, would appear not to be amenable to NSC therapy given that 'replacement' cells would presumably meet a similar fate. Surprisingly, however, NSCs may nevertheless be useful in such cell-extrinsic pathological conditions. It appears that NSCs, particularly in their immature, undifferentiated state, may be more resistant to certain stressors – various toxic metabolites, oxidizing agents – than more mature cells. Furthermore, if the NSCs are not inherently more resistant, they can be readily genetically engineered *ex vivo* to become more resistant.

◀ adult nude mouse 6 days following NSC implantation caudal to tumor. Panel 1 shows a tumor populated as pictured under low power in Figure 32.1 A. Note Xgal$^+$ NSCs interspersed among neutral red$^+$ tumor cells. (2) High-power view of NSCs in juxtaposition to islands of tumor cells. (3–8) Contralateral hemisphere. (2–5) Views through the corpus callosum (c), where βgal+ immunopositive NSCs (arrows) are migrating from their site of implantation on one side of the brain toward tumor on the other. Two NSCs indicated by arrows in panel 3 are viewed at higher magnification in panels 4 and 5 to show the classic elongated morphology and leading process of a migrating neural progenitor oriented toward its target. (6) βgal+ NSCs are 'homing in' on the GFP+ tumor, having migrated from the other hemisphere. In panel 7, and magnified further in panel 8, the Xgal$^+$ NSCs (arrows) have entered the neutral red$^+$ tumor (arrowheads) from the opposite hemisphere. (9 and 10) Intraventricular: A section through the brain tumor of an adult nude mouse 6 days after NSC injection into the contralateral cerebral ventricle. (9) Xgal$^+$ NSCs are distributed within the neutral red$^+$ main tumor bed (edge delineated by arrowheads). (10) At higher power, the NSCs are in juxtaposition to migrating islands of glioblastoma cells. Fibroblast control cells never migrated from their injection site in any paradigm. All Xgal positivity was corroborated by anti-βgal immunoreactivity. Scale bars = 20 μm (1; also applies to 3) 8 μm (2), 14 μm (4 and 5), 30 μm (6 and 7), 15 μm (8), 20 μm (9), and 15 μm (10). (C) Bioactive transgene (cytosine deaminase, CD) remains functional (as assayed by *in vitro* oncolysis) when expressed within NSCs. CNS-1 glioblastoma cells were cocultured with murine CD-NSCs. (1) Cocultures unexposed to 5-FC grew healthily and confluent, (2) whereas plates exposed to 5-FC showed dramatic loss of tumor cells represented quantitatively by the histograms ($* = p < 0.001$). The oncolytic effect was identical whether 1×10^5 CD-NSCs or half that number were cocultured with a constant number of tumor cells. (Subconfluent NSCs were still mitotic at the time of 5-FC exposure and thus subject to self-elimination by the generated 5-FU and its toxic metabolites.) (D) Expression of CD delivered by NSCs *in vivo* as assayed by reduction in tumor mass. The size of an intracranial glioblastoma populated with CD-NSCs in an adult nude mouse treated with 5-FC was compared with that of tumor treated with 5-FC but lacking CD-NSCs. These data, standardized against and expressed as a percentage of a control tumor populated with CD-NSCs receiving no treatment, are in the histograms in panel 1. These measurements were derived from measuring the surface area of tumors; camera lucidas of them are in panels 2–4. Note the large areas of panel 2, a control non-5–2FC-treated, tumor-containing CD-NSCs, and in panel 3, a control 5-FC-treated, tumor-lacking CD-NSCs, as compared with panel 4, the dramatically smaller tumor areas of the 5-FC-treated animal, which also received CD-NSCs (~80% reduction, $* = p < 0.001$), suggesting both activity and specificity of the transgene. (3) The lack of effect of 5-FC on tumor mass when no CD-bearing NSCs were within the tumor was identical to panel 4, the effect of CD-NSCs in the tumor without the gene being employed. *Modified from Aboody KS, et al. 2000. Proc Natl Acad Sci USA 97: 12846–51.*

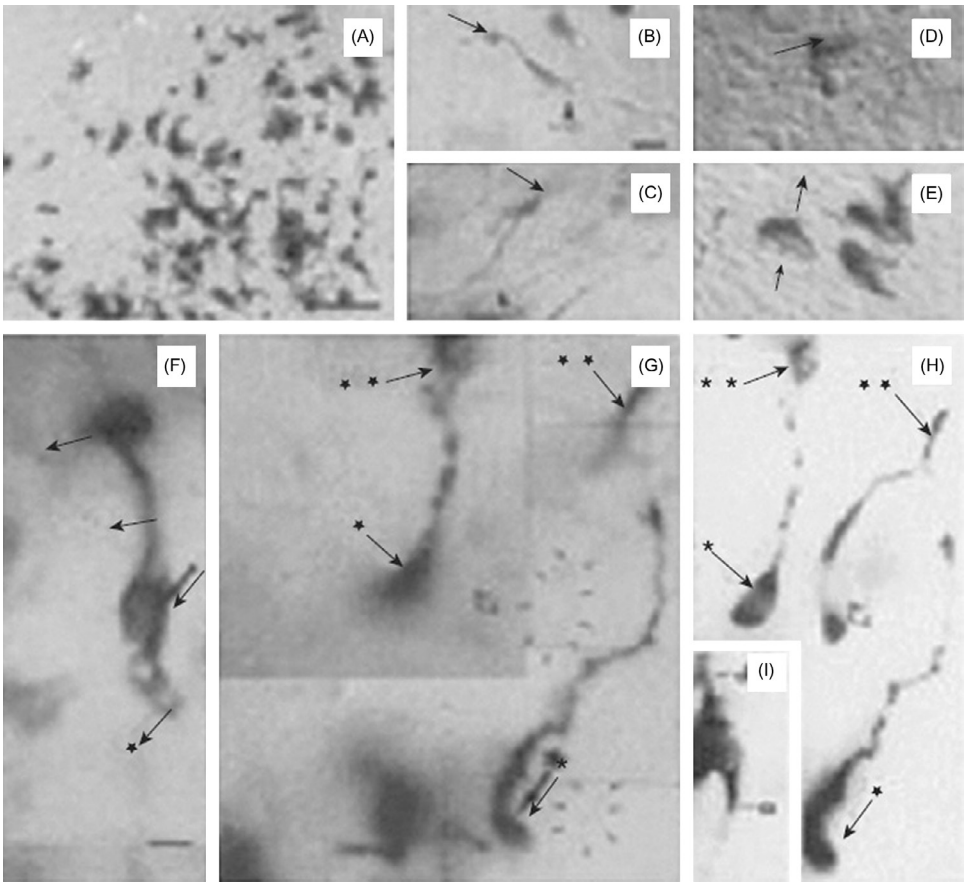

FIGURE 32.3 **Multipotent NSCs acquire neuronal morphology in regions of adult neocortex subjected to targeted apoptotic neuronal degeneration. They differentiate into only glia or remain undifferentiated in intact control cortex.**
(A) Engrafted Xgal$^+$ glia at low magnification 6 weeks following transplantation at 12 weeks; (B–E) higher magnification. (B and C) Donor-derived cells with astroglial features: Small, ovoid cell bodies (arrows) with few, short processes often extending as perivascular end-feet (arrowheads). (D) Small soma of a donor-derived presumptive glial cell (arrow) compared with a much larger (~30 μm), unlabeled, host pyramidal neuron (small arrows). (E) Donor-derived cells with oligodendroglial features (arrow): Multiprocessed, ensheathing neuronal processes (short arrows). (F–I) A total of 15 ± 7% of engrafted cells in regions of neurodegeneration developed neuronal morphology, resembling pyramidal neurons within layer II/III 6 weeks following transplantation at 12 weeks. (F and G) Donor-derived cells with neuronal morphology (large arrows): Large somata (20–30 μm diameter) comparable to residual host pyramidal neurons (small arrows in panel G outline two host neurons, visualized under differential interference contrast (DIC), each with the characteristic large nucleus and prominent nucleolus of a pyramidal neuron), 300–600 μm presumptive apical dendrites positioned between host (small arrows) and donor (large arrows) neurons of similar morphology and size, and presumptive axons. (F) The dark object at the upper end of the presumptive dendrite is another Xgal$^+$ cell out of the plane of focus. (H) Digital confocal microscopy (DCM) image of panel G, collapsing multiple planes of focus. The cell has the characteristic large nucleus of a pyramidal neuron. All but the terminal dendrite of the soma of the cell is out of the plane of focus in panel G. Cells and neurons are indicated to cross-reference views of the same field in panels G and H. (I) Two overlapping donor-derived pyramidal neurons in different focal planes show a characteristic large nucleus, prominent nucleolus, and axon of the overlying cell and a prominent dendrite of the underlying cell, imaged through multiple focal planes of this

Despite their extensive plasticity, NSCs never produce cell types inappropriate to the brain (e.g., muscle, bone, or teeth) or yield neoplasms. The use of NSCs as graft material in the CNS may be considered almost analogous to hematopoietic stem cell-mediated reconstitution of the bone marrow.

Therefore, the biological repertoire of the NSC, if harnessed, may provide multiple strategies for addressing CNS dysfunction. Some of these approaches have already shown promise experimentally in animal models of neurodegeneration. Some illustrative examples are briefly described in the next sections of this chapter.

32.4 GENE THERAPY USING NEURAL STEM CELLS

As stated previously, the ability of NSCs to deliver therapeutic gene products in an immediate, direct, sustained, and perhaps regulated fashion as normal cytoarchitectural components throughout the CNS may overcome some of the limitations of standard viral and cellular vectors. The feasibility of this strategy was first demonstrated in a mouse mutant characterized by a single gene defect in all cells, including those in the CNS, leading to their death. The particular mouse modeled the lysosomal storage disease mucopolysaccharidosis type VII (MPS VII) caused by a deletion mutation of the β-glucuronidase (GUSB) gene. This incurable inheritable condition is characterized by neurodegeneration in mice and by progressive mental retardation in humans. Although the particular disease was rare, it served as a model for neurological diseases whose etiology stemmed from a genetically based loss of function. GUSB-secreting NSCs were implanted into the cerebral ventricles of newborn MPS VII mice, allowing the cells access to the subventricular zone (SVZ), a germinal zone from which the cells were disseminated throughout the brain (creating 'chimeric forebrains'). These enzyme-producing cells, now in residence as normal cerebral constituents, not only metabolized lysosomal storage normally for themselves but also cross-corrected mutant cells throughout the brains of recipient mice (Figure 32.1). Employing a similar strategy, retrovirally transduced NSCs implanted into the brains of fetal and neonatal mice (particularly into periventricular regions) have successfully mediated brainwide expression of other enzymes – for example, β-hexosaminidase, a deficiency of which leads to the pathological accumulation of GM_2 ganglioside.

◀ thick section under DCM. The identification of the previously mentioned cells was supported by immunocytochemical and ultrastructural analysis under electron microscopy. In addition, one could determine that donor-derived neurons received synaptic input from the host and were myelinated by host oligodendrocytes, further supporting their incorporation into the host cytoarchitecture (a: axon; d: dendrite; cells: ∗,◆◆◆, ∗∗ and open arrow; and ◆: neurons). Bars = 25 μm. *Adapted from Snyder EY, et al. 1997. Proc Natl Acad Sci USA 94: 11663–8.*

Findings such as these have helped to establish the paradigm of using NSCs for the transfer of other factors of therapeutic or developmental interest into and throughout the CNS. Although NSCs express baseline amounts of particular enzymes and neuroprotective factors, NSCs can be genetically modified to enhance their production of these molecules or to produce additional molecules that might enhance their therapeutic potential. For example, NSCs have been used to deliver NT-3 within the hemisectioned rat spinal cord, to express NGF and BDNF within the septum and basal ganglia, to provide tyrosine hydroxylase to the parkinsonian striatum, and to express myelin basic protein (MBP) throughout the dysmyelinated cerebrum. In one illustrative set of experiments, NSCs that overexpressed NGF were transplanted into the striatum of a rat lesioned with quinolinic acid, a toxin used to emulate some of the neuronal loss seen in Huntington's disease. Delivery of NGF by the engrafted NSCs appeared to reduce the size of the lesion and to promote the sparing of host striatal neurons. When implanted into the septum of aging rats, NGF-overexpressing NSCs appeared to blunt the typical cognitive decline by preventing age-related atrophy of forebrain cholinergic neurons. Well integrated into the host tissue, the engrafted NSCs continued to produce NGF until nine months after grafting.

That NSCs appear to have a strong affinity for sites of pathology and will migrate extensive distances (even from the opposite cerebral hemisphere) to home in on them, and this makes the NSC unique and valuable as a gene delivery vehicle. For example, a type of pathology particularly elusive to gene therapeutic interventions has been the brain tumor, especially the glioma. Gliomas are so exceptionally migratory and infiltrative that they elude even the most effective surgical, radiation, or gene therapeutic strategy. However, the ability of transgene-expressing NSCs to 'track down' and deliver therapeutic gene products directly to these widely dispersed and invasive neoplastic cells makes them a potentially valuable adjunct in the treatment of these aggressive tumors.

Although this therapeutic approach – exploiting the normal biological behavior of NSCs for transplantation-based gene therapy – is being extended to animal models of many neurological disorders, it is important to recognize that each pathophysiological process, each animal model, and each therapeutic molecule must be assessed and optimized individually.

32.5 CELL REPLACEMENT USING NEURAL STEM CELLS

It is postulated that NSCs persist within the CNS long beyond cerebrogenesis to maintain homeostasis following perturbations in the CNS. Presumably

the mechanisms that allow the endogenous NSC to perform this function are preserved when the cells are isolated from the CNS, expanded in culture, and reimplanted into the damaged CNS. Indeed, it appears that when NSCs are so implanted, they respond by shifting their pattern of differentiation toward replenishing the missing cell type.

The study that first demonstrated this phenomenon was performed on a model of experimentally induced apoptosis of selectively targeted pyramidal neurons in the adult mammalian neocortex. When transplanted into this circumscribed, neuron-depleted region, 15% of the NSCs altered the differentiation path they would otherwise have pursued in the intact adult, non-neurogenic mammalian neocortex (i.e., to become glia); instead, they differentiated specifically into pyramidal neurons, partially replacing that lost neuronal population (Figure 32.3). Of these, a subpopulation spontaneously sent axonal projections to their proper targets in the contralateral cortex. Outside the borders of this small region of selective neuronal death, the NSCs yielded only glia. Thus, neurodegeneration appeared to create a milieu that recapitulated embryonic development cues (e.g., for cortical neurogenesis), and the NSCs were sufficiently sensitive to detect and respond to this micromolecular alteration – possibly to therapeutic advantage.

Apoptosis is implicated in a growing number of both neurodegenerative and normal developmental processes. Whether this differentiation shift occurs only in response to signals associated with apoptosis or in response to other types of cell death as well remains to be determined. Most neurological diseases are characterized by a mixture of neuropathological processes, making dissection of the key stimuli complex. For example, we have observed that following an ischemic insult, NSCs will robustly repopulate infarcted regions of the postdevelopmental cortex and differentiate into cortical neurons in these regions. However, even hypoxic-ischemic cerebral injury – the quintessential example of necrotic and excitotoxic injury – is characterized by an apoptotic phase. An added level of complexity may be the tempo at which apoptotic signals are elaborated – an acute burst may be more instructive than a more languid burst.

Studies are ongoing to identify the key molecules that direct NSC fate during and following various kinds of neural injury. Intriguingly, we are starting to learn that cytokines released during an inflammatory reaction – those emanating from microphages and microglia as well as from parenchymal damage (e.g., SDF1-α) – play a pivotal role in 'beckoning' NSCs. Coincidentally, we are starting to recognize that many processes regarded as neurodegenerative – including amyotrophic lateral sclerosis (ALS), Alzheimer's disease, tumors, and stroke – are characterized by a prominent inflammatory signature. Although each is different in its etiology, region of involvement, and course,

inflammation may be their common denominator from the 'viewpoint' of the NSC.

32.6 'GLOBAL' CELL REPLACEMENT USING NEURAL STEM CELLS

The targeted-apoptosis model described in the previous section exemplifies a circumscribed type of neural cell loss. However, the pathologic lesions of many neurological disorders are often widely – even globally – dispersed throughout the brain. Such conditions include the neurodegenerative disorders of childhood (e.g., inborn errors of metabolism, storage diseases, leukodystrophies, and neuronal ceroid lipofuscinoses) and hypoxic-ischemic encephalopathy as well as some adult CNS diseases (e.g., multiple sclerosis, Alzheimer's disease, and ALS). Treatment for these disorders requires widespread replacement of genes, cells, or both as well as the regeneration, protection, or both of broad networks of neural circuitry. The ability of inherently migratory NSCs to integrate into germinal zones from which they can be 'launched' and the inclination of NSCs to travel long distances to home in on pathologic regions makes these cells ideally – and perhaps uniquely – suited for this task.

Mouse mutants characterized by CNS-wide white matter disease provided the first models for testing the hypothesis that NSCs might be useful against neuropathologies requiring widespread neural cell replacement. The oligodendrocytes – the myelin-producing cells – of the dysmyelinated *shiverer* (*shi*) mouse are dysfunctional because they lack MBP, a molecule essential for effective myelination. Therapy, therefore, requires widespread replacement with MBP-expressing oligodendrocytes. NSCs transplanted at birth (employing the intracerebroventricular implantation technique described previously for the diffuse engraftment of enzyme-expressing NSCs to treat global metabolic lesions) resulted in engraftment throughout the *shi* brain with repletion of significant amounts of MBP. Of the many donor cells that differentiated into oligodendrocytes, a subgroup myelinated ~40% of host neuronal processes. In some recipient animals, the symptomatic tremor decreased (Figure 32.4). Therefore, 'global' cell replacement was shown to be feasible for some pathological conditions if cells with stem-like features are employed. This approach has been extended to other myelin-impaired animal models, such as the experimental allergic encephalomyelitis mouse model of multiple sclerosis as well as rodent mutant models of Palezeus-Merzbacher, Krabbe, and Canavan leukodystrophies. It was demonstrated that neural progenitor-stem cells of human origin have a similar remyelinating capacity in the *shi* mouse brain. Viewed more parochially, the ability of NSCs to remyelinate is of significant importance because impaired myelination plays such an important

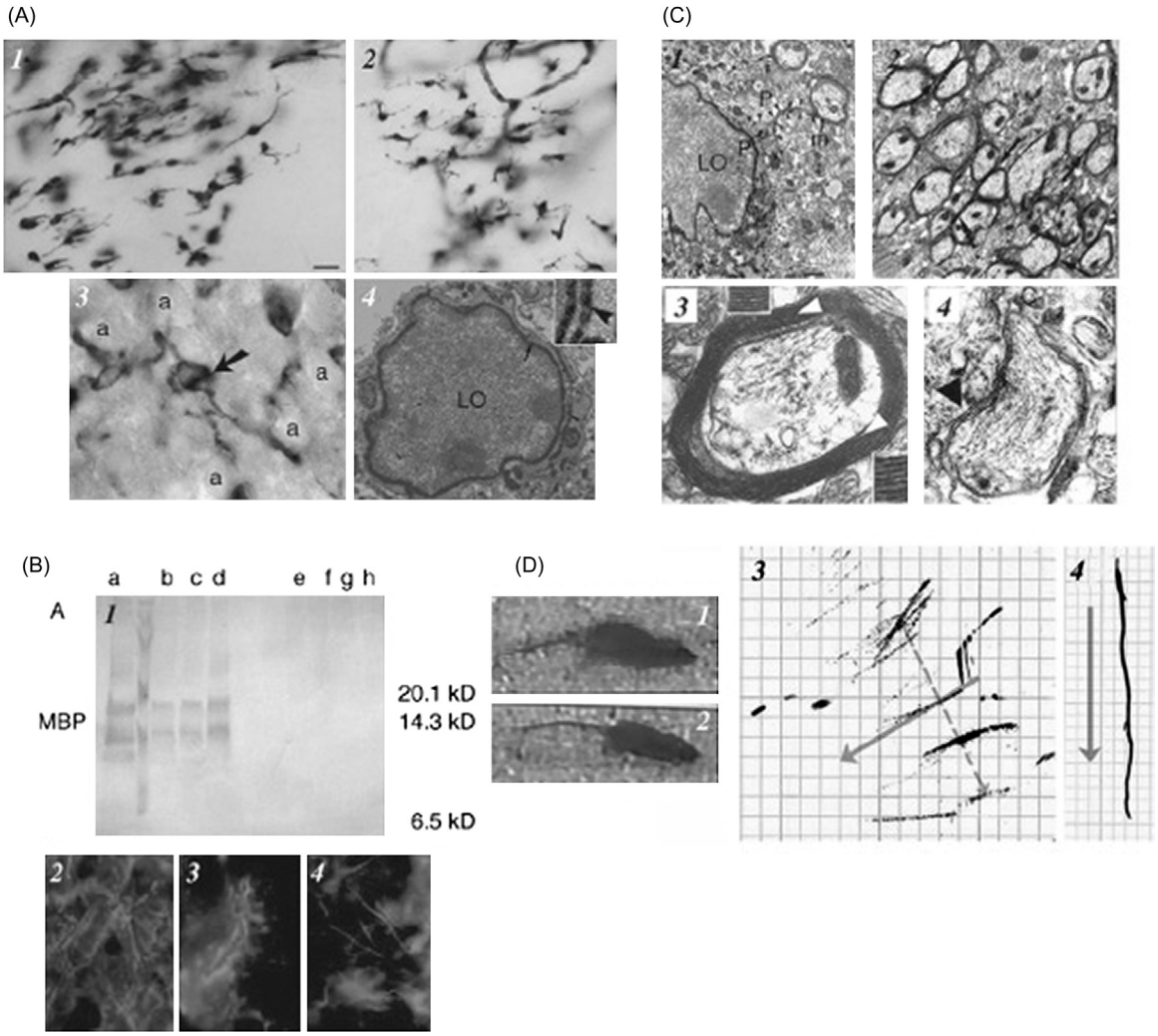

FIGURE 32.4 **'Global' cell replacement is feasible using NSC transplantation: evidence from the dysmyelinated shi mouse brain.**

(A) NSCs engraft extensively throughout the *shi* dysmyelinated brain, including within white tracts, and differentiate into oligodendrocytes. *LacZ*-expressing, βgal-producing NSCs were transplanted into newborn *shi* mutants and analyzed systematically at intervals between 2 and 8 weeks following engraftment. Coronal sections through the *shi* brain at adulthood demonstrated widely disseminated integration of $Xgal^+$ donor-derived cells throughout the neuraxis, similar to the pattern seen in Figure 32.1 A in the MPS VII mutant mouse. (1 and 2) Donor-derived $Xgal^+$ cells (higher magnification) in sections through the corpus callosum possessed characteristic oligodendroglial features (small, round, or polygonal cell bodies with multiple fine processes oriented toward the neural fiber tracts). (3) Close-up of a donor-derived anti-β immunoreactive oligodendrocyte (arrow) extending multiple processes toward and beginning to enwrap large, adjacent axonal bundles (a) viewed on end in a section through the corpus callosum. That cells such as those in panel A 1–3 and in panel B 2–4 were oligodendroglia was confirmed by the electron micrograph in panel A 4 and in panel C, demonstrating their defining ultrastructural features. A donor-derived $Xgal^+$ oligodendrocyte (LO) can be distinguished by the electron dense Xgal precipitate

role in many genetic (e.g., leukodystrophies and inborn metabolic errors) and acquired (e.g., traumatic, infectious, asphyxial, ischemic, and inflammatory) neurodegenerative processes. However, viewed more broadly, the ability of the NSC to replace this particular type of neural cell throughout the brain bodes well for its ability to replace other classes of neural cell across the broad terrains often demanded by other categories of complex neurodegenerative diseases.

◀ typically localized to the nuclear membrane (arrow), endoplasmic reticulum (ER) (arrowhead), and other cytoplasmic organelles. The ER is magnified in the inset to demonstrate the unique crystalline nature of individual precipitate particles. (B) MBP expression in mature transplanted and control brains. (1) Western analysis for MBP in whole-brain lysates. The brains of three transplanted *shi* mutants (lanes 2–4) expressed MBP at levels close to those of an age-matched unaffected mouse (lane 1, positive control) and significantly greater than the amounts seen in untransplanted (lanes 7–8, negative control) or unengrafted (lanes 5–6, negative control) age-matched *shi* mutants. (Identical total protein amounts were loaded in each lane.) (2–4) Immunocytochemical analysis for MBP. (2) The brain of a mature unaffected mouse was immunoreactive to an antibody to MBP (revealed with a Texas red-conjugated secondary antibody). (3 and 4) Age-matched engrafted brains from shi mice similarly showed immunoreactivity. Because untransplanted *shi* brains lack MBP, MBP immunoreactivity has classically been a marker for normal donor-derived oligodendrocytes in transplant paradigms. (C) NSC-derived 'replacement' oligodendrocytes are capable of myelination of *shi* axons. In regions of MBP-expressing NSC engraftment, *shi* neuronal processes became enwrapped by thick, better-compacted myelin. (1) Two weeks after transplant, a donor-derived, labeled oligodendrocyte (LO), recognized by extensive Xgal precipitate (p) in the nuclear membrane, cytoplasmic organelles, and processes, was extending processes (arrowheads) to host neurites and was beginning to ensheathe them with myelin (m). (2) If engrafted *shi* regions (1) were followed to 4 weeks, the myelin began to appear healthier, thicker, and better-compacted (arrows) than that in age-matched untransplanted control mutants. (3) By 6 weeks after transplant, these matured into even thicker wraps; ~40% of host axons were ensheathed by myelin (white arrowheads; MDLs are evident) that was dramatically thicker and better-compacted than that of *shi* myelin (4, black arrowhead) from an unengrafted region of an otherwise successfully engrafted *shi* brain). (D) Functional and behavioral assessment of transplanted *shi* mutants and controls. The *shi* mutation is characterized by the onset of tremor and a 'shivering gait' by postnatal week 2 or 3. The degree of motor dysfunction in animals was gauged in two ways: by blindly scoring periods of standardized, videotaped cage behavior of experimental and control animals and by measuring the amplitude of tail displacement from the body's rostral–caudal axis (an objective, quantifiable index of tremor). Video freeze-frames of (1) unengrafted and (2) successfully engrafted *shi* mice. (1) The whole body tremor and ataxic movement observed in the unengrafted symptomatic animal causes the frame to blur, a contrast with (1) the well-focused frame of the asymptomatic transplanted *shi* mouse. Of transplanted mutants, 60% evinced nearly normal-appearing behavior (2) and attained scores similar to normal controls. (3 and 4) Whole body tremor was mirrored by the amplitude of tail displacement (dotted gray arrow), measured perpendicularly from a line drawn in the direction of the animal's movement. Measurements were made by permitting a mouse, whose tail had been dipped in India ink, to move freely in a straight line on a sheet of graph paper. (3) Large degrees of tremor cause the tail to make widely divergent ink marks from the midline, representing the body's axis (solid gray arrow). (4) Absence of tremor allows the tail to make long, straight, uninterrupted ink lines on the paper congruent with the body's axis. The distance between points of maximal tail displacement from the axis was measured and averaged for transplanted and untransplanted *shi* mutants and for unaffected controls (dotted gray arrow). Panel 3 shows data from a poorly engrafted mutant that did not improve with respect to tremor, whereas panel 4 reveals lack of tail displacement in a successfully engrafted asymptomatic mutant. Overall, 64% of transplanted *shi* mice examined displayed at least a 50% decrement in the degree of tremor or 'shiver.' Several showed no displacement. *Modified from Yandava BD, et al. 1999. Proc Natl Acad Sci USA 96: 7029–34.*

32.7 NEURAL STEM CELLS DISPLAY AN INHERENT MECHANISM FOR RESCUING DYSFUNCTIONAL NEURONS

The examples cited previously highlight that an abnormal environment can direct the behavior of a grafted NSC. However, they leave the impression that the exogenous NSC alone fills gaps. The situation is more complex and richer. We are beginning to learn that the NSC and the injured host engage in a dynamic series of ongoing reciprocal interactions, each instructing the other. Under instruction from exogenous NSCs, the injured host nervous system also contributes to its own repair. These important stem cell phenomena were first illustrated in a few examples, as follows.

The effect of NSCs in directly rescuing endangered host neurons was first evinced in a series of experiments in aged rodents in which the nigrostriatal system was impaired (Figure 32.5). Parkinson's disease is a degenerative disorder characterized by a loss of midbrain dopamine (DA) neurons with a subsequent reduction in striatal DA. The disease, in addition to incapacitating thousands of patients, has long served as a model for testing neural cell replacement strategies. Transplantation therapy for this CNS disorder has a long history. It was the neural disease first treated clinically by neural transplantation, using primary tissue from human fetal ventral mesencephalon to replace DA-expressing cells. Indeed, in this disease, the limitations of fetal tissue grafts in not only rodent and primate models of Parkinson's disease but also in clinical trials were first recognized. These limitations include, on one hand, short graft survival and limited integration of the grafts and, on the other hand, the possibility of unregulated DA production in improper regions leading adversely to dyskinesias. Given the storied role of Parkinson's disease in the development of cellular therapies, it is appropriate that a model of this disease should have also played a pivotal role in revealing a little-suspected but powerful therapeutic action that NSCs may play in preserving degenerating host cells by some heretofore unheralded mechanisms that are nevertheless inherent to stem cell biology.

In the hope that NSCs might spontaneously differentiate into DA neurons when implanted into a DA-depleted region of the CNS, unmanipulated murine NSCs were implanted unilaterally into the substantia nigra of aged mice exposed systemically to high-dose 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin that produces a persistent impairment of mesencephalic DA neurons and their striatal projections. The NSCs not only migrated from their point of implantation and integrated extensively within both hemispheres but also were associated with a dramatic reconstitution of DA function throughout the mesostriatal system. Although there was

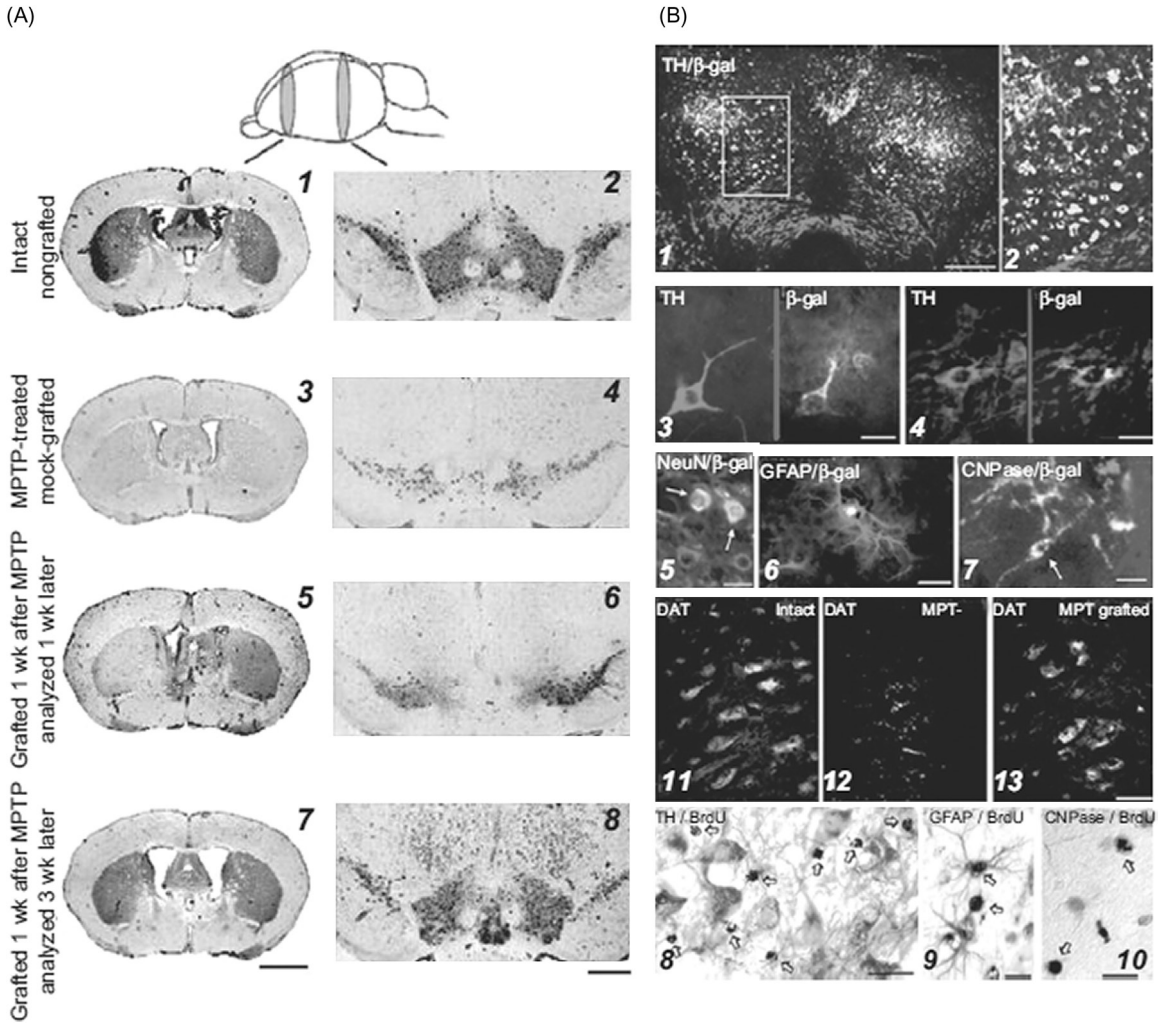

FIGURE 32.5 **NSCs possess an inherent mechanism for rescuing dysfunctional neurons: evidence from the effects of NSCs in the restoration of mesencephalic dopaminergic function.**

(A) TH expression in mesencephalon and striatum of aged mice following MPTP lesioning and unilateral NSC engraftment into the substantia nigra-ventral tegmental area (SN-VTA). A model that emulates the slow dysfunction of aging dopaminergic neurons in SN was generated by giving aged mice repeated high doses of MPTP. Schematic indicates the levels of the analyzed transverse sections along the rostrocaudal axis of the mouse brain. Coronal sections are through the striatum in the left column and through the SN-VTA area in the right column. Immunodetection of TH (black cells) shows the normal distribution of DA-producing TH+ neurons in (2) coronal sections in the intact SN-VTA and (1) their projections to the striatum. Within one week, MPTP treatment caused extensive and permanent bilateral loss of TH immunoreactivity in both (3) the mesostriatal nuclei and (4) the striatum. Shown in this example, and matching the time point in 7 and 8, is the situation in a mock-grafted animal 4 weeks after MPTP treatment. Unilateral (right side) stereotactic injection of NSCs into the nigra is associated, within 1 week after grafting, with substantial recovery of TH synthesis within (6) the ipsilateral DA nuclei and (5) their ipsilateral striatal projections. By 3 weeks after transplant, however, the asymmetric distribution of TH expression disappeared,

spontaneous conversion of a subpopulation of donor NSCs into dopaminergic neurons in DA-depleted areas, contributing to nigral reconstitution, most (80–90%) dopaminergic neurons in the 'reconstituted midbrain' were actually host cells that had been 'rescued' by factors produced constitutively by the NSCs with which they were juxtaposed and that had not become neurons. These chaperone cells constitutively produce substantial amounts of neurosupportive agents. One such prominent molecule was GDNF, a factor known to be neuroprotective of ventrally located neurons (including DA neurons and spinal-ventral horn cells). A similar observation is beginning to emerge from the implantation of human NSCs into the MPTP-lesioned, subhuman primate model of Parkinson's disease.

A sense for the extent of the cross talk also became evident when examining rodent models of hypoxia-ischemia, a common cause of neurological

◀ producing TH immunoreactivity in (8) the midbrain and (7) striatum of both hemispheres that approached the immunoreactivity of (1 and 2) the intact controls and gave the appearance of mesostriatal restoration. Similar observations were made when NSCs were injected 4 weeks after MPTP treatment (not shown). Bars: 2 mm (left), 1 mm (right). Note the ectopically placed TH+ cells in panel 8. These are analyzed in greater detail with the entire SN in B. (B) Immunohistochemical analyses of TH, DAT, and BrdU+ cells in MPTP-treated and grafted mouse brains. The initial presumption was that the NSCs had replaced the dysfunctional TH neurons. However, examination of the reconstituted SN with dual βgal and TH ICC showed that (1 and 3) 90% of the TH+ cells in the SN were rescued, host-derived cells, and (4) only 10% were donor-derived cells. Most NSC-derived TH+ cells were just above the SN ectopically (blocked area in panel 1; enlarged in panel 2). These photomicrographs were taken from immunostained brain sections from aged mice exposed to MPTP, transplanted 1 week later with NSCs and sacrificed after 3 weeks. The following combinations of markers were evaluated: (1–4) TH with βgal, (5) NeuN with βgal, (6) GFAP with βgal, (7) CNPase with βgal, (8) TH with BrdU, (9) GFAP with BrdU, and (10) CNPase with BrdU. Anti-DAT-stained areas are revealed in the SN of (11) intact, (12) mock-grafted, and (13) NSC-grafted brains. Fluorescence filters for Alexa Fluor 488 and Texas red and a double-filter for both types of fluorochromes were used to show antibody binding. (3, 4, and 8–10) Single-filter exposures; (1, 2, and 5–7) double-filter exposures. (1) Low power overview of the SN-VTA of both hemispheres. Most TH+ cells within the nigra are of host origin (~90%), a much smaller proportion (~10%) are donor derived (close-up in panel 4). Although a significant proportion of NSCs differentiated into TH+ neurons, many of these resided ectopically, dorsal to the SN (boxed area in panel 1 enlarged in panel 2; and a high-power view of the donor-derived cell that was also TH+ in panel 3), where the ratio of donor-to-host cells was inverted: ~90% donor derived compared with ~10% host derived. Note the near absence of a βgal-specific signal in the SN-VTA, whereas ectopically, many of the TH+ cells were double labeled and thus NSC derived. (5) NSC-derived non-TH neurons (NeuN +, arrow), (6) astrocytes (GFAP +), and (7) oligodendrocytes (CNPase +, arrow) were also seen, both within the mesencephalic nuclei and dorsal to them. (10) The DAT-specific signal suggests that the reconstituted mesencephalic nuclei in the NSC-grafted mice were functional DA neurons comparable to those seen (8) in intact nuclei but not (9) in MPTP-lesioned, sham-engrafted controls. This further suggests that the TH+ mesostriatal DA neurons affected by MPTP are functionally impaired. Note that (9) sham-grafted animals contain only punctate residual DAT staining within their dysfunctional fibers, whereas DAT staining (8) in normal and (10) in engrafted animals was normally and robustly distributed both within processes and throughout their cell bodies. (11–12) Any proliferative BrdU+ cells after MPTP insult, graft, or both were confined to glial cells, whereas (11) the TH+ neurons were BrdU. This finding suggested that the reappearance of TH+ host cells was not the result of neurogenesis but rather the recovery of extant host TH+ neurons. Bars = 90 μm (1), 20 μm (3–5), 30 μm (6), 10 μm (7), 20 μm (8–10), 25 μm (11), 10 μm (12), and 20 μm (13). *Modified from Ourednik J, et al. 2002. Nat Biotechnol 20: 1103–10.*

disability in adults and children. Hypoxia-ischemia causes much of its damage from extensive loss of cerebral parenchyma and the cells and connections that reside there. When NSCs are implanted into these regions of extensive degeneration (particularly when transiently supported by biodegradable scaffolds), robust reciprocal interactions ensue spontaneously between the exogenous implant and the injured host brain substantially reconstitute parenchyma and anatomical connections as well as reduce parenchymal loss, secondary cell loss, inflammation, and scarring. Similar results are observed in the hemi-resectioned adult rodent spinal cord in which evidence of an upregulated host neuronal regenerative response is noted, resulting in significant functional improvement.

Indeed, the ability of engrafted NSCs to exert a protective and regenerative influence on degenerating host neural systems because of their intrinsic expression of trophic factors is being observed in an increasing number of conditions. For example, the implantation of murine and human NSCs into the spinal cords of the SOD1 transgenic mouse model of ALS, a disease characterized by virulent motor neuron degeneration, has been pivotal in protecting these ventral horncells from death, preserving motor and respiratory function, blunting disease progression, and extending life. NSCs can similarly protect other neuron pools, promote motor axonal outgrowth following traumatic spinal cord injury, preserve infarcted regions of cerebrum, induce vascularization of reconstituted regions of cortical parenchyma, and inhibit inflammation and scarring following traumatic or ischemic insult.

Together, these observations suggest that exogenous NSCs may not only replenish inadequate pools of endogenous NSCs to compensate for missing neural cells but also reactivate or enhance endogenous regenerative and protective capacities. It also highlights a heretofore unanticipated mechanism by which NSCs may exert a therapeutic effect – to be added to their more traditional roles in direct cell replacement and gene therapy.

32.8 NEURAL STEM CELLS AS THE GLUE THAT HOLDS MULTIPLE THERAPIES TOGETHER

Experimentally, one may eliminate a particular cell type, lesion a particular region of CNS, knock out a particular gene, or choose a mouse strain in which, by chance, certain mutations have spontaneously occurred. However, most human neurodegenerative diseases are not as 'clean.' They are quite complex. And complex diseases, such as those affecting the nervous system, will require complex and multifaceted solutions – including pharmacological, gene and molecular, cell replacement, tissue engineering, angiogenic, anti-inflammatory, antiapoptotic, proregenerative, proneurite outgrowth-promoting therapies. The NSC, as a key player in a set of fundamental

developmental mechanisms, may serve as the glue that holds many of these strategies together. The ways in which they may be intelligently, effectively, safely, and practically orchestrated in actual patients will require a good deal of careful investigation.

For example, as the sophistication of our knowledge about disease processes grows, we are beginning to learn that more than one neural cell type probably needs to be replaced in a disease. For example, in a disease like ALS, a disorder characterized by progressive motor neuron degeneration, we are beginning to learn that astrocyte replacement may be just as critical as motor neuron replacement. Conversely, in multiple sclerosis, a white matter disease characterized by oligodendrocyte degeneration, replacing neurons and their axonal connections may be critical for the restoration of function. These same caveats may apply to many diseases in which replacement of multiple cell types may be the key to neurological reconstitution and to damaged milieu reconstruction. Because of their ability to develop into multiple integral, cytoarchitectural components of many regions throughout the host brain, NSCs may be able to replace a range of missing or dysfunctional neural cell types within the same region. This is important in the likely situation in which return of function may require the reconstitution of the milieu of a given region (e.g., not just the neurons but also the chaperone cells – the glia and support cells) to nurture, detoxify, guide, and/or myelinate the neurons. As noted previously, the NSCs, especially in particular differentiation states, express certain genes of intrinsic interest (many neurotrophic factors, lysosomal enzymes, angiogenic factors, anti-inflammatory molecules, antioxidants, etc.), or they can be engineered *ex vivo* to do so.

The challenge remains, however, to coordinate these multifaceted therapies so that they work in concert synergistically and not at cross-purposes.

32.9 SUMMARY

The ability of NSCs to migrate and integrate throughout the brain as well as to disseminate a foreign gene product is of great significance for the development of new therapies for neurodegenerative diseases in humans. Lethal, hereditary neurodegenerative diseases of childhood, such as the gangliosidoses, leukodystrophies, neuronal ceroid lipofuscinoses, and other storage diseases, result in lesions throughout the CNS. Diseases of adult onset (e.g., Alzheimer's disease) are also diffuse in their pathology. Even acquired diseases such as spinal cord injury, head trauma, and stroke are broader in their involvement than typically assumed. Such abnormalities may benefit from the multifaceted approach NSCs may enable. First, they may replace a range of cell types – not only neurons to reconstitute neural connections but also oligodendrocytes to elaborate myelin and astrocytes to serve trophic,

guidance, protective, and detoxification functions. Second, NSCs may deliver exogenous genes that might restore normal metabolism, complement a factor deficiency, support the survival of a damaged host neuron, neutralize a hostile milieu, counteract a growth-inhibitory environment, promote neurite regrowth, and reform stable and functional contacts. As noted previously, may molecules are produced inherently by NSCs when they are in particular states of differentiation. The production of other molecules might require *ex vivo* genetic engineering. NSCs appear to have their greatest therapeutic effect when used in the early stages of a degenerative process or in the subacute phase following injury.

A major requirement for the better use of NSCs will be a better understanding of the pathophysiology of the diseases to be targeted – that is, knowing what aspects require repair and which cell type or types require replacement or rescue.

Another challenge will be determining how to exploit stem cell biology for chronic conditions – in other words, how the acute milieu can be recreated in the chronic environment such that NSCs behave therapeutically there as well.

Among the methodological hurdles will be to devise how, when, where, and with what frequency to deliver NSCs to adults with disseminated diseases in regions not fed by readily accessible germinal zones – for example, the spinal cord in which the central canal is no longer patent. Linked to this is the need for a better understanding of the methods for augmenting yet controlling the propagation and the phenotype specification of NSCs *in vitro* and *in vivo*.

Another question has come to dominate the stem cell field: What is the most effective way to obtain NSCs for therapy? Should they be obtained directly from regions of neuroectodermal origin, and if so, should that be from a fetal source? Can an adult source be equally effective? Should stable lines of NSCs be established that can be used for all patients, or should NSCs be abstracted from each patient to be used as an autograft on a case-by-case basis? The degree to which the immune system presents a barrier to stem cell transplantation will likely determine the answers to this question and its subquestions.

If ES cells (from the inner cell mass of blastocysts) are directed to become NSCs *in vitro*, will such cells be equally as safe and effective?

The success in isolating stem-like cells from the CNS (the first 'solid organ stem cells' discovered) and their therapeutic potential also gave rise to the search for and successful isolation of stem-like cells from other non-neural organ systems, including bone marrow mesenchyme, muscle, skin, retina, and liver, for the purposes of repairing those tissues. Whether such non-neural stem cells can yield neural stem cells – through metaplasia or transdifferentiation – remains unresolved and exceptionally controversial. Can NSCs be derived efficiently and effectively from non-neural organs with outcomes as good as from NSCs themselves?

A better understanding of fundamental NSC biology will be required before human NSCs can be transplanted efficaciously in true clinical settings. Nevertheless, progress in this regard is being made. Several NSC lines have been established from the human fetal telencephalon and spinal cord that seem to emulate many of the appealing properties of their rodent counterparts: They differentiate *in vitro* and *in vivo* into neurons, astrocytes, and oligodendrocytes; they follow appropriate developmental programs and migrational pathways similar to endogenous precursors following engraftment into developing rodent and subhuman primate brain (Figure 32.6); they express foreign genes *in vivo* in a widely disseminated manner; and they can replace missing neural cell types when grafted into various mutant mice or rodent models of injury. The principal differences between human NSCs and mouse NSCs so far seem to be the length of the cell cycle (up to four days in the human) and the predilection of human NSCs to senesce (after ~100 cell divisions), obstacles that are being actively addressed. If human NSCs behave in lesioned subhuman primate brains with safe and effective engraftment and foreign gene expression as they seem to do in rodents, then human trials might be warranted for testing their value against certain genuine clinical neurodegenerative diseases. Through a careful and circumspect series of experiments and trials, we may learn whether we, indeed, have found within nature's own toolbox a powerful and versatile therapeutic tool – one of the goals of experiments that started more than 15 years ago.

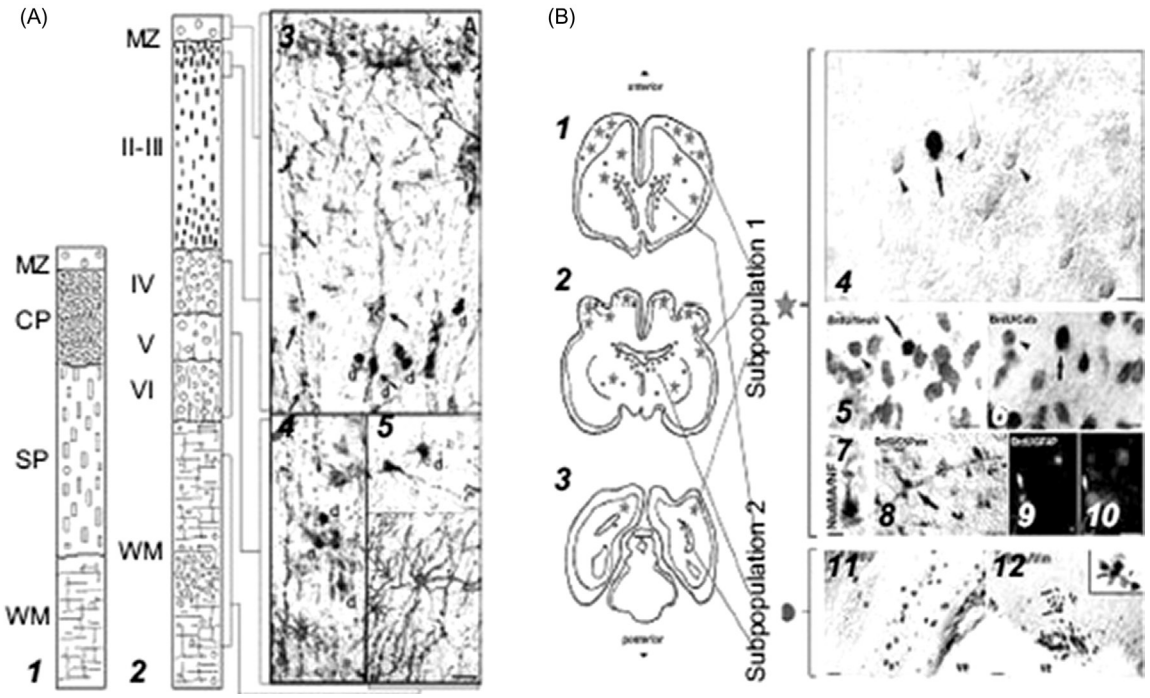

FIGURE 32.6 **Clonal human NSCs migrate from the ventricular zone (VZ) into the developing neocortex.**
Schematics of the developing monkey neopallium (A) at the time of transplantation. (1) 12–13 wpc and (2) at the time of death (16–17 wpc). (3–5) Photomicrographs from selected locations spanning the neopallium. (Their location relative to the schematic is indicated by brackets.) (3) Injected into the left lateral ventricle and having integrated throughout the VZ, the human NSC-derived cells (d), identified by their BrdU immunoreactivity (black nuclei), migrated along the monkey's radial glial processes through the neopallial wall to reach their temporally appropriate destination in the nascent superficial layers II and III (3), where they detached from the radial glia and took up residence as neurons. Arrows indicate climbing (donor- and host-derived) cells positioned along the processes of the vimentin-positive host radial glia. Some cells (inset) are still attached to these fibers and in the process of migration. (4 and 5) Immature, donor, human NSC-derived astrocytes intermixed with host-derived astrocytes in deeper cortical lamina, having differentiated as expected for that site and time. (B) Segregation of the fates of human NSCs and their progeny into two subpopulations in the brains of developing Old World monkeys. Schematics (left) and photomicrographs (right) illustrate the distribution and properties of clonal human NSC-derived cells. Human NSCs (labeled with BrdU) dispersed throughout and integrated into the VZ. From there, clonally related human NSC-derived cells pursued one of two fates. Those donor cells that migrated outward from the VZ along radial glial fibers into the developing neocortex constituted one pool or subpopulation. (4–12) The differentiated phenotypes of cells in this subpopulation (1) (stars in the schematic), particularly in layers II and III. (4) An human NSC-derived BrdU+ cell (black nucleus, arrow) – likely a neuron according to its size, morphology, large nucleus, and location – is shown (under Nomarski optics) intermingled with the monkey's similar neurons (arrowheads) in neocortical layers II and III. The neuronal identity of such donor-derived cells is confirmed by immunocytochemical analysis. High-power photomicrographs of human donor-derived cells integrated into the monkey cortex double-stained with antibodies against BrdU and cell type-specific markers: (5) NeuN and (6) calbindin for neurons (arrows, donor-derived cells; arrowheads, host-derived cells). (8) CNPase for oligodendroglia (arrow). (BrdU+ is the black nucleus in the CNPase+ cell; the arrowhead indicates the long process emanating from the soma.) (9, 10) GFAP for astroglia antibody to BrdU revealed through fluorescein; (9) antibody to GFAP revealed through Texas red. The human origin of the cortical neurons is independently confirmed in panel 7, where the human-specific nuclear marker NuMA (black

nucleus) is colocalized in the same cell with neurofilament immunoreactivity. Progeny from this same human NSC clone were allocated to a second cellular pool – subpopulation 2 (dots in the schematic; arrows in panels 9 and 10) – that remained mainly confined to the SVZ and stained only for an immature neural marker. (Vimentin colocalized with BrdU is easily seen in inset (arrows); the arrowhead indicates the host vimentin-positive cell.) Some members of subpopulation 2 were identified within the developing neocortex (dots) intermixed with differentiated cells. Panels 9 and 10 use immunofluorescence; the other immunostains use a DAB-based color reaction. Scale bars = 30 μm (4–6) and 20 μm (7–12). (d: human NSC-derived cells, MZ: marginal zone, CP: cortical plate, SP: subplate, WM: white matter, and II–VI: cortical layers.) *Modified from Ourednik V, et al. 2001. Science 293: 1820–4.*

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CHAPTER 33

Adult Progenitor Cells as a Potential Treatment for Diabetes

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33.1 IMPORTANCE OF β-CELL REPLACEMENT THERAPY FOR DIABETES AND THE SHORTAGE OF INSULIN-PRODUCING CELLS

Despite the success achieved with pancreas transplants, the supply of insulin-producing cells, which can now only be obtained from cadaveric donors, is inadequate for the number of those with diabetes who could benefit from this treatment. In the United States, only about 3,000 usable cadaver pancreases become available each year, yet the annual incidence of Type 1 diabetes is about 30,000 cases, with more than 10 times as many people developing Type 2 diabetes. Clearly, a new source of insulin-producing cells must be found if this important treatment is to become available to those in need.

