Stem Cell Biology and Regenerative Medicine

Justin Ainscough
Shinya Yamanaka
Takashi Tada *Editors*

Nuclear Reprogramming and Stem Cells

Humana Press

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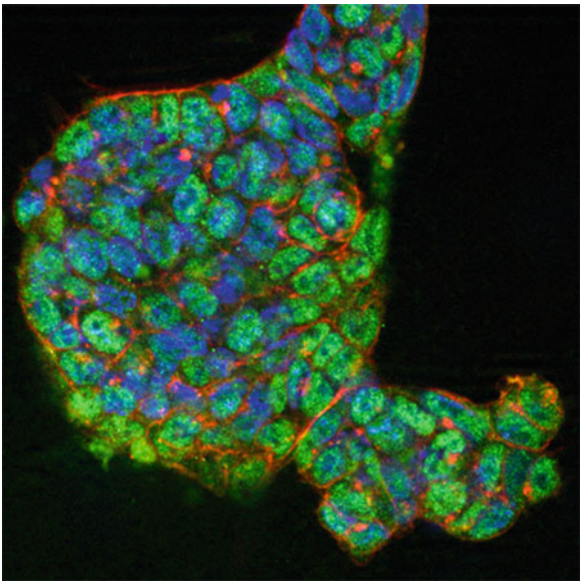

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Title page image shows expression of a DNA replication protein in mouse embryonic stem cells, courtesy of Sumia Bageghni and Erin Greaves, Leeds, UK.

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Preface

Rarely has a field of research generated such intense interest and division between individuals as the field of stem cell biology. This has become, without rival, the most awe inspiring area of biological research in recent times, and can only increase in importance as the potential impact on human health is realised. The field is evolving on a weekly basis with dramatic new discoveries bringing the possibility of personalised stem cell therapeutic intervention ever closer. Inevitably, with such rapid development, it is not possible to cover everything in a single collection of work. Instead, we hope that this book will provide a representative and informative glimpse into the world of stem cell biology as the field stands in 2011, and will be of interest to inquisitive lay readers and students, as well as academic researchers.

Stem cell research has historically relied on materials derived from embryos, often human. Consequently, many ethical issues have been raised that require sensitive and balanced handling by researchers and governments alike. In some countries stem cell research was all but blocked; however, the rules of the game are changing. Now the pioneering work of a few insightful individuals is becoming the foundation for an exploding field in which a new generation of talented scientists are making their own incremental contributions.

Some of the earliest documented attempts at manipulating cellular identity and generating potential for new life were made in the 1950s, with the nuclear transfer experiments of Robert Briggs and Thomas King. Their work was closely followed by that of John Gurdon whose pioneering experiments resulted in the first surviving adult animals to be cloned from somatic cells. It was over 30 years before the next major leap forward in this area of research, with the creation of Dolly the sheep by Ian Wilmut and colleagues. Since Dolly was the product of a single somatic cell isolated from her genetically identical donor "mother" the only genetic material held in common with her birth mother was their mitochondrial genome. The potential ramifications of this are discussed in Chap. 8 of this book. Following announcement of Dollys' arrival, the scientific community soon realised that the possibilities were now endless. The original articles from John Gurdon and Ian Wilmut and his colleagues, as well as other selected seminal pieces of work, are reprinted at the end of this book to enable the reader easy access.

Meanwhile, in the 1980s Martin Evans and colleagues developed ground-breaking technology for isolation and maintenance of mouse embryonic stems cells, with human embryonic stem cells appearing on the scene almost 2 decades later. The advent of embryonic stem cell research opened the doors to an array of new avenues aimed at exploiting the flexibility of the embryonic environment. At the beginning of this century Masako and Takashi Tada used cell fusion techniques to reprogram a somatic genome by exposing it to factors in embryonic stem cells (discussed in Chap. 6). However, the major driver for the current intensity of research was the demonstration, in 2006 by Shinya Yamanakas' team, that somatic cells can be directly reprogrammed to acquire a stem cell-like identity with only minimal coaxing (discussed in Chap. 7). Reprogramming is now so commonplace that it can be performed using commercially available kits with the most basic of facilities. With one stroke, this breakthrough eliminated two major obstacles blocking the application of stem cell-based therapeutics: the ethical issues associated with the use of materials derived from human embryos and the very real complication of immunological incompatibility. However, as genetic manipulation is still required, the use of reprogrammed materials for therapeutic intervention remains restricted. At the time of writing, major efforts are directed towards improving these technologies to make them safe for use in humans.

In brief, following Introductions from eminent scientists in the field, Chap. 3 of this book discusses when, where and what cells are subjected to nuclear reprogramming during progression through normal mammalian development. Chapters 4–7 discuss different experimental approaches used to reprogramme a fully differentiated somatic nuclear genome into a genome with freshly acquired pluripotency. Chapter 8 is devoted to the importance of the mitochondrial genome in this process and why this should not be ignored when producing reprogrammed cells for medical application. Chapters 9 and 10 discuss some of the core factors that play key roles during the process of nuclear reprogramming, while Chaps. 11–16 discuss the reprogramming of pluripotent cells down specific cellular lineages. In these chapters, emphasis has been placed on differentiation down neural lineages, in particular those of the eye, and on development of pancreatic beta cells. Both of these areas have developed significantly over the last few years bringing closer the possibility of using an individual's own cells to treat common ocular and diabetic complications. Chapter 16 discusses the enormous efforts that are being made to manipulate cellular identity towards interventions for diseases of the cardiovascular system. Chapter 17 discusses how reprogramming strategies can be used for development of disease-specific cell lines that more accurately represent specific diseased states, for use in both basic research and testing of new drugs. Finally Chaps. 18 and 19 come with warnings, and discuss the fact that reprogramming strategies are often associated with destabilisation of the genome and do not always yield consistent results. These chapters drive home the fact that reprogrammed cells must be rigorously evaluated before being used in humans.

Not so long ago the reprogramming of a human cell, so that it might become a useful part of a different functioning entity, was unimaginable. This collection of works has been drawn together at a time when a great deal is known about how to

achieve this remarkable feat. Some of the key genetic components essential to the reprogramming process have been identified, and methodologies are rapidly evolving and improving. However, there is still much to learn and, undoubtedly, significant progress will have been made before this book is widely available. It is intended, therefore, to offer a brief introduction to this thought provoking area of research and provide a background for how we have come to be where we are now. Interested readers can progress in this field by directly accessing new publications from the talented individuals who have contributed the following chapters. It is a privilege to have had the opportunity to work with Takashi and Shinya in compiling this book, at such a critical time for this field of research.

Leeds, UK Justin Ainscough

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Chapter 1
Introduction

John Gurdon and Azim Surani

As cells proceed in normal development from embryonic and fetal stages to an adult state, they become increasingly committed to their differentiated state. Hardly ever do cells reverse this process and go back from a differentiated state to an embryonic one. Nevertheless, there are experimental procedures by which this reversal of differentiation can be induced. These include somatic cell nuclear transfer to eggs and oocytes and the fusion of adult with embryonic cells as was shown over 50 years ago. Nevertheless, the field of nuclear reprogramming received an enormous stimulus when it was shown by Takahashi and Yamanaka in 2006 that some fully differentiated cells could be induced by transcription factor overexpression (iPS) to form multipotential ES cells. These can then be directed into a wide range of differentiated states unrelated to that of the original cells. Although changes in cell differentiation by overexpression of particular transcription factors had been shown many years before, the sucess with which the reversal to an iPS state took place was a surprise to everyone. The iPS-derived embryonic stem cells have the ability to proliferate indefinitely as embryonic stem cells but are then able to differentiate into many different somatic cell types as well as germ cells.

Somatic cell nuclear transfer can achieve reprogramming with high efficiency in the mouse without the need for exogenous transcription factors, although unfertilized human eggs are unobtainable in any number, and this route is not seen as likely to be therapeutically useful. The fusion of a somatic cell with an embryonic stem cell can effectively reverse the differentiation of the specialized cell, but it has not been possible to efficiently remove the embryonic cell contribution to the reprogrammed fused cell. Therefore, in both somatic cell nuclear transfer to eggs and in heterokaryon cell fusion, the techniques are primarily valuable for identifying the function of normal, natural cell components with reprogramming activity. On the other hand, the efficiency of embryonic stem cell derivation by iPS is very low, but

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holds great promise for therapeutic application in the future. Eventually it may be desirable to combine the benefit of natural reprogramming components of eggs and cultured cells with the induced pluripotency procedure. In "Nuclear Reprogramming and Stem Cells," the editors have assembled a wide range of chapters from authors working in many different aspects of this topic. While the principle of reprogramming by these routes is established, very little is at present understood about the mechanisms involved, and the authors of chapters in this book have summarized what can be said on mechanisms at the present time.

Before nuclear reprogramming and stem cell derivation can be turned to therapeutic benefit, considerable advances will be needed in several areas. Notably, rapid progress is currently being made in techniques that provide transcription factor overexpression without integrating viruses or other agents into the genome and in reducing the probability of cancer resulting from stem cell conversion. However, the efficiency of iPS production is still low and might therefore require a considerable amount of amplification of the resulting stem cells. This in turn would increase the likelihood of genomic errors in the amplified ES cells, and these errors could adversely affect the value of the derived cells. Derivation of embryonic stem cells directly from blastocysts also occurs from very few cells that need to be amplified. Nonetheless, currently the most significant use of iPS is to use patient-specific cells to model human diseases in vitro, which provide both basic knowledge concerning a variety of disease processes, and to use these cells to screen for therapeutic agents.

In most instances, the use of iPS in cell therapy will require considerable additional work. For instance, the integration of stem cells that function well in vitro into a functioning recipient organism does not yet work well. Much more will have to be understood about the way in which in vitro derived cells can be incorporated into tissues of a living host without loss of function.

The chapters in this book make a very valuable contribution to our present understanding of the state of the art in this exciting and promising field.

Chapter 2
Introduction: Stem Cells – What Next?

Ian Wilmut

An extraordinary amount has been learned during recent decades about the mechanisms that regulate early development and this understanding has provided the intellectual basis for exciting technical innovations. These in turn are already providing important new opportunities in research and therapy. While it is true that the present methods have limitations that must be overcome, it also seems very likely that there are important additional opportunities that have not yet been explored.

During the last 10 years a great deal has been learned about the signalling networks that maintain cell phenotype. In particular, the networks in pluripotency have been analysed extensively. Following the identification of Nanog as a key regulatory gene (Chambers et al. 2003), Chambers and Masui and their colleagues, along with several other laboratories, have characterised its interaction with other genes, such as SOX2 and Oct4 (reviewed by Chambers and Tomlinson 2009). These studies have revealed several other genes that are members of the network and also shown that pluripotent cells are poised in an oscillating state ready either to differentiate or to retain their pluripotent status (Kalmar et al. 2009).

This volume demonstrates just how far we have already come in a relatively short time. Only 2 decades ago, very few people believed that differentiated cells could be reprogrammed to have the characteristics of embryonic or foetal cells. The birth of Dolly, the cloned sheep, in 1996 demonstrated that the fate of cells is far more adaptable than had previously been imagined (Wilmut et al. 1997). Subsequent research by Shinya Yamanaka, Jamie Thomson and their colleagues has opened up a fertile field of enquiry (Takahashi and Yamanaka 2006; Yu et al. 2007). The first methods were established that make it possible to obtain pluripotent cells from skin cells of patients. If the person has an inherited disease then those pluripotent cells can provide cells of the type that are affected in the inherited disease. For the first time, comparisons may be made between diseased and healthy cells and vital

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understanding gained of the molecular mechanisms that lead to the development of disease symptoms. Soon, we will see the first drugs able to provide effective treatment for degenerative and genetic diseases. There are hundreds of inherited human diseases that are not understood and for which there is no treatment (http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim).

However, present methods of reprogramming have a number of limitations. The most commonly used methods rely upon retroviral vectors to introduce the transcription factors. Pluripotent cells produced in this way have been used effectively to understand the molecular basis of at least one inherited disease, familial dysautonomia (Lee et al. 2009), but such cells could not be used in therapy. New protocols have been described more recently that use non-integrating vectors or the direct introduction of proteins or RNA encoding those proteins (Kim et al. 2009; Warren et al. 2010).

There are numerous reports of iPS cells having an epigenetic memory of the cell type from which they were derived (see Sullivan et al. 2010 for a review). In addition, there is evidence of considerable and reproducible variation between laboratories in the characteristics of cells produced by application of the same protocol (Newman and Cooper 2010). It is also interesting to note that there are great differences in the speed and efficiency of reprogramming depending upon the method used. Where the Yamanaka protocol requires several weeks to achieve full reprogramming, either cell fusion or nuclear transfer achieve reprogramming within hours or days. After fusion of a somatic cell and a pluripotent cell, nuclear reprogramming occurs in the great majority of hybrids within 1 day (Han et al. 2008). Similarly, normal pre-implantation development of mouse embryos produced by somatic cell nuclear transfer must reflect substantial reprogramming within a day or two. These differences could reflect the presence of the Yamanaka factors at a higher concentration in the recipient oocyte or, their greater access to key regions of the crucial regulatory genes or the presence of other factors that are able to influence the reprogramming process. Of course all three and perhaps other mechanisms may come into play. But evidence that there are indeed other factors is provided by the work of Alberio and Johnson (2011, Bian et al. 2009) and of Scholer and colleagues in a recent publication (Singhal et al. 2010).

Together, these observations suggest that it will be possible to establish protocols that do not involve integrating vectors and to refine the protocols in other ways in order to obtain a more reproducible outcome. However, it may also be necessary to establish laboratory procedures at higher standards of accuracy and reproducibility than are generally used in academic research laboratories and to consider the use of robotic systems in order to obtain a reproducible product.

Public discussion of the potential uses of stem cells in human medicine concentrates upon cell therapy. To focus exclusively upon this application is to overlook the enormous potential value of stem cells in research and drug discovery. The ability to study in the laboratory cells that are genetically identical and physiologically very similar to those in a patient that are affected by an inherited disease is providing extraordinary opportunities to study inherited degenerative diseases (see earlier). There are hundreds of inherited human diseases, some of which are

mercifully very rare. Unfortunately, very few of these diseases have effective treatments. By production of iPS cells and their differentiation into the affected lineage, it is possible to compare cells from patients and unaffected relatives and study differences in gene expression and cell function between the cells from the two donor groups. This innovative application of stem cells will provide new treatments for some human diseases in the near future.

The emerging methods for direction of cell phenotype depend absolutely for their effectiveness and use upon the availability of methods for accurate culture and characterisation of different cell types. There is still a need for simplification of protocols for maintenance of pluripotent cell cultures. These populations may then be induced to differentiate to the required cell type and notable contributions to the derivation of cardiomyocytes, retinal pigment epithelial cells, pancreatic beta-cells and cells of different neural lineages are described in this volume. However, in all cases it is fair to say that only a proportion of cells attain the desired terminal lineage and this fact imposes significant limitations on their use for therapy. In some cases, the cells reach the foetal stage of maturation rather than the adult state of the specific cell type and in addition pluripotent cells may remain in culture. The recent ultimately successful application to the FDA by the Geron Corporation for clinical trial of embryo stem cell derivatives for spinal cord repair is reported to have involved 20,000 pages of information. No doubt this was to establish both efficacy and the level of risk of teratoma formation.

There is a different approach which may offer some advantages in the development of cell therapies. Rather than reprogramme cells to pluripotent status why not derive multi-lineage progenitor cells? Such cells would retain the ability to form the cell types of interest, but are expected to pose a far smaller risk should they be inadvertently transplanted into a patient. A number of groups apparently have this strategy in mind and the first publications are appearing. In one recent publication, haematopoietic cells were obtained from fibroblasts after expression of Oct4 and selection of appropriate culture conditions (Szabo et al. 2010).

As has often been the case in the past the most rapid progress may involve a combination of basic research and empirical analysis of the best available means of achieving a particular technical objective. As is heralded by the chapters in this book, we can expect further significant progress in the years to come.

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Chapter 3
Inherent Nuclear Reprogramming in Mammalian Embryos

Ai Khim Lim, Barbara B. Knowles, Toshie Kai, and Daniel M. Messerschmidt

Abstract In the current literature, the term "nuclear reprogramming" is defined as either the switch of the gene expression state from one cell type to another or the change of a differentiated, specialized cell into a developmentally more primitive but more pluripotent state. Experimentally, nuclear reprogramming can be achieved by somatic cell nuclear transfer (SCNT) into oocytes, by cell fusion, or by introduction of specific transcription factors into a cell. The epigenome of the target cell becomes altered and a gain of developmental potential ensues. During embryogenesis, nuclear reprogramming is achieved inherently through complex epigenetic processes. Here we focus on the natural, forward process of differentiation in the developing mouse embryo and discuss the key epigenetic reprogramming events involved.

3.1 Introduction

The development of mammalian organisms is a one-way street. Fertilization of the oocyte by the sperm initiates a process characterized by constant loss of developmental potential with progression of time. The zygote, or one-cell stage embryo, is capable of giving rise to all cell types including the extraembryonic tissues, and

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is therefore defined as totipotent. In the mouse the totipotent state is unique to the zygote and to the single blastomeres generated after the first cleavage, but is rapidly lost thereafter. Although blastomeres of later stages (8–16 cells) are still able to contribute to all embryonic and extraembryonic lineages, they are unable to give rise to a whole organism as individuals. Now, at the morula stage, cells gradually become restricted and contribute to either the trophectoderm lineage or the inner cell mass. This commitment is irreversible and the first major restriction in potential cell fates. While trophectoderm cells are considered multipotent, since they can give rise to an array of cell types in the developing placenta, inner cell mass cells are considered to be pluripotent. They are competent to contribute to the three germ layers, namely endoderm, ectoderm and mesoderm and, importantly, to the germline. Like the totipotent state, the pluripotent state is transient and is lost as cells progress to the next developmental stage. Eventually, the fetal and adult organism loses pluripotent cells, establishing pools of multipotent, somatic stem cells required for tissue maintenance. The cells of the germline are a remarkable exception. As the sole source of genetic information for the next generation, the germ cells must be maintained in an epigenetically reprogrammable state.

Importantly, with few physiological and pathological exceptions, the genomic content of the totipotent zygote is identical to that of its pluripotent progeny, terminally differentiated somatic cells and premeiotic germ cells, and this content remains unchanged throughout development. Thus, the difference in the developmental potential of these cell types is imposed by a cell-specific transcriptional state that is ultimately dependent on the epigenetic changes of the genome.

3.2 Epigenetic Mechanisms

Epigenetic changes of the genome are controlled by DNA methylation, histone modifications, and in a broader sense, small, noncoding RNAs (see later section for discussion). Both DNA methylation and histone modifications affect the structure of the chromatin rather than the sequence. Thus, these modifications can be erased and reestablished without mutating the genome. DNA is methylated on the cytosines of CpG dinucleotides by DNA methyltransferases (DNMTs, Table 3.1). The extent of cytosine methylation, predominantly on CpG islands of promoters, reflects the expression status of the gene, with hypermethylation keeping the gene silent and hypomethylation allowing high gene expression. Posttranslational modifications, such as acetylation, deacetylation, methylation, and demethylation, on lysine and arginine residues of histone tails are mediated by histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), and histone demethylases (Table 3.1). Depending on the amino acid residues modified on histone tails, and the extent of these

Table 3.1 Function of chromatin modifying enzymes

Enzymes		Function and consequence	References
DNA methyltransferases	DNMT3a	De novo methylates CpG islands Establishes imprints in male & female germ cells	Okano et al. (1999)
	DNMT3b	De novo methylates CpG islands after implantation Demethylates microsatellite DNA	
	DNMT3L	Lacks intrinsic DNA methyltransferase activity Interacts with DNMT3a/b to modulate their activities	Hata et al. (2002)
Histone acetyltransferases		Mediates histone tail acetylation Destabilizes chromatin, hence activates transcription	Ogryzko et al. (1996) reviewed by Nakatani (2001)
Histone deacetylases		Removes histone tail acetyl groups Condenses chromatin, hence represses transcription	Taunton et al. (1996), reviewed by Ruijter et al. (2003)
Histone methyltransferases		Catalyzes *S*-adenosyl methionine transfer to histone lysines & arginines Modulates epigenetic gene regulation in genomic imprinting & X-chromosome inactivation	Xu et al. (2000); Yao et al. (1998), reviewed by Marmorstein and Roth (2001)
Histone demethylases		Removes methyl groups from histone lysine residues Transcriptional corepression	Shi et al. (2004); Tsukada et al. (2006), reviewed in Tian and Fang (2007)

Table 3.2 Histone modifications and their effects on gene expression

	Histone					
Modification	H3K4	H3K9	H3K27	H3K79	H2AR3	H4R3
Monomethylation	Activation	Activation	Activation	Activation		
Dimethylation		Repression	Repression	Activation		
Trimethylation	Activation	Repression	Repression	Activation Repression		
Acetylation		Activation			Repression	Repression

modifications, transcription factors or repressors are recruited to activate or repress gene expression, respectively (Table 3.2). For instance, trimethylation of lysine 9 and lysine 27 residues of histone H3 (H3K9 and H3K27) has a repressive function, while H3K4me3 and H3K9 acetylation (H3K9ac) is generally associated with gene activation.

3.3 First Wave: Early Epigenetic Reprogramming: The Egg, the Sperm, and the Zygote

Both egg and sperm are highly specialized, unipotent cells, which are transcriptionally inactive in their mature state. Consequently, their DNA is highly methylated and heterochromatinized. After fertilization the genome, or at least loci relevant for very early development, must be activated to ensure the proper onset of embryogenesis. These changes occur in a sequence of well-defined events, beginning with replacement of the highly basic protamines by histones in the paternal pronucleus, as soon as the sperm genome begins to decondense (Reik et al. 2003; Santos et al. 2002) (Fig. 3.1). Notably, despite extensive replacement of histone complexes by protamines during spermatogenesis, up to 15% of histones are retained in human spermatozoa (Gatewood et al. 1990) and in the mouse approximately 4% of the mature spermatozoa genome still contains histones, which bear transmittable chromatin modifications (Hammoud et al. 2009). Such nucleosomes consist of either canonical or variant histone proteins including the testes-specific H2B (TH2B) and are found primarily at loci specifying transcription factors and signaling molecule that guide embryonic development. Histones are also enriched on the promoters of maternally and paternally imprinted genes, although H3K4me3 modifications colocalize only to paternally expressed genes (Hammoud et al. 2009). It remains to be seen whether

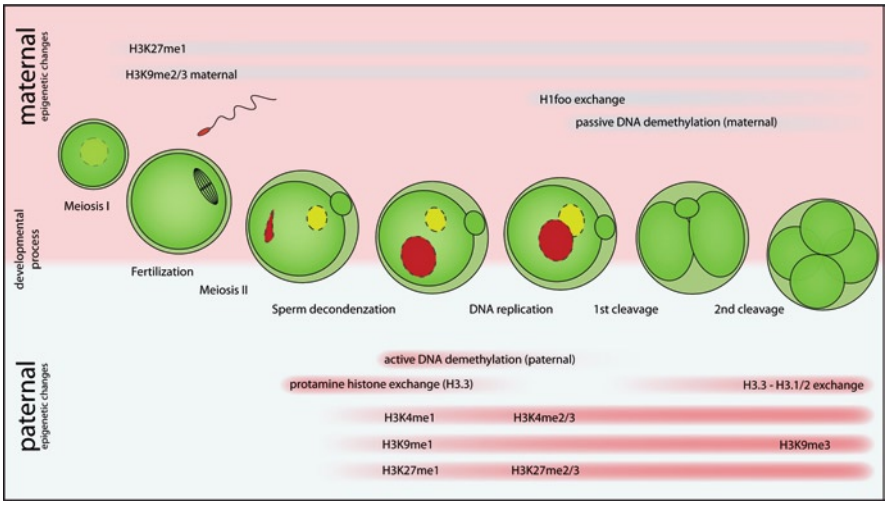

Fig. 3.1 Early epigenetic reprogramming: oocyte, sperm, and early embryo. Maternal and paternal genomes undergo characteristic epigenetic modifications in a timely controlled manner. Maternal DNA is readily packed into modified nucleosomes carrying distinctive modifications. On replication, the somatic form of H1 replaces the oocyte specific histone H1FOO. Maternal DNA is demethylated passively over several cleavage stages. Protamines in the paternal pronucleus are replaced independent of DNA replication by histones from the maternal protein pool and rapidly modified by methylation. Upon replication, H3.3 is replaced by H3.1/H3.2. DNA-demethylation occurs actively after sperm decondensation and protamine-histone exchange

the presence of (modified) histones in the sperm genome is of functional relevance, e.g., to prime embryonic transcription, or indeed whether these histones remain bound to the paternal genome after pronuclear decondensation. Other histone variants located at pericentric regions in sperm, for instance, are rapidly exchanged after fertilization (Govin et al. 2007; Wu et al. 2008).

Protamine exchange by histones stored in the oocyte takes place after polar body extrusion and decondensation of both the maternal and paternal genomes (Morgan et al. 2005). As this exchange is replication-independent, the paternal pronucleus harbors the H3.3 variant, which can be deposited by DNA synthesis-independent mechanisms utilizing the chaperone HIRA. Only later, during DNA replication are these H3.3 variants exchanged with H3.1/H3.2 core histones (Fig. 3.1).

The histones incorporated into the paternal pronucleus, as compared to the maternal pronucleus, are highly acetylated although it is unclear whether this is a passive effect of a greater abundance of acetylated histones in the maternal protein pool, or an active modification process (Adenot et al. 1997; Santos et al. 2002). Immediately after incorporation, the histones in the paternal pronucleus become monomethylated at H3K4, H3K9, and H3K27 (Erhardt et al. 2003; Lepikhov and Walter 2004; Santos et al. 2005). Marks such as H3K9me3 are virtually absent from the paternal genome (Fig. 3.1).

By contrast, the maternal chromatin is already prepacked with histones and highly organized before fertilization. Marks associated with the active chromatin state (e.g., acetylation and H3K4 methylation), as well as marks associated with a repressive chromatin state (e.g., H3K9me2/me3 and H3K27me1) are found in the germinal vesicle. Egg chromatin differs from somatic chromatin by the presence of a specialized, oocyte-specific H1 linker-histone (H1FOO) (Tanaka et al. 2001), which is speculated to mediate a less rigid chromatin state (Saeki et al. 2005). H1FOO is replaced in the chromatin during the first cleavage and it is not known if, and how much, this exchange contributes to the activation of the embryonic genome.

The differences between histone modifications and nucleosome composition in chromatin of maternal and paternal origin observed after fertilization persist in the 2-cell stage, but are no longer detectable in 4-cell stage or later embryos. However, they may be the origin of another difference between the paternal and maternal pronuclei, at the level of DNA methylation, which persists beyond the 16-cell, morula stage. Shortly after histones are incorporated into the paternal pronucleus in the zygote, the DNA from the paternal genome undergoes a rapid and active demethylation process, completed before the first round of replication. Although the maternal genome is also highly methylated, it is not affected by this active demethylation (Fig. 3.1). What protects the maternal genome is unknown. Differences in the demethylation dynamic of the DNA may be related to the differential modifications of the core histones mentioned above, and also nucleosome/histone interacting factors such as the maternally inherited *Stella/PGC7*. STELLA is recruited to the maternal nucleus in the zygote, where it protects several maternally methylated, imprinted gene loci (Nakamura et al. 2007). Moreover, it has been suggested that the presence of the replication-independent H3.3 variant in the paternal pronucleus

is associated with the asymmetric demethylation of DNA (Torres-Padilla et al. 2006; van der Heijden et al. 2005).

Clearly, the capacity for active demethylation of the paternal genome lies within the oocyte, as any introduced genome can be demethylated after SCNT into the egg. This process is poorly understood functionally, yet in principle, two possible mechanisms, either bona fide demethylation removing the methyl group from the C5 position of the cytidine ring or removal of the cytidine base itself (indirect demethylation), are possible. Bona fide demethylation requires breaking very stable C–C bonds. However, the deletion of the only candidate enzyme shown to be capable to catalyze such a reaction, MBD2, has no effect on the active demethylation of the paternal genome (Bhattacharya et al. 1999; Ng et al. 1999; Santos et al. 2002). A new study implies involvement of the transcription-elongator complex in direct demethylation, pointing to a function of the SAM (radical *S*-adenosylmethionine) domain of ELP3 (Okada et al. 2010). Indirect demethylation pathways involve DNA repair and several DNA–repair machinery components, such as the cytidine deaminases, AID and APOBEC1, or GADD45α-associated nucleotide-excision repair (Barreto et al. 2007; Bhutani et al. 2010; Morgan et al. 2004).

It is currently unknown whether the asymmetries between maternal and paternal chromatin are of any developmental consequence. The rapid demethylation of the paternal genome could simply reflect plain accessibility of "nude" DNA after protamine removal. It has been shown in other species that the demethylated state is short-lived, as it is quickly reversed by de novo methylation (Lepikhov et al. 2008; Park et al. 2007). Further, injection of round spermatids, which contain histones and are not (or are only partially) demethylated, into the oocyte does not significantly interfere with normal development (Polanski et al. 2008). Finally, some experimental evidence pointing toward an overall hypomethylation of promoters in sperm questions the importance of genome-wide demethylation for gene regulation during very early development (Farthing et al. 2008).

3.4 Second Wave: Cleavage Stages and Lineage Segregation in the Blastocyst

Following fertilization and nuclear reprogramming, the zygote enters into DNA replication and undergoes the first cleavage divisions. At this point the maternal genome, which has been protected from active demethylation, undergoes gradual passive demethylation until the morula stage. With each cleavage division, DNA methyl marks are progressively diluted, perhaps aided by exclusion of the maternal form of DNMT1 (DNMT1o) from the nucleus until the 8-cell stage (Bestor 2000; Howell et al. 2001; Monk et al. 1987). Thus, at the time the first lineage decision is made, the maternal and paternal genomes are no longer distinguishable other than by their parent-of-origin specific imprints, which are protected from demethylation.

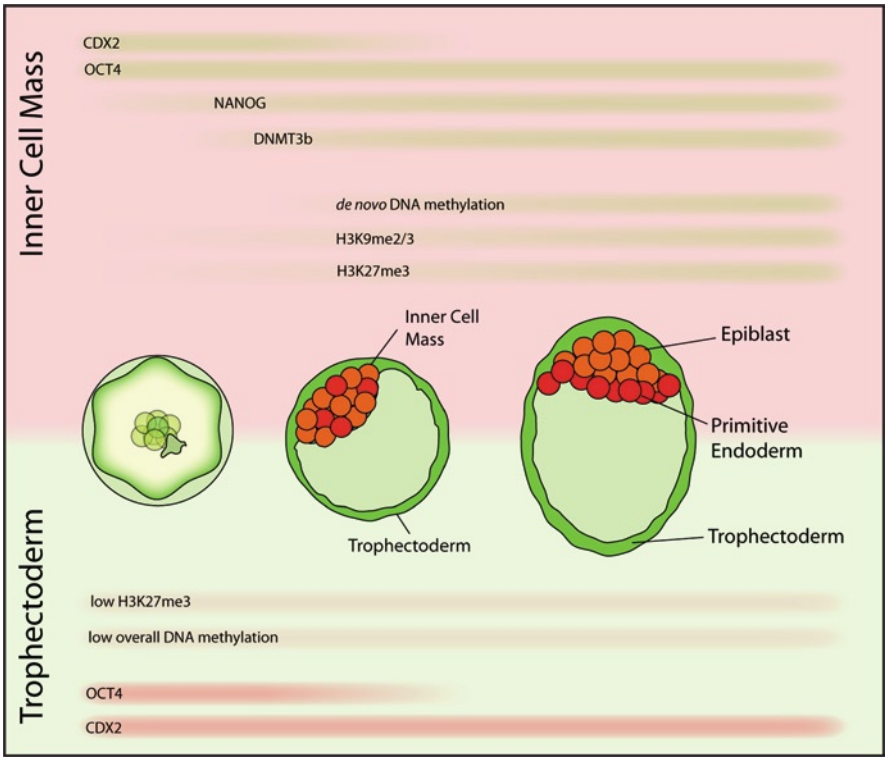

Fig. 3.2 Lineage segregation in the blastocyst. In the preimplantation embryo, lineage-specific transcription factors drive trophectoderm and inner cell mass lineage commitment. CDX2 enforces trophectoderm and OCT4/NANOG inner cell mass differentiation. Epigenetic modifications reinforce this lineage commitment with de novo DNA methylation?, due to DNMT3b expression in the inner cell mass. Repressive histone-modifications (H3K9me3, H3K27me3) are introduced onto developmentally regulated genes in the inner cell mass

Whereas the first wave of epigenetic reprogramming results in the totipotent zygote, the second wave culminates in the emergence of the pluripotent epiblast of the blastocyst. How this is achieved in the first 2–3 days of development is being studied in extensive detail in the mouse embryo. It is widely accepted that the interplay of lineage-specific transcription factors and epigenetic modifications drives the segregation of trophectoderm and inner cell mass, beginning as early as the 8-cell stage (Rossant and Tam 2009). This is followed by a second segregation of the inner cell mass into the epiblast and the primitive endoderm at the early blastocyst stage (Gardner and Beddington 1988).

Genetically, a well-understood network of transcription factors acts by promoting or suppressing lineage commitment (Fig. 3.2). Particularly, the pluripotency genes *Oct4, Sox2*, and *Nanog* direct cells into the pluripotent epiblast lineage (Mitsui et al. 2003; Niwa et al. 2000; Yuan et al. 1995), while *Cdx2* and *Eomes* are essential

for a functional trophectoderm (Ralston and Rossant 2008) and GATA transcription factors are required for primitive endoderm differentiation (Koutsourakis et al. 1999; Morrisey et al. 1998). In particular, the reciprocal repressive action of OCT4 and CDX2 is essential in driving the trophectoderm/inner cell mass segregation, whereas NANOG and GATA4/6 regulate the epiblast/primitive endoderm (Chazaud et al. 2006; Messerschmidt and Kemler 2010) (Fig. 3.2).

Lineage-specific epigenetic modifications are equally important to drive and support fate adoption. Several HMTs have been shown to be essential for early embryonic development. Prominent among them is EZH2, the enzymatically active component of the PRC2-complex, which imposes the repressive mark H3K27me3 on differentiation genes in the embryonic lineage (O'Carroll et al. 2001). Along the same lines, the euchromatic H3K9 HMT, SETDB1, is also required for maintenance of the epiblast (Dodge et al. 2004). Consequently, the epiblast lineage shows higher levels of H3K27me3 and H3K9me3 (Fig. 3.2).

In addition, de novo methylation, initiated in the morula, contributes to or enforces cell-fate decisions. Immunohistochemical detection of 5-methylcytosine revealed that the trophectoderm and inner cell mass adopted different methylation states. The trophectoderm retains the low ground state imposed by active and passive demethylation during the cleavage stages, compared to the inner cell mass, which shows extensive de novo methylation (Manes and Menzel 1981; Santos and Dean 2004; Santos et al. 2002). The responsible enzyme introducing this imbalance could be the DNA methyltransferase, DNMT3b, which is restricted to the inner cell mass lineage in the blastocysts (Watanabe et al. 2002). By contrast, embryonic stem cells, pluripotent stem cells derived from the epiblast, cannot maintain their lineage restriction and differentiate to trophectoderm derivatives if they lack DNMT3a or DNMT3b. This disproportion of DNA methylation is also maintained in the future derivates of the trophectoderm (placenta) and inner cell mass (the embryo), respectively (Chapman et al. 1984; Rossant et al. 1986). Whole genome-wide methylation analysis by high-throughput and deep sequencing technologies, however, have revealed that methylation differences in trophectoderm and inner cell mass might be restricted to intergenic regions. Thus, gene promoters could be an exception to this asymmetry, explaining the overall similar transcriptional activity between the two lineages (Farthing et al. 2008; Tanaka et al. 2002).

3.5 DNA Demethylation: Imprinting and Retrotransposons

Although referred to as "genome-wide" demethylation, retrotransposons, heterochromatin in and around the centromeres and imprinted genes are exceptions that escape the erasure of the 5-methylcytosine marks. Imprinted genes add another layer of asymmetry to the early zygote because they are methylated in a parent-specific fashion. These marks are preserved, despite active or passive DNA

demethylation of both the paternal and maternal genomes following fertilization. Over a hundred genes have been shown to be imprinted to date, and a recent report suggests the number of genes whose expression exhibits a parent-specific bias might be highly underestimated, at least in the brain (Gregg et al. 2010). The developmental benefits of, and mechanisms for, establishing imprinting are unknown. Imprinted genes are often organized in chromosomal domains, and their respective imprinting control regions are marked by monoallelic methylation to activate or silence specific genes on the respective parental alleles (Kaneko-Ishino et al. 2003; Reik and Walter 2001b).

Remarkably, only three imprinting control regions in the mouse genome are methylated during spermatogenesis, whereas many more are methylated in the oocyte regardless of later maternal or paternal expression of the "targeted" gene. It has been hypothesized that this imbalance is the result of drastic demethylation of the paternal genome whereas the prevalence of methylation imprints in the maternal genome evolved to protect imprinting marks (Reik and Walter 2001a). In contrast to the case in primordial germ cells (PGCs), where DNA methylation marks on imprinted loci are completely erased, even the paternal marks are retained in the early embryo to avoid over- or under-expression of developmentally important genes (Morison et al. 2005).

A major portion of the mammalian genome is composed of interspersed repeats and transposable elements. Mammalian retrotransposons include short interspersed nuclear elements (SINEs), long interspersed nuclear elements (LINEs), and endogenous retroviruses [long terminal repeat (LTR)-type retrotransposons]. While studies have shown that retrotransposons are actively demethylated in the embryonic germline (Hajkova et al. 2002; Lees-Murdock et al. 2003), a class of endogenous LTR-retroviral elements, the intracisternal A-type particle (IAP) elements, appears to be largely resistant to demethylation during preimplantation development (Lane et al. 2003). IAP-resistance to demethylation may have evolved as a protective mechanism to preserve the genome from random retrotransposition. Alternatively, the epigenetic inheritance of IAP-methylation may affect the expression state of neighboring genes.

3.6 Third Wave: Epigenetic Reprogramming of the Germline

At E7.25 when germ cell fate is established, both somatic cells and PGCs display similar levels of DNA methylation, histone marks, and exhibit monoallelic expression of imprinted genes (Fig. 3.3). These marks are continually maintained in the somatic cells but altered in migratory PGCs. In developing germ cells (E11.5–E12.5), global DNA demethylation takes place and consequently, imprinted genes are biallelically expressed (Hajkova et al. 2002; Szabo and Mann 1995). This erasure of parental imprints is necessary to restore the naïve state of the germ cell so that new imprints can be established according to the sex of the germ cell's host.

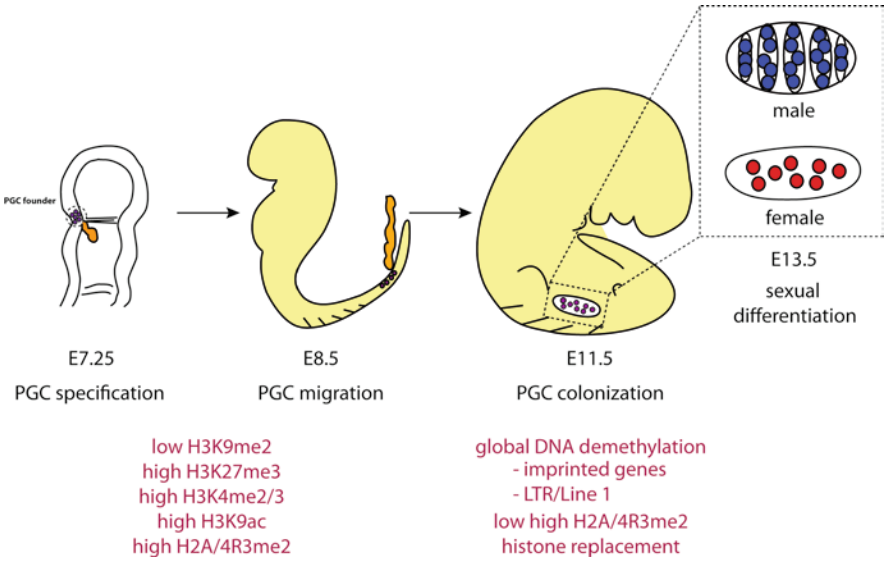

Fig. 3.3 Chronological events during germ cell development in mouse embryo. At E7.25, the proximal epiblast cells receive BMP signals from the extraembryonic ectoderm, marking these cells as the PGC founders. These cells relocate to the posterior proximal end of the embryo and divide mitotically to form a cluster of approximately 40 PGCs. As development continues, PGCs migrate toward the genital ridges at E9.5. During migration, PGCs are still sexually bipotential and upon colonization of the genital ridges at E11.5, they begin parent-of-origin, sex-specific differentiation

3.7 DNA Methylation Is Linked to Changes in Chromatin Structure

Hajkova et al. (2008) suggest that DNA demethylation and the subsequent erasure of imprints are linked to changes in chromatin structure. At E8.5, early migratory PGCs could already possess unique histone marks distinct from those of the surrounding somatic cells. These include low H3K9 dimethylation (H3K9me2) and high H3K27 trimethylation (H3K27me3), H3K4me2, H3K4me3, H3K9 acetylation (H3K9ac), and H2A and H4R3 symmetrical dimethylation (H2A/H4R3me2s) (Fig. 3.3) (Ancelin et al. 2006; Hajkova et al. 2008; Seki et al. 2007). The erasure of H3K9me2 appears to be linked to repression of a H3K9 methyltransferase, G9a-like protein (GLP) (Seki et al. 2007) while upregulation of H2A/H4R3me2s is mediated by a BLIMP1-interacting factor, protein arginine methyltransferase 5 (PRMT5) (Ancelin et al. 2006).

The global erasure of repressive marks during epigenetic reprogramming of migratory PGCs potentially results in transcriptional misregulation of the germ cell genome. To protect the germ cell genome from deregulated transcription, RNA polymerase II-dependent transcription is reduced until the repressive mark,

H3K27me3, is sufficiently reestablished (Seki et al. 2007). It is likely that the increased H3K27me3 also serves as a form of protection to maintain chromatin in a repressive state during global histone modification.

Upon colonization of the genital ridges at E11.5, germ cells are further programmed and geared toward sex-specific differentiation. First, global demethylation of the germ cell genome takes place. As such, the differentially methylated regions of imprinted genes, including maternally methylated *Snrpn*, *Peg3*, *Lit1*, and *Igf2* and paternally methylated *H19* and *Rasgrf1*, are synchronously demethylated (Hajkova et al. 2002; Li et al. 2004; Yamazaki et al. 2005). These parental imprints are then reestablished differentially in males and females. In the case of males, this is completed at around birth and in females, reestablishment of imprints only takes place after birth during oocyte growth. An undesirable consequence of global demethylation of the embryonic germ (EG) cell genome is derepression of retrotransposon expression, such as Line 1 and IAP elements. While these retrotransposons are mostly remethylated and repressed, at E17.5 or after birth in the male and female germline respectively, Line 1 elements remain unmethylated and transcripts from the defective mouse retrovirus *Mouse transcript* (MT) are highly expressed until after fertilization (Peaston et al. 2004). Differential expression of retrotransposon-host chimeric genes, as observed during the oocyte-to-embryo transition and in preimplantation embryos, may also trigger sequential reprogramming of the germ cell genome (Peaston et al. 2004).

Second, global DNA demethylation is accompanied by histone changes at E11.5. The BLIMP1/PRMT5 complex is translocated into the cytoplasm, H2A/H4R3me2 methylation is downregulated, and transcriptional repression of genes is relieved (Ancelin et al. 2006). At the same time, rapid loss of histone H1 and replacement by histone variant H2A.Z are observed in the germ cells (Hajkova et al. 2002, 2008). This is accompanied by concomitant loss of both repressive (H3K9me3, H3K27me3, and H2A/H4R3me2s) and transcriptionally active (H3K9ac) histone marks, as well as accumulation of the histone chaperones, nucleosome assembly protein 1 (NAP1), and HIRA, in the germ cell nuclei (Hajkova et al. 2008). The DNA chromocentres disappear and proteins that are normally associated with heterochromatin, such as heterochromatin protein 1 (HP1), alpha-thalassemia/mental retardation syndrome X-linked (ATRX), and polycomb (PCL/M33), are also lost (Hajkova et al. 2008), suggesting the "loosening" of closed chromatin. Taken together, genome-wide DNA demethylation in germ cells appears to be linked to histone replacements, loss of histone modifications, and changes in nuclear architecture.

The above-described epigenetic changes take place during the G2 phase of the cell cycle and are therefore independent of DNA replication. The DNA-repair pathway is likely to be involved in replacing methylated cytosines with their unmethylated counterparts. Indeed, in a recent report by Hajkova et al. (2010), chromatin remodeling in germ cells was described to be mechanistically linked to the base excision repair (BER) pathway. X-ray repair complementing deficiency repair in Chinese hamster cells (XRCC1), a soluble nuclear factor that binds single-stranded DNA, is largely bound to the chromatin in germ cells during global DNA demethylation at E11.5 (Hajkova et al. 2010), suggesting that single-stranded DNA breaks

are present in the germ cell chromatin that is undergoing demethylation. Indeed, in *Stella*-null zygotes where demethylation occurs in both the maternal and paternal pronuclei, XRCC1 is detected in both pronuclei as compared to the wild-type zygote where only the paternal pronucleus undergoes demethylation (Hajkova et al. 2010). Although it has been suggested that DNMT3a/3b removes 5-methyl-cytosine through deamination (Metivier et al. 2008), the lack of these enzymes in E11.5 germ cells precludes the contribution of DNA methyltransferases to global DNA demethylation (Seki et al. 2005).

3.8 Influence of Somatic Environment on PGC Reprogramming

Following global DNA demethylation of the germ cell genome, de novo DNA methylation of imprinted genes takes place in a sex-specific manner. The influence of the somatic environment on imprinting in germ cells is still controversial (Durcova-Hills et al. 2006; Iwahashi et al. 2007; Tada et al. 1998). Using an in vitro system, Iwahashi et al. (2007) demonstrated that isolated male and female fetal germ cells are capable of maintaining their characteristic methylation patterns of imprinted genes in the absence of supporting somatic cells, suggesting that epigenetic reprogramming is autonomously regulated in germ cells. On the other hand, Durcova-Hills et al. (2006) showed that the extent of methylation of a paternally imprinted gene *H19* is strikingly different between sex-reversed XY female germ cells (in vivo) and cultured EG cells (Tada et al. 1998). Hence, the absence of a somatic environment in cultured EG cells may lead to differential epigenetic modifications in vitro.

3.9 Nuage and Reprogramming

Covalent modifications of histone tails in somatic cells regulate cellular processes. In the germline, it has recently been reported that tudor domain-containing proteins such as fly Tudor, mouse TDRD1, TDRD2, TDRD6, TDRD7, TDRKH, and STK31 recognize symmetrical arginine methylation on Piwi subfamily proteins such as mouse MILI, MIWI, and MIWI2, and fly Aubergine and Argonaute 3, through their interaction with the arginine methyltransferase, PRMT5 (Chen et al. 2009; Kirino et al. 2009, 2010; Vagin et al. 2009). Many of the tudor domain-containing proteins and Piwi subfamily proteins localize to a unique germline structure, known as the nuage (or chromatoid body in mouse). The nuage/chromatoid body is an electron-dense structure that is well conserved in many animal germline cells. In recent years, several tudor domain-containing proteins and Piwi subfamily proteins that localize to the nuage/chromatoid body have been demonstrated to repress the expression of retrotransposons through regulating the production of a unique class of 30 nucleotide long small RNAs, known as the Piwi-interacting RNAs (piRNAs)

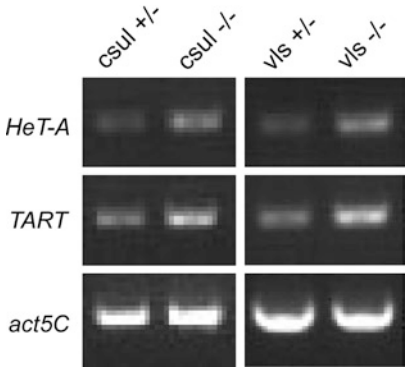

Fig. 3.4 Upregulation of retrotransposons in capsuleén and valois *Drosophila melanogaster* mutant ovaries. Telomeric retrotransposon transcripts, *HeT-A* and *TART*, are derepressed in *Drosophila* ovaries lacking expression of Capsuleén (mouse *Prmt5*) and Valois (mouse *Mep50*)

(Aravin et al. 2007, 2009; Brennecke et al. 2007, 2008; Chen et al. 2007; Kuramochi-Miyagawa et al. 2008, 2010; Lim and Kai 2007; Ma et al. 2009; Shoji et al. 2009; Soper et al. 2008). Interestingly, in fly ovaries, mutants for *capsuleén* (mouse PRMT5) and its cofactor *valois* (mouse Methylosome protein 50, MEP50) exhibit derepression of retrotransposons *HeT-A* and *TART* (Fig. 3.4). Since PRMT5 has been described as a player in epigenetic programming of mouse germ cells (Ancelin et al. 2006) and fly *capsuleén* is reported to regulate piRNA production (Kirino et al. 2009), it is plausible that piRNAs have an epigenetic role during germ cell reprogramming. Furthermore, a recent study by Brennecke et al. (2008) has demonstrated that the lack of maternal piRNA inheritance compromises retrotransposon silencing. Collectively, studies in fly and mouse would suggest that the nuage/chromatoid body is directly or indirectly linked to epigenetic programming and to the control of endogenous viruses in the genome. It is therefore intriguing to speculate about the role(s) of piRNAs and the nuage/chromatoid body in epigenetic programming of the mouse germ cell genome.

The promoter region of a gene known to be expressed in the mouse nuage/chromatoid body, *Mouse vasa homolog* (*Mvh*), contains CpG islands that undergo "developmental" demethylation as do the differentially methylated regions of imprinted genes at E11.5 (Maatouk et al. 2006). Cytosine demethylation of *Mvh* coincides with the period of global DNA demethylation of the germ cell genome, when retrotransposon expression is rampant. Possibly, the expression of *Mvh* during global DNA demethylation is critical to regulate retrotransposon expression. Indeed, several studies have reported that elevated retrotransposon expression in the nuage/chromatoid body mutant testes, *Mvh*, *Mili*, *Miwi2*, and *Maelstrom* (*Mael*), is due to defective de novo DNA methylation and piRNA production (Kuramochi-Miyagawa et al. 2008, 2010; Soper et al. 2008).

Combining the results from flies and mammals implicates the small RNAs, particularly piRNAs, in epigenetic programming of the mouse germ cell genome. Since several proteins known to participate in piRNA-mediated silencing are localized to the nuage/chromatoid body, it will be exciting to elucidate how piRNAs and the nuage collaborate with other players such as DNMTs and the chromatin modifiers to orchestrate reprogramming of germ cells.

3.10 Coda

Epigenetic regulatory mechanisms are indispensable for reprogramming of somatic cells during SCNT into oocytes and for germ cells following fertilization, as well as for activation of the embryonic genome and for normal gametogenesis. Are these processes governed in the same manner, i.e., are the same molecules and mechanisms used, or have different tissue and developmental stage-specificities evolved to fine tune the process? This chapter provides insight into the fragmentary knowledge of inherent reprogramming, providing a sliver of light on which to build a platform of understanding.

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Chapter 4
Epigenetic Reprogramming During Somatic Cell Nuclear Transfer and the Development of Primordial Germ Cells

Lyle Armstrong and Majlinda Lako

Abstract There are few circumstances in which the phenotype of differentiated cells generated during normal embryonic development can revert to a less developed or more developmentally plastic state. Somatic cell nuclear transfer and more recently the derivation of induced pluripotent stem cells are two examples of this rare phenomenon and we discuss the mechanisms by which these techniques may be able to effect epigenetic reprogramming of a differentiated somatic cell in addition to a few of the shortcomings of these techniques.

4.1 Introduction

An embryonic stem cell is a truly fascinating entity because it provides us with a "snapshot" of a brief window in embryonic development when all cells that comprise the early embryo's very simple architecture are equally capable of becoming any of the cell types needed for the later stages of development. This property is referred to as pluripotency and is the exclusive preserve of embryonic stem cells, certain embryonic carcinoma cell lines and possibly those cells that represent the very early stages in the development of the organism's germline, the so-called primordial germ cells (PGCs). The second of these types is a rare aberrant form of the last (PGCs) and so we can probably ignore them for any purposes that involve nuclear reprogramming but from the other two classes we can learn a great deal about the molecular basis of this process.