33.2 POTENTIAL OF ADULT STEM-PROGENITOR CELLS AS A SOURCE OF INSULIN-PRODUCING CELLS

Along with the promise of embryonic stem cells, much attention has been paid to stem-progenitor cells and their potential to develop into insulin-producing cells (Table 33.1). This chapter is focused upon adult stem-precursor cells. However, the cell replacement route toward the normalization of glucose levels in people with diabetes competes with other technologies, including a mechanical insulin delivery system linked to a glucose sensor. Progress in obtaining a mechanical system has been slowed by the difficulty in developing an adequate glucose sensor. Additionally, xenotransplantation, with pigs appearing to be the most promising source, continues to be challenging.

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Table 33.1 Candidate Sources of Insulin-Producing Cells	
Adult Precursor Cells	
Pancreatic origin	Duct cells
	Nestin-positive cells
	Other precursor–stem cells
	Acinar transdifferentiation
Hepatic origin	Transdifferentiation of hepatocytes directly to insulin-producing cells
	Stimulation of islet neogenesis from hepatic precursor cells
Bone marrow origin	Questionable potential of true bone marrow stem cells
	Possible pluripotency of other marrow elements
Other sources of precursor–stem cells	Intestine, skin or brain may be sources
Cellular bioengineering	Multiple cell types may be candidates for bioengineering
Embryonic Stem Cells	
Embryonic Germ Cells	
Xeno source	
	Pigs
	Other sources may be possible

33.3 DEFINING β-CELLS, STEM CELLS, AND PROGENITOR CELLS

For the purposes of this chapter, a β-cell is defined as a cell with the phenotype of a mature insulin-producing cell found in pancreatic islets. It is possible to have insulin-producing cells that are immature and lack the full phenotype of a true β-cell. Some of these are β-cell precursors that contain some insulin, which at some point can be called young β-cells. There are cells, however, containing insulin that will never become β-cells, such as cells that have been identified in the thymus, brain, and yolk sac.

It is also necessary to distinguish *stem*, *progenitor*, and *precursor* cells. All new insulin-producing cells originate from precursor cells, which are not necessarily true stem cells. Stem cells can be defined as precursor cells capable of indefinite self-renewal. An embryonic stem cell is pluripotent, being capable to differentiating into the three embryonic germ layers – ectoderm, endoderm, and mesoderm – and then any cell type of the body, even oocytes. Typical adult stem cells such as those found in the bone marrow, intestine, and skin are multipotent, being able to form a variety of cell types restricted

to a defined lineage. For example, cells originating from bone marrow stem cells are of mesodermal origin and form blood cell elements and endothelial cells. Then there are precursor cells which have more restricted products. Hepatic oval cells that can form either new hepatocytes or bile duct epithelia are usually considered liver stem cells and have been suggested to be derived from bone marrow. However, most liver regeneration occurs from the expansion from existing hepatocytes, so they have been considered facultative progenitor cells. Facultative progenitor cells may also include pancreatic duct cells that can form pancreatic acini and probably islets.

33.4 NEW β-CELLS ARE FORMED THROUGHOUT ADULT LIFE

Maintenance of the β-cell mass is a dynamic process of continuing apoptosis balanced by the formation of new β-cells through the replication of existing β-cells, and probably neogenesis, the formation of new islets from precursor cells. The neonatal period in rodents is characterized by active β-cell replication and neogenesis, and near the time of weaning there is an increase in β-cell apoptosis that leads to a remodeling of the endocrine pancreas. By measuring the β-cell replication rate and β-cell mass and making assumptions about neogenesis and apoptosis, it has been possible to estimate the lifetime of a rat β-cell as approximately 58 days. The endocrine pancreas of rats has considerable capacity for regeneration as shown by the 90% pancreatectomy. Even by four weeks after this surgical reduction in β-cell mass, the β-cell mass has increased from 10% to 42% of controls through the combined contributions of β-cell replication and neogenesis. However, there seem to be important species differences in how the β-cell mass expands. Mice with genetically induced peripheral insulin resistance develop gigantic islets mainly through β-cell replication. Humans with the insulin resistance of obesity have increased β-cell mass, but their islets are not particularly enlarged. This finding, with the apparent low rate of β-cell replication, suggests that neogenesis is an important contributor to the increase in mass in adult humans. Another mechanism for increasing β-cell mass, β-cell hypertrophy, has been found in rats in a variety of situations including glucose infusions, pregnancy, and partial pancreatectomy.

33.5 WHAT IS THE CELLULAR ORIGIN OF ADULT ISLET NEOGENESIS?

Although it is clear that new islets are formed in the adult pancreas, the source of these new islets continues to be a matter of debate. One hypothesis is that islets are derived from differentiated duct cells in the pancreas, with these cells serving as facultative progenitor cells. Others argue that new islets

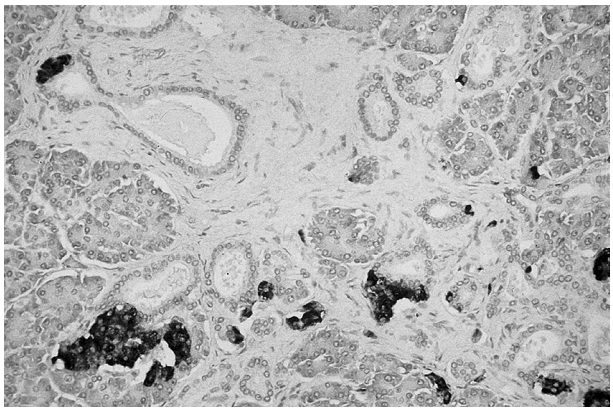

FIGURE 33.1

New islets formed by neogenesis, or differentiation from precursor cells, are seen as the budding of islet cells (shown by immunoperoxidase staining for dark brown insulin) from ducts. The autopsied human pancreas is from a 48-year-old obese man with impaired glucose intolerance. *Reproduced from Butler AE, et al. 2003. Diabetes 52: 102–10 with the permission of the American Diabetes Association.*

come from some other, yet-to-be-identified precursor cells, which may or may not be true stem cells.

33.5.1 Arguments Favoring the Ductal Origin of New Islets

Morphologically, new islets bud from the ducts, breaking through the epithelial basement lamina and migrating from the duct. The process in the adult pancreas closely resembles embryonic new islet formation, which also is seen as budding from ducts (Figure 33.1). The retention of the ability to differentiate into islet cells in adult duct tissue was shown experimentally; islet hormone cells were observed following transplantation of adult pancreatic ducts and fetal mesenchyme. In the rat insulin promoter: interferon gamma transgenic mice, newly formed islets were often seen within the lumen of the ducts, again suggesting their origin from ducts.

Other evidence supporting the concept is that after partial pancreatectomy in rats, ductal cells rapidly replicate and dedifferentiate with a marked increase in Pdx1 protein, a factor known to be important for pancreas development and β-cell function. This return to a more embryonic phenotype precedes the differentiation of whole new lobes of pancreas. Yet another finding strengthening this hypothesis is the marked change in composition of porcine neonatal pancreatic cell clusters transplanted under the kidney capsule of immunodeficient mice. When transplanted, most cells can be stained with the duct marker cytokeratin 7, but after several months, 94% of the cells are

β-cells. During this engraftment, as well as in the neonatal pancreas, cells costained for both insulin and cytokeratin 7 are found, suggesting residual duct markers as duct cells become β-cells. This finding is extended by gene profiling with microarrays of new versus mature islets seven days after partial pancreatectomy in rats. These new islets, which can be identified by markers of new lobe formation, are about three days old. Their β-cells contain a variety of duct markers, supporting the duct precursor hypothesis.

Other supporting data are that human duct-cell-rich fractions remaining after islet isolation and purification can be cultured and can form new islets after exposure to various growth and environmental factors. It must be noted, however, that although these new islets bud from duct cells with a cystic structure, it has not been proved that the precursor cells are duct cells. However, PANC1, a human pancreatic duct cell line, has been manipulated in culture to give islet hormone-expressing cells. Similarly, studies from the Peck group have reported cells derived from mouse pancreatic duct cells that can be markedly expanded and still contain insulin, albeit in small amounts. Although results have been presented that these cells could reverse diabetes in mice, concerns have been raised about whether these results are compatible with the low insulin content of the cells.

A hypothetical argument supporting the concept of duct cells serving as facultative precursor cells comes from estimating how many precursor cells are needed to form a new islet. After partial pancreatectomy in rats, fully formed new islets can be seen 72 hours after surgery, with these new islets consisting of 1,000–1,500 cells. No increased replication in the pancreas is seen until 24 hours after surgery, so they must form within 48 hours. With cell cycle times in rodents being 10–20 hours, 16 starting cells with a doubling time of 10 hours (five doublings over 48 hours), would be required to make 1,000 cells. If the doubling time were 12 hours, then 64 cells would be required. Because so few candidate non-duct cells can be found morphologically in this area, these calculations fit with the local abundance of duct precursor cells. Moreover, this concept fits with the demonstration that β-cells in islets are of polyclonal origin.

33.5.2 Arguments for Non-duct Cells Being Islet Precursor Cells

Because of the intense interest in identifying islet precursor cells, a variety of studies have made a case for the presence of precursor cells that originate from islets. One postulate for a β-cell precursor in islets comes from streptozocin-treated rats in which cells stained for both insulin and somatostatin are thought to differentiate into β-cells. Although these studies are provocative and potentially important, more work is required to understand whether these are true pathways for β-cell regeneration.

There has been considerable controversy about whether the intermediate filament protein, nestin, which is known to be present in neural stem cells, is also found in islet precursor cells. Work with mouse embryonic stem cells suggested that insulin-producing cells could be generated through nestin-containing precursors, but most of these cells have been found to be apoptotic and artifactually stained for insulin. Another study reported on isolated cells stained for nestin derived from human islets. These cells also have the capacity for expansion and express a variety of islet markers, although they too have little insulin. Adding to the controversy are studies using lineage tracing in transgenic mice, which suggest that islet cells are not derived from nestin-expressing precursors. Additional data have shown that nestin-positive cells in the pancreas co-localize not with endocrine or epithelial markers but with mesenchymal, endothelial, and stellate cell markers.

There has only been one lineage-tracing study addressing the ductal origin of new islet cells. This study, which used two sets of cell-specific Cre transgenic mice crossed with floxed reporter gene mice, found no evidence of a ductal origin for islets after birth. With crosses with inducible Pdx1-Cre mice, no duct cells were labeled after embryonic day 12.5 (E12.5), but all islets were labeled, leading to the conclusion that the adult duct population was segregated at an early age and did not contribute to islet formation. However, the expression level of *Pdx1* in ducts after E13 is known to be markedly reduced from that in β-cells or earlier embryonic ducts, so the expression level of the Cre may not have been adequate to mark the ductal cells. The second set of experiments using postnatal, inducible NGN3-Cre mice did not find any labeled cells within the ducts when studied 1, 4, or 7 days after induction. Here, the main criticism is that the labeled cells may have moved rapidly from the duct and by the time of examination were no longer within the ductal compartment. Further lineage-tracing studies are needed to resolve the issue of ductal origin of the islets after E12.5.

33.6 TRANSDIFFERENTIATION OF NONISLET CELLS TO ISLET CELLS

There are now a variety of studies supporting the concept that other cells can be directed to become insulin-producing cells. Strictly speaking, the concept that differentiated duct cells can form new islets, as discussed previously, could be considered a form of transdifferentiation. Current claims about transdifferentiation include pancreatic acinar cells, hepatocytes, and bone marrow. The possibility that transdifferentiation within pancreatic tissues occurs is not surprising in view of the ease with which the phenotype of islet and pancreatic cell lines can shift from one predominant gene product to another. Examples include: clonal rat insulinoma (RIN) cells varying their expression of insulin, glucagon, and somatostatin over time in culture;

a pancreatic adenocarcinoma cell line (AR42J) that can be pushed to produce insulin by either hepatocyte growth factor or a combination of betacellulin and activin A; and growing evidence that progenitor cells in the adult pancreas can form hepatocytes and that liver cells can form pancreatic cells.

33.7 PANCREATIC ACINAR CELL TRANSDIFFERENTIATION

It has been difficult to prove that differentiated adult pancreatic cells are converted to insulin-producing cells. Suggestive evidence is provided by studies in a rat duct ligation model of regeneration in which gastrin infusions produce changes consistent with transdifferentiation of acinar cells to duct cells that can then serve as precursors for β-cell neogenesis. The same pathway for neogenesis has been postulated to occur in rats receiving glucose infusions.

33.8 BONE MARROW CELLS AS A SOURCE OF INSULIN-PRODUCING CELLS

There has been much interest in the possibility that circulating bone marrow cells could serve as precursors for a variety of cells scattered throughout the body. However, several fundamental issues are raised. One is whether hematopoietic stem cells (HSCs) can transdifferentiate into nonhematopoietic cells as has been suggested by various studies. There remains the possibility that cells other than true HSCs derived from bone marrow can serve as circulating stem-precursor cells and that there may be true transdifferentiation or cell fusion. It was recently shown that an impressive repopulation of destroyed liver tissue can be generated from bone marrow cells, but this process was shown to be caused by cell fusion. Nonetheless, a provocative report appeared suggesting that cells from the bone marrow can become glucose-responsive insulin-secreting cells in islets without this being caused by fusion. The question of the potential of bone marrow cells remains open, especially considering the finding by Verfaillie and coworkers that adult bone marrow cells can be pluripotent, capable of progressing to ectoderm, mesoderm, and endoderm fates. Another way in which bone marrow cells could contribute to β-cell formation is as a facilitator rather than a precursor. C-kit-positive adult bone marrow cells were given to mice made diabetic with streptozocin and there was evidence of regeneration of the endocrine pancreas and return to normoglycemia.

33.9 LIVER AS A SOURCE OF INSULIN-PRODUCING CELLS

As suggested previously, the liver, being of endodermal origin, might be expected to be a prime candidate for transdifferentiation. One of the first

suggestions of this promise was the finding of the Ferber group that *in vivo* transduction of hepatocytes in mice with an adenovirus containing Pdx1 could induce endogenous Pdx1 with the associated expression of other β-cell markers and substantial amounts of insulin. A similar approach in mice employing helper-dependent adenoviruses expressing betacellulin and neuro-D appeared to produce enough insulin in the liver to reverse streptozocin diabetes. These results were interpreted not as the transdifferentiation of hepatocytes but as the promotion of islet neogenesis in the liver. The use of adenoviruses expressing Pdx1 in these experiments resulted in hepatitis, which contradicts the Ferber work. Long-term exposure to high glucose drove hepatic oval cells to an islet phenotype. Another provocative finding was that human fetal liver cells, transduced with human telomerase and then Pdx1, produced a remarkable amount of stored and secreted insulin. These cells not only secreted insulin in a regulated manner but also reversed diabetes when transplanted into immunodeficient diabetic mice.

33.10 ENGINEERING OTHER NON-β-CELLS TO PRODUCE INSULIN

The possibility that non-β-cells might be engineered to make insulin and even secrete insulin in response to glucose has been pioneered by the Newgard group using cultured cells. This approach employs the introduction of genes expressing insulin and proteins involved in glucose recognition, but there is concern about what level of engineering will be required to obtain the complex and sophisticated phenotype of β-cells. It may be that the introduction of transcription factors serving as master genes will be required to obtain β-cells with adequate function for therapy. Another interesting example of this approach had insulin expressed in the cells of the intermediate lobe of the pituitary in transgenic mice. Remarkably, not only were these cells able to secrete insulin, they also were resistant to autoimmune destruction. The engineering of gastric inhibitory polypeptide (GIP)-secreting K-cells in the intestine to produce insulin is another novel approach. These cells can secrete insulin with GIP into the blood stream after the oral administration of glucose. Concern must be raised about how this strategy could produce insulin in a manner precise enough to be useful clinically.

We should be open-minded to the possibility that various stem or precursor cells might be converted to useful insulin-producing cells. Neural or skin stem cells would seem to be a challenge because of their ectodermal origin, but we do not understand the restrictions of germ line fates well enough to rule out this possibility. Intestinal stem cells are of endodermal origin, which makes them attractive targets. Hopes for this pathway are raised by the

demonstration that intestinal epithelioid IEC-6 cells can produce and secrete insulin after transfection with Pdx1 followed either by transplantation or by treatment with betacellulin.

33.11 ATTEMPTS TO DELIVER INSULIN THROUGH CONSTITUTIVE RATHER THAN REGULATED SECRETION

The essence of this concept is that glucose could be used to induce the promoter of a gene that could drive the synthesis of insulin followed by the constitutive release by a cell such as the hepatocyte. An example of this approach employed an adeno-associated virus to transduce liver with the L-type pyruvate kinase promoter that responds to glucose to induce the production of single-chain insulin. The advantage of single-chain insulin is that it does not need to be cleaved by the convertases normally needed for proinsulin to become biologically active insulin. It was shown that high plasma glucose levels could stimulate increases of plasma insulin sufficient to bring glucose levels of diabetic mice into the normal range. Important questions have been raised, however, about whether this approach could be clinically useful. A recent perspective article pointed out that the timing of insulin release by a gene-dependent constitutive secretory mechanism seems highly unlikely to be useful for Type 1 or even Type 2 diabetes, in which more rapid insulin delivery or suppression will be needed to cope with the normal dynamics of metabolism found with eating, fasting, and exercise.

33.12 SUMMARY

The quest to find insulin-producing cells that might be used for transplantation is intense. Rapid improvements in our understanding of the mechanisms of cellular development and an array of potential stem or precursor cell candidates provide fuel for optimism that adult cells could solve the problem of diabetes.

FOR FURTHER STUDY

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CHAPTER 34

Burns and Skin Ulcers

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34.1 INTRODUCTION

The epidermis of the skin is a constantly renewing stratified squamous epithelium. It consists mostly of keratinocytes, but also of Langerhans cells, melanocytes, and Merkel cells resting on a supporting dermis that contains the nerve and vascular networks, which nourish the epidermis. The dermis is also the location of epidermal appendages, fibroblasts, mast cells, macrophages, and lymphocytes. Epidermal stem cells are responsible for the ability of the epidermis to replace itself, both in normal circumstances and in traumatic skin loss, such as from burns and skin ulceration.

34.2 BURNS AND SKIN ULCERS – THE PROBLEM

As with many medical conditions, it is difficult to quantify the burden and impact of these two conditions in a meaningful and tangible way. A study estimating the lifetime costs for injuries in the United States in 1985 rated fire and burns as the fourth largest cause of lifetime economic loss, at $3.8 billion. Only motor vehicles, firearms, and poisonings were responsible for greater losses. Burns are also an important cause of injury in the developing world, where more traditional methods of lighting and cooking, such as oil lamps and open fires, are still commonplace. Skin ulcers may be caused by several pathological processes, including infection, trauma, diabetes, and venous ulcer disease. Chronic venous ulceration is a common cause of skin ulcers, with an estimated prevalence of 1–1.3%. Skin ulcers are also difficult and expensive to manage, because of their slow rates of healing and the requirement for expensive and labor intensive dressing regimes. A cost of care analysis by the Visiting Nurse Association in Boston found the average cost per month in 1992 of an unhealed ulcer to be $1927.89. Diabetic foot

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ulcers, like venous ulcers, often have a chronic course. In one study, the cost estimates for foot ulcer care over a two-year period in a population of type 1 and type 2 diabetics who developed an ulcer was $27,987. These figures, especially when extrapolated to include lost productivity, are almost beyond comprehension. What is easily comprehended, however, is the personal cost and burden borne by patients suffering from burns or skin ulcers.

34.3 EPIDERMAL STEM CELLS

The epidermis of the skin is a multilayered, continuously self-renewing tissue, replaced every 30 to 60 days in human skin. *In vivo* cell turnover studies in mice have shown that all proliferative activity is restricted to the basal layer, which generates the mature functional suprabasal keratinocytes. Thus, epidermal stem cells reside within the basal layer, and are characterized by their capacity for self-maintenance and self-renewal. In addition to producing more stem cells, the stem cells generate transient amplifying cells that divide three to five times before producing terminally differentiated keratinocytes *in vivo*. The ability to identify slowly-cycling stem cells *in situ*, visualized as ^{3}H-Tdr or bromodeoxyuridine (BrdU) label-retaining cells, has permitted their localization at specific niches, including the deep rete ridges of the interfollicular epidermis and the bulge region of hair follicles (Figure 34.1). The identification of epidermal stem cells *ex vivo* is a more controversial subject, because there is no unequivocal assay for these cells *in vitro*.

Heterogeneity in the growth capacity of keratinocytes was first reported in 1987; they used clonal analysis to identify cells retrospectively capable of generating large colonies of cells exhibiting limited differentiation in culture, termed *holoclones*. These investigators proposed that holoclones were derived

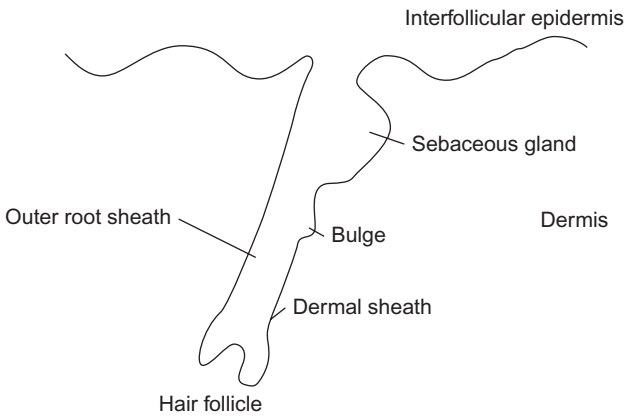

FIGURE 34.1 Epidermis, dermis, and other structures.

from stem cells because of their greater proliferative capacity. This work did not provide a means to prospectively isolate keratinocyte stem cells (KSCs). Subsequent work established that cell-surface markers, specifically β1 integrin, could be used to distinguish basal keratinocytes, with high (β1 bright) or low (β1 dim) proliferative capacity measured in terms of short-term colony-forming efficiency. Despite the initial conclusion that β1 integrin was a marker of KSCs, subsequent work from many laboratories has shown that, although most basal cells are integrin-bright, only a small subpopulation of these exhibit quiescence, as defined by their ability to retain a ^{3}H-Tdr or BrdU label.

Work from our laboratory has further established that ^{3}H-Tdr label-retaining cells can be purified from integrin-bright keratinocytes on the basis of a second cell-surface marker, i.e., CD71, or the transferrin receptor. Thus, epidermal cells exhibiting the phenotype α6(bri)CD71(dim) represent the stem cell population of both human neonatal and adult murine epidermis. This fraction is enriched for label-retaining cells that are small (~9 μm) with a blast-like morphology, display a high nuclear-to-cytoplasmic ratio, exhibit the greatest long-term proliferative capacity to regenerate keratinocytes *in vitro*, and comprise about 5% of total basal cells (Figure 34.2). Moreover, we have demonstrated that the progeny of KSCs can be distinguished by their cell-surface phenotype: transit-amplifying (TA) cells exhibit high levels of CD71 (α6(bri)CD71(bri)), are enriched for actively cycling cells, defined as pulse-labeled cells in murine epidermis, and exhibit intermediate keratinocyte cell regeneration capacity; early differentiating (ED) cells are identifiable as α6(dim) cells exhibiting the poorest long-term proliferative capacity and expressing keratin 10 and involucrin – both markers of keratinocyte differentiation (Figure 34.2). This work permits the prospective isolation of KSCs and their immediate progeny by fluorescence-activated cell sorting (FACS), so that the contribution of distinct classes of epidermal progenitors to tissue regeneration during homeostasis or in wound healing can be directly assessed. This work is an important prerequisite to the development of therapeutic strategies using KSCs for skin conditions, including gene therapy. The identification of growth factors that recruit KSCs to proliferate and regenerate tissue will be important to the development of techniques for rapid *ex vivo* expansion that facilitate earlier transplantation for burn victims.

34.4 STEM CELLS IN BURNS AND SKIN ULCERS – CURRENT USE

34.4.1 Burns

34.4.1.1 Autografts

Epidermal cells have been used in the treatment of burns since the introduction of the split skin graft by Karl Thiersh in the late 1800s. Skin grafting to

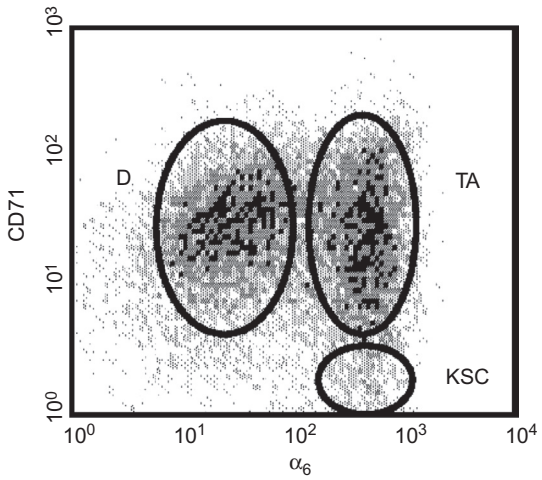

FIGURE 34.2

This FACS plot of primary human, neonatal, foreskin keratinocytes – labeled with antibodies to the cell-surface marker α6 integrin (FITC) and the transferrin receptor (CD71-PE) – reveals phenotypically discrete subpopulations of cells corresponding to stem cells (KSC) with the phenotype α6(bri)CD71(dim), transit-amplifying (TA) cells with α6(bri)CD71(bri), and early differentiating (ED) cells with α6(dim).

cover defects caused by burns or skin ulceration is limited by the area of skin that may be harvested on any one occasion. Full-thickness grafts (including all of the epidermis and dermis) provide good cosmetic results, but require a primary closure of the donor site, limiting the area that may be grafted. To overcome this problem, the use of split skin grafts was developed, whereby epidermis and underlying dermis is shaved from the donor site to provide a graft. The donor site then re-epithelializes from hair follicles, a process that is easily seen with the unaided eye and that takes two to three weeks, after which the donor site can be reharvested. It is thought that stem cells of the hair follicle are responsible for healing both the donor site and the grafted area. Microdissected hair follicle bulges (enriched for stem cells) from transgenic mice expressing the β-galactosidase gene could regenerate interfollicular epidermal tissue, as well as entire hair follicles, when transplanted.

The limitations of skin grafting techniques are the area that can be covered by them, and the many weeks it may take to cover a large area of burn with autologous split skin. Burns of 80–90% are survivable in the short-term with resuscitation, but if coverage of the wounds is delayed because of a lack of grafts, then a high morbidity and mortality is the result.

In the mid-1970s, techniques for serial cultivation of epidermal cells were developed that produced a 1,000- to 10,000-fold greater area of graftable epidermis than the initial biopsy (Figures 34.3 and 34.4). These epidermal

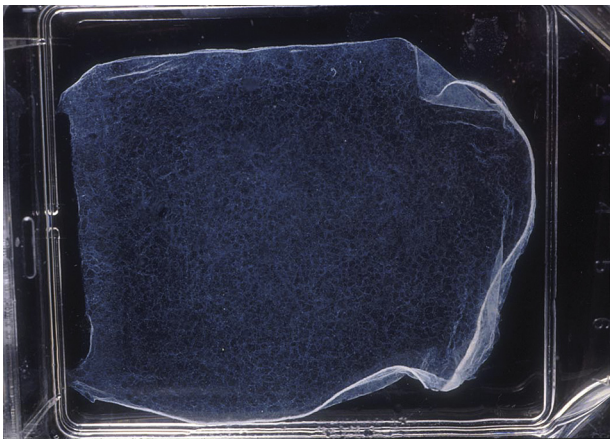

FIGURE 34.3 Detached epithelial graft in a tissue culture flask.
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FIGURE 34.4 Secondary cultures of the epithelium in an incubator.
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sheets can then be grafted onto clean wound beds, but they are sensitive to loss by bacterial infection and blistering. In full-thickness burns where the dermis has been lost, the cultured epidermal autograft may be placed directly onto muscle or fascia (Figure 34.5). These cultured epidermal autografts form a permanent covering, suggesting that the stem cells initially cultured and then transplanted have been maintained as stem cells, and therefore retain their crucial role in epidermal maintenance. Histological examination of the cultured epithelium reveals the structural similarity to normal epithelium

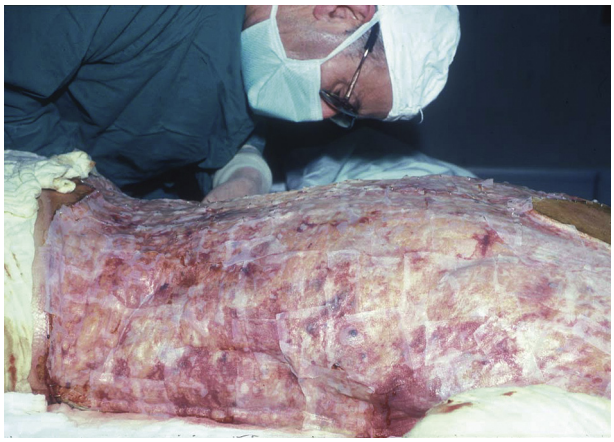

FIGURE 34.5 Application of cultured autograft epidermis to a burns patient.
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in vivo (Figure 34.6). Culturing epidermis is a time- and labor intensive exercise, estimated to cost between $600 and $13,000 per 1% of the body surface area covered (depending on the proportion of the grafts that successfully take).

34.4.1.2 Allografts

The development of allografts has been driven by the lack of available donor sites for split skin grafting in patients with massive burns, and by the time taken to grow cultured autologous skin from these patients. Burn therapy requires coverage of the burned areas to prevent secondary sepsis and other complications. An alternative to split skin grafts is needed that is immediately available, plentiful, effective, and affordable. Cadaveric skin is such an alternative; it is a true allograft and is always eventually rejected by the recipient. As mentioned earlier, skin that lacks a dermis is less able to resist trauma, and is prone to contraction, resulting in a poor functional and cosmetic outcome. Alloderm (Lifecell, Branchburg, New Jersey) is a processed human dermis from which the epidermal and dermal cells have been removed, leaving only the connective tissue matrix. Alloderm can then be applied to burns, and cultured autograft may be placed on top of it. Integra (Integra LifeSciences, Plainsboro, New Jersey) is another dermal substitute developed through the coprecipitation and lyophilization of bovine collagen, chondroitin-6-sulfate, and an artificial epidermal layer of synthetic, polysiloxane polymer. One to two weeks after application, the artificial epidermis is removed, and an ultrathin epidermal autograft (0.003–0.005 inches thick) is placed on the burn area.

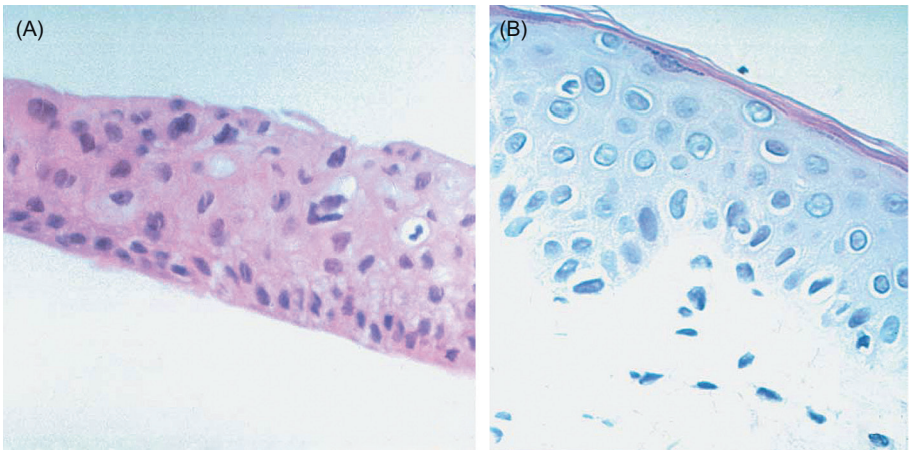

FIGURE 34.6 Side-by-side histological comparison of (A) cultured epidermis and (B) epidermis *in vivo*.
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All currently available examples of artificial dermis lack a vascular plexus for the nourishment of the epidermis, and require host vasculogenesis into the dermis graft to supply nourishment to the grafted epidermis. Efforts have therefore been focused on encouraging the process of vasculogenesis by genetic engineering of grafts to produce growth factors and cytokines vital to this process (see later sections of this chapter).

34.4.2 Skin Ulcers

Therapy for skin ulcers is based on treating the precipitating and perpetuating factors. This includes antibiotic use for infective ulcers, rigorous pressure care for decubitus ulcers, and compression stockings for venous ulcers (for examples of skin ulcers, see Figures 34.7 and 34.8). The use of occlusive dressing techniques has greatly expanded in recent decades, and these form the foundation for the treatment of many ulcers. In spite of advances such as occlusive dressings, ulcer healing often takes months, if not years, to achieve. In burn therapy, the impetus behind the use of stem cells or cultured epidermal autografts is the need to cover large areas quickly. In ulcer therapy, the time constraints are not as severe, and for definitive closure, split skin grafts remain the gold standard. Cultured skin has been used in the treatment of skin ulcers – in particular, in the use of cultured allografts as a 'living dressing.' Cultured, autologous outer root sheath cells used in the treatment of chronic decubitus ulcers have been found to produce an 'edging effect' – the contraction of the chronic wound edges in response to the graft – believed to be caused by a release of growth factors, cytokines, and hormones from the outer root

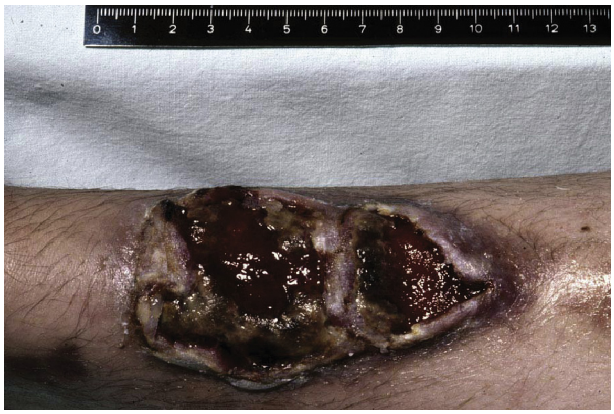

FIGURE 34.7 Leg ulcer.
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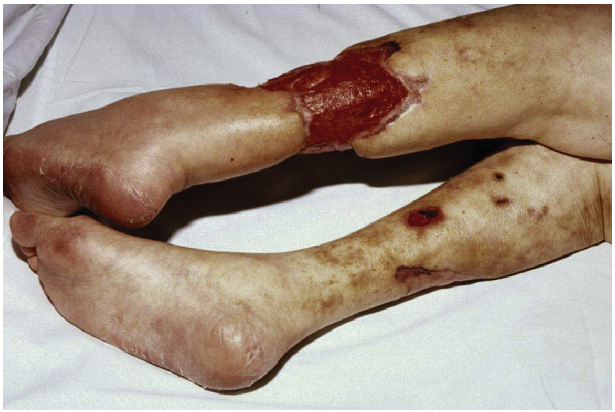

FIGURE 34.8 Circumferential leg ulcer healing by granulation.
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sheath cells. Apligraf (Organogenesis, Canton, Massachusetts) is a cultured, bilayered living skin equivalent derived from neonatal foreskin keratinocytes, fibroblasts, and bovine type I collagen. It is indicated for the treatment of venous ulcers and neuropathic diabetic foot ulcers. Chronic wounds (e.g., those with dormant edges) re-epithelialize when exposed to living allograft material. This edge effect, like that seen with outer root sheath cells, is most probably caused by the presence of stimulatory factors. Chronic wounds heal better after repeated application of skin grafts, suggesting that growth factors in the grafts are responsible. Bioengineered tissues, such as Apligraf, probably act as biological systems for delivering growth factors to wounds.

34.5 RECENT AND FUTURE DEVELOPMENTS

34.5.1 Gene Therapy Approaches in Wound Healing

Epidermal cells can be genetically modified both *in vivo* and *ex vivo*, by both viral and nonviral methods (e.g., recombinant retro- and adenovirus infection, liposomes, plasmid injection, and particle bombardment). Initially, gene therapy of epidermal cells was pursued to correct inherited genetic defects, but it is now used to treat wound healing by using genetically engineered keratinocytes as a source of cytokines and growth factors. Fluid from chronic wounds has been found to be inhibitory to cell proliferation, and to contain degradation products that inhibit keratinocyte migration. The beneficial effect that allografts have on wound healing is thought to be at least partly because of the production of cytokines and growth factors, although the host eventually rejects the allografts. Cultured autografts engineered to produce these cytokines and growth factors in supranormal quantities may expedite wound healing. Epidermal cells can be engineered to express a gene either permanently or transiently. These genes could encode growth factors or cytokines, as mentioned earlier, or they may antagonize some of the inhibitory factors found in chronic wounds.

The vascularization of cultured skin autografts is often delayed when compared with split skin grafting, and may be a contributing factor to the failure of the cultured autograft to take. Cultured skin has been genetically modified *in vitro* to produce vascular endothelial growth factor (VEGF). These modified, cultured skin substitutes have been shown to secrete elevated levels of VEGF, and to have decreased time to vascularization when grafted onto athymic mice.

34.5.2 Tissue Engineering

Tissue engineering of skin is an active area of research and development. Efforts to develop temporary skin substitutes began in the early 1960s. The skin is one of the first organs to have been successfully generated *ex vivo*, and there are several tissue engineered skin equivalents available today. In bioengineered skin, the epidermal component is either cultured allograft or cultured autograft. The keratinocyte sheets are then combined with the dermal component. The dermal component may be acellular, or it may contain allogeneic or autogenic fibroblasts, or other cells. The optimum skin equivalent would be readily available, could be stored or frozen and ready for use, would be inexpensive to make, would have excellent take, and would give good cosmetic results. Such an ideal skin equivalent does not exist. Split skin grafts fulfill many of these requirements but, unfortunately, they are often not available in a sufficient amount. Currently, the culturing of autografts requires at least three weeks, is labor intensive, and is not available in many developing

parts of the world. It would be a great advance if the technique could be made faster and easier to perform. Given our ability to use FACS methodology to separate KSC versus TA populations from the epidermis, we have been testing the hypothesis that the stem cell-enriched keratinocytes may provide faster and more reliable skin regeneration in *in vitro* and *in vivo* transplantation model systems. Our studies indicate that both epidermal stem cells and TA cells can regenerate epithelial tissue in the short-term fairly rapidly, and that dermal cells, and specific components of the basement membrane (i.e., laminins), are critical regulators of this tissue-regenerative capacity. Current studies in our laboratory are aimed at understanding how to recruit greater numbers of epidermal stem cells to proliferate *in vitro*, as well as identifying factors capable of promoting stem cell expansion and renewal *ex vivo*. These studies will be important to the development of cellular therapies using epidermal stem cells as vehicles.

Investigators of epidermal stem cells have the advantage of easy access to their cells of interest. Keratinocytes are also relatively easy to culture and engineer into their normal 'organ form' (i.e., sheets of epidermis). As stated earlier, there is an established role for epidermal stem cells in the therapy of human diseases and injuries. The concept of epidermal stem cells, and their application to health care, could form a conceptual framework for the education of the general public about stem cells, and could help to demystify what is commonly perceived to be a complicated science. Epidermal stem cells are excellent targets for gene therapy seeking to correct genetic deficiencies permanently. This could form the basis of new therapies for previously untreatable genodermatoses (such as epidermolysis bullosa), as well as for burns and skin ulcers.

Epidermal appendages such as hair, sweat glands, and sebaceous glands are often destroyed by burns, and their replacement with bioengineered equivalents is still to be achieved. The ability to develop a hair-bearing skin replacement, for example, would address many of the cosmetic problems caused by burns to the face and scalp.

The stem cells of the dermis have yet to be definitively identified and localized within the skin; this can be attributed to the complex cellular heterogeneity of this tissue. Tissue engineering of a replacement dermis to graft onto burns or other defects lacking a dermis would clearly benefit from the incorporation of dermal stem cells. Given that the dermis provides growth factors regulating epidermal growth and morphogenesis, as well as 'hair inductive' capacity, further elucidation of its molecular, cellular, and functional components is essential to the development of cellular therapies.

The risk of viral transmission remains a concern with allografts, but they can be frozen, thawed, and used when needed. This is very convenient, and contributes to their lifesaving potential. Tissue engineering with bovine or other

sources of collagen must be carefully managed, to reduce the risk of transmission of prion and other diseases to humans. For these reasons, research into cultured autografts will continue, with the goal being a cultured skin equivalent made from epidermal and dermal stem cells, tissue engineered to provide rapid wound coverage with excellent take. The great advances made in the last quarter of a century have saved many lives, in the case of burns, and made lives worth living again, in the case of skin ulcers. Our expanding understanding of stem cells and their manipulation will build on these advances to enable even better treatment of these conditions in the future.

34.5.3 Embryonic Stem Cells

Cell therapy for large burns currently relies, as mentioned, largely on autologous epidermis reconstructed *in vitro*. The biggest limitation of this technique is the availability of cadaver skin used to cover the wounds during the period that autologous cells are being grown, together with the availability of sufficient healthy patient skin to extract and expand keratinocytes in culture for use as autografts. The time taken to grow sufficient amounts of keratinocyte sheets to cover especially large burns increases the risk of infections and dehydration, which could ultimately lead to the patient's death.

The latest discovery for the application of stem cells to treat burns, which has the potential to become a real alternative for treating burns, is the differentiation of embryonic stem cells (ESCs) along the epidermal lineage into keratinocytes. Existing human ESC cell lines were treated for over 40 days with recombinant proteins, i.e., avoiding the use of animal products and thus eliminating the risk of disease transmission. This procedure generated deratinocytes with characteristics of epidermal cells, including the expression of specific keratins and epidermal differentiation. Importantly these hESC-derived keratinocytes (k-hESC) regenerated a pluristratified epidermis *in vitro* and when grafted onto immune compromised mice *in vivo*. Structurally this k-hESC-derived epidermis had a structure consistent with mature skin. Furthermore k-hESCs have been suggested to be immuneprivileged due to a low or no expression of histocompatibility leukocyte antigen (HLA) proteins, which has been suggested before for other hESC derivatives. This advance makes it possible to generate immuneprivileged keratinocyte allografts to cover the wounds of patients awaiting autologous grafts with a reduced risk of rejection by the patients' immune system compared with cadaver skin or biosynthetic matrices. Notably, the hESC-derived keratinocytes could be cultured and amplified for extended periods so that frozen stocks could be readily available at any time to treat burns patients quickly. This implies that keratinocytes derived from ESCs could become a temporary replacement for cadaver skin currently used to cover wounds in large burns during the time needed to grow autografts or even be sufficient for permanent treatment.

However for full-thickness burns, where the skin connective tissue and associated epidermal appendages (i.e., hair follicles and sweat glands) have burned away, a permanent treatment with hESC-derived keratinocytes is not sufficient. Patients with full-thickness burns usually suffer from an inability to regulate body temperature and lack skin elasticity. A combination of different differentiation protocols could be of help. One protocol also generated a dermal equivalent suggesting that the cells were able to differentiate ESC along the mesenchymal lineage, i.e., fibroblasts needed for the dermal compartment missing in full-thickness burns.

Nevertheless the area of replacing skin connective tissue and its associated epidermal appendages by hESC derivatives is in dire need of further investigation and may lead to the development of a combination therapy based on differentiation protocols that specify both fibroblast and keratinocyte lineages in the future.

Genetically modified keratinocytes derived from hESCs could also be created to improve the attraction of specific cell types involved in depositing the dermal matrix, such as fibroblasts and macrophages from neighboring sites and to increase neoangiogenesis to support the regeneration of the epidermis and its connective tissue. Results of a landmark clinical trial using genetically modified epidermal stem cells to treat junctional epidermolysis bullosa, a devastating and often fatal skin disorder have been published. A basement membrane component, i.e., the β3 subunit of laminin 5, was introduced into KSCs selected as holoclones. The expression of the β3 subunit was under the control of the Moloney leukemia virus (MLV) long terminal repeats (LTR) to overcome the deficiency in this protein caused by an inherited mutation.

Human embryonic stem cell based therapies – standardized and produced under Good Manufacturing Practice conditions, could become a real alternative for skin replacement when the uncertainty of genomic instability and tumorigenic capacity of immortalized ESC cell lines can be cleared. Combining these with genetic modifications could increase the potential of this therapy further by improving healing of burns and regeneration of a functional connective tissue. Importantly, the hESC-derived keratinocytes xenografted to immunedeficient mice for 12 weeks by Guenou and colleagues did not form tumors; neither did the autologous epidermis generated by genetically modified keratinocytes to treat junctional epidermolysis bullosa.

In conclusion, there is much hope in the future for improving burns treatment by stem cell technologies and with the aid of discoveries that are being made possible through fundamental research in the areas of ESC lineage specification and gene therapy. Clearly, greater effort is required in the arena of human skin stem cell biology. Recent studies from our laboratory demonstrate that vast proliferative potential lies in the whole of the basal layer of

neonatal skin epidermis, although the minor stem cell population is the most potent tissue reconstituting subset. Importantly, microenvironmental regulation by coculture with specific dermal cells such as pericytes and particular proteins, for example laminin-10/11 and presumably others that remain undiscovered, can promote skin reconstitution of epidermal stem and progenitor cells. These observations need to be translated into the clinic to benefit the expansion of autologous keratinocytes and facilitate earlier treatment of burn patients.

ACKNOWLEDGMENTS

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FOR FURTHER STUDY

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CHAPTER 35

Stem Cells and Heart Disease

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35.1 HEART: A SELF-RENEWING ORGAN

The concept of the heart as a terminally differentiated organ unable to replace working myocytes has been at the center of cardiovascular research and therapeutic developments for the last 50 years. Despite the increasing consistency of accumulating data in favor of the formation of new myocytes in a variety of physiologic and pathologic conditions, the notion of myocyte proliferation in the adult heart continues to be challenged. It is rather remarkable that cardiologists and cardiovascular scientists are willing to believe that ventricular myocytes in human beings can work and live for more than 100 years, until the death of the organism. The possibility of a low turnover of cells as part of cardiac homeostasis is constantly being rejected without any scientific basis. Moreover, myocyte death occurs as a function of age, and the progressive loss of cells in the absence of myocyte multiplication should result in the disappearance of the entire organ over a few decades. Findings demonstrate that the heart belongs to the group of self-renewing organs such as the hematopoietic system, the intestine, the skin, and the brain.

We have documented that the adult rat heart contains a population of undifferentiated cells (Figure 35.1), which express surface antigens typically found in hematopoietic stem cells (HSCs): c-kit, MDR1, and Sca-1. Similar results have been obtained in the canine, pig, and, most importantly, in the human heart. A restricted pool of cells positive for a Sca-1-like epitope has been identified using antibodies against the mouse Ly6A/E protein. Cells with an identical phenotype have been detected in the normal human bone marrow, which also possesses cells positive for both c-kit and Sca-1-like protein. Lineage-negative c-kit-positive (Lin − c-kit^{POS}) cells have been collected from the rat heart and documented to

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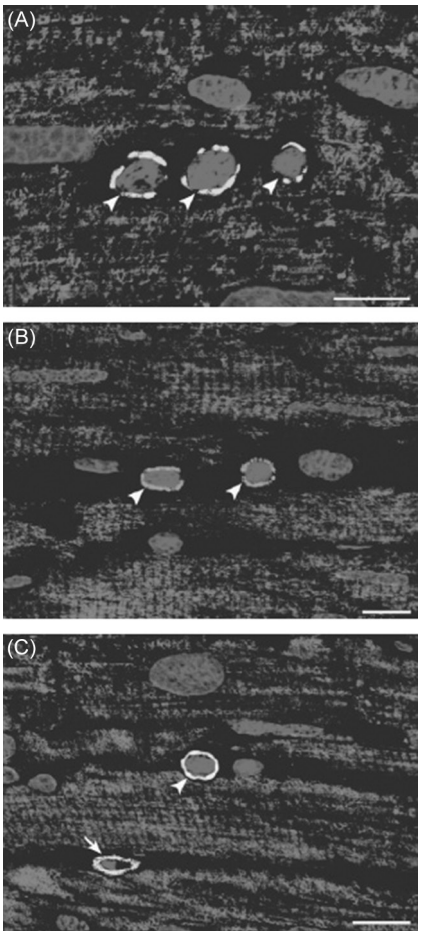

FIGURE 35.1 **Sections of adult rat ventricular myocardium.**
Sections show (A) three c-kit^{POS} cells (arrowheads), (B) two MDR1^{POS} cells (arrowheads), and (C) one Sca-1-like positive cell (arrowhead). The arrow in panel C corresponds to von Willebrand factor labeling of an endothelial cell. Myocytes are identified by α-sarcomeric actin antibody staining (gray areas). Nuclei are labeled by propidium iodide (dark area inside cells). Confocal microscopy; scale bar = 10 μm.

exhibit stem cell characteristics. Thus, as described in this chapter, cardiac stem cells (CSCs) can generate all the components of the myocardium.

As it occurred for the central nervous system (CNS), CSCs can be isolated from the ventricular myocardium and long-term cultures can be developed. So far, only Lin − c-kit^{POS} cells have been shown to have the components of stemness: self-renewal, clonogenicity, and multipotentiality. Properties of MDR1 and Sca-1-like cells remain to be determined. *In vitro*, Lin − c-kit^{POS} cells grow as a

monolayer when seeded in substrate-coated dishes or form spheroids when in suspension, mimicking the biology of neural stem cells. Single clonogenic cells are able to differentiate into myocytes, endothelial cells, smooth muscle cells, and fibroblasts (Figure 35.2). Therefore, primitive cells with properties of stem cells are present in the myocardium, either as a resident population of embryonic origin or as a blood-borne population that continuously seeds the tissue. This arrangement points to a mechanism for the continuous renewal of myocytes and coronary vessels throughout the life of an individual.

35.2 DISTRIBUTION OF CSCs IN THE HEART

Differences in the organization of myocyte bundles and in the levels of mechanical forces exist in the anatomic parts of the heart: the atria and the base, midregion, and apex of the ventricle. Wall stress is higher at the base and midregion of the ventricle and lower at the apex. Stress is further reduced in the atria. These variables may play a critical role in dictating the function and fate of CSCs. Such information is crucial for understanding the effects of structural and physical factors on the behavior of adult CSCs. Although stem cells possess strong cellular defense mechanisms, they are sheltered in specialized structures called niches. Stem cell niches provide a microenvironment designed to preserve the survival and replication potential of stem cells. Primitive cells divide rarely and those endowed in a niche have a much higher probability of self-renewal. For example, the slow-cycling cells of the hair follicle are confined to the bulge, where niches are located, and they generate the epithelia of the follicle and contribute to the renewal of the epidermis. This region ensures good physical protection and is rich in melanin to prevent DNA damage induced by ultraviolet light. Niches are present in all self-renewing organs. Whether CSCs are dispersed in the myocardium or are nested in pockets with a structural organization typical of niches is an important question. Qualitative and quantitative results indicate that CSCs are stored in niches, preferentially located in the atria and apex but also detectable in the ventricle (Figure 35.3). The recognition that stem cells are clustered in specific regions of the heart exposed to moderate and minimal mechanical forces and stored in niches favors the notion that these cells are organ specific. This possibility would speak against the view that stem cells in the myocardium are replenished by cells of bone marrow origin, which migrate chronically to the heart.

The concept of niches was introduced in 1978 by Schofield, who defined a niche as 'a stable microenvironment that might control hematopoietic stem cell behavior.' More recently, the niche has been viewed as:

> 'a subset of tissue cells and extracellular substrates that can indefinitely house one or more stem cells and control their self-renewal and progeny production *in vivo*.'

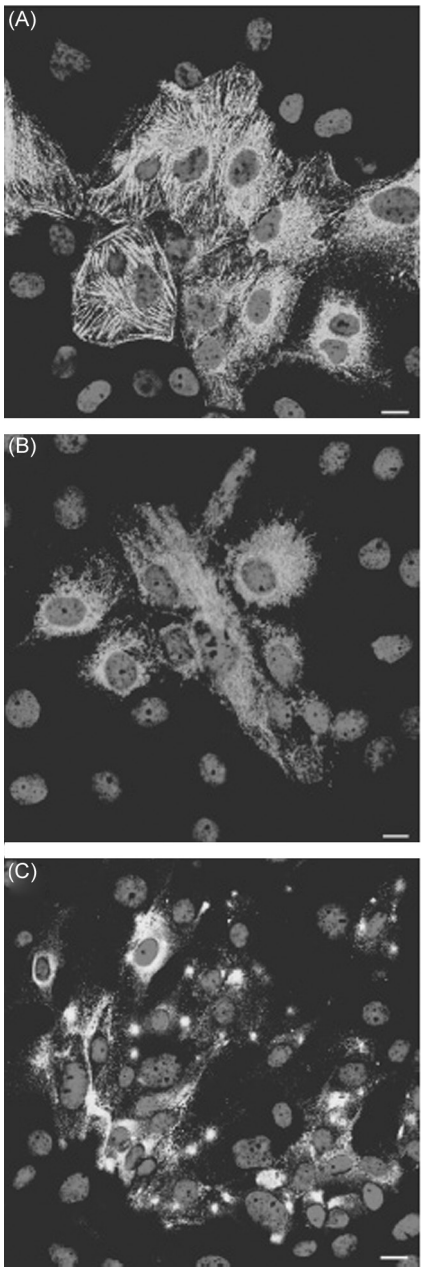

FIGURE 35.2 **Differentiation of cardiac primitive cells *in vitro*.**
(A) Clone-derived myocytes (gray), (B) smooth muscle cells (light gray), and (C) endothelial cells (white); the cytoplasm is recognized by α-sarcomeric actin, α-smooth muscle actin, and von Willebrand factor antibody staining, respectively. Nuclei are labeled by propidium iodide (dark gray spots). Confocal microscopy; scale bar = 10 μm.

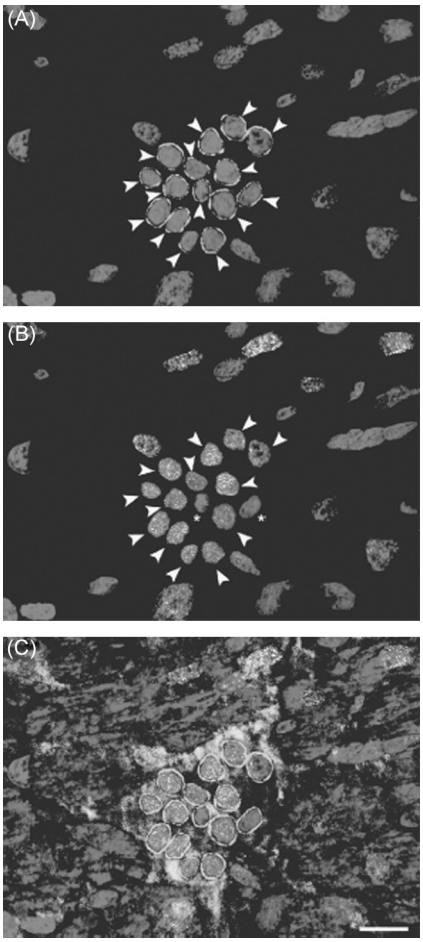

FIGURE 35.3 **Atrial niche in the adult rat heart.**
(A) A cluster of 15 c-kit^{POS} cells (arrowheads), (B) 13 of which express GATA-4 (white). Asterisks indicate two GATA-4– cells. (C) These cells are nested in fibronectin (white). Myocytes are identified by α-sarcomeric actin antibody staining (dark gray). Nuclei are labeled by propidium iodide (gray area inside cells). Confocal microscopy; scale bar = 10 μm.

Stem cells, progenitors, precursors, and early differentiating cells are clustered in the niche and may be coupled through the expression of gap junctions. Gap junctions are intercellular channels formed by individual structural units called connexins. Gap junctions allow cells to communicate with each other and to exchange small molecules. In a manner similar to HSCs, CSCs may use gap junctions to transmit and receive signals for cell survival, proliferation, or differentiation. Gap junctions could couple the CSCs among them with myocytes or adjacent cells that exert a support function similar to that of

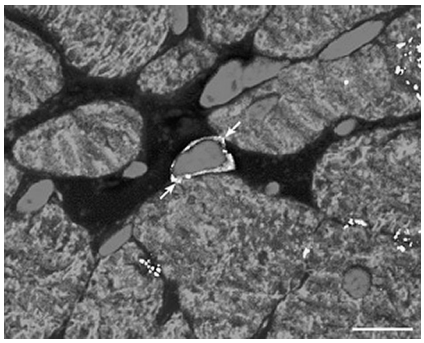

FIGURE 35.4 **C-kit^{POS} cell shows on the surface connexin 43.**
C-kit^{POS} cell (light gray of membrane); connexin 43 (white spots). Connexin 43 is located between the primitive cell and a myocyte (arrows). Myocytes are identified by α-sarcomeric actin antibody staining (gray). Nuclei are labeled by propidium iodide (e.g., the dark area inside the c-kit). Confocal microscopy; scale bar = 10 μm.

stromal cells in the bone marrow. Data from our laboratory support this contention in the heart (Figure 35.4). In summary, the localization of primitive cells in the myocardium is inversely related to the distribution of stress in the anatomic components of the heart.

35.3 REPAIR OF MYOCARDIAL DAMAGE BY NONRESIDENT PRIMITIVE CELLS

Major discoveries have been made concerning the biology of adult stem cells. First, they can differentiate into cell lineages distinct from the organ in which they reside and into cells derived from a different germ layer. These properties were considered to be restricted to embryonic stem cells. Second, adult stem cells can migrate to sites of injury, repairing damage in various organs. Neural stem cells have been identified in selective regions of the brain that, like the heart, was considered a postmitotic organ. Thus, adult stem cells may exist in other unexpected organs, and stem cell behavior is not dictated by the source. HSCs can replace bone marrow and lymphoid organs by migrating to these sites across the vascular endothelium and along stromal pathways. Moreover, HSCs regenerate skeletal muscle, and skeletal muscle stem cells can repopulate the bone marrow. However, these particular skeletal muscle stem cells are of bone marrow origin. HSCs can also differentiate in functioning hepatocytes. Hematopoietic precursors, injected intravenously, reach the brain where they divide, infiltrate the entire organ, and produce the cell types of the CNS. Conversely, CNS stem cells, which derive from the ectoderm, can transdifferentiate into blood cells, which generate from the mesoderm; HSCs can assume the characteristics of CNS cells. Together, this information

supports the notion that injury to a target organ promotes alternate stem cell differentiation emphasizing the plasticity of these cells. This became a controversial issue; it will be addressed later in this section. The controversy is because the early belief in stem cell transdifferentiation has prompted the use of bone marrow cells for the reconstitution of the infarcted myocardium. Additionally, this approach was considered superior to other forms of cellular therapy of the damaged heart.

Effort has been made to restore function in the infarcted myocardium by transplanting cultured fetal myocytes or tissue, neonatal and adult myocytes, skeletal myoblasts, and bone-marrow-derived immature cardiomyocytes. When incorporation of the engrafted cells or tissue was successful, some improvement in ventricular performance occurred. However, these interventions failed to reconstitute healthy myocardium, integrated structurally and functionally with the spared portion of the wall. This defect was particularly evident with skeletal myoblasts, which did not express connexin 43, a surface protein responsible for the formation of ion channels and electric coupling between cells. Moreover, the vascularization of the implants remained an unresolved issue. These problems pointed to the identification of new therapeutic strategies for the regeneration of dead myocardium. The generalized growth potential and differentiation of adult HSCs injected in the circulation or locally delivered in areas of injury suggested that these primitive cells sense signals from the lesion foci, migrating to these sites of damage. Subsequently, homed HSCs proliferate and differentiate, initiating growth processes resulting in the formation of all cellular components of the originally destroyed tissue. On this basis, a population of bone marrow cells enriched with Lin $-$ c-kit^{POS} cells was implanted into the viable myocardium in the proximity of an acute infarct. This was done to facilitate the translocation of these cells to the necrotic region of the left ventricular wall, to reconstitute myocardium, and to interfere with healing and cardiac decompensation. In nine days, numerous small cardiomyocytes and vascular structures developed within the infarcted zone and partially replaced the dead tissue (Figure 35.5). The newly formed young myocardium expressed transcription factors required for myogenic differentiation, cardiac myofibrillar proteins, and connexin 43. Coronary arterioles and capillaries were distributed within the regenerated portion of the left ventricle; these vessels were functionally connected with the primary coronary circulation. For the first time, bone marrow cells were shown to develop myocardium *in vivo*, reducing infarct size and ameliorating cardiac performance.

We now describe the role of cytokines and growth factors in the mobilization of stem cells and their translocation to damaged organs and to the heart in particular. This issue is highly relevant clinically, because its understanding may permit the application of strategies that do not require local

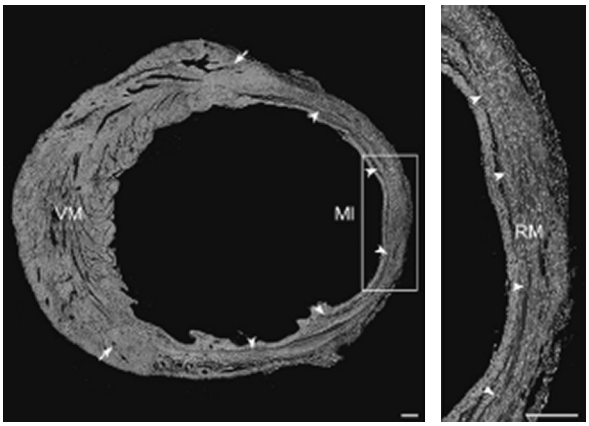

FIGURE 35.5 **Transverse section of an extensive myocardial infarct.**
(left) Infarct was treated with the injection of bone marrow cells in the border zone (arrows). Nine days later, a band of regenerating myocardium was identified (arrowheads). (right) A portion of this band, included in the rectangle, is illustrated at higher magnification. Cardiac myosin heavy-chain labeling of myocyte cytoplasm and propidium iodide staining of nuclei are shown (VM: viable myocardium, MI: myocardial infarct, and RM: regenerating myocardium). Confocal microscopy; scale bar = 300 μm. *From Orlic D, et al. 2001. Nature 410: 701–5 with permission.*

implantation of exogenous stem cells or their preventive storage from the recipient patient. Two critical determinants seem to be necessary to obtain the maximal activation of the therapeutic potential of undifferentiated cells in organs from which they are not derived: organ damage and high levels of primitive cells in the circulation. It has been shown that these two conditions are not always essential. Cell fate transition also has been observed in uninjured organs and following the use of a single bone marrow cell. However, the degree of engraftment of individual cells is much lower and decreases further in unaffected tissues. It is well established that stem cell factor and granulocyte colony-stimulating factor are powerful cytokines that markedly increase the number of circulating HSCs. These mobilized cells can reconstitute the lymphohematopoietic system of lethally irradiated recipient mice, raising the likely possibility that, in the presence of myocardial infarction, they may home to the heart and follow a differentiation pathway that includes myocytes and coronary vessels. As anticipated, this protocol was very successful; tissue reconstitution included parenchymal cells and vascular profiles. Again, *de novo* myocardium was obtained and the repaired area of the wall was functionally competent, improving cardiac pump function and significantly attenuating the negative remodeling of the postinfarcted heart.

Our results on myocardial regeneration after infarction using bone marrow cell implantation or cytokine administration do not address the critical issues

of HSC plasticity and transdifferentiation. Importantly, the hypothesis has been advanced that the donor stem cells might fuse with the parenchymal cells of the host tissue, giving the wrong appearance of transdifferentiation. So far, the accumulated data indicate that the bone marrow contains cells capable of regenerating dead tissue, but the cell or cells in the bone marrow or the cytokine-mobilized cell or cells that have the potential of triggering a reparative response within the infarct has not been determined. Additionally, the contribution of cell fusion cannot be excluded. However, the size of newly formed myocytes is only 1/15 to 1/20 of the surrounding spared myocytes, and this factor argues against cell fusion. Similarly, the donor-derived cells divide rapidly, but tetraploid cells divide slowly and might not divide if the partner cell is a terminally differentiated cardiomyocyte.

35.4 REPAIR OF MYOCARDIAL DAMAGE BY RESIDENT PRIMITIVE CELLS

An important question is whether HSCs have to be considered the stem cells of choice for cardiac repair, or whether resident CSCs could be selectively mobilized and, ultimately, employed to replace damaged myocardium. CSCs can be expected to be more effective than HSCs in rebuilding dead ventricular tissue. This is because HSCs have to reprogram themselves to produce progeny differentiating into cardiac cell lineages. Such an intermediate phase is avoided by the direct activation and migration of CSCs to the site of injury. Moreover, CSCs may be capable of reaching in a short time functional competence and structural characteristics typical of mature myocytes and coronary vessels. It is intuitively apparent that the attraction of this concept and approach is its simplicity. Cardiac repair might be accomplished by merely enhancing the normal turnover of myocardial cells. Although this is a gray area, results in our laboratory have demonstrated that cell regeneration occurs throughout the life span of the heart and the organism. This process continuously replaces old dying cells with new, younger, better-functioning units. Cell renewal is not restricted to parenchymal cells but involves all the cell populations of the heart. For example, the presence of CSCs has provided a logical explanation for the wide heterogeneity among myocytes in the adult ventricular myocardium. Old hypertrophied myocytes are mixed with smaller, fully differentiated cells and cycling, amplifying myocytes. The latter group of cells can create significant amounts of new myocardium by dividing rapidly and differentiating simultaneously until the adult phenotype has been reached.

Stem cells divide rarely, and committed transient amplifying cells are the actual group of replicating cells in self-renewing organs. The less primitive amplifying cells possess a unique property: they undergo rounds of doublings and simultaneously differentiate. Stem cells can divide symmetrically

and asymmetrically. When stem cells divide symmetrically, two self-renewing daughter cells are formed. The purpose of this division is cell proliferation (i.e., expansion of the stem cell pool). When stem cells engage themselves in asymmetric division, one daughter-stem cell and one daughter-amplifying cell are obtained. The objective of this division is cell differentiation (i.e., the production of committed progeny). Stem cells can also divide symmetrically into two committed amplifying cells, decreasing the number of primitive cells.

CSCs undergo lineage commitment, and myocytes, smooth muscle cells, and endothelial cells are generated. CSCs express c-Met and insulin-like growth factor-1 receptors (IGF-1R) and, thereby, can be activated and mobilized by hepatocyte growth factor (HGF) and IGF-1 (Figure 35.6). *In vitro* mobilization and invasion assays have documented that the c-Met–HGF system is responsible for most of the locomotion of these primitive cells. However, the IGF-1–IGF-1R system is implicated in cell replication, differentiation, and survival. These differential effects of HGF and IGF-1 on CSC motility and growth have been confirmed in *ex vivo*, oxygenated Tyrode's solution preparations of nonfixed, perfused, living myocardium by two-photon microscopy. These observations have promoted a series of experiments in which primitive and progenitor cells have been mobilized from the site of storage in the atria to the infarcted ventricular myocardium in rodents. The intense myocardial regeneration induced by this novel form of cellular therapy has been able to rescue animals with infarcts incompatible with life in any species studied so far. This includes mice, rats, dogs, and unquestionably humans. The reconstituted infarcted ventricular wall is composed of contracting cells and blood-supplying coronary vessels, resembling the composition and characteristics of early postnatal myocardial tissue. Since the period for regeneration was very short, it is reasonable to assume that the formed parenchymal cells would develop with time into mature myocytes.

As explained later in the section 'Myocardial Regeneration in Humans,' the transition from putative CSCs to cardiac progenitors, myocyte progenitors, precursors, and ultimately amplifying myocytes has been seen, with aspects resembling clonogenic growth, in the hypertrophied heart of patients with chronic aortic stenosis. The identification of these forms of growth in humans was critical for the significance of the observations made in animal models. The magnitude of growth in the pressure overloaded left ventricle exceeds the extent of cardiac regeneration detected in rodents and provides us with the first demonstration that the human heart can repair itself. Thus, the heart is a self-renewing organ in which the replenishment of its parenchymal and nonparenchymal cells has to be regulated by a stem cell compartment and by the ability of these primitive cells to self-renew and differentiate. This is because regeneration conforms to a hierarchic archetype in which slowly dividing stem cells produce highly proliferating, lineage-restricted progenitor

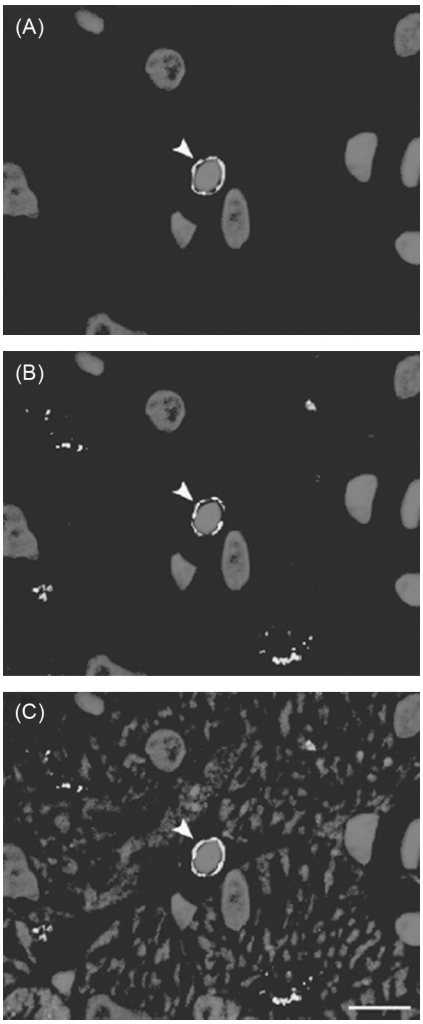

FIGURE 35.6 **C-kit^{POS} cell expresses IGF-1 R.**
(A) C-kit^{POS} cell (arrowhead), (B) IGF-1 R (arrowhead), and (C) colocalization of c-kit and IGF-1 R on the same cell (arrowhead). Myocytes are identified by α-sarcomeric actin antibody staining in panel C (gray). Nuclei are labeled by propidium iodide (e.g., the dark area inside the c-kit and IGF-1 R). Confocal microscopy; scale bar = 10 μm.

cells, which then become committed precursors that, eventually, reach growth arrest and terminal differentiation.

In summary, primitive cells resident in the heart can be activated and translocated to damaged portions of the ventricle where, after homing, they initiate an extensive reparative process leading to the reconstitution of functioning

myocardium supplied by newly formed vessels connected with the primary coronary circulation. This approach is superior to that obtained by the use of bone marrow cells because of its simplicity and immediate accessibility to the sites of stem cell storage in the heart. Additionally, the differentiation of cardiac primitive cells is rapid and does not require the reprogramming necessary for cells used to make blood into a state that would allow them to generate cardiac cell lineages.

35.5 MYOCARDIAL REGENERATION IN HUMANS

According to the dogma, ventricular myocytes in human beings are terminally differentiated cells and their life span corresponds to that of the individual. The number of myocytes reaches an adult value a few months after birth, and the same myocytes are believed to contract 70 times per minute throughout life. Because a certain fraction of the population reaches 100 years of age or more, an inevitable consequence of the dogma is that cardiac myocytes are immortal functionally and structurally. This assumption contradicts the concept of cellular aging and programmed cell death, and the logic of a slow turnover of cells with the progression of life in the mammalian heart. Conversely, several reports have provided unequivocal evidence that myocytes die and new ones constantly form in the normal human heart at all ages. Both processes are markedly enhanced in pathologic states, and the imbalance between cell growth (Figure 35.7) and cell death may be an important determinant of the onset of ventricular dysfunction and its evolution to terminal failure and death of the organism. Observations have indicated that the human heart contains a population of primitive cells prevalently located in the atria mimicking the results in rodents. Through growth and differentiation, primitive cells contribute to the remodeling of the stressed heart by generating myocytes, coronary arterioles, and capillary profiles. These newly formed structures acquire the adult phenotype, are well integrated in the existing myocardium, and become indistinguishable from the pre-existing tissue components. Such a phenomenon has been carefully documented in cardiac chimerism caused by the migration of primitive cells from the recipient to the grafted heart.

A relevant issue is that the growth potential of the diseased human heart decreases in relation to the duration of the overload, suggesting that primitive cells undergo lineage commitment and this process reduces the stem cell pool size. For example, a myocyte mitotic index of nearly 0.08% is detected in the surviving myocardium of the border zone acutely after infarction. However, in postinfarction end-stage failure, this parameter becomes 0.015%. Concurrently, myocyte death, apoptotic and necrotic in nature, markedly increases, exceeding cell multiplication. Therefore, the question is whether

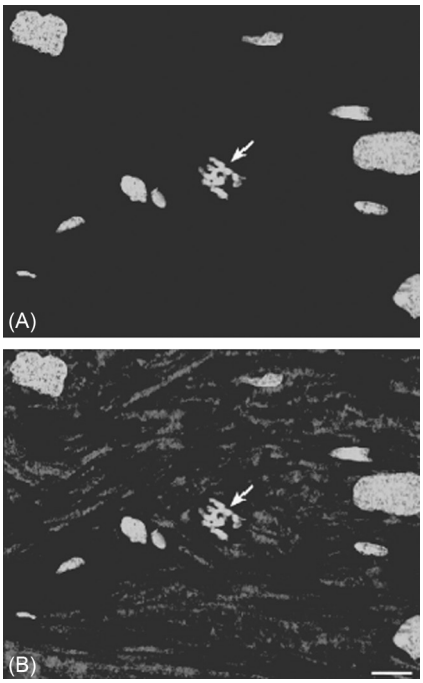

FIGURE 35.7 **Dividing myocyte in the left ventricle of a human heart affected by idiopathic dilated cardiomyopathy.**
(A) Metaphase chromosomes are shown by propidium iodide (arrow and large gray structures).
(B) The myocyte cytoplasm is stained by α-sarcomeric actin antibody (gray). Confocal microscopy; scale bar = 10 μm.

interventions can be applied locally to expand the stem cell compartment, or whether this amplification has to be performed outside of the organ in culture systems. The latter is a less favorable strategy because it requires time and interferes with the urgency of therapy in most patients with advanced cardiac decompensation.

FOR FURTHER STUDY

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CHAPTER 36

Stem Cells for the Treatment of Muscular Dystrophy

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36.1 INTRODUCTION

Muscular dystrophies (MDs) are a family of inherited disorders characterized by progressive muscle wasting leading to a variable degree of mobility limitation, including confinement to a wheelchair and, in the most severe forms such as Duchenne muscular dystrophy (DMD), heart and/or respiratory failure. Lack of one of the several proteins located either at the plasma membrane or, less frequently, within internal membranes increases the probability of damage during contraction, and eventually leads to fiber degeneration, although the molecular mechanisms are not yet understood in detail. Indeed, it is possible that the current 'structural damage' hypothesis may be an oversimplification and the actual pathogenesis is far more complex. Dystrophic muscle is also infiltrated by inflammatory cells, mainly lymphocytes and macrophages, followed by fibroblasts that deposit large amounts of collagen, contributing to progressive sclerosis of the muscle, and adipocytes that replace muscle with fat tissue. The local microcirculation is progressively lost in this process, leading to a hypoxic condition for surviving or regenerated fibers that activates a vicious circle, increasing the chance of further degeneration. Fiber degeneration is counterbalanced by regeneration of new fibers from resident myogenic cells, located underneath the basal lamina and termed satellite cells. Although satellite cells represent the resident stem/progenitor cells in postnatal skeletal muscle and are responsible for muscle regeneration, evidence has accumulated showing that other progenitor cells

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can participate in muscle regeneration. These latter cells are derived from distinct anatomical sites, such as the microvascular niche and/or the interstitium of skeletal muscle and may even reach muscle, through the circulation, from different tissues such as the bone marrow or the adipose tissue. The balance between fiber degeneration and progenitor-mediated regeneration dictates the cellular and clinical outcome. Indeed, recent preclinical evidence strongly supports this model and suggests that contrasting progenitors' exhaustion may represent an approach to alleviate muscle weakness in dystrophic patients. When regeneration is exhausted, skeletal muscle is progressively replaced by fat and fibrous tissue.

Pharmacological options for DMD pediatric patients are currently restricted to steroid treatment, the relative but consistent efficacy of which is supported by a number of recent studies. Steroids target inflammation, which is a consequence of muscle degeneration downstream of the genetic defect. Other drugs under clinical experimentation include nitric oxide (NO) (NCT01350154: Effect of Modulating the nNOS System on Cardiac, Muscular and Cognitive Function in Becker Muscular Dystrophy Patients; ClinicalTrials.gov) and Idebenone (NCT00654784: Efficacy and Tolerability of Idebenone in Boys With Cardiac Dysfunction Associated With Duchenne Muscular Dystrophy; ClinicalTrials.gov) while myostatin-neutralizing antibodies underwent clinical experimentation but did not show efficacy. Other molecules in preclinical investigation include inhibitors of the IKK/NF-κB, myostatin and of the TGFβ signaling pathway, IGF1, as well as chromatin modifying agents. Upregulation of utrophin to compensate for the loss of dystrophin in DMD is also considered, and efforts are currently underway to test recently identified transcriptional activators of utrophin expression.

Additional experimental therapeutic approaches can be schematically grouped in three major areas:

1. **Mutation-specific strategies,** which target the primary genetic defect of mutated dystrophin, i.e., exon-skipping and nonsense codon suppression. They are based upon small molecules that cross the muscle membrane and target mRNA splicing or termination, respectively. Both strategies have undergone phase 2 clinical trials. These agents generate a functional or partially functional protein with the perspective of 'converting' a Duchenne into the milder Becker MD.
2. **Gene-replacement therapy**. 'Skipping' strategies promise a solution for a good proportion of dystrophin mutations, but many others (e.g., mutations in regulatory regions and large deletions) cannot be treated with this approach. Thus, for some patients, the dystrophin gene will have to be replaced rather than repaired. To this aim, several types of vectors (viral and non-viral) have been developed and tested,

but are limited by the large size of the dystrophin gene that prevents its accommodation into most viral vectors currently used in clinics. Therefore mini- or micro-dystrophins have been used, but the results of the latest clinical trial have been unsatisfactory, possibly also because of immune reaction to the protein. A better outcome is seen for limb girdle muscular dystrophies, possibly because of the smaller size and lower immunogenicity of sarcoglycans.

3. **Cell therapy** was based initially on myoblast transplantation and has more recently involved the transplantation of stem/progenitor cells. The recent identification of novel types of stem cells opens new perspectives for cell therapy, the topic of this chapter; however, the limited knowledge of stem cell biology still represents an obstacle that must be overcome in order to devise protocols with a significant prospect of clinical improvement in patients affected by severe forms of muscular dystrophy.

36.2 MYOBLAST TRANSPLANTATION – PAST FAILURE AND NEW HOPE

This field was opened by a pioneer study that showed that intramuscular injection of adult myoblasts would reconstitute with high efficiency dystrophin-positive, apparently normal fibers in dystrophic mdx mice. This result caused immediate hopes for therapy: within a few months in the early 1990s several clinical trials were conducted using intramuscular injection of myoblasts, usually isolated from the biopsy of a parent. Although there were no adverse effects, new dystrophin production was demonstrated in some but not all cases and clinical benefit in none. This is not surprising considering that intramuscular injection in several locations of a single muscle (or at most a few muscles) cannot elicit a general effect, although improved strength of the injected muscles was detected in a minority of the patients treated. Treating muscular dystrophies by intramuscular injection of myoblasts presents several problems, especially for forms of dystrophy that affect the majority of skeletal muscles. First, intramuscular injected cells migrate only short distances from the site of injection, implying that a huge number of injections will have to be performed in order to treat a complete muscle. Second, immune responses toward the injected myoblasts have been described, even in the case of major histocompatibility locus matching. Finally, rapid death of most of the transplanted myoblasts in the first days after injection has been reported, and subsequent studies attempted to improve myoblast survival, proliferation, and differentiation after engraftment. As researchers focused on these aspects, they produced methods that progressively increased survival success of injected myoblasts and in their colonization efficiency. These studies led to a phase 1 clinical trial that has been

completed with encouraging results, even though this method is still limited by the impossibility of delivering myoblasts in all affected muscles. A phase 1/2 trial has begun to evaluate myoblast transplantation throughout the extensor carpi radialis muscle with patients older than 16 years and immunosuppressed for six months. However, in the case of muscular dystrophies that affect only few muscles, such as Oculo-Pharyngeal Muscular Dystrophy, characterized by a typical distribution localized to eyelid and pharyngeal muscles, autologous transplantation of unmodified myoblasts isolated from non-affected muscles (sterno-cleido-mastoidian, or vastus lateralis), has shown good results in preclinical work and has entered clinical experimentation (NCT00773227: Treatment of Dysphagia in Oculopharyngeal Muscular Dystrophy by Autologous Transplantation of Myoblasts; ClinicalTrials.Gov). The trial is a feasibility and tolerance study, and although the first results are encouraging, their analysis is still ongoing especially in terms of swallowing results.

In an effort to improve engraftment of intramuscularly transplanted myoblasts, several groups have succeeded in prospectively isolating 'pure' populations of satellite cells (that give rise to myoblasts in culture) by using a combination of different markers, such as Pax3-GFP, CXCR4 and β1-integrin or α7-integrin and CD34, just to mention a few. It is still unknown whether the different protocols allow isolation of the same cell population, enriched to different extents for a more primitive 'stem-like' fraction. However, all these studies revealed that freshly isolated cells have a much greater capacity to generate dystrophin-expressing fibers in *mdx* mice (which model DMD) than the same cells after *in vitro* expansion. The problem here is that freshly isolated cells from a biopsy are few and by no means sufficient to transplant even a few selected patients' muscles. Recently however, it was reported that culturing myoblasts on biomaterials, rather than on plastic, preserves to a large extent the same engraftment ability of freshly isolated cells.

In a parallel route, several laboratories, including our own, developed strategies to expand *in vitro* myoblasts or other cells (see below) from the patients, transduce them with viral vectors encoding the therapeutic gene, and inject them back in at least a few life-essential muscles of the patients from which they were initially derived. This approach would solve the problem of donor cell rejection but not that of an immune reaction against the vector and the therapeutic gene, a new antigen in genetic diseases. As mentioned above, it is difficult to produce an integrating vector that would accommodate the very large cDNA of dystrophin into patient-derived stem cells. The recent demonstration of preclinical efficacy of transplantation of stem cells transduced with a human artificial chromosome (HAC) containing the whole dystrophin locus (DYS-HAC) in a DMD model, appears as a possible solution to this problem. However, the limited life span of primary cells isolated from

dystrophic patients appeared as an additional problem, and all attempts to solve it, ranging from immortalization with oncogenes or telomerase to myogenic conversion of nonmyogenic cells, have produced interesting results, but are still too preliminary for clinical translation.

In any case, the use of myoblasts to systemically treat patients with muscular dystrophy is limited by their inability to cross the endothelium, which makes their widespread distribution difficult to achieve, systemic delivery impossible, and prevents possible healing of the diaphragm and cardiac muscles – both critical for patient survival.

36.3 UNCONVENTIONAL MYOGENIC PROGENITORS

Tissue-specific transgenic markers allowed the unambiguous demonstration of myogenic stem/progenitor cells originating from tissues other than skeletal muscle (Table 36.1). Upon transplantation, these cells participate in muscle regeneration of wild-type and/or dystrophic mice and also enter the satellite cell pool. The possibility that myogenic differentiation may depend upon fusion (and hence exposure to the dominant activity of myogenic master genes) remains but, for skeletal muscle, this would be part of the physiological morphogenesis of this tissue. A paradigm for this phenomenon is provided by bone-marrow-derived cells. The bone marrow hosts several multipotent cells (Table 36.1), which include hematopoietic stem cells (HSCs), mesenchymal stem cells, multipotent adult progenitor cells, and endothelial progenitor cells. These cells are described in detail in different chapters of this book; here, we briefly review their features which concern their myogenic potential and consequently their possible use in preclinical models of muscular dystrophy, together with other cell types isolated from tissues other than bone marrow (Figure 36.1).

36.3.1 Hematopoietic Stem Cells

In 1998 we reported that murine bone marrow contains transplantable progenitors that can be recruited to an injured muscle through the peripheral circulation and can participate in muscle repair by undergoing differentiation into mature muscle fibers. A transgenic mouse expressing a nuclear lacZ under the control of muscle-specific regulatory elements (MLC3F-nlacZ) only in striated muscle allowed the unequivocal identification of donor-derived-bone marrow nuclei inside regenerated myofibers. The publication of this report raised hopes for clinical translation: it was reasoned that, although the frequency of the phenomenon was low, in a chronically regenerating, dystrophic muscle, myogenic progenitors would have found a favorable environment and, consequently, would have contributed significantly to regeneration

Table 36.1 Properties of Myogenic Stem/Progenitor Cells

Cell Type	Source	Proliferation	*In Vitro Myogenic* Differentiation	Crossing Endothelium (system delivery)	Dystrophin Expression In Vivo
SCs/myoblasts	Skeletal muscle	Variable	Spontaneous	No	+ + +
CD133$^+$	Blood/Skeletal muscle	Low	Induced by muscle cells / Spontaneous	Yes	+ +
EPCs	Vessel wall	Low	Induced by muscle cells	Unknown	NT
HSCs	Bone marrow	Low	Induced by muscle cells	Upon induction	+
MABs	Vessel wall	High	Induced by muscle cells / Spontaneous	Upon induction	+ +
MADs	Adipose tissue	High	Spontaneous	Unknown	+
MAPCs	Bone marrow / Vessel wall	High	Induced by Aza-cytidine	Unknown	NT
MDSCs	Skeletal muscle	High	Induced by muscle cells	Unknown	+ + +
MECs	Vessel wall	High	Spontaneous	Unknown	+
MSCs	Vessel wall	High	Induced by Aza-cytidine	Unknown	+
NSCs	Subventricular zone	High	Induced by muscle cells	Unknown	NT
PICs	Skeletal muscle	High	Spontaneous	Unknown	NT
PSC-derived myogenic cells*	ES/iPS cells	Variable	Spontaneous/Induced by Pax 3/7	Variable	+ + /NT

A schematic and simplified overview of the features known, presumed, or under scrutiny of different adult stem cells in the perspective of use for cell therapy. CD133$^+$ (Ac133$^+$) stem cells; EPCs: endothelial progenitor cells; HSCs: hematopoietic stem cells; MABs: mesoangioblast; MADs: multipotent adipose-derived stem cell; MAPCs: multipotent adult progenitor cells; MDSCs: muscle-derived stem cells; MECs: myoendothelial cells; MSC: mesenchymal stem cells; NSC: neural stem cells; PICs: PW1-positive interstitial cells. NT: not tested.

**This row refers to different papers with different results: see specific section for details.*

of dystrophin-positive normal fibers. This, however, turned out not to be the case. In the following year, other groups showed that mdx mice transplanted with the bone marrow side population, or SP (a fraction of the total cells separated by dye exclusion and containing stem-progenitor cells able to repopulate the hematopoietic system upon transplantation) of syngeneic C57BL/10 mice, developed, within several weeks, a small number of dystrophin-positive fibers containing genetically marked (Y-chromosome) donor nuclei. Even many months after the transplantation, the number of fibers carrying dystrophin and the Y-chromosome never exceeded 1% of the total fibers in the muscle, thus precluding a direct clinical translation for this protocol. Similar results were later obtained in a slightly different animal model, the mdx4cv mutant. Additionally, retrospective analysis in a DMD patient that had undergone bone marrow transplantation confirmed the persistence of donor-derived skeletal muscle cells over many years, again at a low frequency.

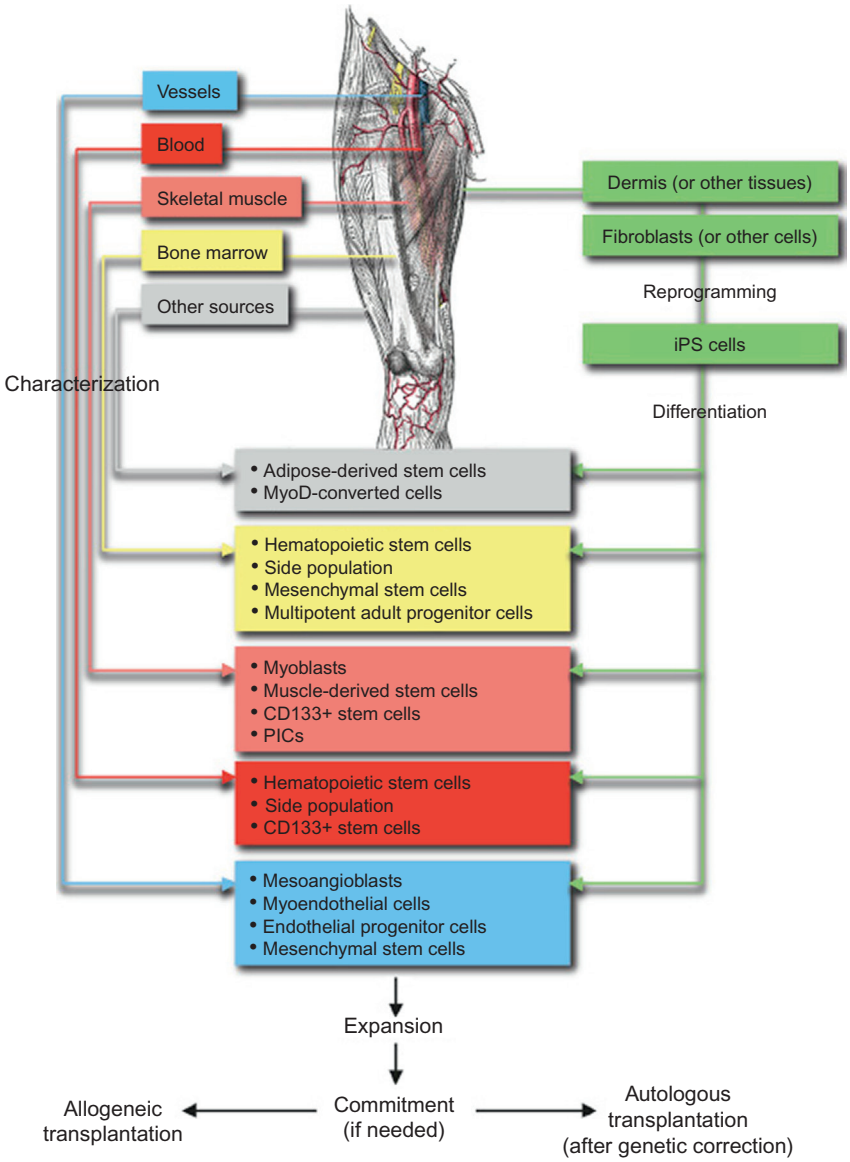

FIGURE 36.1 **Derivation of different stem/progenitor cells for possible use in muscle regeneration.**

Cell types listed in bold underwent or are undergoing clinical experimentation. *Modified from Tedesco FS, et al. 2010. J Clin Invest 120: 11–9. Illustration adapted from H. Gray (1918) Gray's Anatomy of the Human Body, 20th US edn. (source: Wikipedia. Public domain image, expired copyright).*

Reasons for this low efficiency may be:

1. The paucity of myogenic progenitors in the bone marrow;
2. Inadequate transplantation, a procedure optimized for hematopoietic reconstitution;
3. Insufficient signals to recruit myogenic progenitors from the bone marrow;
4. An inadequate environment to promote survival, proliferation, differentiation;
5. Competition by resident satellite cells (that sustain regeneration for most of the *mdx* mouse life span);
6. Difficulties in reaching regenerating fibers because of the increased deposition of fibrous tissue and the reduced vascular bed of the dystrophic muscle.

Although in DMD patients, regeneration by endogenous satellite cells is exhausted much earlier in life than in the mouse, and therefore muscle colonization by blood-borne progenitors may be different, some data suggest that in any case the process occurs with low frequency.

When bone marrow was fractionated into CD45 positive and negative fractions, the muscle forming activity after bone marrow transplantation was associated with the $CD45^+$ fraction, suggesting that a myogenic potential is present in the HSC itself, or in a yet to be identified cell that, however, expresses several markers in common with true HSCs. In the following years, several reports have convincingly demonstrated that bone marrow SP cells can be recruited to dystrophic or regenerating muscle, and can differentiate into skeletal muscle cells upon exposure to differentiating muscle cells or in response to Wnt molecules secreted by recruiting cells; moreover a fraction of SP cells localizes in a position (between the basal lamina and the sarcolemma) typical of satellite cells and indeed expresses markers of satellite cells. Two papers provided final evidence that a single HSC is able to reconstitute the hematopoietic system of a recipient mouse upon bone marrow transplantation, and at the same time gives rise to a progeny that differentiate into skeletal muscle *in vivo*. The two papers, however, disagreed on the mechanism of such a phenomenon: one, in agreement with previous data, identifies donor cells also as *bona fide* 'satellite cells;' the other fails to identify donor-derived satellite cells and claims fusion of a myeloid intermediate progenitor to be the cause of differentiation. All these data have in common a low frequency of engraftment, and recent years have not seen further advances in the use of HSCs for the cell therapy of muscular dystrophy.

On the other hand, circulating human CD133 (also known as AC133) positive cells differentiate into skeletal muscles *in vitro* and *in vivo* when injected into dystrophic immunodeficient mice. A phase 1 trial was conducted with

an autologous transplant of unmodified, and thus still dystrophic, muscle-derived $CD133^+$ cells in eight boys affected by DMD, exclusively to test safety; no adverse events were reported. Furthermore, when $CD133^+$ cells from DMD patients were genetically corrected by lentivirus-mediated exon-skipping for dystrophin exon 51, they were able to mediate morphological and functional recovery in *scid/mdx* mice.

36.3.2 Non-Hematopoietic Cells Derived from Mesoderm

Many different types of mesoderm stem/progenitor cells have been shown to exhibit myogenic potential, usually after drug treatment, genetic modification, or co-culture with myoblasts. In some cases evidence of *in vivo* myogenesis has been documented. Table 36.1 lists such cells, and some of them, such as mesenchymal stem cells (MSCs), endothelial progenitor cells (EPCs) and mesoangioblasts (MABs) are briefly described below.

36.3.2.1 Mesenchymal Stem Cells

MSCs have been shown to be capable of skeletal myogenesis. Although Pax3 activation enabled the *in vitro* differentiation of murine and human MSCs into $MyoD^+$ myogenic cells, these cells failed to cause functional muscle recovery in *mdx* mice, despite good engraftment. The reason for this failure remains unclear.

Initially identified as circulating cells expressing CD34 and fetal liver kinase-1 (Flk-1; also known as VEGFR2), EPCs were shown to be transplantable and to participate actively in angiogenesis in various physiologic and pathologic conditions. It was then shown that freshly isolated human cord blood $CD34^+$ cells injected into ischemic muscles gave rise not only to endothelial but also to skeletal muscle cells in mice. Cells with myogenic potential within the vascular endothelium of human adult skeletal muscle have been identified. These human myoendothelial cells, which represented less than 0.5% of the cells in dissociated adult skeletal muscles, express both myogenic and endothelial cell markers ($CD56^+CD34^+CD144^+CD45^-$), exhibit long term proliferation, have a normal karyotype, and when transplanted into *scid* mice are able to regenerate fibers in injured muscle.

Human, multipotent, adipose-derived stem (hMADS) cells, isolated from adipose tissue, differentiate into adipocytes, osteoblasts, and myoblasts. Recently, the myogenic and muscle repair capacities of hMADS have been enhanced by transient expression of MyoD. The easy availability of their tissue source, their strong capacity for expansion *ex vivo*, their multipotent differentiation, and their immune-privileged behavior suggest that hMADS cells could be an important tool for cell-mediated therapy for skeletal muscle disorders.

Recently Sassoon and colleagues identified a population of interstitial stem cells that express the cell stress mediator PW1 but not other markers such as Pax7. These cells, named PICs, efficiently contribute to skeletal muscle regeneration and generate both satellite cells and PICs. This new cell population seems to be upstream of satellite cells and holds promise for cell therapy, but its potential has not yet been tested in dystrophic animals. Finally, another subpopulation of fibro/adipogenic progenitors resident in muscle was recently described: they generate ectopic white fat in pathological but not in healthy muscle; they proliferate efficiently in response to damage and, while not generating myofibers themselves, they enhance the rate of differentiation of primary myogenic progenitors. Based on these features, these cells may provide a source of differentiation factors, rather than be primary candidates for cell therapy.

36.3.2.2 Mesoangioblasts

MABs are vessel-associated progenitors that express early endothelial markers when isolated from the embryo and pericyte markers when isolated from postnatal tissues. Since MABs are able to cross the vessel wall and are easily transduced with lentiviral vectors, they have been used in preclinical models of cell therapy for muscular dystrophy. Intra-arterial delivery of either wild-type or genetically corrected MABs morphologically and functionally ameliorated the dystrophic phenotype of mice lacking α-sarcoglycan (Sgca), which model limb girdle muscular dystrophy 2D caused by mutations in the *SGCA* gene, in mice lacking dysferlin (a model for LGMD2B), and also in mdx mice. In addition, intra-arterial delivery of wild-type postnatal canine MABs resulted in extensive recovery of dystrophin expression and ameliorated pathologic muscle morphology and function in Golden Retriever dogs which model DMD. Similar cells have been isolated from human postnatal skeletal muscle and shown to represent a subset of pericytes and to give rise to dystrophin-positive muscle fibers when transplanted into *scid/mdx* mice. Moreover, evidence was recently obtained, through cre-lox lineage traces, that pericytes from skeletal muscle contribute to skeletal muscle fiber growth and enter the satellite cell pool, during unperturbed postnatal development of normal mice. Based on these studies, a phase 1/2 clinical trial based upon four consecutive intra-arterial infusions, at escalating doses, of HLA-matched donor-derived mesoangioblast stem cells, started at San Raffaele Hospital in March 2011 (EudraCT no. 2011–000176–33) and results are expected during 2012. While safety will be the primary objective of the study, a possible modification in patients' force of contraction will be measured following mesoangioblast infusions. Given the small number of patients treated (three in 2011 and three in 2012) and the absence of controls (due to the need for immune suppression which would render unethical the inclusion of untreated controls), we have been following the six patients and other 22 DMD patients of similar age for 18 months preceding the start of the trial, with periodic measurements

of their motility and force of contraction. In this way we will be able to compare the changes in force with the previous history of each patient and analyze the possible statistical significance of the variations detected.

36.4 PLURIPOTENT STEM CELLS FOR FUTURE CELL-BASED THERAPIES

Pluripotent stem cells (PSCs) give rise to all cell types of the human body. Specifically, we limit our discussion to embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, as they are, in practice, the two types of PSC most commonly used to direct differentiation towards a given cell type. In particular, iPS cells raise tremendous hopes for the autologous cell therapy of degenerative diseases, offering the possibility to derive patient-specific cells.