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Pluripotency enjoys a fleeting existence in the embryonic development of higher vertebrates. In the zygote and up to about the 32-cell stage embryo, all the cells (blastomeres) are more or less equal in their ability to produce all of the body's cell types and as such may be thought of as totipotent, a fact underlined by their capability to replace blastomeres that have been mechanically removed from the embryo. By the blastocyst stage, differentiation of the outermost blastomeres has taken place to produce the trophectoderm which is an outer layer of larger flattish cells distinct from the inner cell mass that is a protrusion of smaller cells at the base of the embryo. Only these cells will give rise to the developing embryo (the trophectoderm will only produce certain components of the placenta and can never become inner cell mass cells) but even these can no longer be described as totipotent since they lack, in some mammalian species, the ability to produce trophectodermal cells. They are however pluripotent but this first cell differentiation event highlights an important lesson about cell differentiation in that it is almost always unidirectional. Once differentiation has taken place, cells cannot find a way to reverse this process unless something goes wrong with them (such as might occur in cancer cells) and this underlying rule is the basis of all embryogenesis. Development is a one way street populated by vehicles lacking a reverse gear!

And yet there are artificial methods by which we can reverse the process of differentiation. Somatic cell nuclear transfer (SCNT) and lately the process of inducing pluripotency in somatic cells by transfecting with various combinations of pluripotency associated factors such as OCT4, KLF4, c-MYC, LIN28, SOX2 or NANOG, etc. are known to be able to reprogram the genomes of these cells such that they show similar gene expression profiles to those of nature-derived pluripotent cells (Yamanaka 2008; Zhao and Daley 2008). These methods are of enormous potential importance since pluripotent cells are an attractive resource for regenerative medical procedures that involve transplantation of specific differentiated cells into human patients. A great deal of data have been generated on the differentiation of human embryonic stem cells but the possibility of immune rejection of the differentiated cells still remains as a serious disadvantage of this technology although generation of patient-specific or isogenic pluripotent cells may allow us to circumvent this problem.

The use of SCNT to reprogram somatic cells with the aim of generating pluripotent stem cell lines is called therapeutic cloning to distinguish the technique from reproductive cloning whose aim is to produce live offspring that are derived wholly from the genome of the original somatic donor cell. This latter technique was used in the much publicized cloning of the sheep "Dolly" in 1996 by fusion of a mammary gland epithelial cell from a Finn Dorset ewe with the enucleated oocyte of a separate donor, which having resulted in a live birth contradicted the opinion that the somatic cells of adult vertebrates were simply too specialized to revert to a totipotent (or even pluripotent) state (Wilmut et al. 1997). Human reproductive cloning is banned under the laws of many countries but therapeutic cloning is allowed although this is often subject to strict regulatory controls. It must be stressed that despite much international attention no research group has demonstrated that human pluripotent cells can be created using nuclear transfer. The technique works for

some animal species such as mice (Rideout et al. 2001; Kishigami et al. 2006a, b; Wakayama et al. 2008) and the resulting ntESC (nuclear transfer derived ES cells) seem to have similar characteristics to ESC derived from blastocyst stage embryos and crucially they are able to undergo germline transmission in chimaeric mice, which is one of the major determinants of pluripotency of animal ESC (Mizutani et al. 2008). The development of the induced pluripotency method may supplant therapeutic cloning as a means of producing human pluripotent cells (iPSC) but even this technique suffers from several potential disadvantages that may limit its clinical application. Current iPSC technology relies upon the use of retroviral or lentiviral vectors to transfect several pluripotency-associated factors into a somatic cell and the permanent integration of these genes into the host genome may be problematic if the transgenes cannot be efficiently and permanently silenced during differentiation of the iPSC into clinically useful cell types. Moreover, the possibility of site-directed mutagenesis during transgene integration indicates that iPSC produced using this technique are unlikely to find approval for the treatment of human disease. Newer techniques to induce pluripotency in somatic cells will undoubtedly be developed but at the time of writing there is much work needed to establish that iPSC are truly equivalent to embryonic stem cells in terms of their differentiation potentials and their ability to maintain the integrity of their genomes during extended in vitro culture. It is also unclear how the transfection of as few as four pluripotency-associated factors is able to reprogram the genome of a differentiated somatic cell such as a fibroblast to cause it to adopt the genome architecture and gene expression profile of a pluripotent cell. Similarly, our knowledge of the equivalent process during SCNT is scant although at a more advanced state than for iPSC. In the following sections, I intend to review the current state of knowledge regarding nuclear reprogramming in both of these techniques and how we are using embryonic stem cells and PGCs derived from these to answer this most fundamental of biological questions.

4.2 Epigenetic Genome Modification Is the Basis of Differentiation

Epigenetics is the study of changes in gene function, which occur without changes in DNA sequence information and thus is in a sense “outside” of traditional genetics. The genome can be modified either by methylation of specific cytosine residues particularly at CpG islands such as are often found in the promoters of mammalian genes or by post-translational modification of the histone proteins which bind and support the DNA macromolecule in the nucleus in the form of chromatin. This organizes DNA into nucleosomes in which the nucleic acid macromolecule is wound around an octameric protein core consisting of histone proteins H3, H4, H2A and H2B. These structures are important for ensuring that large amounts of DNA can be packaged into the limited volume of the nucleus but more importantly, post-translational modifications of specific amino acids along the N-terminal

polypeptide "tails" of the histone proteins provide a level of information outside that which is encoded in the sequence of nucleotides in the DNA. The pattern of such post-translational modifications controls not only the degree of chromatin compaction (heterochromatinization) but also the accessibility of gene promoters and other control elements meaning that gene transcription is to a large extent under epigenetic control (Robertson and Wolffe 2000; Shilatifard 2006; Cheng et al. 2008; Murayama et al. 2008). Typical examples of the modifications controlling transcription are methylation of specific lysines (K4 and K9) of histone H3, which respectively promote and repress gene activity with the di- and trimethylated states showing the greatest effectiveness. DNA methylation is thought to be important for the regulation of a number of different groups of genes and genomic sequences (Robertson and Wolffe 2000; Bestor 2000). Among the most important are the long terminal repeats of endogenous retroviruses, in which DNA methylation plays an essential role in the silencing of these retrotransposons, thereby maintaining "genome-defence", the differentially methylated regions of imprinted genes, and the inactive X chromosome (Mermoud et al. 2002; Heard 2004).

The pattern of DNA methylation is an indicator of the differentiation state of the cell although there is a paucity of information concerning the rules governing this pattern. Recent data suggest that DNA methylation patterns have a closer correlation to the distribution of histone methylation than to the underlying DNA sequence (Meissner et al. 2008). Modifications of both histone and DNA methylation undergo extensive changes during cell differentiation particularly in regulatory regions other than those found within the core promoters of genes. The regions controlled by DNA methylation appear to fall into two broad classes based upon high or low CpG island density and in embryonic stem cells, the sequences of high CpG density tend to be unmethylated and vice versa. Interestingly genes with high CpG density at their promoters tend to be either ubiquitously expressed housekeeping genes or key developmental genes that are needed in the early stages of pluripotent cell differentiation but nevertheless are tightly repressed in the pluripotent cells. The latter group of genes have a further interesting trick up their sleeves in that their promoters are populated by both activating (H3K4 di- or trimethylation) and repressive (H3K27 trimethylation) histone modifications. (Bernstein et al. 2006). This has been interpreted to mean that such "bivalent" genes are held in an open chromatin conformation that would normally be associated with active transcription while the presence of H3K27 me3 prevents actual transcription taking place. Not all genes in embryonic stem cells fall into this classification and many tissue-specific genes, whose expression is not required until later in development when terminally differentiated cell types are required for the construction of organs, do not show bivalency. The promoters of this type of gene often have low CpG density and tend to be methylated at the embryonic stem cell stage. Their methylation status is not permanent in the earlier stages of development since expression of particular genes from this class may be required depending upon the fate choices made by a cell as it proceeds down its differentiation pathway.

Bivalent chromatin domains are one of the primary characteristics of pluripotent cells and there is little evidence to suggest that they occur in any other cell types.

Since they silence developmental genes while keeping them poised, they tend to resolve upon ES cell differentiation into Lysine 4 or Lysine 27 methylation, in accordance with associated changes in gene expression. The histone modification profile along with patterns of DNA methylation in the CpG islands of differentiated cell type-specific gene promoters is what we seek to remove during nuclear reprogramming by whichever methods we choose and replace this with a histone/DNA methylation pattern similar to those found in pluripotent cells.

4.3 Epigenetic Modification Is Central to Nuclear Reprogramming in Both Somatic Cell Nuclear Transfer and Derivation of iPSC

SCNT as the name suggests involves the insertion of the nuclear genome from a somatic cell into an oocyte from which the genome has been removed (enucleation). The protocols needed to achieve this are outside the scope of this chapter and there are several excellent descriptions of the technique in the literature (Kishigami and Wakayama 2009; Egli and Eggan 2006), so I will pass over the basic SCNT strategy in favour of a discussion of the molecular basis of the reprogramming it can impose upon a somatic genome. Upon transfer of a somatic nucleus to an MII stage (ovulated) oocyte during the cloning process, several essential changes must ensue. First, the somatic nucleus must cease to express its unique repertoire of gene products. Second, that nucleus must become subject to the instructions provided by the oocyte cytoplasm to unfold a new pattern of development-specific gene transcripts, and third, the heritable memory endowed by the chromatin that ensured the characteristics of the donor tissue must be erased. These events must occur in a very short time window. Typically, the time between transfer of the nucleus and activation of the "reconstructed" embryo is only a few hours, so if reprogramming is to occur at all it must be rapid.

Repression of the somatic gene expression programme is a seemingly rapid event during SCNT. Several groups have described the dissociation of transcription factors and other chromatin-associated proteins from somatic genomes during the SCNT process (Gao et al. 2007; Sun et al. 2007; Zheng et al. 2008) followed by their re-association shortly after the formation of the pseudo pronuclei. This also has natural parallels in the latter stages of gamete development and in the early stages after fertilization as evidenced by the dissociation of chromatin-associated proteins from chromatin during the first meiotic cycle of the developing oocyte and their subsequent re-association after pronuclear formation. This correlates with repression of the maternal transcription programme and has been labelled the "erase and rebuild" strategy.

Such removal of chromatin-associated proteins seems to occur in parallel with epigenetic modification of the paternal genome. Epigenetic reprogramming is an essential feature of normal development and is associated with the erasure of some of the epigenetic modifications inherited from the gametes (Oswald et al. 2000).

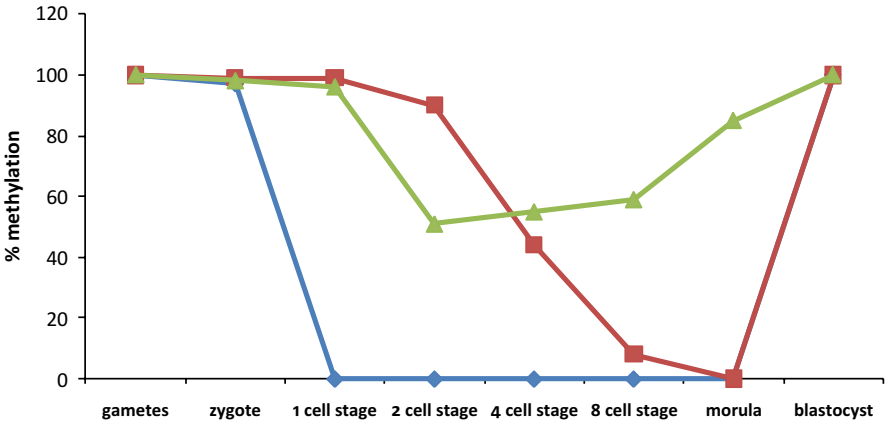

Fig. 4.1 Methylation levels throughout pre-implantation development of normal and nuclear transfer-derived embryos. The paternal genome is highlighted by diamonds, the maternal by squares and that of the SCNT embryo by triangles

Upon fertilization, there is a series of events that involve the incoming sperm as it encounters the egg cytoplasm. The initial event after fertilization is the decondensation of the sperm nucleus, resulting in the unwinding of the tightly packaged sperm DNA held in a unique, almost toroidal, conformation by the sperm-specific protamines (Braun 2001). So highly ordered is this chromatin organization in sperm that it is effectively dehydrated, and hence rehydration is an essential, very early event. Upon decondensation, protamines are replaced rapidly by nucleohistones derived from the oocyte cytoplasm, usually in the first hour after fertilization (Nakazawa et al. 2002), and the DNA is wound onto the histone octamers in an ATP-dependent process (McLay and Clarke 2003). It is during this same time period that rapid and paternal-specific demethylation of the genome takes place in the absence of transcription or DNA synthesis (active demethylation – see Fig. 4.1).

Demethylation of the maternal genome, which is kept separate from the paternal by the formation of pronuclei, takes place more slowly and thus may be passive in nature due to the exclusion of the maintenance methylase enzyme DNMT1 from the nucleus at this stage of development (Cirio et al. 2008). The exact nature of the active demethylation is not well understood.

Active demethylation is operatively defined as loss of methylation in the absence of DNA replication. The speed with which this process occurs strongly suggested that it is mediated enzymatically and there are several candidate genes that could mediate the process. Two strong candidates for such a system (MBD2 and MBD4) are highly expressed in mammalian oocytes. MBD2 (methyl binding domain 2) has demethylase activity (Hendrich et al. 2001) involving methanol as a stable leaving group but the uridine deglycosylase enzyme methyl binding domain protein binding 4 (MBD4) is involved in base excision repair and thus

may also be capable of replacing 5-methylcytosine (Wu et al. 2003). Interestingly, null mutations of these genes in isolation show normal paternal demethylation (Klimasauskas et al. 1994; Cedar and Verdine 1999), indicating a possible redundancy of function or the existence of alternative demethylation pathways. A third possibility is hydrolytic deamination of 5-methylcytosine resulting in the conversion of 5-methylcytosine to thymidine (Morgan et al. 2004); however, this process would require considerable energy input and as such it is the least likely mechanism. Additional support for the involvement of the base excision DNA repair pathway has come to light in the recent publication by Hajkova (Hajkova et al. 2010) although it is still unclear exactly which enzymes and components of this repair pathway are needed for reprogramming the pre-implantation embryonic genome; however, it is clear from this latter study that enzymes responsible for the deamination proposed by Morgan et al. above are not necessary for this DNA repair-mediated demethylation. It is interesting that SCNT appears to be only capable of partial demethylation of the somatic genome (see Fig. 3 in Armstrong et al. 2006).

Not all the methylated sequences of the genome are subjected to loss of 5-methylcytosine. Some "elite" regions such as centromeric satellites and endogenous retroviruses seem to escape perhaps reflecting an over-riding need to silence these features as part of a mechanism defending the integrity of the genome but the greater exception to demethylation is found in imprinted genes. The expression of imprinted genes is dictated by the parent-of-origin and typically one of the alleles derived from either the paternal or maternal gametes will be silent due to DNA methylation within its control regions (Constancia et al. 2000; Frank et al. 2002; Lin et al. 2003; Ferguson-Smith and Surani 2001). Imprinting seems to predominate on genes involved in foetal growth and development of the placenta (Kelsey 2007; Wood and Oakey 2006) and they are particularly sensitive to changes in the environment to which the embryo is exposed highlighted by the increased occurrence of conditions such as Angelman and Beckwith–Wiedemann syndromes in children derived from assisted reproduction technologies (Lucifero et al. 2004). This is probably of much greater importance to the maternal genome because it has by far the greater number of methylated imprinted genes, but the mechanism by which this methylation is maintained remains unknown. It has been suggested that binding of nonhistone proteins to individual imprinting control regions could prevent their methylation in either the paternal or maternal germ line cells (Feil and Khosla 1999). This hypothesis is supported by the binding of the CCCTC binding factor protein to the unmethylated maternal allele of the H19/Igf2 locus in mouse, which seems to maintain its unmethylated status. Introduction of mutations into the binding site for this protein prevented binding of CCCTC binding factor and led to methylation on the maternal allele (Schoenerr et al. 2003; Pant et al. 2003) although it is unclear whether there are factors in the male germline cells which could direct DNA methylation to this imprinting control region (Bowman et al. 2003) or whether the methylated state is simply established by default. Alternatively, such active demethylation arose as a protective response of the maternal genome to reduce the influence of the paternal genome, which may have alleles optimized to the production

of larger, more competitive offspring, thus serving the maternal interest in ensuring survival of larger numbers of offspring overall (Moore and Reik 1996).

The pattern of histone modifications is also partially reprogrammed during SCNT. For example, acetyl groups are removed from many regions immediately after SCNT particularly from H3K9, K14 and K16, but others such as H4K8 and H4K12 seem to be refractory to this process (Wang et al. 2007). Activation restores the levels of acetylation at these lysines, thus it is likely that such deacetylation/reacetylation is part of the mechanism for transcriptional silencing of the somatic gene expression programme. Loss of methylation of some lysines of histone H3 is also observed. H3K9 3me is gradually lost after activation of SCNT embryos implying that the MII oocyte has some active mechanism for removal of such methyl groups although what this may be is not clear at present. Possible candidates for the removal of H3K9 3me and H3K9 2me are the demethylase encoding genes *JMJD1a* and *JMJD2c*, which are positively regulated by Oct4 (Loh et al. 2007). Other candidate reprogramming genes have been described in the literature on the basis of their high expression levels in oocytes at either the germinal vesicle (GV) or MII stages (Oliveri et al. 2007). Of particular interest are those that increase during the transition from the GV stage of oocyte development to MII; these are likely reprogramming candidates since SCNT using MII oocytes is more successful (Gao et al. 2002) and include a range of enzymes that may contribute to epigenetic reprogramming such as the histone arginine methyltransferase PRMT3.

Other proteins known to be involved in epigenetic modification of the genome are present at high levels in mammalian oocytes such as histone deacetylases (HDACs) 1 and 6 (Kim et al. 2003; McGraw et al. 2003), histone acetyl transferases (MYST1 and 4) (McGraw et al. 2007a, b) and the H3K9-specific histone methyltransferase SUV39H to name but a few. The expression of these proteins decreases from the GV stage to MII but that is not to say that their contributions to the reprogramming activity of the oocyte are less significant and their activity is worthy of investigation. Important insights have also been obtained from nuclear transplantation studies such as the observation that Tpt1 seems to be involved in the upregulation of previously silenced Oct4 (Koziol et al. 2007) and bovine oocytes that express higher levels of phosphorylated transcriptionally controlled tumour protein (TCTP) give higher rates of blastocyst formation (Tani et al. 2007). Oct4 seems to play a special role during nuclear transfer since cloned embryos with low global Oct4 levels (either in all their cells or as a mosaic) are less capable of blastocyst development and embryonic stem cell formation.

Recent publications concerning induced pluripotency (iPSC) (Yu et al. 2007; Takahashi et al. 2007; Park et al. 2008; Nakagawa et al. 2008) have underlined the importance of Oct4 and Sox2 which, in conjunction with two of the following four (Nanog and Lin28 or Klf4 and c-Myc), appear to be able to reprogram somatic cells into pluripotent cells, but the exact status of iPS cells remains currently unclear. NANOG and OCT4 have been shown to associate with unique transcriptional repressive complexes (Liang et al. 2008) and small molecules known to influence DNA methylation have been shown to have an impact upon the iPSC method, which supports an epigenetic reprogramming mechanism (Mikkelsen et al. 2008).

Several preliminary studies have begun to dissect the iPSC reprogramming mechanism (Maherali et al. 2007; Stadtfeld et al. 2008) but much remains to be learned about this process. The high level of Oct4 expression in the MII oocyte (Mamo et al. 2008) suggests that the reprogramming mechanisms that operate in SCNT and iPS may have common features. iPS has widely been seen to be the method of choice towards developing individual- and disease-specific pluripotent stem cell lines in the future, and so an understanding of the SCNT mechanism may help us to understand the process that operates in the iPSC technique and is likely to define ways in which the latter may be greatly improved. This possibility arises from the large differences in the rate of epigenetic reprogramming occurring between iPSC, which typically requires continual, high-level expression of the reprogramming factors for a minimum of 10 days in vitro, and SCNT in which DNA demethylation is normally complete (as far as possible) within 24 h. This is supported by earlier experiments in which re-expression of murine Oct4 within 4 days of transferring human lymphocyte nuclei into the oocytes of *Xenopus laevis* (Byrne et al. 2003) and so these observations suggest that oocytes possess additional factors that may be able to expedite epigenetic reprogramming with greater efficiency.

4.4 SCNT Does Not Reprogram the Somatic Genome Efficiently

There is much hope that iPSC will provide a robust and readily applicable technology for the generation of pluripotent cells because SCNT is fraught with many difficulties. Quite apart from the technical difficulty associated with the protocol, there are ethical and logistical issues associated with the use of large numbers of human oocytes that would be needed to generate sufficient numbers of pluripotent cell lines.

In addition to these problems, there is a wealth of evidence suggesting that epigenetic reprogramming during SCNT is rather inefficient. DNA methylation of imprinted loci is very often disrupted in SCNT embryos, and the studies of several groups show that the mouse somatic genome does not respond well to the demethylation activity of the oocyte (Han et al. 2003; Kang et al. 2001, 2002) with the level of methylated DNA in the SCNT embryo being typically higher than in normal embryos. In addition, as shown in Fig. 4.1, the onset of de novo methylation begins much earlier implying that the epigenetic remodelling of the somatic nucleus is inadequate which may be one of the reasons behind the high abortion rate and level of developmental abnormalities in cloned animals. It has been suggested that endogenous DNA methyltransferases (DNMTs) in the somatic nuclei continue to methylate their targets but it is also possible that the epigenetic "architecture" of the somatic chromatin is significantly different to that of the diploid zygote and may therefore be resistant to the DNA demethylating and/or histone modification activity of the oocyte. If this were true we would expect that NT embryos would retain some of the characteristics of the somatic cell donors. In fact this is observed in pre-implantation SCNT embryos derived from myoblast donor cells (Gao et al. 2003)

and additional evidence supports the retention of some epigenetic "memory" from the somatic donor cells (Ng and Gurdon 2005; Sebastiano et al. 2005).

This ineffective reprogramming is not restricted to DNA methylation. To turn off somatic gene expression, the histones at the appropriate loci need to be deacetylated by HDACs followed by methylation of particular lysines (such as lysine 9 on histone H3) to make the repression permanent. The MII stage oocytes of mammals express relatively low levels of HDAC enzymes so it is possible they cannot perform these deacetylation steps efficiently. Conversely, oocytes do express higher levels of the histone acetyltransferases HAT1 and GCN5, so one might imagine that remodelling of chromatin at inactive genes could be possible although this would potentially require the presence of the histone demethylases such as LSD1 that are able to remove the activating H3K4 me3 histone mark. Literature evidence suggests that MII stage mammalian oocytes do express many of the genes required for histone demethylation (JARID1A/1B, JMJD1A, JMJD2B and LSD1) in addition to a range of other enzymes involved in epigenetic modification such as PRMT5 (a protein arginine methyltransferase that mono or dimethylates arginines in histone N-terminal tails) and EHMT1/2 (euchromatic histone lysine methyltransferases that methylate H3K9) (McGraw et al. 2007a, b). These data are based upon quantitative PCR measurements of levels of maternal transcripts (mRNA) that accumulate in the oocyte, which may not necessarily be translated into functional proteins but assuming that this occurs, the oocyte has the theoretical capability to reprogram a somatic genome. However for these enzymes to be effective, they must be able to access the DNA or the N-terminal tails of the histones with which it associates and we are uncertain if the oocyte is able to remodel somatic chromatin to permit this.

Chromatin remodelling happens all the time in somatic cells by repositioning of nucleosomes throughout the genome, which occurs through the activities of multisubunit protein complexes of the SWI/SNF type. The individual subunits of these complexes are thought to be constitutively expressed in somatic cells so it is interesting that the MII oocytes of non-human primates show low or undetectable expression of some subunits. The chromatin accessibility complex (CHRAC) facilitate the entry of transcription factors into chromatin but two of its five components (CHRAC1 and CHRAC17) are only modestly expressed at MII when compared to the levels found in developing embryos (Zheng et al. 2004). The mRNA encoding another CHRAC subunit, ACF1, is more abundant at MII than in somatic cells.

Temporal expression patterns of the SWI/SNF-related transcription regulators show a similarly variable pattern. For example, SMARCA2, SMARCB1 and SMARCAD1 show little difference between the MII oocyte and the cells of later developmental stages but levels of ARID1A, ARID1B, SMARCA4 (Brahma group protein 1) and the bromodomain protein BR140 are greatly different. Interestingly, SMARCA4, ARID1B, CHRAC1 and CHRAC17 are upregulated at the eight cell stage of pre-implantation development in the non-human primate suggesting that they may have a role in the transition from using the maternal transcripts of genes to reliance upon the embryo's own genome (zygotic genome activation). There are low levels of CHRAC1, CHRAC17 and other CHRAC components in the MII oocyte that could contribute to their reprogramming ability after SCNT but it is possible

that other chromatin remodelling proteins that would be needed to remodel a somatic genome to a state where it could be reprogrammed optimally by the histone modification/DNA methyltransferases or demethylases are not present in high enough concentrations.

Activation of the zygotic genome in embryos from natural fertilizations is reflected to a large extent in the SCNT embryo or rather the latter appears to attempt this crucial phase of pre-implantation development in a broadly similar timeframe to the former. This highlights another possible reason for the inefficient reprogramming of a somatic genome during SCNT in that there may not be sufficient time between the first exposure of the somatic nucleus to the oocyte cytoplasm and the pre-implantation stage at which genome activation begins. Irrespective of the limitations to reprogramming, a low number of NT embryos do survive, suggesting that in rare cases it is capable of at least partial resetting of the genome. At present, it is not yet clear whether the oocyte is uniquely competent to remodel and reprogram the wide variety of chromatin modifications, both nucleosomal and otherwise, in a more efficient manner. It may be the case that the reprogramming activity is simply overwhelmed by the enormous task of having to modify or replace somatic histones, remove polycomb complex proteins, and demethylate areas of the genome that may be a lot less accessible than the corresponding areas in gamete-derived genomes. Alternatively, it may be that such reprogramming is actually "forbidden" for the genomes of somatic cells and the few NT embryos that survive may only do so because of a malfunction in the mechanism that prevents reprogramming of a somatic genome.

Investigating the mechanism of this reprogramming effect in SCNT will be a herculean task. With the techniques currently at our disposal, the best we can probably hope for is to demonstrate the interaction between the somatic genome and the proteins that mediate chromatin accessibility and epigenetic modification. This will be useful data but it does not allow us to determine the precise loci that are bound to specific reprogramming complexes as a function of time during SCNT embryo development. We could possibly generate such data from individual embryos using techniques such as PCR analysis of DNA fragments obtained from chromatin immunoprecipitation but the numbers of genomic loci that could be examined will be small. To analyse the whole genome would require very large numbers of embryos which given the technical and logistical problems of performing SCNT is probably not feasible. That notwithstanding, SCNT is valuable for an examination of some aspects of reprogramming that may help us to improve the speed and efficiency of iPSC techniques.

4.5 Epigenetic Reprogramming During Germ Cell Specification

Despite the well-documented abnormalities of SCNT-derived animal embryos that develop to term, those that are capable of reproduction seem to produce relatively normal offspring (Mir et al. 2005) suggesting that passage of a genome through

germ cell development and gametogenesis may be able to remove any epigenetic "problems" left by inefficient SCNT-based reprogramming.

The germline of most mammalian species is specified quite early in development. PGCs are the earliest recognizable precursors of the gametes and are detectable at 7.2 dpc in the developing mouse embryo as a cluster of approximately 50 alkaline phosphatase positive cells located in the extraembryonic mesoderm at the base of the allantois. (Ginsburg et al. 1990). PGCs are present in the hindgut epithelium of the 4-week human embryo (Fujimoto et al. 1977) after which they escape into the neighbouring mesenchymal tissues to begin their migration via the dorsal mesentery to the developing gonads in which they arrive at approximately week 6 of gestation. Their subsequent development depends on the sex of the gonadal environment. PGCs in the developing testes undergo mitotic arrest in which they remain until they initiate meiosis at the onset of puberty to permit their development in spermatozoa but female PGCs enter meiotic prophase upon entry into the genital ridges, which marks the beginning of their development into oocytes, which will be completed before birth.

4.6 Epigenetic Reprogramming Accompanies Germ Cell Development In Vivo

Studies of murine embryos show that the DNA of PGCs resident in the genital ridges is hypo-methylated (Brandeis et al. 1993; Kafri et al. 1992). As we already know DNA demethylation normally begins shortly after fertilization leading to an extensively hypo-methylated genome by the 4-cell stage of pre-implantation development. De novo methylation occurs principally in the inner cell mass of the blastocyst stage embryo, which is proposed to be connected with the process of lineage decision and cell differentiation (Monk 1995; Reik et al. 2001); however, this means that the process of PGC specification must activate mechanisms for the removal of methylated DNA. This process is needed to eliminate parental gene imprints derived from the gametes to permit the developing germ cells to establish their own sex-specific set of gene imprints (Sato et al. 2003), but the demethylation process is not restricted to the imprinted genes (Hajkova et al. 2008).

DNA demethylation is accompanied by chromatin and histone modification changes such as loss of dimethylation of lysine 9 of histone H3 (H3K9me2) and enrichment of methylation of lysine 4 of histone H3 (H3K4me2 and H3K4me3) coupled to increased trimethylation of lysine 27 on Histone H3 (H3K27 me3). Many histone acetylation marks increase especially H3K9ac as well as symmetrical methylation of arginine 3 on histones H4 and H2A (H4/H2AR3me2s) attributed to the BLIMP1–PRMT5 complex5 (Ancelin et al. 2006). Importantly, this chromatin signature is not established in the somatic cells surrounding the PGCs in vivo. However, the changes in the levels of several histone modifications are transient and after E12.5 in mouse embryos, they are similar to those of the surrounding somatic cells while others such as H3K9ac and H4/H2AR3me2 undergo permanent changes.

The changes in histone methylation may be the result of specific modifying enzymes such as SUZ12 or the lysine demethylase LSD1 but in the case of demethylation of H4/H2AR3me2, there is less evidence that this is enzymatic in nature and direct replacement of H4/H2AR3me2 by histone H4 or H2A that are not modified by arginine methylation may be employed. However, there is a paucity of information on the mechanisms that control these PGC-specific epigenetic changes.

PGCs need to suppress somatic gene expression programmes and therefore epigenetic modification of their genomes is likely to be very important for this suppression. PGCs in Blimp1 null embryos have aberrant expression of Hox genes (Kurimoto et al. 2008) that are normally tightly repressed at the PGC stage and this further underlines the importance of the Blimp1 gene product in establishing the chromatin architecture of the PGCs. However, our current knowledge of chromatin modification changes during PGC development is restricted to comparing global differences in the levels of specific DNA methylation or histone modification levels between different stages of PGC development and the surrounding somatic cell types. This work has been highly valuable but even incisive studies such as that of Ancelin et al. (2006) on the functions of the Blimp1-Prmt5 complex as a PGC-specific arginine methyltransferase have only demonstrated binding of this complex to specific control sequences of a single gene. Epigenetic modifying enzymes systems such as Blimp1-Prmt5 are unlikely to undergo non-specific binding to sites across the entire genome since arginine methylation of histone H3 is very often associated with transcriptional repression. The involvement of arginine methylation for the repression of genes central to the somatic development programme is acceptable but many other genes need to be active throughout PGC development; therefore, there must be molecular systems that direct the Blimp1-Prmt5 complex only to specific loci. The same is probably true for other histone N-terminal tail modifying enzymes such as histone acetyl transferases (HATs), histone methyltransferases (Ezh2, Suv39h, G9a, etc.), HDACs and perhaps of equal importance, histone demethylases (Lsd1, jmjd3, etc.). However, investigating the mechanism of epigenetic reprogramming in PGC development is extremely difficult using ex vivo-derived PGCs because of the low availability of these cells from mammalian embryos. The situation is worse in the human model since embryos of the appropriate stage are not routinely obtainable.

Fortunately, it is highly likely that PGCs are among the cell types formed during human embryonic stem cell differentiation. We have a reasonable knowledge of the changes in gene expression that occur from initial specification through migration of the cells via the hindgut endoderm to the developing gonad structures and their final commitment to gamete formation, and we can replicate much of this by differentiating hESC either as embryoid bodies (Kee et al. 2006) or as a monolayer (Tilgner et al. 2008, 2010). Robust protocols that maximize the numbers of PGCs that may be obtained using flow cytometry (FACS) to sort them from other cell types in cultures of differentiating hESC have been developed. The RNA helicase enzyme VASA is specific to germ cell lineages from post-migratory to post-meiotic gametes (Castrillon et al. 2000) and is thus a good indicator of the presence of PGCs. Optimal VASA expression occurs after 3 weeks of monolayer differentiation

and this correlates with the percentage of total cells expressing the cell surface protein stage-specific embryonic antigen 1 (SSEA-1) that is a recognized PGC marker (Kerr et al. 2008). FACS sorting of a single cell suspension derived from monolayer differentiation using an anti-SSEA-1 antibody conjugated to phycoerythrin afforded a population of SSEA-1 positive cells representing candidate PGCs. SSEA-1 positive cells derived from female hESC lines share many characteristics with ex vivo PGCs such as the expression of key genes (VASA, OCT4, NANOG and STELLA) and the synaptonemal complex proteins SCP1 and SCP3 suggesting that they may be preparing for entry into meiosis although the perinuclear location of these proteins revealed by immunostaining suggests that meiosis has not yet begun.

The cell cycle status of the SSEA1 positive population (they are mostly in S-phase) suggests that the majority of the cells may still be in their mitotic expansion phase (McLaren and Southee 1997); however, they seem to be undergoing epigenetic reprogramming since bisulphite sequencing (Clark et al. 1994) indicates that CpG sites of *IGF2/H19* DMR showed the expected decrease of methylation compared to SSEA1 negative and undifferentiated human ESC (see Fig. 4, Tilgner et al. 2008). Mouse PGCs (ex vivo) are characterized by substantial increases in the level of the repressive trimethylated H3 lysine 27 (H3K27 trimethyl) modification that is frequently associated with facultative heterochromatin for genes that should be repressed and after entry into the genital ridges (E12.5) the levels of H3K4 dimethylation (the other modification associated with bivalent chromatin domains) rise sharply. Repressive histone modifications such as methylation of H3K9 are progressively reduced from E8.0 onwards. Similar differences can be seen in the levels of histone modifications at the promoters of several genes between the SSEA1 positive putative human PGC population and their surrounding somatic cells represented by the SSEA1 negative FACS sorted cells (see Fig. 5, Tilgner et al. 2008). Some of the genes examined in this study are known to be expressed early in both embryonic development and differentiating embryonic stem cells (Itskovitz-Eldor et al. 2000; Fehling et al. 2003; Wiles and Johansson 1997; Zhang et al. 2007) and therefore should not be expressed in PGCs in the process of establishing their pluripotent genomes. The absolute levels of H3K4 dimethylation enrichment vary between individual genes, but the general trend is for this histone modification to be greater in our putative PGC population in line with the observations of Seki et al. (2007). H3K9 dimethylation also undergoes a general decrease with the notable exceptions of *HOXA3* and *GATA4;* however, the changes for H3K27 trimethylation can be divided into two groups depending upon the expression requirement of these genes. *VASA, OCT4, NANOG* and *SCP3* are all expressed in the SSEA1 positive population, which correlates with a lower level of H3K27 trimethylation on the promoters of these genes compared to those in the SSEA1 negative cells. Genes which should have lower activity in PGCs such as *BRACHURY, SCL, GATA4* and some members of the *HOXA* cluster show higher H3K273me enrichment which is consistent with observations of in vivo-derived murine PGCs. These data may indicate epigenetic reprogramming of the SSEA-1 positive PGC population to establish their pluripotency and may reflect the creation of bivalent chromatin domains on early developmental gene promoters in a manner similar to that observed for hESC.

4.7 Investigating Epigenetic Reprogramming Using PGCs Derived from hESC

The ability to generate large numbers of PGCs in vitro that are close representatives of ex vivo PGCs permits detailed investigations of the changes in genomic architecture, DNA methylation and histone modification patterns that occur during early germline cell development. It is not unreasonable to obtain 1×10^6 to 1×10^7 cells from the differentiation protocol, and sufficient chromatin should be available from these to permit analysis of DNA fragments from chromatin immunoprecipitation experiments using tiled microarrays (ChIP on chip) (Kim et al. 2007). At the time of writing, this technique yielded large quantities of data that helped us understand how various proteins interact with the genome, which had lent itself rather well to investigate how chromatin remodelling complexes and epigenetic modifying enzymes were able to reprogram the genome of a developing PGC. It would be difficult if not impossible to perform analogous experiments using SCNT embryos, so data resulting from ChIP on chip analyses of PGCs may contribute greatly to our understanding of epigenetic reprogramming.

4.8 Conclusions

The mechanisms by which cells are able to reprogram the functionality of their genomes is possibly one of the most fascinating biological questions of our time, but the prospect of even this knowledge pales next to the possibility that we might be able to turn one cell type into another at will by the manipulation of such epigenetic control systems. The two experimental models I have described in this chapter outline some of the approaches being taken to identify potential epigenetic reprogramming systems but the challenge for the future is not only to develop a fundamental understanding of these but also to develop the means to modulate their activities in a way that is harmless to the target cells. Successful resolution of this challenge will greatly enhance our ability to generate functional cell sources for regenerative medicine.

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Chapter 5
Epigenetic Reprogramming with Oocyte Molecules

Ramiro Alberio and Andrew D. Johnson

Abstract Following the demonstration that somatic cells can be fully reprogrammed to totipotency after transfer into enucleated eggs, the prospect of developing methods of cellular reprogramming for autologous cell therapies has come a little closer. How the egg reprogrammes somatic cells is little understood, and much work has been devoted towards elucidating the basis of these reprogramming events. Here, we have reviewed the current knowledge of reprogramming after nuclear transplantation and we present an experimental approach using the axolotl oocyte as a tool to broaden our understanding of the basic mechanisms of oocyte-mediated nuclear reprogramming, many of which, we assume, are shared by other approaches, such as transcription factor-mediated reversal to pluripotency. We expect that a combination of approaches aimed at understanding the mechanisms of reprogramming will contribute to the development of safe and efficient methods for generating autologous cells for transplantation.

5.1 Introduction

Cellular identity in multicellular organisms is established during embryonic development as a result of the activation of specific transcriptional programmes. A longstanding biological question has been to determine how cells acquire different phenotypes, and whether this process includes the permanent loss of potential to differentiate into other lineages. An answer to this question has been provided in the

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last couple of decades through landmark in vivo experiments (Hochedlinger and Jaenisch 2002; Wilmut et al. 1997). Reprogramming cells by somatic cell nuclear transfer (SCNT) into oocytes provided conclusive evidence that terminally differentiated cells contain all the genetic information required to recapitulate normal development. Thus, it is by different epigenetic regulatory mechanisms that cells acquire and maintain their identity. These epigenetic marks are inherited through cell division and are critical for tissue homeostasis. However, reprogramming by SCNT also highlights the notion that stable regulation of gene expression is reversible and that molecules in the oocyte/egg can erase epigenetic marks and re-establish a pluripotent state. The idea that oocyte molecules can reprogramme cells has triggered the development of numerous strategies aimed at understanding this process and at identifying the key reprogramming molecules. A better understanding of the mechanisms underlying oocyte-mediated reprogramming will contribute to the improvement of newly developed technologies, such as cell fusion and iPS cells (discussed in Chaps. 6 and 7, respectively). At present SCNT is the most efficient method for deriving reprogrammed stem cells. Around 40–50% of SCNT embryos develop to normal blastocysts and about 30–40% of these can give rise to normal ES cells (Kim et al. 2010; Wakayama et al. 2006), thus effective reprogramming of pluripotency occurs in 3.5 days with an overall efficiency of stem cell line derivation of 12–20%. This contrasts with the low efficiency of current iPS cell derivation, ranging from 0.001 to 3% over a period of 3–4 weeks (Huangfu et al. 2008; Maherali et al. 2008; Takahashi and Yamanaka 2006). In this chapter, we discuss recent advances in our understanding of the mechanisms of reprogramming by oocytes, and propose a model for the development of a reprogramming strategy based on the identification of evolutionarily conserved domains of oocyte-specific reprogramming molecules to create a “cocktail of synthetic reprogramming molecules”.

5.2 Oocyte-Reprogramming After Fertilization

Epigenetic reprogramming refers, in broad terms, to alterations in cell fate occurring in response to chromatin modifications, without changes in the DNA sequence. The most important chromatin modifications studied to date include DNA methylation and histone tail modifications, and they will be described in the following sections.

5.2.1 Reprogramming DNA Methylation

The reprogramming capacity of oocytes demonstrated by SCNT is not surprising considering the natural function of oocytes. Egg molecules reprogramme the highly differentiated sperm and egg chromatin to totipotency in the one-cell zygote. The epigenetic basis of this reprogramming begins with the remodelling of sperm chromatin as it enters the egg. This is shown by the rapid exchange of protamines with

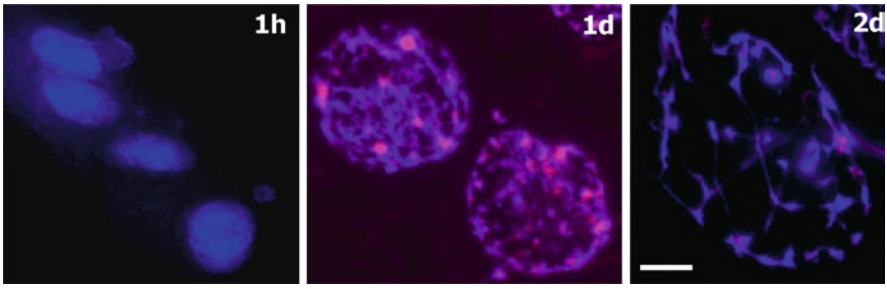

Fig. 5.1 Nuclear decondensation of mammalian cells injected into axolotl or *Xenopus laevis* oocytes. Cells after 1 h, 1 day (1d) and 2 days (2d) were stained with DAPI (*blue*) and propidium iodide (*red*) to show nuclear DNA. Bar: 10 μm

maternal histones before the initiation of DNA replication, indicating an active process of chromatin remodelling. The nuclear chaperones nucleoplasmin (Gillespie and Blow 2000) and HIRA (van der Heijden et al. 2005) participate in the early DNA decondensation and deposition of histones in the forming pronuclei. A recapitulation of these events was also observed in early SCNT experiments (Prather et al. 1990) and after transfer of somatic cells into germinal vesicles (GVs) of amphibian oocytes (Fig. 5.1). Nucleoplasmin promotes decondensation and reorganization of heterochromatic foci of pluripotent cell nuclei exposed to *Xenopus* egg cytoplasmic extracts; however, these effects are not observed in chromatin of differentiated cells, indicating a more complex heterochromatin organization in somatic cells (Tamada et al. 2006). General chromatin remodelling and nuclear decondensation initiated by nucleoplasmin can have apparent positive effects on reprogramming by increasing pregnancy rates after bovine SCNT (Betthauser et al. 2006).

Chromatin remodelling during early embryonic development results in the establishment of epigenetic asymmetry between the parental genomes. The first evidence of these events was the identification of changes in DNA methylation, which occurs rapidly in the paternal pronucleus (Lepikhov et al. 2008; Mayer et al. 2000). DNA methylation of CpG dinucleotides in promoter regions results in the repression of gene expression, but despite its fundamental importance in epigenetic gene regulation, the mechanisms leading to the dynamic demethylation of the DNA remain unknown (Ooi and Bestor 2008). In zygotes, DNA demethylation occurs before the initiation of S-phase, indicating that an active demethylation process is operative in the egg. Efforts to identify a DNA demethylase have failed, and it has been suggested that indirect DNA demethylation via DNA repair may lead to a removal of methylated cytosines (Morgan et al. 2005). Some evidence supporting this hypothesis was provided recently by showing that Gadd45a and XPG, both involved in nucleotide excision DNA repair (NER), are critical for the demethylation of the *Oct-4* promoter when injected into *Xenopus* oocytes (Barreto et al. 2007). Evidence that Gadd45a participates with the deaminase AID and the thymine glycosylase MBD4 during demethylation in zebrafish embryos suggests that a combination of mechanisms may work in concert during embryonic development (Rai et al. 2008).

There is, however, no evidence yet for such a mechanism operating during mammalian development. Interestingly, *Gadd45a* (Engel et al. 2009) and *Gadd45b* (Okada et al. 2010) knockout mice show no differences in overall DNA methylation and develop normally. Although compensatory demethylation carried out by other members of the Gadd45 family cannot be excluded in these zygotes, these findings suggest that *Gadd45* is not required for paternal DNA demethylation. An alternative indirect mechanism triggered by the cytidine deaminases AID and Apobec (both highly expressed in oocytes) has been proposed as another possibility for replication-independent DNA demethylation followed by base excision repair (BER) (Morgan et al. 2004). Single strand DNA breaks detected by the appearance of phosphorylated γH2A.X is evidenced in pre-replicative zygotes, supporting the idea of repair-coupled DNA demethylation (Wossidlo et al. 2010). However, a recent study shows that AID−/− mice undergo normal development, with no alterations in the overall levels of DNA methylation in foetuses, with the exception of PGCs, which show increased DNA methylation (Popp et al. 2010). It is possible that Apobec deaminases compensate for the lack of AID, although the expression levels of this enzyme are extremely low in PGCs (Hajkova et al. 2010). This remarkably unaffected phenotype suggests that other demethylation mechanisms may exist.

More recently, another possible demethylation mechanism has been proposed, based on the discovery that TeT proteins can catalyse the hydroxylation of 5mC to 5hmC (Tahiliani et al. 2009). TeT proteins are highly expressed in ES cells. TeT1 is particularly interesting because it is expressed from the 1-cell stage in the mouse, and is predominant in the ICM. When TeT1 expression is knocked down, the ICM shifts towards a trophoblast fate, indicating that TeT1 is essential for forming and maintaining the ICM. Interestingly, TeT1 maintains pluripotency by preventing re-methylation of the *Nanog* promoter. As a result, when TeT1 is knocked down in ES cells, *Nanog* expression (but not *Oct-4* and *Sox2*) is sharply reduced, and its promoter becomes hypermethylated (Ito et al. 2010). This exciting work provides compelling evidence for another possible mechanism mediating active DNA demethylation.

A question concerning the active demethylation mechanism relates to the dynamics of this event in the context of the nucleosome. Since histones with different modifications provide a level of compaction to the DNA, the general view is that the first step required in the DNA demethylation is to make the DNA accessible to the factors responsible for targeting methylated cytosines. The counterpart to this model is the repair model, as recently proposed for DNA demethylation in primordial germ cells (Hajkova et al. 2008). This model proposes a mechanism similar to that described in plants where methylcytosine-specific DNA glycosylases trigger a DNA repair response. Such glycosylases have not been found in mammals. An alternative possibility is a DNA repair-mediated DNA demethylation that is triggered by histone replacement, which targets the DNA repair complex to the damaged region. This model, which contemplates repair outside of S-phase, is compatible with the NER/histone incorporation reported after UV damage in human cells (Polo et al. 2006), and deserves consideration as a possibility after fertilization or SCNT. Active demethylation of sperm DNA occurs simultaneously with protamine replacement

by histones within the first 6 h after fertilization (Santos et al. 2002), and it has been shown that the H3.3 variant is preferentially incorporated into sperm chromatin during the initial steps of sperm decondensation (van der Heijden et al. 2005). H3.3 is deposited by the histone chaperone HIRA in a replication-independent manner, and this histone variant differs from the canonical H3.1 in that it is associated with transcriptionally active sites in somatic cells. The male pronucleus becomes transcriptionally active before the female pronucleus, thus H3.3 may confer the features of active chromatin with increased histone turnover at the nucleosome (Schwartz and Ahmad 2005; Torres-Padilla et al. 2006). Thus, the increased accessibility created by incorporation of this histone variant may facilitate DNA demethylation via DNA repair.

Active demethylation is followed by a passive demethylation occurring during the cleavage stages, where the newly synthesized DNA remains unmethylated as a result of the exclusion from the nucleus of the oocytes-specific DNA methyltransferase 1 (Dnmt1o) during the first three cleavage divisions (Carlson et al. 1992). In SCNT delayed and incomplete DNA demethylation is observed, suggesting that this may contribute to reprogramming failures and the low success of development in clones (Dean et al. 2001). The use of DNA demethylating agents and histone deacetylase inhibitors contributes to a reduction of DNA methylation in the donor chromatin. Yet, an improvement in full-term development has only been reported after using Trichostatin A (Kishigami et al. 2007; Rybouchkin et al. 2006). One may therefore ask whether the limited capacity of the oocyte to remodel somatic chromatin during early development is related to differences in nuclear configuration compared to that of the mature sperm fully packaged with protamines. Some evidence supporting this concept was shown in experiments using mouse spermatids for oocyte injection. Round spermatids (which have decondensed nuclei and lack protamines) undergo limited active DNA demethylation after injection into oocytes; however, the embryos made under these conditions can develop to term normally, suggesting that the initial wave of active demethylation is not essential for development (Kishigami et al. 2004; Polanski et al. 2008).

Thus, eggs can reprogramme DNA methylation of mature sperm effectively in a very short period of time prior to zygotic S-phase; however, erasure of DNA marks via passive demethylation can compensate for the reduced ability of the egg to demethylate other types of nuclei, such as round spermatids. By contrast, passive DNA demethylation cannot compensate in SCNT, where high levels of DNA methylation are maintained up to the blastocyst stage. Two lines of evidence suggest that reduced chromatin condensation contributes to effective remodelling in the zygote. First, TSA, which promotes maintenance of a loose chromatin configuration, contributes to better development in clones. Second, the fact that round spermatids, which have a decondensed nucleus, are effectively reprogrammed despite undergoing limited demethylation suggests that the DNA methylation mark can be remodelled later in development.

DNA methylation is an epigenetic mark that maintains the inactivation of promoter regions and is thus correlated with transcriptionally inactive genes. In somatic cells, this mark is enriched at cell type-specific promoters. Therefore, the erasure of

methylation marks from chromatin of different somatic cell lineages may prove more challenging to the egg than the demethylation of germ cell (sperm and egg) nuclei. Evidence from other experimental systems, such as iPS, shows that the addition of the DNA demethylating agent *5-aza-2′*-deoxycytidine can enhance the derivation of iPS cells (Mikkelsen et al. 2008), suggesting that DNA methylation must be remodelled in order to obtain reprogrammed cells. These observations point to the idea that DNA methylation reprogramming is an essential component of the targeting events required for an effective reprogramming strategy.

5.2.2 Reprogramming Histone Marks

Covalent histone modifications are heritable epigenetic marks critical for maintaining cellular identity. A distinct pattern of histone marks is established after fertilization and is characterized by parental asymmetry, as observed for DNA methylation. The female pronucleus carries a number of active marks such as H3K4me1/2/3 (Santos et al. 2005; Shao et al. 2008), H3K9/14 ac (Kim et al. 2003), H4K5/8/12/16 ac (Maalouf et al. 2008) and repressive marks H3K9me2/3, H3K27me1 and H4K20me3 (Morgan et al. 2005) all of which do not undergo major changes during pronuclear development. Sperm chromatin, instead, undergoes a major remodelling during the first cell cycle of the zygote. Along with the replacement of protamines with histones, monomethylated histone marks H3K4me1, H3K9me1 and H3K27me1 are predominantly acquired before the initiation of S-phase. Subsequently, during the course of the first cell cycle, histone methyltransferases (HMTs) and acetyltransferases (HATs) associate with the paternal chromatin and are responsible for the enrichment of methyl groups and the appearance of H3K4me3, H3K9me2, H4K5/8/12/16 ac and H3K27me3 marks (Lepikhov and Walter 2004; Maalouf et al. 2008; Santos et al. 2005). Towards the end of the first cell cycle, there is reduced asymmetry between the histone marks of the parental genomes, except for the absence of H3K9me2/3 (Lepikhov and Walter 2004; Santos et al. 2005) and H3K20me3 (Probst et al. 2007) in the male pronucleus. This dynamic remodelling of paternal genomes has been studied after SCNT and important differences with the normal course of reprogramming has been reported, suggesting that failure at these early stages of development are not properly compensated for, and lead to reduced development. The major differences relate to the reduced capacity of the egg to remove active chromatin marks such as H3K9ac (Santos et al. 2003) and H4K5/8/12/16ac (Inoue et al. 2006; Maalouf et al. 2008), as well as repressive marks such as H3K9me3 (Santos et al. 2003). Interestingly, addition of the HDAC inhibitor TSA can lead to an increase in development in clones, suggesting that general chromatin accessibility is reduced in cloned embryos by the HDAC activity of eggs. Thus, maternally inherited factors are responsible for the gradual targeting of chromatin after fertilization. These factors have only been partially identified and include HATs: Gcn5; HMTs: Ezh2, G9a; histone deacetylases (HDAC), DNA binding proteins: Mbd3; numerous chromatin remodelers such as Brg1, Npm2, ATRX;

and polycomb group members: Eed, Suz12, YY1, Ring1b. The interaction of these proteins with transcriptional regulators with sequence specificity will contribute to the establishment of totipotent chromatin in the zygote. What has become increasingly clear is that the efficiency of SCNT is limited in part by the brief window of time that the egg has to reprogramme fully differentiated cells. The forward developmental momentum, represented by cell cycle progression and transcriptional activation of the embryonic genome that is triggered by egg activation, is an impediment to the reprogramming activities of the recipient egg. Thus, studying reprogramming in a cell cycle-independent context may help develop a better understanding of the nature of the reprogramming events.

5.3 Experimental Nuclear Reprogramming

Lessons learned from reprogramming by SCNT have taught us that eggs are inefficient in recapitulating early epigenetic reprogramming associated with normal development after introduction of a somatic nucleus. Clearly, remodelling of the highly specialized sperm nucleus is unique, as shown by the asymmetry of parental genomes in early development. As mentioned above, extending the window for reprogramming in the oocyte by avoiding cell cycle progression after SCNT can facilitate an efficient erasure of somatic epigenetic information and re-establish a pluripotent epigenotype (Fig. 5.2). Oocytes arrested in prophase of meiosis are perfectly suited to test this idea. Prophase oocytes contain a large nucleus, the GV, and they can be used either for injections or to prepare cell extracts. Cells injected into mouse GVs or treated with extracts thereof undergo H3K9me3 and H3K9ac remodelling, and these cells can improve the outcome of live births after SCNT (Bui et al. 2008). However, the difficulty in generating such extracts from mammalian oocytes

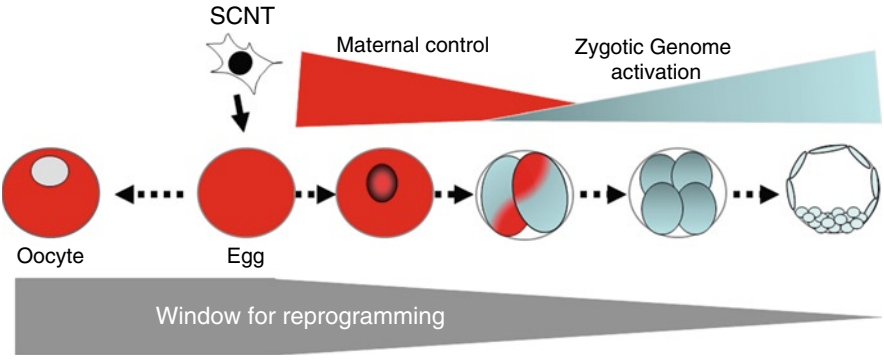

Fig. 5.2 Extending the window of reprogramming. Oocytes arrested in prophase of meiosis offer a reprogramming environment that is independent of cell cycle progression. Significant remodelling events take place when cells are exposed to factors in these oocytes (see text for details)

requires the use of alternative sources. For example, *Xenopus laevis* oocytes and eggs can be obtained easily and they have been used to elucidate fundamental reprogramming events (Gurdon 2006). Mammalian cells injected into *Xenopus* oocytes are epigenetically reprogrammed and reactivate pluripotent gene expression demonstrating that this heterologous system can be used to study nuclear reprogramming. Molecules stored in *Xenopus* GV-stage oocytes trigger *Oct-4* promoter demethylation and gene reactivation (Byrne et al. 2003; Simonsson and Gurdon 2004). The basis for this reprogramming relies in part on the active histone exchange promoted by oocyte molecules that help maintain a highly dynamic chromatin environment in the GV (Stewart et al. 2006). The initial phases of reprogramming can also be visualized by the enlargement of somatic nuclei that accompany nuclear DNA decondensation after incubation in GV oocytes (Byrne et al. 2003 and Fig. 5.1). *Xenopus* oocyte/egg extracts (XOE and XEE, respectively) also have nuclear remodelling activity, as shown by the remodelling of nuclear lamina protein lamin A/C (Alberio et al. 2005; Miyamoto et al. 2007). Importantly, we have shown that oocyte and egg extracts support remodelling activities in very different cellular contexts. Oocyte extracts do not support DNA replication of somatic cells but promote PolII-dependent transcription, whereas egg extracts promote DNA replication but no transcription (Alberio et al. 2005). Thus, the use of GV-arrested oocytes allows the dissection of reprogramming activities in a cell cycle-independent context. We have also extended the observation made with *Xenopus* using oocytes from axolotls, a urodele (salamander) amphibian (Fig. 5.3). Mammalian somatic cells incubated in axolotl oocyte extracts (AOE) reactivate *Nanog* and *Oct-4* expression (Bian et al. 2009). The activation of *Nanog* is rapid and precedes the activation of *Oct-4*. Furthermore, *Nanog* activation fails to occur in fibroblasts incubated in *Xenopus* oocytes (Halley-Stott et al. 2010). This suggested to us that the molecules present in these two types of oocytes had very different reprogramming capacity. As a first step towards identifying reprogramming molecules, we looked for pluripotency factors in axolotl oocytes. Axolotl oocytes express *Oct-4* (Bachvarova et al. 2004), *Sox-2* and *Nanog* (Dixon et al. 2010), and these factors are stored as proteins in the oocyte (Alberio et al. unpublished), indicating that axolotl oocytes combine the powerful remodelling activities normally found in oocytes with the unique transcriptional network proper of an embryonic stem cell, so-called maternal pluripotency. On that basis we propose that the axolotl oocyte constitutes a valuable reprogramming cocktail of molecules that can be used to dissect nuclear reprogramming. We have used AOEs to study reprogramming of somatic cells and we found that they promote the removal of repressive chromatin marks H3K9me3, H3K27me3, and the chromatin-associated protein HP1 (Bian et al. 2009). Furthermore, AOE can re-establish H3K4me3 to the *Nanog* and *Oct-4* promoter of somatic cells. This is in fact not seen in cells incubated in XOE, suggesting that the targeting of these promoters may be mediated by specific transcription factors present in cells expressing the pluripotency transcription factor network. This is further supported by the extensive DNA demethylation of *Nanog* and *Oct-4* promoter occurring during a 6-h period in cells exposed to AOE but not in XOE (Bian et al. 2009) as well as the demethylation of tumour suppressor genes in breast cancer cell lines

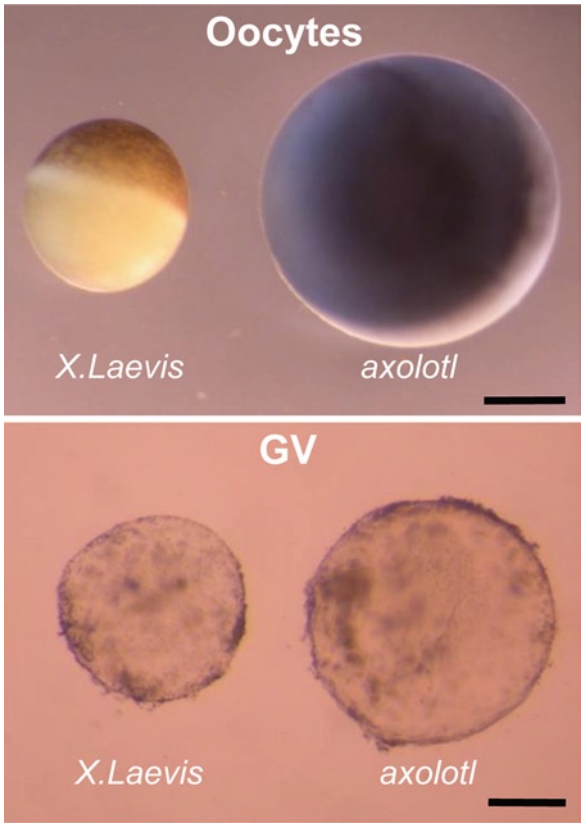

Fig. 5.3 *Xenopus* and axolotl oocytes. Both types of oocytes can be used for numerous manipulations and for preparing extracts that allow the treatment of large numbers of cells. The germinal vesicle can be injected with several dozen cells. Bar, *top*: 0.5 mm, *bottom*: 0.2 mm

(Allegrucci et al. 2011). Further support to the transcription factor targeted/initiated reprogramming comes from our *AxNanog* overexpression experiments in mouse ES cells. Mouse ES cells overexpressing *AxNanog* were fused to mouse neural stem cells (NSC) carrying Oct-4 GFP (Dixon et al. 2010). In these hybrids *AxNanog* induced the reactivation of Oct-4-GFP in NSC with efficiency similar to mouse *Nanog*, demonstrating that an amphibian *Nanog* can participate in the reprogramming of pluripotency, and highlights the fact that evolutionarily distant molecules maintain conserved core components required for transcriptional activation of target genes. Importantly, sequence analysis showed that AxNanog is active as a monomer, and lacks the tryptophan repeat responsible for dimerization in mammalian Nanogs. We established that monomeric AxNanog does not support LIF-independent self-renewal in ES cells, in contrast to dimeric mouse Nanog. Dimerized Nanog molecules promote cell division by recruiting Sall4, Nac1 and Dax1, three cell cycle regulators. AxNanog, by contrast, does not bind to these molecules; however, it binds to other core members of the pluripotency network such as Oct-4 and Hdac1. Thus, the discovery of AxNanog has allowed us to separate two intrinsic activities in mammalian ES cells: (1) self-renewal and (2) pluripotency. As a result

of these experiments, we conclude that the acquisition of dimerization domains in *Nanog* was critical during the evolution of mammals.

Alignment of mouse, human and axolotl *Nanog* shows a high level of conservation in the homeodomain. Interestingly, mouse and human *Nanog* are virtually identical, thus making it very difficult to partition the molecule on the basis of conserved domains. Having sequence information from axolotls allowed us to identify ancestrally conserved regions. The partition of this molecule into sub-components allowed us to construct a synthetic molecule with potent reprogramming capacity. Synthetic *Nanog* induces potent reactivation of *Oct-4* in NSC fused to ES cells overexpressing this molecule, and when used to complement *Xenopus* oocytes synthetic *Nanog* improves reprogramming of mouse *Nanog*.

The proof of this concept suggests that evolutionary distance can be used to identify the fundamental components of transcription factors. Thus, it is possible to accurately predict, for example, transcriptional activator domains, dimerization domains, ubiquitination domains, or repressor domains, and use this information to design small optimized molecules with powerful reprogramming capacity. Use of engineered molecules for reprogramming somatic cells has multiple advantages: (1) they can be engineered to have specific half lives, (2) they can be delivered using protein delivery systems, (3) they can carry specific tags which can be useful for screening purposes.

Here we have presented evidence of how much we have learned about the natural reprogramming abilities of oocytes, and the impact that this knowledge can have in improving reprogramming of somatic cells. It will be, by combining the knowledge arising from multiple strategies an efficient method for reversal of differentiation of somatic cells will result in the development of autologous cell therapies.

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Chapter 6
Cell Fusion-Mediated Nuclear Reprogramming of Somatic Cells

Kunio Hirano and Takashi Tada

Abstract Epigenetic reprogramming of nuclei from differentiated to pluripotent state can be induced by three experimental approaches: nuclear transfer, cell fusion, and transduction of transcription factors. In cell fusion between embryonic stem and somatic cells, stem cell-derived trans-acting factors function to confer pluripotency on somatic cell nuclei by overwriting the epigenotype of the undifferentiated state. Intensive analyses using inter-subspecific hybrid cells revealed that decondensation of nuclear chromatin is a key initial step for acquiring a fully reprogrammed nuclear status. The formation of de-condensed chromatin is a common molecular event seen in cell fusion-mediated reprogramming and the generation of induced pluripotent stem cells via the transduction of transcription factors. Thus, cell fusion is a powerful tool for providing information on the molecular mechanisms of nuclear reprogramming.

6.1 Introduction

Nuclear reprogramming, a change in epigenetic status from the differentiated cell type to the pluripotent cell type, can be induced by three approaches: (i) transplantation of a somatic nucleus into an unfertilized egg, (ii) cell fusion between a somatic cell and an embryonic stem (ES) cell, and (iii) forced expression of transcription factors in somatic cells.

In 1952, Briggs and King experimented with nuclear transplantation (NT) of blastula cells into enucleated eggs in the "Leopard" frog in order to address the

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genomic equivalence of highly diverged cell types. Then, using modified NT methods, cloned albino "*Xenopus*" frogs were generated by the transfer of intestinal epithelial cells from albino feeding tadpoles into wild type oocytes (Gurdon 1962). Since the cloned frogs belonged to Amphibia and there were no reports of cloned mammals, the idea developed that NT cloning is uniquely applicable to Amphibia. In 1997, the birth of the cloned sheep "Dolly" surprised the scientific community, as it was the first cloned mammal. Dolly was produced by NT of somatic cells into unfertilized eggs using a sophisticated technique (Wilmut et al. 1997). Lately, it has been generally accepted that mammalian oocytes of many species including mice, cows, and monkeys retain nuclear reprogramming activity in oocytes (Tada and Tada 2001). NT cloning is the only way of producing a genetically identical clone from an adult. Thus, cloning technology is a useful tool for conserving biological resources, and is a potentially powerful tool for the resurrection of extinct mammals from artificially or naturally frozen cells (Wakayama et al. 2008).

Direct reprogramming of somatic cells to produce pluripotential stem cells is a recently developed technology. Cell fusion between embryonic germ (EG) cells or ES cells and somatic cells demonstrated that the reprogramming activity was resident in the pluripotent stem cells (Tada et al. 1997, 2001). Forced expression of the transcription factors, *Oct4*, *Sox2*, *Klf4,* and *c-Myc*, that had been isolated from an ES cell expression library, triggered direct reprogramming of somatic cells into pluripotent cells, induced pluripotent stem (iPS) cells with epigenetic changes in mice (Takahashi and Yamanaka 2006) and humans (Takahashi et al. 2007; Yu et al. 2007). iPS cells and iPS cell technologies are expected to greatly contribute to biological, pharmaceutical, and medical research; and clinical applications are also predicted. However, the molecular mechanism involved in reprogramming remains largely unknown at this time.

6.2 Pluripotent Stem Cells in Mice

Mouse ES cells derived from blastocyst inner cell mass without carcinogenesis display pluripotency, as shown by their multi-lineage differentiation including germ cells in chimeric mice (Evans and Kaufman 1981). In addition to ES cells, other types of pluripotent cells, including trophoblast stem (TS) cells from extraembryonic cells of blastocysts (Tanaka et al. 1998), epiblast stem (EpiStem) cells from the epiblasts of E5.5–7.5 embryos (Brons et al. 2007; Tesar et al. 2007), EG cells from primordial germ cells of E8.5–12.5 embryos (Matsui et al. 1992), and multipotential spermatogonial stem (mGS) cells (Kanatsu-Shinohara et al. 2004), have been established as multi- or pluripotential stem cells verified by their ability to form teratomas (Fig. 6.1). EG and mGS cells can contribute to the development of chimeric embryos, whereas TS and EpiStem cells cannot. Mouse ES, EG, and mGS cells retain a similar cell phenotype, which is characterized by a large nucleus and a small amount of cytoplasm, and form bowl-shaped colonies on culture dishes with feeder cells. The phenotype of mouse iPS cells resembles that of ES cells and differs from

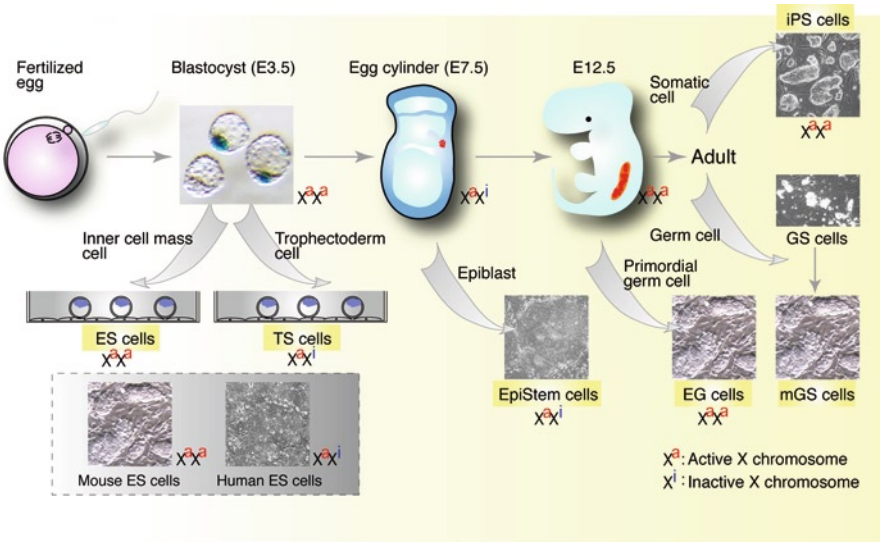

Fig. 6.1 Various pluripotent stem cell lines established from developing mouse embryos and adults. X-chromosome inactivation occurs in some types of pluripotent cell lines, while it does not occur in other cell lines. The morphology of human embryonic stem (ES) cell colonies differs from that of colonies of mouse ES cells. TS cells: trophoblast stem cells, EpiStem cells: epiblast stem cells, *EG cells* embryonic germ cells; *mGS cells* multipotent germline stem cells; *iPS cells* induced pluripotent stem cells

that of EpiStem and TS cells. Furthermore, parental imprints are maintained in ES cells, while they are erased in EG cells from E12.5 embryos (Tada et al. 1997). This variety of stem-cell properties suggests that epigenetic modifications vary between pluripotent stem cells of different origin, even in those with the same genetic background. It seems that the molecular network required for acquiring pluripotency allows a certain degree of divergent epigenetic modification of the genes dispensable for pluripotency.

6.3 Cell Fusion Between Somatic Cells

Cell fusion between two different types of somatic cells was first mediated using Sendai virus (Okada et al. 1957). Later, chemical and electric fusion technologies were developed (Kao and Miehayluk 1974; Zimmermann and Scheurich 1981). The three different methods share a cell fusion mechanism, in which heterokaryons generated by plasma membrane fusion between neighboring cells through membrane restoration after virus, chemical, or electric stimulation-mediated transient plasma membrane breakdown become synkaryon hybrid cells with a giant nucleus through rounds of cell division (Harris 1965) (Fig. 6.2a). The cell fusion technique is a powerful tool for studying genetics.

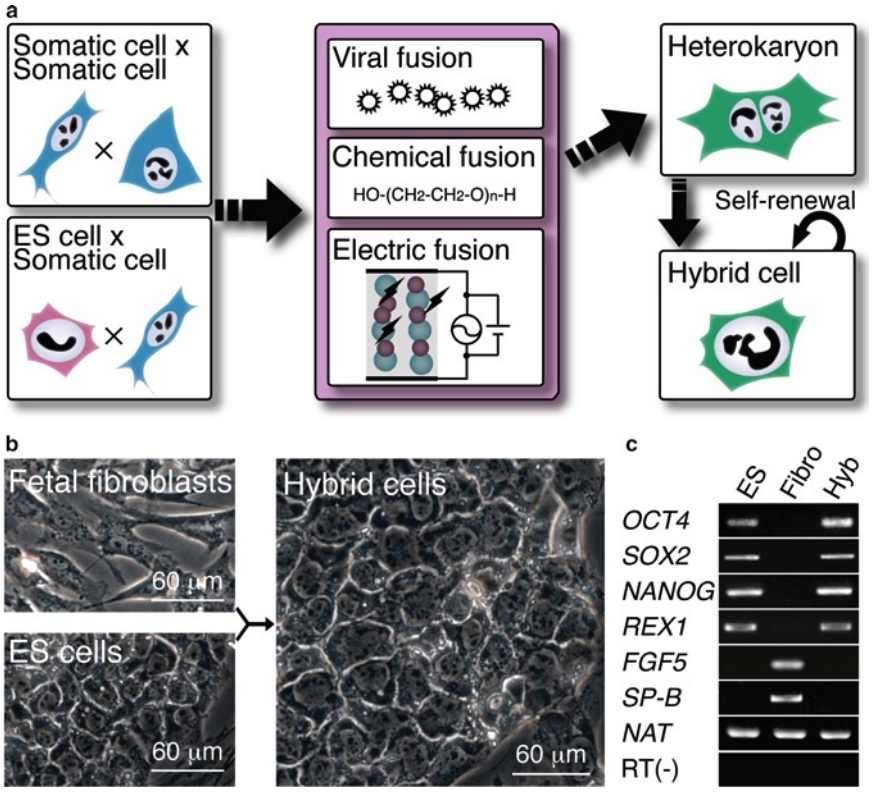

Fig. 6.2 Cell fusion of human embryonic stem (ES) cells and somatic cells. (**a**) Scheme for the generation of hybrid cells derived from two somatic cells, and ES and somatic cells using viral, chemical, or electric cell fusion technologies. (**b**) Morphology of human somatic, ES, and hybrid cells. The TIG1 and TIG3 human somatic cell lines are derived from fetal lung fibroblasts. KhES1 is a human ES cell line. (**c**) Expression of pluripotent and somatic cell marker genes by RT-PCR. *OCT4, SOX2, NANOG,* and *REX1* are pluripotent marker genes, while *FGF5* and *SP-B* are somatic cell marker genes. *NAT* is the control. *ES* embryonic stem cells; *Fibro* fetal lung fibroblasts; *Hyb* hybrid cells derived from ES cells and fibroblasts

Cell fusion techniques have been used as a key method for investigating mechanisms underlying the determination of diverse cell phenotypes. Interspecific heterokaryons between human HeLa cells and mouse Ehrlich ascites tumor cells demonstrated that nuclear properties can be changed through intracellular communication (Harris and Watkins 1965). In the regulation of pigment synthesis, the pigment production of Syrian hamster melanoma cells was quenched when they were hybridized with unpigmented mouse fibroblasts (Davidson et al. 1966). Expression of tyrosine aminotransferase (TAT) in rat heptoma cells was reduced to a very low level in hybrids formed with mouse fibroblasts (Schneider and Weiss 1971). This phenotypic disappearance, which is known as "extinction," is induced by the interaction of regulatory molecules. More interestingly, cell fusion between muscle cells and non-muscle cells

showed remarkable plasticity of gene expression in differentiated cells (Blau et al. 1985). A trans-acting molecule involved in the extinction of TAT activity in hybrids between the rat hepatoma cells and mouse fibroblasts was identified on mouse chromosome 11, which carries the tissue-specific extringuisher-1 (*Tse*-1) locus, using the microcell-mediated chromosome transfer technique (Killary and Fournier 1984). Interestingly, *Tse*-1 plays a role in the extinction of TAT, but not in modulating the gene expression of other hepatic markers, indicating that the expression of tissue-specific genes may be regulated by independent pathway.

The transiently expressed exogenous *Oct4*, *Sox2*, *Klf4,* and *c-Myc* (OSKM) genes function as trans-acting activators or repressors in the reprogramming of somatic cells into iPS cells. The above transcriptional regulatory mechanism resembles that involved in the "extinction" of genes in hybrid cells formed using different types of somatic cells. It would be interesting to examine whether the small number of trans-acting transcription factors can change the entire epigenetic molecular network formed in a nucleus carrying a huge amount of genomic DNA coding for more than 20,000 genes.

6.4 Role of Cell Fusion In Vivo

The development of in vitro cell fusion technology has contributed to our understanding of the genetic mechanisms behind the phenotypic changes induced by transcriptional activation or silencing. We know that in vivo cell fusion occurs during normal development, as seen by the generation of multinucleated cells, syncytia, in the muscle and placenta. Notably, it is likely that cell fusion occurs spontaneously in vivo. Bone marrow-derived cells transplanted into mice demonstrated the ability to contribute to the different tissue lineages including hepatocytes in the liver, Purkinje neurons in the brain, and cardiomyocytes in the heart, after cell fusion (Alvarez-Dolado et al. 2003; Wang et al. 2003; Weimann et al. 2003). In hepatocytes in the liver, hematopoietic donor genomes were reprogrammed into a hepatocyte-like state through spontaneous cell fusion with endogenous hepatocytes. Therefore, it is possible that in vivo reprogramming of donor cells by spontaneous cell fusion with host cells plays a key role in the transdifferentiation of hybrid cells as a means of intrinsically contributing to the development and maintenance of some cell types.

6.5 Nuclear Reprogramming of Mouse Somatic Cells by Fusion with ES Cells

Cell fusion between mouse embryonal carcinoma (EC) cells and thymocytes resulted in the generation of hybrid cells, which have undifferentiated carcinoma cell-like properties including the capability to generate teratomas containing a range

of differentiated cells (Miller and Ruddle 1976). It was, however, unclear whether the EC cell properties, including chromosomal abnormality, which are generated through carcinogenesis, are required for the generation of pluripotent hybrid cells. To address whether pluripotent stem cells established without carcinogenesis have the capability to form the hybrid cells with somatic cells, mouse ES and EG cells with normal karyotypes ($2n=40$) were electrofused with thymocytes, in which cell differentiation was verified by DNA rearrangement of the immunoglobulin genes (Tada et al. 1997, 2001). The nuclear reprogramming of somatic genomes in the mouse ES hybrid cells was shown by several lines of evidence, the activation of the somatic cell-derived pluripotent reporter Oct4-*Gfp* gene, the reactivation of the inactivated X chromosome in female somatic cells, teratoma formation, and chimera formation (Tada et al. 2001). Further intensive analysis of reprogramming with inter-subspecific ES-somatic hybrid cells demonstrated that the reprogrammed somatic genomes functioned similarly to the pluripotent ES genomes with regard to lineage-specific gene expression through in vitro neuronal differentiation and in vivo teratoma formation (Tada et al. 2003). Importantly, these data indicated that mouse ES cells contain factors that are sufficient for inducing the reprogramming of somatic nuclei. Research aimed at identifying pluripotent genes from ES cells, in order to understand the pluripotent molecular network and enable direct reprogramming of somatic cells began using the cDNA subtraction (Hatano et al. 2005) and in silico differential display techniques (Mitsui et al. 2003). Consequently, OSKM were identified as factors sufficient for reprogramming of various types of somatic cells into iPS cells in vitro (Takahashi and Yamanaka 2006).

6.6 Nuclear Reprogramming of Human Somatic Cells by Fusion with ES Cells

Human ES cells with a normal karyotype, $2n=46$, were established from blastocysts as pluripotent cell lines, which demonstrated high telomerase activity, expressed pluripotent marker genes, and displayed differentiation potential towards not only all of the three germ layers but also trophoblast lineage (Thomson et al. 1998). The expression profiles of the pluripotent genes are similar between mouse and human ES cells, while the expression of the cell surface markers SSEA3 and SSEA 4, in vitro differentiation into trophoblast, and the maintenance of an undifferentiated state dependent on basic FGF (fibroblast growth factor) and independent of LIF (leukemia inhibitory factor) are unique to human ES cells. Strikingly, human ES cell colonies show a plate-shaped morphology, which resembles the colony morphology of mouse EpiStem cells rather than the bowl-shaped colony morphology of mouse ES cells. Therefore, to address whether the reprogramming activity was maintained in human ES cells similar to mouse ES cells, human ES cells were chemically fused with human primary fibroblasts (Cowan et al. 2005). Global gene expression and DNA methylation analyses demonstrated that the somatic cell nuclei

were reprogrammed from a differentiated state to an undifferentiated state. Furthermore, human myeloid precursors were efficiently reprogrammed from differentiated state to undifferentiated state through cell fusion with human ES cells (Yu et al. 2006). In the hybrid cells, myeloid precursor-specific antigens were silenced instead of ES cell-specific surface antigens and pluripotent marker genes being activated. The efficiency of differentiation of the hybrid cells was comparable to that of ES cells, suggesting that the complete erasure of somatic memory had occurred in the hybrid cells.

We fused human primary fibroblast lines from fetal lung, TIG1 and TIG3, with the human ES cell line KhES1 (Suemori et al. 2006) using the electrofusion technique (Tada and Tada 2006) (unpublished data) (Fig. 6.2b). Human ES-somatic hybrid cells, which resemble human ES cells in morphology, were stably cultured for a long period with a normal tetraploid karyotype. The expression of pluripotent marker genes in the ES-somatic hybrid cells was comparable to that in ES cells (Fig. 6.2c). The human hybrid cells that were capable of forming teratomas after implantion under the kidney capsule were used as materials in the selective chromosome elimination experiments described below.

6.7 Selective Chromosome Elimination from Pluripotent Hybrid Cells

The nuclear reprogramming activity found in mouse and human ES cells by cell fusion with somatic cells suggested that key factors involved in reprogramming could be isolated from the nucleus or cytoplasm of ES cells. Then, several new technologies including cytoplasmic cell fusion and ES cell factor introduction were applied to the direct reprogramming of somatic cells in culture (Matsumura and Tada 2008). Currently, the forced expression of transcription factors, OSK(M), is the most commonly used method for inducing direct reprogramming of somatic cells into iPS cells.

Here, we introduce the challenge of selective chromosome elimination from the nuclei of living hybrid cells using DNA recombinase (Cre)-dependent chromosome rearrangement technology (Matsumura et al. 2007). To eliminate entire chromosome(s) from the nucleus, a chromosome elimination cassette (CEC) bearing a fluorescence reporter and a drug-resistance gene between oppositely orientated *loxP* sites was designed. Mouse hybrid cells derived from ES cells carrying CEC-tagged chromosome(s) and normal somatic cells were treated with Cre to induce homologous recombination between the *loxP* sites in G1 or G2 phase of the cell cycle in a trans- or cis-targeted recombination manner (Mills and Bradley 2001). Nulli- or di-centric chromosome(s) generated by unequal recombination were selectively eliminated from the nucleus through cell division. Interestingly, targeted elimination of a pair of ES cell-derived chromosome 6s in ES-somatic hybrid cells resulted in the survival of hybrid cells carrying three but not two (somatic) copies of chromosome 6. The additional chromosome 6 was generated by the duplication of

one of the somatic cell-derived chromosome 6s, as shown by chromosome-specific DNA polymorphisms, indicating that three chromosome 6s in tetraploid nuclei are required for the survival and proliferation of the hybrid cells (Matsumura et al. 2007). To analyze whether single chromosome elimination is harmful to the survival and proliferation of cells and/or to the maintenance of pluripotency, the CEC-mediated chromosome elimination technology was applied to human ES-somatic hybrid cells. Consequently, the elimination of an entire chromosome did not give rise to defects in ES-somatic hybrid cells (unpublished data). These data suggest that in mice and humans, undifferentiated cells tolerate a 25% fluctuation in gene expression level. It is likely that gene copy number is an important factor in the survival of diploid cells.

6.8 Molecular Mechanism of Cell Fusion-Mediated Reprogramming

The cell fusion-mediated reprogramming of somatic cells was visualized by reactivation of the somatic cell-derived pluripotent marker gene Oct4-*Gfp* 24–48 h after the fusion event (Tada et al. 2001; Han et al. 2008). Oct4-*Gfp* reactivation during cell fusion occurred considerably faster than it does during iPS cell induction, which takes 10–12 days after OSKM viral transduction (Mikkelsen et al. 2008; Nagata et al. 2009). It is predicted that the reprogramming mechanisms of ES-somatic cell fusion and iPS cell induction are at least in part shared. However, further studies are required to confirm this.

Interestingly, in mouse cell fusion between inter-subspecific donor somatic cells and recipient ES cells that have a high degree of DNA polymorphism, two molecular events, genome-wide de-condensation of nuclear chromatin and the establishment of a pluripotent cell-specific molecular network, were observed by DNA and histone methylation analyses together with gene expression profile switching (Kimura et al. 2004). The genome-wide chromatin de-condensation represented by histone H3 and H4 acetylation and histone H3K4 di- and tri-methylation could be linked with the erasure of somatic epigenetic memory prior to or in parallel with the acquisition of a pluripotent molecular network (Fig. 6.3). A similar situation was detected in the mechanisms involved in iPS cell induction. The histone deacetylase inhibitor valproic acid (VPA) and the activator of the β-Catenin signaling pathway Wnt, which facilitate the formation of open chromatin, promoted the efficient reprogramming of mouse primary embryonic fibroblasts to iPS cells (Tada 2008) (Fig. 6.3). Furthermore, Akt (Nakamura et al. 2008) and β-Catenin (Lluis et al. 2008), which are also involved in forming open chromatin, improved the reprogramming efficiency of embryonic fibroblasts to iPS cells. Therefore, chromatin de-condensation is an important initial step toward the full reprogramming of somatic nuclei in not only cell fusion-mediated reprogramming but also iPS cell induction.

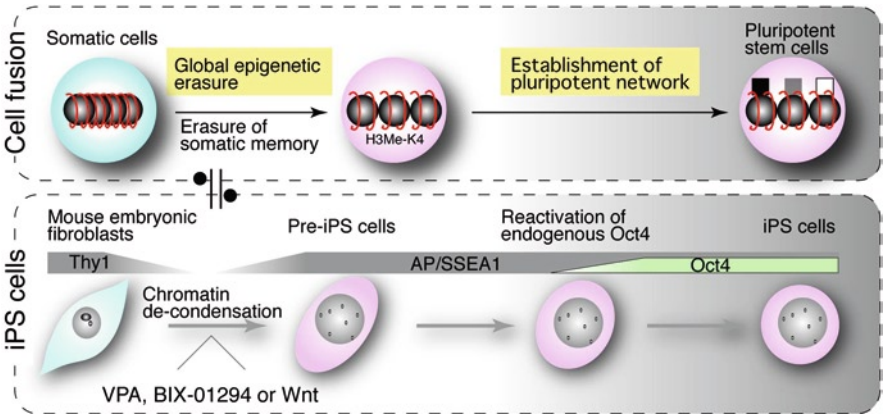

Fig. 6.3 Model of the molecular mechanisms of nuclear reprogramming of somatic cells to pluripotent stem cells by cell fusion and induced pluripotent stem (iPS) cell induction. *Thy1* is a somatic cell marker gene. *AP* alkaline phosphatase; *VPA* valproic acid (histone deacetylase inhibitor); BIX-01294 (histone methyltransferase inhibitor); *Wnt* wingless-int

6.9 Perspective

Direct reprogramming of somatic cells into iPS cells is expected to facilitate the clinical application of personalized pluripotent stem cells to regenerative medicine. Tetraploid hybrid cells are less suitable for clinical application than iPS cells. However, cell fusion is a powerful approach for understanding the molecular mechanisms involved in nuclear reprogramming, as shown by functional analysis of activation-induced cytidine deaminase (AID) (Bhutani et al. 2010). Nuclear transfer, cell fusion, and iPS cell induction are expected to become vital tools for understanding the nuclear plasticity of cells through the modulation of epigenetic modifications.

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Chapter 7
Generation of Induced Pluripotent Stem Cells from Somatic Cells

Masato Nakagawa

Abstract Pluripotent stem cells have been thought to be useful sources for regenerative medicine. Although embryonic stem (ES) cells have abilities to differentiate into several kinds of somatic cells and grow infinitely in vitro, there are several problems with using ES cells for clinical application such as ethical issues and rejection after transplantation. To overcome these problems, pluripotent stem cells were generated from somatic cells by introduction of defined factors. These have been termed induced pluripotent stem (iPS) cells. iPS cells have raised hopes for a new era of regenerative medicine because they can avoid the ethical problems and innate immune rejection associated with ES cells. In this chapter, I look back at the story of generation of iPS cells and discuss recent understanding of iPS cells with our latest data.

7.1 Introduction

Pluripotent stem cells have been thought to be useful sources for regenerative medicine. Although embryonic stem (ES) cells have abilities to differentiate into several kinds of somatic cells and grow infinitely in vitro, there are several problems with using ES cells for clinical application such as ethical issues and rejection after transplantation. To overcome these problems, pluripotent stem cells were generated from somatic cells by introduction of defined factors. These have been termed induced pluripotent stem (iPS) cells. iPS cells have raised hopes for a new era of regenerative

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medicine because they can avoid the ethical problems and innate immune rejection associated with ES cells. In this chapter, I look back at the story of generation of iPS cells and discuss recent understanding of iPS cells with our latest data.

7.2 Generation of iPS Cells

7.2.1 Reprogramming Factors

Reprogramming of somatic cell nuclei, and production of fertile adults from the reprogrammed cells, has been demonstrated in *Xenopus laevis* (Gurdon and Uehlinger 1966). The nuclei of fully differentiated intestinal epithelial cells were isolated and transplanted into unfertilized eggs, which resulted in reprogramming of the injected nucleus and subsequent production of fertile male and female frogs. This study gave rise to the understanding that somatic cells are reversible, and that unfertilized eggs have reprogramming ability (= existence of reprogramming factors). The reprogramming of somatic cell nuclei has also been achieved by in vitro cell fusion experiments. Nuclear reprogramming of mouse adult thymocytes was reproducibly achieved by fusion with ES cells, and the reprogramming was confirmed by V-(D)-J DNA rearrangement of the T cell receptor (Tada et al. 2001). In thymocytes, the Oct3/4-GFP reporter gene is inactivated, but is activated after hybridization at 48 h after cell fusion. Contribution of the thymocyte-ES hybrid cells to early mouse development demonstrated that the reprogrammed cells were pluripotent. The important message from this study was the demonstration that ES cells, like unfertilized eggs, express reprogramming factors.

7.2.2 Mouse iPS Cells

Although it may one day be possible to use human ES cells for cell transplantation therapy, their clinical application faces ethical objections against utilizing human embryos. One solution is to generate pluripotent cells directly from somatic cells. Several important studies have revealed that somatic cells can be reprogrammed, however the molecular mechanisms involved are not clear. In studies using frog or lamb cells, reprogramming has been demonstrated by nuclear transfer into unfertilized eggs. However, the efficiency and reproducibility of reprogramming events are not consistent, and the techniques used are not routine. To resolve these problems, we decided to induce somatic nuclear reprogramming directly using defined factors and simplified methodologies.

We hypothesized that reprogramming factors important for maintenance of ES cell pluripotency should be highly expressed in ES cells, but not in somatic cells.

We therefore searched for candidate genes by in silico analysis. Numerous candidate reprogramming factors were identified, which we named ECATs (ES cell associated transcripts) (Mitsui et al. 2003). Nanog, one of the ECATs, was found to be a very important gene for maintenance of ES cell pluripotency (Mitsui et al. 2003). Nanog-knockout ES cells did not have pluripotency and differentiated into extraembryonic endoderm lineage. The ECATs also contained other genes that have been shown to be important for ES cell pluripotency, Sox2, and Oct3/4 (Boyer et al. 2005; Wang et al. 2006). Finally, we selected and focused on 24 of the putative reprogramming factors for further analysis (Takahashi and Yamanaka 2006).

These factors are classified into three groups. The first group contains Nanog, Sox2, and Oct3/4, which play important roles in maintenance of ES cell pluripotency. The tumor-associated genes, Tcl1, Stat3, and c-Myc, are in the second group. The third group contains ECAT1, Esg1, and Klf4, found in our laboratory to be specifically expressed or function in ES cells. Transduction of all 24 genes into mouse embryonic fibroblasts (MEFs) by retroviral infection induced ES-like cells (Takahashi and Yamanaka 2006). The 24 factors were subsequently refined into four factors, including Sox2, Oct3/4, Klf4 and c-Myc. The ES-like cells showed pluripotency like ES cells and infinite cell growth like cancer cells. We named these cells "induced pluripotent stem" cells, iPS cells. For first-generation iPS cells, the cells were selected for G418 resistance derived from Fbx15-reporter (Fbx-iPSC). Since Fbx-iPSCs could generate chimeric fetuses, but not adult chimera mice, we concluded that Fbx-iPSCs do not have the same properties as ES cells, and are therefore incompletely reprogrammed cells.

To produce iPS cells which could make adult chimeras, we constructed a Nanog-reporter line followed by puromycin resistance for selection of high quality iPSCs (Okita et al. 2007). Adult chimeras were obtained using the second-generation iPSCs (Nanog-iPSCs) and they are competent for germline transmission (Maherali et al. 2007; Okita et al. 2007; Wernig et al. 2007). Fully differentiated cells including tail tip-derived fibroblasts (Takahashi and Yamanaka 2006), hepatocytes (Aoi et al. 2008), and gastric epithelial cells (Aoi et al. 2008), were also reprogrammed into iPS cells by the same four factors. These results indicate that somatic cells can be reprogrammed by defined factors.

7.2.3 Human iPS Cells

Human iPS cells were generated using the same four factors used for mouse iPS cell induction, with some modifications (Takahashi et al. 2007). Since human fibroblasts are not susceptible to infection with the retroviruses used for mouse iPS cell generation, the cells were preinfected with the retrovirus receptor, slc7a1, by lentiviruses, followed by infection with the four factors by retroviruses. Human iPS cells were induced by cultivating with the medium used for primate ES cells. Human iPS cells are indistinguishable from human ES cells in morphology, growth rate, and differentiation activity.

Human iPS cells will enable new advances in regenerative medicine. For cell transplantation therapy, ES cells seemed to be a good source; however, people do not have their own ES cells and thus rejection is highly probable. In contrast, iPS cells are derived from an individual's own fibroblasts (or other cells) and therefore rejection will not occur. Consequently, cell transplantation therapy has the potential to develop rapidly.

Human iPS cells can also be produced from somatic cells of patients that contain genetic mutations for diseases (disease-iPSC). These iPS cells would therefore also carry the genetic mutations. Using disease-iPS cells, it is potentially possible to reproduce characteristics of the disease for investigation by inducing their differentiation into disease-specific abnormal cells. Once generating iPS cells, the disease model cells will be easily produced in vitro. Such cells, as discussed in Chap. 15, will be useful for analysis of pathology, drug screening, toxicology, and study for side effect of drugs. These kinds of approach were not possible before the generation of iPS cells.

7.2.4 Recent Updates

Though reprogramming factors are introduced into many cells, only a few convert to iPS cells. One reason why the majority of cells do not become iPS cells is that some factors obstruct abnormal change, reprogramming in the somatic cells. We focused on p53, which is a suppressor of cancer, and when we introduced reprogramming factors into p53-knock out MEFs, or wild-type MEFs expressing dominant negative p53, the efficiency of iPS establishment was enhanced by 10–20 fold (Hong et al. 2009). iPS cell production was also accelerated by p53 knock-down in human dermal fibroblasts (HDF). We were also able to generate numerous iPS cells from p53-null MEFs by plasmid based ectopic expression of reprogramming factors, which do not integrate into the iPS cell genome and are therefore less likely to cause undesired genetic aberration. Furthermore, iPS cells were obtained and contributed to chimeric mice from terminally differentiated T lymphocytes deficient of p53, but not from wild-type T lymphocytes. Microarray analysis indentified 34 genes regulated by p53. Among them, p21 decelerated iPS cell establishment, and p21 protein was found to be highly expressed during reprogramming. As the p53-p21 pathway affects the cell cycle, cell survival, and plating efficiency, it remains to be clarified whether the p53-p21 pathway suppresses reprogramming directly.

Oxygen tension is known to have a significant influence on the function, survival, and differentiation of neural stem cells, hematopoietic stem cells, and ES cells. Based on these facts, we hypothesized that hypoxic conditions can promote the reprogramming process of iPS cell generation. We transduced reprogramming factors into MEFs and cultivated the cells under hypoxic conditions (Yoshida et al. 2009). We found that hypoxic cultivation increased the efficiency of iPS cell generation. The resultant iPS cells were capable of differentiating into all three germ layers and contributed to the formation of somatic tissues in chimeric mice. We

showed that the hypoxic cultivation also increased the efficiency of iPS cell generation using methods other than virus-mediated gene transduction, such as plasmid expression vectors. Furthermore, we found that exposure to hypoxia increased the efficiency of iPS cell generation from human somatic cells. To elucidate the mechanism of hypoxic enhancement of reprogramming efficiency, further investigation is required.

Many iPS cell clones have been generated using different transcription-factor combinations, cell types, and selection methods. Considering all of the differences in reprogramming methods reported to date, the safety and therapeutic implications of these differences should be thoroughly evaluated before iPS cells are used in cell therapies. We generated neurospheres from 36 mouse iPS cell clones and examined their neural differentiation capacity and teratoma-forming propensity after transplantation into the brains of immunodeficient (NOD-SCID) mice (Miura et al. 2009). We revealed that the neural differentiation capacity and teratoma-forming propensities of neurospheres vary significantly depending on the iPS cells' tissue of origin. Our results highlight the importance of iPS cells' origin as a determinant of safeness.

7.3 Safety of iPS Cells

7.3.1 Tumorigenicity

The first iPS cells were generated from fibroblasts by retroviral transduction of four defined factors. We found this to be a highly efficient and reproducible method for iPS cell induction. Using retroviruses to introduce the reprogramming factors also resulted in a small portion of retroviral vector integration, which led to tumor formation in iPS cell-derived chimeric mice. We found that retroviral c-Myc was activated in the tumor (Okita et al. 2007), and concluded that usage of retroviral c-Myc should be avoided for iPS cell generation. Because of this, we tried to induce iPS cells without retroviral c-Myc to improve their safety, and succeeded using 3F-iPSC (Nakagawa et al. 2008), albeit with low efficiency (Table 7.1). The morphology, growth rate, and differentiation activity were similar to those of four factor-induced iPS cells (4F-iPSCs). In chimeric mice derived from 3F-iPSCs, tumorigenicity was dramatically decreased compared to 4F-iPSC chimeric mice. These results strongly suggest that retroviral c-Myc should be omitted for generation of safer iPS cells. However, according to our recent study, the efficiency of germline transmission for 3F-iPSC is lower than that for 4F-iPSC. The reprogramming would appear to be not fully achieved by three factors alone. Identification of a new factor is required for generation of safer and high quality iPS cells, in preference to c-Myc. For generation of safer iPS cells, several methods have now been reported for transduction of reprogramming factors into fibroblasts. We reported that transfection of plasmid vectors by general transfection reagents could generate mouse iPS cells, and these iPS cells

Table 7.1 Characterization of iPS cells induced by three factors (3F) with or without Myc genes

Reprogramming factors	Chimera mice formation	Tumorigenicity in chimera mice	Germline transmission
3F+L-Myc	+++++	+	+++++
3F+c-Myc	+++++	+++++	+++++
3F alone	+++++	+	+

iPS cells can be generated by introducing three or four factors. All iPS cells result in chimera formation to the same extent. c-Myc but not L-Myc iPS cell-derived chimeric mice show high (+++++) tumorigenicity. The capacity for germline transmission is similar for both c-Myc and L-Myc iPS cells. 3F-iPS cells have low (+) efficiency of germline transmission

were competent for germline transmission (Okita et al. 2008). Generation of iPS cells has also been reported by other groups using other methods (Carey et al. 2008; Gonzalez et al. 2009; Hotta et al. 2009a, b; Kaji et al. 2009; Kim et al. 2009; Lyssiotis et al. 2009; Shao et al. 2009; Soldner et al. 2009; Sommer et al. 2009; Stadtfeld et al. 2008; Woltjen et al. 2009; Zhou et al. 2009; Zhou and Freed 2009). At this time it remains to be determined which method will prove most efficient and effective.

We found that both chimeras and progeny derived from mouse iPS cells showed an increased incidence of tumor formation, primarily due to reactivation of the c-Myc retrovirus (Okita et al. 2007). Subsequently, we and others succeeded in making mouse iPS cells without the c-Myc retrovirus by modifying the induction protocol (Nakagawa et al. 2008; Wernig et al. 2008). Chimeric mice derived from c-Myc-free iPS cells did not show any increased incidence of tumor formation (Nakagawa et al. 2008). However, the efficiency of iPS cell generation is significantly lower without the c-Myc retrovirus. Indeed, c-Myc is utilized in most of the reported methods to generate iPS cells without viral integration (Fusaki et al. 2009; Kim et al. 2009; Okita et al. 2008; Stadtfeld et al. 2008; Woltjen et al. 2009; Yu et al. 2009; Yusa et al. 2009; Zhou et al. 2009; Zhou and Freed 2009). Therefore, c-Myc functions as a double-edged sword in that it promotes both iPS cell generation and tumorigenicity.

7.3.2 Myc Family Genes

The Myc proto-oncogene family consists of three members; c-Myc, N-Myc, and L-Myc (Birrer et al. 1988; Cole 1986; Schwab et al. 1985; Yancopoulos et al. 1985). All three members dimerize with Max and bind to DNA (Blackwell et al. 1993). N-Myc is similar to c-Myc regarding its length, domain structure, and frequent association with human cancers (Malynn et al. 2000). In contrast, the L-Myc protein has a shorter N-terminal amino acid sequence than the other two members, while also possessing a significantly lower transformation activity in cultured cells (Barrett et al. 1992; Birrer et al. 1988; Cole and Cowling 2008; Hatton et al. 1996; Oster et al. 2003). Consistent with this property, only a small number of human cancers have been associated with the aberrant expression of L-Myc.

7.3.3 L-Myc and iPS Cells

Recently, we examined the efficiency of L-Myc for promotion of iPS cell generation (Nakagawa et al. 2010). Despite its weak transformation activity, we found that L-Myc has a stronger and more specific activity in promoting iPS cell generation. In addition, the efficiency of germline transmission was similar to that of c-Myc.

DNA microarray analyses suggested that L-Myc and the transformation-deficient c-Myc mutant regulate different target genes, compared to the wild-type c-Myc. When overexpressed in HDF, L-Myc and the c-Myc mutant suppressed genes that were highly expressed in fibroblasts, in comparison to iPS or ES cells. In contrast, only a small number of gene were selectively activated by L-Myc and the c-Myc mutant. We therefore speculate that the main role of L-Myc protein in promoting iPS cell generation might be to suppress differentiation-associated genes. This finding is consistent with a previous report regarding c-Myc (Smith et al. 2010; Sridharan et al. 2009) and we also found both L-Myc and the c-Myc mutant to be more potent than wild-type c-Myc.

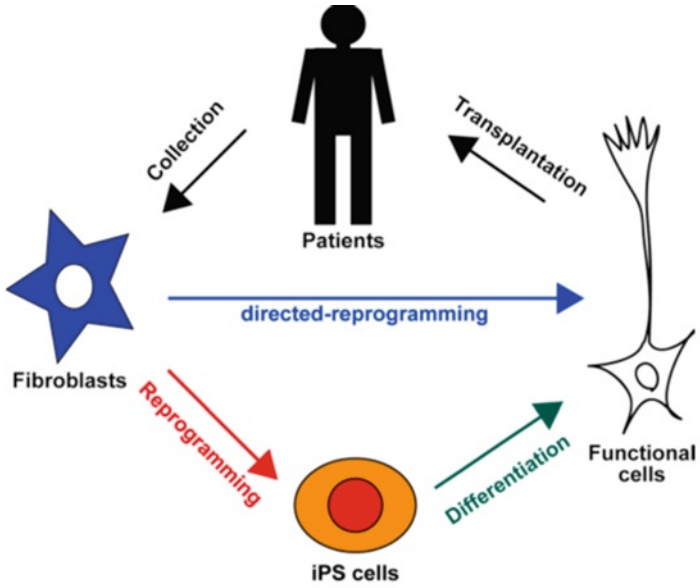
The relation of iPS cell generation and transformation has also become evident by the increased incidence of tumor formation observed in chimeric mice derived from iPS cells (Aoi et al. 2008; Okita et al. 2007). More than 50% of chimeras derived from MEF-derived 4F-iPSCs developed tumors within 1 year after birth. In these tumors, reactivation of the c-Myc retrovirus was detected. In contrast, chimeras derived from iPS cells generated without the c-Myc retrovirus did not show any such increased incidence of tumorigenicity (Nakagawa et al. 2008). Therefore, c-Myc seems to play a major role in the observed tumorigenicity in iPS cell-derived chimeras. We examined chimeric mice derived from L-Myc iPS cell clones, after 2 years. In contrast to c-Myc, the L-Myc retrovirus did not result in any marked increase in either tumorigenicity or mortality (Table 7.1). When compared to chimeric mice derived from Myc-minus iPS cells, L-Myc iPS cells did show a slightly higher incidence of mortality, but not tumorigenicity. This result is consistent with the weak transformation activity of L-Myc.

Although methods of iPS cell induction that do not result in integration of transgenes have been reported (Fusaki et al. 2009; Kim et al. 2009; Okita et al. 2008; Stadtfeld et al. 2008; Woltjen et al. 2009; Yu et al. 2009; Yusa et al. 2009; Zhou et al. 2009; Zhou and Freed 2009), even transient expression of the c-Myc transgene may cause harmful effects on the generated iPS cells. Therefore, the usage of L-Myc should be beneficial for the future clinical applications of iPS cell technologies.