Approximately twenty years ago it was described how ES-cell-derived embryoid bodies (tridimensional structures formed when ES cells are grown in suspension) contained multinucleated muscle fibers which express skeletal muscle genes. Ten years later it was then documented that injection of mouse EBs co-cultured with muscle-derived progenitors in *mdx* mice led to the production of clusters of donor-derived dystrophin-positive fibers. Recent articles now describe the generation of satellite-like cells from mouse ES and iPS cells. These cells regenerate injured and dystrophic muscle and can be also secondarily transplanted, although there are no data concerning functional amelioration upon transplantation.

Myogenic conversion and lineage-specific reprogramming of ESC-derived cells is another intriguing approach. Indeed, mouse ESCs have been converted by the transcription factor MyoD. Recently, researchers achieved *in vivo* skeletal muscle differentiation of purified PDGFRα^{+}/Flk1^{-} progenitors derived from EBs obtained from mouse ES and iPS cells containing an inducible Pax3/7 gene. Moreover, reprogramming of MABs to iPS cells and their differentiation with a similar strategy based upon Pax3/7 expression yielded cells with a stronger myogenic commitment than fibroblast-derived iPS cells, indicating that they own a durable epigenetic memory and paving the way for tailored cell protocols for muscular dystrophies.

As expected, differentiation of human PSCs towards the myogenic lineage is even more complex. A protocol for the systematic derivation of transplantable myoblasts from human ESCs showed the engraftment of these cells in *scid/beige* mice without evidence of clear *in vivo* myogenic differentiation, so their regenerative potential was not comparable to standard embryonic or adult myoblasts. Recently, a combination of HAC and iPSC technologies allowed the generation of genetically corrected human DMD iPSCs by using the DYS-HAC; this will certainly open new scenarios for gene and cell therapy of MDs.

With a strategy similar to that utilized to derive myoblasts from PSCs, it should be possible to derive vessel-associated progenitors that could be delivered through the circulation. Since an increasing number of reports show the contribution of pericytes to skeletal muscle regeneration, it will be interesting to derive these cells from ES/iPS cells, after genetic correction, if needed. Indeed we have recently demonstrated that a similar strategy can be utilized to generate, genetically correct and restore pericyte/MAB-like cells from limb girdle muscular dystrophy; on a parallel route, derivation of vasculogenic pericytes from human ES/iPS cells was shown to ameliorate limb ischemia. Other reports showed the derivation of PDGFRα^{+} mesoderm progenitors from mouse ESCs which, after *in vivo* transplantation, express markers of satellite cells and contribute to muscle regeneration. Unfortunately, these cells were not transplanted in dystrophic animal models. Still other reports described the derivation of mesenchymal precursors from human ESCs; although the multipotentiality of these cells was analyzed only *in vitro*, they were shown to differentiate into different mesoderm lineages, including skeletal muscle.

Despite the promise of these novel strategies for treating degenerative muscle conditions, a number of safety and efficacy issues, some common to other cells (immunogenicity, survival, and differentiation), some specific of ES/iPS cells (such as tumor formation), still need to be solved. The use of standardized protocols for generating iPS cells, together with stringent cytofluorimetric characterization/purification and tumorigenic assays for the derived cell types, will certainly be a fundamental step toward their clinical application.

36.5 FUTURE PERSPECTIVES

As in all other cases of cell therapy, the use of donor cells does not require genetic correction but immune suppression, while the opposite is true, at least in theory, for autologous, genetically corrected cells. In practice an immune reaction may occur against the viral vector and the therapeutic gene, since at least part of its protein product has never been seen by the immune system of the patient. In both autologous or heterologous cell therapy, in order to have reasonable chances of producing significant benefit, cells should be:

1. Derived from an accessible source (e.g., blood, bone marrow, fat aspirate, muscle, or skin biopsy);
2. Isolable from an heterogeneous population on the basis of antigen expression;
3. Able to proliferate *in vitro* for extended periods without loss of differentiation potency;

4. Susceptible to *in vitro* transduction with vectors encoding therapeutic genes (these vectors should themselves meet criteria of efficiency, safety, and long term expression);
5. Able to reach the sites of muscle degeneration-regeneration through a systemic route and in response to cytokines released by dystrophic muscle;
6. Able to differentiate *in situ* into new muscle fibers with high efficiency and to produce physiologically normal muscle cells;
7. Able to escape surveillance by the immune system despite the presence of a new protein (the product of the therapeutic gene) and possibly of some residual antigen from the viral vector.

Unfortunately we are not yet close to satisfying all of these criteria. Nevertheless, in the last years, considerable progress has been made in the identification and characterization of novel classes of stem cells, some of which have been shown to be able to repair, to variable extent, dystrophic muscle in rodents and also in large-animal models. Several trials have started with stem cells for muscular dystrophy and results will become available in the next years. Then we will be in the position to decide whether the direction towards a cure is correct, even though there will be certainly still many years ahead to optimize the current protocols that use donor cells and eventually start trials with genetically corrected, patient's own cells.

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FOR FURTHER STUDY

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CHAPTER 37

Cell Therapy for Liver Disease
From Hepatocytes to Stem Cells

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37.1 INTRODUCTION

Until recently, the only option for treating chronic liver disease or metabolic defects in liver function has been whole organ transplantation. Recently, hepatocyte transplantation has been performed. Although still an experimental therapy, there are some potential advantages for a cell therapy approach to treat liver disease. Some of the advantages of, and problems with, the current treatments for liver disease are listed in Box 37.1.

Despite the unquestioned success of this technique, orthotopic liver transplantation (OLT) requires major surgery and has a significantly long recovery period. The financial costs associated with OLT and subsequent lifelong immunosuppression are considerable. There is a high incidence of complications from the surgical procedure and the concomitant immunosuppression that is required following the organ transplant. Complications can range from simple infections to renal failure, hyperlipidemia, and an increased incidence of skin and other types of cancers following long-term immunosuppression. As with all other organs, the number of liver donors does not nearly equal the number of patients on the waiting list. Patients may wait two or more years for a liver transplant, and there is a death rate of greater than 10% per year of patients on the waiting list. Timing is critical for whole organ transplant. An ABO-compatible liver donor must be available when a patient requires the transplant. Some of the limitations associated with whole organ transplants are addressed with hepatocyte transplants (Box 37.1). Hepatocyte transplants do not require major surgical procedures as they are performed by infusion of cells into the blood supply to an organ such as the liver or spleen. Thus, hepatocyte transplants are less invasive and less costly procedures. Because major surgery is not required, there are fewer complications associated with the procedure.

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BOX 37.1 CURRENT TREATMENTS FOR LIVER DISEASE

Orthotopic liver transplantation

- Major and expensive surgery
- Extensive recovery period
- High incidence of complications
- Expensive maintenance therapy
- Shortage of donor organs
- Timing is critical

Hepatocyte transplantation

- Less invasive and less costly procedure
- Complications fewer and less severe
- Timing of procedure is easier
- Alternative cell sources
- Patient retains native liver
- Graft loss is not necessarily lethal
- Option remains for whole organ transplant

Since cell infusions are minor procedures, there is essentially no recovery period needed. If patients were healthy prior to the procedures, for example a stable metabolic disease patient, they would likely feel no adverse effects from the procedure other than from the placement of a catheter. Hepatocytes can be banked and cryopreserved, so, theoretically, cells could be available at any time for a patient transplant. The timing of a hepatocyte transplant depends on the status of the patient rather than on the availability of a suitable organ. Currently, the source of hepatocytes for hepatocyte transplants is mainly discarded organs not suitable for whole organ transplant. At present, there are not enough hepatocytes to transplant all recipients who would likely benefit from the procedure. However, some inventive new ideas have been proposed, such as the use of segment IV, which can be made available from a split-liver procedure to make more hepatocytes available for transplants. Alternative sources of hepatocytes could also be available in the future. Although many options have been discussed, the most prominent sources are xenotransplants from pigs or other species, immortalized hepatocytes, and most recently stem-cell-derived hepatocytes. Future developments in these areas may make the number of cells available for hepatocyte transplants virtually unlimited.

A significant benefit of hepatocyte transplantation is that the patient retains their native liver. In cases of cell transplants for metabolic disease, the patient's native liver still performs all of the liver functions with the exception of the function that initiates the disease. Patients with ornithine transcarbamylase deficiency (OTC) have a mutation in an enzyme involved in the urea cycle that prevents the metabolism and elimination of ammonia. Although the native liver is not proficient in ammonia metabolism, it is still capable of performing other liver functions; including the secretion of clotting factors, albumin, drug metabolism, and all other metabolic and synthetic processes. A cell transplant need only support the ammonia metabolism for the patient, and will not be required to provide complete liver support. Because all liver functions are not dependent on donor cells, loss of the cell graft or failure

of the cells to function properly will not necessarily be life threatening, especially for a stable metabolic disease patient. Finally, a whole organ transplant always remains as an option for the cell transplant patient. Even if the cell transplant fails to function or is rejected, nothing done as part of the cell transplant procedure would likely interfere with a subsequent whole organ transplant. Prior hepatocyte transplantation does not sensitize the cell transplant recipient to either the donor cells or to an eventual liver graft. Thus, despite sometimes transplanting hepatocytes directly into an immunological response organ, the spleen, no immunological reactions are initiated that are deleterious to the cell transplant or an eventual whole organ transplant.

There are potential disadvantages of hepatocyte transplants as well. First, there are no reports of long-term complete corrections of metabolic liver disease in patients following cell transplantation alone. Because it is a new field, much additional experimentation will be required to determine the full efficacy of cell therapy of liver disease and the length of time for which the cell graft will function. Also, like whole organ transplants, it is believed that cell transplant recipients will require the administration of immunosuppressive drugs. It is likely that lower doses of the drugs will be needed to prevent rejection of cell transplants than are required for whole organ transplants. Because of this, fewer and less severe side-effects from immunosuppressive drugs would be expected, but definitive studies are lacking.

37.2 BACKGROUND STUDIES

37.2.1 Choice of Sites for Hepatocyte Transplantation

Hepatocyte transplants have been conducted for over 20 years. The large numbers of preclinical studies conducted on hepatocyte transplants have firmly established that the transplants are safe and effective. The most common sites for the transplantation of hepatocytes are the spleen and the liver; however, transplants to the peritoneal cavity, stomach, or omentum have been reported. Long-term survival of the cells is readily measured following transplants into the spleen or liver. Most cells transplanted into the peritoneal cavity – intraperitoneal (IP) – are rapidly lost. Following IP transplants, only those cells that nidate near blood vessels and can attract sufficient nutrition survive long-term. Despite the ease of the procedure, IP transplants of hepatocytes have only limited efficacy. Transplants of hepatocytes to the spleen or the liver have been shown to function for the lifetime of the recipient. Hepatocytes survive for long periods of time in the recipient and over time the spleen of an animal can be 'hepatized' to the extent that 80% of the mass of the organ can be replaced with hepatocytes.

The concept of establishing ectopic liver function in the spleen is similar in theory to the bioartificial liver (BAL). In the BAL, the hepatocytes are seeded into and maintained in some form of an extracorporal device. The patient's

blood or plasma is pumped to the device, where it interacts with the hepatocytes across membrane barriers and is then returned to the patient by a second series of pumps. There are reports that BAL can provide short-term synthetic and metabolic support. The ease of transplant of hepatocytes and the abundance of the patient's own natural basement membrane components coupled with the naturally high blood flow make the spleen a useful site for the establishment of short- or long-term ectopic liver function. It is likely that hepatocyte transplants will be easier, cheaper, and more efficient, and will provide the same, or better, level of support as extracorporal devices.

For transplants into the liver, the preferred route for administration of cells is via the portal vein. Cells are infused into the blood supply that feeds the liver, and the hepatocytes are distributed to the different lobes in proportion to the blood flow they receive from the portal vein. Portal vein injections are difficult in small animals, so an alternative method is used in these studies: hepatocytes are injected directly into the splenic pulp. The proportion of the cells that remains in the spleen is determined by the extent to which the outflow through splenic veins is impeded. In studies where the spleen was 'hepatized', the splenic outflow was briefly occluded, which helped retain the cells in the spleen. Alternatively, when the spleen is used as a method to affect a portal vein injection, the splenic veins are left open. It was reported that up to 52% of the cells injected into the spleen traverse to the liver via the splenic and portal veins within a few minutes.

37.3 INTEGRATION OF HEPATOCYTES FOLLOWING TRANSPLANTATION

Integration of hepatocytes into recipient liver is a complex process that requires the interaction of donor and native hepatocytes to form an integrated tissue. The process may be considered in four steps (Box 37.2). Although they are presented as separate, there is considerable overlap of the steps in both time and space. Following infusion into the portal vein, hepatocytes must traverse the endothelium to escape the vascular system. Although the liver has fenestrated

BOX 37.2 INTEGRATION OF DONOR HEPATOCYTES INTO NATIVE LIVER FOLLOWING TRANSPLANTATION

- Filling vascular spaces with donor cells
- Disruption of the sinusoidal endothelium
- Donor cell integration in host parenchyma
- Remodeling of liver via modulation of extracellular matrix

endothelium, under normal conditions the pores, which are in the range of 150 nm, are far too small to provide a simple transit of parenchymal hepatocytes, which range in size from 20 to 50 μm. Infusions of hepatocytes quickly fill the portal veins and embolize secondary and tertiary portal radicals. Portal pressures increase as flow is restricted by hepatocyte plugs in the portal veins. Venograms that were normal prior to cell transplantation become markedly attenuated and show greater filling of vessels proximal to the portal vein, including the mesenteric and splenic vein. If the number of hepatocytes transplanted is in the range of 5% of the total number of hepatocytes in the native liver, the portal hypertension is transient and resolves within minutes to hours.

A proportion of transplanted cells begins to fill sinusoidal spaces and the space of Disse as the endothelium in the region of the transplanted cells begins to degenerate. It is likely that both physical and humoral (growth factors, cytokines) factors are involved in this process. Microscopic analysis of tissue sections reveals that endothelium is breached in many places and donor hepatocytes leave the portal veins in regions where endothelium is incomplete and broken. Reports suggest that most of the hepatocytes that eventually integrate into the recipient liver will have traversed the endothelial barrier by 24 h post-transplant. Cells that remain in the portal vessels are eventually removed by macrophages between 16 and 24 h post-transplant. Other reports suggest that cells may continue to integrate into the parenchyma for 2–3 days following transplantation. Transient hypoxia in the region of the occluded vessels leads to changes in the endothelium as well as both recipient and donor hepatocytes. Endothelium and donor and native hepatocytes all express vascular endothelial growth factor (VEGF) in the areas of hepatocyte integration, a factor known to be induced by hypoxia. It is interesting that VEGF was previously known as vascular permeability factor (VPF). Expression and secretion of VEGF/VPF, a potent angiogenesis factor, is thought to contribute to the reformation of new sinusoids and restoration of the endothelial barrier following cell transplantation.

Passage through the endothelial barrier allows donor hepatocytes to become integrated into recipient parenchyma. Full integration of donor hepatocytes and restoration of full hepatic function is difficult to ascertain. However, careful studies of the expression of antigens and activities localized to specific membrane fractions clearly demonstrate that donor hepatocytes fully integrate into the hepatic plate of native liver, and for hybrid structures between native and donor cells, within 3–5 days following transplantation. The antibody to CD26 recognizes the dipeptidylpeptidase IV (DPPIV) antigen, which is localized to the basolateral membrane of hepatocytes. Antibodies to connexin 32 can be used to visualize gap junctions between adjacent hepatocytes. Likewise, canicular ATPase activity can be used to identify bile cannicular regions between

adjacent hepatocytes. The proper localization of these different antigens and activities requires that the hepatocyte be fully integrated into the hepatic plate and polarized. By 3–7 days post-transplant, hybrid structures could be visualized in recipient liver containing both donor (DPPIV) hepatocytes and recipient ATPase activity or donor DPPIV colocalized with connexin 32. Both studies clearly demonstrate proper integration of donor hepatocytes as well as the re-establishment of intracellular communication (connexin 32) between donor and recipient hepatocytes. Hybrid structures between donor and recipient hepatocytes were shown to be functional by the transport and excretion of a fluorescent conjugated bile acid. Hepatic transport of indocyanine and sulfobromothalein into the bile following hepatocyte transplantation was also reported. Hepatocyte transplants were conducted on Eizai-hyperbilirubinemic rats. These animals have a defect in multidrug resistance protein2 (MRP2), which prevents the normal transport of bile acid conjugates and their excretion into bile. This is a relevant animal model of metabolic disease, as the condition is similar to Dubin-Johnson syndrome in humans. The correction of this transport defect by hepatocyte transplantation is definitive proof of the complete functional integration of donor hepatocytes into recipient liver.

As part of the integration process, there is significant remodeling of the hepatic parenchyma. Matrix metaloprotease-2 (MMP-2) are activated and released in the immediate area of donor cells. It is not clear whether the proteases are produced by the donor or recipients cells or even which cell type is the source of the protease, but the degradation of extracellular matrix components helps to create space for the donor cells. Expression of MMP-2 was detected in and surrounding foci of proliferating donor hepatocytes two months following cell transplantation. Increased production and release of MMP-2 were also observed at the growth edge of nodules of fetal rat hepatocytes proliferating in adult liver following transplantation. While not all of the components of the process are completely understood, it is clear that hepatocytes can be transplanted into the vascular supply of the liver, breach the endothelial barrier, remodel and integrate into hepatic parenchyma, and establish communication with adjacent cells and the biliary tree all within 3–5 days in a process of remodeling that completely retains normal host hepatic architecture.

37.4 CLINICAL HEPATOCYTE TRANSPLANTATION

Hepatocyte transplantation has been employed in clinics in three types of procedures (Box 37.3). Cell transplants have been used to provide short-term liver support to patients who were dying of their disease before a suitable organ could be found. As these patients are already listed for a whole organ transplant, the hepatocyte infusion used is sometimes referred to as a 'bridge' to transplant. A second use for hepatocyte transplants grew out of the attempts

BOX 37.3 OPPORTUNITIES FOR HEPATOCYTE TRANSPLANTATION

- 'Bridge' for patients to whole organ transplantation
- Cell support for acute liver failure
- 'Cell therapy' for metabolic disease

to bridge people to OLT. It was discovered that some patients recovered completely following hepatocyte transplant and no longer required whole organ transplant. The third general use for hepatocyte transplants is for the correction of metabolic liver disease. Each technique will be discussed separately.

37.5 HEPATOCYTE BRIDGE

With the bridge technique, hepatocytes are provided to a patient in acute liver failure or experiencing acute decompensation following chronic liver disease. The majority of these patients are already listed for OLT, and they are in danger of dying before a suitable organ can be found. Hepatocyte transplants have been conducted on these patients in an effort to keep them alive long enough to receive OLT. The primary goal of the bridge transplant is not to substitute for whole organ transplant, but rather to support and sustain the patient until an organ becomes available. Preclinical studies with several different models of acute or chronic liver failure have demonstrated that hepatocyte transplantation can support liver function and improve survival. The results with human hepatocyte transplantation in the clinics also show an increase in the survival of patients following hepatocyte transplantation. There is a 65% survival rate for patients receiving hepatocyte transplants. Although randomized control studies could not be conducted, the preliminary results with approximately 25 patients indicate a survival advantage to those patients receiving cell transplants. In addition to increased survival, there are consistent reports that clinical parameters such as ammonia levels, intracranial pressures, and cerebral blood flow are improved following hepatocyte transplantation. These results indicate that desperately ill patients who receive hepatocyte transplants are more likely to survive long enough to receive OLT than are the nontransplant controls.

Most of the patients who would be candidates for the hepatocyte bridge technique suffer from chronic liver disease and have advanced cirrhosis. Because of the cirrhotic changes in the liver and the accompanying portal hypertension, hepatocytes were not transplanted into the liver (portal vein) in most of the clinical studies. Preclinical studies were conducted where cirrhosis was induced in rats by the administration of phenobarbital and carbon tetrachloride. When hepatocytes were subsequently transplanted into animals

with increased portal pressures and cirrhosis, there was significantly greater intrapulmonary translocation of donor cells, presumably because of portosystemic shunting. These results suggest that serious complications could arise if portal infusion of hepatocytes were conducted on cirrhotic patients with portal hypertension. Indeed, shunting of transplanted hepatocytes to pulmonary vascular beds has been reported in one clinical study. To avoid this possible complication, Fisher and colleagues recommend that hepatocytes be transplanted into the spleen in cirrhotic patients via the splenic artery. Despite the obvious success of the splenic artery route for hepatocyte transplantation, a report suggests that transplantation of hepatocytes by direct splenic puncture results in superior engraftment and fewer serious complications, although long-term engraftment was not studied. Although the method for splenic delivery of cells may not be settled, it is clear that, in cases where physical and/or anatomic abnormalities are present in the native liver, the preferred route for hepatocyte transplantation is to an ectopic site, the spleen.

The promising results reported to date suggest that hepatocyte transplantation is beneficial to patients suffering from severe hepatic insufficiency while awaiting OLT. A logical extension of these results might be for the use of hepatocyte transplants earlier in the process. Rather than wait until the patient is near death and with no immediate prospect for a whole organ transplant, a more preemptive approach might be warranted. Hepatocyte transplants could be performed when patients awaiting OLT become unstable. This would presumably stabilize the patient and avoid or at least delay more serious complications of liver failure. Early intervention might avoid more costly hospitalization and other treatments.

37.6 HEPATOCYTE TRANSPLANTATION IN ACUTE LIVER FAILURE

As described above, hepatocyte transplants have been used as a bridge to OLT. Most of the patients who have been referred for bridge transplants suffered from chronic liver disease and had cirrhotic changes in liver architecture. There is a subgroup of patients referred for OLT who experience acute liver failure. In these patients there is massive loss of hepatocytes over a short period of time, leading to hepatic insufficiency. Except for the dramatic loss of hepatocytes, there is no longstanding pathological change in liver architecture. Since the liver has the capacity for robust regeneration following loss of liver mass, there is considerable interest in trying to correct acute liver failure with hepatocyte transplantation. The hypothesis is similar to the bridge technique, where hepatocyte transplantation is used to provide support at a time of critical and otherwise lethal liver failure. The expectation is that, if the patient survives the acute loss of tissue mass, their native liver will regenerate. If the native

liver regenerates, there will no longer be a need for OLT. An exogenous source of hepatocytes by transplantation would provide support of liver function to prevent lethal hepatic failure. Both donor and native hepatocytes would be expected to participate in the regeneration response. Once the native liver has been fully restored, there might not be a need for donor-derived hepatocytes. If the chimeric liver generated following the transplant is composed predominantly of native hepatocytes, the patient could be safely removed from immunosuppressive therapy. In this manner, the patient receives what amounts to a temporary liver cell transplant. If cell therapy is sufficient, the patient will be spared whole organ transplantation and lifelong immunosuppression. Several preclinical studies support the hypothesis that hepatocyte transplantation can provide sufficient liver function to maintain an animal experiencing acute liver failure. Studies have shown that hepatocyte transplants dramatically improve survival of animals with acute liver failure induced by D-galactosamine.

There are now reports of reversal of acute liver failure in four patients following hepatocyte transplantation. The causes of acute liver failure were hepatitis B-induced liver failure, acetaminophen intoxication, liver toxicity following consumption of poisonous mushrooms, and liver failure of unknown etiology in a pediatric patient. In each case, the patients presented with classic symptoms of acute liver failure, and most were immediately listed for OLT. The number of cells transplanted varied between different procedures but were in the range of approximately 1–5 billion total viable cells. In all cases, cells were transplanted into the portal vein to get a direct transplant into the liver. In general, patients were given fresh frozen plasma prior to placement of the catheter to prevent bleeding. The results are typical of the response to hepatocyte transplantation. There is usually a rapid fall in ammonia levels following the transplant. Circulating levels of clotting factors stabilize following the transplant and then slowly increase over the next two weeks. Fisher and colleagues report that Factor VII levels were 1% of normal prior to transplant and increased to 25% by day 7 and 64% of normal by week 2 after cell transplant. The recovery of the clotting factors is usually rapid enough that, following the cell transplant, no additional fresh frozen plasma is required.

Patients are generally discharged within 2–4 weeks and are judged to experience a complete recovery. The cell transplant recipients ranged in age from 3 to 64 years, indicating that even older patients have sufficient regenerative capacity to be supported by hepatocyte transplantation.

As is observed with donor tissue allografts, hepatocyte allografts produce and secrete human leukocyte antigen-I (sHLA-I) immediately upon implantation. If there is a mismatch between the donor and recipient, the donor-specific sHLA-I can be detected in the circulation and quantified by enzyme-linked immunosorbent assay (ELISA). Donor-specific HLA class I alleles can be

identified and quantified by polymerase chain reaction (PCR) analysis of tissue samples taken at biopsy. When it is determined that the preponderance of cells in the patient's liver are native, the patient can slowly be removed from immunosuppressive therapy. In the cases described to date, the patients recovered completely from liver failure following hepatocyte transplantation without serious adverse consequences and without whole organ transplant and lifelong immunosuppression. Although the numbers of patients are small, the treatment of acute liver failure by hepatocyte transplant has some significant advantages that make further investigation of this novel therapy appropriate.

37.7 HEPATOCYTE TRANSPLANTATION FOR METABOLIC LIVER DISEASE

A common indication for whole organ transplantation in pediatric patients is metabolic liver disease. In these cases, there is usually a genetic defect in an enzyme or protein that is produced in the liver that inactivates a critical liver function. Although all other liver functions are generally normal, the liver is removed and replaced with a liver that can perform the missing function. Because there is usually only one genetic defect associated with each metabolic liver disease, a gene therapy approach to correct the defect would seem appropriate. Unfortunately, gene therapy has met with considerable problems, which have prevented successful use of this experimental technique. Hepatocyte transplantation has been used in attempts to correct the metabolic defects associated with several types of metabolic liver disease (Box 37.4).

In an approach similar to gene therapy, with hepatocyte transplants one tries to seed the patient's liver with cells that are proficient in the enzyme or function missing in the native liver. The goal is to repopulate the liver of the transplant recipient with sufficient numbers of hepatocytes to provide the missing liver function by donor cells.

Large numbers of hepatocytes cannot be infused into the portal system because of the problems with embolism of the portal veins and portal hypertension.

BOX 37.4 CLINICAL TRANSPLANTS FOR METABOLIC LIVER DISEASE

- Familial hypercholesterolemia
- Crigler-Najjar
- Ornithine transcarbamylase deficiency
- Arginosuccinate lyase deficiency
- Citrullinemia
- Factor VII deficiency
- Glycogen storage disease, Type 1a and 1b
- Infantile Refsum disease
- Progressive familial deficiency
- Alpha-1 antitrypsin deficiency
- Carbamoylphosphate synthase deficiency
- Phenylketonuria

Generally, we infuse approximately 2×10^8 cells/kg body weight of the recipient. Infusions of these cell numbers have not resulted in any long-term complications. There is always a transient increase in portal pressures that resolves within hours. While quite experimental, this number was arrived at by an extrapolation from preclinical studies with nonhuman primates. Infusion of $1–2 \times 10^8$ cells/kg into baboons who had previously received a left or right lobectomy was accomplished without serious complications and with only transient increases in portal pressures. Because only a few percent of liver mass can be transplanted at any one time, single hepatocyte transplants cannot be expected to replace a large proportion of liver with donor cells. For this reason, the metabolic diseases that are candidates for cell transplants are those in which the restoration of 10% or less of total liver function or activity is likely to correct the disease. The liver has highly redundant functions. Thus, it is recognized that 10% of a normal amount of gene product or enzyme activity would likely correct the symptoms of most metabolic liver diseases. Exceptions exist, such as hypercholesterolemia, where more than 50% replacement of liver with donor cells would likely be needed to correct circulating low-density lipoprotein levels. However, for most metabolic liver diseases and all of those listed in Box 37.4, it is believed that the replacement of the liver with 10% donor hepatocytes would either be completely corrective or at least ameliorate most of the symptoms of the disease.

In general, hepatocyte transplants work best when the donor cells have a selective growth advantage. There are a number of animal models of liver disease in which the native hepatocytes show an increased death rate as compared with normal liver. In these situations, when cells without the defect are transplanted into the diseased liver, the donor cells have a strong and selective growth advantage over the native hepatocytes. Over time, the liver may become nearly completely replaced with donor cells. In certain human diseases, there might be sufficient selective pressure to strongly favor the replacement of large parts of the liver with donor cells. Such diseases include tyrosinemia Type 1, Wilson's disease, progressive familial intrahepatic cholestasis (PFIC), and alpha-1 antitrypsin deficiency (A1AT). In these diseases, integration of only a small proportion of liver mass by hepatocyte transplantation would likely be necessary because the donor cells would be expected to continue to proliferate in the host liver, and over time replace the diseased cells. Although there are clear examples of this in studies of transplants of laboratory animals, there are no studies with human patients showing comparable results.

Most metabolic diseases such as Crigler-Najjar (CN), OTC deficiency, and all of those diseases listed in Box 37.4 would not be expected to show such selective growth pressure for donor cells. For diseases such as these, multiple transplants over time will be required to populate the liver with 10% donor cells.

A large number of studies with different animal models have shown the efficacy of hepatocyte transplantation to correct metabolic liver disease. Metabolic defects in bilirubin metabolism, albumin secretion, ascorbic acid production, tyrosinemia Type 1, copper excretion, PFIC, as well as other defects in biliary transport similar to Dubin-Johnson syndrome in humans have been shown to be amenable to correction by hepatocyte transplantation. These encouraging results suggested that similar defects in human patients could be corrected by hepatocyte transplantation. The diseases listed in Box 37.4 have been the focus of human trials of hepatocyte transplants.

Hepatocyte transplants were previously shown to result in a rapid correction of ammonia levels. For this reason, urea cycle defects that result in life-threatening hyperammonemia were the first metabolic disease target for hepatocyte transplants. In the initial study, 1 billion viable cells were transplanted into the portal vein of a five-year-old recipient. Portal pressures increased from 11 cm of water prior to cell transplant to 19 cm immediately following the cell infusion, but recovered rapidly. The patient's ammonia levels normalized without medical intervention within 48 hrs of cell infusion, and his glutamine levels returned to normal. Although OTC activity was undetectable prior to cell transplant, measurable OTC activity was detected in a biopsy performed at 28 days. In these studies, 10% of the cells were labeled with indium^{111} prior to infusion into the patient to monitor distribution of the cells. Quantitative analysis of the scintigraphic images showed an average distribution ratio of liver:spleen of 9.5:1. Measurements made prior to cell infusion indicated that free indium was released from hepatocytes at a rate of 10% per hour, and free indium is rapidly cleared from circulation by reticuloendothelial systems such as the spleen. Thus, most of the tracer in the spleen following cell infusion was thought to be free indium, not hepatocytes. Pulmonary radiotracer uptake was consistent with background counts, indicating the absence of portosystemic shunting despite the modest increase in portal pressures observed at the time of transplant. This first transplant for metabolic liver disease indicated that hepatocyte transplantation into the portal vein could be conducted safely in patients with no significant liver pathology, with only a moderate and reversible increase in portal pressures. From the rapid normalization of ammonia levels following hepatocyte transplant, it was concluded that cell transplantation can partially correct the hyperammonemia associated with the disease. Subsequent studies have verified that partial corrections of ammonia levels are possible by cell transplants alone. While complete corrections of OTC deficiency have not been accomplished, these studies indicate that cell transplants provide much-needed metabolic control of ammonia levels. Even in the absence of complete correction, liver cell transplantation should be considered as a bridge to whole organ transplantation for OTC patients to prevent the neurological problems associated with uncontrolled hyperammonemia.

A number of groups have attempted to correct CN syndrome, Type 1 with hepatocyte transplants. The first case was in many ways typical of the results obtained by other groups and will be discussed in greater detail. This disease is caused by a defect in the enzyme that is responsible for the conjugation and eventual excretion of bilirubin. The absence of the enzyme results in severe hyperbilirubinemia, which can lead to central nervous system (CNS) toxicity, including kernicterus. Following the transplantation of approximately 7.5 billion cells into the liver of a 10-year-old female, there was a slow and continuous decrease in circulating bilirubin levels over the first 30–40 days, and bilirubin conjugates were readily detected in the bile. Overall, there was approximately a 60–65% decrease in bilirubin levels compared with pretransplant levels. Because the bilirubin conjugates could only be produced by the donor cells, their detection in the bile demonstrated the robust biochemical function of the transplanted cells and established that donor hepatocytes integrated into the hepatic parenchyma and quickly established connections with the recipient's biliary tree.

Several important findings were gained from this transplant. First, large numbers of hepatocytes could be safely transplanted into the portal vein without complication. Although the total numbers of hepatocytes in liver are difficult to assess, a transplant of 7.5 billion cells represents an estimated 3.5–7.5% of the liver mass, which was transplanted without complication over approximately a 15 hr period. Second, the apparent engraftment and function of hepatocytes in the clinical trials seems to exceed that found in previous animal studies. The transplantation of 3.5–7.5% of liver mass resulted in the restoration of approximately 5% of a normal amount of bilirubin conjugation capacity in the liver. Third, a long-term correction in bilirubin levels was observed. This patient was followed for more than 1.5 years. Fourth, single transplants of hepatocytes are effective in creating partial corrections of the disease, but, given the limitation of transplanting 2×10^8 cells/kg body weight, one cannot transplant sufficient numbers of hepatocytes to achieve a complete correction of metabolic liver disease with one transplant. It is estimated that complete corrections would require 2–4 transplants if each were as successful and efficient as the first. Finally, this was the first unequivocal demonstration of the long-term success of hepatocyte transplantation. Although patients were bridged to transplant and clinical parameters such as ammonia levels rapidly changed following transplantation, many of the previous patients underwent subsequent OLT and the long-term metabolic function of the transplanted cells was difficult to assess.

These studies firmly established that hepatocyte transplants were an effective means of correcting metabolic liver disease. The results of hepatocyte transplants of other patients with CN largely confirm those seen with the first patient.

Partial correction of glycogen storage disease, Type 1 following hepatocyte transplantation has been reported. Improvement was documented by the

patient's ability to maintain blood glucose between meals as well as sustained and higher glucose levels with meals. Hepatocyte transplants achieved a partial correction of infantile Refsum disease, an autosomal recessive inborn error in peroxisome metabolism of very long chain fatty acid metabolism, bile acid, and pipecolic acid.

Improvement in fatty acids metabolism, and reductions in circulating pipecolic acid and bile salt levels were observed. An overall improvement in the health of the patient was evidenced by the report of significant increase in muscle strength and weight gain. Hepatocyte transplantation partially corrected a severe deficiency in the production and secretion of coagulation Factor VII. Following cell transplant, exogenous Factor VII requirement was reduced to 20% of that needed prior to the cell transplant.

Complete correction of a 3.5-year-old female patient with neonatal onset arginosuccinate lyase (ASL) deficiency was achieved. Likewise with OTC deficiency, ASL patients are at risk of brain damage from hyperammonemia. The patient received three sequential hepatocyte transplants over a five-month period. Both freshly isolated and previously cryopreserved hepatocytes were used. At one year post-transplant the patient displayed 3% of normal ASL activity in hepatic biopsy samples. Engraftment of donor cells could be demonstrated by fluorescence *in situ* hybridization for Y chromosome. These results confirm that hepatocyte transplantation can achieve sustained engraftment of donor cells and sustained metabolic and clinical control.

37.8 HEPATOCYTE TRANSPLANTATION – NOVEL USES, CHALLENGES, AND FUTURE DIRECTIONS

37.8.1 Hepatocyte Transplants for Non-Organ Transplant Candidates

Most patients who received a hepatocyte transplant were already listed for a whole organ transplant. The need for liver support is not limited to this group. There are large numbers of patients for whom OLT is not an option. Patients in this group could include alcoholic cirrhotic patients who have not met the required abstinence period, patients with acute liver failure resulting from suicide attempts, and cancer patients. Early case reports have suggested that hepatocyte transplants into the spleen could be useful to restore liver function to end-stage cirrhotic patients. Although both of the patients in the reported study eventually died of concomitant renal failure that was left untreated, the patients were sufficiently improved following the cell transplants that they were able to be discharged from the hospital. Fox and coworkers created an animal model to study the efficacy of hepatocyte transplants to support liver function

in cirrhosis in a more controlled setting. Their studies clearly demonstrated that hepatocyte transplants significantly improve liver function and survival of rats experiencing chronic liver failure following repeated injections of carbon tetrachloride. With millions of patients currently infected with hepatitis viruses, there is clearly a need for additional means to support liver function in these patients. Notwithstanding the difficulties of such clinical studies in cirrhotic patients, cell transplantation should be thoroughly evaluated as a possible support therapy.

In addition to cirrhotic patients who may not be candidates for OLT, there are metabolic liver diseases such as phenylketonuria (PKU) that are not currently referred for OLT. Although some still believe that diseases such as PKU can be adequately controlled by diet, there is evidence of continued and progressive mental deterioration in most patients treated with diet alone. It is likely that cell therapy with hepatocytes would improve control of phenylalanine levels in these patients. Severely affected PKU patients and those not controlled well by diet alone should be given serious consideration for inclusion in hepatocyte transplant protocols, as it seems that the benefits would likely outweigh the risks for these individuals.

An important factor preventing the use of hepatocyte transplants in additional medical centers is the limited availability of hepatocytes. The normal source of cells for hepatocyte transplants is livers with greater than 50% steatosis, vascular plaques, or other factors that render the tissue unsuitable for whole organ transplantation. Better utilization of existing liver tissue could increase the numbers of hepatocytes available immediately. In the USA there are no regulations requiring that donor organs be allocated to transplantation research centers for hepatocyte isolation, and relatively few organs go to centers where hepatocyte transplant is a possibility. Most of the organs not used for whole organ transplant are provided to commercial firms where hepatocytes are isolated for resale or for in-house metabolism and toxicology studies. While most uses of donor liver tissue have merit, simple allocation procedures could be instituted to route the organs to transplant centers for initial review and selection of the most suitable cases for cell isolation. Split-liver procedures have made it possible to use caudate lobe and segment IV for hepatocyte isolation. Depending on the surgical procedure, these portions of liver tissue may remain untransplanted and have been shown to be useful for hepatocyte isolation. Although currently quite hypothetical, most livers that are currently transplanted could be split. A portion such as the left lateral segment could be made available for cell isolation while the remaining liver tissue is utilized as a tissue graft. Because hepatocyte transplantation is not currently the standard of care, such proposals are not presently feasible. However, if the efficacy of hepatocyte transplants were firmly established, the risk and the extra time needed for the split procedure would be outweighed by the benefit of the cell transplants.

Cell transplants rather than OLT could free up the organs that are now used for acute liver failure and metabolic disease patients.

37.8.2 Methods to Improve Engraftment and Repopulation

Hepatocyte transplants will not be able to progress past the small numbers of patients currently being transplanted until sufficient numbers of hepatocytes become available or engraftment and repopulation are significantly improved. It has become evident that pretreatment of the native liver of the transplant recipient to induce regeneration and proliferation of donor hepatocytes may be needed prior to hepatocyte transplantation. Most of the pretreatment conditioning regimens used in studies with experimental animals are too hazardous to be applied in a clinical setting. The two most common approaches that can be applied in the clinic that have been suggested are portal embolization and hepatic irradiation. The theories and literature on these techniques are widely available and will not be presented here. There is another technique that is commonly used in experimental animals that has not been given serious consideration as a pretreatment to hepatocyte transplantation. Partial hepatic resection, more commonly called partial hepatectomy (PH), has been considered too risky for clinical application. Due to improved techniques and instruments and increased activities at experienced centers, liver resection and living donor liver transplantation are now common procedures. With today's advanced surgical techniques and in the hands of surgeons with considerable experience with reduced grafts, split livers, and partial liver resection, this surgical procedure would likely be as safe as portal embolization and hepatic irradiation and should also be considered as a possible pretreatment for hepatocyte transplantation. A partial resection to induce liver regeneration would be a much simpler and safer surgical procedure than those routinely performed in the clinic today. Partial liver resections are most commonly performed to remove malignancies or during living donor transplant procedures. When performing a surgical procedure for tumor removal, the amount of tissue and the location of the procedure are dictated by the location of the tumor(s). Likewise, in the case of living donor liver graft removal, the surgeons need to consider preservation of vessels as well as minimize ischemia injury to the graft during surgery.

Removal of liver tissue to induce liver regeneration will be much safer, as the only concern for the surgeon will be safety of the patient. Since the resected liver tissue will not be used as a tissue graft, the surgeon will not need to be concerned about the vessels in the resected tissue. The amount and exact location of the tissue to be removed can be chosen by the surgeon and the procedure performed in the safest, fastest, and easiest way. Although there are risks associated with both the surgery and anesthesia, these risks would likely be much lower than for living donor liver transplantation. As an example, in

a recent report of 100 donor resections from one center, no life-threatening complications occurred. Although for a different reason, hepatectomy prior to hepatocyte transplantation has already been done in a series of patients transplanted for familial hypercholesterolemia in 1992–1994. These patients underwent left lateral hepatectomy to harvest tissue for hepatocyte isolation and subsequent retroviral transduction of the LDL receptor. Transplantation of transduced autologous hepatocytes was performed on day 3 post-operation. The surgical safety of this procedure was thoroughly studied and reported without any major complications. A point that remains unanswered is to what extent hepatectomy will generate a sufficient signal to improve the engraftment or proliferation of transplanted hepatocytes. The timing of the transplantation after hepatectomy might also be important. Efimova et al. measured serum growth factors in healthy individuals after living related liver donation and showed that HGF increased 12-fold at 2 hrs post-operation and thereafter stabilized at a level that was three-fold higher than pre-operation for an additional five days. Other growth factors such as VEGF and EGF did not change significantly, and TGF-alpha was not detected at all. These data suggest that partial hepatic resection could provide a significant stimulus to donor hepatocytes, and that the effect would last at least five days. Taken together, these data suggest that partial hepatic resection could be a safe and effective pretreatment to hepatocyte transplantation.

In addition, the tissue removed as a pretreatment could be used for cell isolation. One could also, at least in theory, envision use of tissue removed from a patient with a metabolic disease for cell isolation and subsequent domino transplantation to a patient with a different metabolic disease.

37.8.3 Stem Cells and Alternative Cell Sources for Liver Therapy

In addition to attempts to improve engraftment and repopulation of the liver, alternative cell sources for hepatocytes have been proposed. Xenotransplants, immortalized human hepatocytes, stem-cell-derived hepatocytes, and fetal hepatocytes have been proposed as alternative sources of cells for clinical transplants. To date, no alternative cell source has been found that meets all of the requirements for safety and efficacy. There is currently great interest in stem-cell-derived hepatocytes and the possibility that they might become a future source of cells for clinical transplantation.

Proponents for the use of stem cells suggest that, because of their wide availability and small size, stem cells could be a feasible and efficient alternative to hepatocytes for cellular therapy. While it is true that stem cells, once generated, should be available in sufficient numbers for transplant protocols, it is not clear that the small size of the stem cells would actually favor engraftment and

repopulation of target organs. It is pleasing to speculate that the relatively low levels of engraftment of mature hepatocytes following transplant is due to their large diameter, and that the 50–90% of transplanted hepatocytes do not engraft because they temporarily obstruct and get trapped in portal veins or hepatic sinusoids. However, obstruction of the portal vessels and hepatic sinusoids and transient increases in portal pressure may actually be a necessary step in engraftment into the liver parenchyma. Smaller, stem-cell-derived hepatocyte-like cells may actually engraft less effectively than mature hepatocytes. When examined in transplant models, smaller hepatocytes or hepatocyte-like cells were usually less effective than larger, or more mature, hepatocytes. Engraftment and proliferation of mouse ES-derived hepatocyte-like cells and mature hepatocytes were examined and compared directly in the $FAH^{-/-}$ mouse. The $FAH^{-/-}$ is a robust model of metabolic liver disease where transplanted donor ($FAH^{+/+}$) hepatocytes are under strong positive growth selection leading to rapid and effective repopulation of the diseased mouse liver. Mouse ES-derived cells with hepatic features were found to engraft less efficiently than mature hepatocytes and showed very limited capacity for repopulation and tissue formation. Of the cell types most often suggested to become a source of cells for clinical transplants, embryonic stem cell (ES) and induced pluripotent stem cell (iPS) may hold the greatest promise for future therapy. However, to move this potential therapy to the clinics, two significant roadblocks would have to be overcome: efficient and effective hepatic differentiation of the stem cells and removal of the tumorigenic potential of the transplanted cells. To date, neither condition has been met. No published protocols are efficient or effective enough to produce large numbers of mature human hepatocytes that could be immediately used for transplants. The problem of tumor formation from cells in the population that did not undergo hepatic differentiation and the possibility that differentiated hepatocyte-like cells could regress to undifferentiated stem cells following transplantation will have to be overcome before either ES or iPS cells could be considered for clinical protocols. Also, as stated above, ES-derived hepatocyte-like cells showed limited capacity for liver tissue formation following transplantation when compared with mature hepatocytes; thus, much more basic research will be required before ES or iPS-derived hepatocytes are ready for the clinics. Liver stem cells are covered in Chapter 23 and are not discussed here.

Cell types that are currently in clinical practice and could potentially be available for cellular therapy in the near future are those from bone marrow and mesenchymal stromal cells (MSCs). Following an initial publication, there was great excitement at the possibility that bone marrow cells might serve as a source of hepatocytes for the correction of liver disease. Subsequent detailed work has suggested that cell fusion is the principal source of bone-marrow-derived hepatocytes observed in the experimental model. Although there is still some controversy over this issue, the bulk of the more recent data suggests

that bone marrow is not the source of the progenitor cells in the liver and there is little evidence for the conversion of hematopoietic cells to hepatocytes, *in vivo*, in experiments with animals or in a clinical setting. When the presence of X and Y chromosomes was analyzed in liver biopsies taken from sex-mismatched liver transplant recipients (eight female to male and five male to female) the recipient-specific sex chromosome pattern was only detected in the inflammatory cells, and not hepatocytes. This study had a transplant-to-biopsy interval of 4.5 years (range 1.2–12 years), and the authors concluded that recipient engraftment of stem hematopoietic cells is an infrequent feature in long-term grafts. Thus, bone marrow cells may not be a relevant source of hepatocytes for the treatment of liver disease.

MSCs isolated from a variety of tissues including cord blood, skin, and human liver have been proposed as a source of hepatocytes for transplantation. There are now numerous reports of mesenchymal cells adopting hepatic features when cultures are placed under specific conditions or upon transplantation, *in vivo*. Several groups have now reported the expression of hepatic genes and proteins normally expressed in the liver such as albumin, α-1 antitrypsin, α-fetoprotein, fibrinogen, glycogen, and even some more mature hepatic markers such as drug metabolizing genes including CYP3A4. In all cases, the levels of expression of hepatic genes and their functions, when measured, were quite low when compared with authentic human hepatocytes. The utility of MSCs as a source of hepatocytes may depend on their ability to differentiate to cells with a mature hepatic phenotype upon transplantation. When examined carefully, following transplantation into the mouse liver, human cord blood mononuclear cells gave rise to small clusters of hepatocyte-like cells that expressed human albumin and Hep Par, a marker protein found in hepatocytes; however, the cells also expressed mouse cytokeratin 18, suggesting that the clusters of hepatocyte-like cells were the result of cell fusion with endogenous mouse hepatocytes. At the present time, there is no convincing evidence that MSCs can differentiate to cells with a broad range of mature hepatic functions.

Although there is little substantial evidence that bone marrow or MSCs from different sources form mature hepatocytes, *in vitro* or *in vivo*, there is growing evidence that these cells may improve liver function when they are infused into patients with cirrhosis. A remarkable paper by Sakaida and colleagues reported that the intravenous transplantation of bone marrow cells reduced liver fibrosis in a model of cirrhosis induced by treating mice with CCL4, which eventually led to the proposal to use autologous bone marrow therapy for liver cirrhosis. Several groups soon began phase 1 safety and feasibility studies with bone marrow cell infusions in cirrhotic patients. Most of the studies have been uncontrolled investigations of the infusion of bone-marrow-derived mononuclear cells or CD34-selected cells. In most of the studies G-CSF was used to mobilize $CD34^+$ cells. Cells were delivered through a peripheral vein or were infused

directly through a hepatic artery. The most common findings were a slight improvement in liver function as measured by a small decrease in bilirubin levels, which was usually accompanied by a small increase in serum albumin levels. Improvements in the Child-Pugh and/or the MELD scores were also frequently reported. In one study, bone-marrow-derived MSCs rather than $CD34^+$ or unfractionated bone marrow mononuclear cells were infused via a peripheral vein with similar results. It is important to note that the authors observed an increase in mortality and other complications if the MSCs were infused into the hepatic artery of patients with decompensated cirrhosis rather than via a peripheral vein.

In the only controlled trial reported, a minimum of 1×10^8 mononuclear cells from bone marrow aspirates (without G-CSF pretreatment) were infused through the hepatic artery of patients with cirrhosis. Fifteen patients were randomized to each arm of the study. The results indicated that the Child-Pugh score improved in the cell therapy group relative to the controls. The MELD score remained stable in patients receiving the cell therapy, while in the control group the MELD score increased. Serum bilirubin levels were also improved in the treated group. The improvements noted in the different endpoints were only significant for 90 days. The clinical findings suggest that slight improvements in hepatic function as measured by bilirubin and albumin levels, NELD, or Child-Pugh scores are obtained following the transplantation of bone marrow mononuclear cells or partially purified $CD34^+$ cells. It is comforting that similar findings were obtained with different protocols and by different groups. What is not clear from these initial studies is the most useful cell type to transplant and the best route of delivery. Perhaps sustained improvements in liver function could be obtained if these parameters were optimized.

37.9 CONCLUSION

With the exception of bone marrow or bone-marrow-derived stem cells, other stem cell sources have not been employed for the treatment of liver disease. Much more research will have to be conducted before cell types such as ES or iPS can be approved for clinical trials. At the current time, adult stem cells do not seem to show sufficient engraftment, proliferation, and differentiation to hepatocytes to warrant clinical trials with any of the cell types. For now, authentic hepatocytes remain the preferred cell type for the treatment of liver disease. Future work with hepatocyte-based therapy will need to focus on the improvement of engraftment and/or proliferation of donor cells post-transplant. Even 2–4-fold increases in liver repopulation by hepatocytes over the levels obtained with current transplant procedures could lead to substantial improvement in the clinical outcome of patients with liver-based metabolic diseases. It is likely that the incorporation of preconditioning regimens with hepatic resection,

ischemia/reperfusion injury, and/or radiation-induced blockage of the growth of native liver will provide the selective growth advantage to the donor cells necessary to attain the levels of liver repopulation required to normalize the alterations observed in metabolic disease patients. Since these types of studies are currently being planned at medical centers around the world, the efficacy of these modified protocols should soon be apparent.

Hepatocyte transplantation studies conducted in animal models of liver failure and liver-based metabolic disease have proven safe and effective means to provide short- or long-term synthetic and metabolic support of liver function. For certain organ transplant candidates such as those with metabolic liver disease, cell transplantation alone could provide relief of the clinical symptoms. Cell transplant studies in patients with acute or chronic liver failure or genetic defects in liver function clearly demonstrate the efficacy of hepatocyte transplantation to treat liver disease. In virtually all cases, a clinical improvement in the condition of the patient could be documented. No serious complications of hepatocyte transplant have been reported. Although all of the initial reports concerning hepatocyte transplants are encouraging, it must be realized that there are still no reports of long-term and complete corrections of any metabolic disease in patients. The recent report of a complete correction of a patient with a urea cycle defect is most encouraging; however, the length of time that human hepatocytes will function following transplantation has not been determined. Studies in animal models of liver disease have documented that donor hepatocytes transplanted into the spleen or the liver function for the lifetime of the recipient and participate in normal regenerative events. Although it is likely that human hepatocyte transplantation will result in lifelong and normal function of donor cells, this needs to be clearly demonstrated in a clinical study.

Future work will have to be conducted to establish optimal transplant and immunosuppression protocols to minimize complications and maximize engraftment and function. A major problem for clinical hepatocyte transplant is the inability to track donor cells following transplantation. Except for the short-term tracking of hepatocytes pre-labeled with radioactive substances such as indium^{111}, and following differences between donor and recipient-secreted HLA, there are no reports of quantitative and facile methods to detect donor cells. Relatively noninvasive methods will be needed to optimize transplant and immunosuppressive protocols as well as for day-to-day monitoring of the cell graft. None of the problems cited here seems insurmountable. There are now reports of successful hepatocyte transplants from laboratories in many different countries. The cooperative spirit that has developed between the investigators at the different transplant centers should benefit the research field and especially the future recipients of hepatocyte transplants.

FOR FURTHER STUDY

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CHAPTER 38

Orthopedic Applications of Stem Cells

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38.1 INTRODUCTION

Technologic advances have resulted in the advent of a wide array of biomaterials and synthetic growth factors. Clinicians and scientists face the enormous task of generating biologic constructs that emulate the interaction between cells and the complex extracellular matrix they secrete and which encapsulates them. A thorough understanding of the biology of each tissue type (material properties, ratio of extracellular matrix components, and cellular profile) is essential for the construction of functional tissue. The first element of tissue engineering is the use of cells. Different approaches include the use of differentiated lineage-specific cells (osteoblasts, chondrocytes, tenocytes, meniscal fibrochondrocytes, etc.) or the use of progenitor cells.

Pluripotent mesenchymal stem cells (MSCs) with the ability to differentiate into multiple mesodermal lineages have been isolated from bone marrow, adipose tissue, and synovium. Stem cells have the advantage that they are unlimited in supply, easily harvested, and can be cultured into large numbers. Moreover, it is postulated that the induction of stem cells down various mesodermal lineages will result in tissue which more closely resembles native tissue and recapitulates embryonic development. MSCs isolated from human bone marrow aspirates have been shown to retain pluripotentiality and proliferative ability through long-term passaging. Another advantage of using MSCs for tissue engineering strategies is that they can be used in allogeneic transplantation. Human MSCs cells do not induce a mixed lymphocyte reaction when incubated with allogeneic donor lymphocytes and have the ability to suppress an ongoing mixed lymphocyte reaction.

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Allogeneic MSC-based tissue constructs would be limitless in supply and have enormous economic advantages.

A number of growth factors have been described that enhance angiogenesis, promote cell proliferation, and induce differentiation of cells down various mesodermal lineages. The delivery of single doses of recombinant growth factors has been demonstrated to be effective in the healing of segmental bone defects and articular cartilage defects. Similarly, the structural properties of ligaments during the repair process can be improved by the application of growth factors. However, there are many clinical situations in which a sustained exposure to growth factors is necessary. Moreover, leakage of supraphysiologic doses to adjacent sites can pose huge problems such as heterotopic ossification from osteoinductive factors.

In addition to being a powerful tool in cell-based therapy, MSCs may also be useful as a delivery vehicle for growth factors through genetic manipulation. Cells can be transduced with a retrovirus so that the target gene can be integrated into the cell's DNA. This allows for propagation of gene expression when the cell replicates so that long-term expression of the target gene would occur. Alternatively, gene therapy strategies using adenoviral vectors would be useful in clinical situations where only transient gene expression is desired. *In vivo* gene therapy in which the viral vector is introduced to a tissue by direct injection has many disadvantages. A host response to the virus may result in immunologic rejection. Moreover, transduction efficiencies are often low, especially if the target tissue contains slowly growing cells. Diffusion of the vector to adjacent areas may also lead to potential complications. *Ex vivo* gene therapy using MSCs overcomes some of these potential problems. Use of autologous cells as delivery vehicles would prevent the host immune response. In addition, cells can be seeded onto matrix scaffolds customized to the shapes and sizes required at the target tissue.

This chapter provides a brief summary of the biology and properties of various musculoskeletal tissue types, highlights the present tissue engineering strategies, and speculates about the future direction of the application of MSCs in tissue engineering as it applies to each tissue. MSCs can serve as the building blocks for tissue regeneration by their ability to differentiate down various lineages. They also hold great promise as delivery vehicles for sustained release of growth factors to injured tissue sites. The growth factors may enhance tissue repair by promoting migration of MSCs, inducing differentiation of progenitor cells, and enhancing vascularization of the newly formed tissue.

38.2 BONE

Bone has the ability to regenerate itself with functional tissue with properties similar to the original tissue. However, bone tissue engineering still has many

clinical applications, including fracture nonunion, congenital malformations requiring bone-lengthening, tumors, and bone loss secondary to trauma or osseous infections. Moreover, bone regeneration in the setting of bony ingrowth is important in joint arthrodesis for osteoarthritis, spinal fusion, and more rigid fixation of prosthetic implants.

The extracellular matrix of bone is primarily type I collagen and calcium phosphate. Osteoblasts line the periphery and are active in matrix deposition. In the clinical setting, autogenous bone grafts harvested from the iliac crest and fibular grafts are often used in fracture repair. However, some of the disadvantages of harvesting autogenous bone graft are donor site morbidity and their limited supply. Clearly, there is a need for alternative methods of harvesting autologous bone substitutes. Autologous marrow aspirates harvested from the posterior iliac crests have been evaluated as a graft substitute for patients with tibial nonunions when injected into fracture sites. A considerable amount of new bone was evident and the patient was able to fully bear weight at five months. Percutaneous harvest of bone marrow aspirates is less invasive, and bone marrow suspensions may serve as useful bone graft substitutes.

MSCs isolated from bone marrow aspirates and processed lipoaspirate (PLA) cells from adipose tissue differentiate into osteoblasts when cultured in the presence of dexamethasone, ascorbic acid, and beta-glycerophosphate. Bone-marrow-derived MSCs have also shown the ability to form heterotopic bone in animal models. MSCs seeded onto hydroxyapatite (HA) and tricalcium phosphate (TCP) ceramic discs implanted into subcutaneous pockets show evidence of bone formation within the pores of the ceramic scaffolds. Preincubation of human MSCs in a porous ceramics in the presence of osteoinductive medium followed by intraperitoneal implantation into athymic nude mice resulted in the formation of thick layers of lamellar bone and active osteoblasts that line the ceramic surface.

Another preclinical animal study showed the ability of autologous MSCs loaded into HA/TCP carriers to heal a critical-sized segmental defect in a canine model. Significant bone formation occurred at the host-implant interface, and a continuous span of bone was seen across the defect. Evidence of both woven and lamellar bone was seen. Periosteal calluses formed around the implant. During the 16-week period of the study, the callus remodeled and resulted in healing of the defect with bone that was similar in shape and size to the original segment of bone that was resected. Human MSCs loaded onto HA/TCP carriers were implanted into femoral defects in athymic rats in a study similar to the one described previously. Radiographic and histologic evidence of new bone formation was seen at eight weeks. Biomechanical testing showed that cell-loaded ceramic implants had more than twice the stiffness and torque to failure as ceramic implants that had no cells.

Growth factors in the bone morphogenetic protein (BMP) family, including BMP-2 and BMP-7, also induce progenitor cells to differentiate down the osteogenic lineage. Current strategies for bone tissue engineering include the use of osteoconductive matrix devices that promote bony ingrowth and the delivery of osteoinductive growth factors to bony defect sites. Matrix materials include calcium HA, type I collagen gel, polylactic acid polymers, and demineralized bone matrix. Demineralized bone matrices implanted with recombinant human bone morphogenetic protein-2 (*rhBMP-2*) yielded histologic and radiographic evidence of healing. In a study involving a larger animal model, a sheep femoral defect model was used to show the efficacy of *BMP-2* in healing critical-sized long bone defects in a large animal model. A prospective, randomized trial of 450 patients looking at the safety and efficacy of *rhBMP-2* in improving the outcome of open tibial fractures showed that patients treated with intramedullary nailing and *rhBMP-2* had significantly lower risks of delayed union and need for more invasive intervention such as bone grafting and nail exchange. Moreover, they tend to have significantly faster fracture healing and lower risks of infection and need for hardware removal. Gene therapy using genetically manipulated MSCs is an attractive alternative method for delivery of growth factors. Not only can they safely deliver sustained release of growth factors to anatomic sites but their osteogenic potential allows them to serve as substrates for osteoinductive factors and building blocks for newly formed bone.

Regional gene therapy using MSCs as a vehicle for localized expression of osteoinductive proteins has shown promising results in animal models. Human bone marrow stromal cells transfected with adenoviral *BMP-2* leads to more robust, trabecular bone in an athymic rat femoral defect model compared with the thin, lacelike bone that formed in defects filled with matrix carrying localized recombinant human *BMP-2* (*rhBMP-2*). Moreover, the femurs from the adenoviral *BMP-2* showed no statistically significant difference in biomechanical strength with respect to ultimate torque to failure and energy to failure compared with control femurs. The difference in characteristics of the healing response may be related to the increased efficacy from sustained release of *BMP-2* from the adenovirally transfected bone marrow cells compared with the single-dose response seen in the *rhBMP-2* group. Moreover, the implanted bone marrow stromal cells may themselves contribute to osteogenesis in the defect site. Stem cells isolated from adipose tissue were transfected with the *BMP-2* gene and showed rapid induction into the osteoblast phenotype in *in vitro* cell cultures. Collagen matrices seeded with the transduced cells were able to produce heterotopic bone in the hind limbs of SCID mice. Adipose tissue is plentiful and easily accessible and may well serve as another alternative bone graft substitute in an orthopedic surgeon's armamentarium.

Vascular invasion is a vital step in endochondral ossification. Vascular endothelial growth factor (VEGF) is one of the best-characterized angiogenic factors. Studies have also shown that it is essential during embryogenesis, skeletal development, and endothelial function. Muscle-derived MSCs transduced with retroviral *VEGF* was found to work synergistically with cells transfected with BMP-4 to enhance bone healing in critical-sized calvarial defects. VEGF was found to be important for endochondral bone formation through enhancement of angiogenesis, cell recruitment, improved cartilage formation, and accelerated cartilage resorption. This study demonstrated another strategy for use of stem cells in gene therapy-based treatment of skeletal defects.

Several applications of MSCs in the repair of local bone defects have been described previously. Bone-marrow-derived MSCs may also hold great potential for treatment of diffuse musculoskeletal disease. Osteoporosis and osteogenesis imperfecta (OI) are two of the more attractive candidates. OI is a genetic disorder of MSCs characterized by a defect in the type I collagen gene that results in children with growth retardation, short stature, and numerous fractures secondary to fragile bone. Six children have already been enrolled in a clinical trial at St. Jude Children's Research Hospital, which involves the intravenous administration of unmanipulated bone marrow from human leukocyte antigen (HLA)-identical or single-antigen-mismatched siblings. Five of the six patients showed engraftment in one or more sites, including bone, skin, and stroma. More importantly, these five patients all showed acceleration of growth velocity during the six months postinfusion. The authors attributed the increase in growth to the generation of normal osteoblasts from the MSCs that engrafted in skeletal sites.

38.3 CARTILAGE

Osteoarthritis is currently one of the most prevalent chronic conditions in the United States, accounting for as many as 39 million physician visits a year. According to a National Health Interview Survey, it is estimated that approximately 70% of the population older than 65 years will have activity limitation or require medical attention because of osteoarthritis. Moreover, a significant number of adolescents and young adults suffer from chondral defects secondary to trauma, sports-related injuries, and osteochondritis dissecans. Of 31,516 knee arthroscopies, 63% of the knees had chondral lesions, with an average of 2.7 hyaline cartilage lesions per knee. Cartilage has poor intrinsic healing ability, and superficial defects typically do not heal spontaneously. This is related to both the lack of vascular supply and the poor proliferative ability of chondrocytes. When the lesion extends into the subchondral bone, mesenchymal cell recruitment occurs from the synovium and subchondral marrow and a healing response ensues in which the defect is

filled with repair tissue resembling fibrocartilage. Unfortunately, this tissue is structurally inferior to the native cartilage.

Articular cartilage is highly acellular with cell volume averaging only approximately 2% of the total cartilage volume in adults. The extracellular matrix is composed of a highly complex network of collagen fibrils and proteoglycans. Type II collagen is the dominant collagen subtype found in cartilage. The molecule is a triple helix composed of three α1 chains and with multiple cross-links. The collagen fibrils contribute to the tensile strength of cartilage. Load transmission capacity and compressive strength mainly arise from the proteoglycans, the other main constituent of the extracellular matrix of articular cartilage. The core protein of aggrecan contains a large number of chondroitin sulfate and keratin sulfate side chains that become highly hydrated. Aggrecan molecules also contain a hyaluronic acid-binding region.

Clinically, current treatment options for cartilage defects can be categorized into cartilage stimulation and cartilage replacement strategies. Cartilage stimulation techniques include abrasion arthroplasty, subchondral drilling, and the microfracture technique. However, the repair tissue never achieves the hyaline architecture of the native tissue. Osteochondral autografts and allografts represent the other spectrum where the defect is filled with plugs taken from normal regions of articular cartilage. The main disadvantages associated with the use of autografts are paucity of tissue and donor site morbidity and the possible long-term complications. Allografts, on the other hand, face the risk of donor rejection from immunogenicity and disease transmission. Joint prosthesis remains the mainstay for symptomatic relief of pain and improvement of daily function of patients suffering from severe articular cartilage defects. In 1999 alone, more than 244,000 total knee arthroplasties were performed.

One exciting development has been the introduction of tissue engineering strategies for cartilage defects. Cell-based therapy for treatment of cartilage defects is already in use clinically with the use of autologous chondrocytes. Chondrocytes are isolated and culture expanded from arthroscopically harvested cartilage and reimplanted into deep articular cartilage defects. Since 1987, more than 950 patients have been treated with this technique. Long-term (mean 7.5 years) follow-up of patients showed good to excellent rating on the Cincinnati rating score. Biopsy specimens resembled hyaline cartilage with extracellular matrices that consisted primarily of type II collagen and aggrecan. Some of the disadvantages of this technology include a limited supply and donor site morbidity associated with the initial cartilage harvest.

MSCs capable of chondrogenesis are present in bone marrow, periosteum, synovium, and adipose tissue. Human MSCs can be culture expanded more than 1 billion-fold and retain their multilineage potential. Moreover, they

proliferate rapidly and have the advantage that they can recapitulate the embryonic events present in chondrogenesis. The importance of MSCs in cartilage repair is illustrated by the lack of a repair response in defects that do not penetrate subchondral bone. On penetration, recruitment of pluripotential marrow mesenchymal cells ensues. The influence of local cytokines from inflammatory cells and synovial fluid results in differentiation of the progenitor cells into chondrocytes and filling of the space with fibrocartilage.

MSCs were shown to be capable of healing full-thickness articular cartilage defects in a rabbit femoral condyle model. Bone-marrow-derived and periosteum-derived MSCs were isolated from New Zealand White rabbits and embedded into a collagen-based scaffold. The cellular constructs were implanted into 6 mm long × 3 mm wide × 3 mm deep full-thickness cartilage defects in the weight-bearing portion of the medial femoral condyle. Healing of the defect by hyaline cartilage was evident as early as two weeks, with filling of the subchondral space with dense highly vascularized new bone. After four weeks, the neocartilage was thicker, and excellent integration was noted at the interface between the new subchondral bone and the host tissue. Moreover, further analysis of the histologic specimen at various time points showed that the formation of subchondral bone resulted from a recapitulation of the embryonic process of enchondral ossification with progression of chondrocytes to a hypertrophic state, followed by vascular invasion and ossification. No evidence of osteoarthrosis was noted in any of the knees. At later time points, however, significant remodeling of the cartilage occurred with loss of metachromatic staining. Genetic modification of the MSCs may enhance the healing response and allow for better long-term repair tissue.

In another study, periosteal cells transfected with a retroviral vector containing *BMP-7* showed *in vitro* and *in vivo* gene expression for at least eight weeks after seeding onto polymer grafts. When the cell-based constructs were placed into 3 mm circular osteochondral defects in the intertrochlear grooves of rabbit knees, the defects were completely filled with predominantly hyaline-like tissue that persisted for as long as 12 weeks after implantation. The subchondral portion of the defects showed rapid reconstitution of bone. Restoration of the tidemark and formation of subchondral bone were also seen in articular cartilage defects treated with collagen scaffolds containing another member of the transforming growth factor (*TGF*) superfamily *BMP-2*.

As we reach a better understanding of the biology and biomechanical properties of articular cartilage and its response to different growth factors, gene-modified tissue engineering can become a more powerful tool for cartilage resurfacing. A number of growth factors have been described that enhance chondrocyte proliferation and chondrogenic differentiation including fibroblast growth factor-2 (FGF-2), insulin-like growth factor-I (IGF-I), TGF-1,

growth hormone (GH), BMP-7 (also known as osteogenic protein-1), and BMP-2. Induction of chondrogenesis using growth factors can be accomplished by direct injection into the defect site and application of gene therapy techniques.

With direct injections, single supraphysiologic doses of growth factors may often be insufficient for proper healing. Degradation of the proteins may occur as a result of an inflammatory reaction and the recruitment of macrophages and neutrophils. Moreover, dilution and clearance of growth factors by the surrounding synovial fluid occurs over time. Prolonged exposure to growth factors may be necessary for chondrogenic differentiation of resident progenitor cells. In addition, by using chondrocytes or MSCs as a delivery vehicle for growth factors via gene therapy, the cells can in turn be the building blocks for the regenerated cartilage and respond to autocrine and paracrine growth factors. The newly formed cartilage can be a chimera of the original cell construct and new mesenchymal progenitor cells from the recruitment and migration that occurs from the actions of the cytokines secreted by the transduced cells.

It is feasible to transduce bone-marrow-derived mesenchymal progenitor cells with both retroviral and adenoviral TGFβ-1 vectors. These genetically modified progenitor cells were able to undergo chondrogenesis *in vitro* with and without exogenous TGFβ-1 addition. Using a replication-incompetent adenovirus vector expressing IGF-I, chondrocytes, MSCs, and synoviocytes were successfully transfected. Significant increases in proteoglycan production in the extracellular matrix secondary to *IGF-I* induction were noted. Moreover, cells expressed high levels of the *IGF-I* for up to 28 days in culture. Both of these studies verify the possibility of genetically modifying MSCs so that they have the ability to secrete chondroinductive growth factors over sustained time periods.

Animal models for *ex vivo* gene therapy of articular cartilage defects using MSCs have already been carried out. The ability of adenoviral-mediated expression of perichondrial MSCs to heal an articular cartilage defect in a rat model was investigated. The effects of *AdBMP-2* and *AdIGF-1* transfected cells suspended in fibrin glue were evaluated with respect to their ability to heal the defects and integrate with the host tissue. The partial thickness lesions healed in both the *AdBMP-2* and *AdIGF-1* groups with repair cartilage exhibiting hyaline morphology composed of type II collagen in the extracellular matrix but no type I collagen. The defects in the nontransfected cell group primarily filled with fibrous tissue rich in type I collagen. Again, this is consistent with the observations seen in the natural repair process in which MSCs from marrow cavity progress to repair subchondral cartilage defects with fibrocartilage-like tissue. A complication of the *AdBMP-2* group that was not

seen in the *AdIGF-1* group was osteophyte formation secondary to leakage of cells outside the construct.

Chondroprotective approaches to preservation of articular cartilage have also been explored by several groups. Instead of trying to stimulate cartilage repair and replace cartilage defects, an alternative is prophylactic treatment and cessation of disease progression in its earlier stages. Interleukin-1 receptor antagonists (IL-1Ra), tumor necrosis factor (TNF) blockers, and interleukin-4 (IL-4) have all been described to protect against degenerative changes in articular cartilage. The anti-inflammatory drugs infliximab (Remicade) and etanercept (Enbrel), both inhibitors of TNF, have both been approved by the Food and Drug Administration for patients suffering from rheumatoid arthritis. Animal models have already shown the suppression of osteoarthritic changes using gene therapy to effect delivery of IL-1Ra. Transduced MSCs expressing IL-1Ra or antagonists of TNF may both enhance the reparative process through their inherent chondrogenic potential and retard the degradative process in cartilage lesions.

38.4 MENISCUS

The annual incidence of meniscal tears is approximately 60 to 70 per 100,000. The menisci of the knee are semilunar fibrocartilaginous structures that are integral to the normal function of the knee. The extracellular matrix consists of collagenous fibers that are mostly oriented circumferentially, with interspersing of radial fibers that contribute to its structural integrity. The circumferential fibers help disperse compressive forces while the radial fibers protect against tearing from tensile forces. The matrix contains mainly type I, II, and III collagens, with type I collagen being the most prevalent. The cells of the meniscus are fibrochondrocytes because of their chondrocyte-like appearance and synthesis of fibrocartilaginous matrices. The main function of the meniscus is load transmission during weight-bearing. In extension, approximately 50% of the load in the knee is transmitted to the menisci. At 90 degrees of flexion, this increases to almost 90% of the total load. The medial meniscus also acts as an important secondary restraint to anteroposterior translation, especially in the anterior cruciate ligament (ACL) deficient knee. Finally, the meniscus is important in shock absorption and joint lubrication.

Early in prenatal development, the menisci are very cellular and highly vascularized. After skeletal maturity, the vascular zone is generally confined to the peripheral one third of the meniscus. Longitudinal tears in the peripheral zone can generally be repaired, whereas radial tears are usually not amenable to repair. Defects that cannot be repaired are usually debrided to avoid the irritation from loose meniscal flaps. Total meniscectomy is usually

contraindicated because it leads to a number of osteoarthritic changes. Long-term follow-up studies showed that a high percentage of patients with a history of meniscectomy go on to develop knee instability and considerable amounts of radiographic degenerative changes in the knee.

Clearly, there is a need for new treatment options. Currently, meniscal allograft transplantation is one of the few alternatives for replacement of large meniscal defects. Unfortunately, there is a risk of rejection and disease transmission with allograft tissue. Moreover, the graft size of the donor must match the recipient, which can sometimes be difficult. Long-term problems with meniscal allografts include graft shrinkage, decreased cellularity, and loss of normal biologic activity. Other experimental methods for meniscal repair include the use of fibrin clots, collagen-based polymers, and a number of polyurethane-based meniscal prostheses.

MSCs may provide novel treatment strategies in both growth factor based and cell-based treatment options for meniscal tears. A number of cytokines including platelet-derived growth factor (PDGF), BMP-2, hepatocyte growth factor (HGF), epidermal growth factor (EGF), IGF-I, and endothelial cell growth factor have been implicated in the proliferation and migration of meniscal cells within the different zones. Single doses of growth factors may not provide adequate stimulus in the repair tissue. Successful adenoviral-mediated expression of *BMP-2*, *PDGF*, *EGF*, and *IGF-1* has already been demonstrated in culture systems of MSCs and fibroblasts. MSCs can serve as delivery vehicles for sustained release of growth factors that contribute to cell proliferation, recruitment, and differentiation and an increase in localized vascularization. The feasibility of gene therapy in tissue engineered meniscal tissue was supported by evidence of transduced gene expression in meniscal allografts at four weeks. In another study, bovine meniscal cells were transfected with an adenovirus vector encoding *HGF*, seeded onto polyglycolic acid scaffolds, and placed into subcutaneous pouches of athymic mice. Gene expression of *HGF* was associated with a significant increase in neovascularization.

The repair of meniscal defects by fibrocartilage has been advocated by some authors. Lesions in the avascular zone of canine menisci repaired by implantation of polyurethane polymers showed evidence of fibrocartilaginous tissue compared with only fibrous tissue in the control group. In a small clinical series, resorbable collagen scaffolds were implanted into medial meniscus lesions and showed preservation of the joint surfaces and evidence of regeneration of tissue regeneration two years postoperatively. The chondrogenic potential of bone-marrow-derived MSCs has been well-characterized. An alternative strategy in meniscal repair would be a prefabrication of a cartilage-like construct using stem cells and a biodegradable matrix. Data from a rabbit

partial meniscectomy model showed that MSCs embedded in a collagen sponge can enhance the formation of fibrocartilage tissue at the defect site. Although the tissue would not have the identical properties of native menisci, it would serve a similar function in joint surface preservation and load transmission across the knee joint.

38.5 LIGAMENTS AND TENDONS

Ligaments and tendons are bands of dense connective tissue that lend stability and provide movement of joints. Injury that leads to inflammation or tearing of these structures can result in significant functional deficits and development of degenerative joint disease. During the normal healing process, various growth factors such as PDGF, FGF, and TGFβ are released by macrophages and platelets to stimulate fibroblast proliferation and tissue remodeling. These growth factors contribute to proliferation, extracellular matrix secretion, and recruitment of cells. Tissue engineered cellular constructs and delivery of growth factors via gene therapy offer great potential in augmenting the healing process.

In general, most of the dry weight of skeletal ligaments is made up of collagen. Greater than 90% of this is type I, with a small percentage of type III. Glycosaminoglycans and elastin also make up a small proportion of the biochemical makeup. Ligaments are oriented to resist tensile forces along their long axes. Collagen fibril diameters and the number of collagen pyridinoline cross-links correlate with the tensile strength of the healed ligament. Ligaments work in conjunction with muscle-tendon forces, bony intra-articular constraints, and other soft tissues to help stabilize joints and prevent nonphysiologic movements. There are differences in the intrinsic healing capacity of various ligaments. After injury, ligaments heal in a series of stages: hemorrhagic, inflammatory, proliferation, and remodeling. Clinically, medial collateral ligament (MCL) injuries heal reliably without surgical intervention, whereas ACL tears usually do not heal spontaneously. Histologic studies of human ACL tears show that, unlike extrasynovial ligaments, no evidence of bridging occurs between remnants of the ACL from the femoral and tibial sides. Typically, ACL ruptures are treated with a variety of different tendon autografts and allografts. Unfortunately, autograft harvest involves damage to previously healthy tissue, and allografts carry the risk of disease transmission. Moreover, although isolated MCL tears do well with nonoperative treatment, biochemical analysis shows that untreated MCL scar has a higher than normal proportion of type III collagen, higher collagen turnover rates, and increases in total glycosaminoglycan. Histologically, the extracellular matrix never completely approaches the highly organized appearance of normal ligament substance. Studies have also shown that MCL fibroblasts migrate more

rapidly and repopulate cell-free areas more rapidly than those from ACLs. Structural differences exist between cells of the MCL and the ACL.

Growth factors play a large role in the healing and remodeling of musculoskeletal tissue. Exposure to EGF and basic FGF leads to a significantly higher rate of proliferation in fibroblasts from the MCL and ACL. Fibroblast proliferation is a major component of the normal ligament healing process. Similarly, FGF and EGF treatment of ligament fibroblast cultures leads to increased collagen synthesis. IGF-I also stimulates type I collagen synthesis in fibroblasts. However, complex interactions exist between different growth factors because some act synergistically, whereas others antagonize one another. The structural and functional properties of tissues are largely dependent on the composition of the extracellular matrix.

The use of MSCs in gene therapy strategies for ligamentous and tendon injuries has many potential clinical applications. Growth factors can be delivered locally to injury sites that can promote each of the four stages of normal healing. Increased fibroblast proliferation effected by FGF and EGF can be promoted through adenoviral-mediated gene expression. Short bursts of growth factors will enable the healing process to occur more rapidly in the initial stages. TGFβ-1 promotes wound healing in many animal models. Its ability to augment cellular proliferation and increase secretion of collagen in the extracellular matrix hold great promises as a useful target gene for regional growth factor delivery. Animal studies have already shown that use of MCLs exposed to PDGF leads to stiffness and breaking energy values similar to their respective controls. Moreover, combination treatment with IGF-I and FGF further enhances these structural properties. In a canine model, basic FGF leads to increased neovascularization and better orientation of collagen fibers in a partially ruptured ACL. The feasibility of using cells as a delivery vehicle of growth factors into the knee joint was confirmed by a study showing cell-seeded collagen scaffolds that remained viable for up to four weeks when implanted in the knee joint.

In addition to the use of autografts and allografts, treatment options that have been proposed for tendon defects include use of synthetic polymers and acellular biodegradable scaffolds. Scaffolds serve as a conduit for recruitment of cells during the initial healing process. As they degrade over time, the mechanical loading forces are transferred to the new repair tissue. Despite the promising data regarding repair strength and biomechanical properties, the immunogenicity and biocompatibility of the materials over time are not well understood. It would be ideal to use autologous sources of cells in combination with biodegradable matrices as a tissue engineered construct to reconstruct ligaments or tendons.

Fibroblasts generate tension and change their orientation along tensile forces when cultured in three-dimensional collagen gels. MSCs display a similar

fibroblastic property. The combination of MSCs with biodegradable scaffolds could be useful for bridging of large tendon defects. Autologous cell-based tendon repair was performed in a hen flexor tendon model, in which tenocytes seeded onto polyglycolic acid scaffolds were able to heal a 3 to 4 cm defect. The healed tissue resembled native tendon grossly and histologically. Biomechanically, the experimental group had 83% of the breaking strength of the normal tendon. Similarly, MSC-based repairs of Achilles tendon in animal models showed improvement of the biomechanics and function of the tendon. However, the histologic appearance and biomechanical strength are inferior to normal tendon controls.

In one study, collagen gels were seeded with MSCs in a full-thickness patellar tendon defect model to compare its effectiveness with the natural repair process. At 26 weeks after the original implantation, composite constructs showed significantly higher moduli and maximum stresses compared with the natural repair group. When compared with native tissue, however, the maximum stress was only one fourth of the control. Moreover, 28% of the cell-matrix constructs formed bone in the tendon repair site.

Rabbit MSCs were seeded onto a pretensioned polyglyconate suture to create a contracted construct. At 40 hours, cell nuclei were spindle shaped and a cell viability of approximately 75% was noted in the constructs. Repair tissue treated with MSCs had a significantly larger cross-sectional area at the repair site than the contralateral control (suture only) and untreated native tissue. Cell-seeded repairs showed superior biomechanical properties with a two-fold increase in load-related properties. Compared with native tissue, the treated tendons had almost two thirds of the structural properties at 12 weeks. A rapid rate of increase in load-related material properties may reflect the remodeling stage of healing mediated by the MSCs. Histologically, increases in tenocytes and collagen crimping pattern also occurred over time.

The two studies reviewed previously demonstrate the potential of MSCs to help augment the tendon repair process. Tissue engineered constructs combining stem cells with biodegradable scaffolds enhance the biomechanical properties of the repair tissue and remodel over time to more closely resemble the complex organization of normal tendon. Preincubation of the constructs *in vitro* with growth factors may produce better biologic substitutes. Moreover, transfection of MSCs with genes such as *TGFβ-1*, *EGF*, and *FGF* may have a synergistic effect and lead to stronger structural repair tissue. Successful transfection of myoblasts and ACL fibroblasts with adenovirus and subsequent introduction into the rabbit ACL has been demonstrated. Expression of the *lacZ* reporter gene was noted at seven days and persisted for as long as six weeks. *BMP-12* gene transfer into lacerated chicken tendon

resulted in augmentation of ultimate force and stiffness of the repaired tissue. Mesenchymal progenitor cells form tendon-like tissue ectopically when transfected with the *BMP-12* gene.

38.6 SPINE

Posterolateral lumbar intertransverse process fusions are very commonly performed for spinal disorders secondary to degenerative changes and trauma. However, the nonunion rates have been reported to be as high as 40% with single-level fusions and even higher in multiple-level procedures. Currently, nonunions are prevented by application of instrumentation such as pedicle screws, rods or plates, and various interbody fusion devices to achieve better correction of deformities and biomechanical stability during the healing period. However, a significant number of nonunions and pseudoarthrosis still exist in fusions with instrumentation, and some have shown no statistically significant decrease in nonunions with use of screw fixation.

More recently, a large number of clinical studies have compared the efficacy of osteoinductive proteins with autogenous iliac crest bone graft in lumbar fusions. In a small randomized clinical series, osteogenic protein-1 (OP-1), also known as BMP-7, was as effective as autogenous bone graft in achieving single-level lumbar fusions. In a rabbit model, *BMP-7* was found to overcome the inhibitory effects of nicotine on spinal fusion. In a prospective, randomized, clinical trial, Texas Scottish Rite Hospital (TSRH) pedicle screw instrumentation with rhBMP-2 with TSRH pedicle screw instrumentation and rhBMP-2 without instrumentation were compared and the groups with rhBMP-2 had 100% fusion rates compared with only 40% (2/5) fusion in the TSRH pedicle screw instrumentation only group. Improvement of the Oswestry score at follow-up was highest in the rhBMP-2 only group. The use of *rhBMP-2* as an adjunct in fusion using lumbar interbody devices was demonstrated in a sheep model. Recombinant BMP-2 was shown to be superior to autogenous bone graft. Minimally invasive spinal fusion and relief of discogenic back pain was possible in a series of 22 patients undergoing laparoscopic placement of *rhBMP-2*.

Direct introduction of growth factors into the intervertebral space for spinal fusion, however, does have its risks. Heterotopic bone formation could occur from leakage of osteoinductive proteins outside its carrier into the surrounding tissue. This could be devastating, especially in the setting of dural tears. Moreover, most osteoinductive growth factors are rapidly metabolized so that single-bolus injections may not achieve ideal efficacy in clinical settings. Gene therapy using MSCs offers the ability to deliver sustained release of growth factors to a local site. Successful spinal fusion has been shown in rats using bone

marrow cells transfected with a novel osteoinductive protein, LIM mineralization protein-1 (*LMP-1*). LMP-1 is thought to be a soluble osteoinductive factor that induces expression of other BMPs and their receptors. Adenoviral-mediated regional gene therapy using MSCs expressing *BMP-2* achieved radiographic evidence of fusion in a rat model by four weeks postoperatively. Moreover, the group receiving *AdBMP-2*-transfected cells showed repair with coarse trabecular bone compared with thin, lacelike bone in the rhBMP-2 group. The long-term sustained release of osteoinductive proteins was perhaps more effective in producing a stronger biologic response than a single supraphysiologic dose. Another possibility could be that the MSCs have differentiated down the osteogenic lineage and are now contributing to the new bone in the fusion mass.

Similar to cartilage repair strategies, another potential strategy in the treatment of spinal disorders would be the suppression of disc degeneration. Fraying, splitting, and loss of collagen fibers in the intervertebral discs and calcification of the cartilage in the endplates occur with age. Matrix metalloproteinases and aggrecanases are also thought to be important in extracellular matrix degradation in the disc. Proteoglycans are important for maintenance of disc height and its compressive ability, and their preservation is essential for the load-bearing capacity of intervertebral discs. The effects of various growth factors on proliferation and proteoglycan synthesis were measured in annular pulposus and nuclear pulposus cells isolated from canine intervertebral discs and it was found that TGFβ-1 and EGF induced a five-fold increase in proteoglycan production. Retardation of programed cell death in annulus cells and a dose-dependent increase in proteoglycan synthesis have been shown in discs exposed to IGF-1. Delivery of *TGFβ-1* and/or *IGF-1* to intervertebral discs using MSCs as a vehicle for gene therapy, therefore, represent two possible strategies in the treatment of disc disorders.

Successful retroviral-mediated gene transfer of *lacZ* and *IL-1Ra* of cultured chondrocytes has been performed by harvesting endplate tissue cartilage from patients with degenerative discs and the reintroducing genetically modified cells into the disc space. This same strategy can be applied using MSCs that can be easily isolated from the iliac crest. As mentioned previously, adenoviral-mediated expression of *IGF-1* by MSCs has already been successfully shown. Gruber and coworkers showed the efficacy of *in vivo TGFβ-1* gene transfer to intervertebral discs and its ability to increase the level of proteoglycan synthesis.

38.7 SUMMARY

MSCs hold great potential for the development of new treatment strategies for a host of orthopedic conditions. Animal models have demonstrated the wide spectrum of clinical situations in which MSCs could have therapeutic

effects. Clinical application of the principles of cell-based tissue engineering is already seen with the Carticel (Genzyme, Cambridge, MA) program that uses culture expanded autologous chondrocytes in the repair of articular cartilage defect. The multilineage potential and plasticity of MSCs allow them to be building blocks for a host of nonhematopoietic tissues including bone, cartilage, tendon, and ligament. Advances in fabrication of biodegradable scaffolds that serve as beds for MSC implantation will hopefully lead to better biocompatibility and host tissue integration. Minimal toxicity has been observed in animal models involving genetically manipulated stem cells transduced with retroviral and adenoviral vectors. Gene therapy using stem cells as delivery vehicles is a powerful weapon that can be used in a plethora of clinical situations that would benefit from the osteoinductive, chondroinductive, proliferative, and angiogenic effects of growth factors.

FOR FURTHER STUDY

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CHAPTER 39

Embryonic Stem Cells in Tissue Engineering

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39.1 INTRODUCTION

One of the major obstacles in engineering tissue constructs for clinical use is a limit on available human cells. Stem cells isolated from adults or developing embryos are one current source of cells for tissue engineering. The derivation of human embryonic stem (hES) cells in 1998 generated great interest regarding their potential application in tissue engineering. Embryonic stem (ES) cells can grow indefinitely in culture, and can generate the differentiated cells of all adult tissues. However, despite their therapeutic potential, both adult and ES cells present several challenges associated with their clinical application. For example, although adult stem cells can be directly isolated from the patient and are therefore immunologically compatible with the patient, they are typically hard to isolate and grow in culture. In contrast, ES cells can be easily grown in culture and differentiated to a variety of cell types, but ES-derived cells may be rejected by the patient, and undifferentiated ES cells may form tumors.

The goal of this chapter is to analyze the potential of ES cells in tissue engineering. The chapter explains the importance of ES cells as a source of cells for tissue engineering by using examples from current research in the field. Furthermore, the chapter explains some fundamental principles and seminal work in tissue engineering.

39.2 TISSUE ENGINEERING PRINCIPLES AND PERSPECTIVES

Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences to developing biological substitutes. These are typically composed of biological and synthetic components which restore,

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maintain, or improve tissue function. Tissue-engineered products would provide a lifelong therapy and would greatly reduce the hospitalization and health care costs associated with drug therapy, simultaneously enhancing the patients' quality of life.

In general, there are three main approaches to tissue engineering:

1. To use isolated cells or cell substitutes as cellular replacement parts
2. To use acellular materials capable of inducing tissue regeneration
3. To use a combination of cells and materials (typically in the form of scaffolds)

Although host stem cells could be involved in all of these approaches, ES cells can be directly involved in the first and third approaches.

39.2.1 Isolated Cells or Cell Substitutes as Cellular Replacement Parts

Isolated cells have been used as a substitute for cell replacement parts for many years. The first application of stem cells as a cellular replacement therapy was associated with bone marrow transplantation or blood transfusion studies, in which donor hematopoietic stem cells repopulated the host's blood cells. Other stem cells have demonstrated their potential in various diseases. For example, bone marrow-derived cells have been shown to:

1. Generate endothelial progenitor cells used to induce neovascularization of ischemic tissues;
2. Regenerate myocardium;
3. Give rise to bone, cartilage, and muscle cells; and
4. Migrate into the brain to produce neurons.

In addition, myoblasts isolated from skeletal muscle restored heart muscle function when injected into the heart, and neural stem cells that resulted in treatment of Parkinson's disease are examples of other potential adult stem cell-based therapies. Tissue engineering products based on cells have been developed in the form of skin substitutes through the use of allogeneic cells (from companies such as Organogenesis and Advanced Tissue Sciences). In addition, the injection of mesenchymal stem cells is under way for cartilage and bone regeneration.

ES cells provide an alternative source for cellular substitutes. *In vitro*, ES cells have been shown to generate cells of hematopoietic, endothelial, cardiac, neural, osteogenic, hepatic, and pancreatic tissues. Although ES cells provide a versatile source of cells for the generation of many cell types, so far only a few experiments have demonstrated the use of ES cells to replace functional loss of particular tissues. One such example is the creation of dopamine-producing

cells in animal models of Parkinson's disease. These ES cell-derived, highly enriched populations of midbrain neural stem cells generated neurons that showed electrophysiological and behavioral properties similar to neurons. Although the functional properties of neurons derived from hES cells still need to be investigated, it has been shown that hES cell-derived neural precursors can be incorporated into various regions of the mouse brain, where they differentiate into neurons and astrocytes. Also, hES cells which differentiated into neural precursors were shown to migrate within the host brain and differentiate in a region-specific manner. ES cells were also tested for future use in the heart. It was shown that murine ES cell-derived cardiomyocytes were morphologically similar to neighboring host cardiomyocytes. In addition, mouse ES cells transfected with an insulin promoter (driving expression of the *neo* gene, a marker for antibiotic resistance) have been shown to generate insulin-producing cells that can restore glucose levels in animals. Although these functional data were obtained using genetically modified ES cells, insulin production from ES cells suggests that these cells may potentially be used for the treatment of diabetes.

ES cells have also been shown to produce functional vascular tissue. Early endothelial progenitor cells isolated from differentiating mouse ES cells were shown to generate three blood vessel cell components: hematopoietic, endothelial, and smooth muscle cells. Once injected into chick embryos, these endothelial progenitors differentiated into endothelial and mural cells and contributed to the vascular development. We have shown that hES cells can differentiate into endothelial cells and have isolated these cells using platelet endothelial cell adhesion molecule-1 (PECAM-1) antibodies. *In vivo*, when transplanted into immunodeficient mice, the cells appeared to form microvessels.

39.2.2 Using Combinations of Cells and Materials

Tissue engineering approaches that use cells and scaffolds can be divided into two categories: open and closed systems. These systems are distinguished based on the exposure of the cells to the immune system upon implantation.

39.2.2.1 Open Systems

In open tissue engineering systems, cells are immobilized within a highly porous, three-dimensional scaffold. The scaffold could consist of synthetic or natural materials or composites of both. Ideally, this scaffold provides the cells with a suitable growth environment, optimum oxygen and nutrient transport properties, good mechanical integrity, and a suitable degradation rate. The use of scaffolds provides a three-dimensional environment, and brings the cells close so that it provides them with sufficient time to enable self-assembly and the formation of various components associated with the tissue microenvironment.

Ideally, the material degrades as the cells deposit their extracellular matrix molecules. The materials used for tissue engineering are either synthetic biodegradable materials – such as poly(lactic acid), poly(glycolic acid), poly(lactic-glycolic) acid (PLGA), poly(propylene fumarate), and polyarylates – or natural materials such as hydroxyapatite, calcium carbonate, collagen, or alginate. Natural materials are typically more favorable to cell adherence, but the properties of synthetic materials – such as degradation rate, mechanical properties, structure, and porosity – can be better controlled.

Open tissue engineering systems have been successfully used to create several biological substitutes, such as bone, cartilage, blood vessels, cardiac, smooth muscle, pancreatic, liver, tooth, retina, and skin tissues. Several tissue-engineered products are in clinical trials for Food and Drug Administration (FDA) approval. Engineered skin or wound dressing and cartilage are two of the most advanced areas with regards to clinical potential. For example, a skin substitute that consists of living human dermis cells in a natural scaffold consisting of type I collagen has already received FDA approval to be used for a diabetic foot ulcer. In addition, tissue-engineered cartilage and bone are also in clinical stages, and bladder and urologic tissue are being tested in various stages of research.

Despite the ability of stem cells to differentiate into cells with the phenotypic and morphological structure of desired cell types, there have been few scaffold-based tissue engineering studies that use ES cells. For adult stem cells, scaffolds have been used with mesenchymal stem cells, neural stem cells, and oval cells. One such example is the transplantation of neural stem cells onto a polymer scaffold which is subsequently implanted into the infarction cavities of mouse brains injured by hypoxia-ischemia. These stem cells generated an intricate meshwork of many neurites and integrated with the host. We have seeded neural stem cells onto specialized scaffolds, have demonstrated spinal cord regeneration, and have improved hind-leg function of adult rats from a hemisection injury model. Also, mesenchymal stem cells have been differentiated on polyethylene glycol or PLGA scaffolds and have been shown to generate cartilage or bone depending on the medium conditions.

ES cells may be differentiated in culture; desired cell types may be selected and subsequently seeded onto scaffolds. We have used this technique to study the behavior of ES cell-derived endothelial cells in tissue engineering constructs. Human ES cell-derived endothelial progenitors seeded onto highly porous PLLA/PLGA biodegradable polymer scaffolds formed blood vessels that appeared to merge with the host vasculature when implanted into immunodeficient mice (Figure 39.1).

There may be other approaches to using ES cells or their progeny with scaffold-based tissue engineering systems. For example, it may be possible to directly differentiate ES cells on scaffolds in culture. Finally, it may be

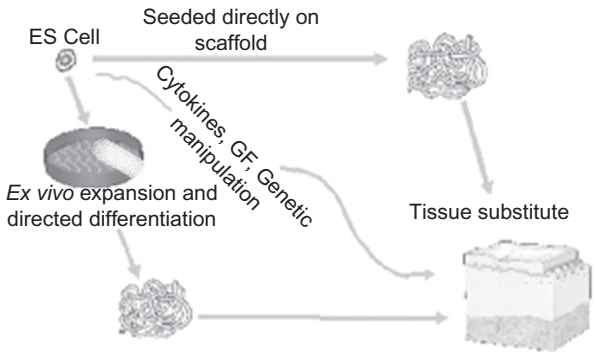

FIGURE 39.1 **Approaches for using ES cells for scaffold-based tissue engineering applications.**

ES cells can be used in tissue engineering constructs in a variety of methods. ES cells can be expanded in culture and then seeded directly onto scaffolds, where they are allowed to differentiate. Alternatively, stem cells can be directed to differentiate into various tissues and enriched for desired cells prior to the seeding of the cells onto scaffolds.

possible to differentiate genetically engineered ES cells seeded onto scaffolds *in vivo* (Figure 39.2).

Coercing cells to form tissues while differentiating is an important issue that has not been explored greatly. This may be achieved by seeding ES cells directly onto the scaffolds followed by inducing their differentiation *in situ*. Porous, biodegradable polymer scaffolds can be used to support the ES cells, and they are a promising system for allowing the formation of complex three-dimensional tissues during differentiation. The scaffold provides physical cues for cell orientation and spreading, and pores provide space for remodeling of tissue structures. These scaffolds should ideally provide the cells with cues to guide their differentiation into desired cell types. The possible advantages of this system could involve the assembly of the cells as they differentiate. This differentiation pattern may mimic the developmental differentiation of the cells much more closely and therefore may induce differentiation into desired tissue. Ultimately, *in vitro* differentiated constructs can potentially directly be used for transplantation.

An approach that has not been considered as an alternative to *in vitro* differentiation of ES cells is to use the adult body's microenvironment to induce the differentiation of ES cells. *In vivo* differentiation of ES cells is not yet a feasible option because of the tumorigenic nature of ES cells as well as the heterogeneous cell population that results from their nondirected differentiation. However, it may be possible to use a cell's apoptotic response mechanism to induce selective pressure for the desired cells *in vivo*. Thus, genetically modified ES cells that undergo apoptosis upon differentiation into undesirable cell types

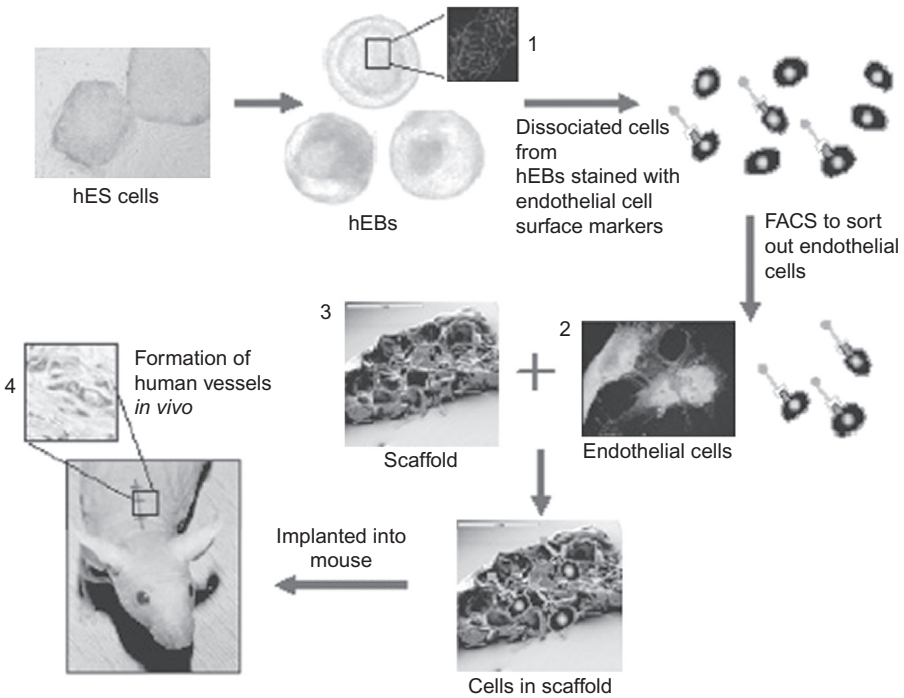

FIGURE 39.2 **Embryonic endothelial cells on scaffolds *in vivo.***
(1) Confocal image of vessel network formation within a 13-day-old human embryoid body (hEB), stained with PECAM-1 antibodies. The hES cells were induced to form EBs in which differentiation into endothelial cells and the formation of vessel-like network were observed. Embryonic endothelial cells were isolated from hEBs by staining dissociated EB cells with endothelial surface marker and sorting out positive cells using flow cytometry (FACS). (2) Isolated embryonic endothelial cells grown in culture stained with PECAM-1 and VWF antibodies. The isolated endothelial cells were seeded (3) on polymer scaffolds and implanted into immunodeficient mice. (C) Scanning electron microscopy of PLLA–PLGA scaffolds. (4) Immunoperoxidase staining of seven-day implants with anti-human PECAM-1 antibodies showing vessels lined with human endothelial cells. The embryonic endothelial cells appeared to form vessels *in vivo*.

could be used to direct the differentiation of these cells, and similar approaches could be adopted to control the proliferative behavior of these cells.