7.4 Generation of iX Cells (X = Any Somatic Cell)

7.4.1 History of Direct Conversion (Reprogramming)

In the reprogramming experiments using frogs and lambs, eggs were utilized, which harbor many proteins and other factors. Since many things are involved in somatic cell reprogramming, it is difficult to explain the mechanism of reprogramming by



Reprogramming	Time for generation	Methods from reprogram to differentiation	Quality of functional cells	Possibility of tumorigenicity after transplant
iPS	Long	Many procedures	Homogenous	Yes ? (contamination of stem cells)
Directed	Short	Easy, simple	Heterogeneous	No ?

Fig. 7.1 Reprogramming technologies for therapeutic application. The functional cells for clinical application can be prepared by two reprogramming technologies, iPS and directed-reprogramming. There are merits and disadvantages associated with both methods

nuclear transfer. Using cell culture systems, treatment with the DNA demethylating agent, 5-azacytidine, converted MEFs to myogenic, adipogenic, and chondrogenic cells (Taylor and Jones 1979) (this type of change is not "reprogramming" but "conversion"). From this result, the conversion of some cells to other types of cells by only a single gene has been reported. The gene is MyoD, master gene for regulation of myogenesis (Davis et al. 1987). Introduction of MyoD alone converted mouse fibroblasts, adipoblasts, or monkey kidney cells to myoblasts. This study suggested that cells could be converted to another lineage of cells only by ectopic expression of inducing factors (Fig. 7.1).

7.4.2 Induced Neuronal (iN) Cells

It has recently emerged that somatic cells may be reprogrammed to pluripotent stem cells in vitro or directly reprogrammed to another somatic cell type in vivo.

After the report of in vivo directed cell reprogramming, induced somatic cells were generated in vitro (Vierbuchen et al. 2010). The authors expressed defined factors in mouse fibroblasts resulting in conversion to neuronal cells. The defined factors were only three, and were classified into neuronal-lineage-specific transcription factors. These cells were named as induced neuronal (iN) cells. In iN cells, neuron specific genes were expressed and functional synapses were also generated. This technique is very useful for production of disease-specific abnormal cells in terms of time taken for generation.

7.4.3 Induced Cardiomyocyte (iCM) Cells

The direct reprogramming of fibroblasts into cardiomyocytes has also been reported recently (Ieda et al. 2010). First cardiomyocyte-inducing factors were sought using fluorescence-activated cell sorting (FACS) screening of concentrated cardiomyocytes. Fourteen factors were identified as primary candidates, and these were systematically refined into only three factors, Gata4, Mef2c, and Tbx5. The induced cardiomyocytes (iCMs) exhibited spontaneous calcium ion flux, electrical activities, and beating. Cardiac fibroblasts, infected with retroviruses to express these three factors, were transplanted into NOD-SCID mouse hearts resulting in "in vivo reprogramming" to cardiomyocytes. Thus, the results indicate that functional cardiomyocytes can be reprogrammed from somatic cells by introduction of defined factors.

7.4.4 Potential of Directly Reprogrammed iX Cells

The directed-reprogramming technology seems to be developing rapidly after the reports of iPS cell generation. We can obtain differentiated somatic cells from fibroblasts by directed-reprogramming in a short period of time compared to iPS cell technology. Moreover, there is no risk of contamination of undifferentiated pluripotent stem cells in the transplantation. This will vastly reduce the risk of tumor formation. However, the characteristics and safety aspects of iX cells must be examined in more detail, before they could be used for clinical application.

7.5 Conclusions

Nuclear reprogramming has been a major dream for many researchers for a very long time. The trials started with the frog, and this study yielded cloned animals and iPS cells. Reprogramming may occur in differentiated cells by activation of key reprogramming factors, however they are normally repressed by methylation of genomic

DNA or chromatin modifications. However, once the reprogramming factors are activated, the cells can change their properties to other cell types (reprogramming or conversion). Thus, reprogramming might be easier than we thought. Researchers need to identify the exact combinations of reprogramming factors to use and will undoubtedly find many combinations for many cell types in the future.

Directing cell reprogramming is also a very promising technique for obtaining disease-specific abnormal cells. For example, it can be envisaged that disease model cells for neurological disorders can be generated from patients skin fibroblasts directly. Further study is required for application of these cells. The iPS cell technology has now become well established by many researchers and competitive studies. Ultimately, human iPS cell technology is likely to have a significant impact on the regenerative medicine possibilities that will become available to the next generation.

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Chapter 8
The Consequences of Reprogramming a Somatic Cell for Mitochondrial DNA Transmission, Inheritance and Replication

Justin C. St. John and Keith H.S. Campbell

Abstract Reprogramming promotes the generation of animals and pluripotent stem cells from a variety of somatic cells, which was not previously possible through natural or in vitro fertilisation. Whilst much of the debate on reprogramming has been related to epigenetic regulation, chromosomal gene expression and the establishment of pluripotency, the regulation and expression of the genes encoded by the mitochondrial genome have been largely ignored. The maternally inherited mitochondrial genome encodes 13 key proteins of the electron transfer chain, which is the cell's major generator of ATP through the biochemical process of oxidative phosphorylation (OXPHOS). OXPHOS is essential for driving a large number of cellular functions. Mitochondrial DNA (mtDNA) rearrangements and depletion can lead to phenotypes that are either severely debilitating or lethal. Following natural fertilisation, mtDNA is maternally inherited and its copy number is strictly regulated during early development and differentiation. This ensures that specialised cells acquire the appropriate numbers of mtDNA to meet their specific requirements for OXPHOS-generated ATP. We discuss how somatic cell nuclear transfer disrupts the strict control of mtDNA replication and how this will affect cellular function in specialised cells. We discuss how the transfer of the somatic cell results in the loss of maternal-only inheritance of mtDNA and the consequences of this. We further highlight the importance of choosing the appropriate recipient oocyte, as the electron transfer chain is highly dependent on chromosomally- and mtDNA-encoded genes and their compatibility is essential to cellular and offspring function and survival.

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8.1 Introduction

ART or assisted reproductive technologies are used in humans and animals to overcome a range of natural barriers to reproduction and provide a means to maintain fertility by preserving germ cells and embryos. In animals, these techniques have allowed the storage and dissemination of specific genetic traits; however, an increasing need for more diverse assisted reproductive technologies has resulted in approaches which require a nucleus from a differentiated cell to be reprogrammed as in the technique of somatic cell nuclear transfer (SCNT) or animal cloning. One of the key problems associated with some assisted reproductive technologies is the maintenance of the tightly regulated transmission and replication of mitochondrial DNA (mtDNA). There is now evidence indicating that the replication, transmission and inheritance of the mitochondrial genome do not occur in a manner similar to that associated with natural fertilisation, i.e. maternal transmission from the mtDNA population present in the metaphase II oocyte. These unregulated events result in the random transmission of either one or two populations of mtDNA. Here, we discuss the transmission of mtDNA and how the degree of reprogramming of the somatic nucleus may influence these outcomes. We specifically relate this to SCNT as this technique has been exploited to a greater extent for both basic and applied research and for animal production.

8.2 The Necessities for Reprogramming the Somatic Nucleus

SCNT involves the transfer of a single somatic nucleus into the cytoplasm of an enucleated recipient oocyte (Campbell et al. 1996) (see Fig. 8.1). The reconstructed oocyte is artificially activated to mimic fertilisation and then transferred to a suitable surrogate recipient for subsequent development, usually following a period of in vitro or in vivo culture. In order to support embryonic and foetal development, the somatic nucleus must be "reprogrammed" by the recipient cytoplasm to recapitulate the temporal and spatial patterns of gene expression required for development. The nature and timing of the mechanisms involved in this so called "reprogramming" event are slowly being elucidated.

Reprogramming does not involve changes in DNA sequence but rather in epigenetic modification of the chromatin to eradicate genetic memory and control gene expression. Epigenetic memory may involve DNA methylation or post-translational modification of histones including methylation, phosphorylation, acetylation or ubiquitination and such modifications can control gene expression and confer cellular specialisation. Consequently, removal of these epigenetic marks not only provides the potential for the naïve cell to express markers of pluripotency but also empowers the cell with the potential to commit to any specific fate by allowing genes specific to one lineage to be expressed and thus suppressing gene expression associated with other lineages.

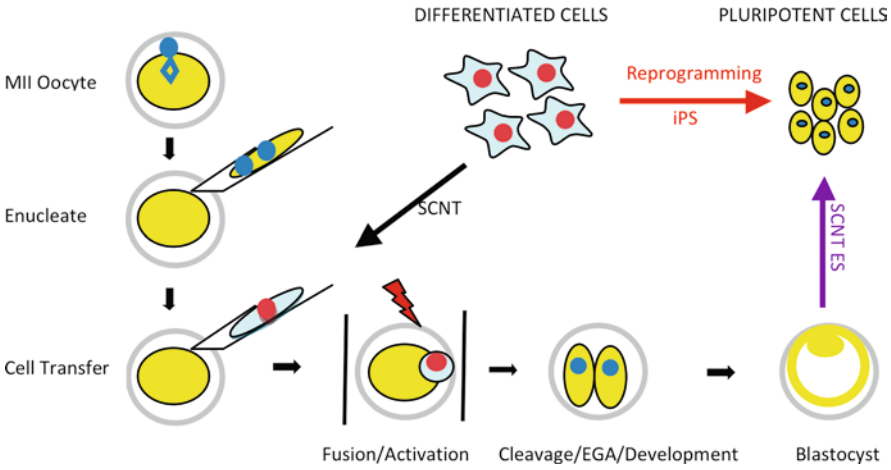


Fig. 8.1 Somatic cell nuclear transfer and the isolation of SCNT ES and iPS cells. In SCNT, a differentiated somatic cell is fused to or injected into an enucleated oocyte or cytoplast. The reconstructed oocyte is then activated and cultured to the blastocyst stage for the isolation of ES cells. iPS cells are generated by the over-expression of a range of factors in differentiated cells and then once colonies of "pluripotent" cells have formed, they are harvested and further cultured

Studies of SCNT embryos have demonstrated that reprogramming of a somatic nucleus is mediated by oocyte cytoplasmic factors prior to activation and continues during the early stages of preimplantation development such that at the blastocyst stage, embryonic stem (ES) cells can be isolated from the pluripotent inner cell mass (ICM) cells (Wakayama et al. 2001). Studies of developing embryos and ES cells have demonstrated that a number of genes are associated with undifferentiated or pluripotent cells for example, Oct-4, Lin28, Nanog, Klf4, c-Myc and Sox2 (reviewed in Chen and Daley 2008). Subsequently, over-expression of some of these genes was demonstrated to directly reprogramme somatic cells to an undifferentiated state, so called induced pluripotent (iPS) cells (Takahashi and Yamanaka 2006) (see Fig. 8.1).

Both iPS cells and SCNT derived ES cells offer significant advantages for research and therapy. First, in both cases they have the potential to provide patient-specific stem cells for treatment purposes. The use of differentiated and/or genetically corrected cells derived from iPS or SCNT ES cells should ensure that the transplanted cells are not rejected because they carry the same HLA-matching as for the patient. Second, disease specific stem cells could be produced from individuals and used for basic research into the molecular and cellular basis of such diseases, and to determine the pharmaceutical approaches to the treatment of disease in a patient-specific manner.

SCNT also has wider implications in the pharmaceutical and the agricultural industries providing not only a method for the asexual multiplication of selected animals, but also a route for their genetic modification. Transgenic offspring can be generated for a range of uses; for instance in the biopharmaceutical industry,

animals can be engineered which produce human proteins in their milk or other bodily fluids i.e. sheep producing human factor IX, a clotting factor used for treatment of haemophilia (Schnieke et al. 1997); in agriculture, transgenesis may be used to confer resistance to disease i.e. cattle resistant to Staphylococcal mastitis have been engineered by expression of a bacterial peptide in mammary tissue (Wall et al. 2005); in addition, production of specific proteins may have industrial applications i.e. the production of spider's silk in the milk of transgenic goats (Williams 2003). Equally so, specific lines or breeds of livestock can be generated which would carry specific genetic traits and the use of SCNT technology allows these traits to be propagated in a much faster fashion as multiple rounds of cross breeding are not necessary; simply the transfer of a somatic cell from one animal into the oocyte of another would suffice.

The technique of SCNT is however, associated with a range of problems. Often, the somatic cell is not completely reprogrammed with a large body of documented evidence indicating that imprinting and other types of epigenetic disorders are associated with abnormal foetal development and reduced survival of offspring (e.g. see Cibelli et al. 2002). Although such outcomes are not always the case, they appear to be restricted to within the first generation. Subsequent generations, following the breeding of cloned offspring with other animals, do not inherit these disorders (Shimozawa et al. 2002; Kasai et al. 2007). The isolation of functional ES cells from SCNT embryos in both mice and monkeys (Wakayama 2003; Wakayama et al. 2001; Byrne et al. 2007) demonstrates that a significant degree of reprogramming occurs early in development. These observations coupled to numerous other studies clearly indicate that the power of the oocyte to reprogramme early on is a key factor, as is also demonstrated following sperm–egg fusion. Indeed, prior to activation of the embryonic genome (EGA), development of fertilised oocytes and early embryos is dependent on maternally inherited mRNAs and proteins present in the oocyte (Braude et al. 1988). Embryos reconstructed by SCNT are highly dependent on these maternal factors to facilitate reprogramming of the somatic chromatin and mediate the processes of DNA replication and cell division during the early cell cycles. In general, SCNT has been carried out using an oocyte from the same species (intraspecific); it is likely that an oocyte from another species (interspecies) will be less efficient in reprogramming a somatic donor nucleus and controlling early division cycles (Chung et al. 2009). The ability of the oocyte cytoplasm to demethylate DNA varies between species, and in interspecies SCNT (iSCNT) embryos demethylation may not occur, for instance in pig to rabbit SCNT (Chen et al. 2006). Furthermore, many studies of inter-specific SCNT report developmental arrest around the time of EGA, while microarray analysis of gene expression in inter-specific human-cow SCNT embryos demonstrate that EGA does not occur (Li et al. 2008). Nevertheless, the completion of this process for those embryos that do successfully develop to blastocyst is highly likely to be affected by low levels of oxidative phosphorylation (OXPHOS) – derived ATP, especially as the later stages of preimplantation development are OXPHOS – rather than glycolysis-dependent due to the genetic incompatibility of the proteins with the electron transfer chain (ETC) (Bowles et al. 2007a). A further major problem associated with these technologies is the regulation of mtDNA transmission and replication

very early on during development, which can have a wide impact on the functional capability of those stem cells derived or offspring generated.

8.3 What Is MtDNA?

MtDNA is located within the inner membrane of the mitochondrion, and is present in nearly all eukaryotic cells. It encodes 13 of the 70+ subunits of the ETC (Anderson et al. 1981; Fig. 8.2), the intracellular apparatus that generates the vast majority of cellular ATP through the process of OXPHOS (Fig. 8.3). In this respect, OXPHOS contributes 32 molecules of ATP to every 2 produced by glycolysis (Pfieffer et al. 2001). Unlike any other cellular apparatus, the ETC is dependent on proteins encoded by both the mitochondrial and nuclear genomes. Characteristically, the size of the mitochondrial genome varies between and within a species. For example, in the pig, it is approximately 16.7 kb in size (Ursing and Arnason 1998) while in human the mtDNA genome is 16.6 kb (Anderson et al. 1981) and in mouse 16.3 kb (Bibb et al. 1981). This genome also incorporates 22 tRNAs, 2 rRNAs and one non-coding region, the D-Loop (Fig. 8.2). These differences result in sequence variations between species within the coding and non-coding regions (Mitomap 2011). There are also

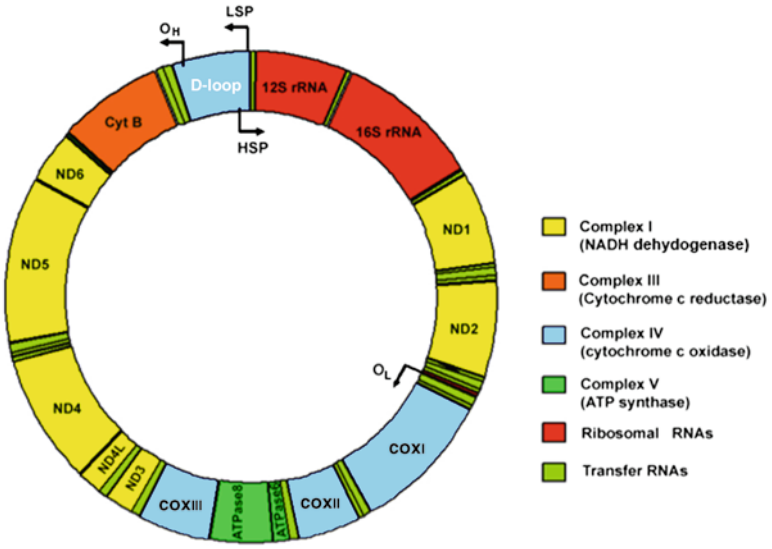
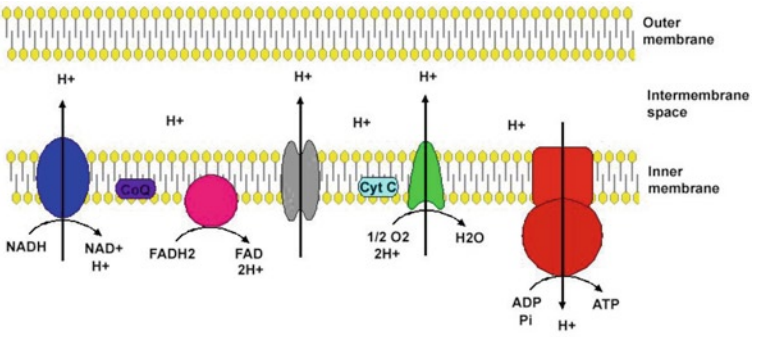


Fig. 8.2 The mitochondrial DNA genome. mtDNA encodes: ND 1–6 and ND4L of complex I; CytB of complex III; COX I to III of complex IV; and ATPase 6 and ATPase 8 of complex V of the ETC. It also encodes 2 rRNAs (12S and 16S rRNAs) and 22 tRNAs. The D-loop comprises the H-strand promoter region (HSP), the L-strand promoter region (LSP), and the origin of H-strand replication (O_H). A second regulatory region, located between ND2 and COXI, is the site of the origin of L-strand replication (O_L)



Complex	I	II	III	IV	V
nDNA subunits	38	4	10	10	14
mtDNA subunits	7	0	1	3	2

Fig. 8.3 The electron transfer chain (ETC). The ETC comprises nuclear DNA and mtDNA-encoded subunits. Electrons flow through the first four complexes and the energy released is used by complexes I, III and IV to generate an electrochemical gradient which mediates the production of ATP through complex V. Complex I (ND); coenzyme Q (CoQ); complex II (SD); complex III (cytochrome c reductase); cytochrome C (CytC); complex IV (COX); complex V (ATPase)

differences between and within breeds and strains of a species (Mitomap 2011). The differences within a species define maternal lineage and can also confer specific haplotypes that are often regional (Ruiz-Pesini et al. 2000; Montiel-Sosa et al. 2006) but also specific to certain genetic traits, such as fertility and meat quality within, for example, the livestock industry (reviewed in Bowles et al. 2007a).

The correct expression of genes from both mitochondrial and nuclear genomes is essential to cellular function and to the wellbeing of the offspring. To this extent, a reduction of mtDNA copy number, or mutation or deletion of the mtDNA within a cell type, tissue or organ can be severely debilitating or even lethal (Wallace 1999). Diseases related to mtDNA-depletion syndrome, such as infantile mitochondrial myopathy (Poulton et al. 1994), familial mtDNA-associated liver disease (Spelbrink et al. 1998), fatal childhood myopathy (Larsson et al. 1994), skeletal muscle and mitochondrial encephalomyopathy disorders (Siciliano et al. 2000) and ocular myopathy, exercise intolerance and muscle wasting (Siciliano et al. 2000), tend to be driven by decreased expression or mutation to chromosomally-encoded genes that restrict the transcription and/or replication of the mitochondrial genome. Diseases associated with the mtDNA genome tend to be organ- or tissue-type specific largely due to the random patterns of mtDNA segregation that occur during embryonic development (Shoubridge and Wai 2007). They also tend to be related to tissues that are high-ATP users, such as neuronal and muscle tissue (McFarland et al. 2007). The onset of mtDNA disease is also dependent on the degree of mutant/deletion load. For example, in myoclonic epilepsy and ragged red fibres (MERRF), a cell or

tissue would characteristically harbour 85% mutant load (Boulet et al. 1992) while others, such as Leber's Hereditary Optic Neuropathy (LHON), require approximately 60% mutant loading (Chinnery et al. 2001).

8.4 Why Is the Regulation of MtDNA Transmission Important?

In order to ensure maximum efficiency for ATP production, the mammalian embryo has evolved mechanisms for regulating the transmission of mtDNA. In those offspring generated from oocytes fertilised with sperm from the same breed or strain, i.e. intraspecific crosses, mtDNA is inherited through the population present in the oocyte, as sperm mtDNA is normally eliminated just after fertilisation through a developmentally regulated, ubiquitin-mediated process that first labels the mitochondrion protein of spermatogonia with ubiquitin. This label persists throughout spermatogenesis and spermiogenesis for the subsequent targeted disruption of sperm mitochondria in the oocyte (Sutovsky et al. 1999). However, this outcome does not apply to embryos and subsequent offspring generated through sperm and oocytes from different strains and breeds, i.e. inter-specific crosses, thus resulting in the transmission of sperm mtDNA (Sutovsky et al. 1999). Consequently, the resident population of mtDNA tends to be identical, i.e. homoplasmic, following intraspecific crossing but potentially heteroplasmic in cases of inter-specific crossings.

The maternal and homoplasmic transmission of mtDNA from one generation to the next is further maintained by ensuring that the mtDNA content present in the oocyte just prior to fertilisation is diluted as each newly divided blastomere is formed. This is mediated by the nuclear-encoded mtDNA-specific replication factors, such as DNA Polymerase Gamma (Polg) and mitochondrial transcription factor A (Tfam) not being expressed prior to the blastocyst stage (Bowles et al. 2007b; Spikings et al. 2007). Indeed, in larger mammals, during preimplantation development between the four cell and morula stage, there is a considerable reduction in mtDNA copy number and these numbers are only replenished at the blastocyst stage, when up-regulation is required to match the embryo's increased need for OXPHOS-derived ATP (Houghton et al. 1996). This increase in mtDNA copy number is most likely confined to the outer, trophectodermal cells (Houghton et al. 1996; Spikings et al. 2007), which also appear to have increased mitochondrial density. This is in contrast to their ICM counterparts that appear to have fewer mitochondria and mtDNA copies and reduced POLG expression and are thus more likely to still function through glycolytic metabolism (Houghton et al. 1996; Spikings et al. 2007).

This continual dilution of mtDNA ensures that low numbers of mtDNA copies are present as early post-implantation cells are given the cue to differentiate into specific cell lineages. Consequently, primordial germ cells (PGCs), which are first laid down just after gastrulation at E6.5 in the mouse, possess approximately 200 copies

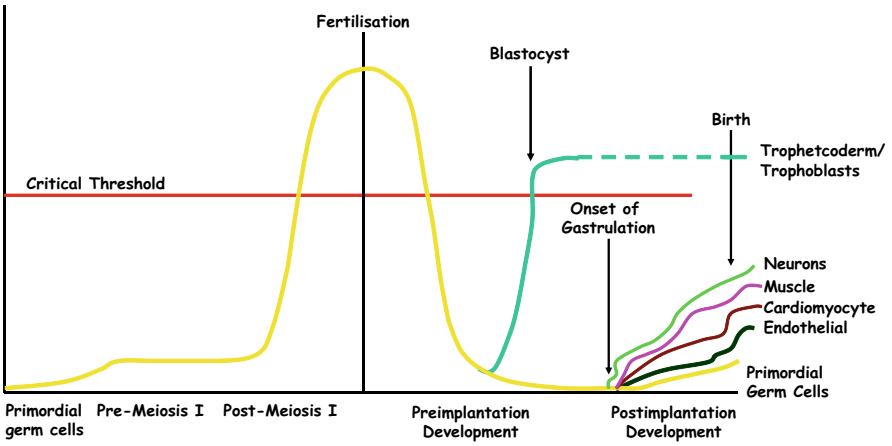


Fig. 8.4 Cyclical replication of the mtDNA genome. mtDNA copy number is cell type specific and is specific to the energy requirements of individual cell types. PGCs possess very few mtDNA molecules as they have been "filtered" by the mitochondrial genetic bottleneck (Hauswirth and Laipis 1982) and then increase their copy number as they mature during oogenesis and reach or exceed a specific number (critical threshold) for fertilisation to take place. Oocytes falling below this threshold will be developmentally incompetent and will not fertilise (Spikings et al. 2007; *red line*). MtDNA replication is inactive during preimplantation development and, along with a large number of mtDNA molecules being lost in larger mammals, the large number of cell divisions results in very few mtDNA copies being present, thus establishing the mtDNA "set point," in each cell of the inner cell mass (ICM), the cells that give rise to ESCs or early foetal cells. As cells commit to specific lineages, post-implantation and following the loss of pluripotent gene expression, they will adopt the required numbers of mtDNA molecules to support their requirements for OXPHOS-derived ATP. Trophectodermal cells, however, increase their mtDNA content in the pre-implantation blastocyst, so that they can support the metabolic requirements of implantation. This cyclic behaviour passes from one generation to the next as the PGCs are laid in the next female embryo to ensure, as far as possible, the homoplasmic, maternal-only transmission of mtDNA

(Wai et al. 2008). These genomes are then clonally amplified during oogenesis (Smith and Alcivar 1993) resulting in mature fertilizable oocytes possessing more than 200,000 copies (Spikings et al. 2007; Almeida-Santos et al. 2006; Fig. 8.4). As with somatic cell function, growth, maturation and subsequent development of the fertilizable oocyte is also associated with mtDNA copy number as those oocytes with significantly fewer copies tend not to fertilise or the resultant embryo arrests within the first two divisions. This is probably due to insufficient mitochondria being present to fuel the early stages of preimplantation development that would be dependent on OXPHOS rather than glycolysis (Houghton et al. 1996).

In those early pluripotent post-implantation cells that give rise to somatic cells and gametes, mtDNA replication appears to be associated with replenishment of mtDNA copy following cellular division rather than increased mtDNA copy number (Facucho-Oliveira et al. 2007). Based on evidence from knockout Polg (Hance et al. 2005) and Tfam (Larsson et al. 1994) mice, it appears that mtDNA replication needs to be initiated either prior to E8.5 ($Polg^{-/-}$) or E10.5 ($Tfam^{-/-}$).

Failure to do so results in embryonic death, while the heterozygous knockouts exhibit mtDNA-depletion syndromes. Murine ESCs appear to regulate mtDNA replication in a similar manner where continued pluripotent gene expression is associated with mtDNA turnover as opposed to increased copy number. In this respect, undifferentiated ESCs would possess between 30 and 50 copies of the mtDNA genome (Facucho-Oliveira et al. 2007; Facucho-Oliveira and St John 2009), which establishes the mtDNA "set point" (see Fig. 8.4) (St John et al. 2010) and this pattern continues until commitment to differentiation has been clearly established (Facucho-Oliveira et al. 2007; Facucho-Oliveira and St John 2009). At this point, cells will adopt specific numbers of mtDNA copies to ensure that they have sufficient molecules present to support the cellular requirements for OXPHOS-derived ATP (Fig. 8.4).

8.5 Are Similar Patterns of MtDNA Transmission Observed Following SCNT?

The process of SCNT involves the transfer of a nucleus and part or all of the somatic cytoplasm into the recipient oocyte creating a so called cybrid (cytoplasmic hybrid) containing mtDNA from donor and recipient cells. During subsequent development, the donor mtDNA is either eliminated, resulting in homoplasmic transmission of recipient oocyte mtDNA, or persists resulting in heteroplasmy, i.e. donor cell and recipient oocyte mtDNA. A number of studies have demonstrated that the transmission of donor cell mtDNA following SCNT is random with contributions to preimplantation embryos ranging from 0 to 63% (Meirelles et al. 2001) and similarly from 0 to 59% to live offspring (Takeda et al. 2003). This vast range in levels of transmission tends to be independent of whether intra- or inter-specific SCNT is used to generate embryos and offspring (St John et al. 2004). For example, donor cell mtDNA has been detected in bovine embryos derived by both intra- (Do et al. 2002) and inter-specific NT (Meirelles et al. 2001) though not in all cases (Meirelles et al. 2001; Takeda et al. 2003); and in caprine embryos (Jiang et al. 2004) and porcine offspring (Takeda et al. 2006) derived by inter-specific SCNT. This variable rate of donor cell mtDNA transmission appears to be independent of the original contribution of mtDNA to the SCNT process. In order to understand why this persists at levels inconsistent with its original contribution, we generated intra- and inter-specific SCNT ovine embryos using donor cells depleted to 0.01% (residual – $mtDNA^R$) and 0.1% of their original mtDNA content; and age-matched non-depleted ($mtDNA^+$) cells (Lloyd et al. 2006). In each combination, donor cell mtDNA persisted to the hatched blastocyst. We demonstrated that the persistence of donor cell mtDNA is related to the continued expression of the mtDNA-specific replication factors, *Polg,* both its catalytic (*PolgA*) and accessory (*PolgB*) subunits, and *Tfam* (Bowles et al. 2007b), during preimplantation development, which is in stark contrast to their in vitro fertilised counterparts. This arises as the donor cells also express these factors prior to SCNT and thus carry these factors over into the

oocyte as SCNT is performed and continue to transcribe these factors, indicating incomplete reprogramming of the somatic genome. Consequently, there is the potential for the donor cell to rescue its mtDNA population during the early stages of preimplantation development, especially when donor cell mtDNA is in close proximity to its host nucleus, thus ensuring its preferential replication.

Similarly, in iSCNT embryos, donor cell mtDNA has been detected in preimplantation embryos. In human-bovine cybrids, it has been demonstrated to be present up to the 16-cell stage (Chang et al. 2003) and in a small minority of caprine-ovine embryos (4/40; range = 2–20+ cell embryos; Bowles et al. 2007b), while it is present at the blastocyst stage in macaque-rabbit fusions (Yang et al. 2003). Again, in this small number of heteroplasmic caprine-ovine iSCNT embryos, we observed significant increases in donor cell mtDNA reinforcing the concept that occasionally in iSCNT embryos selective replication does take place, mediated through the continued expression of mtDNA-specific replication factors during preimplantation development (Bowles et al. 2007b). However, a clearer generalisation, based on the human-bovine (Chang et al. 2003) and caprine-ovine crosses (Bowles et al. 2007b), is that donor cell mtDNA tends to be eliminated as embryos divide through preimplantation development. Consequently, blastocysts will most likely harbour very few, if any, copies of donor cell mtDNA resulting in any subsequent ES cells derived from these embryos being "true" cybrids, i.e. possessing chromosomal genetic material from one source and mtDNA from another.

8.6 The Consequences of Failing to Regulate Nucleo-Mitochondrial Compatibility

As both nuclear and mtDNA-encoded genes contribute to the ETC, cellular function is highly dependent on a high degree of compatibility between these two genomes. We would predict that large-scale genetic diversity between the nuclear- and mtDNA-encoded genes of the ETC would contribute to the high levels of failure to develop to the blastocyst stage due to a dysfunctional ETC that will generate insufficient levels of ATP. This is especially apparent as, although many interspecies embryos do not develop past the EGA, those that do often fail to persist to blastocyst when they depend on ATP generated through OXPHOS rather than glycolysis. Indeed, once the interspecies embryo has completed EGA, and reached the morula stage, the nuclear-encoded subunits of the ETC will have been synthesised in readiness to assemble ETCs with those subunits encoded by the mtDNA located within the recipient oocyte. As the embryo develops towards blastocyst, it will become increasingly dependent on ATP generated through OXPHOS in the ETC rather than glycolysis (Houghton 2006) and, depending on the compatibility of these factors, this may well result in insufficient ATP being generated. Indeed, interspecies somatic cell cybrids demonstrate how developing embryonic cells are likely to be affected. For example, the fusion of a mouse karyoplast to a rat cytoplast does not affect replication, transcription or translation of rat mtDNA by murine nuclear-encoded factors.

However, OXPHOS function is compromised which results in decreased levels of ATP output, which, in turn, affects cellular function (McKenzie and Trounce 2000). Similar outcomes have been observed in cybrids from human and other closely related primates (Barrientos et al. 1998). The degree of loss to cellular function will, however, be more detrimental to those cells with high demands for OXPHOS-derived ATP, such as neurons, muscles and cardiomyocytes. Consequently, any resultant ESC line harbouring a mutation related to a specific disorder that can be derived through iSCNT will have OXPHOS deficiencies and function inefficiently. If the intent is to derive ESC models of disease using iSCNT, then poorly matched nuclear and mtDNA-encoded genes of the ETC would introduce a second experimental variable into the model that may allow false conclusions to be drawn (Bowles et al. 2007a). For example, the outcomes of a study related to a drug that would modulate Ca^{2+} in synaptic function in genetically impaired neurons will be biased if those ESCs derived from iSCNT are not able to support themselves through OXPHOS and are dependent on ATP derived from glycolysis. Consequently, the pharmacokinetics of the drug could not be truly determined.

8.7 What Are the Consequences of Introducing Somatic Mitochondria and Donor Cell MtDNA into Oocytes?

Mixing of mtDNA genomes, namely from the donor cell and recipient oocyte, can be detrimental to embryonic development. Although it has been argued that the use of cytoplasmic transfer, i.e. the transfer of mitochondria from an oocyte of a younger donor to an oocyte from an older woman with repeated fertilisation failure, improves embryonic outcome and gives rise to live offspring (Cohen et al. 1997), severe developmental abnormalities have been associated with this approach. For example, one offspring was diagnosed with pervasive development disorder, while one selective and one natural abortion took place due to the foetuses harbouring XO-syndrome (Barritt et al. 2001).

In addition, somatic mitochondria appear to adversely affect embryonic development. For example, development rates to blastocyst of parthenogenetically activated oocytes supplemented with somatic mitochondria were lower in comparison to non-supplemented controls and those supplemented with oocyte cytoplasm (Takeda et al. 2005). This suggests that somatic, differentiated mitochondria do not have the potential to dedifferentiate into embryonic type mitochondria. Indeed, the elimination of donor cell mtDNA enhances embryonic development. To this extent, ovine SCNT blastocysts generated using $mtDNA^R$ primary foetal fibroblasts as nuclear donors had significantly higher cell numbers as compared to control $mtDNA^+$ blastocysts (Lloyd et al. 2006). Furthermore, in caprine-ovine iSCNT embryos, significantly more $mtDNA^R$ embryos reach >20-cell stage of preimplantation development than $mtDNA^+$ embryos (Bowles et al. 2007b).

This effect is further exacerbated when different breeds and strains are used in, for example, cattle and pigs that have sequence variations in the coding genes. In

inter-specific bovine (Steinborn et al. 2002) and porcine (St John et al. 2005) SCNT offspring, variations in the mtDNA sequences of the fusion partners can result in potential differing amino acid synthesis within one offspring. Consequently, the degree of conformational change induced by these two mtDNA genomes in the subunits of the ETC will determine their suitability for ATP production, and is most likely a function of the genetic distance between the two sources of mtDNA. As many offspring do survive with heteroplasmy, compensatory mechanisms may exist where the mtDNA heteroplasmy may be reversed at the transcriptional level so that mtRNA may be representative of only one population of mtDNA in high-ATP using tissues such as lung. Such an outcome was observed in foetal tissue generated through hand-made cloning, a derivation of SCNT, where multiple cytoplasts were fused to a single somatic cell (Bowles et al. 2008). We have now circumvented this problem by generating homoplasmic sheep by using somatic cells specifically depleted of their mtDNA and demonstrated that the offspring were homoplasmic for recipient oocyte mtDNA only thus inheriting their mtDNA in a manner similar to offspring derived through fertilisation (Lee et al. 2010).

8.8 Is There an Association Between the Genetic Distance of Donor Cell MtDNA and Recipient Oocyte MtDNA?

A comparison of the effects of mtDNA haplotype between two breeds of cattle demonstrated that one mtDNA lineage produced a greater number of blastocysts following SCNT (Bruggerhoff et al. 2002) while the other had significantly improved developmental competence following IVF (Tamassia et al. 2004). We have sought to clarify the relationship between donor cell and recipient oocyte mtDNA through hand-made cloning. In this instance, the donor cell appears to favour a slightly genetically more diverse mtDNA haplotype to that of its own, especially in those embryos that progress to term and survive, and thus possess fully differentiated and functional cells (Bowles et al. 2008). Furthermore, we observed that divergence up to 0.04% was tolerable for development to the blastocyst stage in ovine SCNT embryos, but again no development was recorded for very closely related fusions. However, by increasing genetic distance tenfold to generate caprine-ovine-SCNT embryos, development to blastocyst was not achieved (Bowles et al. 2007b).

8.9 Conclusions

Although the vast majority of scientific endeavour associated with reprogramming concentrates on epigenetic regulation of the nuclear genome and activation of pluripotency, mtDNA outcomes need to be considered in order that the pluripotent cells produced by this route acquire very low numbers of the mtDNA genome so that they can adopt the appropriate complement as they differentiate into specialised cells. This will ensure that such specialised cells will be able to match their specific requirements

for ATP production, especially those cells dependent on OXPHOS-produced ATP. In ESCs derived from reconstructed oocytes, it is essential to eliminate the toxic somatic mitochondria accompanying the donor cell and to ensure that homoplasmic transmission takes place. Furthermore, somatic mitochondria may lack sufficient plasticity, and consequently this could be disadvantageous for somatic cells induced to dedifferentiate in a non-oocyte environment, such as for iPSCs or somatic cell-ESC fusions.

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Chapter 9
The Function of Nanog in Pluripotency

Nicholas Mullin and Ian Chambers

Abstract The transcription factors Oct4, Sox2 and Nanog form a triumvirate of regulatory proteins governing efficient maintenance of pluripotent cell identity. However, in contrast to Oct4 and Sox2, whose protein levels are relatively constant in undifferentiated mouse ES cells, Nanog levels fluctuate widely. Here, we discuss the effect of altering the dose of Nanog on self-renewal efficiency. The key role of Nanog as a self-renewal rheostat and the fact that fluctuations in Nanog level allow entry of Nanog-low cells into a differentiation-prone state are presented in relation to interactions of Nanog not only with itself but also with its partner proteins.

9.1 Introduction

A current view of the network of transcription factors responsible for the maintenance of pluripotency places the triumvirate of Nanog, Oct4 and Sox2 at centre stage (Boyer et al. 2005; Loh et al. 2006). Oct4 and Sox2 act together co-operatively to bind DNA at composite Oct-Sox sites (Yuan et al. 1995; Ambrosetti et al. 1997, 2000; Niwa 2001). Interactions between side chains on both the Sox protein and the Oct protein stabilize binding of the binary complex to DNA (Williams et al. 2004). In addition, chromatin immunoprecipitation studies (reviewed by Chambers and Tomlinson (2009)) have revealed that Nanog binds in close proximity to Oct4 and

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Sox2 binding sites throughout the mouse and human ES cell genomes (Boyer et al. 2005; Loh et al. 2006). The co-localized binding of additional pluripotency transcription factors to such sites suggests the combinatorial action of these factors in transcriptional control (Chen et al. 2008; Kim et al. 2008; Chambers and Tomlinson 2009).

The level of Oct4 protein in ES cells is critical; elevation above 150% of the wild-type level results in differentiation into cells expressing markers of endoderm and mesoderm while reduction below 50% of the wild-type level results in trophectoderm differentiation (Niwa et al. 2000). Studies with inducible Sox2 null cells demonstrate that the importance of Sox2 in self-renewal is not simply in global binding of composite Oct/Sox enhancers but rather in controlling the expression of transcription factors such as Nr2f2 and Nr5a2 that regulate Oct4 expression. Indeed, pluripotency can be rescued in *Sox2*$^{-/-}$ ES cells by over-expression of Oct4 (Masui et al. 2007). The reader is referred to Chap. 10 for further discussion on Sox2.

Nanog was initially identified as a key regulator of pluripotency due to its ability, when over-expressed in mouse ES cells, to confer LIF-independent self-renewal (Chambers et al. 2003; Mitsui et al. 2003). Nanog also plays a role in reprogramming somatic cells to pluripotency. Experiments in which cell hybrids are formed between ES cells and somatic cells demonstrated that increasing the expression of Nanog in either of the fusion partner cells increased the efficiency with which the differentiated nucleus becomes reprogrammed to a pluripotent state (Silva et al. 2006). A fivefold increase in the expression of Nanog in ES cells (Yates and Chambers 2005) resulted in an approximately 200-fold increase in the yield of reprogrammed hybrid colonies (Silva et al. 2006). Subsequent experiments have shown that infection of somatic cells with a mixture of recombinant retroviruses encoding Oct4, Sox2, Klf4 and c-myc is sufficient to reprogramme somatic cells to an induced pluripotent stem (iPS) cell state (Takahashi and Yamanaka 2006; Okita et al. 2008). These pioneering studies have been confirmed by several other labs who have expanded upon the initial discoveries; iPS cells are the subject of recent reviews (Yamanaka 2008; Hochedlinger and Plath 2009) and are further discussed throughout this book. Subsequent analyses have reported that Nanog can act to reprogramme human somatic cells when used in combination with Oct4, Sox2 and lin28 (Yu et al. 2007). The initial observation that Nanog was not one of the exogenous proteins required to reprogramme fibroblasts to pluripotency was surprising. However, the fact that Nanog does not necessarily act as one of the initiating factors in induced pluripotency does not mean that Nanog has no role in this process. Indeed, partially reprogrammed cells lack Nanog expression (Sridharan and Plath 2008). Analysis of the reprogramming capacity of *Nanog*$^{-/-}$ cells indicates that in the absence of Nanog, somatic cells can reach a partially reprogrammed state. However, in order to progress to a fully reprogrammed pluripotent state in which cells can form germ-line competent chimaeras following aggregation with host blastocysts, Nanog needs to be re-introduced to the *Nanog*$^{-/-}$ somatic cells (Silva et al. 2009).

9.2 The Role of Nanog in Development

The importance of Oct4, Sox2 and Nanog is demonstrated by the phenotype of the corresponding gene knock outs. For both Oct4 and Nanog, deletion results in pre-implantation lethality (Nichols et al. 1998; Mitsui et al. 2003; Silva et al. 2009). *Sox2*$^{-/-}$ embryos die at the peri-implantation stage, with survival, until this point, suggested to result from long-lived maternal stores of Sox2 transcript (Avilion et al. 2003).

Nanog expression is first detectable in morulae with levels remaining high in early-mid stage blastocysts. At E3.5, most, if not all, ICM cells express Nanog. One day later, the ICM is composed of a salt-and-pepper distribution of Nanog-expressing cells intermingled with cells expressing GATA factors (Chazaud et al. 2006; Dietrich and Hiiragi 2007; Silva et al. 2009). Development of *Nanog*$^{-/-}$ embryos fails at this stage (Mitsui et al. 2003); *Nanog*$^{-/-}$ ICMs do not re-activate the inactive paternal X-chromosome and do not generate pluripotent cells (Silva et al. 2009). This requirement for Nanog in specification of pluripotency in vivo is entirely in line with the necessity of Nanog during reprogramming of somatic cells to a fully pluripotent state (Silva et al. 2009). The failure to re-activate the inactive X-chromosome is of interest in relationship to studies in ES cells showing that repression of the cis-regulator of X-chromosome inactivation, Xist is mediated by Oct4, Sox2 and Nanog (Navarro et al. 2008). In *Nanog*$^{-/-}$ ES cells this repression is only partially alleviated, due to continued Oct4 expression (Navarro et al. 2008). This may indicate a difference between initiation and maintenance of Xist repression. Following implantation, Nanog expression is restricted to the epiblast with a gradient of expression such that highest levels are present proximally and posteriorly (Hart et al. 2004). Pluripotent epiblast stem cell lines (EpiSCs) have been established from the post-implantation epiblast and these cells express an overlapping set of transcription factors, including Oct4, Sox2 and Nanog, compared to ES cells (Brons et al. 2007; Tesar et al. 2007). EpiSCs depend upon FGF2 and activin for maintenance in vitro. Expression of Nanog in response to activin has led to the suggestion that Nanog is the critical downstream mediator of activin action in EpiSCs (Brons et al. 2007; Vallier et al. 2009). It will therefore be interesting to determine whether deletion of Nanog from EpiSCs causes their differentiation or death. Expression of Nanog is rapidly eliminated as epiblast cells delaminate and enter the primitive streak. However, to date there is no evidence that Nanog expression is important here. The fact that Nanog-null cells can contribute differentiated derivatives to each of the three primary germ layers (Chambers et al. 2007) suggests that Nanog may either have no unique role at this point in development or that Nanog-null cells can be rescued by the surrounding wild-type cells.

Nanog is also expressed in primordial germ cells (PGCs) (Yamaguchi et al. 2005). During PGC development, large-scale epigenetic reprogramming events occur including DNA demethylation and erasure of imprinting. The initial step in this process is the establishment of a chromatin state similar to that of ES cells (Hajkova et al. 2002, 2008), and is associated with the expression of pluripotency

genes including Nanog, Oct4 and Sox2. To investigate whether Nanog plays a role in PGC development, the behaviour of cells in embryonic chimaeras formed by Nanog-null cells and wild-type cells was examined. Nanog-null cells can enter the PGC development programme and migrate to the genital ridges where they can be detected at E11.5 as Oct4-expressing cells with weak but detectable expression of Mvh. However, Nanog-null PGCs can no longer be detected 1 day later at E12.5. That this phenotype was due to loss of Nanog was established by reparative homologous recombination of *Nanog* and the demonstration that the resulting cells could contribute to the Mvh expressing population at E12.5 (Chambers et al. 2007). Subsequently, an inducible RNA knock-down study reported an increase in apoptosis in PGCs showing reduced Nanog expression (Yamaguchi et al. 2009).

9.3 Nanog Acts a Rheostat

Oct4 and Sox2 both show a relatively uniform pattern of staining by immunofluorescence in ES cell cultures. In contrast, immunofluorescent staining of ES cells demonstrates a mosaic pattern of Nanog expression (Chambers et al. 2007) reminiscent of the salt-and-pepper expression of Nanog in Oct4-expressing cells of the pre-implantation epiblast (Dietrich and Hiiragi 2007). Indeed, in a fraction of undifferentiated Oct4-positive cells, Nanog is undetectable by antibody staining (Chambers et al. 2007). To investigate the significance of these differences in levels of Nanog, cell lines were generated that were heterozygous or null for Nanog (Chambers et al. 2007). Comparison of the self-renewal capacity of these cell lines together with the parental lines demonstrated that the capacity to form colonies composed entirely of undifferentiated cells is directly correlated to Nanog dosage. Thus, a step-wise drop was observed in the number of self-renewing colonies formed at clonal density as the Nanog alleles were deleted. This reduction in self-renewal capacity is consistent with other studies that demonstrate diminished self-renewal upon deletion or knock-down of Nanog (Mitsui et al. 2003; Hatano et al. 2005; Ivanova et al. 2006). However, *Nanog*$^{-/-}$ ES cells retain undifferentiated colony forming capacity and can be maintained in culture for many months. Daily medium changes help maintain the undifferentiated state, reflecting the propensity of null cells to differentiate (Chambers et al. 2007). Subsequent morula aggregation assays indicated that these Nanog-null cells were able to contribute to all three primary germ layers. Thus, these experiments clearly demonstrate that Nanog is not essential in order to maintain pluripotency in ES cells. These results were unexpected given the requirement for Nanog, like Oct4, for specification of pluripotent cells during pre-implantation development. This could indicate a different requirement for Nanog during specification rather than maintenance of pluripotency. Alternatively, it may reflect an inability to shield Nanog-null cells in the embryo from differentiation or apoptotic signals.

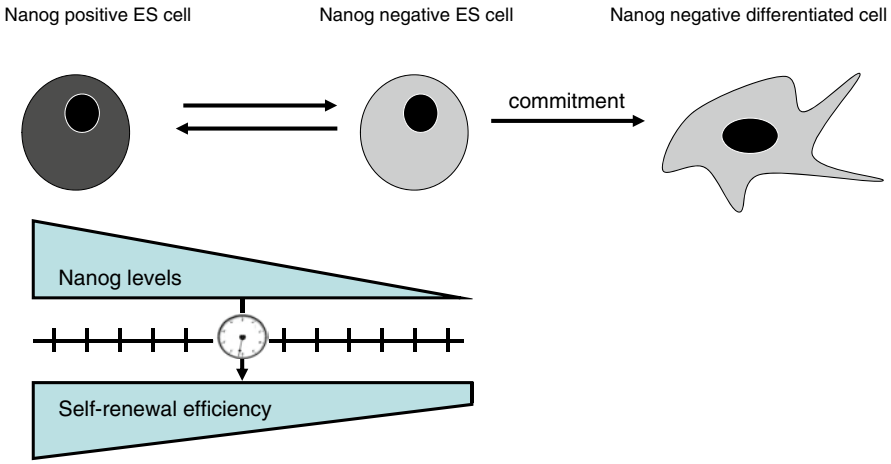

Fig. 9.1 Model of relationship between Nanog levels and resistance to differentiation. Cells expressing high levels of Nanog are resistant to differentiation cues. Low levels of Nanog are correlated with the ability of cells to respond to uncharacterized commitment cues and progress towards a differentiated cell type

Further insight into the role of Nanog with respect to self-renewal was obtained by visualizing mosaic expression of Nanog using a GFP reporter knocked into the *Nanog* locus in ES cells (Chambers et al. 2007). Experiments showed that ES cells exist predominantly in a Nanog-high or Nanog-low state with a range of cells with intermediate Nanog levels. Furthermore, the situation is dynamic as single cells in a Nanog-low state regain high Nanog expression when re-plated in culture (Chambers et al. 2007). The importance of the level of Nanog is demonstrated by the fact that these Nanog-low cells are more prone to differentiation. When *Nanog:GFP* cells are sorted into GFP negative and GFP positive populations and placed back in culture, the GFP negative cells lose expression of SSEA1, a surface marker of undifferentiated ES cells, faster than GFP positive cells (Chambers et al. 2007).

Together, these observations led to the hypothesis that Nanog acts as a rheostat. In this model, the level of Nanog expression dials in the self-renewal efficiency (Fig. 9.1). By fluctuating between high and low levels of Nanog, ES cells move between a state in which they are resistant to differentiation and one in which they are responsive to differentiation cues. Thus, the variable expression level of Nanog may directly correspond to a variable resistance to differentiation. In the absence of differentiation cues, re-expression of Nanog returns Nanog-low cells to the differentiation resistant state. In this way Nanog stands apart from Oct4 and Sox2, levels of which do not fluctuate widely. Moreover, the indispensability of Oct4 and Sox2 indicates that they have housekeeping functions in the maintenance of pluripotency. In contrast, Nanog acts to modulate self-renewal efficiency. In the absence of Nanog, ES cells become primed to receive differentiation cues but do not cross the Rubicon of commitment.

9.4 Nanog Dimerization: Implications for DNA Binding

While the above analyses have informed us about the role of Nanog, detailed biophysical techniques are required to provide a molecular understanding of how Nanog functions. The development of a recombinant system for Nanog expression has opened up such avenues of investigation. Analytical ultracentrifugation showed that, in solution, Nanog exists as a dimer with a K_d for self-interaction lying in the low-micromolar range (Mullin et al. 2008). Co-immunoprecipitation experiments suggested that the region involved in Nanog dimerization was one containing ten repeats of a pentapeptide motif in which tryptophan is conserved at the same position within each repeat (the tryptophan repeat or WR). That the WR alone was sufficient for interaction with Nanog was demonstrated by direct binding between a purified maltose binding protein-WR fusion and purified Nanog. Further experiments in which the dimerizing tryptophan repeat region was deleted demonstrated that the ability to dimerize is essential in order to confer cytokine-independent self-renewal upon transfected ES cells (Mullin et al. 2008). These results were subsequently confirmed using an approach in which constructs consisting of two Nanog molecules joined by a flexible linker ("tandem dimers") were expressed (Wang et al. 2008a). In this situation, intra-molecular interactions are favoured and forced dimers predominate. When expressed in ES cells the forced dimer supported cytokine-independent self-renewal to a greater extent than a tandem dimer in which the second tryptophan repeat was removed (Wang et al. 2008a). However, whether the forced dimer has enhanced function compared to wild-type Nanog has not been reported. While these results identify WR as the dimerization domain, the identity of the critical residues responsible for dimerization remains to be determined. Although the tryptophans are clear candidates, other amino acid residues have a repetitive occurrence within the WR and identification of critical residues will be important in order to illuminate the mechanism of action of the dimer.