39.2.2.2 Closed Systems

One of the main difficulties associated with open tissue engineering systems is the potential immunological issues associated with the implanted cells. Closed systems aim to overcome this difficulty by immobilizing cells within polymeric matrices that provide a barrier for the immunological components of the host. For example, cells can be immobilized within semipermeable membranes that are permeable to nutrients and oxygen and can provide a barrier to immune

cells, antibodies, and other components of the immune system. Furthermore, the implants can be either implanted into the patient or used as extracortical devices. Closed tissue engineering systems have been used particularly for the treatment of diabetes, liver failure, and Parkinson's disease. This system may prove to be particularly useful in conjunction with ES cells, since the immobilization of ES cells within a closed system may overcome the immunological barrier that faces ES cell-based therapies. For example, ES cell-derived β-cells can respond to insulin, or domain-producing neurons can be used in clinics without the fear of rejection. In addition, closed systems protect the host against potentially tumorigenic cells as it limits the cells within the polymeric barrier. Currently, engineering and biological limitations such as material biocompatibility, molecular weight cutoff, and the immune system's reaction to shed antigens by the transplanted cells are some of the challenges that prevent these systems from widespread clinical applications.

39.3 LIMITATIONS AND HURDLES OF USING ES CELLS IN TISSUE ENGINEERING

Despite significant progress in the field of tissue engineering, there are several challenges limiting the use of ES cells in tissue engineering. These challenges range from understanding stem cell biology to questions concerning controlling stem cell fate, solving engineering challenges on scale-up, and answering business questions related to feasibility and pricing.

39.3.1 Directing the Differentiation of ES Cells

Perhaps the biggest challenge in using ES cells in clinical applications is the lack of knowledge in directing their differentiation ability. All studies that have shown the generation of specific cell types have not shown a uniform differentiation into a particular cell type. This may be attributed to the intrinsic property of ES cells to differentiate stochastically in absence of proper temporal and spatial signals from the surrounding microenvironment.

Techniques can be used to increase the ratio of cells that give rise to the desired lineages, including genetic and microenvironmental manipulations. Genetic techniques can be categorized into positive or negative regulators. The positive regulators include the constitutive or controlled expression of transcription factors that have been shown to derive the differentiation into particular tissues. For example, the overexpression of the Nurr transcription factor has been shown to increase the frequency of ES cells that differentiate into functional neural cells. Alternatively, the negative regulators could be incorporated to induce the apoptosis of cells that differentiate to varying pathways. For example, neomycin selection and suicide genes activated by certain transcription

factors can be used. Clearly, these techniques will benefit from further understanding of inner workings of transient cells and knowledge of the differentiation pathways and lineages. Further analysis into stem cell and progenitor hierarchy through high-throughput analysis of microarray or proteomics data should accelerate this process.

Another important criterion is the functionality of ES cell-derived cells as a source of tissues. The importance of rigorous testing has become clear in studies in which nestin-positive, putative pancreatic cells stained positive for insulin using antibodies because of cellular uptake from the surrounding medium. Thus the incorporation of protein and functional tests should accompany the morphological and phenotypic analysis often used in ES cell literature to characterize differentiated cells.

39.3.2 Isolating the Desired Cell Types for Therapy

One of the main problems with ES cell-based therapies is finding suitable techniques to isolate desired cells from the heterogeneous population. One approach is to allow random differentiation of ES cells followed by isolation using a cell surface marker. We have used this method for the isolation of ES cell-derived endothelial cells using the PECAM-1 receptor. Also, ES cell-derived hematopoietic progenitors have been isolated in a similar manner using the CD34 marker. Another potential method is through reporter gene knock-in modifications. These modifications have already been used on ES cells to allow the labeling of cells at various stages of differentiation. The use of other techniques, such as magnetic separation or neomycin selection, must be further examined for selecting various ES cell-derived progeny.

39.3.3 Scale-Up of ES Cells in Tissue Engineering

Although laboratory-scale ES cell cultures have been shown to produce differentiated progeny for both rodent and human ES cells, it is generally acceptable that these culturing methods are not feasible for the large-scale production of ES cells for therapeutic applications. The production of a sufficient quantity of differentiated cells from ES cells is an important challenge in realizing their clinical potential. The large-scale production of ES cells will likely be specific to the type of tissue being generated and must remain reproducible, sterile, and economically feasible. Furthermore, this scale-up process must maintain appropriate control over bioprocess conditions – such as mechanical stimuli, medium conditions, and physicochemical parameters (such as temperature, oxygen, pH, and carbon dioxide levels) – as well as growth factor and cytokine concentrations.

ES cell differentiation protocols have generally used two-dimensional cultures, embryoid bodies (EBs), or both. Although each technique provides specific

advantages, the differentiation of ES cells in EBs produces a wider spectrum of cell types. This has been attributed to the EBs' ability to better mimic the temporal pattern of cell differentiation as seen in the embryo. However, in some applications, the combined use of EBs and adherent cultures has resulted in better cell yields. For example, to induce ES cells to differentiate to cardiomyocytes, an EB formation in suspension cultures followed by a differentiation in adhesion cultures has been shown to optimize the percentage of cells that generate cardiomyocytes. Similarly, the production of hepatocytes has been shown to be induced first by culturing the cell in EBs then by culturing on two-dimensional cultures.

The formation of EBs in labs has been generally performed using techniques that have not been ideal for large-scale production. For example, many studies have used the 'hanging drop protocol,' in which ES cells are placed within a hanging drop and allowed to form an aggregate that can then be differentiated. Other techniques have formed EBs by placing the cells on nonadherent tissue culture dishes, which once more limit the quantity of cells produced. A technique that may allow the scale-up of the EB cultures is the use of suspension cultures using spinner flasks. Such cultures have been shown to enhance the supply of oxygen and nutrients to the cells within the EB by exposing the surface of the cell aggregate to a continuous supply of fresh medium.

To prevent the difficulties associated with EB heterogeneity, EBs have been immobilized in alginate microbeads. The microencapsulation of cells within these microbeads resulted in the differentiation of cells into cardiomyocytes and smooth muscle cells. In addition, ES cells may be adhered to beads with desired extracellular matrices and differentiated. This approach also enhances the transportation of medium and oxygen to the cells, in comparison to two-dimensional cultures, and provides additional mechanical stimuli, which may be an improved alternative to two-dimensional culture systems.

To enhance the supply of medium to tissue-engineered scaffolds or EBs, methods other than passive diffusion may be required, such as the use of perfusion systems in which the medium is flown through the scaffold. Perfusion bioreactors have already been developed for a variety of tissue engineering applications, such as cartilage and cardiac. For example, perfusion through scaffolds has been generated in rotating wall vessels or by pumping medium directly through the scaffolds to grow chondrocytes for cartilage generation.

It is known that mechanical forces affect the differentiation and functional properties of many cell types; thus, ES cell-based cultures that aim to direct the differentiation of ES cells require proper mechanical stimuli for the tissue. Our understanding of the effects of mechanical stimuli on ES cell differentiation is still primitive, but tissue engineering systems have been developed that incorporate the effects of mechanical forces. For example, functional autologous

arteries have been cultured using pulsatile perfusion bioreactors. Thus, the use of mechanical stimuli may further enhance the ability of these cells to respond to exogenous signals. Other environmental factors that may be required include the use of electrical signals and spatially regulated signals to induce the differentiation and allow maturation of the desired tissues. Hopefully, with time such techniques will become particularly important in allowing scaled-up ES cell-based tissue engineering applications. The development of bioreactors that control the spatial and temporal signaling that induces ES cell differentiation requires a collaborative effort between engineers and biologists and is currently in the early stages.

39.3.4 Tissue Engineering Limitations

Synthetic scaffolds that support tissue growth by serving as the extracellular matrix for the cells do not represent the natural extracellular matrix associated with each cell type and tissue. ES cells and their progeny during development reside in a dynamic environment; thus, synthetic or natural substrates that aim to mimic the developing embryo must present similar signaling and structural elements. Several approaches are under development that may yield useful for scaffolds in which ES cells are seeded. For example, the use of 'smart' scaffolds that release particular factors and/or control the temporal expression of various molecules released from the polymer could be used to induce the differentiation of ES cells within the scaffolds. For example, by dual delivery of vascular endothelial growth factor-165 and platelet-derived growth factor-BB – each with distinct kinetics – from a single, structural polymer scaffold, it has been shown that a mature vascular network can be formed. An alternative approach to modifying the surface exposed to the cells is to immobilize desired ligands onto the scaffold. For example, RGD peptides, the adherent domain of fibronectin, can be incorporated into polymers to provide anchorage for adherent cells.

Another difficulty with the current materials is their lack of control over the spatial organization within the scaffold. To create tissues that resemble the natural structure of biological tissues, the spatial patterning of cells must be recapitulated. For ES cells differentiated in scaffolds, this modeling and structure may be directly obtained as cells differentiate. The spatial arrangement of cells grown in EBs is typically organized with cells of particular tissues appearing in clusters. For example, blood precursors occur in the form of blood islands similar to their normal appearance in embryonic development. In the system in which ES cell-derived cells are plated onto scaffolds, spatial rearrangement can occur through direct patterning or cell 'reorganization.' In the direct cell patterning system, cells can be seeded into the scaffold at particular regions within the scaffold. For example, the direct attachment of two cell types on different

sides of the scaffold has been used to generate cells of the bladder. Cell patterning techniques, as have been developed using soft lithography for controlled coculture of hepatocytes and fibroblasts, could also be adapted for tissue engineering scaffolds to allow more controlled and complex cell patterning.

39.4 SUMMARY

ES cells have generated a great deal of interest as a source of cells for tissue engineering. However, several challenges exist in making ES cell-based therapy a reality. These include directing the differentiation of ES cells (using controlled microenvironments or genetic engineering), ensuring their safety and efficacy *in vivo*, ensuring the cells are immunologically compatible with the patient and will not form tumors, improving protocols for isolating desired cell types from heterogeneous populations, and enhancing current tissue engineering methods. Further research is required to control and direct the differentiation of ES cells. With the development of methods to generate tissues of various organs, this may lead to realizing the ultimate goal of tissue engineering. We are getting close to a day when ES cells can be manipulated in culture to produce fully differentiated cells that can be used to create and repair specific organs. Clearly, significant challenges remain, and the ability to overcome these difficulties does not lie within any scientific discipline but rather involves an interdisciplinary approach. Innovative approaches to solving these challenges could lead to improved quality of life for a variety of patients who could benefit from tissue engineering approaches.

FOR FURTHER STUDY

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CHAPTER 40

Ethical Considerations

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40.1 INTRODUCTION

There is consensus in the scientific community that hES cell research holds great promise for developing new treatments for a variety of serious and currently untreatable disease conditions. However, because hES cell research requires the manipulation and destruction of human embryos, it has also been a focus of ethical controversy and opposition. In the course of these debates, several challenging ethical questions have been raised. Scientists, clinicians, or patients involved in hES cell research or therapies must formulate their answers to these questions. Society, too, must address them to determine the extent to which hES cell research may require oversight and regulation. Here, I present these questions and examine some of the leading answers that have been proposed to them.

40.2 IS IT MORALLY PERMISSIBLE TO DESTROY A HUMAN EMBRYO?

Human embryonic stem (ES) cell lines are made by chemically and physically disaggregating an early, blastocyst-stage embryo and removing its inner cell mass. At this stage, the embryo is composed of approximately 200 cells, including an outer layer of differentiated placental material and the undifferentiated (totipotent or pluripotent) cells of the inner cell mass. The embryo inevitably dies as a result of this procedure. Hence the question: Can we intentionally kill a developing human being at this stage to expand scientific knowledge and potentially provide medical benefit to others?

At one end of the spectrum are those who believe that, in moral terms, human life begins at conception. For those holding this view, the early embryo is morally no different from a child or adult human being. It cannot be used in research that is not to its benefit, and it cannot be used without

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its consent. Furthermore, proxy consent by parents in such cases is inadmissible since it is an accepted rule of pediatric research that parents may not volunteer a child for studies that are not potentially beneficial and that risk the child's life. Many Roman Catholics, evangelical Protestants, and some Orthodox Jews take the position that life (morally) begins at conception, and they oppose hES cell research.

At the other end of the spectrum are those who believe that the embryo is not yet fully a human being in a moral sense. They hold a *developmental* or *gradualist* view of life's beginning. They do not deny that the early embryo is alive and has the biological potential to become a person. Nevertheless, they believe that other features are needed for the full and equal protections we normally accord children and adults and that these features only develop gradually across the full term of gestation. These features include such things as bodily form and the ability to feel or think. The early embryo, they maintain, does not have these features or abilities. They note as well that the very early embryo lacks human individuality, since it can still undergo twinning at this early stage, and two separate embryos with distinct genomes can still fuse to become a single individual. The very high mortality rate of such embryos (most never implant) also reduces the force of the argument from potentiality. Those who hold this view do not agree on the classes of research that warrant the destruction of embryos, but most support some form of hES cell research. Their reasoning is that, although the early embryo may merit some respect as a nascent form of human life, the lives and health of children and adults outweigh whatever claim it possesses.

Each individual faced with involvement in hES cell research must arrive at his or her own answer to this first question. Legislators and others must also wrestle with these issues. Because American law (and the laws of most other nations) does not regard the early embryo as a person meriting the legal protections afforded to children and adults, it is hard to see how one can justify legal or regulatory prohibitions on privately financed hES cell research or clinical applications. Such prohibitions would interfere with individual liberty on grounds inconsistent with the lesser view of the embryo shown elsewhere in the law. However, because publicly funded research rests on narrower political considerations, including the way that a majority chooses to spend public funds, it may be expected that public support for hES cell research will depend on how a majority of citizens answer this first question.

40.3 SHOULD WE POSTPONE hES CELL RESEARCH?

Some who oppose the destruction of human embryos maintain that hES cell research should be deferred at least until science provides a better view of the

likely benefits of adult stem cell research. They maintain that such research is as promising as hES cell research, and they argue that the moral acceptability of this alternative justifies any delay in the availability of therapies. Others add that scientific uncertainty and the ethically controversial nature of this research warrant a moratorium on hES cell studies in both the private and the public sectors. Scientific studies in this area continue to be equivocal, sometimes supporting and sometimes contradicting claims regarding the plasticity, the ability to immortalize, and the usefulness of adult stem cells. This raises the question of whether it is justified to delay the development of therapies and cures for children and adults to protect embryos and respect the sensitivities of those opposed to embryo research. Many people feel that such a delay is not warranted and that it is scientifically and morally preferable to keep open multiple pathways to stem cell therapies. In the words of the National Research Council:

> 'The application of stem cell research to therapies for human disease will require much more knowledge about the biological properties of all types of stem cells.'

40.4 CAN WE BENEFIT FROM OTHERS' DESTRUCTION OF EMBRYOS?

It might seem that a negative answer to the first question ends discussion. If the embryo is morally as human as you and me, what could justify the use of cells derived from its deliberate destruction for other people's benefit? However, research or therapy has many steps, not all of which involve the destruction of embryos. This raises the question of whether downstream researchers, clinicians, or patients may *use* the stem cell lines that others have derived. Ethically, this is a question of whether we can ever benefit from deeds with which we disagree morally or regard as morally wrong.

This question arises partly because most embryos used to produce stem cell lines are left over from infertility procedures. Couples using *in vitro* fertilization (IVF) routinely create more embryos than can safely be implanted. There are hundreds of thousands of these embryos in cryogenic freezers around the country and around the world. Since few frozen embryos are made available for adoption, most of these supernumerary embryos will be destroyed. In 1996, British law mandated the destruction of 3,600 such embryos. This destruction continues regardless of whether some embryos are diverted to hES cell research or not.

Why is it morally wrong to benefit from others' wrongdoing? One answer is that by doing so, we encourage similar deeds in the future. This explains why we are morally and legally prohibited from receiving stolen goods or why it

may be wrong to benefit from research produced by scientists who choose to ignore human-subject constraints. However, it seems less objectionable to benefit from others' wrongdoing when their deeds are independently undertaken, when our choices are not connected to theirs, and when these choices do not encourage the wrongful deeds. For example, few people would object to using the organs from a teenage victim of a gang killing to save the life of another dying child. The use of such organs benefits one young person and does not encourage teen violence. Similar logic might apply to stem cell research: using spare embryos remaining from infertility procedures. A downstream researcher, clinician, or patient may abhor the deeds that led to the existence of an hES cell line, including the creation and destruction of excess human embryos in infertility medicine. But nothing that a recipient of an hES cell line chooses to do is likely to alter, prevent, or discourage this continuing creation or destruction of human embryos or to make the existing lines go away. Those who use such embryos also know that if they refuse to use an hES cell line, they forego great therapeutic benefit. A researcher will fail to develop a lifesaving or health-restoring therapy. A clinician's decision may threaten a patient's life. People in this position will struggle with the question of whether it is worthwhile to uphold a moral ideal when doing so has no practical effect and when it risks injury to others.

Religious views on the question of whether one may ever benefit from others' wrongdoing are diverse. The Roman Catholic moral tradition, with its staunch opposition to complicity with wrongdoing, presents different answers, including some that permit one to derive benefit in particular cases. This suggests that some researchers, clinicians, or patients who morally oppose the destruction of human embryos may nevertheless conscientiously conclude that they can use hES cell lines derived from embryos otherwise slated for destruction.

It is noteworthy that in his August 2001 address to the nation, President George W. Bush adopted a conservative version of the position that allows one to benefit from acts one morally opposes. Stating his belief that it is morally wrong to kill a human embryo, the president nevertheless permitted the use of existing stem cell lines on the grounds that the deaths of these embryos had already occurred. Presumably, the president believed that it would not encourage further destruction of embryos to permit this use. The president did not go so far as to permit the use of lines derived from embryos slated for destruction. However, it is possible that if existing lines prove inadequate, as some scientists fear, many people would support a slightly more expansive version of this willingness to benefit from what one regards as objectionable deeds. The reasoning here would be that such embryos are unavoidably slated for destruction, and their use will not, by itself, encourage the creation or destruction of other embryos.

40.5 CAN WE CREATE AN EMBRYO TO DESTROY IT?

A fourth question takes us into even more controversial territory. Is it ever morally permissible to deliberately create an embryo to produce a stem cell line? This was done in the summer of 2002 at the Jones Institute in Norfolk, VA. Those in favor of this research defend it on several grounds. First, they say that in the future, if we seek to develop stem cell lines with special properties and perhaps closer genetic matches to tissue recipients, it will be necessary to produce stem cell lines to order using donor sperm and eggs. Second, they argue that it is *ethically* better to use an hES cell line created from embryos that have been produced just for this purpose, with the full and informed consent of their donor progenitors, than to use cell lines from embryos originally created for a different, reproductive purpose.

Those who believe the early embryo is our moral equal oppose the deliberate creation of embryos for research or clinical use. They are joined by some that do not share this view of the embryo's status but who believe that it is morally repellant to deliberately create a potential human being only to destroy it. Such people argue that this research opens the way to the 'instrumentalization' of all human life and the use of children or adult human beings as commodities. Some ask whether such research does not violate the Kantian principle that we should never use others as 'a means only.'

On the other side of this debate are those who believe that the reduced moral status of the early embryo permits its creation and destruction for lifesaving research and therapies. The proponents of this research direction ask why it is morally permissible to create supernumerary embryos in IVF procedures to help couples have children but morally wrong to do the same thing to save a child's life. They are not persuaded by the reply that the status of the embryo is affected by its progenitors' intent, such that it is permissible to create excess embryos for a 'good' (reproductive) purpose but not for a 'bad' (research) purpose. They point out that the embryo is the same entity. We do not ordinarily believe that a child's rights are dependent on its parents' intent or degree of concern. They conclude that it is not parental intent that warrants the creation of excess of embryos in such cases but the embryos' lesser moral status and the likelihood of significant human benefit from their use. These same considerations, they believe, justify deliberately creating embryos for stem cell research.

40.6 SHOULD WE CLONE HUMAN EMBRYOS?

A fifth question is whether we are willing to support human cloning for stem cell research. This question arises in connection to a specific stem cell

technology known as 'human therapeutic cloning.' It involves the deliberate creation of an embryo by somatic cell nuclear transfer technology (cloning) to produce an immunologically compatible (isogenic) hES cell line.

Immune rejection could occur if the embryo used to prepare a line of hES cells for transplant does not share the same genome as the recipient. This would be the case whether the cell line was created from a spare embryo or from one made to order. Therapeutic cloning offers a way around this problem. In the case of a diabetic child, the mother could donate an egg whose nucleus would then be removed. A cell would be taken from the child's body and its nucleus inserted into the egg cytoplasm. With stimulation, the reconstructed cell would divide, just like a fertilized egg. If the resulting embryo were transferred back to a womb, it could go on to birth and become a new individual – a clone of the child. But in therapeutic cloning, the blastocyst would be dissected and an hES cell line prepared. Growth factors could be administered to induce the cells to become replacement pancreatic cells for the child. Because these cells contain the child's own DNA and even the same maternal mitochondrial DNA, they would not be subject to rejection. Research has shown that the small amount of alien RNA from the mitochondria of an egg other than the mother's might not provoke an immune rejection.

Although this is a very promising technology, it raises a host of novel questions. One is whether the embryonic organism produced in this way should be regarded as a 'human embryo' in the accepted sense of that term. Those who believe that 'life begins at conception' tend to answer this question affirmatively, even though cloned 'embryos' are not the result of sexual fertilization. They base their view on the biological similarities between cloned and sexually produced embryos and on the argument that both have the *potential* to become a human being. Nevertheless, the very high mortality rate of cloned embryos suggests significant biological differences from sexually produced embryos. Furthermore, if their status rests on their potential, this potential is more than significantly reduced; in an era of cloning, some degree of potentiality attaches to all bodily cells.

The promise of this technology rests on the ability to make stem cell lines 'to order' for a specific patient. If you hold the view that the cloned organism is morally equivalent to a human embryo, therapeutic cloning research and therapies again raise the question of whether it is morally permissible to deliberately create an embryo in order to destroy it.

Another question often raised in this context is whether therapeutic cloning will create an enormous demand for human eggs. If it does, some believe this may create substantial problems of social justice, since collecting these eggs

may involve hundreds or thousands of women, many of whom are likely to be poor women of color attracted by the financial rewards of egg donation. Those who discount this problem offer several arguments. They point out that even if many eggs are needed for therapeutic cloning procedures, they are likely to be provided by relatives of patients. This reduces the magnitude of a market in oocytes. Others observe that therapeutic cloning is likely to be 'transitional research.' As such, it may lead the way to direct somatic cell reprogramming, eliminating the need for eggs altogether.

Finally, there is a moral question specific to cloning itself. The more scientists are able to perfect therapeutic cloning, the more likely it is that they will sharpen the skills needed to accomplish reproductive cloning, which aims at the birth of a cloned child. There is a broad consensus in the scientific and bioethics communities that, at this time, the state of cloning technology poses serious health risks to any child born as a result of it. There are also serious, unresolved questions about the psychological welfare of such children. Finally, there is the possibility that embryos created for therapeutic cloning research might be diverted to reproductive cloning attempts. All these concerns raise the question: Do we really want to develop cloning technology for the production of isogenic stem cells if doing so hastens the advent of reproductive cloning?

In 2001 and again in 2003, the US House of Representatives answered 'no' to this question and passed a bill introduced by Rep. James Weldon that banned *both* reproductive and therapeutic cloning. Similar bills have been introduced in the Senate. Although the Senate initiatives have been stalled for some time, this may soon change. If a Senate bill passes, therapeutic cloning research and therapies will be outlawed in the United States. Similar prohibitions are either in effect or being considered for passage in continental Europe. This would leave only a relatively small number of countries, including Great Britain, Israel, and China, in which such research would be allowed.

Those who oppose these prohibitions believe that therapeutic and reproductive cloning research can be decoupled. They observe that strict regulations and governmental oversight, of the sort provided in Great Britain by the Human Fertilisation and Embryology Authority, make it unlikely that embryos produced for therapeutic cloning will be diverted to reproductive purposes. They also point out that several researchers or groups with minimal qualifications in cloning research have announced their intent to clone a child or have even tried to do so. Such attempts are likely to continue regardless of whether therapeutic cloning research is banned. As a result, a ban on therapeutic cloning will not protect children and will only have the negative effect of interrupting beneficial stem cell research.

40.7 WHAT ETHICAL GUIDELINES SHOULD GOVERN hES CELL AND THERAPEUTIC CLONING RESEARCH?

Mention of the need to prevent the diversion of cloned embryos to reproductive purposes raises the larger question of what guidelines should apply to the conduct of hES cell research and therapeutic cloning research. In August 2000, the US National Institutes of Health released a series of guidelines for hES cell research that never went into effect because they were largely preempted by President Bush's decision to limit hES cell research to existing cell lines. Guidelines have also been developed by the Chief Medical Officer's Expert Group in Great Britain and by private ethics boards at the Geron Corporation and Advanced Cell Technology in the United States. The various recommendations share several features.

40.7.1 Donor Issues

Because hES cell and therapeutic cloning research require a supply of human gametes or embryos, steps must be taken to elicit the informed consent of donors, to protect their privacy, and to minimize any risks to which they might be subject. Informed consent requires that donors fully understand the nature of the research being undertaken and that they explicitly consent to that research. For example, it is morally impermissible to elicit sperm, eggs, or embryos for the production of hES cell lines without informing donors that an immortalized, pluripotent cell line might result that could be widely used in research or therapeutic applications. If there are likely to be commercial benefits flowing from the research, donors must also be informed of this, and their rights (if any) in such benefits should be clearly specified. If the research involves therapeutic cloning, both egg and somatic cell donors must be informed that a cloned embryo and a cell line with the egg donor's mitochondrial genetic material and a somatic cell donor's nuclear DNA will result.

In conducting research, efforts should be made to preserve donor privacy by removing identifying information from gametes, embryos, and cell lines and keeping this information apart in a secure location. In view of the controversy surrounding much of this research, donors can be subjected to harassment or embarrassment if their association with the research is revealed without their consent.

Ovulation induction is an invasive medical procedure with known and undetermined risks. Not only must egg donors be informed of these risks, but steps also must be taken to preserve the voluntary nature of their consent. This includes preventing them from being pressured into producing excess eggs or embryos for research in return for discounts on infertility services. It also includes avoiding undue financial incentives. Although it is

unreasonable to expect even altruistically motivated donors to undergo the inconvenience and risks of these procedures without some form of compensation, payment should not be so high as to lead a donor to ignore the risks involved. For Advanced Cell Technology's therapeutic cloning egg donor program, its Ethics Advisory Board set a payment level similar to that established for reproductive egg donors in the New England area. Payments were also prorated for the degree of participation in the program to allow donors to drop out at any time. Levels of stimulatory medications were maintained on the low side of current regimens, and payments were never attached to the number of eggs harvested. Additional protections ensured that donors had the educational level and backgrounds needed to appreciate the risks involved. A study monitor was employed to ensure that donors' consent was free and informed.

40.7.2 Research Conduct

Guidelines also apply to the actual conduct of research. These include the requirement that no embryo used in hES cell or therapeutic cloning research be allowed to develop beyond 14 days *in vitro*. This limit is based on the substantial changes that occur at gastrulation, which marks the beginning of individuation and organogenesis. Also required is supervision and accountability of all staff and scientists involved in this research to prevent any diversion of gametes or embryos to reproductive purposes.

40.7.3 Transplantation Research

If and when hES cell lines become available for research in transplant therapies, researchers and institutional review boards will have to address ethical questions raised by their use. For example, if such lines were cultured on mouse or other feeder layers, as almost all current lines are, there will be a safety question regarding the possible introduction of exogenous retroviruses or other pathogens into the human population. Basing their judgments on adequate preliminary animal studies, researchers and oversight bodies will have to assess the risks of rejection, including graft-versus-host disease. Issues surrounding the tumorigenicity of hES cells will also have to be resolved. Experience with fetal cell transplants for Parkinson's disease shows that cells can behave in unexpected ways when transplanted into the human body. None of these problems are insurmountable, however, and their existence itself constitutes an argument for continued research in this area under careful ethical oversight.

40.8 SUMMARY

Answering all of the questions I have identified would require an ethical treatise. However, by noting that I currently serve in a *pro bono* capacity as

chairman of the Ethics Advisory Board of Advanced Cell Technology, I indicate my own answers to the most controversial of these questions. I would not have accepted this position if I did not believe that hES cell and therapeutic cloning research is both therapeutically important and ethically acceptable. In my view, the moral claims of the very early embryo do not outweigh those of children and adults that can be helped by hES cell and therapeutic cloning technologies. I also do not believe that therapeutic cloning research will lead to reproductive cloning, which we should take firm steps to forbid. I recognize that others may disagree with these conclusions. As our debates move forward, continuing dialog about these questions and clearer scientific research results will bring us closer to a national consensus on these issues.

FOR FURTHER STUDY

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CHAPTER 41

Overview of the FDA Regulatory Process

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41.1 INTRODUCTION AND CHAPTER OVERVIEW

The field of regenerative medicine encompasses a breathtaking array of interdisciplinary scientific approaches which address a broad spectrum of clinical needs. Recent advances in scientific knowledge related to cell biology, gene transfer therapy, biomaterials, immunology, and engineering principles applicable to biological systems place the regenerative medicine community in a position to address a number of challenging and critical health needs. These include treatment of disease conditions resulting from pancreas, liver, and kidney failure; structural cardiac valve repair; skin and wound repair; and orthopedic applications. Scientific challenges confronting this field include expanding the knowledge base in each discipline as well as developing an interdisciplinary approach for identifying and resolving key questions. The Food and Drug Administration's (FDA's) regulatory review process mirrors the scientific challenges with regard to development of review paradigms that cross scientific disciplines.

This chapter will provide a brief historical review of the FDA and its organizational structure, as well as discussing topics pertaining to the regulation of regenerative medicine products, including possible regulatory pathways for combination products and relevant jurisdictional issues. Sources of information concerning FDA regulatory policies important to regenerative medicine product developers will also be discussed. It is essential for individuals,

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institutions, and companies, collectively referred to in FDA regulations as sponsors, responsible for the clinical trials of regenerative medicine products to be aware of FDA regulatory policies and how to obtain the necessary information. Suggestions will also be provided as to how to effectively engage FDA during the development of a novel regenerative medicine product.

41.2 BRIEF LEGISLATIVE HISTORY OF FDA

Medical products regulated by FDA include human and animal drugs, medical devices, and biological products. Among the therapeutic agents of biological origin regulated by FDA are vaccines, gene therapies, and cellular therapies, including products derived in whole or part from human tissue and xenotransplants, and blood products. In addition to medical products for human use, FDA regulates food other than meat and poultry, radiation-emitting products for consumer, medical, and occupational use, cosmetics, medical products for animal use, and animal feed.

FDA's role in medical product regulation extends throughout the entirety of the product life cycle. Depending on the product category, this may mean oversight, including review and inspection, of clinical trials, of the premarket product approval/clearance process, of manufacturing controls, controls over labeling, and registration and listing requirements. Once a product is marketed, FDA continues its oversight in a variety of ways, including inspections, mandatory and voluntary post-approval (e.g., phase 4) studies, and surveillance of adverse events reported to FDA.

FDA laws and regulations have developed over time, prompted partly in response to serious medical adverse events or by other public health and safety concerns. Early regulation of biological products was prompted in part by the death of 13 children in 1901 following administration of diphtheria antitoxin prepared from a source contaminated with tetanus. In response, Congress passed the Biologics Control Act in 1902. This act provided for regulation of viruses, serums, toxins, and analogous products; required licensing of manufacturing establishments and manufacturers; and provided the government with inspectional authority. The Act focused on requiring control of manufacturing processes for producing biological products, reflecting the extent to which the starting source material and the manufacturing process defined the final product.

In 1906, Congress passed the Federal Food and Drugs Act proposed in part in reaction to the meat-packing industry conditions described in Upton Sinclair's book *The Jungle*. While the primary focus of the Act was on food safety, the law also required that drugs be provided in accordance with standards of strength, quality, and purity unless otherwise specified in the label.

Premarket review of new drugs was not required until the passage of the 1938 Food, Drug, and Cosmetic Act (FD&C Act), which repealed the earlier 1906 Federal Food and Drugs Act. In 1937, the sulfa drug Elixir Sulfanilamide, previously available only in tablet or powder form to treat streptococcal infections, was marketed as a liquid using diethylene glycol, an analog of antifreeze, as a formulating solvent. This change in formulation, which was made without the requirement for premarket review, resulted in over 100 deaths, many of whom were children, prompting the passage of the 1938 FD&C Act. The 1938 Act also put medical devices and cosmetics under FDA authority and authorized factory inspections.

The Public Health Service Act (PHS Act), passed in 1944, incorporated the 1902 Biologics Control Act and is the present legal basis for licensing of biological products. Because most biological products also meet the definition of 'drugs' under the FD&C Act, they are also subject to regulation under that Act.

The requirement for premarket demonstration of efficacy and the authority for FDA oversight of clinical trials were provided by the Kefauver-Harris amendments to the FD&C Act in 1962. These amendments were prompted in part by the tragic adverse events resulting from use of thalidomide as a non-addictive prescription sedative. This drug, not approved as a sedative in the USA, when taken by pregnant women during the first trimester, resulted in thousands of birth defects for children born outside this country.

The Medical Device Amendments to the FD&C Act were passed in 1976, following reports of safety issues with respect to the Dalkon Shield intrauterine device. The Medical Devices Amendments included risk-based requirements for premarket notification or approval of medical devices. Prior to 1976, FDA authority was limited to taking action against marketed devices found to be unsafe or ineffective.

41.3 LAWS, REGULATIONS, AND GUIDANCE

The previous section summarized the history of laws that form the underpinning of FDA medical product regulation. This section provides a brief description of how laws are made and implemented, the procedures for promulgating regulations, and a description of how FDA develops and uses guidance documents.

Laws are created as an outcome of legislative activity conducted in the US Senate and House of Representatives resulting in passage of a bill. Once Congress passes a bill, it becomes law if signed by the President. If the President vetoes the bill, it becomes law if two-thirds of the Senate and House of Representatives vote in its favor. A federal law also is denoted as a public law and may contain a name, such as the FD&C or PHS Acts. These

laws are then incorporated into the US Code (USC) which is updated every six years, with supplements published regularly to incorporate changes to statutes between updates. Drugs, biologics, and device laws can be found in the US Code at:

- Drugs and Devices: Title 21 Chapter 9
- Biologics: Title 42 Chapter 6A.

When laws are passed, government agencies, such as FDA, often implement them by promulgating regulations. Sometimes, an agency may elect to promulgate regulations on its own whereas other laws may explicitly require an agency to issue regulation. The process for making regulations must be performed in accordance to the Administrative Procedures Act (Title 5, USC, Chapter 5). This Act generally requires agencies, such as FDA, to provide public notice and opportunity for comment as part of the rule-making process.

FDA regulations are contained in the Code of Federal Regulations (CFR). Regulations for drugs, biologics, devices, and tissues, along with related regulations, may be found in various parts of Title 21 of the CFR. The following is a list of key regulatory provisions:

- Drugs: 21 CFR Parts 200–299, 300–369
- Biologics: 21 CFR Parts 600–680
- Devices: 21 CFR Parts 800–898
- Human Cells, Tissues, and Cellular and Tissue-based Products: 21 CFR Parts 1270/1271
- Recalls: 21 CFR Part 7
- Informed Consent/Institutional Review Boards: 21 CFR Parts 50/56
- Financial Disclosure by Clinical Investigators: 21 CFR Part 54
- Good Laboratory Practice for Nonclinical Laboratory Studies: 21 CFR Part 58
- Good Guidance Practices: 21 CFR Part 10

Guidance documents are non-binding publications that describe FDA's interpretation of policy pertaining to a regulatory issue or set of issues related to:

1. The design, production, labeling, promotion, manufacturing, and testing of regulated products;
2. The processing, content, and evaluation or approval of submissions; and
3. Inspection and enforcement policies.

Guidance documents, which are developed in accordance with Good Guidance Practices found at 21 CFR 10.115, are intended to clarify FDA's current thinking related to regulatory issues and procedures. Unlike regulations and laws, guidance documents are not enforceable. Therefore, sponsors may elect to choose alternate approaches that still comply with existing laws and

regulatory requirements. In most cases, guidance documents are issued in draft for public comment before implementation. In cases where prior public participation is not feasible or appropriate, FDA may issue a guidance document for immediate implementation without first seeking public comment. Many of the guidance documents referred to in this chapter, although available to the public, may be in draft form. The publication of draft guidance documents is 'Not for Implementation' and reflects FDA's efforts to convey up-to-date information to those involved in the developing field of regenerative medicine.

When considering the development of a guidance document, FDA may freely discuss related issues with the public. In fact, FDA may hold a public meeting, advisory committee meeting, or workshop to obtain input on scientific issues. Finally, after receiving public input, FDA will evaluate submitted comments and finalize the document. Guidance documents are a useful way for FDA to communicate current thinking to the public. Within the arena of regenerative medicine, it is of value to be aware of both product-specific and cross-cutting guidance documents. Some of the more pertinent guidance documents to this field, such as those related to preclinical testing, manufacturing practices, and clinical trial design, are discussed in this chapter. In addition to guidance documents, FDA may refer to guidelines published by the International Conference on Harmonization (ICH). ICH is an international effort to harmonize regulatory requirements. ICH guidelines, similar to FDA guidance documents, are non-binding.

41.4 FDA ORGANIZATION AND JURISDICTIONAL ISSUES

Scientific development of regenerative medicine products involves extensive testing and planning prior to the initiation of clinical trials. It can be helpful for individuals and organizations involved in product development to engage in early dialog with the appropriate FDA review unit in order to receive and consider FDA's comments on the design of the preclinical and clinical development plan. This section describes FDA's organizational structure and provides basic information regarding jurisdictional decisions made to determine the appropriate regulatory pathway for a broad range of products.

FDA consists of seven centers and the Office of the Commissioner. Three of the centers are responsible for regulating medical products for humans. The Center for Biologics Evaluation and Research (CBER) has jurisdiction over a variety of biological products, including blood and blood products, vaccines and allergenic products, and cellular, tissue, and gene therapies, as well as some related devices. The Center for Devices and Radiological Health

(CDRH) has jurisdiction over diagnostic and therapeutic medical devices, administration of the Mammography Quality Standards Act (MQSA) program, and ensuring safety of radiation-emitting products. The Center for Drug Evaluation and Research (CDER) has jurisdiction over a variety of drug products, including small-molecule drugs, and well-characterized biotechnology-derived drug products that include monoclonal antibodies and cytokines.

For many medical use products it is clear which center within FDA shall have primary jurisdiction for the premarket review. For other products, including some technologically novel products under development, determining which center has jurisdiction for review may be unclear. Important starting points for determining product jurisdiction are the formal regulatory definitions of biological products, drugs, devices, and combination products, as well as contacts with the agency. The formal definitions are as follows:

- **Biological Product** [42 USC 262(i)]: A virus, therapeutic serum, toxin, antitoxin, vaccine, blood, blood component or derivative, allergenic product, protein (except for any chemically synthesized polypeptide) or analogous product, or arsphenamine or derivative of arsphenamine (or any other trivalent organic arsenic compound), applicable to the prevention, treatment, or cure of a disease or condition of human beings.
- **Drug** [21 USC 321(g)(1)]: (A) articles recognized in the official US Pharmacopeia, official Homeopathic Pharmacopeia of the USA, or official National Formulary, or any supplement to any of them; and (B) articles intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease in man or other animals; and (C) articles (other than food) intended to affect the structure or any function of the body of man or other animals; and (D) articles intended for use as a component of any articles specified in clause (A), (B), or (C).
- **Device** [21 USC 321(h)]: An instrument, apparatus, implement, machine, contrivance, implant, *in vitro* reagent, or other similar or related article, including any component, part, or accessory, which is:
  1. Recognized in the official National Formulary, or the US Pharmacopeia, or any supplement to them;
  2. Intended for use in the diagnosis of disease or other conditions, or in the cure, mitigation, treatment, or prevention of disease, in man or other animals; or
  3. Intended to affect the structure or any function of the body of man or other animals, and which does not achieve its primary intended purposes through chemical action within or on the body of man or other animals and which is not dependent upon being metabolized for the achievement of its primary intended purposes.

- **Combination Product** [21 CFR 3.2(e)]:
  1. A product composed of two or more regulated components, that is, drug/device, biologic/device, drug/biologic, or drug/device/biologic, that are physically, chemically, or otherwise combined or mixed and produced as a single entity;
  2. Two or more separate products packaged together in a single package or as a unit and composed of drug and device products, device and biological products, or biological and drug products;
  3. A drug, device, or biological product packaged separately that according to its investigational plan or proposed labeling is intended for use only with an approved individually specified drug, device, or biological product where both are required to achieve the intended use, indication, or effect and where upon approval of the proposed product the labeling of the approved product would need to be changed, for example, to reflect a change in intended use, dosage form, strength, route of administration, or significant change in dose; or
  4. Any investigational drug, device, or biological product packaged separately that according to its proposed labeling is for use only with another individually specified investigational drug, device, or biological product where both are required to achieve the intended use, indication, or effect.

FDA's Office of Combination Products (OCP), located in the Office of the Commissioner, has broad administrative overview responsibilities covering the regulatory life cycle of drug-device, drug-biologic, and device-biologic combination products. When jurisdiction is uncertain, sponsors may contact OCP for assignment of primary regulatory review responsibility for combination and other medical products. Jurisdictional determinations are made following a formal submission process called a Request for Designation (RFD). The appropriate FDA center jurisdiction is determined by considering the primary mode of action (PMOA) of the product. The definition of PMOA is clarified in the final rule issued by the FDA on August 25, 2005. Additional information regarding combination products and product jurisdiction are available at the OCP website.

41.5 APPROVAL MECHANISMS AND CLINICAL STUDIES

There are several premarket approval pathways for medical products, depending on whether the product is a drug, biological product, or device. Approval pathways, explained in more detail below, include the Biologics License Application (BLA) for biologics and New Drug Application (NDA)

for drugs. The Premarket Approval Application (PMA), Humanitarian Device Exemption (HDE) and 510(k) clearance mechanism are various regulatory pathways used for medical devices. Clarification on the type of application needed for a particular regenerative medicine product may be helpful to the sponsor early in development, to enable the sponsor to discuss the data needed for a marketing application during the planning stage.

A BLA is an application for licensure under the PHS Act; the approval standards set forth in the statute are demonstration that the product is safe, pure, and potent. Further information concerning the licensure of biological products is provided in 'Guidance for Industry: Providing Clinical Evidence of Effectiveness for Human Drugs and Biologic Products.' A PMA is an application for approval for most Class III medical devices; the sponsor must show reasonable assurance of safety and effectiveness. Under medical device regulation a product can also gain approval as an HDE, which is not a full marketing approval but requires demonstration of safety and probable benefit. To qualify for this type of application, a sponsor would need to first receive a designation from FDA Office of Orphan Products Development that the device is a Humanitarian Use Device (HUD), intended for treatment or diagnosis of a disease or condition that affects or is manifested in fewer than 4,000 individuals per year in the USA. The 510(k) clearance process applies to products that are 'substantially equivalent' to a Class I or II (or in a few cases, a Class III) device already on the market.

Many, but not all, combination products are approved or cleared under one marketing application. For example, depending on the specific facts, including the primary mode of action of the product, a combination biological device could be licensed under the biologics authorities or approved under the medical device authorities. Following approval of a marketing application there are also postmarketing requirements such as reporting. In addition, modifications to the product or labeling may require prior approval. FDA has published regulations and guidance documents that address submission and approval processes for modifications to marketed products. Compliance with manufacturing requirements is also an ongoing sponsor obligation. FDA has issued a draft guidance document entitled 'Draft Guidance for Industry and FDA: Current Good Manufacturing Practice (cGMPs) for Combination Products' which provides direction on applicable manufacturing requirements for combination products. Due to the relatively new nature of regenerative medicine and its developmental status, postapproval topics will not be further discussed in this chapter.

In circumstances when clinical investigation is needed to evaluate the safety and efficacy of an investigational product prior to marketing approval, an Investigational New Drug (IND) application is required for drugs and

biologics, and an Investigational Device Exemption (IDE) is generally required for devices. For both types of applications, the sponsor needs to submit a description of the product and manufacturing process, preclinical studies, a clinical protocol, information on any other prior investigations such as human clinical studies, and a rationale for the study design. An Institutional Review Board (IRB) review and informed consent are also required. FDA has 30 days to review the application to determine if the study may proceed. The contents are specifically laid out in FDA regulations for each type of application. Requirements for the content of an IND can be found at 21 CFR 312.23 and for an IDE at 21 CFR 812.20.

For some products, there may be applicable guidance with respect to developing the manufacturing or the preclinical data to support the study. For example, the 'Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Somatic Cell Therapy Investigational New Drug Applications (INDs)' discussed in the following section provides information on characterization and manufacturing of a cellular product to be submitted in an IND. Applicable regulations and guidance should be further consulted for information on adverse event reporting, labeling, study conduct and monitoring, and other topics related to requirements for conducting an IND. For information on general clinical study design and conduct issues, FDA has many guidance documents that may be helpful. For some indications there may be guidance documents that apply across technologies, such as the 'Guidance for Industry: Chronic Cutaneous Ulcer and Burn Wounds – Developing Products for Treatment.' In addition, guidance documents not directly applicable for a specific product, indication, or technology may be worth consulting, as the documents may provide some insights into general clinical issues such as assessment parameters that may be of value.

41.6 MEETINGS WITH INDUSTRY, PROFESSIONAL GROUPS, AND SPONSORS

Although the terminology and procedures may vary, all three FDA Centers performing medical product review encourage meetings with sponsors to address questions prior to a regulatory submission and at specific developmental milestones. When requesting a formal or informal meeting with FDA it is helpful to provide background information as well as specific discussion questions. Further information about formal meetings, such as what to include in a meeting request, and what type of information to include in the information package submitted prior to the meeting, is provided in 'Guidance for Industry: Formal Meetings with Sponsors and Applicants for prescription drug user fee Act (PDUFA) Products.' Early stage device meetings

are addressed in 'Early Collaboration Meetings under FDA Modernization Act (FDAMA); Final Guidance for Industry and CDRH Staff.'

FDA also interacts with organizations representing a group of interested parties (e.g., International Society for Cellular Therapy, American Association for Blood Banks, and Pharmaceutical Research and Manufacturers of America), which provides an opportunity to discuss topics of interest to FDA and the organization. These interactions can be very valuable for FDA and stakeholders as they are a way to better understand general issues of concern, as opposed to product-specific discussions with individual firms. In addition to such interactions and meetings with individual sponsors, FDA has various advisory committees that review available data and information, and make recommendations related to a variety of issues, many of which are pertinent to the field of regenerative medicine. Advisory committees will be discussed further in the 'Advisory Committee Meetings' section.

41.7 REGULATIONS AND GUIDANCE OF SPECIAL INTEREST FOR REGENERATIVE MEDICINE

The topics discussed thus far have been of general applicability for medical product regulation: marketing pathways, clinical trial regulation, meetings, guidance development, and related topics. This section will review a few topics of particular interest to the scientific community engaged in development of regenerative medicine products: FDA regulations on human tissue products, product characterization for cellular products, FDA policy and guidance on xenotransplantation, and gene therapy.

41.7.1 Regulation of Human Cells and Tissues Intended for Transplantation

An understanding of the regulations applicable to cells and tissues is important for developers of regenerative medicine products since human cells or tissues comprise the whole, or are a key component, of many products.

In 1997, noting the fragmented approach to regulation of human cell and tissue-based products, FDA issued the 'Proposed Approach to the Regulation of Cellular and Tissue-Based Products.' This document proposed a tiered risk-based approach to regulation of these products. According to the proposed approach, products posing less risk would be subject to the rules designed to minimize communicable disease risks, and additional regulatory requirements would be imposed on those products posing additional risk. The proposed approach to regulation of human tissues was implemented in three parts, collectively referred to as the 'tissue rules': Establishment Registration and Listing, Donor Eligibility, and Good Tissue Practices (GTP). The complete set of rules went into effect on May 25, 2005, and is codified in 21 CFR

Part 1271. The tissue rules derive from the statutory authority of section 361 of the PHS Act, which addresses control of spread of communicable diseases. Because the tissue rules apply to all human cellular and tissue-based products, it is important for sponsors of regenerative medicine products to be aware of these rules, as well as the specific additional requirements for biologics or devices that may apply depending on the particular regulatory pathway for their products.

With some exceptions that are noted in the tissue rules, human cells or tissue intended for implantation, transplantation, infusion, or transfer into a human recipient are regulated as a human cell, tissue, and cellular and tissue-based product (HCT/P). Examples of HCT/Ps are: musculoskeletal tissue, skin, ocular tissue, human heart valves, dura mater, reproductive tissue, and hematopoietic stem/progenitor cells. Tissues specifically excluded are: vascularized organs, minimally manipulated bone marrow, blood products, xenografts, secreted or extracted products such as human milk and collagen, ancillary products, and *in vitro* diagnostic products.

The tissue rules require that tissue establishments do the following:

- register and list their HCT/Ps with FDA (21 CFR 1271 Subparts A and B);
- evaluate donors through screening and testing, to reduce risk of transmission of infectious diseases through tissue transplantation (21 CFR 1271 Subpart C);
- follow Current Good Tissue Practices to prevent the spread of communicable disease (21 CFR 1271 Subpart D).

Additional requirements for reporting, labeling, inspections, importation, and enforcement are described in 21 CFR 1271 Subparts E and F; these provisions apply only to HCT/Ps regulated solely under section 361 of the PHS Act, and therefore would not apply to most regenerative medicine products.

The Establishment Registration rule defines the circumstances under which a product would be subject to the tissue rules only (21 CFR 1270.10), and when there would be additional regulatory oversight such that a BLA, PMA, or other marketing application would be required (21 CFR 1271.20). Products that meet the following conditions are regulated by FDA solely under the tissue rules:

1. The HCT/P is not more than minimally manipulated;
2. The HCT/P is intended for homologous use;
3. The HCT/P is not combined with a drug or device (with certain exceptions); and
4. The HCT/P does not have a systemic effect and is not dependent upon the metabolic activity of living cells for its primary function (except for autologous use or allogeneic use in a first or second degree blood relative, or reproductive use).

If any of these four conditions is not met, a marketing application is required. The Tissue Reference Group (TRG) handles various inquires from stakeholders concerning application of the tissue rules including generating recommendations for consideration for CBER, CDRH, and OCP regarding whether specific HCT/Ps meet the criteria specified in 21 CFR 1271.10 for regulation solely under section 361 of the PHS Act. Additional information and documents regarding these rules, as well as electronic forms for registration and listing, can be found on FDA's website.

A joint FDA-CDC (Center for Disease Control and Prevention) workshop held in 2007 on the Processing of Orthopedic, Cardiovascular and Skin Allografts is of relevance to the regenerative medicine field. The workshop discussed pertinent information regarding current clinical practices and expectations for the graft, effect of processing on graft management, and adverse event assessment/management and challenges associated with current microbiological methods used for pre- and post-processing cultures and with disinfection/sterilization of tissues. Some of the manufacturing topics discussed at the workshop are addressed in guidance for industry entitled: 'Guidance for Industry: Current Good Tissue Practice (CGTP) and Additional Requirements for Manufacturers of Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps).'