Results from X-ray and NMR analyses of many homeodomain proteins indicate that while dimerization is a common property of homeodomain proteins, in the vast majority of non-plant cases it is the homeodomain itself that forms the dimerization interface (Banerjee-Basu et al. 2001). This contrasts with the situation in Nanog where the homeodomain is monomeric in solution (Jauch et al. 2008) and where the region involved in dimerization has been shown to be separated from the homeodomain by more than 40 amino acid residues (Mullin et al. 2008). Therefore, Nanog dimerization may be more akin to that of some plant homeodomain proteins in which the dimerization domain is separate from the homeodomain (Chan et al. 1998; Palena et al. 1999).

A number of studies have investigated the DNA sequences bound by Nanog (Mitsui et al. 2003; Loh et al. 2006; Jauch et al. 2008). The work has shown that in common with many homeodomains a "TAAT" motif supports binding. The identity of the two bases following this core is more variable between homeodomain proteins and is dependent upon the amino acid residues within the third α-helix of the homeodomain that contacts the DNA. Experiments in which all combinations of

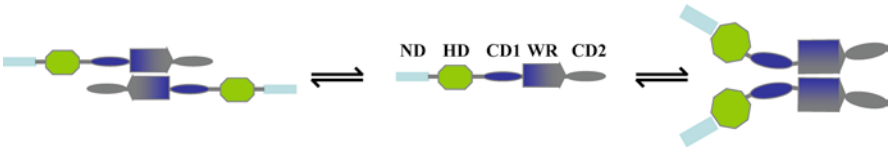

Fig. 9.2 Cartoon of possible mechanisms of dimerization of Nanog. *ND* N-terminal domain; *HD* homeodomain; *CD*1 C-terminal domain 1; *WR* tryptophan repeat; *CD*2 C-terminal domain 2

bases at positions five and six were tested for binding to Nanog showed that a sequence of TAATGG gave the highest binding affinity (Jauch et al. 2008) consistent with the influence of a glutamine residue at position 50 of the Nanog homeodomain upon target site selection (Chaney et al. 2005).

The K_d for the equilibrium between monomeric and dimeric Nanog is in the micromolar range and thus, from a thermodynamic viewpoint, dimeric Nanog will predominate. This has clear functional implications with regard to the ability of the molecule to interact with DNA. Examples of both parallel and anti-parallel dimer orientations exist among known homeodomain structures with parallel cases being more common (Rose et al. 2000; Kasahara et al. 2001). Both DNA affinity and specificity will be affected by the relative orientation of Nanog subunits because the relative spatial disposition of the homeodomains will differ in parallel and anti-parallel configurations. This will affect the ability of the dimer to interact with a pair of DNA cognate sites within a stretch of linear DNA (Fig. 9.2). In a parallel arrangement, the homeodomains are likely to be relatively close in space and to recognize closely spaced DNA sequences. Conversely an anti-parallel arrangement would likely result in relatively well-spaced homeodomains and DNA recognition sequences. Therefore, determining the direction of dimerization of Nanog will be an important step towards understanding how Nanog interacts with DNA recognition sites at the target genes that mediate Nanog function.

9.5 Protein Partners of Nanog

The arrangement of monomers within the dimer is also likely to influence the interactions of Nanog with its protein partners. Several protein molecules have been demonstrated to interact with Nanog and in a small number of cases the regions of the molecules involved in the interaction have been mapped (Table 9.1). It is noteworthy that in almost all cases, the methods used to identify interacting partners of Nanog do not distinguish between direct and indirect interactions. The process of Nanog dimerization will have a profound bearing on protein–protein interactions. First, the dimer interface could hide residues used by monomeric Nanog for partner interactions. Second, a Nanog dimer may present a completely novel molecular surface compared to a monomer. The latter scenario is precisely that by which HNFα1 interacts with DCoH (the dimerization co-factor of HNFα1) with distinct residues in each subunit of the HNFα1 dimer binding to separate residues on DCoH (Rose et al. 2000). A

Table 9.1 List of known protein interactors of Nanog

Nanog partner protein	Partner protein family	Partner protein function	Site of interaction	Obligate interaction with dimer	Independent verification	References
Sall4	Spalt like protein	Transcription factor	N-terminal region of Sall4 Homeodomain of Nanog/tryptophan repeat[a]	✓	✓	Wu et al. (2006); Liang et al. (2008); Wang et al. (2008a)
Oct4	POU transcription factor family	Transcription factor	N-terminal half of Nanog	×	✓	Wang et al. (2006, 2008a); Zhang et al. (2007); Liang et al. (2008)
Sox2	HMG box protein	Transcription factor	ND	ND	×	Mallanna et al. (2010)
Zfp281	Zinc finger protein	Transcription factor	ND	✓	✓	Wang et al. (2006, 2008b)
Nr0b1	Orphan nuclear receptor	Transcriptional regulator	ND	✓	×	Wang et al. (2006, 2008a)
Esrrb	Orphan nuclear receptor	Transcription factor	Interaction via DNA-binding domains of both proteins	ND	×	Wang et al. (2006); Liang et al. (2008)
Zfp198	Zinc finger protein	Transcription factor	ND	✓	×	Wang et al. (2006)
Sall1	Spalt family	Transcription factor	ND	ND	×	Wang et al. (2006)
REST	Kruppel family	Transcription factor	ND	ND	×	Wang et al. (2006)
Sp1	Zinc finger protein	Transcription factor	ND	ND	×	Wang et al. (2006)
Elys	AT hook DNA-binding domain family	Transcription factor	ND	ND	×	Wang et al. (2006)
Smad1	SMAD	Transcriptional regulator	MH1 domain of SMAD1	ND	×	Suzuki et al. (2006)
Tif1β	TRIM family	Transcriptional regulator	ND	ND	×	Wang et al. (2006)
Nac1	BTB containing family	Transcriptional regulator	ND	×	×	Wang et al. (2006, 2008a)
Mybbp	–	Transcriptional regulator	ND	ND	×	Wang et al. (2006)

Rif1	N/A	DNA damage response factor	ND	ND	✓	Wang et al. (2006); Liang et al. (2008)
HDAC1	Histone deacetylase	Chromatin modification	ND	ND	×	Liang et al. (2008)
HDAC2	Histone deacetylase	Chromatin modification	ND	ND	✓	Wang et al. (2006); Liang et al. (2008)
MTA1	–	Transcriptional regulator	ND	ND	×	Liang et al. (2008)
MTA2	–	Transcriptional regulator	ND	ND	×	Liang et al. (2008)
Gata2da	Zinc finger protein	Transcriptional regulator	ND	ND	×	Liang et al. (2008)
Gata2db	Zinc finger protein	Transcriptional regulator	ND	ND	×	Liang et al. (2008)
Pml	Zinc finger protein	Transcriptional regulator	ND	ND	×	Liang et al. (2008)
Sin3a	–	Transcriptional regulator	ND	ND	×	Liang et al. (2008)
LSD1	Flavine monoamine oxidase family	Transcriptional regulator	ND	ND	×	Liang et al. (2008)
SMARCA2	SNF2/Rad54 helicase family	Transcriptional regulator	ND	ND	×	Liang et al. (2008)
SMARCA4	SNF2/Rad54 helicase family	Transcriptional regulator	ND	ND	×	Liang et al. (2008)
BAF180	HMG box protein	Transcriptional regulator	ND	ND	×	Liang et al. (2008)
Med12	–	Transcriptional regulator	ND	ND	×	Tutter et al. (2009)

Independent verification indicates whether the protein has been shown to interact with Nanog in published studies from more than one laboratory

[a] The results of two studies suggest different sites of interaction on Nanog. One means of reconciling these results is the possibility that the mutations in the tryptophan repeat which abolish the Nanog-Sall4 interaction have an effect on the presentation of the homeodomain to Sall4

similar situation may be illustrated by the interaction between Nanog and Sall4. This interaction has been reported to be mediated by the homeodomain based on the ability of a deletion series of GST-Nanog mutants to pull down Sall4 from transfected 293T cell lysates (Wu et al. 2006). In a separate study, tryptophan residues within the tryptophan repeat were required to co-immunoprecipitate Nanog and Sall4 from transfected 293T cells (Wang et al. 2008a). One explanation of these seemingly disparate results is that, for wild-type Nanog it is only in the context of the dimer that the homeodomain is presented in the correct manner to interact with Sall4. The mutations within the tryptophan repeat that abolish (directly or indirectly) the interaction with Sall4 also eliminate the interaction of Nanog with several other known partners (Dax1, Zfp281 and Zfp198) (Wang et al. 2008a). Although these interactions have not yet been mapped it is possible that at least for some, it is the dimerized form of Nanog that recognizes these partner proteins. Another Nanog partner whose site of interaction has been mapped is Nac-1 (Ma et al. 2009). In this case, when fused to Gal4, the tenth WR repeat alone can interact with Nac-1. However, it is not clear whether Nac-1 interacts with monomeric and dimeric Nanog equally well.

Given the number of proteins identified as Nanog interactors it would be a surprise if all of them recognize the same form of Nanog. A scenario can therefore be envisaged where monomeric and dimeric Nanog each have distinct protein partners. Alteration of the steady state equilibrium between the two forms of Nanog would then alter the spectrum of proteins that bind to Nanog with downstream functional consequences. Given the fact that phosphorylation can alter equilibria (Amster-Choder and Wright 1992; Hunter and Karin 1992; Zhong et al. 1994; Lambert et al. 1999), and that Nanog exists in ES cells in multiple phosphorylated states (Yates and Chambers 2005), phosphorylation is one means by which the dimerization equilibrium and affinity for various partners could be altered.

Many of the proteins that interact with Nanog have already been identified as having a role in pluripotency. Moreover, most proteins that interact with Nanog are regulators of transcription. A large proportion of the interactors contain DNA-binding domains and have either been demonstrated to act as classical transcription factors or are inferred to have such activity. Some interactors have no DNA-binding domains but are nonetheless involved in transcriptional regulation; for instance Nr0b1 (Niakan and McCabe 2005) and the Med12 component of the Mediator complex (Tutter et al. 2009). Other Nanog interactors include proteins that are part of transcriptional repression complexes such as Sin3A and NuRD (Wang et al. 2006; Liang et al. 2008).

The complexity of Nanog-mediated protein–protein interactions is illustrated by Table 9.1. With its multiple binding partners, Nanog may be considered a “hub” protein. Hubs may be classified as “party hubs” which interact with multiple molecules simultaneously via multiple binding sites, or “date hubs” which only interact with a single partner molecule at a time (Han et al. 2004; Wilkins and Kummerfeld 2008) via a common binding site (Fig. 9.3). The pertinence of such a classification to a discussion of Nanog becomes clear when the case of a hub protein whose levels fluctuate is considered. Changes in the levels of a party hub over a limited range will not modulate the spectrum of proteins bound, assuming non-limiting amounts of interactors, but will simply result in differing amounts of hub-interactor complex. In contrast, in the case of a date hub expressed at limiting levels, the affinities of the

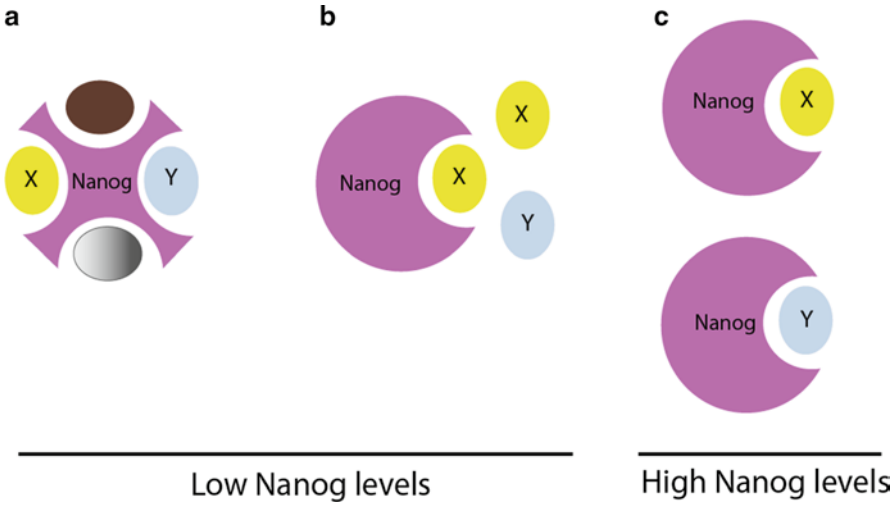

Fig. 9.3 Representation of Nanog as a party and date hub. As a party hub (**a**) Nanog would be able to interact with multiple binding partners simultaneously (e.g.interactors X and Y). However, as a date hub, when levels of Nanog are limiting (**b**), Nanog will preferentially interact with the protein partner with highest affinity (interactor X). Only when Nanog levels increase (**c**) will the interaction between Nanog and the lower affinity partner Y become significant

various competing interactors for the binding site impose a hierarchy on the hub protein interactions. Thus, in the simple case of two interactors, X and Y, which have high and low affinity for the hub, respectively, when there are limiting levels of the hub then protein interactor X binds preferentially. As the hub protein concentration rises above the concentration of X then interactor Y is able to bind the hub protein. It is therefore possible that in cells with fluctuating Nanog levels, distinct spectra of proteins are bound to Nanog at different times and that these spectra change in a defined manner as the Nanog concentration alters. Both party hub and date hub models can potentially explain the rheostatic function of Nanog in ES cell self-renewal and indeed it is possible that Nanog may act as both a party and a date hub for different proteins simultaneously. However, if the critical interactions of Nanog occur through action as a date hub, then the identity of interactor Y will be critical in mediating Nanog function.

9.6 Concluding Remarks

Nanog is arguably the key component in the network of factors responsible for determining efficient maintenance of pluripotency. Fluctuations in the level of Nanog protein indicate that regulation of Nanog is likely to involve multiple systems all of which must be coordinated to produce the dynamic expression pattern observed. Furthermore, the inter-connection between many of the proteins involved in self-renewal implies that a deeper understanding of the biology of Nanog can be

expected to illuminate features of other system components involved in the maintenance of pluripotent cell identity. Given that many of the proteins involved also perform roles in production of induced pluripotent cells, future advances can be expected to teach us more about potential distinctions between "maintaining" and "imposing" pluripotent cell identity.

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Chapter 10
Function of Oct3/4 and Sox2 in Pluripotency

Shinji Masui

Abstract Transcription factors regulating pluripotency have been extensively studied, and of these, *Oct3/4* and *Sox2* have been found to play pivotal roles in the molecular mechanism to maintain pluripotency. Both *Oct3/4* and *Sox2* are necessary to maintain pluripotency in vivo and in ES cells. Oct3/4 and Sox2 can synergistically activate Oct-Sox enhancers, by which many pluripotent stem cell-specific genes are regulated. Many other factors are involved in the regulation of *Oct3/4* and *Sox2* expression, but these two also regulate themselves by auto-regulatory mechanisms. Possible mechanisms of induction of iPS cells are discussed.

10.1 Introduction

The transcription factors that govern pluripotency are of interest to many researchers. A variety of fields have contributed to the analysis of these factors, and the functionality of two of the best-known transcription factors, Oct3/4 and Sox2, has been analyzed from numerous angles. The importance of these factors was dramatically illustrated when Yamanaka and colleagues produced iPS cells, proving that expression of *Oct3/4* and *Sox2* can be a sufficient condition for conferring pluripotency (Takahashi and Yamanaka 2006).

The mechanism by which pluripotency is sustained involves a variety of other molecules in addition to Oct3/4 and Sox2. However, those molecules have been thoroughly reviewed elsewhere (Jaenisch and Young 2008; Niwa 2007).

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Here I will summarize our current understanding of the Oct3/4 and Sox2 molecules, based on studies of their function in embryonic stem (ES) cells, and will describe a paradigm for the transcription network by which pluripotency is stipulated, focusing primarily on these two molecules.

10.2 *Oct3/4*

10.2.1 Expression Pattern and Function in Early Development

In the mouse early embryo, as cleavage progresses to the blastocyst developmental stage (E3.5), the outer layer develops into the trophectoderm (TE) while the inner layer forms an inner cell mass (ICM) of pluripotent stem cells (Yamanaka et al. 2006). ES cells are cultured from the ICM (Evans and Kaufman 1981; Martin 1981), and in 1990 three separate research groups reported *Oct3/4* as a new POU family gene (a type of transcription factor that has a homeodomain) that was highly expressed in pluripotent cells including germ cells, ICM, ES cells, and embryonal carcinoma (EC) cells (Okamoto et al. 1990; Rosner et al. 1990; Scholer et al. 1990) (Fig. 10.1a). Of these three early reports, two designated this factor as Oct3, and one

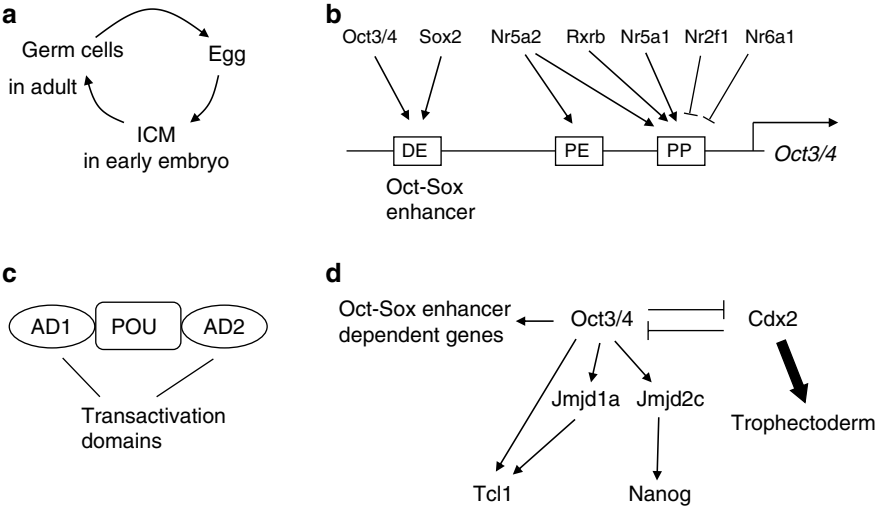

Fig. 10.1 *Oct3/4* expression and function (**a**) *Oct3/4* is expressed specifically in pluripotent lineages. (**b**) Expression of *Oct3/4* is regulated by a variety of factors in addition to the Oct-Sox enhancers. *DE* distal enhancer; *PE* proximal enhancer; *PP* proximal promoter. (**c**) Oct3/4 domain structure. The DNA binding domain consists of a POU domain and two transactivation domains. AD1 and AD2 indicate the activation domains 1 and 2, respectively. (**d**) Oct3/4 functions in ES cells. In addition to repressing TE differentiation by regulating Cdx2 functions, Oct3/4 directly or indirectly regulates the expression of *Tcl1* and *Nanog*, maintaining proliferation and undifferentiated status, respectively. Oct3/4 works with Sox2 and other Sox family members to activate Oct-Sox enhancers, and also activates pluripotency-specific genes

called it Oct4, so that today both terms are in use. In 1998, results were reported for a knockout study of *Oct3/4* showing that ICM pluripotency was lost in the knockout blastocysts, and thus that *Oct3/4* was essential in maintaining pluripotency (Nichols et al. 1998).

Oct3/4 is highly expressed in ICM, and although not expressed in TE, it is highly expressed temporarily in the primitive endoderm (PrE), which is formed on the ICM surface at the late blastocyst stage (Palmieri et al. 1994). In studies using ES cells, for which the amount of *Oct3/4* expression is under drug-inducible control, a reduction of Oct3/4 protein to less than 50% of wildtype levels results in differentiation into TE, and an increase to 150% or above leads to differentiation into PrE-like cells (Niwa et al. 2000). Therefore, in order to maintain pluripotency, it is necessary to strictly regulate *Oct3/4* expression so that it remains between 50 and 150% of wildtype. The mechanism of differentiation induced by an overdose of Oct3/4 has not yet been clearly elucidated, whereas the differentiation process in repression of *Oct3/4* has been analyzed in detail.

When *Oct3/4* repression results in the differentiation of ES cells into TE, upregulation of the transcription factor gene *Cdx2* (Caudal type homeo box 2) constitutes one of the factors in the molecular mechanism of that differentiation. *Cdx2* is expressed in TE in the early embryo (Niwa et al. 2005), and from findings in *Cdx2* knockout mice we know that *Cdx2* is essential for correct TE formation (Strumpf et al. 2005). Through the forced expression of *Cdx2*, the ES cells can be differentiated to form TE, so *Cdx2* is assumed to be a differentiation master gene for TE. Protein–protein interaction between Oct3/4 and Cdx2 results in mutual regulation of function. If there is more Cdx2 present, the expression of *Oct3/4* will be repressed simultaneously with the induction of expression of TE differentiation factors (including *Cdx2* itself and also *Eomesodermin*, another transcription factor which is necessary for TE formation and forced expression of which will result in TE differentiation) (Niwa et al. 2005).

10.2.2 Regulation of Oct3/4 Expression

The *Oct3/4* locus (also termed *Pou5f1*) is known to contain three regulatory regions: the proximal promoter (PP), proximal enhancer (PE), and distal enhancer (DE) (Fig. 10.1b). DE activity is seen in ICM and ES cells, while PE activity is seen in pluripotent stem cells at a slightly more advanced developmental stage, such as in the postimplantation epiblast and in EpiSC (Tesar et al. 2007; Yeom et al. 1996). DE is activated synergistically by Oct3/4 and Sox2 (described below) (Okumura-Nakanishi et al. 2005), and PE is activated by Nr5a2 (Lrh1, Liver receptor homolog 1) (Gu et al. 2005). In the *Nr5a2* knockout embryo, the epiblast expression of *Oct3/4* cannot be sustained, and the mutation is embryonic lethal (Gu et al. 2005). PP is regulated by a variety of nuclear receptors. The known transcriptional activators for PP include Nr5a1 (Sf1, steroidogenic factor 1) (Barnea and Bergman 2000), Rxrb (Retinoid X receptor beta) (Ben-Shushan et al. 1995), and Nr5a2 (Gu et al. 2005),

and the known transcriptional repressors include Nr2f1 (Coup-tfI, Chicken ovalbumin upstream promoter-transcription factors 1), Nr2f2 (Coup-tfII) (Ben-Shushan et al. 1995), and Nr6a1 (Gcnf, Germ cell nuclear factor). When *Nr6a1* is knocked out from an embryo, *Oct3/4* expression is derepressed in somatic lineages of that embryo, identifying Nr6a1 as an indispensable factor for restricting *Oct3/4* expression in cells of pluripotent lineage (Fuhrmann et al. 2001). As a posttranscriptional mechanism to regulate Oct3/4 protein level, in differentiating cells *Oct3/4* mRNA is bound by miR-470, which reduces the translational efficiency (Tay et al. 2008). In addition to transcriptional/translational regulation, activity of the Oct3/4 protein is also regulated. Nr0b1 (Dax1, dosage-sensitive sex-reversal adrenal hypoplasia congenital critical region on the X chromosome gene 1) binds to Oct3/4, and inhibits Oct3/4 binding to its target genes (Sun et al. 2009). Ubiquitinization mediated by the E3 ubiquitin ligase Wwp2 results in negative regulation of Oct3/4 transcription activity (Xu et al. 2004).

10.2.3 Oct3/4 Functional Domains

The Oct3/4 protein is configured of the N-terminal transcriptional activation domain, the central POU domain, and the C-terminal transcriptional activation domain (Fig. 10.1c). Study of *Oct3/4* knockout ES cells, with the application of an *Oct3/4*-transgene rescue system, has shown that self-renewal of the ES cells can occur if the POU domain is present along with either the N-terminal or the C-terminal transcriptional activation domain, which can even be replaced by a heterologous transcriptional activation domain (Niwa et al. 2002). In pluripotent stem cells, endogenous transcription activity similar to that of E1A is sometimes termed "E1A-like activity," which is not currently well understood. Findings using an artificial reporter gene containing an octamer-binding sequence have shown that, in differentiated cells, Oct3/4 does not have obvious transcriptional activation activity, and that simultaneous expression of the adenovirus E1A protein leads to increased transcriptional activation (Scholer et al. 1991). In 1993, Shimazaki et al. reported that co-expression of *Oct3/4* and *E1a* could activate a pluripotent stem cell-specific enhancer, and revert differentiated EC cells to a morphologically undifferentiated state (Shimazaki et al. 1993).

10.2.4 Regulation of Downstream Genes by Oct3/4

Oct3/4 also regulates expression of a great many downstream genes other than the TE differentiation-inducing factors such as *Cdx2*. Examples that have been studied in detail are the Oct3/4 regulation of *Tcl1* (T-cell-mediated lymphoma breakpoint 1) and the regulation of genes involved in cell proliferation (Matoba et al. 2006) (Fig. 10.1d).

Oct3/4 also regulates histone demethylase genes *Jmjd1a* (jumonji domain containing 1A) and *Jmjd2c*, which in turn regulate the expression of the *Tcl1* and *Nanog* genes respectively, contributing to cell proliferation and stabilization of the pluripotent state (Loh et al. 2007). Through the activation of Oct-Sox enhancers, Oct3/4 regulates expression of a set of pluripotent stem cell-specific genes (see below).

10.2.5 Oct3/4 in Other Organisms

The *Oct3/4* ortholog is widely distributed in mammals. Functionally similar *Oct3/4*-like genes have been found in *Xenopus laevis* (*Xlpou60, 25, 91*) (Morrison and Brickman 2006), with functional differences to the Zebrafish paralog (*Pou2*) (Lunde et al. 2004; Reim et al. 2004). There are no reported findings for *Drosophila* or for nematodes or other *Protostomia*. The *Oct3/4* ortholog has been reported to be a gene specific to mammals that first appeared in the *platypus* (Niwa et al. 2008).

10.3 *Sox2*

10.3.1 Expression Pattern and Function in Early Development

In 1995 Sox2 was identified as a factor that, along with Oct3/4, binds to the enhancer region of the *Fgf4* gene that is specifically expressed in pluripotent stem cells (Yuan et al. 1995). *Sox2* is expressed in germ cells, early embryo ICM, and neural tissues (Fig. 10.2a). In the *Sox2* knockout early embryo, as for *Oct3/4*, pluripotency is lost at the ICM stage, indicating that *Sox2* is essential for maintaining pluripotency (Avilion et al. 2003). The knockout ICM differentiates primarily into TE, with a small amount of differentiation into extraembryonic endoderm (ExEn). *Sox2* knockout ES cells also differentiate mainly into TE (Masui et al. 2007). Overexpression of *Sox2*, through the reduction of Oct-Sox enhancer activity, represses expression of the Sox2-regulated gene group and induces differentiation (Kopp et al. 2008).

10.3.2 Regulation of Sox2 Expression

The *Sox2* gene has enhancers at the 5′ terminal and 3′ terminal sides (Fig. 10.2b). The 5′-enhancer, HS1/SRR1, has two octamer binding sites, and is regulated by Oct3/4 (Catena et al. 2004). The 3′-enhancer, designated SRR2, is a typical Oct-Sox enhancer with an octamer- and Sox-binding sequence (described below) (Tomioka et al. 2002).

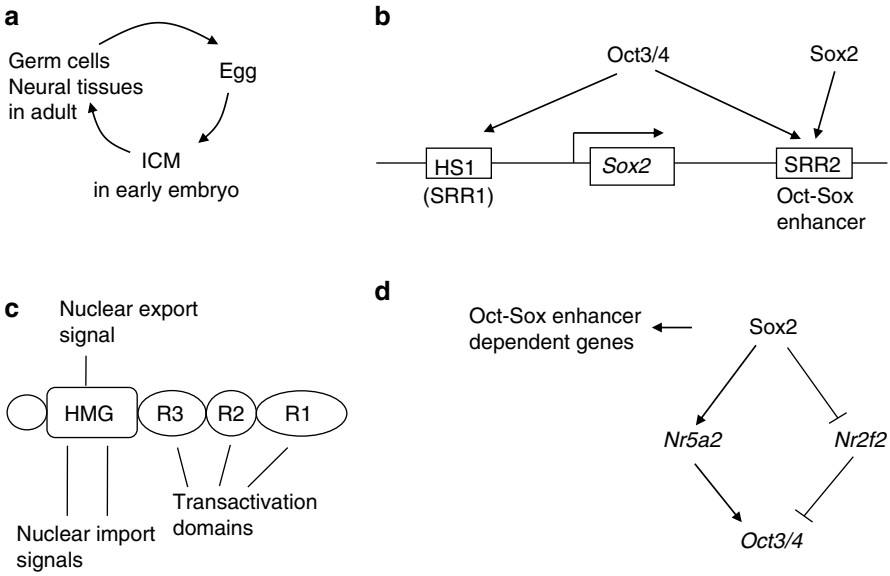

Fig. 10.2 *Sox2* expression and function (**a**) *Sox2* is expressed specifically in pluripotent lineages and in the nervous system (**b**) *Sox2* regulatory regions. SRR2 (Sox regulatory region 2 has an Oct-Sox motif, and functions as a major enhancer. HS1/SRR1 has two octamer motifs and is regulated by Oct3/4 (**c**) Sox2 domain structure. HMG domain has DNA binding activity. Transactivation domain can be divided into three subdomains (**d**) Sox2 functions in ES cells. Through its regulation of genes such as *Nr5a2*, Sox2 plays an essential role in maintaining *Oct3/4* expression. Redundantly with other Sox factors, Sox2 contributes to Oct-Sox enhancer activation and also activates pluripotency-specific genes

Both of these enhancers are active in both the pluripotent stem cells and the neural stem cells (Miyagi et al. 2004; Zappone et al. 2000). As for *Oct3/4*, the translational efficiency of *Sox2* mRNA is regulated by miR-134, of which expression is upregulated upon induction of differentiation (Tay et al. 2008).

10.3.3 Sox2 Functional Domains

Sox2 consists of an HMG domain and the transcriptional activation domain at the C-terminal end (Fig. 10.2c), both of which are required for activation of the target enhancers (Ambrosetti et al. 2000). The HMG domain mediates nuclear-cytoplasmic shuttling of Sox2, and may provide another mode of the activity regulation. There are two nuclear localization signals in the HMG domain (Li et al. 2007), by which Xpo4 (exportin 4) facilitates nuclear import (Gontan et al. 2009). Sox2 activity is negatively regulated by nuclear export, which is promoted by acetylation of the nuclear export signal in the HMG domain (Baltus et al. 2009).

10.4 The Oct-Sox Enhancer

Researchers have found that the structure of the enhancer region in the *Fgf4* gene involves an octamer motif (consensus with other genes; WBHWGCAT) adjoining a Sox motif (WWCAAWG) (the Oct-Sox enhancer), and that Oct3/4 and Sox2 work together to activate the Oct-Sox enhancer (Yuan et al. 1995). The next step has been to analyze the regulatory regions in the variety of genes that are expressed specifically by pluripotent stem cells, and it has become clear that most of the genes under consideration (*Utf1*, *Nanog*, *Oct3/4*, *Sox2*, *Fbxo15*, and *Lefty1*) are regulated by Oct-Sox enhancers (Kuroda et al. 2005; Nakatake et al. 2006; Nishimoto et al. 1999; Okumura-Nakanishi et al. 2005; Tokuzawa et al. 2003; Tomioka et al. 2002).

Findings from crystal structure analysis have been reported for the binding of the POU/HMG domain to DNA having an Oct-Sox motif (Remenyi et al. 2003). The results show a tertiary structure that varies depending on slight variations in the Oct-Sox sequence, and there are also variations in the amino acid residue on the HMG domain that binds to the POU domain.

Sox2 is sufficient for Oct-Sox enhancer activation, but is also dispensable. Investigations using inducible *Sox2* KO ES cells showed that repression of *Sox2* did not produce an immediate decrease in Oct-Sox enhancer activity, indicating that Sox2 is not required for this activity (Masui et al. 2007). In addition to *Sox2*, ES cells also strongly express *Sox4*, *11*, and *15*, which can contribute to Oct-Sox enhancer activation (Maruyama et al. 2005; Masui et al. 2007), and since pluripotent stem cell formation can be shown to occur in single knockout mice (*Sox4*, *11*, or *15*) (Lee et al. 2004; Schilham et al. 1996; Sock et al. 2004), it appears that these Sox factors operate redundantly. That is to say, *Sox2*, *4*, *11*, and *15,* (with the possible inclusion of other Sox factors) work together to carry the responsibility for Oct-Sox enhancer activation (see Fig. 10.3).

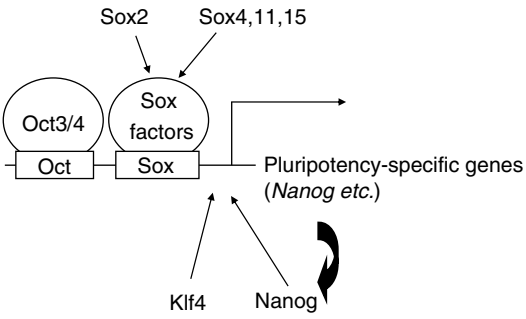

Fig. 10.3 Pluripotency-maintaining mechanism controlled by Oct3/4 and Sox2. Through the Oct-Sox enhancers, pluripotency-specific genes are activated by Oct3/4 and Sox2 and by Sox4, 11, and 15. These include *Nanog* as well as *Oct3/4* and *Sox2* themselves. Expressed Nanog works with Oct3/4 and Sox2 to encourage the activation of pluripotency- specific genes. Klf4 can also work with Oct3/4 and Sox2, so a similar mechanism may be operating at the time of iPS cell induction

Regardless of this functional redundancy among Sox family members, however, if *Sox2* is knocked out of ES cells they will finally differentiate into TE-like cells. It is known that pluripotency can be maintained under these conditions if there is forced expression of *Oct3/4* simultaneously with the knockout of *Sox2* (Masui et al. 2007). The results from such studies underscore the primary role of Sox2, mediated by activation of genes such as *Nr5a2* and *Nr2f2*, in maintaining *Oct3/4* expression levels (Fig. 10.2d).

10.5 Colocalization as Core Network Factors

Findings from a global chromatin immunoprecipitation (ChIP-on-chip) study show that Oct3/4 and Sox2 work with Nanog (reviewed in Chap. 9) to regulate downstream genes (including these three themselves) required for maintaining pluripotency, and to regulate the expression of genes such as Pax and the Hox family that are necessary for embryonic development (Boyer et al. 2005; Loh et al. 2006). Polycomb repressor complexes (PRC1 and PRC2) have been shown to co-localize with Oct3/4, Sox2, and Nanog to regulate such expression (Boyer et al. 2006; Endoh et al. 2008; Lee et al. 2006). Thus, these transcription factors may recruit polycomb repressor complexes to repress the differentiation program, presenting a model for contributing to the maintenance of pluripotency. The expression levels of the downstream genes are fine-tuned by microRNAs including miR-290-295, and their expression in turn is regulated by transcription factors including Oct3/4, Sox2, Nanog and Tcf3 (Marson et al. 2008).

10.6 Oct-Sox Role in Induction of iPS Cells

In 2006, Yamanaka et al. of Kyoto University reported that they had successfully introduced four factors (Oct3/4, Sox2, Klf4, and c-Myc) into differentiated cells to induce pluripotent stem cells (Takahashi and Yamanaka 2006). Subsequently c-Myc was shown to be unnecessary (Nakagawa et al. 2008). The iPS cells are not obtained within a few days of the introduction of Oct3/4, Sox2, and Klf4 into differentiated cells; instead, 2–3 weeks are required before the cells are identifiable (reprogrammed) as iPS cells. This suggests that these 3 factors do not bear the sole and entire responsibility for stipulating pluripotency, but that other important factors also contribute to pluripotency through a chain of expression-inducing events.

There is an inconsistency inherent in the need for Sox2. Findings from ES cell studies show that pluripotency can be maintained by the forced expression of Oct3/4 even if Sox2 has been knocked out (Masui et al. 2007). However, iPS cells cannot be produced without the introduction of Sox2 (using only Oct3/4 and Klf4 in the absence of either endogenously expressed Sox2 or a chemical compound) (Takahashi and Yamanaka 2006). This inconsistency can be attributed to the existence of certain genes whose expression is induced by Sox2 in differentiated cells, while such genes are expressed independently of Sox2 (or redundantly through other Sox factors) in the pluripotent stem cells. The Oct-Sox enhancer-dependent genes such

as Nanog can be considered candidates for this role. As noted above, Oct-Sox enhancer activation requires both Oct3/4 and Sox2 (or another Sox factor), while pluripotent stem cells can be activated by other Sox factors. It is possible that iPS cells can be established by the use of Sox15 as well as by Sox2 (Nakagawa et al. 2008). However, the simple addition of Nanog to Oct3/4 and Klf4 is not sufficient for the production of iPS cells (Takahashi and Yamanaka 2006), indicating the involvement of multiple Oct-Sox enhancer-dependent genes.

Assuming that Klf4 works with Oct3/4 and Sox2 to activate Oct-Sox enhancers (Nakatake et al. 2006; Sridharan et al. 2009), we can envisage the following model for the induction of iPS cells. When Oct3/4, Sox2, and Klf4 are introduced, these factors bind to the regulatory regions represented in the Oct-Sox enhancer, only slightly activating expression of the pluripotency-specific gene group. Within the differentiated cells, the gene chromatin is in an inactive state, so expression efficiency is initially low. Epigenetic factors such as the Polycomb repressor complex tend to separate from the chromosome during cell mitosis, so the transcription of those factors might occur first in cell populations undergoing active mitosis (Egli et al. 2008). The more numerous the types of binding at the pluripotent stem cell locus with other pluripotency-specific transcription factors in addition to Oct3/4 and Sox2, such as Nanog, the more expression there will tend to be (Chen et al. 2008; Kim et al. 2008). That is to say, if the expression of pluripotency-specific transcription factors such as *Nanog* is induced by the activity of genes such as Oct3/4 and Sox2, this will put into operation a positive feedback mechanism that will strongly express intrinsic *Oct3/4*, *Sox2*, and other pluripotency-specific transcription factor groups, leading to the upregulation of epigenetic factors stipulating pluripotency, such as *Jmjd1a*, and finally (stochastically) resulting in stability for the pluripotency transcription network.

10.7 Conclusion

Oct3/4 and *Sox2* have been extensively studied in recent years, and there has been considerable clarification of the major targets of Oct3/4 and Sox2, as well as the function of inducing pluripotent stem cells that these molecules (Oct3/4 and Sox2) possess. Further detailed analysis of the process for establishing a pluripotent stem cell network will advance our understanding of the network functions controlled by Oct3/4 and Sox2.

On the other hand, although *Oct3/4* and *Sox2* are expressed both by epiblasts and by germ cells, we have very little insight into the functions involved in sustaining these cells. Further studies are eagerly awaited.

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Chapter 11
Generation of Neural Cells from Pluripotent Stem Cells

Takuya Shimazaki and Hideyuki Okano

Abstract Recent advances in the establishment and controlled differentiation of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have generated new model systems to access the biology of early mammalian, including human, development and to investigate new approaches for regenerative medicine. Among the studies using pluripotent stem cells (PSCs), neural differentiation and its applications have received the most focus, because of the relative ease of neural induction in vitro and the high expectation for transplantation therapeutics to treat neurological disorders. The discovery of iPSCs increased the potential for developing efficient systems for drug discovery and screening, because they enable different types of functional neurons and their precursors to be generated in vitro from patients with genetic disorders. This chapter describes recent advances in protocols for the neural differentiation of PSCs that were designed using findings from basic embryology, and their possible applications.

11.1 Introduction

Restoration of the central nervous system (CNS) functions that are lost in neurological disorders, including Parkinson, Huntington's, and Alzheimer diseases, multiple sclerosis, and brain and spinal cord injuries has been highly desired as an ultimate cure. However, the fact that the CNS normally has a very limited capacity for regeneration has made this goal very difficult to achieve. The identification of mammalian neural stem cells (NSCs) and the establishment of protocols for their amplification and differentiation in vitro and in vivo, have raised expectations for

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their clinical application in cell replacement therapies to treat these CNS disorders. There have already been a number of reports on the transplantation of in vitro-expanded neural progenitor cells (NPCs), including NSCs, and the mobilization of latent NPCs in vivo, in animal models of several neurological disorders, in which at least some functional recovery was achieved (Rossi and Cattaneo 2002; Goldman 2005).

Ideally, NSCs could be used to treat a variety of neurological disorders by replacing specific types of neurons. However, the developmental potential of in vitro-expanded and adult NSCs is not yet well understood. While the developmental potential of NSCs in vivo is likely to be temporally and spatially restricted during development, that of NSCs expanded in vitro seems to be broad and inconsistent (Morrison 2001; Merkle and Alvarez-Buylla 2006; Miller and Gauthier 2007; Allen 2008). There are reports of the differentiation of NSCs expanded in vitro into certain types of projection neurons, such as midbrain-type dopaminergic (DA) neurons and forebrain-type cholinergic neurons, that are normally born only during the early period of CNS development (Ling et al. 1998; Yan et al. 2001; Wu et al. 2002). However, it is unclear whether the function and characteristics of these in vitro-generated neurons are identical to those of their native counterparts born in vivo. Moreover, the in vitro expandability of NSCs is probably limited (Ostenfeld et al. 2000), and dependent on the region from which the cells originated (Hitoshi et al. 2002).

ESCs, which are truly pluripotent, and their derivatives represent an ideal population to use as an unlimited source for transplantation therapeutics; however, their use is hampered by the controversial ethical issues associated with the clinical application of human ESCs (Vogel and Holden 2003). The successful creation of human iPSCs (Takahashi et al. 2007; Yu et al. 2007) appears to resolve many of the divisive ethical issues associated with the use of human embryos in research and applications. Several dozen groups have already established differentiation protocols for obtaining various types of neurons from these PSCs (Stavridis and Smith 2003; Erceg et al. 2008; Gaulden and Reiter 2008; Schwartz et al. 2008).

11.2 Neural Induction

To design ways to control the differentiation of various types of neural cell from PSCs, it is necessary to understand the mechanisms underlying CNS development, that is, how various types of neurons are generated at the proper place and time in vertebrate development. The nerve networks controlling animal behavior are formed through the development of various types of neurons, each of which express particular neurotransmitters and their receptors, and are born at and project to specific sites.

In development of the vertebrate nervous system, which consists of the peripheral nervous system (PNS) and the CNS, neural induction is initiated with the induction of the neural plate, at the gastrula stage. The research on neural induction dates back to the discovery of the organizer by Spemann and Mangold. These embryologists found that the dorsal blastopore lip in the early gastrula embryo of the newt is capable of inducing the neural plate in the region destined to become epidermis, and called it an “organizer” (Spemann and Mangold 1924). It was later shown in the

1930s that the area corresponding to this organizer exists in most vertebrates, including mammals. After 60 years of intensive effort to identify the molecular substance secreted by the organizer for neural induction, Noggin, Follistatin, and Chordin were identified in the 1990s. Soon afterwards, the actions of these proteins were further clarified as the masking factors of bone morphogenic proteins (BMPs), which obstruct neural induction, rapidly leading to the "default model" of neural induction (Stern 2005). However, the simple default model is still debated, because a positive effect of FGFs through the MAPK pathway and both positive and negative effects of Wnt signaling on neural induction are also reported to occur, depending on developmental stage (Gaulden and Reiter 2008). Perhaps the initial specification of neuroectoderm identity can occur independently of FGF signaling, but later establishment of the definitive NSC identity from the neuroectoderm requires it (Dang and Tropepe 2010).

The first neural induction from ESCs was achieved without using information gained from experimental embryology. The classic and most general differentiation method for ESCs is embryoid body (EB) formation through floating culture, which induces the differentiation of the three germinal layers. It was found that the exposure to retinoic acid (RA) during EB formation facilitates neural differentiation (Bain et al. 1995). This induction method was originally established as a differentiation system for embryonic carcinoma cells (Sherman et al. 1981). It is still uncertain how RA functions to promote neural induction.

A number of protocols for the neural induction of ESCs, including human ESCs, have been reported (Stavridis and Smith 2003; Erceg et al. 2008; Gaulden and Reiter 2008; Schwartz et al. 2008). Most of these protocols can be classified roughly into three groups: EB formation, co-culture with stromal cells as a feeder layer, and direct differentiation (Fig. 11.1). The EBs can be formed either in the presence or in the absence of serum. In the presence of serum, which normally contains BMPs (Kodaira et al. 2006), the inhibition of BMP signaling using BMP antagonists such as Noggin facilitates neural induction (Okada et al. 2008). The inhibition of Nodal signaling during EB formation may also facilitate neural induction. A lack of *Cripto*, a Nodal co-receptor, or the overexpression of *Lefty* or of a truncated form of *Cerberus,* which are Nodal inhibitors, in ESCs results in increased neural induction (Vallier et al. 2004; Sonntag et al. 2005; Smith et al. 2008). Axiomatically, the addition of BMPs or Nodal during EB formation inhibits neural induction (Finley et al. 1999; Xu et al. 2002; Vallier et al. 2004). The increased neural induction of ESCs by inhibiting Wnt signaling has also been reported by several groups (Aubert et al. 2002; Lindsley et al. 2006; Doble et al. 2007; Verani et al. 2007). On the other hand, the neural differentiation of ESCs has also been shown to require β-catenin signaling, which is the canonical Wnt signaling pathway (Otero et al. 2004). This discrepant action of Wnt signaling might be a reflection of its stage-dependent action in vivo described above, although the ESC lines and culture conditions of these studies were also different.

The mechanisms responsible for the highly efficient neural induction of ESCs on stromal feeder cells are still unknown. One possibility is the selective survival of the neural lineage, since neural induction usually requires serum-free conditions (Kawasaki et al. 2000). In fact, the direct differentiation protocols, in which ESCs

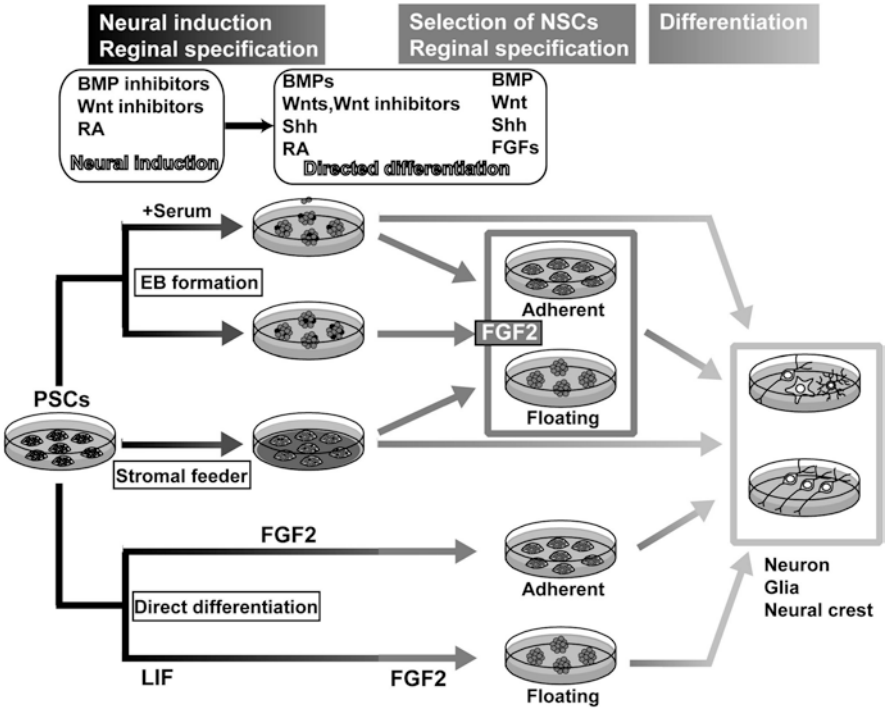

Fig. 11.1 Protocols for neural differentiation of pluripotent stem cells. Neural cells can be efficiently differentiated from PSCs through EB formation, co-culture with stromal feeder layers, or direct differentiation in chemically defined media. In these protocols, neural induction is often enhanced in the presence of BMP inhibitors, Wnt inhibitors, or RA. Sequential addition of secreted factors that regulate regional identities of NSCs during neural induction and selection of NSCs facilitate directed differentiation of regional specific types of neurons and neural crest lineage cells. NSCs can be amplified in the presence of FGF2, which is a mitogen for NSCs

are differentiated into the neural lineage through neurosphere formation or adherent culture, which is accompanied by much cell death, are also conducted without serum (Tropepe et al. 2001; Ying et al. 2003). Normally, direct differentiation requires the cultures to be at a low cell density, supporting the "default model" of neural induction from ESCs. Indeed, Nestin expression, a marker of neural lineage, is induced quickly, a few hours after ESCs are plated in PBS at a very low density (Smukler et al. 2006).

11.3 Regional Specification and Directed Differentiation

To generate specific types of neurons efficiently from ESCs, it is necessary to direct the specification of the NSCs to follow courses that occur regionally in vivo, during neural induction in vitro. In the vertebrate ontogeny, the regional identities of NSCs

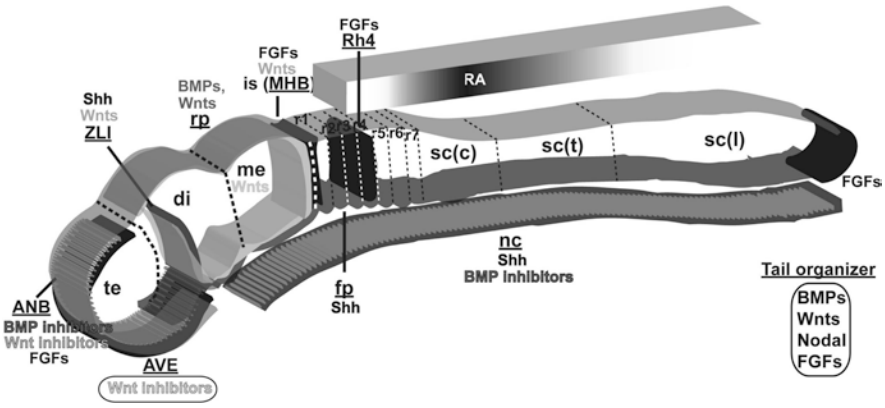

Fig. 11.2 Regionalization of nervous system by secreted factors. Regional specification of NSCs regulated by various secreted factors during development results in site-specific generation of specific types of neurons. *te* telencephalon; *di* diencephalon; *me* mesencephalon; *is* isthmus; *r* rhombomere; *sc* spinal cord; (*c*) cervical; (*t*); thoracic; (*l*) lumber; *fp* floor plate; *rp* roof plate; *nc* notochord

are probably specified chiefly by the gradients of various inductive signals secreted from various places in the embryo at the appropriate time (Fig. 11.2) (Jessell 2000; Altmann and Brivanlou 2001).

First, basic regionalization along the antero-posterior (A-P) and dorso-ventral (D-V) axes of the embryo occurs until the end of gastrulation, along with the differentiation of the three germinal layers. In the mouse, Wnt/β-catenin signaling plays important roles in this process. In the β-catenin-deficient mouse, formation and migration of the anterior visceral endoderm (AVE), which is essential for head formation, including the anterior neuroectoderm, does not occur (Huelsken et al. 2000). Moreover, neither this mutant nor the Wnt3-deficient mouse forms a node, resulting in a lack of posterior morphogenesis (Liu et al. 1999; Huelsken et al. 2000). Thus, Wnt/β-catenin signaling is essential for the initial A-P axis formation in the early embryo. Nodal signaling also contributes to the initial A-P axis formation in a similar fashion (Arnold and Robertson 2009).

Analyses of various vertebrates, mostly using zebrafish, revealed that in the period of neural plate formation after the gastrula stage, five signaling centers (local organizers) that control the detailed regionalization along the A–P axis are formed. These are, the anterior neural boundary (ANB), located at the anterior edge of the neural plate (the boundary between the neural plate and the surface ectoderm); the zona limitans intrathalamica (ZLI), which is the boundary between prosomeres 2 and 3 in the forebrain; the midbrain–hindbrain boundary (MHB), which is the boundary between the midbrain and hindbrain; rhombomere 4 (Rh4) in the hindbrain; and the tail organizer at the tail end of the neural plate. Wnt/β-catenin signaling is also involved in forming these local organizers. The head formation by AVE is probably regulated by the secretion of Wnt antagonists (Cer1, Dkk1, Sfrp1, Sfrp5), leading to the formation of the ANB, which is required for the regionalization of the

forebrain (Kemp et al. 2005; Marikawa 2006). On the other hand, a concentration gradient of Wnt8 secreted from the tail organizer is essential for regionalization in the posterior part, and it appears to govern the position of the MHB in the zebrafish (Rhinn et al. 2005). In addition, Wnt genes have also been suggested to be involved in the positioning of the ZLI (Braun et al. 2003).

The local organizers described above control the detailed regionalization of the nervous system by secreting various factors. ANB secretes FGF3, FGF8, and Tlc, another Wnt antagonist, which regulates formation of the telencephalon (Houart et al. 2002). It has been suggested that Tlc inhibits the Wnt signal secreted from the posterior diencephalon that promotes the formation of the diencephalon but suppresses the formation of the telencephalon (Houart et al. 2002). Wnt8b and sonic hedgehog (Shh), expressed in the ZLI, control the regionalization of the diencephalon (Lim and Golden 2007). FGF8 expressed in the dorsal midline of the diencephalon controls regionalization of diencephalic subdomains (Kataoka and Shimogori 2008; Martinez-Ferre and Martinez 2009). FGF8 is also expressed in the MHB and controls the regionalization of the midbrain and hindbrain (Nakamura et al. 2008). Rh4, a boundary formed in the hindbrain, first controls the formation of its neighboring boundaries (rh3, 5, and 6) by expressing FGF3 and FGF8 (Walshe et al. 2002). In addition, RA is a well-known factor that induces posteriorization of the embryo and probably regulates the regionalization of the posterior neural plate in cooperation with Wnt and FGF signals (Deschamps and van Nes 2005).

Similar to the A–P axis, secreted factors also regulate regionalization along the D–V axis of the nervous system. The known factors are BMPs and Wnt, which are initially secreted from the surface ectoderm and roof plate, and regulate the dorsal pattern formation, required for the generation of the neural crest lineage and the dorsal types of neurons, and Shh and Noggin, which are initially secreted from the notochord and the floor plate, and regulate the ventral pattern formation, which is required for generation of the ventral types of neurons, such as motor neurons and DA neurons (Wilson and Maden 2005).

There have already been many reports showing the directed differentiation of specific types of neurons born in the various regions of the nervous system from ESCs using protocols based on the findings from embryology described above (Fig. 11.2). Renoncourt et al. (1998) found that the neurons generated from mouse ESCs through EB formation in the presence of RA contain many spinal cord types, including motor neurons. Later, Wichterle et al. (2002) increased the efficiency of the motor neuron generation by adding Shh, which induces ventralization of the nervous system, using a similar culture system. These in vitro-generated motor neurons were able to extend their axons to skeletal muscles and form synapses after transplantation into the chicken embryonic spinal cord. In addition, we were able to control the regionalization of NSCs along both the A–P and D–V axes by exposing EBs to various concentrations of RA, Wnt3a, BMP4, and Noggin (Okada et al. 2008). Lee et al. (2000) efficiently generated DA neurons by selectively culturing the neural precursor cells from EBs with FGF8 and Shh. The addition of LeftyA, which is a Nodal antagonist, and Dkk1 during EB formation in the absence of serum (SFEB) enhances the generation of telencephalic types of neurons (Watanabe et al. 2005). This telencephalic population can be further regionalized along the A–P and D–V axes by additional

exposure to FGF8 or FGFR3-Fc (an FGF inhibitor) and to Shh or Wnt3a and/or BMP4, respectively (Watanabe et al. 2005; Eiraku et al. 2008). BMP4, Wnt3a, and FGF8 induce cerebellar types of neurons including Purkinje cells in SFEB cultures (Su et al. 2006). On the other hand, the strict removal of exogenous patterning factors during an early differentiation step of SFEB culture induces the efficient generation of rostral hypothalamic-like progenitors (Wataya et al. 2008). The control of regionalization by various secreted factors during neural induction on PA-6 or MS5 feeders has also been shown; in these systems, DA, serotonergic and motor neurons, neural crest cells, and others are efficiently generated by adding FGF8, FGF4, RA, SHH, BMP, and other factors (Barberi et al. 2003; Mizuseki et al. 2003). Thus, the recapitulation of in vivo CNS regionalization has been shown to be a rational way to achieve the directed differentiation of specific types of neurons.

11.4 Temporal Specification and Glial Differentiation

Research investigating the in vitro differentiation of ESCs into neural lineages has paid much less attention to glia than to neurons. Glia can be generated from in vitro-expanded NSCs or glial progenitors derived from the embryonic or adult CNS. Furthermore, glia only consist of astrocytes and oligodendrocytes, unlike neurons, which exhibit a number of subtypes. However, there is much less of an advantage to using ESC-generated glial cells for clinical applications, so there has been less incentive to develop them. With respect to developmental biology research, however, the in vitro differentiation of glia from ESCs may provide very important information about the mechanisms of glial development.

In the developing CNS, NSCs first generate neurons, and then glia (Merkle and Alvarez-Buylla 2006; Miller and Gauthier 2007). This temporal regulation is probably largely regulated by intrinsic mechanisms encoded within the NSCs (Shen et al. 2006; Namihira et al. 2008). The cis-regulatory element of the glial fibrillary acidic protein (GFAP) gene, one of the markers of astrocytes, is epigenetically silenced in NSCs, to repress its expression in the early stage when only neurons are generated (Namihira et al. 2008). This finding suggests that the differentiation potential of NSCs is controlled by regulation of gene expression at the chromatin level.

On the other hand, the control of NSC differentiation potential by specific transcription factors is also reported. In the pMN domain of the developing spinal cord, motoneurons and oligodendrocytes are generated sequentially. However, in mice lacking *Sox9,* a transcription factor with an HMG type of DNA-binding domain, the generation of motoneurons extends to the period when oligodendrocytes are normally generated (Stolt et al. 2003). The knock-down of *Coup-tfI* and *II*, members of an orphan nuclear receptor family, results in extended neurogenesis by NSCs throughout the CNS in vivo and in vitro (Naka et al. 2008). The Coup-TFs are also required for the proper sequential generation of specific subtypes of neurons by NSCs (Naka et al. 2008). Despite these findings, there is still only fragmentary information regarding the mechanisms that control the temporally regulated developmental potential of NSCs.

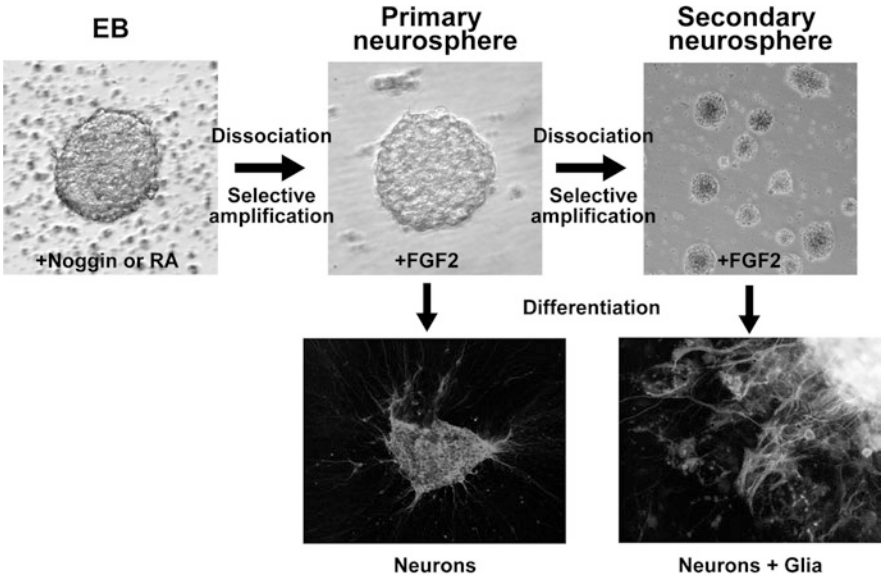

Fig. 11.3 Temporal regulation of differentiation potential of NSCs derived from ESCs. NSCs induced in EB derived from ESCs in the presence Noggin or RA can be selectively amplified as neurospheres, floating cell aggregates grown from NSCs (Reynolds and Weiss 1992), in the presence of FGF2. The primary neurospheres mostly differentiate into neurons, then glial differentiation appears after the subsequent passage and secondary neurosphere formation

In vitro systems of neural differentiation from ESCs should be very useful for elucidating these unknown mechanisms. This is because many aspects of physiological CNS development, including the temporal specification of NSCs, can be recapitulated using ESCs. We recently developed a system in which NSCs are induced from ESCs, through EB formation, and selectively amplified with proper temporal specification; they differentiate almost solely into neurons first, then gliogenesis appears during sequential passages (Naka et al. 2008; Okada et al. 2008) (Fig. 11.3). This system works using iPSCs, as well (Miura et al. 2009). This in vitro system enables comprehensive analyses of early CNS development by DNA microarray, proteomics, and other methods, and the functional screening of the involved genes by gain-of-function and loss-of-function studies. The involvement of Coup-TFs in the temporal specification of NSCs was discovered using this system.

11.5 Possible Applications and Use of iPSCs

There have already been many attempts to use PSC-derived neural cells to treat animal models of several neurological disorders. Parkinson's disease is the most studied target for cell replacement therapeutics, since the transplantation of DA neurons into the striatum, the target site of midbrain DA neurons, was found to

ameliorate the motor defect in a rat model of Parkinson's disease lesioned via 6-hydoxydopamine injection into the striatum (Brundin et al. 1988). Moreover, clinical trials with fetal midbrain tissue grafted into the striatum of patients have resulted in some improvement (Deierborg et al. 2008). DA neurons derived from mouse and primate sources including human ESCs and iPSCs have been shown to integrate and function, resulting in functional recovery in several animal models (Deierborg et al. 2008; Wernig et al. 2008; Swistowski et al. 2010). However, in some cases, the ESC-derived DA neurons did not work, due to their poor survival or differentiation (Deierborg et al. 2008). Therefore, successful transplantation requires an appropriate environment for ESC-derived DA neurons and their precursors.

Transplantation of neural or oligodendrocyte progenitors derived from mouse and human PSCs has also been shown to improve motor function in animal models of spinal cord injury, with enhanced remyelination by oligodendrocytes that differentiated from the grafts near the site of transplantation (Barnabé-Heider and Frisén 2008; Tsuji et al. 2010). In the case of spinal cord injury, the mechanism for the functional recovery by the transplantation of a progenitor population has not yet been fully clarified.

Potential targets for transplantation therapy by NPCs derived from PSCs include brain ischemia, multiple sclerosis, and lysosomal storage disorders. The transplantation of NPCs from various origins including PSCs into animal models of these diseases has resulted in some functional recovery (Pluchino et al. 2003; Ishibashi et al. 2004; Fukuhara et al. 2006; Lee et al. 2007; Aharonowiz et al. 2008; Takahashi et al. 2008; Chen et al. 2010). In addition to transplantation, iPSCs derived from patients with familial or spontaneous neurodegenerative diseases may be useful for examining their pathology at the molecular level and for drug discovery research (reviewed further in Chap. 17).

11.6 Concluding Remarks

Although the use of PSC-derived neural cells for clinical applications, drug discovery, and to increase our understanding of CNS development has a promising future, there are still many problems to be solved. The most important issue is that there are marked line-to-line differences in the differentiation potentials and global gene expression profiles of PSC cultures (Kim et al. 2007; Sharova et al. 2007; Müller et al. 2008; Osafune et al. 2008). This forces researchers to test many lines to secure the interpretation of the results from an experimental system. The differences may be due to genetic variation, specifically, to the varied epigenetic status of many developmentally regulated genes. This may cause subtle variation in the expression levels of genes that regulate differentiation into a specific lineage (Allegrucci et al. 2007; Pannetier and Feil 2007). To overcome this problem, a standardization of PSCs with respect to their global gene expression profile and epigenetic status is required. Attention must also be paid to genomic alterations occurring in PSC lines during culture (Maitra et al. 2005).

Another important issue is the purity of the desired types of cells. Contamination with undifferentiated PSC-like cells increases the risk of tumor formation after transplantation. To reduce this contamination as much as possible, the desired cells can be isolated by methods such as fluorescence-activated cell sorting-mediated purification using specific cell-surface markers or genetic modification to express fluorescent proteins, or by the use of selective drugs with the corresponding genetic modifications during culture (Klug et al. 1996; Yoshizaki et al. 2004; Pruszak et al. 2007). The purification of a specific population is often required in in vitro studies to eliminate the effects of other types of cells that do not usually coexist with the cells of interest in vivo. However, for therapeutic applications, a pure population may not always be desired. For instance, the functional recovery achieved by the transplantation of NPCs in brain ischemia and multiple sclerosis may be largely due to multiple factors exhibiting anti-inflammatory and/or trophic effects from a transplant containing a mixed population of cells (Aharonowiz et al. 2008; Takahashi et al. 2008). Thus, we are still just getting underway toward the systematic use of PSC-derived neural cells.

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Chapter 12
Noncell Autonomous Reprogramming to a Pluripotent State

Sowmya Parameswaran, Sudha Balasubramanian, and Iqbal Ahmad

Abstract In 2006, Takahashi and Yamanaka discovered that somatic cells could be reprogrammed to a pluripotent state by the expression of a defined set of exogenous transcription factors. This represents a significant breakthrough for the practical use of stem cells in regenerative medicine. Since then, several strategies have been used to generate induced pluripotent (iPS) cells that include nucleic acid and nonnucleic acid-based approaches, with and without epigenetic modifications. The purpose of these different approaches for generating iPS cells, besides understanding the underlying mechanism, is to develop a facile method for reprogramming without genetic alteration, suitable for clinical use. Here, we discuss different strategies for generating iPS cells, with an emphasis on a recent noncell autonomous approach to reprogram somatic progenitors that regenerate cornea to a pluripotent state through the recruitment of endogenous transcription factors.

12.1 Introduction

Direct reprogramming of somatic cells to a pluripotent state holds significant implications in treating intractable degenerative diseases by ex vivo cell therapy. In addition, the reprogrammed cells can serve as a model for diseases and discovery of drugs and genes. The advent of reprogramming cells to pluripotency dates back to 1952, when Briggs and King successfully transferred the nuclei of early blastocysts into the eggs of the amphibian, *Rana pipiens*, resulting in normal embryos (Briggs and King 1952). The study inferred that the technique could not

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be applied to more specialized tissues due to a loss of plasticity during cell differentiation. However, the reprogramming of somatic cells was initiated by John Gurdon in 1962, who transferred the nuclei of intestinal cells from adult frogs into oocytes (Gurdon 1962). The resulting zygotes successfully developed into embryos that hatched into tadpoles. This study demonstrated the existence of pluripotency-inducing factors, and that the process of differentiation is reversible. It paved the way for reprogramming by somatic cell nuclear transfer (SCNT), which culminated in the first successful cloning of a mammal, Dolly the sheep (Wilmut et al. 1997). The application of SCNT as a strategy for autologous therapy in humans, though fascinating, is not without obstacles, as it is technically demanding and has ethical concerns due to the requirement of a large number of human oocytes. One alternative is cell fusion, in which human embryonic stem (ES) cells are fused with human fibroblasts generating hybrid cells that contain both the somatic and human ES cell's chromosomes in a single nucleus (Tada et al. 2001; Kimura et al. 2004; Cowan et al. 2005). Although these cells shed valuable light on the mechanism of pluripotency, their clinical utility is considered to be marginal at best, due to their tetraploid nature. Therefore, the direct reprogramming of somatic cells toward pluripotency using defined transcription factors is heralded as a groundbreaking development, addressing serious technical and ethical barriers associated with SCNT and cell fusion. This strategy, therefore, represents a significant breakthrough toward the practical use of stem cells in regenerative medicine.

12.2 Reprogramming by Exogenous Transcription Factors

12.2.1 Nucleic Acid-Based Approach

In 2006, Takahashi and Yamanka, in their seminal work, demonstrated that retrovirus-mediated expression of four defined transcription factors, namely *Oct4, Klf4, Sox2*, and *c-Myc* (OKSM), in mouse fibroblasts caused these cells to acquire pluripotent potential. These induced cells were termed "induced pluripotent stem" (iPS) cells. However, these cells were germline incompetent. A year later, using the same approach of induction but selecting cells for the activation of endogenous *Nanog*, Yamanaka's lab isolated iPS clones that were germline competent (Okita et al. 2007). The initial methods for exogenous expression of OKSM required transduction through retroviral (Takahashi and Yamanaka 2006; Takahashi et al. 2007a, b; Okita et al. 2007; Park et al. 2008; Wernig et al. 2007; Maherali et al. 2007), constitutive lentiviral (Yu et al. 2007; Blelloch et al. 2007), or inducible lentiviral (Brambrink et al. 2008; Wernig et al. 2008; Stadtfeld et al. 2008a, b; Maherali et al. 2008; Hockemeyer et al. 2008) vectors. The major disadvantages associated with the use of viral vectors include the risk of genomic integration and insertional mutagenesis (Maherali and Hochedlinger 2008).

Since then, different strategies have been employed to overcome these disadvantages that range from the use of nonintegrating viral vectors (Stadtfeld et al. 2008a), plasmid-based transient ectopic expression (Okita et al. 2008) to the excision of vectors following their integration in the genome (Yusa et al. 2009; Woltjen et al. 2009; Kaji et al. 2009; Soldner et al. 2009). A predictable drawback of the last strategy for reprogramming is the possibility of random genomic alterations due to the integration/excision events.

12.2.2 Nonnucleic Acid-Based Approach

In 2009, an important finding from Shen Deng's lab suggested that DNA vector-free reprogramming of somatic cells is a possibility (Zhou et al. 2009). They demonstrated that mouse embryonic fibroblasts could be reprogrammed by direct transduction of OKSM proteins, containing a poly-arginine protein transduction domain fused to their C-terminus. This reprogramming, which also included exposure of cells to the histone deacetylase (HDAC) inhibitor valproic acid (VPA), produced pluripotent cells that formed chimeras and contributed to germline cells. In a variation of this approach, Kwang-Soo Kim's group demonstrated that fetal human fibroblasts could be reprogrammed to a pluripotent state when they are cultured in the presence of extracts from genetically engineered HEK293 cells expressing OKSM proteins with protein transduction domain consisting of human immunodeficiency virus transactivator of transcription (HIV-TAT) (Kim et al. 2009a). The major disadvantages cited for protein transduction-based reprogramming are short half-life of the factors that necessitate multiple applications, and the difficulty in purifying the proteins (Maherali and Hochedlinger 2008).

12.3 Reprogramming by Noncell Autonomous Recruitment of Endogenous OKSM Factors

The maintenance and differentiation of stem cells are profoundly influenced by cells that make up the cellular niche. This noncell autonomous regulation of stem cells helps maintain them in a quiescent state, and contributes toward their temporal reprogramming, required for generating stage-specific cell types during histogenesis. For example, in the developing mammalian retina, where the multipotential nature of neural stem cells was first established, stem cells generate seven different cell types through evolutionarily conserved stages of early and late histogenesis, in response to their interactions with the changing environment. A corollary to this observation is that retinal stem cells which have progressed to the late stage and programmed to generate late-born neurons such as rod photoreceptors can be

reprogrammed to generate early-born neurons such as the retinal ganglion cells (RGCs) if placed back in the niche present during early histogenesis. This premise was tested by placing late retinal stem cells in an environment simulating early histogenesis, which influenced these cells to activate genes required for the specification and differentiation of RGCs, and consequently, they acquired a RGC phenotype (James et al. 2003). Such reprogramming of older stem cells by a younger environment has been demonstrated during the rejuvenation of aged muscle progenitors by exposure to factors present in the serum of young animals (Conboy et al. 2005). While these and several other observations (Basta et al. 2004; Yang et al. 2009; Pan et al. 2008) have demonstrated the influence of the environment on programming of stem cells within the context of a particular tissue, or transdifferentiation into another somatic lineage, whether they can be reprogrammed into an early embryonic lineage remains an open question. This question was tested in adult stem cells that regenerate cornea, a transparent and refractive epithelium in the front of the eye. These are called limbal stem cells, as they are present in the basal layer of a circular tissue called the limbal epithelium, which surrounds the cornea. Limbal stem cells are derived from the embryonic ectoderm which possesses a default neural potential, i.e., unless instructed by the epidermalizing influence of the environment, presumably through bone morphogenetic protein (BMP) signaling, it acquires a neural phenotype (Hemmati-Brivanlou and Melton 1997). It was observed that adult limbal stem cells, when removed from their niche, acquired neural potential, and the efficiency of reprogramming increased with the neutralization of the BMP signaling by the antagonist, Noggin (Zhao et al. 2008). The noncell autonomous reprogramming of adult limbal stem cells to an early embryonic lineage suggested that, given the conducive environment, they might be pushed back even further to the stage of cells in the inner cell mass present in blastocysts. Another fact in favor of this premise was that these cells expressed three of the four OKSM genes, *Sox2*, *Klf4*, and *cMyc*. The seminal observation of Gail Martin, where embryonal carcinoma (EC) cell conditioned medium (CM) was used to generate and maintain ES (Martin 1981) cells, suggested that the conducive environment for reprogramming adult limbal stem cells toward pluripotency might be provided by EC/ES CM. Additionally, the observation that ES cells in low density culture adopt neural fate suggests a noncell autonomous mechanism for the maintenance of pluripotency (Smukler et al. 2006) and therefore provides validity for their use as inducers for reprogramming limbal stem cells. When rat limbal stem cell-derived neurospheres are cultured in the presence of mouse ESCM, they first flatten, and by the fifth day in culture, they generate colonies resembling those obtained from the mouse ES cells in morphology and size (Fig. 12.1a). Biochemical analysis of the limbal stem cell-derived colonies revealed expression of Oct4 and Nanog (Fig. 12.1b, c), similar to mouse ES cells. These cells also expressed the stage-specific embryonic antigen-1 (SSEA-1) and possessed alkaline phosphatase activity, two definitive characteristics of ES cells (Fig. 12.1d, e). These observations suggest that limbal stem cells acquire biochemical features of ES cells under the inductive influence of ESCM.

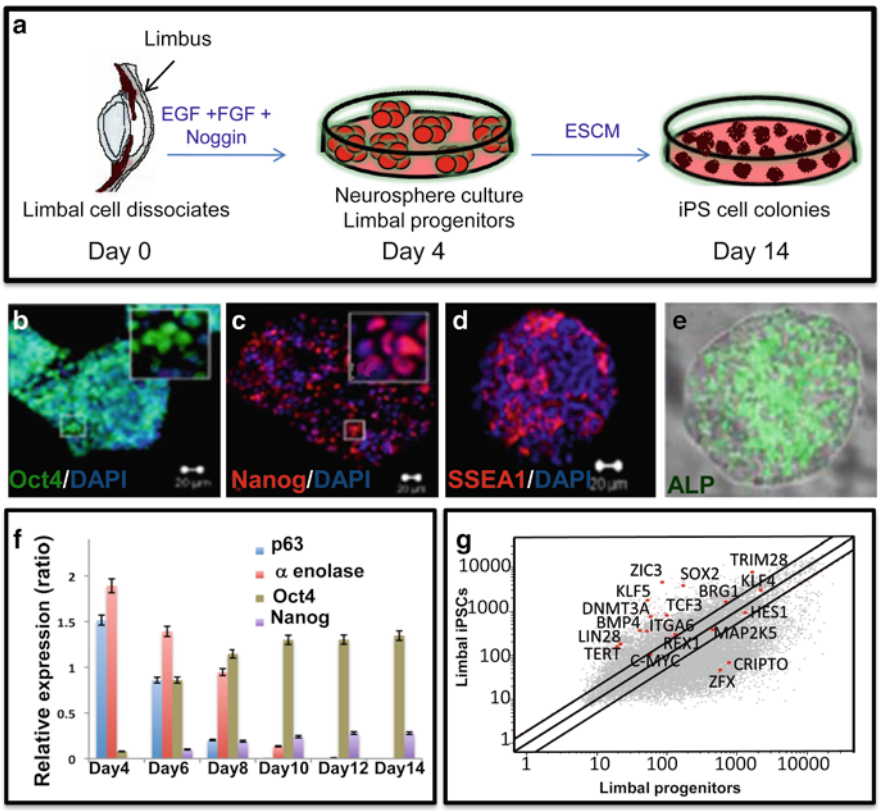

Fig. 12.1 Reprogramming of limbal progenitors. (**a**) Schematic representation of a two-step noncell autonomous reprogramming of limbal progenitors, where limbal cells are enriched from cell dissociates as neurospheres, followed by the generation of limbal iPS cells under the influence of ES cell conditioned medium (ESCM). The resulting iPS cell colonies express immunoreactivities corresponding to (**b**) Oct4, (**c**) Nanog and (**d**) SSEA1, and (**e**) possess alkaline phosphatase activity. (**f**) Induction of *Nanog* expression is preceded by expression of *Oct4* when limbal neurospheres are cultured in the presence of ESCM. Conversely, there is a temporal attenuation of the expression of the limbal progenitor genes, *α-enolase* and *p63*. (**g**) A comparative global gene expression analysis of rat limbal iPSCs and rat limbal progenitors after 14 days in culture shows the up-regulation of key pluripotency network genes in the former relative to the latter. *Red lines* indicate the linear equivalent and twofold changes in gene expression levels between the samples. *Red dots* indicate the 18 regulatory genes. Figure adapted with permission from Balasubramanian et al. (2009)

12.3.1 Reprogramming and Its Efficiency

The examination of the temporal expression patterns of OKSM genes in limbal stem cell-derived colonies provides the first indication of reprogramming toward pluripotency. The limbal stem cells at 0 days in culture express *Klf4, Sox2,* and *cMyc*, in addition to limbal stem cell genes, *p63* and *α-enolase.* In the presence of ESCM, the expression of KSM genes increases with the time in culture, but more importantly,

the expression of *Oct4* and *Nanog*, which is absent in the beginning, can be detected by the second day in culture and increases temporally thereafter, the expression of *Nanog* lagging behind that of *Oct4* (Fig. 12.1f). The fact that the expression of *Oct4* precedes the induction of *Nanog* suggests the recapitulation of the core pluripotency transcriptional network, where *Oct4* and *Sox2* are upstream of *Nanog* (Jaenisch and Young 2008). That the reprogramming includes the reciprocal inhibition of limbal-specific genes is demonstrated by a temporal decrease in the expression of *p63* and *α-enolase* (Fig. 12.1f). In addition, a comparative global gene expression profile shows that the induced limbal stem cells express 14 out of 18 (77.7%) key genes that underlie the regulatory network of pluripotency (Marson et al. 2008; Do and Scholer 2009) relative to uninduced controls (Fig. 12.1g). However, a comparison of the expression of *Oct4, Klf4, Sox2, c-myc, Nanog*, and *Lin28* expression by Q-PCR reveals that these genes are expressed at significantly lower levels in induced limbal stem cells relative to mouse ES cells, suggesting that the limbal iPSCs were similar, but not identical to mouse ES cells. The reprogramming efficiency by the noncell autonomous approach, as determined by the Nanog –GFP^{+ve} colonies, was 0.0025%, better than three- (0.001%) and two- (0.001%) factor retroviral transduction of human fibroblasts but lower than the efficiency of reprogramming obtained by four- (0.1%) and three- (0.01%) factor retroviral transduction of mouse embryonic fibroblasts (Lowry and Plath 2008). The efficiency was also lower (0.014%) than that obtained by single factor retroviral transduction of adult neural stem cells (Kim et al. 2009b).

12.3.2 Proof of Pluripotency

The limbal iPSCs possess the ability to differentiate along all three embryonic lineages like ES cells in side-by-side comparisons (Fig. 12.2a). For example, in the absence of LIF in a hanging drop culture, limbal iPSCs generate embryoid bodies (EBs) in the same time frame (2 days in culture) as mouse ES cells, and are indistinguishable in the expression of markers of embryonic ectoderm (e.g., OTX2), endoderm (e.g., SOX17), and mesoderm (e.g., Brachyury) (Fig. 12.2b). When these EBs are subjected to established protocols for the differentiation of ES cells into specific cell types belonging to the three embryonic lineages, they generate neurons, cardiomyocytes, and hepatocytes similar in biochemical and molecular features to those that are derived from the ES cells (Fig. 12.2c). The resulting cell types are functional, addressing the concern that cell type-specific marker expression may reflect a stress-related response and not pluripotent potential (Jaenisch and Young 2008). For example, whole cell recording of the limbal iPSC-generated neurons reveals AMPA receptor-mediated inward currents and fast acting inward currents, due to voltage-gated sodium channel, blocked by tetradotoxin. The limbal iPSC-generated cardiomyocytes display rhythmic contractions, L-type Ca^{++} currents, and depolarizing characteristics of functional ventricular myocytes. Hepatocytes generated by limbal iPSCs secrete albumin and functional factor VII two- to threefold more than primary rat hepatocytes. Together, these results suggest that rat limbal

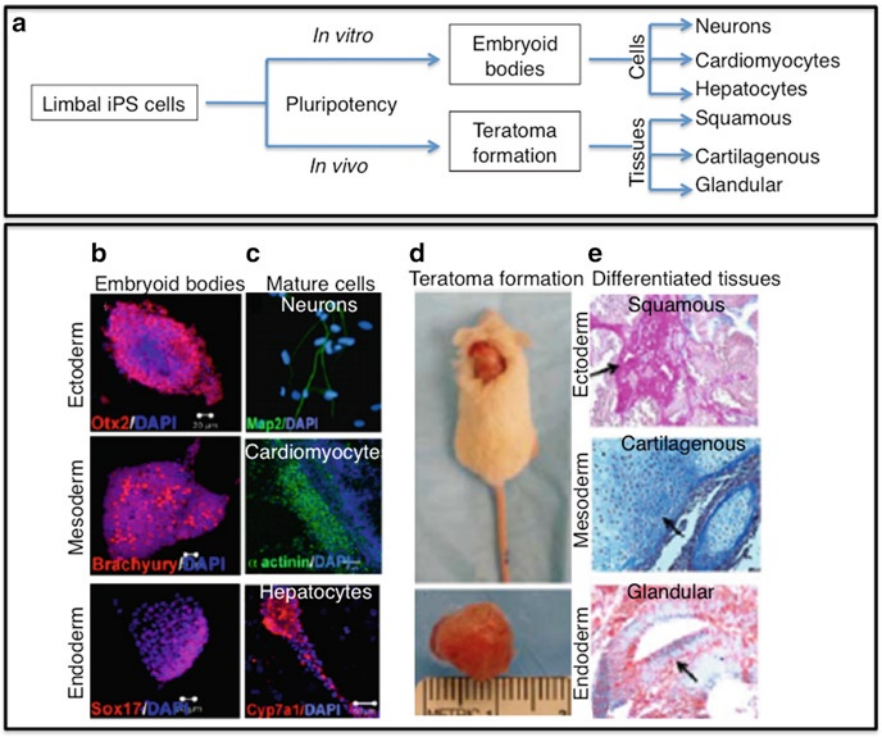

Fig. 12.2 Pluripotency of limbal iPS cells. (**a**) Schematic representation of tests for pluripotency of limbal iPS cells. The in vitro tests involved formation of embryoid bodies (EBs) with primitive lineage markers and their ability to generate cell types belonging to three different lineages. The in vivo test involved generation of teratomas with tissues of all three lineages. (**b**) EBs obtained from limbal iPS cells express the primitive ectoderm marker, Otx2, the primitive mesoderm marker, Brachyury, and the primitive endoderm marker, Sox17. (**c**) EBs subjected to established specific differentiation protocols generate neurons, cardiomyocytes, and hepatocytes, which are functional (Balasubramanian et al. 2009). (**d**) Limbal iPS cells injected in NOD-SCID mice generate teratomas, containing tissues of (**e**) ectoderm, mesoderm, and endoderm lineage. Figure adapted with permission from Balasubramanian et al. (2009)

iPSCs are as equally pluripotent as mouse ES cells, and are capable of generating functional cells of three different lineages in vitro. Additionally, iPSCs are equally pluripotent as mouse ES cells in the generation of teratoma (Fig. 12.2d). Although teratoma formation does not test the ability of cells to promote normal development, it demonstrates the potential of generating cell types of all three embryonic lineages in vivo. When limbal iPSCs are injected subcutaneously in NOD SCID mice, they develop into teratomas within 3 weeks, and except for their smaller size, they are morphologically similar to those that develop from mouse ES cells. The limbal iPSC-derived teratomas contain squamous (ectoderm), glandular (endoderm) and cartilaginous (mesoderm) tissues similar to those of ES cells (Fig. 12.2e). The formation of chimeras, the evidence that iPSCs contribute to normal development, with limbal iPSCs has not been successful yet. This failure could be attributed to

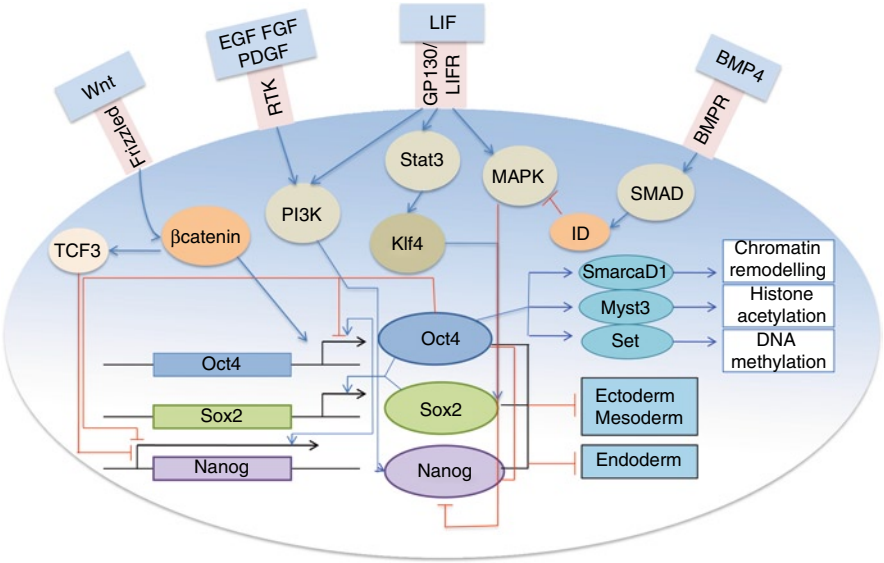

Fig. 12.3 Signaling pathways involved in pluripotency. Signaling pathways that might be involved in the non-cell autonomous reprogramming of limbal progenitors to limbal iPSCs are depicted. Signaling mediated by diffusible ligands recruit different intercellular effectors to alter the expression of Oct4, Sox2, and Nanog, establishing the regulatory feedback circuit to initiate and maintain self-renewal and pluripotency. Cross-talks between these pathways may influence the levels and efficiency of reprogramming. For example, the pluripotency circuit could be strengthened by indirect ID-mediated activation of Nanog. Oct4-mediated regulation of epigenetic signature by Smarca D1 (involved in chromatin remodeling), Myst 3 (involved in Histone acetylation) and SET (SET translocation; involved in Histone methylation) may further promote reprogramming. Differentiation into different germ layer is prevented by the concerted activation and actions of Oct4, Nanog and Sox2

strain, partial reprogramming, and/or efficiency of reprogramming. Studies on ES cells have reported that karyological instability may be the major reason for the lack of contribution to chimeras by individual ES cell clones (Longo et al. 1997; Nagy et al. 1993; Li et al. 2005a, b). In addition, the state of a single gene cluster may influence chimeric potential of iPS cells. For example, aberrant silencing of maternally imprinted genes in the Dlk1-Dio3 cluster may have an impact on chimerism and tetraploid complementation tests (Stadtfeld et al. 2010).

12.3.3 Enhancing Reprogramming Efficiency

The identification of factor(s) that mediate noncell autonomous reprogramming is the key to increasing reprogramming efficiency. This will require identification of the ESCM fraction where inducing activities are localized, followed by proteomics to

identify secretory proteins. This approach could be coupled with transcription profiling of limbal stem cells and mouse ES cells to identify transcripts encoding putative ligands (ES cells) and cognate receptors (limbal stem cells) in an approach similar to the identification of signaling pathways involved in the differentiation of mesenchymal stem cells along different lineages (Ng et al. 2008). Some of the putative signaling pathways that might be engaged in the noncell autonomous reprogramming of limbal iPSCs by ES cells are illustrated in Fig. 12.3. The reprogramming is accompanied by changes in epigenetic status. For example, upon induction by ESCM, there is a general decrease in the number of methylated CpG dinucleotides in the promoters of *Oct4* and *Nanog* in rat limbal iPSCs. Therefore, small molecule-based epigenetic change, along with ESCM induction, is likely to enhance the efficiency of noncell autonomous reprogramming. Small molecules or factors that induce chromatin modifications by affecting either DNA methylation or histone acetylation have been shown to greatly improve the reprogramming efficiencies of both murine and human somatic cells (Huangfu et al. 2008a, b). VPA, an inhibtor of HDAC, improves the reprogramming efficiency of mouse and human iPS cells by 50- to 100-fold in the presence of OKSM transcription factors (Huangfu et al. 2008a, b). 5-azacytidine (AZA), which blocks the maintenance of CpG methylation at promoters of pluripotency genes such as *Oct4*, has been observed to complete the reprogramming of partially reprogrammed cells (Mikkelsen et al. 2008). BIX-01294 inhibits the G9a-histone methyltransferase-mediated repression of *Oct4* and promotes the reprogramming of neural progenitors into iPS cells (Kubicek et al. 2007). Like VPA (Huangfu et al. 2008a), it also facilitates the induction of pluripotency using fewer than four exogenous factors (Shi et al. 2008a). Small molecules affecting signaling pathways such as Wnt, MAPK, and TGFβ have been employed in improving reprogramming of iPSCs. For example, the GSK3β inhibitor, CHIR99021, facilitates the reprogramming of mouse embryonic fibroblasts, transduced by 2 factors (Oct4 and Klf4) (Li et al. 2009), and the MEK inhibitor PD0325901 converts partially reprogrammed iPS cells to fully reprogrammed iPS cells (Shi et al. 2008b). Ascorbic acid has been used for increasing the efficiency of reprogramming by inhibiting the p53 pathway, known to hinder somatic cell reprogramming (Esteban et al. 2010). To test whether small molecule-based epigenetic changes may influence noncell autonomous reprogramming, limbal stem cells were induced by ESCM in the presence of VPA. The treatment led to a threefold increase in the reprogramming efficiency, suggesting that small molecule-based epigenetic modifications have similar effect on noncell autonomous reprogramming as nucleic acid-based approaches.

12.3.4 Amenable Cell Types

All somatic cells are not equally amenable to reprogramming even by forced expression of OKSM. For example, keratinocytes are more amenable to reprogramming than fibroblasts (100-fold more efficient), presumably due to higher expression of endogenous cMyc (Aasen et al. 2008; Maherali et al. 2008; Singh and Dalton 2009).

The rate of reprogramming may also differ based on the different cell types; for example, human foreskin keratinocytes and adipose stem cells were reprogrammed in the presence of four factors by the 10th and 18th day of reprogramming, respectively (Aasen et al. 2008; Sun et al. 2009), while the neural progenitor cells were reprogrammed in the presence of the four factors along with MAPK and GSK3b inhibitors in 12 days (Li et al. 2009). In the case of noncell autonomous reprogramming of limbal stem cells, the unidentified activities elaborated by ES cells effectively influence the maintenance of the expression of all four iPS cell genes, OKSM. An interesting question is whether these activities would have a similar influence on differentiated somatic cells. It is likely that progenitors will be more susceptible to noncell autonomous reprogramming than somatic cells if one considers recent findings, which show that hematopoietic stem cells are reprogrammed by OKSM with a significantly higher efficiency than B and T cells (Eminli et al. 2009). KSM expressing neural stem cells can be reprogrammed by exogenous *Oct4* alone (Kim et al. 2009b), and the *Oct4*-expressing adult germline stem cells can be reprogrammed under culture conditions similar to ours (Ko et al. 2009). It is quite likely that the ES cell-derived activities were effective in reprogramming because the limbal progenitor genome was rendered malleable to these activities due to prior expression of KSM. The induction of *Oct4* might represent the tipping point where OCT4 and SOX2 initiated a concerted activation of *Nanog* and NANOG, in turn, stabilized the core transcriptional network for a pluripotent state (Jaenisch and Young 2008). Such sequential induction of genes and stabilization of their expression may involve co-operative interactions between iPS cell transcription factors and chromatin remodeling enzymes whose function and/or expression is open to extrinsic influences, as suggested by a significant increase in the reprogramming efficiency of limbal progenitors brought about by VPA.

12.4 Conclusions

Reprogramming of somatic cells represents a significant breakthrough for the practical derivation of patient-specific stem cells for autologous cell therapy. In addition, the reprogrammed cells can serve as a model for understanding the mechanisms underlying disease processes and discovery of drugs and genes. Since the seminal observation of Takahashi and Yamanaka in 2006 that a defined set of exogenous transcription factors can reprogram somatic cells to pluripotency, the field of reprogramming of somatic cells that emerged from John Gurdon's experiment in 1962 has moved with a breakneck pace, a testimonial to its importance in understanding development, disease, and regenerative medicine. Significant progress has been made through different approaches that will soon lead to a facile method of reprogramming somatic cells without genetic alteration for practical clinical use. One of the approaches in that direction is the noncell autonomous reprogramming by recruiting endogenous pluripotency genes through extrinsic factors and small molecule-based epigenetic regulation.

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Chapter 13
Toward Regeneration of Retinal Function Using Pluripotent Stem Cells

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Abstract Embryonic stem (ES) cells have the ability to maintain pluripotency, while during development cells undergo progressive and irreversible differentiation to become specialized adult cell types. Remarkably, in spite of restriction in potential, adult somatic cells can be reprogrammed and returned to the naïve state of pluripotency found in the early embryo simply by forcing expression of a defined set of transcription factors. These induced pluripotent stem (iPS) cells are molecularly and functionally similar to ES cells, and as such, provide powerful in vitro models for development, disease, and drug screening, as well as material for cell replacement therapy. Transplantation of photoreceptors or retinal pigment epithelia derived from human ES cells can restore some visual function, and patient-specific iPS cells may lead to customized cell therapy. In this chapter, we review current progress on retinal regeneration, focusing on the therapeutic potential of pluripotent stem cells.

13.1 Introduction

The main cause of blindness in advanced countries is retinal impairment, usually resulting from cell loss. Indeed, in most central nervous system (CNS) diseases, functional impairment is caused by cell loss. Thus, recovery of lost cells is an important treatment strategy for these diseases. Neurogenesis occurs in two regions of the adult mammalian brain (Lledo et al. 2006; Zhao et al. 2008a), with Müller glia serving as retinal progenitors following injury in the adult retina (Ooto et al. 2004;

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Osakada et al. 2007). However, since self-repair in the CNS is very limited, many research efforts have focused on transplantation of new neurons into damaged or diseased CNS tissue as a promising treatment approach.

Visual impairment is usually caused by specific loss of different cell populations within the retina. For instance, glaucoma is a retinal degenerative disease in which the retinal ganglion cells (RGCs) forming the optic nerve are selectively lost. In retinitis pigmentosa, photoreceptors are lost due to genetic mutations (Hartong et al. 2006). In age-related macular degeneration (AMD), degeneration of the retinal pigment epithelium (RPE) is followed by loss of photoreceptors (Rattner and Nathans 2006). Since first-order neurons are selectively affected in retinitis pigmentosa and AMD, the neural circuitry mediating higher order visual processing is maintained in the early phase of degeneration. Thus, transplantation of photoreceptors or RPE cells may permit recovery of visual function (Haruta et al. 2004; Lund et al. 2006; MacLaren et al. 2006; Lamba et al. 2009), which may be facilitated by the proximity of synaptic target cells to the first-order neurons. In contrast, in the case of glaucoma, optic nerve regeneration requires replacement of RGCs and reconstruction of distant synaptic connections.

In order to achieve retinal regeneration by transplantation, the following steps must be optimized: (i) preparation of donor cells, (ii) transplantion of donor cells in the subretinal space, (iii) integration of donor cells into the host tissue, and (iv) recovery of retinal function. Pluripotent stem cells, such as ES cells and induced pluripotent stem (iPS) cells, may be used as a source of donor cells. However, generating retinal cells from pluripotent cells by controlled differentiation requires a detailed understanding of eye development. Moreover, to integrate transplanted cells into host tissue and to restore retinal function, the normal mechanisms of circuit assembly in the developing retina will likely need to be reactivated. Thus, questions that require further investigation include: How do axons and dendrites make the appropriate connections? How are complex neural circuits assembled? How are light signals converted into neural signals? How is visual information transduced to the brain?

In addition, diseased host tissue may exhibit pathological conditions that differ from normal physiological conditions. To repair the tissue efficiently, these host conditions, including the immune response, must be manipulated. For instance, inflammation affects the survival, synapse formation, and neural activity of transplanted cells, and except in cases of auto-transplantation, immune rejection occurs following cell transplantation. Optimization of pharmacological conditions and transplantation materials are therefore crucial for successful transplantation. Here, we review the progress to date in retinal regeneration research.

13.2 Development and Physiology of the Retina

13.2.1 Retinogenesis and Synaptogenesis

The eye is derived from three types of tissue during embryogenesis: the neural ectoderm gives rise to the retina and the overlying RPE, the mesoderm produces the cornea and sclera, and the lens originates from the surface ectoderm. During embryogenesis,

the eye develops as a result of interactions between the surface ectoderm and the optic vesicle, an evagination of the diencephalon (forebrain) (Fig. 13.1a). The optic vesicle is connected to the developing CNS by a stalk that later becomes the optic nerve. Upon contacting the surface ectoderm, the optic vesicle epithelium forms a lens placode (Fig. 13.1b), which subsequently invaginates, pinches off, and eventually becomes the lens. During these events, the optic vesicle folds inward to form a bilayered optic cup (Fig. 13.1c, d). The outer layer of the optic cup differentiates into the RPE, whereas the inner layer differentiates into the neural retina (Fig. 13.1e). The iris and ciliary body develop from the peripheral edges of the retina. The sclera is derived from mesenchymal cells of neural crest origin that migrate to form the cornea and trabecular meshwork of the anterior eye chamber (Fig. 13.1f, g).

During development, retinal progenitors change their competency under the control of intrinsic regulators (such as transcription factors) and extrinsic regulators (such as neurotrophic factors). Within the retina, seven types of cells differentiate from common progenitors in the following temporal sequence: RGCs, cone photoreceptors, amacrine cells, and horizontal cells, followed by rod photoreceptors, bipolar cells, and Müller glia (Fig. 13.1h, i). These cells comprise three cell layers: rod and cone photoreceptors in the outer nuclear layer (ONL); horizontal, bipolar, and amacrine cells and Müller glia in the inner nuclear layer (INL); and ganglion and displaced amacrine cells in the ganglion cell layer (GCL). All retinal cells are born at the outer surface of the retina, adjacent to the pigmented epithelium. Postmitotic cells migrate a distance to occupy their final positions within the retina, and then establish synaptic connections to other neurons.

In the mouse retina, synapses between RGCs and amacrine cells appear in the inner plexiform layer (IPL) during the first postnatal week. Then, rods and cones establish synapses with horizontal cells in the outer plexiform layer (OPL). Finally, during the second postnatal week, bipolar cell dendrites contact photoreceptors and horizontal cells in the OPL, and their axons contact amacrine cells and RGCs in the IPL.

RGCs first form conventional synapses with amacrine cells between P3 and P20, then develop ribbon synapses with bipolar cells starting at P11, around the time of eye opening, reaching a maximum density around P20. In conjunction with this temporal sequence of synapse formation, waves of electrical and calcium activity propagate across the retina. RGCs exhibit spontaneous bursts of action potentials during the propagation of retinal waves. Retinal waves between P1 and P10 are driven by cholinergic transmission, while subsequent waves are glutamate dependent, correlating with formation of synapses between RGCs and bipolar cells. These retinal waves are implicated in the refinement of ganglion cell projection patterns.

13.2.2 Neural Circuits in the Visual Pathway

In the mammalian retina, 95% of photoreceptors are rods, and the remaining 5% are cones. Rod photoreceptors are more sensitive and thus function under dark-adapted conditions, and their responses saturate at high light levels. Cone photoreceptors are less sensitive and function under light-adapted conditions. Rod and cone photoreceptor

a
diencephalon
optic vesicle
b
epidermis
lens placode
c
optic cup
d
RPE
optic stalk
lens vesicle
neural retina
e
RPE
optic stalk
lens epithelium
neural retina
cornea
f
RPE
neural retina
lens epithelium
cornea
g
RPE
optic nerve
neural retina
lens
cornea
h
RPE
photoreceptor cells
horizontal cells
Muller glia
bipolar cells
amacrine cells
retinal ganglion cells
ONL
INL
GCL
i
Müller glia
Bipolar cells
Rod photoreceptors
Amacrine cells
Cone photoreceptors
Horizontal cells
Ganglion cells
Mouse
E10 E12 E14 E16 E18 P1 P3 P5 P7 P9 P11
Human
6 w 8 w 10 w 12 w 14 w 16 w 18 w 20 w
Retinogenesis

Fig. 13.1 (**a–f**) Development of the eye. Mouse embryos at E9.5 (**a**), E10.0 (**b**), E10.5 (**c**), E11.5 (**d**), E13.5 (**e**) and E18.5 (**f**). (**g**) Adult eyes, (**h**) cell types and layers in the adult retina. *RPE* retinal pigment epithelium; *ONL* outer nuclear layer; *INL* inner nuclear layer; *GCL* ganglion cell layer. (**i**) Genesis of seven types of retinal cells during development. Retinal ganglion cells and horizontal cells differentiate first, followed by cone photoreceptors, amacrine cells, rod photoreceptors, bipolar cells and, finally Müller glia, with overlap in the appearance of these different cell types. The sequence of cell genesis in the vertebrate retina is highly conserved among many species

signaling differs from that of almost every other neuron in the CNS. In the absence of light, the photoreceptors continuously release the excitatory neurotransmitter glutamate. A light stimulus activates the photo-transduction cascade in the outer segment of the photoreceptor, causing a decrease in the level of intracellular cGMP and closure of cGMP-gated cation channels. This leads to hyperpolarization of the photoreceptor, resulting in decreased glutamate release from the photoreceptor terminal in the OPL. Both rod and cone photoreceptors employ this general cascade, although the molecules that function in the G protein cascades differ.

Importantly, photoreceptors do not produce action potentials, but instead have graded potentials that are modulated around a mean level. In most neurons in the CNS, release of glutamate is dependent on voltage-dependent calcium channels. However, the release of glutamate from the photoreceptor is graded, resulting in graded potentials in the bipolar cells and horizontal cells.

Photoreceptors contact the dendrites of both bipolar cells and horizontal cells. The synaptic terminals of rods (rod spherules) and cones (cone pedicules) contain synaptic ribbons that are associated with high rates of neurotransmitter release. Bipolar cells are excitatory inter-neurons that use glutamate as a neurotransmitter and are specialized for sustained transmitter release. Hyperpolarizing bipolar cells hyperpolarize in response to reduced glutamate release from photoreceptors, while depolarizing bipolar cells instead depolarize in response to glutamate. Other cells in the retina are horizontal cells, which are laterally extensive inter-neurons in the outer row of the INL, and amacrine cells, which form a morphologically and physiologically diverse group of mostly inhibitory inter-neurons located in the inner row of the INL. Most amacrine cells use GABA, glycine, or both acetylcholine and GABA as neurotransmitters and provide feedback inhibition to bipolar cell terminals and feed-forward inhibition to ganglion cells. Ganglion cells are physiologically and morphologically diverse, with an estimated 15–20 different types. They can be classified by many physiological and anatomical criteria, including size, response, receptive field, color, ON or OFF, conduction velocity, morphology, blanch pattern, stratification, coupling, and coverage.

Vision is initiated in rod and cone photoreceptors when light is converted into an electrical signal. Visual information is processed in parallel pathways known as the midget pathway, the parasol pathway, the blue-ON/yellow-OFF pathway, and the rod pathway (Field and Chichilnisky 2007) (Fig. 13.2). Cone photoreceptors make synapses to ON or OFF cone bipolar cells, which connect to midget RGCs, parasol RGCs, or bistratified RGCs. Interestingly, the neural circuits in the rod pathway differ from those in the cone pathway (Masland 2001; Wassle 2004). Rod photoreceptors connect to ON rod bipolar cells, which subsequently send the signal to AII amacrine cells. The AII amacrine cells send the information to ON or OFF cone bipolar cells, which synapse to ON or OFF ganglion cells, respectively.

In addition, lateral inhibitory pathways modulate the excitatory signaling in the vertical pathway. First, lateral inhibition occurs in the OPL via feedback from horizontal cells at the photoreceptor-bipolar cell synapse. In the IPL, amacrine cells mediate lateral inhibition via feedback, feed-forward, and serial inhibition. Finally, other pathways also modulate retinal function, including dopaminergic control of light adaptation and gap junctions.