FDA has issued 'Guidance for Industry: Eligibility Determination for Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products' to assist establishments making donor eligibility determinations with complying with the Donor Eligibility rule (21 CFR 1271 Subpart C). This guidance also incorporates and finalizes the content of 'Guidance for Industry, Preventive Measures to Reduce the Possible Risk of Transmission of Creutzfeldt-Jakob Disease (CJD) and Variant Creutzfeldt-Jakob Disease (vCJD) by Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps).'

41.7.2 Human Cellular Therapies

Many products in development for tissue repair or replacement are composed of cells or cells combined with a scaffold. The cell or tissue source and manufacturing process may vary greatly for different products. Despite the diversity in products, there are regulatory considerations that apply to all cellular preparations being developed as investigational regenerative medicine products intended for early phase clinical studies. Among these considerations are three that will be discussed briefly: control of the source material, demonstrated control of the manufacturing process, and characterizations of the cellular product that results from the manufacturing process.

The cell source will vary for different products and may be autologous or allogeneic, undifferentiated stem/progenitor cells, or terminally differentiated

cells. Assuring the safety of source cellular materials used during manufacture of an investigational regenerative medicine product begins by determining the eligibility of the donors selected to provide the source material through screening and testing. This screening and testing is part of the tissue rules described earlier in this chapter. Autologous products are not required to comply with the donor screening and testing requirements in the tissue rules. However, if autologous tissue either is positive for specific pathogens or has not been screened or tested, it is recommended that manufacturers document if tissue culture methods could propagate or spread viruses or other adventitious agents to persons other than the recipient. Donor eligibility determination is required for all allogeneic donors of cells and tissues.

In addition to the possibility of infectious disease transmission, there are other aspects of the cell source that may raise substantial concerns. In addition to screening and testing donors for communicable disease agents, according to the 'ICH Guidance on Quality of Biotechnological/Biological Products: Derivation and Characterization of Cell Substrates Used for Production of Biotechnological/Biological Products,' FDA has suggested that sponsors consider the importance of evaluating donor medical history information and the relevance of conducting specified molecular genetic testing as part of an overall comprehensive assessment program to establish the suitability of a specific cellular preparation for use in the manufacture of a regenerative medicine somatic cellular product. The rationale for and feasibility of collecting additional information about molecular genetic testing were discussed in a public meeting of FDA Biological response modifiers Advisory Committee (now known as Cellular, Tissue and Gene Therapies Advisory Committee (CTGTAC)) convened July 13–14, 2000 on the topic of 'Human Stem Cells as Cellular Replacement Therapies for Neurological Disorders.' A description of the physiological source of the cellular material, including tissue of origin and phenotype such as hematopoietic, neuronal, fetal, or embryonic, conveys important information about the cells and their critical attributes.

Control of the manufacturing process helps provide assurance of the consistent, reproducible production of the cellular component. Often, manufacturing will involve a multistep process that must be performed using aseptic techniques to prevent introduction of microbial contamination. Many types of reagents may be used to manufacture the cellular component of a product, including those that promote cellular replication, induce differentiation, and those used to select targeted cell populations, specifically, serum, culture medium, peptides, cytokines, and monoclonal antibodies. It is essential that reagents be properly qualified. Demonstration of manufacturing control is evidenced by strict adherence to standard operating procedures and quality control assessment of manufacturing intermediates as well as testing of the final cellular preparation.

Due to inherent biological complexity it is unlikely that a unique biomarker or other single analytical test will be sufficient to permit full characterization of a cellular product. Accordingly, as recommended in 'Guidance for Industry: Guidance for Human Somatic Cell Therapy and Gene Therapy,' FDA asks sponsors to provide documentation that their testing paradigm developed for the final cell product encompasses a multiparametric approach that may involve biological, biochemical/biophysical, and/or functional characterization. Tests developed to demonstrate identity of the cell product (physical and chemical characteristics, identify the product as being what is designated on the label), purity (freedom from contaminants including residual reagents and unintended cell populations), and potency/biological activity (the specific ability of the cells, as indicated by appropriate laboratory tests, to effect a given result) should be conceived to determine the degree to which the characteristics of the manufactured cell preparation conform to desired and specified criteria. This process can be challenging for a number of reasons. For example, the mechanism of action associated with a cell product may be incompletely understood and this constrains the ability to develop a specific potency assay. Direct assessment of potency for a cellular preparation may not be possible due to a lack of appropriate *in vitro* or *in vivo* assay systems. On February 9–10, 2006, FDA CTGTAC discussed this challenging topic and obtained input on alternative approaches for performing potency assessments of cellular therapy products. FDA's recommendations for developing tests to measure potency for cell and gene therapy products can be found in 'Guidance for Industry: Potency Tests for Cellular and Gene Therapy Products.'

In summary, assuring the safety of cell products that in and of themselves constitute a regenerative medicine product or that constitute a component of a product requires demonstrated control over each facet of the manufacturing process. This assurance begins with acquisition of the source material and is carried forward through manufacturing and characterization of the final cellular preparation using specified analytical tests based in large measure on the intrinsic biological properties of the cell product.

41.7.3 Xenotransplantation

The success of allogeneic organ transplantation has increased the demand for human cells, tissues, and organs. Scientific advances in the areas of immunology and molecular biology coupled with the growing worldwide shortage of transplantable organs have led to increased interest in xenotransplantation. In addition to the potential use of xenotransplantation to address the shortage of human organs for transplantation, there are increasing efforts to utilize other xenotransplantation products in the treatment of disease. An example of this is the use of encapsulated porcine pancreatic islet cells for the treatment of type 1 diabetes. Along with the promise of xenotransplantation are a number

of challenges, including the potential risk of transmission of infectious agents from source animals to patients, and the spread of any zoonotic disease to the general public. In addition, the potential exists for recombination or reassortment of source animal infectious agents, such as viruses, with nonpathogenic or endogenous human infectious agents, to form new pathogenic entities. These considerations demonstrate the need to proceed with caution in this area.

The US PHS Agencies including FDA, National Institutes of Health (NIH), Centers for Disease Control and Prevention (CDC), and Health Resources and Services Administration (HRSA) have worked together to address the risk of infectious disease transmission, publishing the 'PHS Guideline on Infectious Disease Issues in Xenotransplantation.' This Guideline discusses xenotransplantation protocols, animal source, clinical issues, and public health issues. Following publication of the PHS Guideline, FDA published a Guidance document entitled 'Guidance for Industry: Source Animal, Product, Preclinical and Clinical Issues Concerning the Use of Xenotransplantation Products in Humans' to build on the concepts in the PHS Guideline, and provide more specific advice regarding xenotransplantation product development and production, and xenotransplantation clinical trials.

Xenotransplantation is defined in the PHS Guideline and FDA Guidance as any procedure that involves the transplantation, implantation, or infusion into a human recipient of either live cells, tissues, or organs from a nonhuman animal source or human body fluids, cells, tissues, or organs that have had *ex vivo* contact with live nonhuman animal cells, tissues, or organs.

Examples of xenotransplantation products provided in FDA Guidance are:

- Transplantation of xenogeneic hearts, kidneys, or pancreatic tissue to treat organ failure, implantation of neural cells to ameliorate neurological degenerative diseases;
- Administration of human cells previously cultured *ex vivo* with live nonhuman animal antigen-presenting or feeder cells;
- Extracorporeal perfusion of a patient's blood or blood component through an intact animal organ or isolated cells contained in a device to treat liver failure.

Medical products that do not contain living cells are not considered to be xenotransplantation products by this definition. Therefore, products that include some common animal-derived components such as collagen, small intestinal submucosa (SIS), and heart valves do not automatically fall under this category. When a product does meet the definition, xenotransplantation guidelines are applied as appropriate to the specific product. FDA encourages any potential sponsor of a xenotransplantation product to familiarize themselves with available documents that can be found on FDA's website.

41.7.4 Gene Therapy

FDA regulates human gene therapy products as biological products. The field of gene therapy holds great promise for treating a wide array of illnesses, from genetically inherited diseases such as cystic fibrosis or hemophilia, to heart disease, wound healing, AIDS, graft versus host disease, and cancer. The use of gene therapy in the area of tissue repair and tissue engineering is also being investigated.

There are a number of safety issues associated with gene therapy, some of which are unique to this area. Safety issues specific to gene therapy trials include generation of replication competent virus, vector, as well as transgene-associated immune responses, toxicity associated with transgene expression, and inadvertent germline transmission of vector. Two examples of gene therapy-specific risks are instructive:

1. High doses of adenovirus vector particles have been shown to induce toxicity under certain circumstances, and resulted in the death to a study subject in 1999; and
2. Genomic integration of retroviral vectors has been shown to result in genotoxicity.

In the latter case, five children developed leukemia and one died as a direct result of altered gene expression after vector integration. Detailed recommendations from FDA regarding what type of information to submit in an early phase study of gene therapy products are available in FDA 'Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs).' This guidance covers product manufacturing and characterization information (including components and procedures), product testing (including microbiological testing, identity, purity, potency, and other testing), final release testing criteria, and product stability, giving specifics in these areas that are pertinent to gene therapy. Suggested preclinical testing includes tests designed to describe localization, and persistence of gene expression. For vectors intended for direct *in vivo* administration, demonstrating the extent of dissemination and gonadal distribution is suggested.

Gene therapies may differ from conventional drugs in that vector and transgene expression may persist for the lifetime of the subject. In these cases, there is a risk of delayed adverse effects. Indeed, the previously mentioned leukemias in a clinical study of gene therapy for the treatment of X-linked severe combined immunodeficiency (SCID) did not occur until approximately three years after exposure to the retroviral vector. These events highlight the need to assess long-term risk in research subjects. FDA has discussed these issues, noting that the assessment of risk is based on the persistence of vector sequences, the potential for integration into the host genome, and transgene-specific

effects. FDA has published 'Guidance for Industry: Gene Therapy Clinical Trials – Observing Subjects for Delayed Adverse Events' which addresses the duration and types of observations to be performed based on the patient population and the risks presented by the gene therapy product.

Although regulatory authority for gene therapy trials rests with FDA, NIH serves an important complementary role. In addition to funding a number of gene therapy research studies, NIH provides an important forum for open public deliberation on the scientific, ethical, and legal issues raised by recombinant DNA technologies and its basic and clinical research applications. This is accomplished through the Recombinant DNA Advisory Committee (RAC), an expert advisory committee to the NIH Director. Clinical studies discussed in this forum include studies funded by NIH, as well as industry-funded studies conducted at clinical sites receiving NIH funding for recombinant DNA research.

41.7.5 Cell-Scaffold Combination Products

Cell-scaffold combination products often face unique product development challenges because of their inherent complexity. These products often combine metabolically active cells and extracellular matrix or other scaffold components into complex three-dimensional structures, making the manufacturing, characterization, and study of these products a challenge. Such a complex product that is derived from chemically or physically combining multiple entities cannot be defined solely by the characteristics of the individual components alone. Other factors such as product assembly and the resulting cell-scaffold interactions play critical roles in determining final product characteristics. Furthermore, these products are commonly designed to remodel *in vitro* during processing in bioreactors and/or *in vivo* post-transplantation during clinical use, thereby precluding complete functionality testing by product release. Packaging, shipping, and shelf-life for these dynamic cell-scaffold products are also nontrivial considerations.

As with other products, product safety and efficacy of cell-scaffold products need to be supported by a combination of appropriate *in vitro* and *in vivo* preclinical testing. FDA draws upon its extensive experience in regulating mammalian cell products and other tissue-derived products in evaluating product safety and efficacy. Many of the important tests needed for the individual components, such as sterility, mycoplasma, pyrogenicity/endotoxin, scaffold characteristics, cellular viability, identity, and purity, are applicable for the combined product as well. Demonstration of product potency and/or performance is also necessary and may require the development of new scientific techniques and assays. A critical consideration for developers of cell-scaffold combination products is determining which tests need to be conducted on individual components prior to assembly and which are most relevant after

product assembly. As each product is often unique, with its own set of potential safety issues for a specific clinical indication, preclinical evaluation of these products in the appropriate animal models provides important data to establish controls for product development and manufacture. In addition, it is strongly recommended that sponsors engage FDA early in the product development process to obtain feedback on key considerations.

For many innovative products, such as cell-scaffold combinations used for regenerative medicine, the final product and instructions for use can be expected to undergo iterative modifications over time. Consequently, refinement of the product by the sponsor and review of product modifications by FDA will be an ongoing process. It is critical for the sponsor to have a good understanding of the product and key scientific and/or clinical issues that can affect safety and efficacy of the product, including the establishment of appropriate manufacturing controls to ensure product quality and consistency. When changes in composition or manufacturing of the cell and/or scaffold component of the combination product are made, it is essential that the sponsor fully evaluate the impact of such changes to the final product's quality and function.

41.7.6 Case Study: Cell-Scaffold Wound Healing Skin Constructs

Some of the earliest successes in developing cell-scaffold combination products for medical use produced skin-like constructs for wound management. These products are worth a special mention in this chapter because there are a number of FDA-approved products currently on the market. These products are often composed of keratinocyte and/or fibroblast cells combined with a scaffold (e.g., animal collagen, glycosaminoglycans, or gauze). Cell-scaffold wound dressings for the skin that function primarily as physical wound coverings have generally been reviewed by CDRH.

Aspects of product manufacturing that are common to these products are cell, tissue, and scaffold material sourcing, product processing, in-process and final product tests, and quality control procedures to ensure lot-to-lot safety, effectiveness, and consistency. Considerations regarding product shipment and shelf-life are nontrivial in the manufacture and distribution of these skin constructs that contain living cells. Preclinical evaluation of these products, including study in animal models for wound healing, provide important data to establish controls in product development and manufacture.

Apligraf and Orcel are two examples of commercially available bilayered co-culture constructs composed of allogeneic neonatal keratinocyte and fibroblast cells on bovine collagen scaffolds. Apligraf is indicated for treatment of venous insufficiency and diabetic foot ulcers and Orcel is approved

for treating split-thickness donor site wounds on burn patients. An HDE for Orcel use in a patient with recessive dystrophic epidermolysis bullosa (DEB) as an adjunct in covering wounds and donor sites after the surgical release of hand contracture and deformities was approved in 2001. Lastly, Epicel consists of autologous keratinocytes (ranging from two to eight cell layers in thickness) layered on petrolatum gauze. Notably, Epicel is defined as a xenotransplantation product because, as part of the manufacturing process, the keratinocytes are co-cultivated with a feeder layer of proliferation-arrested, murine 3T3 fibroblasts. The HDE for Epicel was approved in 2007 for use in patients who have deep dermal or full-thickness burns comprising a total body surface area of greater than or equal to 30%.

Information about the clinical performance of each product is available in the published literature and product labeling. Summaries of Safety and Effectiveness Data (SSEDs) that provide information about the clinical studies, as well as other studies supporting approval, for each of these approved products are available on FDA's website using a searchable database (using the search term 'product code MGR'). Information on approved HDEs and associated Summary of Safety and Probable Benefit are also available on FDA's website.

41.7.6.1 Preclinical Development Plan

For device and drug clinical trials, the goal of preclinical development studies is to establish a scientific rationale for the clinical investigation and to demonstrate an acceptable safety profile. Traditional pharmacology/toxicology safety studies are important to identify potential toxicity in target organs and tissues, and to obtain information on effective safe starting doses in humans as well as establishing a safety profile for dose escalation and/or clinical monitoring. For cellular therapy, gene therapy, and cell-scaffold combination products there are often additional product-specific safety questions that might need to be addressed prior to initiation of a clinical trial. For example, what is their potential to undergo unanticipated undesired changes in their characteristics, such as malignant transformation? For cell-scaffold constructs, are there safety issues associated with the implantation procedure or potential construct failure? Animal models have limitations that are confounded by anatomical as well as physiological differences between the animals and humans. For some products, *in vitro* analyses may play a critical role in product characterization and evaluation for safety, efficacy, and potency. Non-destructive product testing and rapid characterization tests may also aid in product development. Many of these products, because of their novelty, do not have an established paradigm for preclinical evaluation, and sponsors are therefore encouraged to discuss their development plan with FDA early in the development process.

FDA may hold public discussions at an advisory committee meeting, at workshops, or engage the public in guidance development, on areas of importance to researchers developing these novel products. For example, the goals, challenges, and methods of *in vitro* analyses of cell-scaffold products were discussed at an FDA-NIST co-sponsored workshop on December 6, 2007. FDA also held a discussion at an advisory committee meeting of the CTGTAC on April 10, 2008 regarding preclinical safety testing of cellular therapies derived from human embryonic stem cells (hESCs). These meetings are important for FDA to gain scientific insight to inform policy, and can be informative for researchers who are considering these types of issues.

41.7.6.2 Clinical Development Plan

The goal of the clinical development program is to establish product safety and efficacy. In the field of regenerative medicine, variability in the product, as well as the patient, poses unique challenges in clinical trial design and conduct. An additional challenge is the need, with many of these products, to observe their integration into the host over a prolonged period. Specific feedback regarding the adequacy of certain proposed studies and predictors of clinical benefit can be provided to sponsors through the use of meetings with FDA at various development time points, and the use of a Special Protocol Assessment (for products regulated as a biologic) or an Agreement Meeting (for products regulated as a device) prior to initiation of their Phase III studies.

As with preclinical issues, FDA may hold workshops or advisory committee meetings to discuss pertinent topics that affect clinical development. FDA convened a workshop on Nov 2, 2010 regarding cell and gene therapy clinical trials in pediatric populations to gather information from IRBs, gene and cellular therapy clinical researchers, and other stakeholders regarding best practices related to cell and gene therapy clinical trials in pediatric populations, and challenges and considerations in trial review.

41.8 FDA's STANDARDS DEVELOPMENT PROGRAM

Since its inception, the development and use of standards have been critical to the mission of FDA. The use of standards in FDA medical product regulation began with the 1906 Federal Food and Drugs Act. Drugs, defined in accordance with the standards of strength, quality, and purity in the US Pharmacopeia and the National Formulary, could not be sold in any other condition unless the specific variations from the applicable standards were plainly stated on the label. In current times, Federal government agencies, including FDA, are encouraged, when practical, to use voluntary consensus

standards, whether domestic or international, when performing regulatory activities in lieu of government-unique standards which are developed by the government for its own uses. Standard-setting activities include the development of performance characteristics, testing methodology, manufacturing practices, product standards, scientific protocols, compliance criteria, ingredient specifications, labeling, or other technical or policy criteria.

As with guidance document development, in which Good Guidance Practices describe FDA's procedures for developing and using guidance documents, there are specific regulations that describe FDA participation in outside standard-setting activities. Regulations governing this participation can be found in 21 CFR 10.95. Additionally, FDA's Staff Manual Guide (SMG) 9100.1 establishes agency-wide policies and procedures related to standards management activities to assure a unified approach to standards within FDA. Constructive FDA participation in organizations responsible for developing standards applicable to the products regulated by the agency is considered essential.

The FDAMA of 1997 provides for the recognition of national and international standards in medical device reviews for IDEs, HDEs, PMAs, and 510(k)s. A 'recognized consensus standard' is a consensus standard that FDA has evaluated and recognized, in full or in part, for use in satisfying a regulatory requirement and that FDA has published in a Federal Register notice. A 'consensus standard' is a standard developed by a private sector standards body using an open and transparent consensus process. Conformance with recognized consensus standards is strictly voluntary for a medical device manufacturer, who may choose either to conform to applicable recognized standards or to address relevant issues in another manner. Standards may also be used in support of non-device applications when appropriate and not in conflict with FDA regulation or Guidance. As the policies regarding the use of standards in the review process by FDA Centers involved in the regulation of regenerative medicine products may vary, it is prudent for sponsors to check with FDA staff, or the center websites, regarding the applicability of a particular standard to a specific area of product development. Lists of recognized standards, Guidance Documents, and Standard Operating Policies and Procedures (SOPP) can be found on FDA's website.

In regenerative medicine, CDRH and CBER work actively with standards development organizations such as the American Society for Testing and Materials International (ASTM International). ASTM Committee F04 on Medical and Surgical Materials and Devices Division IV is actively engaged in development of standards for tissue engineered medical products (TEMPs). F04 Division IV consists of six subcommittees: (1) Classification and Terminology, (2) Biomaterials and Biomolecules, (3) Cells and Tissue

Engineered Constructs, (4) Assessment, (5) Adventitious Agent Safety, and (6) Cell Signaling. Currently, the ASTM TEMPs group has developed more than 25 published standards, including standard guides and test methods, and has approximately 30 draft standards under preparation. The first of these standards were developed for substrates, biomaterials, natural materials such as collagen, alginate, and chitosan, terminology, cells and cell processing, bone morphogenetic protein, assessment of adventitious agents, and test methods for characterizing biomaterials. These standards are reviewed on a regular basis by the appropriate ASTM subcommittee to ensure that the standards reflect the current scientific knowledge and FDA regulatory practices. Some examples of approved standards with which the TEMPs group was involved include standards for the classification of therapeutic skin substitutes, characterization and testing of biomaterial scaffolds (e.g., collagen, hyaluronan, chitosan), immobilization/encapsulation of living cells or tissue in alginate gels, quantification of cell viability within biomaterial scaffolds, and preclinical *in vivo* assessments of repair in articular cartilage and in critical size segmental bone defects.

Another standards development organization with which FDA is involved is the International Standards Organization (ISO), a nongovernmental international organization that develops consensus standards in collaboration with both the private and public sector. Standards for regenerative medicine/tissue engineering products are developed in TC 150 subcommittee (SC) 7, Tissue Engineered Medical Devices, and in TC 194, Biological Evaluation of Medical Devices. TC 194 SC 01 is responsible for Tissue Product Safety, and within SC 01 are four Working Groups (WGs): WG 01 Risk Assessment, Terminology, and Global Aspects; WG 02 Sourcing Controls, Collection, and Handling; WG 03 Elimination and/or Activation of Viruses and Transmissible Spongiform Encephalitis (TSE) Agents; and WG 04 TSE Elimination. For regenerative medicine products regulated as medical devices or combination products that contain a device component, the ISO 10993 series of standards (10993–1 through 20) describe extensively the biocompatibility testing that is typically performed for the device component (standards are available through ISO's website: http://www.iso.org).

FDA is actively engaged in standards development since it can facilitate the development and maintenance of guidance for industry, address issues that cannot be normally addressed in FDA guidance documents (e.g., proprietary methods for tests or processes, critical reviews of emerging fields), facilitate product design, and lead to international harmonization of practices. These benefits can cumulatively improve the efficiency of product development by the regenerative medicine community and thereby impact public health. Thus, FDA plays a critical role in providing support for standards development activities.

41.9 ADVISORY COMMITTEE MEETINGS

As mentioned in section 'Meetings with Industry, Professional Groups, and Sponsors' above, because of the diversity of innovative technology evaluated by FDA review staff, FDA makes use of expert scientific advisory committees or panels (for medical devices) to complement its internal review process. These advisors provide outside advice to contribute to scientific regulatory decision making. Outside experts can be asked to review data, or make recommendations about study designs across a product or clinical area; outside advisors can also be helpful at earlier stages of product development. Expertise on the advisory committee often includes scientific, statistical, and clinical experts, as well as consumer representation, patient advocates, and industry participation. Most meetings are public and there is an opportunity for public participation in the form of public comment.

There are 32 FDA advisory committees currently, with the areas of responsibility for the committees divided along product lines. The advisory committee for cellular, tissue, and gene therapy products (CTGTAC) has discussed in recent years a number of areas that are of potential interest to product developers in the regenerative medicine area, including:

- Hematopoietic stem cells for hematopoietic reconstitution (February 2003)
- Allogeneic islet cell therapy for diabetes (October 2003)
- Somatic cell cardiac therapies (March 2004)
- Cellular products for joint surface repair (March 2005)
- Potency measures for cell, tissue and gene therapies (February 2006)
- Cellular therapies derived from human embryonic stem cells – considerations for preclinical safety testing and patient monitoring (April 2008)
- Animal models for porcine xenotransplantation products intended to treat type 1 diabetes or acute liver failure (May 2009)
- Clinical issues related to FDA draft guidance 'Preparation of IDEs and INDs for Products Intended to Repair or Replace Knee Cartilage' (May 2009)
- Testing of replication competent retrovirus/lentivirus in retroviral and lentiviral vector based gene therapy products (November 2010)
- Cellular and gene therapy products for the treatment of retinal disorders (June 2011)

The presentations for each topic, as well as a transcript of the discussion, are available on FDA's website referenced at the end of this chapter.

The Medical Devices Advisory Committee consists of 18 panels that cover the medical specialty areas. Panel meetings are held regularly to discuss specific products. A complete list of upcoming Medical Devices Advisory Committee

panel meetings and searchable archive of past meetings with agendas and materials can be found on FDA's website.

41.10 FDA RESEARCH AND CRITICAL PATH SCIENCE

FDA recognizes the complexity of the scientific issues related to regenerative medicine products. FDA research laboratories play an important role in helping ensure that the agency stays abreast of the rapid change and development affecting the entire field as well as addressing specific regulatory science questions. In 1992, CBER researchers began systematic efforts to uncover mechanisms that control the behavior of cells subjected to various environmental stimuli, particularly those encountered during normal wound healing, regeneration, and prenatal development. Those studies led to the discovery of several growth factors and feedback mechanisms that help control these pathways. Other research addressed the interactions between hematopoietic and mesenchymal cell lineages both *in vitro* and *in vivo*. The success of those efforts led to the recruitment of additional investigators in these areas. In 2004, FDA introduced the Critical Path Initiative to identify and support research priorities that are expected to advance innovation in medical products. The Critical Path Opportunities List 'presents specific opportunities that, if implemented, can help speed the development and approval of medical products' (US Department of Health and Human Services, Food and Drug Administration, 2006), and is available on FDA's website. A number of the research topics on the Critical Path Opportunities List have applications to regenerative medicine, such as developing characterization tools for cell therapy and tissue engineering, biomarkers for cardiovascular disease, and advanced imaging technologies. FDA labs are actively engaged in these and other research questions that will facilitate the advancement of the field of regenerative medicine.

A priority of the Critical Path Initiative is updating and modernizing techniques, to ensure that the agency and the research community have the tools necessary to bring safe and effective products to market. Efforts include promoting collaborations spanning multiple centers and regulatory jurisdictions across FDA as well as among FDA and other relevant organizations (e.g., other agencies, academic organizations, regulated industry). Developing research collaborations and the requisite infrastructure to support those and other efforts will facilitate review of combination products, which are often seen in regenerative medicine.

A recent example of collaborative work across FDA labs is a multi-investigator project at CBER using a battery of state-of-the-art analytic techniques chosen

to provide complementary data on cell state and seeking to develop new biomarkers for cell therapies. The project aims to characterize mesenchymal stromal cells from a number of perspectives, including genetic stability, proteomic and phosphoproteomic analysis, microRNA analysis, mRNA profiling by microarray and quantitative polymerase chain reaction (PCR) analysis, chromatin immunoprecipitation, and examination of the potential for cells to mature and contribute to the formation of organs and tissues. Furthermore, the study will look for molecular differences between cells from early versus late passage numbers. Importantly, the same cells that go through this panel of tests will also be implanted in a mouse model of hindlimb ischemia, allowing for correlation of product characterization data with the *in vivo* outcome with regard to localization, differentiation, and functionality. This FDA research project may yield information that will be useful for product characterization, in-process testing, lot release criteria, developing comparability and stability protocols, and predicting cell fate and function after receiving a cell therapy.

One of the major issues associated with the clinical use of cellular therapies is predicting what happens to the cells after injection. Another FDA Critical Path research study at CBER will advance cell therapy by helping to develop methods for *in vivo* tracking and imaging of neural stem cells (NSCs) after transplantation. NSCs from adult, fetal, and embryonic sources have been proposed as treatments for degenerative conditions such as Parkinson's disease, and for repair of tissues damaged by stroke and spinal cord injury. Magnetic resonance imaging and single photon emission computed tomography are being used to qualitatively and quantitatively determine cellular location and persistence of engraftment. The goal of this project is to develop methods for evaluating biomarkers that may be predictive of NSC function.

FDA's research projects often involve collaborations with other federal and academic partners to employ new technologies to help address regulatory science questions. For example, a collaboration between FDA and the National Institute of Standards and Technology is using automated microscopy to characterize the differentiation of mesenchymal stem cells (MSCs). The goal is to improve the safety of MSC products by developing robust assays that can be used for in-process and lot release testing.

FDA critical path research also helps address some of the challenges faced in both product development and product evaluation. For example, following the observation of unexpected toxicity of adenoviral vector gene therapy in a clinical trial, CBER research provided insight into how adenovirus vectors cause toxicity and developed an animal model for gene therapy in the context of preexisting liver disease. CBER researchers/regulators also worked with a consortium from industry and academia to develop reference material for

adenoviral vector particles. FDA research is ongoing to understand the nature of toxicity of systemically delivered adenovirus and mechanisms for vector clearance to improve the safety of gene therapy trials.

Some FDA labs are engaged in research projects related to tissue. CDRH scientists are studying the effects of mechanical and electrical stimulation on cardiac cell cultures and how the parameters modulate cellular physiology. A CBER/CDRH collaboration is examining the relationship between encapsulation of chondrocytes in a scaffold material, with and without mechanical stress, on the status of several signaling pathways.

Additional FDA research projects are studying medical devices with implications for the regulation of regenerative medicine products. A CDRH research effort is examining the effects of contact with certain types of materials on cardiac cells, which has implications for biocompatibility regulations for regenerative medicine products with structural and other device components. An additional research project associated with cardiovascular disease is a collaboration between CDRH and CDER using cardiac myocyte cultures to study toxicological effects of certain drugs on cardiac tissue. A collaborative project between CDRH and other government researchers is examining the ability of wavelength-specific light to promote nerve regeneration in laboratory and animal models.

In summary, FDA research labs, with support through the Critical Path Initiative, provide an important source of in-house expertise in regenerative medicine and other cutting-edge technologies and research areas. Although by no means exhaustive, the examples provided above demonstrate the diverse range of topics under investigation at FDA. Especially in consideration of the rapid change and development of the regenerative medicine field, Critical Path research efforts ensure that FDA stays abreast of the current innovations. FDA's research programs provide an important source of the latest science to inform the regulatory process and bring safe regenerative medicine products to market.

41.11 OTHER COMMUNICATION EFFORTS

Due to the highly interdisciplinary nature of regenerative medicine, FDA recognizes the need to build collaborative efforts both outside of and within the agency to successfully ensure medical product safety and efficacy while helping to speed innovativations that advance public health. In order to accomplish this goal, FDA has established a number of collaborative efforts with other federal agencies, professional societies, regulatory agencies, and other groups in an effort to clearly communicate regulatory expectations and to

ensure that current policies and policies in development are informed by current science.

For example, FDA has established Memoranda of Understanding (MOU) agreements with two different NIH institutes that involve FDA scientific review staff and NIH extramural research program officers. The MOUs co-signed by the National Institute of Neurological Disorders and Stroke (NINDS) and the National Heart, Lung and Blood Institute (NHLBI) incorporate safeguards to protect from disclosure shared, nonpublic information such as trade secrets and confidential commercial information, identities of study participants and other personal information, privileged and/or predecisional agency information, research proposals, progress reports, and/or unpublished data or information protected for national security reasons. Under these MOU agreements, participants are able to hold unfettered discussions and exchange information that enables the respective agencies to maintain currency with respect to ongoing scientific activities that could impact regenerative medicine from both the laboratory and clinical research perspectives. By identifying gaps in knowledge related to the state of available scientific information and fostering familiarity with FDA regulatory expectations, interagency MOU interactions contribute to the identification of promising basic research with the potential for clinical translation. Furthermore, these interactions facilitate collaborative efforts that maintain the active dialog between the FDA and the scientific community. A recent example of this type of effort is the jointly sponsored FDA-NIH public workshop entitled 'Pluripotent Stem Cells in Translation: Early Decisions' held on March 21–22, 2011. This workshop covered clinical translational topics that need to be considered early in the scientific development of pluripotent stem cell-based products.

Another example of interagency collaboration in the regenerative medicine arena is the Multi-Agency Tissue Engineering Science (MATES) Interagency Working Group in which FDA is a partner. Spanning more than a dozen federal agencies, this partnership is designed to provide a forum to facilitate communication and coordination across the government regarding activities in tissue engineering and regenerative medicine. The full strategic plan can be found at the MATES website.

Interagency communication and collaboration also extend to the international community as well, and FDA maintains interactions with a number of international regulatory agencies. For example, FDA has a regular dialog with the European Medicines Agency (EMA) on a bimonthly basis regarding Advanced Therapy Medicinal Products, defined by the European Directive to include gene therapy medicinal products, somatic cell therapy medicinal

products, and tissue engineered products. FDA staff has visited EMA and also hosted visitors from EMA and other regulatory agencies.

Equally important as these interagency efforts are the intra-agency efforts that promote coordination of regenerative medicine-related regulatory and scientific activities within the FDA. A pertinent example is the FDA Commissioner's Fellowship Program (CFP) which was established in 2008 to attract and retain new talent to the agency. Within its annual cohort of 50 fellows, a multicenter regenerative medicine fellowship program has been established. Regenerative medicine fellows work across both CBER and CDRH, to facilitate cross-agency collaboration and conduct research projects related to the regulation of regenerative medicine products.

Lastly, FDA has made efforts to communicate to the research community to explain the FDA regulatory process and policies. FDA staff participates frequently in seminars at scientific conferences and via webinars hosted by external groups such as the California Institute of Regenerative Medicine (CIRM). Also of note is the online FDA educational series that explains in detail the submission process for a cell or gene therapy investigational study application.

41.12 CONCLUSION

The field of regenerative medicine is an exciting field with scientific advances leading to the promise of future therapies for current unmet medical needs for patients. The FDA regulatory approach to medical products evaluation includes an ongoing assessment of how the science of those products informs regulatory policy.

FDA looks to continue ongoing dialog with the scientific community and product sponsors to continue to develop science-based regulatory review policies that are robust and predictable in order to meet the needs of the challenging array of products that are on the horizon.

FOR FURTHER STUDY

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CHAPTER 42

It's Not about Curiosity, It's about Cures

Stem Cell Research – People Help Drive Progress

Mary Tyler Moore and S. Robert Levine

42.1 CHOOSING LIFE

> Ah, but a man's reach should exceed his grasp. Or what's a heaven for?
>
> **Robert Browning, 'Adrea del Sarto'**

Many of you know that I have had Type 1 (juvenile) diabetes for more than 40 years. As a consequence, I struggle every day, like millions of others, to do what happens naturally for people who do not have diabetes: achieve a balance between what I eat, the energy I expend, and the amount of insulin I inject. Although to most, metabolic balance is as automatic as breathing, to people with Type 1 diabetes, like me, it requires constant vigilance, constant factoring and adjusting, frequent finger sticks to check blood sugars, and multiple daily insulin injections just to stay alive. Even with the greatest of care and closest of personal scrutiny, I find that I am often unable to achieve good balance – my sugars are dangerously low or frighteningly high. Yes, dangerous and frightening – because, frankly, serious lows can lead to seizures, coma, and death, and highs, over time, result in life-limiting and life-shortening complications like blindness, amputation, kidney failure, heart disease, and stroke. Diabetes is an all too personal time bomb that can go off; today, tomorrow, next year, or 10 years from now – a time bomb affecting millions, like me, that must be defused.

This reality is made all too clear by the recent sudden death of a young friend, Danielle Alberti. Danielle was 31. She was an aspiring artist. Although rapidly losing her vision because of diabetic retinopathy, Danielle stuck to her dream of being a painter and was pursuing her career when she, like too many young adults with Type 1 diabetes, developed kidney failure. People with diabetes-related kidney failure do not do well on dialysis, so kidney transplant was her only real option. With her doctor's guidance, she and her

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mother decided to return home together to Australia where her chances for a transplant were greater. However, Danielle did not survive the flight. She died at 30,000 feet, seeking comfort in her mother's arms – her last words, 'Mum, hold me.'

Most of us share at least a piece of this experience – our loved ones, in times of pain or need, reaching out, looking to us for comfort, for a way to stop their suffering. At that moment, we each would do anything in our power to change their reality, to take their pain from them. When given the choice or the power to effect change, we all would choose to protect the lives of those we love. This is the quest we join, together, when we contemplate the promise of stem cell research, debate its proper methods, and work toward making our hopes a reality – curing disease and disability through stem cell-derived therapies. We choose the idea of a better life and reach beyond our grasp to achieve it.

42.2 SIZE OF THE PROMISE

> 'It is not unrealistic to say that [stem cell] research has the potential to revolutionize the practice of medicine and improve the quality and length of life.'
>
> **Dr. Harold Varmus, Nobel laureate and former Director of the US National Institutes of Health (NIH)**

Judgment does not only require choosing between the good and the bad. It often challenges us to balance more than one good or choose between the bad and the worse. Good judgment, therefore, demands that we make efforts to understand the relative impact of our choices, to come to an understanding of the greater good.

However, measuring the promise of stem cell research, for me, does not start with the recitation of the literally hundreds of millions of people who could benefit from the insights gained and therapies derived, it starts with understanding its potential for you and me, our parents and children, our friends and families, neighbors, and coworkers.

For people with Type 1 diabetes, we look first to stem cell research as a means to help us replace the insulin-producing cells of the pancreas that are destroyed by our disease. However, it also may provide insights into the genetic basis for diabetes, including the differences between Type 1 diabetes, which is an autoimmune disease like lupus and multiple sclerosis, and the more common, obesity-related, Type 2 diabetes. It may also provide solutions to the devastating complications of diabetes: blindness, kidney failure, amputation, and cardiovascular disease. For people with Parkinson's disease,

FIGURE 42.1
Juvenile Diabetes Research Foundation (JDRF) founding moms, Lee Ducat and Carol Lurie.

stem cell research holds the promise of replacing destroyed specialized brain cells and thereby freeing patients from the prison of disease-induced rigidity. For spinal injury patients, it offers the potential for regeneration of neural tissue, which would reconnect the pathways of sensation and motor control and allow them to walk again, or talk again, or hug their child again. For people with heart failure, stem cell research may mean sleeping through the night without struggling for breath, dancing with a spouse, working in the garden, or sustaining one's job and independence. For the person with macular degeneration it might offer sight. Stem cell research offers hope for people with a great diversity of illnesses, for people of all ages and genders and all backgrounds. It offers hope for each of us, and that hope is not measured by numbers, it is very personal.

42.3 PERSONAL PROMISES FUEL PROGRESS

I have had the privilege of serving as the International Chairman of the Juvenile Diabetes Research Foundation (JDRF) (www.jdrf.org) since the mid 1980s (Figure 42.1). JDRF was founded in 1970 by the parents of children with Type 1 diabetes. They were not satisfied with the only option offered to their children by health professionals – a lifetime of insulin injections just to stay alive, and the constant fear of the life-stealing complications they would face in the future. Insulin was not a cure; they knew it, and they wanted someone to do something about it.

So they challenged the established professional associations to do something, to think anew, and to invest in more research (especially Type 1 diabetes research). They were brushed aside but not bowed. They may have been 'just moms and dads,' but they had a purpose that was highly personal. Each of them had promised their child, their loved one, that they would do all they could to find a cure. They intended to keep that promise, and they have. In the years since its founding, JDRF has grown to be the largest charitable contributor to diabetes research in the world, providing nearly $1.7 billion in direct funding since 1975, including well over $625 million in the last five years alone. However, the impact of this 'people-driven' effort to find a cure has been far greater than just the dollars that they have raised for research. JDRF families (supported by an extraordinary professional staff, many of whom have a direct connection to diabetes) have been key leaders in public advocacy that has resulted in the following:

1. The Diabetes Research and Education Act (mid 1970s) that established The National Institute of Diabetes Digestive and Kidney Diseases at the National Institutes of Health (NIH) and called for substantive increases in funding of diabetes research. At the time the NIH was only investing $18 million per year in diabetes research; it now spends more than $1 billion per year.
2. Congressional earmarks for research into the genetics of diabetes and diabetes-related kidney disease (1980s).
3. The lifting of the ban on fetal tissue research in 1993, which President Clinton did 'for Sam' a young man with Type 1 diabetes.
4. The doubling of the NIH budget in five years. Between 1998 and 2003 the budget increased from approximately $13 billion per year to more than $26 billion per year.
5. The establishment of the Congressionally mandated Diabetes Research Working Group that reports to Congress periodically on progress in diabetes research, on research needs and opportunities for the future, and on adequacy of funding (1998).
6. The Special Diabetes Initiative (1998–2013) that will, by fiscal year 2013, have provided more than $1.89 billion in supplemental funding for special initiatives in Type 1 diabetes research (on top of usual NIH appropriations) and will provide an equal dollar amount (more than $1 billion) to fund special initiatives in diabetes care and education for Native Americans with diabetes.
7. Food and Drug Administration (FDA) approval of Continuous Glucose Monitoring Systems (2006).
8. FDA approval of a computer-based diabetes simulator for use as an alternative to animal testing of Type 1 diabetes control strategies (2008).

FIGURE 42.2
Mary Tyler Moore (with Michael J. Fox) testifying before Congress in support of federal funding of stem cell research. *Photo courtesy of Larry Lettera/Camera 1.*

9. Executive Order 13505 (2009) removing barriers to responsible scientific research involving human stem cells.
10. The 2012 publishing of FDA draft guidance on Artificial Pancreas Systems.

JDRF was also a founder of the Coalition for the Advancement of Medical Research (CAMR), a diverse coalition of health- and research-related organizations committed to sustaining federal funding of stem cell research. Working through CAMR and independently, JDRF volunteers and staff played a crucial role in convincing the George W. Bush administration and congressional leadership not to support a total ban on embryonic stem cell research in the United States (Figure 42.2) and more recently leading public advocacy in building bi-partisan support for passage of the Castle-DeGette Stem Cell Research Enhancement Act. JDRFers were also lead proponents of California's $3 billion stem cell research/regenerative medicine initiative, as well as other state-based stem cell research programs.

The experience of the JDRF along with the HIV and AIDS community, the women's health movement, the Michael J. Fox Foundation for Parkinson's Research and other grass roots organizations proves that in their quests to find a cure for their children and loved ones for whatever pains them, in their personal wars against disease, moms and dads, partners and spouses, and people who care will never give up. In fact, people personally affected by illness are the natural and necessary leaders of any global cure movement. They understand the urgency and are uniquely willing do anything required to ensure that their loved ones are freed of the burden of disease as soon as possible. For them, 'failure is not an option,' because their very survival is at stake.

42.4 HOPE VERSUS HYPE

> I am not discouraged, because every wrong attempt discarded is another step forward.
>
> **Thomas Edison**

What we know about any area of health or science is as much the 'wisdom' accumulated through countless errors as it is the outcome of our research 'successes.' Furthermore, progress is often gained as much through the accidental collision of an unexpected finding and a willing mind as it is through the careful and detailed application of all that is 'known.'

What, then, drives the hope versus hype discussions regarding stem cell research? Do we know enough to project possibilities? Yes. Do we know enough to make assertions of a particular outcome by a date certain? No. Should this diminish our commitment to pushing the field forward to prove its potential? Certainly not. Too much is at stake for us to delay or to apply unreasonable constraints because we are worried we might be wrong or might be overestimating the potential. What if we are underestimating it?

Just like with any endeavor at the frontier of new worlds, there is risk in taking the next steps. However, we cannot shrink from these risks. Rather, we must – with proper deliberation and due consideration of the risks – chart our course, prepare ourselves for dealing with the unexpected, and move forward.

42.5 GIVING LIFE

> If you save one life, you save the world.
>
> **The Talmud**

I understand that embryonic stem cell research raises concerns among people of good will, each trying to do what is right based on their very personal religious and moral beliefs. I have not shied from that personal soul searching, nor has JDRF in its policy making, nor should anyone. I have found comfort in my heartfelt view that human stem cell research is truly life affirming. It is a direct outcome of a young family making a choice, without coercion or compensation, to donate a fertilized egg not used for *in vitro* fertilization, for research. An egg that otherwise would have been discarded or frozen forever. Because of the great potential of stem cell research, donating unused fertilized eggs is much like the life-giving choice a mother, whose child has died tragically in an automobile accident, makes when donating her child's organs to save another mother's child. It is the true pinnacle of charity to give so totally, so freely of oneself, to give life to another. Public support for stem

cell research is an extension of this affirmation of life and is the best way to ensure that it is undertaken with the highest of ethical standards.

42.6 PEOPLE DRIVE PROGRESS

> I know of no safe depository of the ultimate powers of the society but the people themselves; and if we think them not enlightened enough to exercise their control with a wholesome discretion, the remedy is not to take it from them, but to inform their discretion by education.
>
> **Thomas Jefferson**

In science, politics, even religion, the public often cedes decisions, important decisions, to an 'expertocracy' or to dogma. Perhaps this is out of respect, humility, fear, or unfamiliarity – even out of a presumption of incapacity. It is, however, a well-informed public that is most capable of making decisions in its own interest and that, uniquely, has the power to effect change. It is, therefore, a true test of leadership to cede discretion back to people and bring comfort to public decision making through careful and objective expert counsel, access to a broad base of information, and support for taking specific actions to achieve goals.

This approach has defined the JDRF success. From its inception, the JDRF has been unique in the way that it conducts its review of research grants and how it decides what research is funded. Scientific experts (peer reviewers) are joined by people personally affected by diabetes (lay reviewers) in the discussion of all proposed research. Scientific merit is established in these peer/lay collaborative review sessions. Then the lay reviewers meet separately to discuss which of the meritorious grants are most responsive to the needs of people with diabetes – that is, which are most likely to have the greatest impact on finding a cure or reducing the burden of diabetes and its complications. The funding decisions are made in the context of cure goals and research priorities established by the JDRF Board and Research Committee, which are themselves predominantly made up of people personally affected by diabetes. These goals and priorities are established via a process of knowledge mapping, which is conducted periodically, by JDRF scientists, experts and volunteers. Knowledge mapping identifies the current state of science along the many potential paths to a cure, where obstacles remain, and where there is the greatest opportunity for JDRF investment to make a difference.

The experience at JDRF infers that decisions regarding embryonic stem cell research are best made in the open, with the full engagement of the public and with particular attention to presentation of the broadest breadth of available information and opinion. We can be confident that the powers of society

FIGURE 42.3
Mary Tyler Moore at the Juvenile Diabetes Research Foundation's (JDRF's) Children's Congress of 2001. Joined on stage by Larry King, Tony Bennett, John McDonough, George Nethercutt, R-WA, Alan Silvestri, and child delegates from 50 states. *Photo courtesy of Larry Lettera/Camera 1.*

(in overseeing the conduct of science) can be safely ceded to the discretion of a well-informed populace.

42.7 BETTER HEALTH FOR ALL

> William Bradford, speaking in 1630 of the founding of the Plymouth Bay Colony, said that all great and honorable actions are accompanied with great difficulties, and both must be enterprised and overcome with answerable courage. If our progress teaches us anything, it is that man, in his quest for knowledge and progress, is determined and cannot be deterred.
>
> **President John F. Kennedy, speech at Rice University, September 12, 1962**

People like me who struggle daily with disease or disability, and the people who love us, recognize the difficulties of scientific advancement, accept the challenges, and everyday answer with courage – if not for our own sake, for our children and our children's children. We are motivated not by curiosity, but by our dedication to finding cures. New therapies derived from embryonic stem cell research, conducted with public support by scientists from all areas of the globe, and made available to all who might benefit, are part of our broader vision of better health for all (Figure 42.3).

FRIENDS IN NEED AND DEED

LISA, 12 Years Old, El Paso, Texas

Lisa has had Type 1 diabetes since she was five years old.

> 'It makes me sad to watch my parents cry when they tell people about my diabetes,' she says. 'I feel really sad when I think about the stuff that can happen if a cure isn't found soon. I don't want to go blind and never again see the faces of the people I love or the really pretty sunsets in El Paso. I don't want my legs amputated, because I love to dance. I don't want to die early and have heart and kidney disease. I want a cure before any of these terrible complications can happen.'

NICHOLAS, 4 Years Old, Boca Raton, Florida

Nicholas was diagnosed with Type 1 diabetes when he was 20 months old. Most nights, his mother, Rose Marie, must intervene in some way to keep Nicholas' blood sugar in the normal range.

> 'As I hold him in the middle of the night, trying to coax him awake so I can feed him, I realize just how delicately his life hangs in the balance,' Rose Marie says. 'I rededicate myself to doing everything within my power to find a cure for this disease which robs my son, and millions of others like him, of a healthy, carefree childhood, and which carries the constant threat of danger – like a thief in the night lurking to strike.'

ASHLEY, 10 Years Old, Medina, Washington

Ashley was diagnosed with Type 1 diabetes at the age of seven, right after a ballet rehearsal for a performance with the Pacific Northwest Ballet. She is passionate about a cure.

> 'I want to find a cure for diabetes so no other children or families have to experience what my family goes through EVERY day. I want to find a cure so that I don't have the terrible headaches I get from the insulin and so I don't go low anymore; it feels so bad and it makes it hard to think in school. I want to find a cure so I can be a good mother some day.'

KYLIE, 12 Years Old, Ogden, Utah

Now 12, Kylie was diagnosed with Type 1 diabetes at age eight.

> 'Over Christmas vacation last year, I had a friend in my fifth grade class who died,' she says. 'He had diabetes just like me. During the middle of the night he went into insulin shock and never woke up.'

Kylie fears the same thing could happen to her.

> 'I would love to have a guarantee that the rest of my life will be long and normal, but I know that there is a lot of work still to be done before they can find a cure.'

BRENNAN, 6 Years Old, and TANNER, 8 Years Old, Round Rock, Texas

Brennan, 6, was diagnosed with Type 1 diabetes at the age of two, just seven months before his older brother Tanner was diagnosed.

> 'Each day is filled with adversity and challenge,' mom Amy says. 'Will the disease win today? Will Brennan succumb to too-low blood sugar levels and lose consciousness? Will Tanner have his first seizure?'

Even when the day goes well, Amy says she and her husband John are still on duty all night.

> 'Diabetes doesn't sleep… so neither do we. We keep a parent's vigil every four hours, no matter how tired we are from the day's battle. Imagine being adrift on a boundless ocean. You are tired of swimming, of trying to keep afloat, but to give yourself up to sleep would mean certain death.' she says. 'Sufferers of diabetes – and their parents – are adrift. They cannot rest from their vigilance, even in the dead of night. To relax completely, to give in to the peace of an eight-hour sleep that healthy people take for granted would invite seizures, unconsciousness, blindness, kidney failure, or heart disease.'

COREY, 11 Years Old, Secaucus, New Jersey

Corey, has had Type 1 diabetes since the age of five. Sitting at home and complaining about diabetes, he says, won't change things or get your voice heard or get us closer to a cure. Since the age of seven, he has spoken at schools and fundraisers to tell his story of what it is like to be very young and have to live with diabetes.