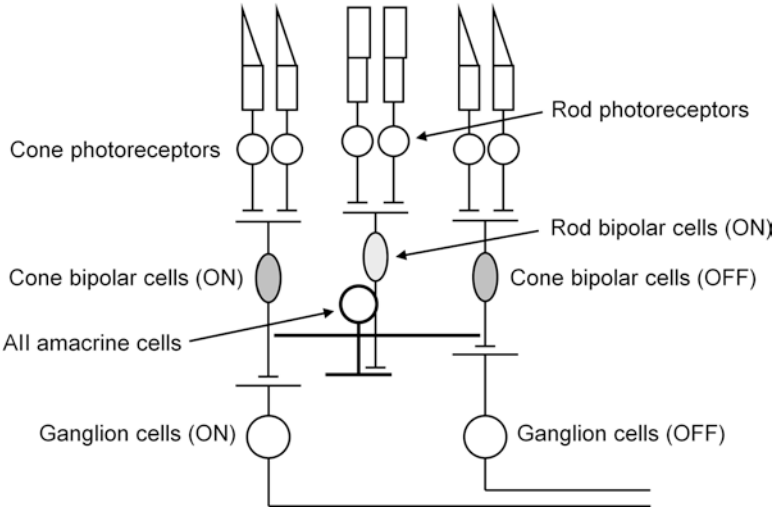

Fig. 13.2 Cone and rod pathway in visual transduction. Cone photoreceptors contact ON and OFF bipolar cells, which in turn contact ON and OFF ganglion cells, respectively. In contrast, rod photoreceptors send input to ON rod bipolar cells, which in turn synapse with AII amacrine cells. ON signals enter the cone pathways via gap junctions between AII amacrine cells and ON cone bipolar cells. OFF signals are produced by a glycinergic synapse between AII amacrine cells and OFF cone bipolar cells

Visual information leaves the eye via the optic nerve, which is composed of RGC axons (Callaway 2005). There are several classes of RGCs that project to the LGN. Midget RGCs comprise about 80% of these neurons and terminate in the parvocellular layers, parasol RGCs make up about 10% and project to the magnocellular layers, and bistratified RGCs, which form synapses in both the upper and lower sublaminae of the IPL, comprise the remaining 10% and project to the intercalated or koniocellular layers. Each class of LGN neurons then projects to a specific subdivision of primary visual cortex. 4Calpha layers in the visual cortex receive inputs from magnocellular neurons, and 4C/3 layers are innervated by parvocellular neurons. Koniocellular neurons project to layers 2 and 3, specifically to regions known as "blobs" that stain densely for the enzyme cytochrome oxidase.

13.3 Retinal Transplantation

13.3.1 Donor Cells for Transplantation

There are several candidate sources for photoreceptor transplantation (Table 13.1). Cells from the developing retina can be used as a donor source for transplantation, but use of human fetal tissue presents ethical problems. Moreover, the quantity of available fetal retinal cells is limited. Thus, we turned to in vitro expansion of retinal cells derived from stem/progenitor cells.

Table 13.1 Potential of various donor cells for transplantation therapy

Cell type	Photoreceptor	Quantity	Integration	Immue rejection	Ethical problem	Tumor formation
Brain-derived neural progenitor	×	△	△	△	△	○
Photoreceptor (P5)	◎	×	◎	△	×	○
Retinal progenitor	◎	△	◎	△	×	○
Ciliary body	○	△	△	△	◎	○
Iris	○	△	△	◎	◎	○
ES cell	◎	◎	◎	○	×	△
iPS cell	◎	◎	◎	◎	◎	×

◎ good; ○ acceptable; △ marginal; × bad

In the rat, adult neural stem cells are present in the subventricular zone of the lateral ventricle and the subgranular zone of the hippocampal dentate gyrus. These stem cells continually generate new neurons that functionally integrate into preexisting neural circuits (Lledo et al. 2006; Zhao et al. 2008a). Neural stem cells also exist in humans, suggesting that they could be harnessed for transplantation therapy. Thus, we examined whether stem cells in the adult rat hippocampus could differentiate into retinal neurons after transplantation into the developing eye (Takahashi et al. 1998). Within 4 weeks of transplantation, the adult stem cells were integrated into the retina and exhibited morphologies and positions characteristic of Müller, amacrine, bipolar, horizontal, and photoreceptor cells. However, none acquired end-stage markers unique to retinal neurons. We concluded that adult brain-derived stem cells cannot adopt retinal fates even when exposed to the cues present during retinal development. Although the brain and the retina are both generated from the ectodermally derived neural tube, neural progenitors in different CNS regions differ in their competence to generate specific types of mature neurons.

We next examined the potential of progenitors in the embryonic retina. Although retinal progenitors from rat fetal tissue can be expanded in vitro, and can differentiate into various types of retinal neurons, they lose their ability to differentiate into photoreceptors following massive expansion (Akagi et al. 2003).

The somatic progenitors in adult eye tissue are another potential source of donor cells. The ciliary marginal zone has been reported to contain stem cells even in adults (Ahmad et al. 2000; Tropepe et al. 2000). When cultured in vitro, these cells give rise to retinal neurons, including photoreceptors. Iris-derived cells have also been reported to generate retinal neurons (Haruta et al. 2001; Asami et al. 2007). Adult tissues have the advantage that they can be used as autografts, which do not cause immune rejection. Autologous iris tissue can be feasibly obtained with peripheral iridectomy. Unlike the hippocampus, both the ciliary margin and iris derive from the optic vesicle and optic cup, suggesting that they may be more competent than brain stem cells to generate retinal neurons. Cells differentiated from adult somatic progenitors in the eye express several photoreceptor marker proteins, but not all the genes responsible for photoreceptor function. Thus, it is likely that the generation of functional photoreceptors requires a recapitulation of the normal process of retinal development.

ES cells are pluripotent cells derived from the inner cell mass of blastocyst stage embryos. They can maintain an undifferentiated state indefinitely in vitro and differentiate into derivatives of all three germ layers: the ectoderm, endoderm, and mesoderm. These characteristics make ES cells an attractive potential donor source for degenerative diseases such as Parkinson's disease, spinal cord injury, and diabetes, as well as retinal degeneration. While somatic progenitors derived from the cilliary body or iris are limited in both differentiation potential and proliferation capacity, human ES cells can generate a large number of retinal cells. Based on our knowledge of embryonic development, we have developed methods of inducing retinal differentiation of ES cells in vitro (Kawasaki et al. 2000; Kawasaki 2002; Mizuseki et al. 2003; Ikeda et al. 2005; Watanabe et al. 2005; Ueno et al. 2006; Osakada et al. 2008).

13.3.2 Retinal Differentiation of ES Cells

During early embryogenesis, the retinal primordium forms in the rostralmost region of the diencephalon expressing Six3. The transcription factor Rx, an early marker of the eye field, plays an essential role in the specification of the retinal primordium within the Six3+ rostral CNS. During early embryogenesis (E10.5), Rx expression coincides with Pax6 expression in neural retinal progenitors, whereas Rx+ cells in the floor of the ventral diencephalon are negative for Pax6. The RPE is Rx− and Pax6+. Thus, the neural retinal lineage during early development is characterized by Rx/Pax6 co-expression. We first attempted to induce Six3+ rostralmost CNS progenitors and Rx+/Pax6+ retinal progenitors by applying exogenous patterning signals. Because the extracellular patterning signals that induce the retinal primordia have not yet been identified, we took a candidate approach to identify soluble factors that induce Rx/Pax6 expression (Ikeda et al. 2005).

In serum-free and feeder-free aggregate culture (SFEB culture), strong expression of Six3 was found on culture day 5, but not in cells cultured with the caudalizing factor RA. The strongest induction of Rx+/Pax+ cells was observed when ES cells were treated with Dkk-1, Lefty-A, FCS, and Activin (referred to as SFEB/DLFA cells). We also tested the effects of Shh, Wnt, BMP4, Nodal, IGF, FGF-1, FGF-2, and FGF antagonists, but observed only marginal effects, if any, on Rx induction in this culture system.

The RPE expresses Mitf, Pax6, and Otx2, but not Rx, during retinal development. We therefore tested whether RPE differentiation was observed in SFEB/DLFA culture by examining the expression of the early RPE marker Mitf. Mitf+ cell aggregates were observed in SFEB/DLFA cultures, whereas SFEB-treated cells rarely expressed Mitf. Consistent with the in vivo expression profile of RPE markers, most Mitf+ cells in the SFEB/DLFA culture were Pax6+ and Otx2+. Thus, we conclude that the SFEB/DLFA treatment preferentially induces differentiation of retinal progenitors from mouse ES cells.

Next, we examined photoreceptor differentiation of SFEB/DLFA-treated retinal progenitors (Osakada et al. 2008). Inhibition of Notch signaling has been shown to

promote photoreceptor differentiation in vivo (Jadhav et al. 2006). In mouse ES cells, purified Rx+ cells expressed Notch (Notch1–4) and its downstream mediators Hes1, Hes5, and Hey1. Inhibition of Notch signaling in these cells by the γ-secretase inhibitor DAPT increased the frequency of Crx+ (cone rod homeobox protein gene) photoreceptor precursors generated from Rx-GFP+ cells. We then examined whether Crx+ photoreceptor precursors that were efficiently generated using DAPT could further differentiate into rod and cone photoreceptors. On day 28, 20–25% of ES-derived cells expressed red/green opsin or blue opsin, cone-specific pigment proteins that are indispensable for color vision. In contrast, fewer cells expressed rhodopsin, the rod-type visual pigment. To optimize conditions for rod differentiation, we next tested several soluble factors that are reported to promote rod differentiation from embryonic or neonatal retinal progenitors (Levine et al. 2000). When acidic FGF, basic FGF, taurine, Shh, and retinoic acid were added, a large number of cells (15–20%) expressed the rod photoreceptor marker rhodopsin.

We have also established a defined culture method for monkey and human ES cells that does not require serum (Osakada et al. 2008). Serum-free, feeder-free suspension culture combined with application of Dkk-1 and Lefty-A induced retinal progenitors positive for RX, PAX6, MITF, and CHX10 on culture days 30–40. Subsequently, the progenitors generated hexagonal pigment epithelium polarized with apical microvilli and basal membranes (Fig. 13.3a, b). The pigment cells expressed RPE-65 and CRALBP, formed ZO-1+ tight junctions, and exhibited phagocytic function on day 100. However, photoreceptor differentiation occurred only infrequently under these conditions. Additional treatment with retinoic acid and taurine significantly promoted differentiation of ES cell-derived progenitors into photoreceptors positive for CRX, NRL, RECOVERIN, RHODOPSIN, BLUE OPSIN, or RED/GREEN OPISN on day 140 (Fig. 13.3c, d). The induced photoreceptors expressed genes responsible for phototransduction.

It is important to note that because xenogenic factors may cause immune rejection following transplantation, the generation of differentiated cells from human ES cells or iPS cells without contamination from animal-derived substances is essential for the clinical application of transplantation strategies (Martin et al. 2005). We have recently established a chemical compound-based method that can induce retinal specification of ES cells and iPS cell. This represents another significant step towards clinical application of retinal transplantation therapy.

13.3.3 Strategy for Photoreceptor Transplantation

The developmental stage of transplanted retinal precursors determines the ability of cells to integrate into diseased retinas. For instance, post-mitotic rod precursors from P3-6 retinas can integrate into normal or degenerating adult retinas following transplantation, whereas proliferating progenitors or stem cells cannot (MacLaren et al. 2006). The grafted P3-6 photoreceptors are able to form synaptic connections and improve visual function. In addition, human ES cells that are committed to

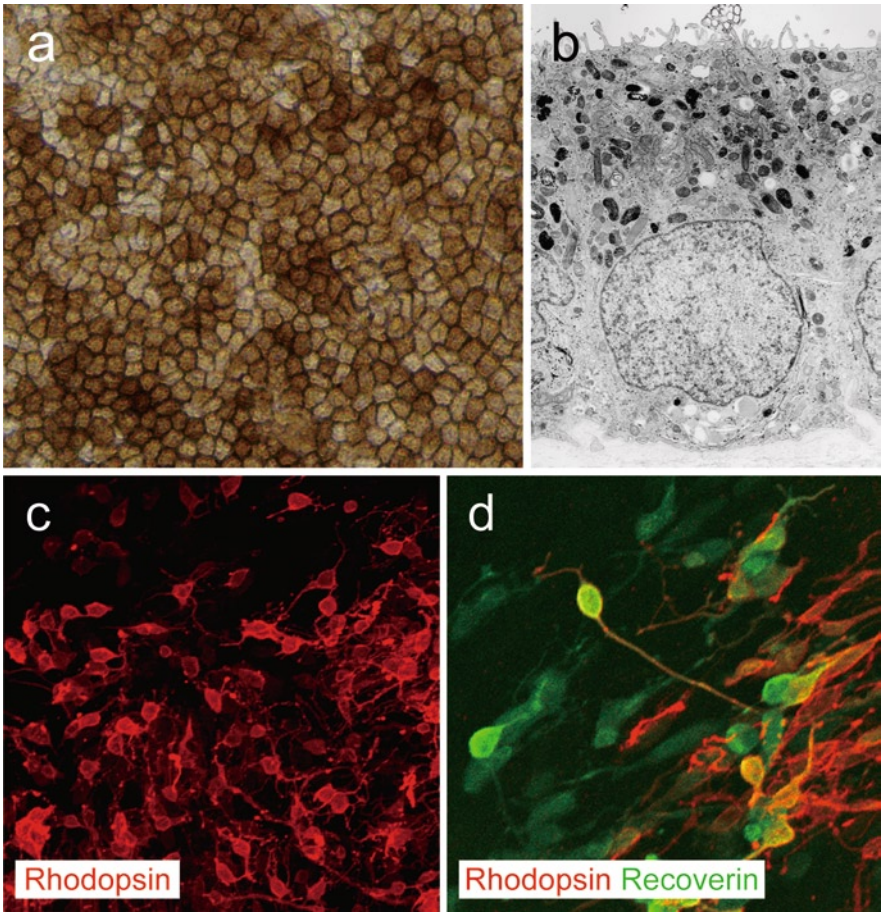

Fig. 13.3 Generation of RPE and photoreceptors from human ES cells. (**a**) Hexagonal pigmented cells derived from human ES cells. (**b**) Electron micrograph of human ES cell-derived RPE. (**c**) Rhodopsin + photoreceptors derived from human ES cells. (**d**) Human ES cell-derived rhodopsin + cells co-express recoverin

retinal fate migrate toward the ONL and differentiate into photoreceptors that appear to make synapses with host bipolar cells (Lamba et al. 2009). Thus, transplanted cells must already be committed to photoreceptor fate for successful integration.

We have been able to differentiate ES cells into RPE cells and photoreceptors under defined culture conditions. However, contamination of transplanted cells with undifferentiated ES cells or iPS cells (see later) leads to tumor formation (Fukuda et al. 2006). Thus, purification to remove undifferentiated cells is required for donor cell preparation. Selection of specific types of ES cell-derived progenitors for transplantation into host mice can be easily achieved using mouse ES cells with knocked-in fluorescent proteins or antibiotic-resistance genes at specific marker

loci. However, knock-in technology is not suitable for human ES cells or iPS cells. Thus, identification of surface antigens marking P3-6 photoreceptors is crucial for further transplantation studies.

Finally, the host environment is also critical for photoreceptor transplantation. Retinal degeneration is characterized by the formation of glial scars and microglial activation, which may impede integration and survival of transplanted cells. Robust integration of transplanted retinal cells into the retina of host mice deficient for both vimentin and glial fibrillary acidic protein has been reported (Kinouchi et al. 2003). Moreover, chondroitinases and matrix metalloproteases-2 that degrade the extracellular matrix in the diseased retina aid in the integration of transplanted photoreceptors (Suzuki et al. 2006, 2007). Disruption of the outer limiting membrane also increases photoreceptor integration following transplantation (West et al. 2008). Therefore, in addition to immunosuppression, the host retinal environment must be modulated for successful transplantation.

13.3.4 Strategy for RPE Transplantation

RPE cells are essential for maintenance of retinal function, particularly for outer segment shedding, supplying nutrients to the retina, and maintaining the blood-retinal barrier. In AMD, RPE cell damage leads to secondary loss of photoreceptors. We have demonstrated that transplantation of RPE cells derived from monkey ES cells can restore visual function in a retinal degeneration model, RCS rats (Haruta et al. 2004). Human ES cell-derived RPE cells have also been shown to be effective for recovery of visual function in RCS rats (Lund et al. 2006).

As is the case for photoreceptor transplantation, tumor formation and immune response must be suppressed for RPE cell transplantation to be clinically applicable. Methods to purify donor RPE cells to exclude undifferentiated cells must be developed, and administration of immunosuppressant drugs may be necessary for allografted human ES cells. However, the exciting possibility of using customized, patient-specific iPS cells differentiated into RPE cells may circumvent this problem.

Finally, improved techniques for effective transplantation need to be explored. For instance, transplantation of RPE sheets rather than cell suspensions may improve functional recovery. In addition, development of specialized surgical tools will be required for human RPE transplantation.

13.4 Advent of iPS Cells

13.4.1 Direct Reprogramming of Somatic Cells

The pluripotency of ES cells has raised the possibility that they might be used to treat various degenerative diseases. However, the clinical application of human ES cell therapy faces ethical difficulties concerning the use of human embryos, as well

as tissue rejection following implantation. One way to circumvent these issues is to generate pluripotent cells directly from somatic cells. Development normally proceeds irreversibly from embryo to adult as cells progressively differentiate into their final, specialized cell types. However, given that adult somatic cells are genetically identical to ES cells, it is theoretically possible to reprogram them to return to the naïve state of pluripotency found in the early embryo. Indeed, somatic cell nuclear transfer (cloning) and fusion of somatic cells with ES cells are able to achieve this feat (Yamanaka 2008; and discussed further in Chaps. 4–6). However, these techniques have technical, ethical, and logistical barriers that preclude the use of the resulting pluripotent cells in both research and therapy. In contrast, direct reprogramming of somatic cells by defined factors would enable the generation of patient-specific cells of any lineage without the use of embryonic material.

In 2006, iPS cells were generated from mouse embryonic and adult fibroblasts by forcing expression of just four transcription factors, Oct3/4, Sox2, Klf4, and c-Myc (Takahashi and Yamanaka 2006). These iPS cells were established by selecting for Fbx15 expression. These Fbx15-iPS cells are similar to ES cells in morphology, proliferation, and teratoma formation, but showed clear differences from ES cells in their global gene expression profile and differentiation potential.

To establish fully reprogrammed cells, Nanog was used to select induced cells (Okita et al. 2007). Mouse embryonic fibroblasts (MEFs) were isolated from Nanog-GFP reporter mice and transgenic *Oct3/4, Sox2, Klf4*, and *c-Myc* were introduced. After a few weeks, ES-like colonies were observed and were selected for GFP expression. Nanog-GFP iPS cells showed ES cell-like morphology and gene expression profiles. These cells also had the potential to differentiate into all three germ layers following injection into nude mice. Moreover, they were able to contribute to the germline of adult chimeric mice and to live late-term embryos when injected into tetraploid blastocysts (Okita et al. 2007). However, approximately 20% of the offspring developed tumors, due to reactivation of the *c-Myc* transgene. Thus, although iPS cells competent for germline chimeras can be obtained from MEFs, retroviral introduction of *c-Myc* should be avoided for clinical application.

Human iPS cells can also be established from adult dermal fibroblasts by introducing the same four transcription factors (Takahashi et al. 2007; Yu et al. 2007). Human iPS cells form flat, tightly packed colonies and exhibit a morphology similar to that of human ES cells, characterized by large nuclei and scant cytoplasm. They express hES cell-specific surface antigens, including SSEAs, TRAs, and NANOG protein. Human iPS cells also show the same growth potential, gene expression patterns, telomerase activity, and epigenetic status as human ES cells. They were able to differentiate into all three germ layers in embryoid bodies and teratomas. Additionally, they differentiated directly into dopaminergic neurons and functional cardiomyocytes in vitro. These data suggested that pluripotent cells could be generated from not only mouse but also human fibroblast culture with the addition of just a few defined factors.

It should be noted that the retroviral transcripts of the four transcription factors are eventually silenced, while the endogenous expression of pluripotent genes is turned on. In fact, differentiation of the iPS cells requires silencing of the exogenous genes (Stadtfeld et al. 2008b). It appears that exogenous factors activate expression

of endogenous genes that regulate pluripotency. While lentiviruses permit the transduction of non-dividing cell types with high expression levels, unlike retroviruses they are poorly silenced in the pluripotent state. At the selection step of iPS cell cloning, it is important to carefully determine whether the exogenous genes have been adequately silenced. Differentiation efficiency, cell types, and tumor formation depend on the particular iPS cell clone. Selection of a genuine iPS cell clone is of critical importance.

13.4.2 Clinical Grade Preparation of iPS Cells

From the therapeutic point of view, direct reprogramming of somatic cells to generate iPS cells provides an invaluable resource for regenerative medicine, enabling the generation of patient-specific cells of any lineage without the use of embryonic material (Hanna et al. 2007; Wernig et al. 2008). Clinical application of human ES cells faces difficulties regarding the use of human embryos, as well as tissue rejection following transplantation. iPS cells have the potential to resolve these problems. However, they present difficulties of their own. For example, the potential for tumor formation due to contaminating, undifferentiated cells remains. In addition, a non-viral establishment method needs to be found, and a chemically defined, pathogen-free culture method for establishment, maintenance, and differentiation of cells is necessary.

The increased tumorigenicity caused by reactivation of the c-Myc retrovirus presented an obstacle for clinical application. However, c-Myc turns out to be dispensable for generation of both mouse and human iPS cells, since Oct3/4, Sox2, and Klf4 can also induce pluripotency. Elimination of c-Myc from the iPS induction protocol sharply reduces tumorigenesis, as measured by cancer-related deaths of chimeric mice derived from iPS cells (Nakagawa et al. 2008). Interestingly, the iPS cells produced by forced Oct3/4, Sox2, and Klf4 expression are more homogenous than those generated by Oct3/4, Sox2, Klf4, and c-Myc expression.

The production of iPS cells by retroviral- and lentiviral-based delivery of the reprogramming factors may result in multiple proviral integration sites, which poses the danger of insertional mutagenesis. To solve this problem, various vectors have been tested for generation of iPS cells. Nonintegrating lentiviruses were used to generate iPS cells; however, integration of the vector was detected in iPS cell clones (Mali et al. 2008). Mouse iPS cells were also generated from fibroblasts and liver cells using adenoviral vectors (Stadtfeld et al. 2008c). These cells were reprogrammed to a pluripotent state without integration of the adenoviral vector, as measured by both PCR and Southern blotting. However, the efficiency of reprogramming was lower than that observed with retroviral infection. Transgene expression had to be maintained for at least 8 days, suggesting that continuous high-level expression of the reprogramming factors may be difficult with adenovirus.

Viral integration into the host genome increases the risk of tumorigenicity. Recently, iPS cells were successfully generated from mouse fibroblasts by transient transfection of plasmids, without using viral vectors (Okita et al. 2008). Repeated

transfection over 7 days of a polycistronic vector carrying *Oct3/4*, *Klf4*, and *Sox2* and a separate plasmid carrying *c-Myc* can reprogram fibroblasts. Interestingly, 2A self-cleaving peptides support efficient polycistronic expression from a single vector. The order of genes in this system is critical, with highest efficiency observed with *Oct3/4*, *Klf4*, and *Sox2* in sequential order. However, forced c-Myc expression in these cells caused tumorigenesis in chimeric mice.

Another strategy to avoid insertional mutagenesis is the identification of small molecules that can take place of the reprogramming transgenes. Indeed, c-Myc and Klf4 can be replaced by the histone deacetylase inhibitor valproic acid, enabling the generation of human iPS cells with transgenic expression of Oct3/4 and Sox2 (Huangfu et al. 2008b). Additional factors can enhance the reprogramming process. For example, Wnt3a (Marson et al. 2008), valproic acid (Huangfu et al. 2008a, b), the histone methyltransferase inhibitor BIX01294 (Shi et al. 2008b), the DNA demethylating agent 5-anacytidine (Mikkelsen et al. 2008), the L-type calcium channel agonist BayK8644 (Shi et al. 2008a), and a combination of p53 siRNA and Utf1 cDNA (Zhao et al. 2008b) can be used to promote iPS cell formation. Because small molecules are safe, cheap, and never integrate into the genome, they seem ideal for clinical applications. Moreover, the replacement of c-Myc function with valproic acid may avoid tumorigenic effects caused by c-Myc expression. However, it is unknown whether small molecules alone can replace all of the transcription factors that are necessary for reprogramming.

Most early studies of iPS cell formation used fibroblasts, since nuclear transfer studies showed that adult fibroblasts are amenable to reprogramming. However, iPS cells have since then also been established from other cell types, including stomach cells (Aoi et al. 2008), liver cells (Aoi et al. 2008; Stadtfeld et al. 2008c), pancreatic beta cells (Stadtfeld et al. 2008a), lymphocytes (Hanna et al. 2008), keratinocytes (Aasen et al. 2008; Maherali et al. 2008), and neural progenitors (Eminli et al. 2008; Kim et al. 2008). These studies indicate that the efficiency and kinetics of reprogramming and the factors required for reprogramming depend on cell type. For instance, stomach and liver cells can be reprogrammed with low-level viral integration (Aoi et al. 2008), and keratinocytes can be reprogrammed more quickly and efficiently than fibroblasts (Aasen et al. 2008; Maherali et al. 2008). Cells obtained from tissues likely to have acquired DNA damage, such as skin cells, might be less suitable for clinical application. Several studies have already reported the successful generation of iPS cells from patients with ALS, muscular dystrophy, and Huntington's disease (Dimos et al. 2008; Park et al. 2008). It appears that older cells and those with mutations can reprogram in the same way as normal, young cells. For clinical use, we must identify target cell types that can propagate easily, can express introduced reprogramming factors, can be reprogrammed efficiently, and can be safely isolated from the human body.

For clinical application of cell transplantation, safety and effectiveness must be evaluated using in vivo animal models. For this purpose, larger animals that are more closely related to humans, such as rabbits, pigs, and monkeys, are useful for preclinical studies. The mechanisms of direct reprogramming seem to be conserved among species; rat and monkey iPS cells have been generated by retrovirus-mediated transduction of the same four factors, Oct3/4, Sox2, Klf-4, and c-Myc, that were used to

generate mouse and human iPS cells (Liu et al. 2008; Li et al. 2009; Liao et al. 2009). Because monkeys are closely related to humans, preclinical studies using monkey iPS cells are key. Autografts and allografts of monkey iPS cell-derived cells will greatly advance our understanding of the safety and effectiveness of transplantation.

13.5 In Vitro Culture of Pluripotent Stem Cells

13.5.1 In Vitro Model for CNS Development

During vertebrate embryogenesis, nervous tissue arises from uncommitted ectoderm during gastrulation. Subsequently, the CNS anlage is patterned to acquire regional specification along the rostral-caudal and dorsal-ventral axes. At an early phase of rostral-caudal specification, the neural tube is subdivided into the forebrain (telencephalon and diencephalon), midbrain, hindbrain, and spinal cord.

In contrast to what is known about the mechanisms of neural induction and CNS regional specification in amphibians, comparatively little is known about these processes in mammals, in part because good experimental systems for in vitro neural differentiation, comparable to the *Xenopus* animal cap assay, are still lacking in mice. Disruption of the *Wnt3* gene in mice is known to cause defects in posterior body structures, while embryos null for the Wnt antagonist Dkk-1 exhibit anterior truncations. In these cases, however, it is not possible to distinguish direct effects on CNS development from secondary effects caused by mesodermal defects.

Mammalian ES cells can differentiate into all embryonic cell types when injected into blastocyst-stage embryos. This pluripotency can be recapitulated in vitro by floating culture of ES cell aggregates, or embryoid bodies (Keller 1995). Embryoid bodies (EBs) frequently contain ectodermal, mesodermal, and endodermal derivatives, but must undergo selective differentiation to generate CNS tissues. We have reported two efficient in vitro systems for neural differentiation of ES cells: the stromal cell-derived inducing activity (SDIA) method (Kawasaki et al. 2000) and the serum-free floating culture of embryoid body-like aggregates (SFEB) method (Watanabe et al. 2005). In these methods, ES cells differentiate into neural precursors at >90% efficiency, while the remaining cells are mostly E-cadherin+, non-neural ectodermal cells. Importantly, mesodermal cells are not detected in these cultures.

In response to exogenous patterning signals such as Wnt, Shh, BMP4, and RA, ES cell-derived neural progenitors differentiate into a wide range of neural cells that correlate with their positions along the dorsal-ventral and rostral-caudal axes (Fig. 13.4). Differentiation of SDIA- or SFEB-treated ES cells in vitro reasonably mimics the natural course of in vivo neurogenesis, as judged by the temporal requirement of patterning signals and the spatiotemporal expression of molecular markers (Mizuseki et al. 2003; Watanabe et al. 2005; Eiraku et al. 2008). Cells characteristic of forebrain, midbrain, hindbrain, and spinal cord tissue could be generated in these cultures (Kawasaki et al. 2000; Mizuseki et al. 2003; Ikeda et al. 2005; Watanabe et al. 2005; Su et al. 2006; Osakada et al. 2008; Wataya et al. 2008).

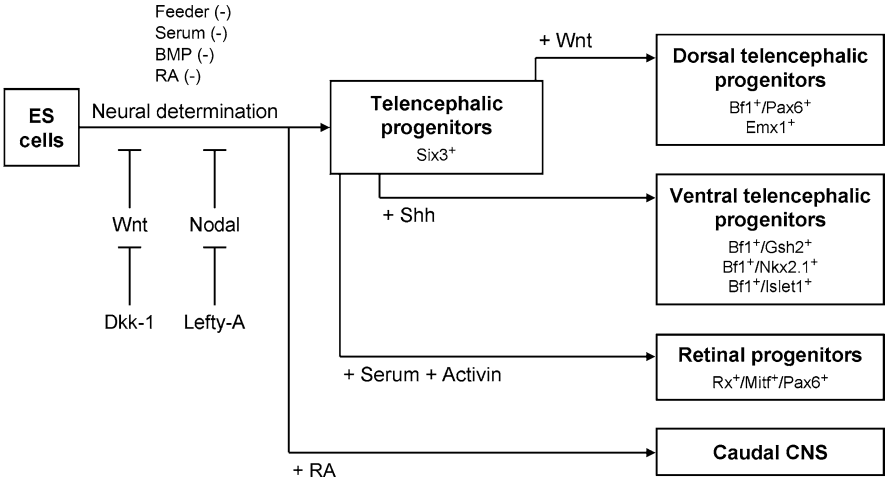

Fig. 13.4 Directed differentiation of ES cells into various neural progenitors in response to patterning signals ES cells differentiate into the rostral-caudal and dorsal-ventral ranges of neuroectodermal derivatives in response to patterning signals. SFEB and soluble factor treatment induce telencephalic, retinal, cerebellar and caudal neural progenitors. *RA* retinoic acid

Thus, SDIA and SFEB culture of pluripotent stem cells serve as in vitro model systems for analyzing neural induction, CNS patterning, and neural differentiation in mammals.

13.5.2 In Vitro Models for Drug Screening and Disease

Stem cell technology has the potential to revolutionize drug discovery, making models available for primary screening, toxicity evaluation, and metabolic profiling. Mouse ES cells are already in use in drug discovery, and high-throughput screening is developing. The availability of human models is highly desirable. Thus, human ES cells and iPS cells will be strong tools for drug discovery (see Chap. 17 for further discussion).

iPS cells can be generated from patients with various diseases and their properties analyzed (Dimos et al. 2008; Ebert et al. 2009; Park et al. 2008). For instance, iPS cells from a spinal muscular atrophy patient exhibit selective cell death of differentiated motor neurons (Ebert et al. 2009). Thus, iPS cells may be used as a tool to study disease mechanisms, to screen drugs, and to develop new therapies.

For genetic diseases, iPS cells provide a new opportunity to analyze the molecular pathways that lead to disease pathogenesis at the cellular level. In addition, although diagnostic analysis of the pathogenic process has been difficult, we should be able to predict whether or not cell degeneration will occur in any given patient by differentiating the patient's iPS cells and examining whether or not they die. Moreover, drug effects detected during clinical treatment can be re-analyzed using iPS cells from patients, permitting personalized optimization of drug treatment.

13.6 Concluding Remarks

iPS cell technology represents a great advance in the field of regenerative medicine. For clinical applications, the combination of reprogramming factors and their delivery method, the choice of target cells, and the identification of genuine pluripotent cells must be carefully optimized. Nevertheless, ES cells are still important for regenerative medicine, since patients with genetic disorders may require transplantation of normal ES cell-derived cells.

At present, the methods developed for transplantation of photoreceptors and RPE are at different stages. RPE transplantation is closer to clinical application, because the FDA has already approved transplantation of RPE derived from human ES cells. Autograft and allograft studies using iPS cell-derived cells will greatly help our understanding of the safety of such transplantations, including rejection and tumorigenesis. Thus, the next step in RPE transplantation research will focus on evaluating safety and effectiveness in monkey and humans as well as the development of surgical methods.

Transplantation of retinal cells from human ES cells has been reported to restore some visual function in a photoreceptor degeneration model, Crx-deficient mice (Lamba et al. 2009), but this study was performed without selection of retinal cells or photoreceptors. For clinical applications, retinal cells must be purified to remove undifferentiated cells. In addition, to promote retinal regeneration, we must elucidate the mechanisms underlying the integration of transplanted cells into preexisting neural networks. Promotion of synapse formation to the correct targets may enhance recovery of function, and a better understanding of the pathological host conditions will be essential for regeneration of retinal function.

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Chapter 14
Reprogramming Towards Pancreatic β-Cells

Kevin Docherty

Abstract Reprogramming towards β-cells is described here in the context of novel cell-based therapies for the treatment of type 1 diabetes (T1D) and potentially type 2 diabetes (T2D). The overall aim is to reverse the decline (T2D) or total absence (T1D) of β-cells as seen in these diseases. Strategies adopted are based on the huge increase in understanding of the developmental biology of the mouse pancreas that has taken place during the past 20 years or so. It is envisaged that the generation of patient-specific iPS cells may prove valuable in understanding the relative contribution of β-cells and other tissues to the causes of the disease. Closely related tissues such as liver and other cells of the pancreas have been shown to exhibit certain plasticity in culture and this has been exploited to facilitate reprogramming using a small number of pancreatic transcription factors, including Pdx1, Ngn3, NeuroD1 and MafA.

14.1 Introduction

The driving force behind the development of strategies aimed at reprogramming towards pancreatic β-cells relates to the demand for novel cell-based therapies as a potential cure for diabetes mellitus. There are two common forms of diabetes, type 1 diabetes (T1D) and type 2 diabetes (T2D), as well as a number of rarer conditions that exhibit diabetes-like symptoms. Both T1D and T2D are associated with a decrease in the number or function of the insulin-secreting β-cells of the islets of Langerhans; in response to a toxic metabolic milieu in the case of T2D or attack from the immune system in the case of T1D (Donath and Halban 2004). There are

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about a million islets dispersed throughout a normal healthy pancreas, comprising ~2% of the total mass of the pancreas. The size of an islet can vary quite markedly, but an average islet would contain about 3,000 cells including β-cells that secrete insulin, α-cells that secrete glucagon, and less abundant δ-cells that secrete somatostatin, PP-cells that secrete pancreatic polypeptide and even rarer ghrelin-secreting ε-cells. The cells of the islet act in concert to control fuel metabolism. Thus, insulin plays a major role in glucose homeostasis through its effects on the uptake and storage of glucose, while glucagon acts as a counter-regulatory hormone through its effects on glucose and fat mobilisation in the fasting state. Somatostatin and pancreatic polypeptide act to fine tune the release of insulin and glucagon.

In healthy adults, β-cells have a long lifespan and proliferate little (Finegood et al. 1995). It is generally accepted that maintenance of β-cell mass occurs mainly through cell replication (Dor et al. 2004), although, more controversially, alternative mechanisms, including neogenesis from putative progenitor cells may also occur (Bonner-Weir and Sharma 2006; Docherty 2009). In T1D, the immune system becomes activated, lymphocytes infiltrate the islets and the β-cells are specifically destroyed through the actions of cytokines and other co-stimulatory molecules. The symptoms of the disease appear when up to 90% of the β-cells have been destroyed. At this point, the damage is irreversible and the further decline in β-cell mass leads to a lifetime dependence on multiple daily insulin injections. The major therapeutic challenges are, in the short term, avoiding unexpected attacks of hypoglycaemia that have a major impact on the daily life of people with diabetes, and in avoiding the debilitating long-term complications of the disease that affect eye, kidney and peripheral nerve function.

Recent successes in islet transplantation (Shapiro et al. 2000), albeit limited (Ryan et al. 2005), have been hindered by a dependence on the availability of cadaveric tissue, and stimulated a drive towards generating a replenishable supply of islets from stem cells. Despite huge advances in the differentiation of embryonic stem (ES) cells towards pancreas, it has been impossible at this stage to generate a fully functional β-cell, i.e. one that secretes meaningful amounts of insulin in response to glucose in the physiological concentration range (Baetge 2008). It has, however, been possible to reproducibly generate pancreatic progenitor cells that can develop further into functional β-cells following engraftment in mice (Kroon et al. 2008). Although this in vivo differentiation step can take up to 10 weeks, it is believed by many that if a safe encapsulation device can be developed, then one could start addressing issues of scale-up with a view towards clinical trials of ES cell-derived pancreatic progenitors.

At the same time, the demonstration that non-β-cells can be reprogrammed into glucose-responsive β-cells has opened up additional therapeutic options, whereby new β-cells, potentially resistant to autoimmunity, could be generated in vivo in people with diabetes who have no residual β-cells. These findings could also be applied to the generation of an exogenous supply of autologous β-cells for transplantation, while the ability to generate patient-specific induced pluripotent stem (iPS) cells may provide important insights into the relative importance of β-cells and insulin-responsive tissue to the development of T1D and T2D (Nishikawa et al. 2008;

Tateishi et al. 2008; Alipio et al. 2010). This chapter will focus on advances in reprogramming of pancreatic and closely related non-pancreatic cells as well as mesenchymal stem cells towards β-cells. Since the strategies involved depend very much on the use of transcription factors that control β-cell development and function, I will first briefly describe the developmental biology of the pancreas with emphasis on the role of those transcription factors that will be discussed later.

14.2 Transcription Factors in the Developing β-Cell

In the mouse, the pancreas is of endodermal origin, first appearing as dorsal and ventral outgrowths at around embryonic day 9.5 (E9.5), in a similar region to the primitive foregut from which the liver also arises (Bernardo et al. 2008). The pancreatic endoderm is marked by expression of the homeodomain transcription factor Pdx1, which along with the basic helix loop helix (bHLH) protein Ptf1a is crucial for pancreatic development (Fig. 14.1). The importance of Pdx1 is emphasised by the Pdx1 knock-out mouse (Jonsson et al. 1994), and humans that are homozygous for mutations in Pdx1 (Stoffers et al. 1997), in which the pancreas fails to form.

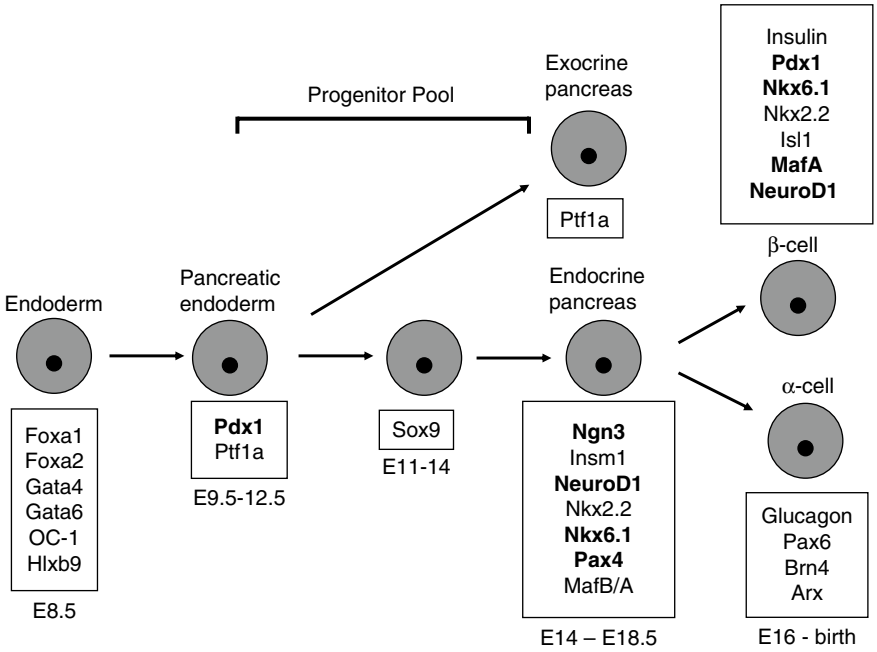

Fig. 14.1 Simplified schematic showing the major stages in the development of the mouse pancreas along with the transcription factors expressed in each cell type. The transcription factors that have played a role in reprogramming are in *bold*

Following a period of expansion, the two lobes of the pancreas fuse at around E13 and individual cells differentiate into acinar (that produce digestive enzymes), duct (that guide secreted pro-enzymes to the duodenum), and centro-acinar (that link acini to the ducts) cells of the exocrine pancreas, as well as cells of the endocrine pancreas. The individual endocrine cells delaminate from the epithelial tubular lattice and cluster to form islets of Langerhans.

Acinar and endocrine cells share a common progenitor after the duct lineage has separated (Gu et al. 2002). The endocrine lineage is determined by expression of the bHLH factor Ngn3 (Gradwohl et al. 2000). Within this lineage, there exists an inhibitory cross regulatory circuit between the transcription factor Arx and the paired domain protein Pax4, with Arx favouring α- and PP-cell formation and Pax4 β-cell formation (Collombat et al. 2007). The bHLH factor NeuroD1 (β2) along with the homeodomain proteins Nkx2.2, Nkx6.1, Pdx1, and the bZIP protein MafA act as β-cell differentiation factors.

14.3 Reprogramming Strategies

There are a number of variables that would determine strategies in terms of therapeutic reprogramming for T1D. One major consideration would be the fact that tissues closer in developmental origin to β-cells, such as pancreas or liver, would presumably be more amenable to reprogramming. On the other hand, if one were aiming for ex vivo reprogramming for autologous transplantation, then accessible tissue such as skin, bone marrow, or fat would be an appropriate starting point. These would also have the advantage that the end-product might function like a β-cell, i.e. express sufficient quantities of preproinsulin, which would be efficiently processed to insulin, stored in secretory granules, and released in response to changes in glucose concentration, whilst lacking many of the features of the β-cell that make it a target for the autoimmune response. It is worth noting in terms of ex vivo reprogramming that an abundant source of exocrine tissue would also be readily available as a by-product of islet transplantation. For in vivo reprogramming, the determining factor would be accessibility of the target tissue, which would make the liver a prime site, especially since this is currently the preferred route of delivery for transplanted islets.

14.4 Reprogramming from Pancreatic Cells

It has been known for some time that cells of the pancreas can exhibit, under certain circumstances, a degree of plasticity (Fig. 14.2). Thus, when adult acinar cells are cultured, they can undergo a process of dedifferentiation, whereby they gain embryonic and ductal characteristics (Rooman et al. 2000). When treated with the glucocorticoid analogue dexamethasone, these dedifferentiated cultures adopted a

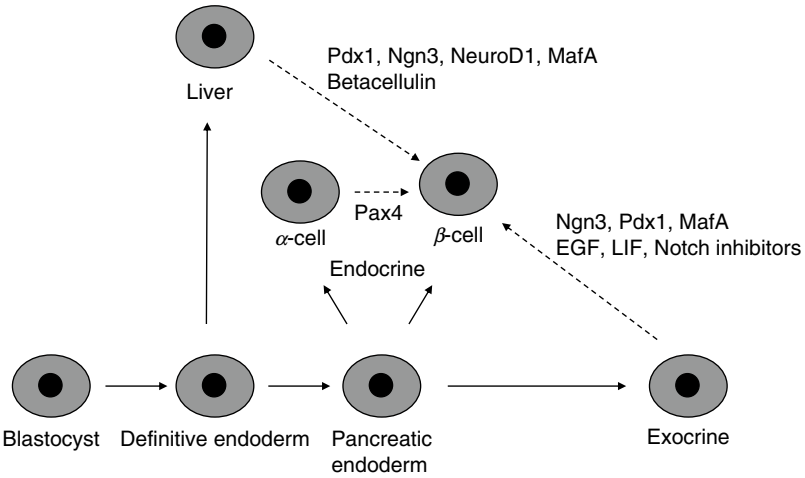

Fig. 14.2 Schematic depicting the various approaches for reprogramming towards β-cells. As described in the text the liver and pancreas are derived from a similar region of the foregut endoderm. The pancreatic endoderm then gives rise to exocrine and endocrine tissue. The β- and α-cells appear to arise from separate lineages although there is some debate about this. The transcription factors and growth factors that have been used either in reprogramming or shown to be involved in the plasticity of liver, α-cells and exocrine cells towards β-cells are indicated (*broken arrows*)

hepatocyte-like phenotype (Lardon et al. 2004) through a process that involved activation of C/EBP-β (Shen et al. 2000), whilst a combination of EGF and leukaemia inhibitory factor (LIF) induced formation of β-like cells (Baeyens et al. 2005). There is indirect evidence that acinar to β-cell conversion can also occur in vivo in rodents in response to injury to the pancreas; however, genetic lineage tracing experiments using amylase or elastase-driven Cre recombinase show conflicting results. Whilst amylase-Cre mice underwent acinar to β-cell conversion in mice treated with EGF and nicotinamide (Minami et al. 2005), the elastase-Cre mice showed that acinar cells give rise only to acinar cells using several models of pancreas injury (Desai et al. 2007). Further evidence of plasticity within the pancreas comes from the identification of Ngn3-postive cells in adult mouse pancreatic ducts, and the presence of stellate cells expressing stem cell markers in explant cultures of rat pancreas. The Ngn3-positive duct cells could undergo a process of neogenesis to form new islets following pancreatitis induced by duct ligation (Xu et al. 2008), although the role of this process in the maintenance of β-cell mass is the subject of some debate (Kushner et al. 2010; Solar et al. 2009). The stellate cells could be isolated using drug selection, and when treated with a cocktail of growth factors, these cells differentiated into β-like cells that expressed insulin (Mato et al. 2009).

Further studies showed that mature endocrine cells, and in particular α-cells, may exhibit a similar degree of plasticity. Thus loss of β-cells in developing mouse embryos, through timed inactivation of Pdx-1, leads to increased α-cell hyperplasia and the appearance of cells that co-express insulin and glucagon (Gannon et al. 2008). More recently, a mouse model, in which total or partial removal of β-cells

could be achieved by administration of diphtheria toxin, was used to show that 99% ablation of β-cells in adult mice resulted in overt diabetes. If these mice were kept alive using exogenous insulin, then β-cell regeneration ensued through transdifferentiation of mature α-cells as confirmed by genetic lineage tracing (Thorel et al. 2010). Pax4 may be involved in this process, as demonstrated using transgenic mice in which Pax4 was conditionally and ectopically expressed using a number of pancreas-specific promoters. This single transcription factor could force putative endocrine precursor cells or mature α-cells to respecify towards β-cells. In fact this redifferentiation of α-cells following ectopic expression of Pax4 was sufficient to restore functional β-cell mass and cure diabetes in animals treated with the β-cell toxin streptozotocin (Collombat et al. 2009). It will now be of interest to see whether viral delivery of Pax4 to α-cells of streptozotocin diabetic mice can also restore β-cells and normoglycaemia. Because abundant numbers of α-cells are present in the islets of people with T1D, they could potentially represent a target for Pax4 viral-mediated in vivo reprogramming. However, α-cells play an important role in counter balancing the hypoglycaemic effects of exogenous insulin and the technical problems of maintaining the correct ratio of α-cells to reprogrammed β-cells might be prohibitive. On the other hand, the identification of this plasticity between α-cells and β-cells should lead to a search for the environmental factors involved in effecting this cellular transition.

Acinar cells have been reprogrammed to β-cells through a specific combination of three transcription factors, namely Ngn3, Pdx1 and MafA (Zhou et al. 2008). The approach used was similar to that in the original iPS study (Takahashi and Yamanaka 2006). Nine transcription factors, that exhibited β-cell developmental defects when mutated, were delivered using adenoviral vectors into the pancreas of immunocompromised adult mice. One month later, the pancreases were examined by immunocytochemistry for extra-islet insulin positive cells. Individual transcription factors were then removed from the pool one at a time. Removal of Nkx2.2, Nkx6.1 and Pax4 (surprisingly given its role in α-cell reprogramming) had no effect on the number of insulin positive extra-islet cells. Within the remaining pool of six, Ngn3, Pdx1 and MafA were absolutely required. NeuroD1 could partially substitute for Ngn3 but single or combinations of two factors had no effect. Reprogramming was relatively rapid with insulin positive cells first appearing at day 3 after viral delivery. Lineage tracing confirmed that the new β-cells originated from acinar cells and that this was through direct conversion rather than via a process of dedifferentiation and redifferentiation. Importantly, the new insulin positive cells remained insulin positive after loss of the virally provided factors. They exhibited many of the properties of β-cells and were able to partially reverse the symptoms of diabetes in streptozotocin-treated mice. This important study may have huge implications for treating diabetes, not so much through in vivo reprogramming because of the risk of inflammation and resultant pancreatitis, but more because of its role in ex vivo reprogramming. At the moment, multiple consecutive islet transplants are required to reverse the symptoms of diabetes. During the islet isolation procedure, ~98% of the tissue is discarded. If this abundant source of acinar cells could be efficiently reprogrammed, then it could be stored and used as a plentiful supply of "top-up" cells that could be given to the islet recipient as required.

14.5 Reprogramming from Liver Cells

The liver is viewed as a potential candidate for reprogramming because of the close developmental similarity and mutual plasticity between the liver and the pancreas. Liver and ventral pancreas arise from adjacent regions of the endoderm and become established through FGF and TGFβ signals emanating from the mesoderm (Wandzioch and Zaret 2009). The plasticity from pancreas to liver is seen in regenerating pancreas of hamsters, in copper depletion-repletion rats and in transgenic mice overexpressing KGF under the control of the insulin promoter (Krakowski et al. 1999; Rao et al. 1986; Scarpelli and Rao 1981). The conversion of liver to pancreas has been observed in transgenic mice and Xenopus in which Pdx1, or Pdx1VP16, a chimaeric protein that contains a strong transcription activation domain from the VP16 protein of Herpes simplex, has been ectopically expressed using liver-specific promoters (Horb et al. 2003; Miyatsuka et al. 2003).

Ferber was the first to show that adenoviral mediated administration of Pdx1 to the liver of streptozotocin diabetic mice resulted in expression of insulin in the liver at low levels, but in sufficient quantities to reverse the symptoms of diabetes induced by streptozotocin (Ferber et al. 2000). In this model the reprogrammed liver cells were resistant to both β-cell toxins (Ber et al. 2003) and autoimmune attack (Sapir et al. 2005). The expression of Pdx1 in the reprogrammed cells persisted well beyond the few days during which the adenoviruses were active, presumably because Pdx1 was able to regulate its own expression (McKinnon and Docherty 2001) and establish regulatory networks leading to long-term (up to 8 months) ectopic insulin production and normoglycaemia in diabetic animals (Ber et al. 2003). Subsequent studies showed that ectopic expression of Pdx1 alone might not be sufficient to induce insulin-secreting cells in the liver and that hyperglycaemia induced by streptozotocin along with partial hepatectomy leading to liver regeneration increased the efficiency of the process (Koizumi et al. 2004).

A major problem, however, with using Pdx1 as a reprogramming agent is that it functions as a master gene for pancreas development and not only produces insulin-secreting cells but also exocrine cells. Thus, although transfer of adenoviral vectors containing Pdx1 to the liver of streptozotocin diabetic mice lowered blood glucose levels, the newly generated acinar cells in the liver produced digestive enzymes that led to auto-digestion, hepatitis and death (Kojima et al. 2003), a result that was in accord with the studies in transgenic mice in which Pdx1 was expressed in the liver under the control of the albumin promoter (Miyatsuka et al. 2003). However, in the same study, streptozotocin-induced diabetes could be partially reversed by adenovirus-mediated delivery of NeuroD1, and completely reversed by a combination of NeuroD1 and Betacellulin (a member of the EGF family of growth factors) without producing hepatitis (Kojima et al. 2003). Inclusion of MafA in the transcription factor cocktail appears to enhance reprogramming (Kaneto et al. 2005), which is consistent with a role for MafA in the terminal stages of differentiation. More recently, reversal of streptozotocin-induced diabetes in mice has been achieved by adenoviral-mediated delivery of Ngn3 to the liver (Yechoor et al. 2009a), with enhanced effects using Ngn3 in combination with Betacellulin (Yechoor et al. 2009b).