'I've found that while millions have this disease, many people don't have a clue about what it really means to live with it each day,' he says. He talks about the adjustments and fears that diabetes has brought to him and his family, adding that 'it has not made me afraid to do what I want to do in life, but I know it will make things harder.' At the moment of his sixth grade graduation, Corey says, 'I want to leave a legacy to help … other children as they strive to live a normal life and hope they'll never forget that we will find a cure.'

EMILY, 15 Years Old, Houston, Texas

Diagnosed with Type 1 diabetes nine years ago, Emily says each day is a battle. At first the finger pricks and shots were the most difficult thing that Emily had to handle, but now she has bigger fears. 'Now the hardest part is facing the reality that there is no cure, and that my life could be determined by this awful disease.' Emily is a straight A student and plays on her school's field hockey team. Her goal is to one day be an orthopedic surgeon – and to help find a cure for diabetes. 'I and many others will not be able to rest until we find a cure,' she says. 'A cure for diabetes will not just happen; it must be pursued, researched, and fought for.'

Glossary

Acellular approach to tissue engineering One of three main approaches encompassed by tissue engineering, the acellular approach aims to use acellular materials capable of inducing tissue regeneration.

Actual functional stem cells The cells on which the tissue is ultimately dependent for day-to-day cell replacement.

Adipose-derived stem cells (ASC) MSC-like cells derived from adipose tissue rather than bone marrow. ASC are quite similar to bone marrow-derived MSC morphologically and immunophenotypically, however, ASC form more CFU-Fs when plated in culture.

Adult stem cells Stem cells that exist in the organism after birth. Adult stem cells replenish specific tissues in the body and vary in their potency.

AF mesenchymal stem cells (AFMSCs) A subpopulation of AF cells (0.9-1.5% of total AF cells in second and third trimester AF) that differentiate toward mesoderm-derived lineages (i.e., adipogenic, chondrogenic, myogenic, and osteogenic).

AF stem cells A subpopulation of AF cells (0.1-0.5% of total AF cells in second and third trimester AF) that express the pluripotency marker Oct4 at both transcriptional and proteic levels.

Allograft (specific to Chapter 34) Cultured allogeneic keratinocyte graft grown from a donor's skin cells, which is eventually rejected by the recipient's immune system (may also have a dermal component).

Amniotic fluid Fluid that appears during second week of gestation and separates the epiblast (future embryo) from the amnioblasts (future amnion), thus forming the amniotic cavity.

Amniotic fluid cells (AF cells) Cells that are found in the amniotic fluid, derived from both extra-embryonic structures (placenta and fetal membranes) and embryonic and fetal tissues.

Anagen The growth phase of the cyclical function of the hair follicle.

Angioblast The progenitor cell of EPC. Angioblasts will give rise to EPC that upon stimulation with angiogenic factors such as VEGF and PlGF are mobilized from bone marrow to peripheral blood. Once in peripheral blood, EPC can be recruited to sites of active neovascularization, as seen in wounds, diabetic retinopathy, and tumors.

Anterior-Posterior (A-P) axis specification The leading hypothesis of A–P axis specification states that anterior fates are established as defaults during early neural induction, and FGF, Wnt, and retinoid signals are essential for establishing posterior cell fates

Apligraf® Commercial product (Organogenesis, Canton, MA) that is a cultured, bilayered living skin equivalent derived from neonatal foreskin keratinocytes, fibroblasts, and bovine type I collagen.

Asymmetric cell division In stem cell self-renewal, asymmetric cell division generates one stem cell and either one differentiated progeny or a stem cell with a restricted capacity for differentiation.

Autograft (specific to Chapter 34) Cultured autologous keratinocyte graft grown from the recipient's own skin cells, not rejected by the recipient's immune system.

Barrett's Metaplasia A clinical situation in which intestinal cells are found in the tissue of the lower end of the esophagus. In the strictest terms, it is the conversion of stratified squamous epithelium to columnar epithelium, and is characterized by the presence in biopsy material of acid mucin-containing goblet cells

Basic fibroblast growth factor (bFGF or FGF2) Growth factor whose signaling pathway signaling appears to be of central importance in the self-renewal of human ES cells. bFGF allows the clonal growth of human ES cells on fibroblasts in the presence of a commercially available serum replacement. At higher concentrations, bFGF allows feeder-independent growth of human ES cells cultured in the same serum replacement.

Bioartificial liver Hepatocytes are seeded into and maintained in some form of an extracorporal device. The patient's blood or plasma is pumped to the device, where it interacts with the hepatocytes across membrane barriers and is then returned to the patient by a second series of pumps.

Biologics Control Act of 1902 This Act provided for regulation of viruses, serums, toxins, and analogous products; required licensing of manufacturing establishments and manufacturers; and provided the government with inspectional authority.

Bone The extracellular matrix of bone is primarily type I collagen and calcium phosphate. Bone has the ability to regenerate itself with functional tissue with properties similar to the original tissue.

Bone morphogenetic protein (BMP) family A family of growth factors that induce progenitor cells to differentiate along the osteogenic lineage.

Bulge A specialized area of the hair follicle in which epithelial skin stem cells(ESSC) are thought to reside. Cells in the bulge possess the ability to differentiate, at least under stress conditions, into different cell lineages to regenerate not only the hair follicle but also the sebaceous gland and the epidermis.

Cap cells Support cells that reside at the at the tip of the Drosophila ovary

Cardiac stem cell Cell surface proteins that mark stem cell populations in other tissues are also found on a subpopulation of undifferentiated precursor cells in the adult heart. These primitive cells can be detected by Sca-1, which is involved in cell signaling and cell adhesion. Sca-1 is not specific for stem cells because it is found on the surface of hematopoietic stem cells and other cell types.

Cardiac stem cell activation CSCs express c-Met and insulin-like growth factor-1 receptors (IGF-1R) and, thereby, can be activated and mobilized by hepatocyte growth factor (HGF) and IGF-1. In vitro mobilization and invasion assays have documented that the c-Met–HGF system is responsible for most of the locomotion of these primitive cells. However, the IGF-1–IGF-1R system is implicated in cell replication, differentiation, and survival.

Cardiac stem cells (CSC) Cells that can generate all the components of the myocardium. CSCs can be isolated from the ventricular myocardium and long-term cultures can be developed. So far, only Lin − c-kitPOS cells have been shown to have the components of stemness: self-renewal, clonogenicity, and multipotentiality.

Cardiomyocytes The cell type that forms the myocardium. The capacity of embryonic and fetal cardiomyocytes to re-enter the cell cycle appears to be largely lost once the heart is developed. In contrast to mammalian skeletal muscle, which regenerates injured tissue, the heart does not appear to retain equivalent reserve cell populations to promote myofiber repair.

Catagen A non-growth phase of the cyclical function of the hair follicle. During catagen phase, some cell types within it undergo cell death and the hair follicle undergoes structural change.

Cavitation After compaction, the outer cell layer becomes the presumptive trophoectoderm and forms a true epithelial layer. Ion pumps in the epithelial layer actively form a fluid-filled cavity that positions the inner cell mass to one pole of the embryo.

CD26/dipoptidylpeptidase IV (CD26/DPPIV) An enzymatic protein found on the surface of HSCs whose targets include stromal-cell-derived factor-1 (SDF-1/CXCL12; a chemotactic and homing chemokine) and colony-stimulating factors (CSFs). CD26/DPPIV inhibition is being evaluated in clinical trials as a means of enhancing engraftment of donor CB.

Cell autonomous disease state Disease states in which the pathology is restricted to a particular cell whose life span is short-circuited but within an extracellular milieu otherwise normal.

Cell cycle regulation Strategies by which the duration of each phase of a cell cycle is regulated. A complete cell cycle consists of four phases, designated as Gap1 (G1), Synthesis (S), Gap 2 (G2) and Mitosis (M).

Cell nonautonomous disease state Disease states in which where normal cells die because of an inhospitable microenvironment.

Cell therapy A therapeutic modality in which living cells are used. Cell therapies are commonly used in regenerative medicine approaches to disease or injury.

Cellular approach to tissue engineering One of three main approaches encompassed by tissue engineering, the cellular approach aims to use isolated cells or cell substitutes as cellular replacement parts.

Central nervous system (CNS) The brain and spinal cord comprise the central nervous system. The main function of the CNS is to integrate the information that it receives from, and coordinate the activity of, all parts of the bodies of animals having bilateral symmetry. During development, all the cells of the CNS are derived from a small set of neuroepithelial cells.

Chimerism The result of introducing donor cells (usually embryonic) into a recipient at a very early stage of embryonic development (usually blastocyst). An organism is chimeric when donor cells become established into the developmental process and are integrated into tissues and organs.

Chronic myeloid leukemia (CML) A classical pathologic condition of adult hematopoietic stem cells that is caused by the BCR/ABL oncoprotein. Introduction of BCR/ABL into differentiated murine ES cells provided the first definitive demonstration of the embryonic HSC (e-HSC) that arises in vitro during ES differentiation.

Circulating stem cells Cells hypothesized to home to the heart in response to damage. The existence of such cell populations has gained credibility from observations of sex mismatched cardiac human transplants in which a female heart is transplanted into a male host. The presence of differentiated host cells (containing a Y chromosome) in the transplanted tissues proves the existence of migratory precursor cells that are induced to differentiate by the cardiac milieu.

c-kit ligand (KL) Also known as stem cell factor, mast cell factor, or steel factor. KL is expressed along the primordial germ cell (PGC) migratory pathway and in the genital ridges and plays a role in PGC survival.

Clonality A characteristic that describes how a population of cells (e.g., culture or cell line) was derived. A clonal population is generated from a single cell.

Closed systems One of two categories of combination approaches to tissue engineering in which cells are immobilized within polymeric matrices that provide a barrier for the immunological components of the host. For example, cells can be immobilized within semipermeable membranes that are permeable to nutrients and oxygen and can provide a barrier to immune cells, antibodies, and other components of the immune system. Furthermore, the implants can be either implanted into the patient or used as extracorporal devices.

Coalition for the Advancement of Medical Research A diverse coalition of health- and research-related organizations committed to sustaining federal funding of stem cell research, founded by the Juvenile Diabetes Research Foundation.

Colony-forming assay An assay used with stock ES cell lines to test media components for suitability; especially useful for evaluating sera from different sources or different batches produced by the same supplier.

Colony-forming unit fibroblasts (CFU-F) The CFU formed by plastic-adherent fibroblast-like cells that are the initial in vitro isolates of a preparation of mesenchymal stem cells (See ISCT-defined minimal criteria for human MSC)

Columnar cells The most abundant epithelial cells in the intestine. Columnar cells are called enterocytes in the small intestine and colonocytes in the large intestine.

Combination approach to tissue engineering One of three main approaches encompassed by tissue engineering, the combination approach aims to use a combination of cells and materials (typically in the form of scaffolds) to induce tissue and/or organ regeneration. There are two categories of the combination approach: open and closed systems.

Common bile duct Bile secreted by hepatocytes is collected in a branched collecting system, the biliary tree, which drains into the duodenum via the common bile duct.

Compaction A process that occurs at the 8-cell to 16-cell stage in mammals where blastomeres flatten, maximize their cell-cell contacts, and become polarized. The cytoplasm forms two distinct zones in preparation for the next round of cell division that is asymmetric, forming and inner and outer cell layer

Conception view of when life begins A moral view that espouses the belief that in moral terms, human life begins at conception. For those holding this view, the early embryo is morally no different from a child or adult human being.

Cord blood (CB) Blood isolated from the umbilicus at birth. CB is typically banked as an HLA-typed source of hematopoietic stem cells (HSC). In recent years, CB has been used as a source of (HSC) for more patients than bone marrow.

Current Good Manufacturing Practices (cGMPs) Regulations enforced by the US Food and Drug Administration (FDA). cGMPs provide for systems that assure proper design, monitoring, and control of manufacturing processes and facilities. Adherence to the cGMP regulations assures the identity, strength, quality, and purity of drug products by requiring that manufacturers of medications adequately control manufacturing operations.

Cyclin-Dependent Kinase Inhibitors (CKIs) Proteins that inhibit the activity of CDKs.

Cyclin-Dependent Kinases (CDKs) Cell cycle progression is regulated by the sequential activation and inactivation of CDKs. CDKs act on other proteins by phosphorylating amino acid residues at specific locations.

Cytosphere A floating cluster of cells in vitro, the ability of cells in culture to form cytospheres is a characteristic of any actively propagating cell of any lineage when maintained in serum-free medium without an adherent substrate.

Default hypothesis of neural induction The dominant model of neural induction, which states that neural tissue is formed spontaneously in the absence of bone morphogenetic protein (BMP) signaling during early gastrulation. Exposure to BMP causes epidermal differentiation.

Defined reprogramming factors Reprogramming of somatic cells without fusion was first accomplished with the defined reprogramming factors Oct3/4, Sox2, Klf4, and c-Myc that are expressed abundantly in ES cells; induced pluripotent stem cells were the result.

Definitive hematopoiesis Mediated by a distinct class of hematopoietic stem cells that arise in the para-aortic region of the developing embryo proper. Definitive hematopoiesis generates mature myeloid and lymphoid lineages for the life of the animal.

Demineralized bone matrix A form of bone tissue preparation that is regulated by the FDA as either a tissue product under the jurisdiction of Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps) or as a medical device under the jurisdiction of the Center for Devices and Radiological Health (CDRH).

Density gradient separation A technique classically used to separate mononuclear cells (MNC) from red blood cells from bone marrow isolates. MSC are derived from MNC collection that is seeded onto tissue culture plates and incubated in media containing 10% fetal bovine serum.

Developmental (or gradualist) view of when life begins A moral view that espouses the belief that the embryo is not yet fully a human being in a moral sense. They do not deny that the early embryo is alive and has the biological potential to become a person. Nevertheless, they believe that other features are needed for the full and equal protections we normally accord children and adults and that these features only develop gradually across the full term of gestation.

Diabetes Research and Education Act Established by The National Institute of Diabetes Digestive and Kidney Diseases (NIDDK) at the National Institutes of Health (NIH) and called for substantive increases in funding of diabetes research.

Differentiated cells Progeny of stem cells that are more restricted in their potency relative to the parent cells.

Dividing transit cells The amplifying cells derived from stem cells that continue to divide several times before undergoing terminal differentiation (maturation) into the functional cells of the tissue.

DNA methylation After implantation, a wave of de novo DNA methylation occurs, leading to epigenetic reprogramming (finished by E6.5 in the mouse). DNA methylation affects the entire genome to a different extent in embryonic and extraembryonic lineages and may be responsible for the observed loss of the ability to contribute to chimeras

Dnmt3l A key gene involved in the initiation of parental imprints, which acts together with the de novo DNA methylase enzyme, Dnmt3a.

Dorsal-Ventral (D-V) axis specification The leading hypothesis of D-V axis specification states that D–V identity is determined by the antagonistic action of Sonic hedgehog (SHH) secreted ventrally form the notochord and floor plate and of BMPs from the roof plate.

DYS-HAC A DNA molecule consisting of a human artificial chromosome (HAC) containing the entire dystrophin (DYS) locus. This molecule was constructed as a possible solution to the difficulty of introducing the large dystrophin gene into cells via exogenous DNA transfer.

Dystrophic muscle Muscle that is infiltrated by inflammatory cells, mainly lymphocytes and macrophages, followed by fibroblasts that deposit large amounts of collagen, contributing to progressive sclerosis of the muscle, and adipocytes that replace muscle with fat tissue.

Dystrophin A key protein in the membrane-bound dystrophin glycoprotein complex (DGC), which is thought to fortify the plasma membrane of the muscle against the intense shearing forces that are generated during daily exercise. The dystrophin gene is mutated in people with Duchenne's muscular dystrophy.

Dystrophin A rod-shaped cytoplasmic protein and a vital part of a protein complex that connects the cytoskeleton of a muscle fiber to the surrounding extracellular matrix through the cell membrane. The dystrophin gene is one of the longest human genes known, covering 2.2 megabases (0.07% of the human genome) at locus Xp21. The primary transcript measures about 2,400 kilobases and takes 16 hours to transcribe; the mature mRNA measures 14.0 kilobases. The 79 exons code for a protein of over 3500 amino acid residues.

Early embryo stem cells (ES cells) Derived directly from the inner cell mass of preimplantation embryos after the formation of a blastocyst. This population of cells would

normally produce the epiblast and eventually all adult tissues. When derived from mice, these cells can also produce germ-line chimeras.

Embryogenesis The process by which a complex organism derives from a single cell, the fertilized egg.

Embryoid bodies Cystic teratoma-like structures consisting of semi-organized tissues representing all three embryonic germ layers. Formed when embryonic stem cells are removed from culture conditions that inhibit differentiation, and are allowed to aggregate and differentiate.

Embryonic carcinoma cells (EC cells) The first pluripotent stem cell to be characterized. Discovered during the study of morphologically undifferentiated cells isolated from teratocarcinomas, the EC cell was identified as capable of forming a self-sustaining teratocarcinoma containing as rich a variety of tissues as its parent tumor when transplanted into a histocompatible adult host.

Embryonic germ cell-derived embryoid bodies (EG-derived EBs) Like EBs generated from embryonic stem (ES) cells, EG-derived EBs contain differentiated cells representing all three germ layers, as well as mixed-cell populations of less differentiated progenitors and precursors.

Embryonic germ cells (EG cells) Cells derived from the primordial germ cells that form the genital ridges and can be identified at embryonic day 6.5 in mice. This population of cells would normally produce germ cells and adult gametes. The most notable difference between ES and EG cells is that EG cells may have significant imprinting of specific genes, which renders them incapable of producing normal chimeric mice.

Embryonic germ cells (EG cells) Pluripotent stem cells derived from primordial germ cells (PGCs) that arise in the late embryonic and early fetal period of development. EG cells have been derived from several species, including mouse, pig, chicken, and human.

Embryonic stem cell A cell population (often clonal – defined in Chapter 2) that is derived from cells dissociated from embryos at a very early stage of development (e.g., blastocyst)

Embryonic stem cell cultures ES cell cultures are essentially primary cultures because in an intact embryo, there is no maintenance of the ES cell phenotype. In vitro culture of ES cell lines is therefore challenging because of the need not to apply selection pressures to the culture that would encourage the growth of variants.

Embryonic stem cells (ES cells) Derived from the inner cell mass of the mouse blastocysts, ES cells are karyotypically normal (unlike embryonic carcinoma cells). Clonal cell lines, introduced into mice, can contribute at high frequency to a variety of tissues in chimeras, including germ cells and thus provided a practical way to introduce modifications to the mouse germ line.

Endocrine cells of the intestine An abundant cell population distributed throughout the intestinal epithelium; these cells secrete peptide hormones in an endocrine or paracrine manner from their contained dense core of neurosecretory granules.

Endothelial progenitor cells (EPC) Cells probably derived from the same hemangioblast precursors of HSCs but they take a separate path of differentiation in the bone marrow. Co-mobilization of EPC and HSC contributes to the revascularization processes.

Epiblast The outer layer of an embryo that appears after blastocyst stage but prior to gastrulation and the segregation of the germ layers. Therefore, cells derived from the epiblast are capable of forming germ layers, ectoderm, endoderm, and mesoderm.

Epidermal proliferative unit The functional group of proliferative basal cells derived from a single stem cell, together with the distally arranged functional differentiated cells.

Epidermal stem cells Slow-cycling and self-renewing cells found in the basal layer of the epidermis and responsible for its constant repopulation.

Epigenetic regulation Regulation of transcription that occurs at the level of chromatin and/or DNA methylation.

Epigenetic reprogramming Heritable, yet reversible, modifications of chromatin and DNA methylation that affect cell fate. The reversibility of these modifications is why it is possible to change the phenotypic characteristics of cells and restor totipotency to somatic nuclei under specific conditions.

Explant culture A method of initiating primary cultures where the source tissue of the cells is not subjected to enzymatic digestion prior to culture.

Facultative progenitor cells (specific to Chapter 33) Cells capable of expanding to replace missing or destroyed cells from the same organ or tissue (e.g., hepatocytes, pancreatic duct cells)

Federal Food and Drugs Act of 1906 While the primary focus of the Act was on food safety, the law also required that drugs be provided in accordance with standards of strength, quality, and purity unless otherwise specified in the label.

Feeder cells Cells used to facilitate the culturing of other cells. Typically, feeder cells are inactivated to prevent further proliferation. Cells requiring feeder cells during culture are typically grown on top of the feeder layer. Although methods of separating feeder cell components from the cultured cells have improved, "feeder-free" culture methods are more compatible with the safety precautions required for the therapeutic use of cultured cells in humans.

Food, Drug, and Cosmetic Act (FD&C Act) of 1938 A repeal of the Federal Food and Drugs Act of 1906, this Act initiated the practice of premarket review of new drugs. The 1938 Act also put medical devices and cosmetics under FDA authority and authorized factory inspections.

Fucosylation A type of glycosylation that enzymatically adds fucose sugar residues to the extracellular portion of membrane proteins. Modifying fucosylation status of CB donor cells is being evaluated in clinical trials as a means of enhancing engraftment.

Gap 0 (G0) A resting phase where the cell has left the cell cycle and has stopped dividing.

Gap junctions Intercellular channels formed by individual structural units called connexins. Gap junctions allow cells to communicate with each other and to exchange small molecules.

Gastric glands Tubular glands formed by the epithelial lining of the stomach. The gastric glands are divided into foveolus, isthmus, neck, and base regions.

Gastrulation The stage of embryonic development just after E6.5 in the mouse where the primitive streak and the three definitive germ layers are formed. The primitive streak defines the future posterior side of the embryo.

Genetic lineage tracing The use of permanent labeling, usually DNA recombination, to mark a defined cellular population and trace its progeny.

Genomic reprogramming The restoration of totipotency to a somatic nucleus that is transplanted into an oocyte as a result of the action of maternally inherited factors contained within the oocyte. Components within the oocyte have the property to alter the somatic nucleus so that it can recapitulate the entire developmental program, and thus give rise to an exact genetic copy or clone of the individual who donated the transplanted nucleus. This definition is the most widely understood meaning of genomic programming

Germ cell specification – germ plasm In lower animals (e.g., *Drosophila* and *C. elegans*), germ cell specification occurs via inheritance of preformed germ plasm.

Germ cell specification – stem cell model In mammals, germ cells are derived from pluripotent epiblast cells in response to signaling molecules from the extraembryonic ectoderm.

Germ-line transmission When donor stem cells become integrated into the recipient's sperm or eggs, thereby generating progeny carrying the donor stem cell's genetic contribution.

Glomerulus The unique structure of the glomerulus is intricately linked to its ability to retain large macromolecules within the circulating bloodstream while allowing for rapid diffusion of ions and small molecules into the urinary space. The glomerulus consists of four major cell types: the endothelial cells of the microvasculature, the mesangial cells, the podocyte cells of the visceral epithelium, and the parietal epithelium.

Goblet cells The mucin-secreting cells of the intestinal epithelium. Goblet cells contain mucin granules, which make the cells rather swollen, thus the name "goblet." Found throughout the colonic epithelium.

Graft versus host disease (GVHD) A condition that follows tissue transplant in which the graft becomes the source of antibodies that trigger a rejection response against the host tissue.

Granulocyte-colony stimulating factor (G-CSF) A hematopoietic growth factor that is effective in mobilizing HSC from bone marrow to peripheral blood. In fact, transplantation of G-CSF-mobilized stem cells harvested from peripheral blood is replacing bone marrow biopsy.

Hair follicle An appendage of the epidermis. The hair follicle is a complex structure made of at least eight different cell types. The hair shaft is located in the middle of the follicle and grows upward, "breaking" the surface of the skin. Each follicle produces hair in a cycle of growth and non-growth phases.

Heart The heart is the first fully differentiated structure to form and function during vertebrate development. The primitive heart tube, composed of contracting cardiomyocytes lined by a layer of endocardial cells, ensures the establishment of a circulatory system that is critical to support rapid rates of embryonic growth.

Hemangioblast (blast colony-forming cell, BL-CFC) A long-theorized, but only recently identified, embryonic cell representing a common progenitor of both the endothelial and hematopoietic lineages. Can be readily detected in differentiating cultures of embryonic stem cells as the first mesodermal element committed to the hematopoietic lineage. Assayed by colony formation in methycellulose supplemented with vascular endothelial growth factor and stem cell factor (the BL-CFC).

Hematopoietic stem cells (HSC) Characteristically quiescent, multipotent cells with the capacity for both self-renewal and differentiation. After development in the fetus, HSCs reside in adult bone marrow and serve to replenish lymphoid, megakaryocytic, erythroid, and myeloid hematopoietic lineages throughout adulthood.

Hepatic artery One of two afferent blood supplies to the liver. The hepatic artery supplies oxygenated blood to the liver.

Hepatocyte transplants An infusion of donor hepatocytes into the blood supply of the liver or spleen – less invasive and costly than orthotopic liver transplant. Hepatocyte transplants have been conducted for over 20 years.

Hepatocytes The main "workhorse" cell type of the liver. The liver is responsible for the intermediary metabolism of amino acids, lipids, and carbohydrates; the detoxification of xenobiotics; and the synthesis of serum proteins. In addition, the liver produces bile, important for the intestinal absorption of nutrients as well as the elimination of cholesterol and copper. All of these functions are primarily executed by hepatocytes.

Holoclone A colony of cultured epithelial cells thought to be produced by keratinocyte stem cells.

HSC mobilization Stem cell numbers in peripheral blood are very low compared with those in the bone marrow. Mobilization of HSCs from bone marrow into peripheral blood can be achieved by hematopoietic growth factors.

Hub cells Support cells that reside at the apical tip of the Drosophila testis

Human embryoid body-derived (Human EBD) cells Human EBD cells are capable of considerable cell proliferation and express a variety of lineage-specific markers. Human EBD cell cultures have a normal and stable karyotype and normal patterns of genomic imprinting, including X-inactivation.

Human MAPC phenotype The phenotype of human MAPC at the cell surface is CD31, CD34, CD36, CD44, CD45, HLA class I, HLA-DR, c-Kit, Tie, VE-cadherin, VCAM, and ICAM-1 negative. Human MAPCs express very low levels of β2-microglobulin, AC133, Flk1, and Flt1, and high levels of CD13 and CD49b.

Human therapeutic cloning The deliberate creation of an embryo by somatic cell nuclear transfer technology (cloning) to produce an immunologically compatible (isogenic) hES cell line.

Hydroxyapatite (HA) A naturally occurring mineral form of calcium apatite, HA can be found in teeth and bones within the human body. It is commonly used as a filler to replace amputated bone or as a coating to promote bone ingrowth into prosthetic implants.

Immunosurgery A method of isolating embryonic stem cells from the embryo. It involves removing the zona pellucida with Acid Tyrode's solution, incubating the embryo in an antibody that binds to the trophectoderm, and then lysing the trophectoderm cells with complement.

In vitro fertilization (IVF) Combining human eggs and sperm outside the body to produce an embryo that can be implanted into a woman's uterus and result in the birth of a child. Without the development of IVF, the derivation of human ES cells would not have been possible.

Induced pluripotent stem cell Usually produced as a clonal (defined in Chapter 2) cell line from somatic cells into which a cocktail of specific genes has been introduced by one of several techniques. The resultant cell line has many characteristics in common with embryonic stem cells, particularly potency, but without involving the destruction of an embryo.

Informed consent Requirements that patients undergoing medical procedures, enrolling in clinical trials, and/or donors of tissue fully understand the nature of the procedures and/or research being undertaken and that they explicitly consent to participating or contributing to that activity.

Inner cell mass cells (ICM cells) Although ES cells are derived from ICM cells, it does not mean that ES cells are the in vitro equivalent to ICM cells, or even that ICM cells are the immediate precursor to ES cells.

Inner ear sensory organs Inner ear sensory organs subserve hearing and balance and are differentiated according to their function. There are three major classes of sensory organs: macula, crista, and acoustic.

Intestinal epithelium Differentiated cells that line both the small and large intestines. Overall, four main epithelial cell lineages exist in the intestinal epithelium: the columnar cells, the goblet cells, the endocrine cells, and Paneth cells in the small intestine. Other less common cell lineages are also present, such as the caveolated cells and membranous or microfold cells.

Intestinal subepithelial myofibroblasts (ISEMF) These cells form a fenestrated sheath that encloses both the intestinal crypts and the gastric glands. The ISEMFs are closely applied to the intestinal epithelium and play a vital role in epithelial–mesenchymal interactions.

Iris The shutter that opens and closes to allow more or less light to penetrate the eye. It includes a pigmented epithelial layer derived from the margin of the optic cup that is continuous with the RPE.

ISCT-defined minimal criteria for human MSC (1) MSCs must be plastic-adherent when maintained in standard culture conditions and CFU-Fs, (2) MSCs must express CD105, CD73, and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79alpha or

CD19, and HLA-DR surface molecules, and (3) MSCs must differentiate to osteoblasts, adipocytes, and chondroblasts in vitro.

Islet precursor cells The search for a definitive islet precursor cell has been an area of intense research interest as a potential treatment for Type 1 diabetes.

Juvenile Diabetes Research Foundation JDRF was founded in 1970 by the parents of children with Type 1 diabetes. JDRF has grown to be the largest charitable contributor to diabetes research in the world, providing nearly $1.7 billion in direct funding since 1975, including well over $625 million in the last 5 years alone.

Karyotype The number and appearance of chromosomes in the nucleus of a eukaryotic cell. Retaining a normal karyotype is a prerequisite for maintenance of totipotency and germ-line transmission of ES cells used for genetic engineering and gene-targeting projects.

Kefauver–Harris Amendments The requirement for premarket demonstration of efficacy and the authority for FDA oversight of clinical trials were provided by the Kefauver–Harris amendments to the FD&C Act in 1962. These amendments were prompted in part by the tragic adverse events resulting from use of thalidomide as a nonaddictive prescription sedative.

Leukemia inhibitory factor (LIF) A protein that when added to the media of hepatic cells in culture can condition the hepatic cells to transdifferentiate upon a change in media. LIF is removed and glucose is added at high concentration, causing the hepatic cells to transdifferentiate into several types of pancreatic cells, including glucagon, insulin, and pancreatic polypeptide-expressing cells.

Ligaments and tendons Bands of dense connective tissue that lend stability and provide movement of joints. Injury that leads to inflammation or tear of these structures can result in significant functional deficits and development of degenerative joint disease.

LIN28 LIN28 is an RNA-binding protein and negatively regulates Let7 microRNA (miRNA) families. LIN28 seems to indirectly enhance reprogramming efficiency through Let7 families.

Liver Liver is an organ that naturally regenerates in adult humans after partial hepatectomy. The liver consists of several separate lobes and represents about 2% of human and 5% of mouse body weight. It is the only organ with two afferent blood supplies. The main cell types resident in the liver are hepatocytes, bile duct epithelium, stellate cells (formerly called Ito cells), Kupffer cells, vascular endothelium, fibroblasts, and leukocytes.

Mechanical dispersion A method of preparing embryonic stem cells for initial plating or passage during expansion. It involves the use of a thin capillary pipette, the end of which is equivalent in diameter to the size colony desired for plating.

Medical Device Amendments The Medical Device Amendments to the FD&C Act were passed in 1976, following reports of safety issues with respect to the Dalkon Shield intrauterine device. The Medical Devices Amendments included risk-based requirements for premarket notification or approval of medical devices. Prior to 1976, FDA authority was limited to taking action against marketed devices found to be unsafe or ineffective.

Meniscus (plural menisci) Semilunar fibrocartilaginous structures that are integral to the normal function of most joints in the body. The extracellular matrix consists of collagenous fibers that are mostly oriented circumferentially, with interspersing of radial fibers that contribute to its structural integrity. The circumferential fibers help disperse compressive forces while the radial fibers protect against tearing from tensile forces.

Mesangioblasts Vessel-associated progenitors that express early endothelial markers when isolated from the embryo and pericyte markers when isolated from postnatal tissues. Since MABs are able to cross the vessel wall and are easily transduced with lentiviral vectors, they have been used in preclinical models of cell therapy for muscular dystrophy.

Mesenchymal stem cells (MSC) The precise definition of MSCs remains a matter of debate. Nevertheless, to date MSCs are widely defined as a plastic-adherent cell population that can be directed to differentiate in vitro into cells of osteogenic, chondrogenic, adipogenic, myogenic, and other lineages. As part of their stem cell nature, MSCs proliferate and give rise to daughter cells that have the same pattern of gene expression and phenotype and, therefore, maintain the "stemness" of the original cells.

Metanephros (or metanephric) The metanephric kidney is the adult kidney that is formed at the caudal end of the nephric duct when an outgrowth, called the ureteric bud or metanephric diverticulum, extends into the surrounding metanephric mesenchyme.

Metaplasia The conversion of one cell type to another; it encompasses conversions that are part of the normal differentiation processes.

Mitomycin C A chemical used to mitotically inactivate cells for use as feeder layers in the culturing of embryonic stem cell lines.

Morality of benefitting from the deeds of others Those who oppose benefitting from the deeds of others that are deemed morally wrong hold the view that by doing so, we encourage similar deeds in the future. Those who support such benefit point to instances where the choices made to perform the objectionable deed are unconnected with the choices made to derive benefit from the accomplished results of the objectionable deed.

Morula-derived ES cells ES cells can be derived from the morula stage of embryonic development at much lower frequency. The advantage of using morula (or blastomeres) is that the embryo is not destroyed. Such cell lines could prove useful to the child resulting from the transfer of a biopsied embryo, as they would be genetically matched to the child.

Mouse MAPC phenotype The phenotype of mouse MAPC is B220, CD3, CD15, CD31, CD34, CD44, CD45, CD105, Thy1.1, Sca-1, E-cadherin, MHC classes I and II negative, epithelial cell adhesion molecule (EpCAM) low, and c-Kit, VLA-6, and CD9 positive.

Multipotent Adult Progenitor Cells (MAPC) Stem cells having the ability to differentiate into the three germ layers at the clonal, or single-cell, level, isolated from the bone marrow of mouse and rat. A Rosa26 mouse-derived MAPC line contributed to many somatic tissues of the mouse when injected into the blastocyst. MAPC are distinct from both mesenchymal stem cells (MSC) and embryonic stem cells (ES)

Multipotent or pluripotent A level of potency (defined in Chapter 1) where a stem cell is able to generate multiple cell types as progeny during mitotic division.

Multipotent Stem Cells Not the same as MSC, which are mesenchymal stem cells. During gastrulation, the pluripotent cells in the inner cell mass become restricted first to a specific germ layer and then to a specific tissue. The latter persist throughout adult life, and are termed multipotent stem cells.

Muscle stem cell (MuSC) The fundamental unit of muscle regeneration.

Muscular dystrophies (MDs) A family of inherited disorders characterized by progressive muscle wasting leading to a variable degree of mobility limitation, including confinement to a wheelchair and, in the most severe forms such as Duchenne muscular dystrophy (DMD), heart and/or respiratory failure.

Myocardial infarction Commonly known as a heart attack, myocardial infarction results from the partial interruption of blood supply to a part of the heart muscle, causing the heart cells to be damaged or die.

Myofibers Large, terminally differentiated, multinucleate cells formed by the fusion of multiple mononucleate MsSC. Myofibers can generally be grouped into two different types based on function, fast or slow contracting, a distinction that depends largely on the composition of myosin heavy chain (MyHC) isoforms they express.

Myosin Myosins comprise a family of ATP-dependent motor proteins and are best known for their role in muscle contraction and their involvement in a wide range of other eukaryotic motility processes. They are responsible for actin-based motility. The term was originally used to describe a group of similar ATPases found in striated and smooth muscle cells.

Naïve pluripotency Self-renewal that occurs in a leukemia inhibitory factor (LIF)-dependent manner and generates daughter cells capable of contributing to embryonic development after injection into blastocysts. Mouse ES cells are examples of cells having naïve pluripotency.

Nanog First isolated and named by Ian Chambers after a Scottish legend, Tir na nÓg, Nanog is a transcription factor, found in the nucleus, that is important for maintaining the ability of pluripotent stem cells to self-renew rather than differentiate. It is one of the most important transcription factors for stabilization of pluripotent state in mouse ES cells.

Nephric duct In mammals, there is a graded evolution of renal tubule development along the nephric duct, with the most anterior, or pronephric tubules, being very rudimentary, and the mesonephric tubules becoming well developed with glomeruli and convoluted proximal tubule-like structures.

Neural retina Formed from the inner cup of the optic vesicle, the neural retina contains the rod and cone cells that are active under dim light and daylight conditions, respectively.

Neural stem cells (NSC) The most primordial cells of the nervous system. They generate the array of specialized cells throughout the CNS (and probably the peripheral, autonomic, and enteric nervous systems as well).

Neurogenin3 The use of permanent labeling, usually DNA recombination, to mark a defined cellular population and trace its progeny.

Nonessential amino acids (NEAA) A cell culture media supplement, typically mixed with Dulbecco's modified Eagles medium (DMEM) to optimize conditions for ES cell line growth.

Oct4 Transcription factor required for formation and survival of pluripotent cells in the embryo. Oct4 is active as a maternal factor in the oocyte and remains active in embryos throughout the preimplantation period. Oct4 can form a heterodimer with Sox2 and bind DNA together. Early determinant of cell fate in preimplantation embryos. Expression of Oct4 is required to form inner cell mass cells; mouse ES cells lacking the Oct4 gene differentiate to trophoectoderm, whereas a twofold increase in Oct4 expression leads to endoderm and mesoderm formation.

Open systems One of two categories of combination approaches to tissue engineering in which cells are immobilized within a highly porous, three-dimensional scaffold. The use of scaffolds provides three-dimensional environments and brings the cells close so that it provides the cells with sufficient time to enable self-assembly and the formation of various components associated with the tissue microenvironment. Ideally, the material is degraded as cells deposits their extracellular matrix molecules.

Optic vesicle An evagination of the neural tube where the diencephalon and telencephalon meet. The vesicle forms a two-layered optic cup.

Organ of Corti An acoustic inner ear sensory organ that is the most complex structurally and in frequency sensitivity in mammals.

Organizer A location in the three-dimensional space of the gastrulating embryo from which induction signals emanate. The neural plate is derived from the dorsal ectoderm and is induced by organizer signals derived from the underlying notochord.

Orthotopic liver transplantation (OLT) Replacement of a patient's liver with a liver harvested from an ABO-compatible cadaveric donor, requiring major surgery and lifelong immunosuppression.

Otic placode The entire vertebrate inner ear derives from the otic placode, a thickening of the dorsolateral surface ectoderm immediately lateral to the hindbrain.

OXTR Gene that encodes the oxytocin receptor and is involved in uterine maturation and contraction. Part of the unique gene expression signature of AFMSCs.

p53 and p21 Suppression of these proteins can accelerate cell proliferation and inhibit senescence of target cells and dramatically increase the efficiency of induced pluripotent stem cell colony production. However, constitutive suppression of the p53 and p21 pathways increases the genomic instability of iPS cells, so transient suppression is used.

Pancreas The adult pancreas contains three major cell types: exocrine cells, organized in acini, which secrete digestive enzymes; duct epithelial cells which flush these enzymes to the duodenum; and endocrine cells, organized in the islets of Langerhans, that secrete hormones to the blood.

Pancreatic and duodenal homeobox gene-1 (Pdx1) A transcription factor gene essential for pancreas development. During embryogenesis, Pdx1 is expressed in stem/progenitor cells, giving rise to all cell types in the adult pancreas. During postnatal life, its expression becomes restricted to beta cells.

Pancreatic beta cell (specific to Chapter 33) A cell with the phenotype of a mature insulin-producing cell found in pancreatic islets. It is possible to have insulin-producing cells that do not meet these conditions, but those cells would not meet this definition's requirements.

Pancreatic beta cells Cells residing in the pancreatic structure known as the islets of Langerhans that produce insulin. Beta cells are the target of autoimmune attack in Type 1 diabetes.

Paneth cells Located almost exclusively at the crypt base of the small intestine and ascending colon, Paneth cells contain large apical secretory granules, and express several proteins – including lysozyme, tumour necrosis factor, and the antibacterial cryptins (small molecular weight peptides related to defensins).

Partial hepatectomy The removal of specific intact lobes of the liver, leaving behind undamaged the remaining lobes. The residual lobes grow to compensate for the mass of the resected lobes, although the removed lobes never grow back. The process is completed within 1 week.

Pax family Pax proteins are part of a family of transcription factors. Pax2 and Pax8 are two of the earliest markers specific to intermediate mesoderm and appear to function redundantly in nephric duct formation and extension.

Pdx1 A pancreatic transcription factor, expressed early in the endoderm prior to overt morphological development of the pancreas, that plays a fundamental role in the development of the pancreas during embryogenesis. Pdx1, when co-expressed with a VP16 protein or as a Pdx1-VP16 fusion protein in hepatocytes, will cause transdifferentiation to produce both endocrine and exocrine cells of the pancreas (e.g., insulin, glucagon, and amylase-expressing cells).

Penetrance The degree to which donor cells contribute to the tissues in a chimera. Low penetrance indicates that very few donor cells are retained in the organism whereas high penetrance indicates that many tissues and organs are derived from donor cells. It is possible that an organism manifests 100% penetrance.

Peripheral nervous system (PNS) The nerves and ganglia located outside the brain and spinal cord. The main function of the PNS is to connect the CNS with the limbs and organs. The PNS consists of the somatic nervous system and the autonomous nervous system. During development, all the cells of the PNS are derived from a small set of neuroepithelial cells.

Polarity cues Microenvironment that orients daughter cells to a location outside the stem cell niche to an alternative environment that encourages differentiation

Porta hepatis An anatomic structure that defines the area where the hepatic artery, portal vein, and common bile duct enter or exit the liver.

Portal vein One of two afferent blood supplies to the liver. The portal vein brings venous blood rich in nutrients and hormones from the splanchnic bed (intestines and pancreas).

Potency A characteristic of stem cells that describes the range of differentiated cell types that can be produced by mitotic division.

Potential stem cell Cell that retains the capacity to function fully as a stem cell if necessary. Normally, these cells are displaced with time into the dividing transit population, but they retain the undifferentiated status of the ultimate stem cell until such time as they are displaced into the dividing transit populations.

Precursor cells (specific to Chapter 33) Cells capable of more limited self-renewal than stem cells, but still capable of producing differentiated cells of multiple lineages.

Primed pluripotency Self-renewal that occurs in the presence of fibroblast growth factor 2 (Fgf2) and Activin and generates daughter cells that are rarely capable of contributing to embryonic development after blastocyst injection. Mouse epiblast stem cells are examples of cells having primed pluripotency.

Primitive hematopoiesis The first wave of embryonic blood development. It occurs in the yolk sac and consists principally of nucleated erythrocytes that express embryonic globins. The primitive wave is believed to supply the needs of the embryo.

Primordial germ cells (PGC) PGC are the sole means of genetic transmission between parent and offspring, as they generate eggs and sperm. PGC do not survive well under standard tissue culture conditions, and are not pluripotent stem cells in vivo or in vitro.

Prostaglandin E (PGE) Treating CB with a short-term pulse of PGE is being evaluated in clinical trials as a means of enhancing engraftment of donor CB.

Public Health Service Act of 1944 Incorporated the 1902 Biologics Control Act and is the present legal basis for licensing of biological products. Because most biological products also meet the definition of "drugs" under the FD&C Act, they are also subject to regulation under that Act.

Quality Assurance A system of procedures that are performed on a scheduled basis (daily, weekly, monthly, etc.) where the observations are recorded, logged, and analyzed on a regular basis.

Quiescence A characteristic of cell populations where the majority of cells are in G0.

Rat MAPC phenotype The phenotype of rat MAPC, the phenotype is CD44, CD45, MHC classes I and II negative, but CD31 positive.

Reprogramming Refers to one of several types of manipulation that result in a significant change in the cell type of recipient cell (e.g., transferring a somatic cell nucleus into a amphibian oocyte)

Reprogramming factors When somatic cells were fused with enucleated oocyte in studies that yielded cloned animals from frog and sheep somatic cells, it indicated the existence of reprogramming factors in the oocyte.

Retinal pigmented epithelium (RPE) Formed from the outer cup of the optic vesicle, the RPE is a single layer of epithelial cells heavily pigmented to capture stray light that passes through the retina.

Satellite cell The original term used to describe the cell type that was eventually identified as a MuSC. Anatomically defined in 1961 by transmission electron microscopy (TEM) studies of the peripheral region of muscle fibers in the tibialis anticus muscle of the frog, the discovery of the satellite cell heralded the birth of the field of muscle regeneration.

Sebaceous gland (SG) An appendage of the epidermis that is located in the upper portion of the hair follicle, just above the arrector pili muscle and is made of fat-containing cells that will release their lipid content into the hair canal.

Self-maintenance probability The probability that stem cells make other stem cells on division. It applies to populations of stem cells rather than individual cells. In steady state it is 0.5, but during situations where stem cell populations expand it can be between 0.5 and 1.0.

Self-renewal A type of mitotic division in which the daughter cell is identical to the parent cell.

Skin The outer covering of the body that is composed of two main tissues: the epidermis and its appendages largely composed of specialized epithelial cells (keratinocytes) and the dermis, largely composed of mesenchymal cells.

Skin ulcers Chronic wounds of the skin that may be caused by several pathological processes, including infection, trauma, diabetes, and venous ulcer disease. Chronic venous ulceration is a common cause of skin ulcers, with an estimated prevalence of 1–1.3%. Skin ulcers are also difficult and expensive to manage, because of their slow rates of healing and the requirement for expensive and labor-intensive dressing regimes.

Somite Bilaterally paired blocks of mesoderm that form along the anterior-posterior axis of the developing embryo in segmented animals. In vertebrates, somites give rise to skeletal muscle, cartilage, tendons, endothelial cells, and dermis.

Special Diabetes Initiative A fifteen-year (1998–2013) initiative that will provide more than $1.89 billion in supplemental funding for special initiatives in Type 1 diabetes research (on top of usual NIH appropriations) and will provide an equal dollar amount (more than $1 billion) to fund special initiatives in diabetes care and education for Native Americans with diabetes.

Stella A maternally inherited factor found in oocytes that is necessary for normal preimplantation development.

Stellate cells Stellate cells represent about 5–10% of all hepatic cells. In addition to storing vitamin A, they are essential for the synthesis of extracellular matrix proteins and produce many hepatic growth factors that play a vital role in the biology of liver regeneration.

Stem cell A cell that can produce a daughter cell by mitotic division in one of two ways: either the daughter cell is a replicate of the parent stem cell (self-renewal – defined in Chapter 2) or it is differentiated cell – defined in Chapter 2).

Stem cell (specific for Chapter 2) A clonal, self-renewing cell population that Is multipotent and thus can generate several different cell types (not applicable in all instances)

Stem cell (specific to Chapter 33) Precursor cells capable of indefinite self-renewal and capable of producing cells from the three embryonic germ layers – ectoderm, endoderm, and mesoderm.

Stem cell niche A subset of tissue cells and extracellular substrates that can indefinitely house one or more stem cells and control their self-renewal and progeny production in vivo.

Stem cell niche hypothesis The integration of intrinsic factors and extrinsic cues provided by the surrounding environment that regulates the balance between self-renewal and differentiation.

Stemness A characteristic of stem cells that refers to common molecular processes that underlie the core stem cell properties of self-renewal and the generation of differentiated progeny. A comprehensive definition of stemness has not been achieved to date; however, many genes that are enriched in stem cells have been identified and shown to be critical to the maintenance of stemness.

STO cells A type of 3T3 cell line commonly used as a feeder cell line for ES cell culture.

Stromal vascular fraction (SVF) A preparation made from adipose tissue after treatment with collagenase. SVF is believed to parallel the mononuclear cell fraction obtained from bone marrow via density gradient separation.

Support cells Non-stem cells that reside within a stem cell niche for the purpose of providing critical self-renewal signals, maintenance signals, or both. Also can provide anchoring function to keep stem cells within the niche via cell-cell interaction.

Symmetric cell division In stem cell self-renewal, symmetric cell division generates two stem cells

Telogen A non-growth phase of the cyclical function of the hair follicle – a resting phase.

Telomere integrity A hallmark of progenitor cells is the maintenance of chromosomal telomere integrity through the action of telomerase. Telomerase activity in the adult myocardium is restricted to small interstitial cells that express Sca-1 but lack other markers of hematopoietic stem cells (c-kit, CD45, CD34) or endothelial progenitor cells (CD45, CD34, Flk-1, Flt-1).

Teratocarcinoma Discovered by Barry Pierce, teratocarcinomas are malignant tumors that were initially obtained as a result of mutations that affected differentiation of male or female germ cells.

Teratoma An encapsulated tumor, usually benign, that results from the abnormal development of pluripotent cells. Teratomas consist of tissue or organ components resembling normal derivatives of all three germ layers. Teratomas occur naturally, but in stem cell research, teratomas have been induced by introducing pluripotent cells into recipient organisms. Teratoma formation has also been used as an assay to demonstrate the pluripotency or stemness of a particular population of cells or clonal cell lines.

Tissue engineering An interdisciplinary science that involves the use of biological sciences and engineering to develop tissues that restore, maintain, or enhance tissue function.

Tissue homeostasis A state where the self-renewal of stem cells and the production of daughter cells via differentiation is in balance.

Tongue proliferative unit A modified version of the epidermal proliferative unit identified in the filiform papillae on the dorsal surface of the tongue.

Transdifferentiation The conversion of one differentiated cell type to another; it is a subset of metaplasia. These events, because they involve differentiated cells, are not part of normal differentiation processes. There is some controversy in the field at present as to what exactly defines a transdifferentiation event. Some believe that such events are artifacts of cell culture. However, one well-documented example that is observed in mice is a pancreas-liver transdifferentiation.

Tropism A characteristic of cells that makes them able to be attracted to a specific location within an organ or body across distance. Stem cells often display tropism toward areas of injury or degeneration.

Type 1 (juvenile) diabetes A form of diabetes in which the insulin-producing beta cells of the pancreas are destroyed by autoimmune reaction.

Unipotent or progenitor A level of potency where a stem cell can produce only one type of differentiated cell. A progenitor cell is more constrained in its differentiation potential or capacity for self-renewal than most stem cells.

Unsegmented, or intermediate mesoderm A region of mesoderm that forms distal to the somites along the anterior-posterior axis in vertebrates. The intermediate mesoderm gives rise to the kidney during development.

Wilms tumor An embryonic kidney neoplasia that consists of undifferentiated mesenchymal cells, poorly organized epithelium, and surrounding stromal cells. The Wilms tumor suppressor gene, WT1, is an early marker of metanephric kidney tissue and is essential for its survival.

Wnt genes Wnt genes encode a family of secreted peptides that are known to function in the development of many tissues. Wnt4 activation is an early event in the mesenchyme that is induced to form kidney in the embryo that participates in separating stromal lineage from epithelial lineage development.

Xenotransplants When an organ or tissue used for transplant comes from a different species than the recipient (e.g., pig tissue transplanted into human)

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