The reprogrammed cells exhibited many of the hallmarks of functional β-cells and formed neo-islets that produced the four major islet hormones. They appeared to arise from hepatic oval cells, a term loosely used to describe a heterogeneous population of activated liver progenitors capable of multiple liver lineages (Duncan et al. 2009).

In summary, rare progenitor-like cells in the liver, possibly located within the biliary duct epithelium (Eberhard et al. 2008) can be programmed to functional β-like cells, and in some cases neo-islets, using combinations of Pdx1, NeuroD1, Ngn3 and MafA. There are, however, major discrepancies between the published studies suggesting that additional parameters such as mouse strain and method of delivery (Wang et al. 2007) warrant more detailed investigation.

14.6 Reprogramming from Mesenchymal and Other Stem Cell Populations

There are a number of studies demonstrating that mesenchymal stem cells isolated from several sources, including bone marrow (Moriscot et al. 2005; Karnieli et al. 2007) and adipose tissue (Timper et al. 2006; Lin et al. 2009), can be induced to express β-cell markers following treatment with growth factors or ectopic expression of Pdx1 (Limbert and Seufert 2009). In some cases, the reprogrammed cells have been shown to reverse diabetes in animal models. Cells present in the umbilical cord (Denner et al. 2007; Sun et al. 2007; Parekh et al. 2009) and amniotic fluid (Gage et al. 2010) have also been reprogrammed towards β-like cells. More controversially, blood mononuclear cells have been reprogrammed into hepatocyte-like and islet-like cells (Ruhnke et al. 2005), while bone marrow-derived haematopoietic cells (HSCs) have been shown to differentiate into insulin positive cells in diabetic mice models (Ianus et al. 2003). These latter studies have been difficult to replicate and probably reflect local HSC effects on islet regeneration (Hess et al. 2003), thus emphasising some of the limitations of the streptozotocin-diabetic mouse model.

14.7 Vectors

At present, the vectors commonly used in reprogramming towards β-cells are either adenovirus or adeno-associated virus (AAV). AAV is a single-stranded DNA virus, which unlike adenoviral vectors does not elicit a significant inflammatory response, making it a particularly promising vector for in vivo reprogramming. Several novel AAV serotypes have been identified each with different tissue specificities. In terms of in vivo reprogramming of acinar cells it is particularly interesting that serotype 5, which was previously shown to transduce cultured human pancreatic islets (Flotte et al. 2001), was capable of transducing pancreatic acinar cells, with very little expression in endocrine cells, when injected in the pancreas of mice (Wang et al. 2004).

However, a major problem with the use of viruses is the observed toxicity that can occur after systemic administration. This has been addressed to some extent with the use of helper-dependent adenoviruses, which lack all adenoviral genes and thus do not cause chronic toxicity, whilst retaining long-term expression when delivered locally to the liver. Nevertheless, there is a requirement for non-viral approaches to reprogramming such as the use of transcription factors that have been modified to contain protein transduction domains (PTDs), comprising short sequences of cationic amino acids. PTD-containing proteins are rapidly taken up into cells at concentrations as low as 100 nM. The mechanism of uptake is not completely understood but appears to occur through strong electrostatic interactions between the PTD domain and negatively charged heparin sulphate chains of membrane-bound proteoglycans followed by micropinocytosis and release of the PTD-containing protein into the cytoplasm. The most commonly used PTD is the 11 amino acid peptide derived from the TAT protein of the TAT/HIV transactivator protein (Gump and Dowdy. 2007). Administration of a TAT/Ngn3 fusion protein to cultured embryonic day (E) 9.5 and E13.5 pancreatic explants resulted in efficient uptake and nuclear localisation and an increased level of endocrine differentiation compared to control samples (Dominguez-Bendala et al. 2005). Interestingly, PTD domains are present in Pdx1, Ngn3, NeuroD1 and Pax4. The antennapedia-like PTD of Pdx1 has been most extensively characterised in studies in which it was shown to facilitate uptake of Pdx1 into ductal and islet cells (Noguchi et al. 2003). The clinical importance of these findings was further emphasised by a proof of principle study in which intraperitoneal injection of recombinant Pdx1 into streptozotocin diabetic mice restored euglycaemia through a combination of β-cell regeneration and liver reprogramming (Koya et al. 2008).

14.8 Mechanisms Involved in Reprogramming Towards β-Cells

Activation of EGF receptor-mediated signalling appears to be important in the in vitro transdifferentiation of pancreatic acinar cells into insulin-secreting β-like cells through pathways that involve PI3-kinase and the establishment of E cadherin-mediated intercellular junctions (Minami et al. 2008). Thus when spheroid formation in isolated acinar cells was inhibited by using a neutralising antibody against E-cadherin, induction of genes specific for pancreatic β-cells was significantly suppressed, whilst transcription factors associated with early pancreatic development such as HNF6 and Foxa2 remained up-regulated. Notch signalling may also be involved in the dedifferentiation process as seen in in vitro cultures of rat acinar cells (Rooman et al. 2006), possibly acting through its inhibitory effects on Ngn3. In similar studies, inhibition of Notch signalling resulted in efficient reprogramming of approximately 30% of acinar to β-cells that were able to correct glycaemia in immunodeficient mice rendered diabetic following treatment with alloxan (Baeyens et al. 2009). These results suggest that γ-secretase inhibitors that act on Notch signalling pathways, and which have been used for some time in the in vitro

differentiation of ES cell towards β-cells (D'Amour et al. 2006), may also have a role in reprogramming of acinar cells for clinical purposes. Finally, a number of targeted gene knock-out and misexpression studies in transgenic mice have implicated the tumour suppressor gene Men1 (Lu et al. 2010), c-myc (albeit reprogramming towards adipocytes) (Bonal et al. 2009) and Akt/PKB signalling (Elghazi et al. 2009) in regulating acinar cell plasticity.

14.9 Conclusions

The detailed understanding of the developmental biology of the murine pancreas that has come about in the past 20 years, combined with more recent breakthroughs in iPS technology and cellular reprogramming, has laid the foundation for recent attempts to develop novel cell-based therapies for T1D, and in the long term T2D. The ability to reprogramme accessible tissue such as skin fibroblasts from patients with T1D or T2D towards β-cells, using protocols that have been optimised on human ES cells, could theoretically provide a supply of autologous β-cells that

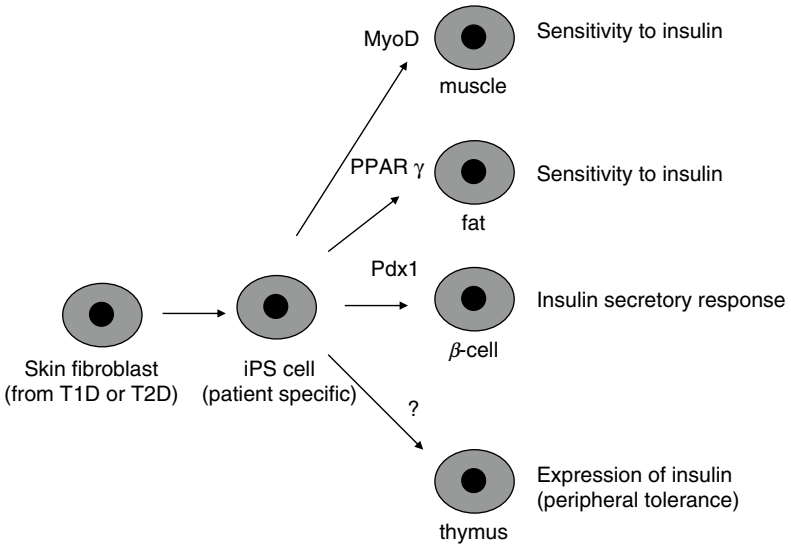

Fig. 14.3 Schematic showing the potential use of reprogramming in generating disease specific tissues. Most of what we know about T1D and T2D comes from measuring hormones and metabolites in the blood of patients infused with various reagents or from examining pathological specimens. However, it is difficult with these tests to differentiate between effects of the immune system and β-cells in T1D, and the β-cell and insulin target tissue in T2D. By generating patient specific tissues using available differentiation protocols based on the expression of key transcription factors for pancreas (Pdx1), fat (PPARγ), muscle (MYOD and other myogenic regulators), and in time from thymus (once key regulators have been identified), important insights into the aetiology of the disease can be expected

could be used for transplantation in the absence of immunosuppression. In reality, however, such individualised medicine could not be made cost effective for the huge numbers of people with diabetes. On the other hand, a cell bank containing a limited number of MHC-homozygous iPS cells could have important therapeutic applications for a wide number of diseases (Taylor et al. 2005). Until such a bank is created, it is more likely that patient-specific iPS cells will be used to help elucidate the role of β-cells and other tissue in both T1D and T2D in the absence of confounding immune and environmental effects (Fig. 14.3).

The most important implication of this new technology is likely to be in reprogramming (or regenerating) β-cells from liver or pancreas. Huge progress has been made in recent years on cellular plasticity but further work is needed on developing new alternatives to viral-mediated transduction and in understanding the mechanisms involved. One of the major current problems with islet transplantation is the requirement for multiple transplants and the poor performance of the graft over time. I therefore see the redundant acinar tissue generated during the islet isolation procedure as a prime target for ex vivo reprogramming. These newly derived insulin-secreting cells could be stored and used as a patient-specific top-up in the early months following the transplant and potentially over many years to maintain or replace the function of the original islet graft.

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Chapter 15
Pancreatic Plasticity and Reprogramming: Novel Directions Towards Disease Therapy

Spencer Willet and Chris Wright

Abstract Generating new β-cells de novo, or regenerating them in vivo, as a cellular therapy, is becoming more tenable as a realistic goal for treatment of the devastating disease of diabetes. One suitable approach towards generating β-cells is to use combinations of instructive and permissive cues to induce the transdifferentiation of terminally differentiated cell types into functional β-cells, which is called reprogramming. Interesting cellular transformations reported recently during development and regeneration suggest that a wide variety of differentiated cell types in the human body may be amenable to such reprogramming. In this chapter, we highlight findings and potential approaches using cell types that are developmentally related to β-cells, and the known molecular players that may be used to control β-cell-directed transdifferentiation. Further investigation with genetic model systems, coupled with the translation to clinically sound reprogramming methods, could lead to efficient reprogramming of specific cell types, with a massive impact on human health.

15.1 Introduction

The remarkable ability of adult human fibroblasts to be transformed into cells with the qualities of embryonic stem cells (Takahashi et al. 2007) immediately raises the question of whether the developmental clock of all adult differentiated cell types can be rewound, taking them back to full pluripotency. But, it also leads to increased conjecture on the plausibility of relatively easily engaging a regulated transformation, with a therapeutic direction, of terminally differentiated cell types into related

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or unrelated cells. As we shall argue below, transformation between cell types within the same organ might be relatively easy compared to other transitions, because of a lineally related closeness in their "inherent control features," fundamentally related to similarities in their epigenomic imprint. We should be open minded as to whether or not all differentiated cell types can be shifted into a state of enforced plasticity, rather than all the way back to the full-blown iPS (induced pluripotent stem cell) condition. We furthermore need to be aware of novel ways of recognizing and tracking plasticity, and of turning it on and off at will. In some organs, with the pancreas being a prime example, some cell types (that is, acinar cells) predominate to such an extent that reprogramming only a small fraction of them to an endocrine β-cell fate would not harm overall digestive enzyme production, but it could restore β-cell mass to functional levels. (An alternative argument is that it would be easier to undergo enzyme supplementation than provide the more critical function of insulin in a tightly controlled manner.) With these propositions in mind, we will in this chapter discuss issues arising related to the recently published reprogramming to insulin-expressing cells from acini (Zhou et al. 2008), which, although they are lineally related "cellular cousins," raise the possibility that similarly successful β-cell production might be obtained with the more easily accessible fibroblasts. We know that fibroblasts, after all, can be redifferentiated relatively easily to form neurons and cardiomyocytes in culture (Ieda et al. 2010; Vierbuchen et al. 2010). We also will cover the interconversion between endocrine cell types of the islet of Langerhans (for example, α to β cell), as well as the more general topic of endoderm to pancreas reprogramming. Questions that are common to reprogramming in vivo and directed differentiation in vitro include whether it is better to aim for the restoration or production of β-cells, or of more complete islet organoids. The latter may function better in the fine-level homeostatic regulation of blood glucose, while the former may be easier to produce in "pure populations," which could at least be used as a stop-gap therapy on our way to a more complete restoration of full pancreatic endocrine function.

The pancreas is an endoderm-derived glandular organ that secretes digestive enzymes into the duodenum (exocrine function), or hormones into the blood to regulate blood sugar levels and fuel storage and use (endocrine function). Diabetes (type I or II) is caused by the loss or dysfunction of the insulin-secreting β-cells, one of the critical component cell types of the endocrine islet of Langerhans. There is either profound β-cell autoimmune destruction (type I) or "β-cell exhaustion" stemming from an incapacity to keep pace with a massively reduced insulin sensitivity in the other organs and peripheral tissues (type II). The most widely applied treatment for the disease, which largely works well, is dose-controlled insulin therapy by injection or pump. Recently, however, a number of patients have experienced at least temporary almost complete remission by the transplantation of islets procured from cadaveric sources (for example, see Shapiro et al. 2000). Diabetes is being increasingly targeted as a disease open to treatment by cellular therapy arrived at via the directed differentiation of ESC, or controlled reprogramming in vivo, because it is fundamentally caused by an inability of a single cell type, the β-cell, to keep up with the body's demands for insulin. The success of islet transplantation indicates the intrinsic merit of this approach. There are, however, several caveats. First, transplantation regimens

include longstanding administration of immunosuppressants, which is bad enough in itself, but some of the drugs that are used were recently found to affect β-cells adversely (Nir et al. 2007). Second, the transplanted islets may not last that long, requiring a return to insulin dosing or additional transplantation (see, for example Ryan et al. 2001, 2005) and, third, there is a very limited cadaver supply stream. A clean and scalable resource would be desirable, hence the extreme interest and large funding towards methods for generating islets or β-cells in vitro, using directed differentiation from ES or iPS cells, and the allied upsurge in interest in potentially reprogramming cells directly within the patient. Patients with type 1 diabetes may still require development of an immunosuppressive regimen or a method of protecting the transplanted cells from immune attack, but this concern might be less important in type II diabetes, especially with autologous sourcing as will be described below.

Forward programming (ES-cell directed differentiation), which essentially attempts to recapitulate the entire normal process of pancreas organogenesis and endocrine cell formation in vitro, still has significant hurdles. Normal differentiation moves along a program of still poorly understood and potentially complicated intercellular interactions within hard to define and fairly dynamic cellular niches. Therefore, the compositions, sequence and timing of application of the various cocktails of inducing factors are unlikely completely correct and, moreover, there could well be three-dimensional cell-cell, or even cell-matrix, associations that it is hard to know how to reproduce in vitro. The same concerns apply even to the promising reports of differentiation induced in response to small molecules that were plucked from chemical library screening (Borowiak et al. 2009; Chen et al. 2009). For example, the most successful ESC differentiation (D'Amour et al. 2006) still unfortunately leaves the final endocrine cells formed as present in small numbers, immature and essentially non-glucose-responsive. Human ES cells undoubtedly can turn into β-cells, as evidenced from transplantation and maturation of ESC-derived prepancreatic-stage cell clusters into mice, which led to the formation of islet cells with excellent glucose responsiveness (Kroon et al. 2008). With respect to reprogramming in vivo, specific cell types could present unique challenges with respect to the cell-targeting methods and the factors required to reprogram them into, in our case, pancreatic β-cells. Even if the target cell is developmentally closely related to the β-cell, beginning as, say, an endocrine α-cell or exocrine acinar cell, it is unknown if they will be, finally, the most appropriate for generating totally authentic β-cells. In addition, we must develop ways to apply any chosen reprogramming technology in a clinically relevant manner, meaning that it is clean, precise and free of side-effects. When thinking deeply about the developmental and gene-regulatory networks that control reprogramming and that are associated with producing a fully functional normal β-cell, exciting issues include the nature of epigenomic priming as it relates to cell fate choice and maintenance, as well as the induction of the cellular niches that are involved in the birth and maturation of this cell type. There are also possible roles for community effects between developing endocrine cells that occur during the assembly of multicellular islets (Fig. 15.1), and intercellular interactions with the pancreatic vasculature and neurons. Ultimately, from a restorative angle, one can

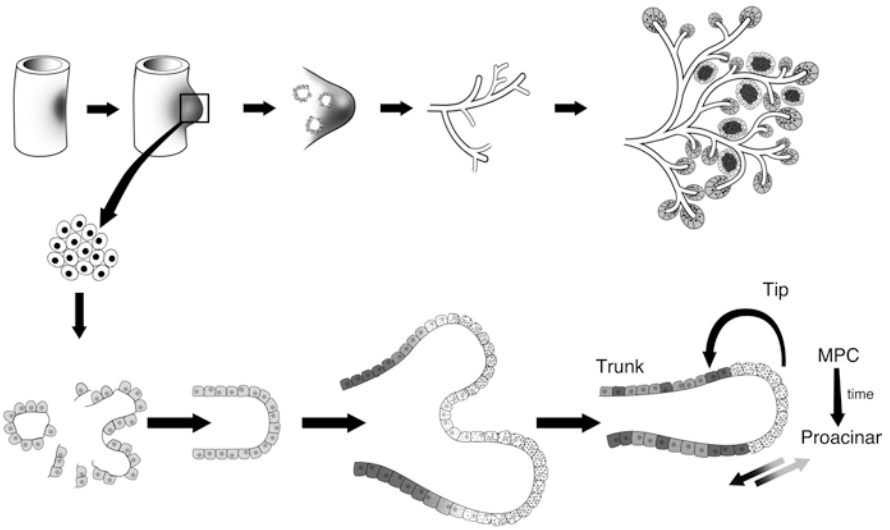

Fig. 15.1 Model for pancreas development. Only one of the pancreatic buds from the foregut endoderm is shown, as a localized bulge of progenitors that represent the pancreas primordium. This unpolarized epithelium expands somewhat in size, until at about 11.5 days of gestation (E11.5), certain cells initiate apical polarization and begin to recruit surrounding cells to form multiple microlumens. Microlumen coalescence pulls essentially all pancreatic progenitors into a common assembly of a tubular epithelial arbor. The final structure after differentiation will have clusters of acinar cells capping the termini of the increasingly more finely branched ducts. Centroacinar cells lie at the duct/acini interface. Islets of various sizes (five shown here) are distributed throughout the organ. Islet endocrine cells account in mice for approximately 2% of the pancreatic volume. Insulin (β) cells are in the core of the islets, with glucagon (α), somatostatin (δ), ghrelin (ε), and pancreatic polypeptide (PP) cells, in variable fractions around the islet periphery, forming a mantle. In a more detailed view (*bottom row* of the schematic), a cluster of progenitors initiate lumen formation and conjoin into a single epithelial branch, which then undergoes progressive, rapid compartmentalization into tip and trunk domains, with different final-fate competencies. MPC at the tips proliferate, driving branch outgrowth into adjacent mesenchymal stroma, and leaving in their wake (*large curved arrow*) progenitors with combined competence for endocrine and duct fates. Initially there may be substantial "softness" in the tip/trunk compartment boundary, suggested here by the transitional zone with overlapping trunk (*gray*) and tip (*stippling*) character. Not indicated here is a putative progenitor heterogeneity that we propose exists within the early anlage (the early "solid" bud stage), with cells having greater tip or trunk predispositions, that could facilitate assembly of an oriented tip-trunk architecture. MPC activity is maintained for some time in the tip region, but over time these MPC evolve to a proacinar state, before moving onwards to a final acinar fate. There may be stage-dependent plasticity between tip and trunk character in cells at the border region, shown here as a "plasticity equilibrium" (*bidirectional shaded arrows*), before cells adopt a complete trunk quality more definitively. In mouse pancreas organogenesis, tip cells lose their MPC quality and commit to the acinar lineage by embryonic day 14.5. Endocrine cells emerge only from the trunk region. Their specification is followed by delamination and congregation into islets; cells left behind form the mature duct epithelium

ask about the completeness of reprogramming to be evoked: does one need an absolutely complete β-cell, in every nuanced aspect, or can we make do with cells that are "good-enough" in their ability to sense glucose, release insulin, and thereby restore euglycemia.

15.2 Pancreas Development

It may not be necessary to understand all aspects of β-cell development in vivo to achieve the goal of generating functional cells. In fact, one hope is that unbiased chemical library screens may provide short-cuts through the process, and such molecules might have greater penetration and precision of dose control, which might increase the efficiency of pushing large numbers of cells through specific steps of differentiation. These are active areas of research, with many possible outcomes. However, a tenable position is that determination of the most important instructive steps (Fig. 15.2), or nodal points, where railroad-switching-type events control the flux down subsequent cell differentiation tracks, together with learning how and when to apply developmentally relevant factors, will facilitate the factory-style production of unlimited supplies of mature β-cells. One concern is the degree to which we may be inappropriately limited if we believe strongly that human pancreas development follows exactly the same rules as in the mouse, and greater access to and definition of the developmental process in humans is required. (One method might be the xenotransplantation of human fetal pancreas fragments into humanized mice and dissecting the endocrine ontogenic process therein.) Even with this caveat, in this review, our focus on mouse pancreas development is warranted and pragmatic,

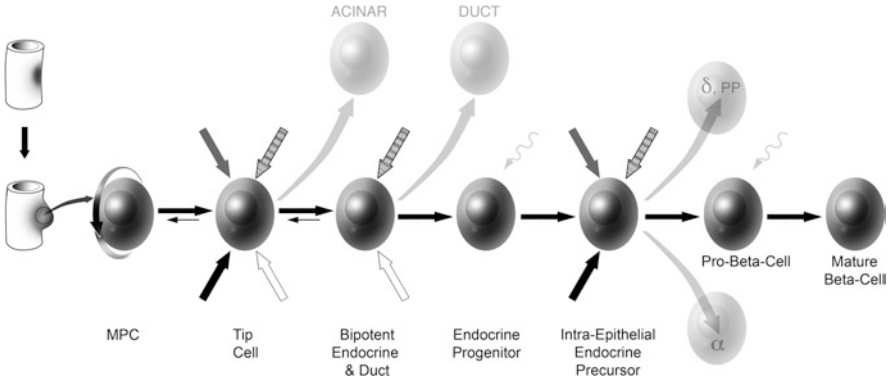

Fig. 15.2 Pancreatic endocrine cell derivation: a hypothetical program of instruction and self-assembly. β-cell formation is envisioned as a series of choices and facilitated steps that lead progenitors down the β-cell track. MPC stem-cell-like self-replicating behavior (*circular arrow*) occurs within the "pancreas-allocated" progenitors in the initial pancreatic anlage (Fig. 15.1), with *equilibrium arrows* indicating the transition to MPC behavior, which then becomes "compartmentalized" to cells in the tip region. Decisions taken in response to several extrinsic influences (intercellular signals, adhesion and matrix interactions, diffusible factors; differently *shaded block arrows*) instruct movement forward to a bipotent endocrine/duct state, while remaining tip MPC cells eventually alter their transcription factor code and move towards the acinar fate. Specific signals again instruct the formation of endocrine cell competence; here conceptualized as two of the first set of signals (another selection operating at the downstream β-cell choice step). Steps that are intra-epithelial and self-assembling, such as the endocrine progenitor to committed intra-epithelial endocrine precursor cell, or the pro-β-cell to mature-β-cell step, may still involve permissive extrinsic signals (*wavy arrows*) to facilitate the transition

and our relatively close mammalian ancestry makes it likely that the fundamental rules gleaned by increasingly rigorous genetic studies in rodents are appropriate to humans. One does, however, need to remember that specific differences between mouse and humans: for instance, many believe that in human pancreas development, there is no "primary transition" – a process described below – and that birth of all endocrine cell types may occur over a single broad phase (Polak et al. 2000; Piper et al. 2004). Such observations mean that we need to remain aware of potential areas where the control mechanisms producing the final endocrine cells from progenitors vary moderately or massively between species. Next, we very briefly review pancreas organogenesis and the endocrine cell developmental process in the mouse, as the backdrop for the recent reprogramming discoveries that are the main focus of this chapter.

In the mouse, all pancreatic cells arise from two buds originating in the posterior foregut endoderm. The dorsal pancreatic bud begins as a local thickening of endoderm at embryonic day 8.75 (E8.75), with an overt ventral bud forming several hours later. During early pancreas development, two critical processes occur. First, progenitors that are allocated (by a still-undefined process) to the pancreas fate expand in number to generate sufficient numbers to go on to form the mature organ. Reducing the early-stage allocation or progenitors concomitantly reduces organ size (Stanger et al. 2007). Second, progenitors need to be maintained actively in an undifferentiated state; unregulated "precocious" differentiation during this period depletes the progenitor pool and compromises organ size (Apelqvist et al. 1999; Jensen et al. 2000a; Bhushan et al. 2001; Hart et al. 2003; Norgaard et al. 2003). The first wave of endocrine cells, primarily glucagon cells with fewer Insulin^{LO} cells (cells with lower levels of insulin than mature β-cells), appear during this "primary transition" stage. The immature Ins^{LO} cells are by molecular and functional criteria thought of as distinct from those generated in the secondary transition (Pictet and Rutter 1972; Herrera 2000).

The pancreatic epithelium undergoes complex morphological changes during its early development, changing from a simple bud, through a simple pseudo-stratified epithelium, to a complex tubular network (Fig. 15.1). Initially, the only implied polarity would be in cells that contact the basement membrane encasing the epithelial buds. At E11.5, pioneer microlumens begin to appear within the body of the buds, such that apical polarity develops in a subset of bud cells (Fig. 15.1). Local spreading of these microlumens occurs by the recruitment of additional cells and intercellular extension of the aligned apical polarization, and inter-lumen fusion is quite rapidly followed by a dramatic transformation of the buds into a tubular network that ramifies into the surrounding pancreatic mesenchymal layer (Yee et al. 2005; Kesavan et al. 2009).

The many branches of this epithelial arbor comprise two functional compartments: the tip and trunk. The sharpness of the boundary between these two domains, and whether cells near this boundary can transit easily from one domain to another, is unclear (Fig. 15.1). A likely oversimplified model has the tip domain containing the pool of multipotent progenitor cells (MPCs) that, as a population, have the capacity to produce all of the future endodermally derived cells: acini, duct and endocrine cells.

For a limited time, MPC tip proliferation extends the arbor, leaving in their wake trunk cells, which is a bipotent pool with duct and endocrine potential (Zhou et al. 2007; Solar et al. 2009). At around E14.5, the tip MPC begins to undergo a switch to the pro-acinar condition. The tip is therefore temporally dynamic, initially containing MPC, and then becoming progressively committed to the proacinar fate, a process that is becoming understood at a molecular and biochemical level in terms of the action of dynamic transcriptional networks (Masui et al. 2007).

The secondary transition (beginning at approx. E13 in the mouse) is the beginning of the progenitor differentiation phase of pancreas development, and is accompanied by a large increase in the birth of insulin-producing β-cells and proacinar cells (Pictet and Rutter 1972). Endocrine progenitors arise from within the trunk domain and quickly move into a transient phase of containing high levels of the transcriptional "endocrine-triggering" factor, Ngn3 (Gradwohl et al. 2000; Schwitzgebel et al. 2000; Jensen et al. 2000b; Gu et al. 2002), and there is a definite impact of the Notch (and possibly other) lateral-inhibition-type pathways on the endocrine birth process, as described in more detail below. Following birth within the epithelium, endocrine progenitors delaminate and coalesce to form islets, possibly by a process related to EMT (epithelial-mesenchymal transformation), although how delamination is induced precisely is still to be elucidated. After about E14.5, acinar cells are mainly generated by the duplication of existing acinar cells. Endocrine progenitor birth in the trunk domain ends by the beginning of postnatal life (Zhou et al. 2007; Solar et al. 2009). Acinar cells and endocrine cells go through a maturation process lasting into postnatal life, and there are periods of significant expansion of endocrine cell numbers, likely controlled by self-replication at birth and weaning, likely in accord with changes in the animal's nutrition (Desai et al. 2007; Dor et al. 2004; Finegood et al. 1995; Teta et al. 2007).

15.3 Endocrine Specification

The current model of endocrine cell specification involves Notch-regulated lateral inhibition (see Kopan and Llagan 2009 for in-depth Notch pathway review), whereby trunk epithelial cells adopt high levels of Ngn3 ($Ngn3^{HI}$) and commit to the endocrine lineage. Whether this step is more or less stochastic or is somehow regionally programmed is also still being worked out. $Ngn3^{HI}$ cells autonomously increase their expression of Notch ligands, which signal to neighbor cells to suppress their acquisition of Ngn3 expression and the endocrine fate. All pancreatic endocrine cells (alpha, which secrete glucagon; beta, insulin; delta, somatostatin; epsilon, ghrelin; PP, pancreatic-polypeptide-producing) are born from $Ngn3^{HI}$ endocrine precursors, which are, as a pool, largely mitotically quiescent. From studies in the mouse, their bulk behavior is to give rise, in a 1:1 manner, to the final islet endocrine cells. A small proportion of post-Ngn3 islet precursors do divide (self-replicate), but with a limited potential under normal physiological conditions (Desgraz and Herrera 2010). And so, in reference to the main thrust of this chapter, a programming theme

emerges: pancreatic organogenesis represents, in essence, the emergence of lineally related sister cells (α and β cells, for example), or cousin cells (acinar vs. β cells) from a common ancestry. While the factors affecting lineage diversification are incompletely characterized (Fig. 15.1; Kordowich et al. 2010; Cleaver and MacDonald 2009; Oliver-Krasinski and Stoffers 2008), their developmental linkage may be reflected by core epigenomic programming similarities and overlap, and these properties may be functionally useful in deriving methods to drive the desired reprogramming transitions. One attractive proposal is that direct inter-cousin transitions might be enforced more easily than ones that involve driving a pancreatic cell fully back to an iPS condition, and then forward again relying on any of the currently complex series of exogenous signaling and chemical cocktails that have been reported, which are probably still far from optimized. In the spirit of provoking an efficient move towards therapeutic application, we also suggest that it will be important for the field to move away from technically clever, highly detailed descriptions of the epigenomic landscape for the effector genes that are associated with specific cell states, and in the direction of making it more obvious how we will use the mountains of such data to be able to gain control over the differentiation or reprogramming process.

The timing and level of Ngn3 expression are crucial factors in driving entry into the endocrine program. By using hypomorphic alleles of Ngn3, Wang et al. (2010) demonstrated that the move to $Ngn3^{HI}$ was necessary to commit cells irreversibly to the endocrine lineage. Cells with moderate to low levels of Ngn3 can avoid endocrine cell fate commitment, leading to a sampling and commitment to acinar or duct fate. A general idea emerging, and we have detected evidence in vivo in support of this behavior, is that low levels of Ngn3 (the $Ngn3^{LO}$ condition) pre-empt $Ngn3^{HI}$, and that the $Ngn3^{LO}$ state is a metastable, relatively plastic, and uncommitted cell state, after which the elevation of Ngn3 works like a one-way railroad switch pushing progeny cells into the islet cell lineage.

The stage and context within which Ngn3-expressing cells arise dictates their endocrine progenitor competence. Early on, in the bud stage, they become mainly α-cells. Later, endocrine progenitors develop competence to become β- and PP-cells, with δ-cells being born somewhat later (Johansson et al. 2007). Thus, the majority of β-cells making their way into the final islets are born during the secondary transition, arising from a specialized epithelium that has currently poorly defined but likely precisely interactive signaling and structural features. Defining the in vivo β-cell birth niche more completely is an extremely active area of research. How the cellular context of endocrine progenitors is linked to the intra-epithelial or delamination-migratory state, or the timing of allocation to specific endocrine cell fates, has not been established. Furthermore, the maturation of pro-endocrine cells to the adult form, which is particularly important for the β-cell (for example, see Fig. 15.2), continues into postnatal life with transcriptional regulators of maturation becoming a hot-topic focus area (see below) (Nishimura et al. 2006).

The $Ngn3^{HI}$ endocrine progenitors move forward into mature endocrine cells that express a single hormone in approximately 2 days, during which time a cascade of transcription factors directs cells towards the appropriate endocrine cell type (Fig. 15.3). For this review, we introduce the concept that these transcription factors

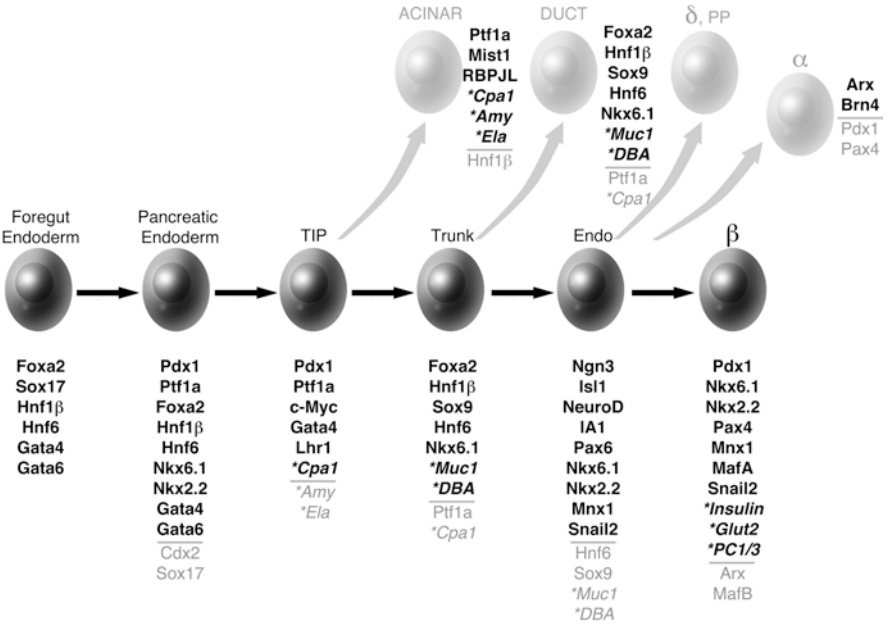

Fig. 15.3 Transcription factor cascade to the β-cell lineage. Nontranscription factors are indicated by an *asterisk* before the italicized designator. All others represent transcription factors; many are referred to in the main text. *Bold black* designators are expressed in the cell types they sit below (or to the *right* for acinar and duct); *gray font* indicates lack of expression. Gene lists for each cell type are not exhaustive and simply used to highlight focus areas of interest, or marked (often instructive) differences in the programs. All pancreatic cell types are born from a localized region (induced by unknown signals) of the foregut endoderm, as shown in Fig. 15.1. Pancreatic progenitors specifically express Ptf1a, Pdx1, Nkx6.1 and Nkx2.2, but not Cdx2 or Sox17, which are expressed in the adjacent duodenum and biliary system domains. The tip domain is distinguished from the trunk by the lack of Hnf1β. Cells in the initial tip domain do not express the digestive enzymes of mature acinar cells; Elastase (Ela) and Amylase (Amy) being examples. The digestive enzyme Cpa1 (Carboxypeptidase A1) is expressed in tip cells prior to, as well as after commitment to the acinar differentiation program. Tip cells are $Ptf1a^{+}Pdx1^{+}Cpa1^{+}$, and have relatively high levels of cMyc ($cMyc^{HI}$). The trunk domain does not express the tip markers Ptf1a and Cpa1, and instead expresses Sox9, which much later becomes lost from the majority of mature ductal cells. Embryonic trunk and mature duct cells are also marked by reactivity with DBA (the Dolichos biflorus agglutinin, which binds to surfaces of trunk and mature duct cells) and the apical membrane protein Muc1 (Mucin 1). Endocrine cells undergo commitment via a transient high level of Ngn3, and rapidly lose trunk cell markers. Following endocrine commitment, several transcription factors facilitate the generation of appropriate endocrine cell numbers and the proper ratio of the various endocrine cell types that are involved in creating a normal islet of Langerhans. Of note, α-cells and β-cells specifically express Arx or Pax4, respectively

can be divided into functional classes based largely on the effect on endocrine cell ontogeny in loss and gain of function studies (see Oliver-Krasinski and Stoffers 2008 for greater depth on each factor). In general, three classes of regulator may be divided, based upon current phenotypic analysis: general endocrine, lineage allocation, and maturation. General endocrine factors operate downstream of Ngn3 and facilitate the

formation of proper endocrine cell numbers, which includes potential survival and proliferation effects, and permit endocrine cells to move forward to become mono-hormone-producing cells. Mouse mutants of NeuroD1 have arrested endocrine cell development during the secondary transition, with reduction in all endocrine cell types. The remaining endocrine cells fail to form islets (Naya et al. 1997). Other examples of such deficiencies are Insulinoma-associated antigen 1 (IA1) (loss of IA1 arrests endocrine precursors prior to hormone expression) and Isl1 (Isl1 is required for the proliferation and survival of post-Ngn3 endocrine cells) (Gierl et al. 2006; Mellitzer et al. 2006; Du et al. 2009). Lineage-specific factors control the flux of endocrine progenitors towards specific hormone type(s). As a consequence of removing such factors, endocrine cell numbers are largely unaffected, but fractional islet allocations are often severely altered. Nkx2.2 mutants highlight the extreme lineage switching caused by single factor removal. Total endocrine cell number is almost normal but β cells are replaced by large numbers of the normally minor ghrelin-producing ε cell (Sussel et al. 1998; Prado et al. 2004) (other important examples, Pax4 and Arx, are described in the next section). Maturation factors seem to control later aspects of the final move towards physiological readiness (although there may be some overlap here with a harder to decipher role in the preceding phase of lineage allocation). Loss of these factors causes little or no defect during endocrine cell lineage diversification, but postnatally or in adulthood, the cells show aberrant function. For example, a recent report on insulin-cell-specific deficiencies in NeuroD1 suggests that it is essential for the proper activation of both insulin genes in mice (Gu et al. 2010). MafA mutants have no effect on endocrine cell development but mice become glucose intolerant postnatally (Zhang et al. 2005). Sequential triggering of all three classes of factors is necessary to generate the correct numbers of functional mono-hormone endocrine cells. The phasing of the autonomous and nonautonomous signaling steps of endocrine cell ontogeny, occurring in the framework of a three-dimensional epithelial structure, can be regarded as a pipeline for endocrine cell formation that produces the cellular building blocks of islets. Again, the epigenomic imprint cannot be overemphasized here: it likely limits the avenues open to a specific progenitor, yet lineage connections may open possibilities for conversion between effectively closely related cell types.

15.4 Alpha to Beta Reprogramming

Models for cellular differentiation pathways (similar to those shown, for example, in Fig. 15.2) are often drawn as a series of essentially one-way forward steps. But, a substantial challenge is that such diagrams will undoubtedly often need to be modified in order to accommodate plasticity, which may be substantial at certain choice points, such that the forward steps would be more correctly represented as equilibria, with cells progressing along a series of variably restricted plasticities (Fig. 15.2). Several remarkable cellular transformations in the pancreas raise the possibility of an induced (facultative) high level of cellular plasticity, and not

restricted to intermediate developmental stages, but between even mature cell types. Endocrine cell plasticity was noticed in rare pancreatic endocrine cell tumors from humans. Pancreatic endocrine tumors have a wide phenotypic spectrum classed on whether they secrete hormones, and which hormone(s) they secrete (Metz and Jensen 2008). Interestingly, one-third of human pancreatic endocrine tumors contain multiple hormones suggesting transdifferentiation during tumorigenesis, unless there is an uncommon concurrent tumorigenesis from multiple endocrine cell types (Heitz et al. 1982; Liu et al. 1992; Kapran et al. 2006). Serial transplantation in rats led to clonally derived tumor cells being able to diversify towards multiple endocrine tumor types (Madsen et al. 1993). Lu et al. (2010) conditionally inactivated the tumor-suppressor Men1 (Multiple Endocrine Neoplasia 1) to initiate tumorigenesis in glucagon cells, causing aggressive tumors to arise from α-cells. Their lineage tracing showed that these cells adopted expression of other endocrine hormones, including insulinomonas and mixed islet tumors (Lu et al. 2010). The switching of endocrine tumor type could in part be due to destabilization of the normal epigenomic state as a result of enhanced proliferation.

Pancreatic injury and regeneration has begun to be informative regarding adult cellular plasticity. A predominant concept in the field is that the generation of additional β-cells in the adult pancreas largely arises from replication of existing β-cells (Dor et al. 2004; Teta et al. 2007), although minor neogenetic sources cannot be ruled out, which is an issue still under enthusiastic exploration. One expectation, however, is that secondary pathways for generating β-cells might be increasingly accessed under conditions where the normal β-cell complement is reduced or removed. Thorel et al. (2010) used selective expression of the DT (diphtheria toxin) receptor and systemic DT administration to achieve near total β-cell destruction in mice. With almost total destruction (99%) of β-cells, Cre-based lineage tracing demonstrated that α-cells could generate apparently authentic β-cells, with the α-cells moving through a bihormonal glucagon^{+}/insulin^{+} state during passage to the glucagon^{-}/insulin^{+} state (Thorel et al. 2010). Understanding the molecular mechanisms of *α*- to β-transdifferentiation in this model could lead to discovery of the endogenous signaling pathways that could then be engaged (perhaps by pharmacological intervention) to coax a controllable amount of such transformation under more normal physiological conditions. It will certainly be interesting to understand why this reprogramming is not engaged by slightly less β-cell loss, and if small molecule screening could lead to rational therapeutic intervention in humans.

Both the above studies demonstrate an inherent but cryptic plasticity within endocrine cells and further demonstrate that adult α-cells can become β-cells, although it is unknown if only subpopulations selectively carry this property. The forced expression of key developmental transcription factors can lead to dramatic transformation of endocrine progenitors. During endocrine ontogeny (summarized in Fig. 15.3), the central regulator of α-cell specification is the transcription factor Arx. Arx mutations block access to the α-cell lineage without affecting endocrine cell number, and in this condition, cells fated to become α-cells were suggested by cell counting (no lineage tracing) to instead become β- or δ-cells (Collombat et al. 2003). This lineage switch is complementary to that observed in Pax4 mutants,

which lose β- and δ-cells and concomitantly increase α-cells (Sosa-Pineda et al. 1997). A simple take-home message here is that Pax4 and Arx drive transitional state cells to the β and α fate, respectively. Two separate subsequent studies moved on to test the sufficiency of Pax4 and Arx as inducers of their respective cell fates in general endocrine-committed progenitors or lineage-committed endocrine precursors. Arx mis-expression in embryonic endocrine cells, using a transgenic Pax6 promoter activation approach, caused the loss of β/δ-cell characteristics and increased α- and PP-cells. Insulin promoter-based Arx mis-expression in embryonic or adult β-cells also converts β-cells to α- or PP-cells (Collombat et al. 2007). Mis-expression of Pax4 in pancreatic or endocrine progenitors caused increased numbers of cells to adopt the β-cell identity, reducing flux to the α, δ, or PP fate, and glucagon promoter mis-expression of Pax4 in α-cells diverted them to β-cells (Collombat et al. 2009). From a therapeutic reprogramming perspective, finding ways to engage specifically, controllably, and perhaps only in a fraction of them, the expression of "β-cell trigger factors" such as Pax4, or of inhibiting Arx in mature α-cells, could be enough to reprogram enough cells into β-cells to ameliorate the effects of diabetes. Our own studies indicate that other factors, such as Pdx1 (which is expressed at the highest levels in the β-cell lineage), show a stage-dependent ability to effect an α- to β-transformation (Yang and Wright, unpublished data).

One interesting claim from the persistent Pax4 overexpression study (Collombat et al. 2009) was the continuous recruitment of new α-cells from a purportedly ductal cell location. This phenomenon was attributed to the lack of glucagon signaling because mouse mutants for other glucagon-signaling components also develop islet hyperplasia, with increased α-cells but without the large-scale transdifferentiation (Furuta et al. 1997; Gelling et al. 2003). Taking a medical reprogramming perspective again, some thought might be given to the possible intervention by inducing glucagon-signaling deficiencies to activate glucagon cell neogenesis, and some way of selectively targeting only the newly arising α-cells for reprogramming.

15.5 Exocrine to Beta Reprogramming

Spontaneous reprogramming from adult exocrine cells has a historical non-molecular era precedent, with dramatic transformations induced by relatively mild nongenetic treatments. Rats on a long-term copper-deficient diet lost nearly all pancreatic acinar cells, and recovery from this diet led to a surprisingly large fraction of the pancreas becoming hepatocytes – a conversion to a nearby organ cell type (Rao et al. 1986). Another interesting transformation involved inactivating c-Myc in acinar cells, reported as causing transdifferentiation to adipocytes (Bonal et al. 2009), usually a mesoderm-derived tissue type. The vast majority (98%) of the pancreas is exocrine (acinar plus duct), and as suggested above, acinar and ductal cells could represent a practical cell of origin for partial reprogramming to endocrine function, without compromising overall organ function. From a developmental standpoint, the last common progenitor for endocrine and acinar cells disappears at E14.5, and

a tiny pool of common duct-and-endocrine progenitors finally disappears sometime after birth (Zhou et al. 2007; Solar et al. 2009). It remains to be seen if any of the transformations above have cell-type subpopulation selectivity, or if there is the induction of facultative multipotential behavior in a subset of cells within the organ having specialized properties, sensitivities and predispositions. One candidate is the putative facultative progenitor population lodged at the interface between acinar and duct cells, the centroacinar cell or terminal duct cell, which is now becoming better defined and has been bravely proposed as an adult-stage multipotent regenerative cell type (Rovira et al. 2010).

Chronic pancreatitis and pancreatic cancer often lead to a focal loss of acini and their replacement by hyperplastic duct epithelium. Endocrine cells found around and within these hyperplastic ducts led some to suggest that adult exocrine cells can produce endocrine cells, possibly at a higher rate after damage (Esposito et al. 2007; Gu and Sarvetnick 1993; Phillips et al. 2007; Song et al. 1999). In one model of duct hyperplasia caused by TGF-α overexpression, the primary source of hyperplastic ducts was concluded to be transdifferentiated adult acinar cells, and elastase or villin promoter-based lineage tracing suggested that any endocrine cells detected in/near these structures had in fact not been sourced from acini or hyperplastic duct cells (Blaine et al. 2010). Other models of hyperplastic duct formation suggest that acinar to ductal transdifferentiation is a minor component of hyperplastic duct formation, but in at least some of the studies the endocrine lineage was not analyzed (Strobel et al. 2007).

Pancreatic duct ligation (PDL) is another method that generates tissue damage and cellular responses, generating a tissue environment similar to that in acute pancreatitis. A ligature around the main pancreatic duct results in distal damage, acinar involution, and an associated inflammatory response. Xu et al. (2008) concluded that this injury induced fairly large numbers of Ngn3-positive cells, from a duct epithelium source, and suggested that these $Ngn3^+$ cells could give rise to proper endocrine cells in vivo and in vitro. On the other hand, Solar et al. labeled mature duct cells prior to PDL and concluded that Hnf1β-expressing duct cells cannot produce β-cells following PDL in vivo (Solar et al. 2009). It remains to be seen if these divergent findings are resolved by a core mechanism, in both cases, of centroacinar/acinar to duct epithelium transformation, with the "new epithelium" being competent to give birth to $Ngn3^+$ cells. An even blunter type of injury is caused by straightforward surgical excision of part of the pancreas, which induces a tissue proliferative response also sometimes referred to as "regeneration." Rigorous evidence for the reprogramming of cell types towards an endocrine fate remains to be reported in this model, but both acinar cells and β-cells self-duplicate following this type of injury (Dor et al. 2004; Desai et al. 2007).

The rate of "neogenetic" formation of endocrine cells from duct or acini under normal physiological conditions in vivo is probably quite low, although the methods for estimating this rate and the precise cellular origin are still being hotly debated and optimized (Dor et al. 2004; Solar et al. 2009; Inada et al. 2008). Under injury conditions, it is possible that there are primary or accessory roles for inflammatory-cell-related relatively broad release of cytokines. For example, triggering

liver cells to begin to express genes of the pancreatic program seems to be encouraged by, and even require, an inflammatory response to the capsid of the adenovirus used to introduce the initial triggering factors (Wang et al. 2007). Immediate questions arise such as: which cytokines or other signals are involved? We also need to consider more detailed studies to address the nature of any secondary influences from non-endoderm-derived tissues, such as the pancreatic vasculature, neurons or even stromal cells. While Ngn3$^+$ cells are induced following PDL, there may be a blockade (perhaps the expression level cannot become high enough) that prevents their large-scale differentiation towards the endocrine lineage in the injured pancreas. It was noted that placing Ngn3$^+$ cells induced by PDL within endocrine-cell-deficient (Ngn3-null) pancreatic explants caused more efficient endocrine cell differentiation than if the cells were left in vivo (Xu et al. 2008). Under PDL in particular, β-cell mass is not reduced by much, despite the massive acinar involution, and the tissue's "call" to produce new β-cells might therefore not be very loud (a notion also relevant to the discussion above regarding the β-cell destruction studies of Thorel et al. 2010). Nonetheless, there have been suggestions that cultures of dissociated pancreatic explants in vitro can show acinar cells turning into endocrine cells, although the signals causing such reprogramming are unknown, and proving the cellular origin by lineage tracing could arguably be much improved in order to make this conclusion more watertight (Baeyens et al. 2009; Minami et al. 2005).

With these various findings as the background, it was considered by many readers remarkable that acinar cells could be reprogrammed into β-cells in vivo by injecting an adenovirus-encoded cocktail of three transcription factors (Ngn3, Pdx1 and MafA), an "enforcement" process reminiscent of the methods first used to induce iPS properties in mature cells. In this method, Ngn3 likely induces general endocrine competence, while Pdx1 assists in the move to the β-cell, and not other endocrine fates, with MafA augmenting Pdx1 function and also ensuring progression towards the fully mature β-cell condition (Gannon et al. 2008; Ahlgren et al. 1998; Gradwohl et al. 2000; Vanhoose et al. 2008).

The observation reported by Zhou et al. (2008) was of sporadic induction of Insulin$^+$ cells, morphologically and functionally akin to β-cells (their formation was associated with an improved glucose homeostasis in diabetic mice), and lineage-tracing data using Cpa1-based tracking systems showed that at least some of the new β-like cells were acinar derived. The cellular competence for this 3-factor reprogramming was not determined. It is likely now being investigated if all acinar cells show equivalent competence, if there is any response from the centroacinar cell type, if duct and other cells are resistant, and how nearby areas of gut epithelium might respond or not to the transcription factor cocktail. At first sight, this transformation might seem miraculous, but both acini and β-cells are well tailored to extremely high output cargo packaging, storage, and secretion (enzymes or hormone, respectively), and are lineally related, as already described (Fig. 15.1) – so this transition could be rationalized as "simply" changing the upper level management in a very efficient secretion factory.

Ruminating on how such observations might lead to β-cell-directed reprogramming intervention on the human exocrine pancreas, perhaps a combination of in vitro culturing and forced transient transduction of β-cell factors would be fruitful. Of course, developing safer ways to access and remove relevant tissue from patients would be necessary, because a pancreatitis-type response is a well-known principal side-effect of pancreatic surgery. Additional speculative yet plausible methods might include drug-based ways of relaxing the resistance of mature cells to plasticity, or of enhancing the reprogramming response to such factors. It may even be possible to develop unstable membrane transport sequence-tagged transcription factors (as used in iPS reprogramming; Kim et al. 2009), with appropriate tissue targeting, to trigger endogenous epigenomic reprogramming.

15.6 Endoderm to Pancreas Programming

The effects of removal or forced expression of key transcription factors involved in pancreas organogenesis will continue to be revealing as to the points in the developmental program where large-scale or residual plasticity exists. Such information would hopefully be able to be leveraged with respect to directing cells in a desired direction. It is entirely possible, given the current lack of definitive knowledge on the pancreas-commitment process, that progenitors from other gut regions, taken at a stage before organ commitment, might also be accessible and suitable for induced reprogramming. We are also interested, as we discuss here, in generating a list of adult-stage cells from nearby organs that can be reprogrammed to adopt pancreatic endocrine functions. The degree of plasticity maintained in the various differentiated cell types amongst the foregut organs may not necessarily be related to the developmental plasticity so far exemplified by the limited genetic analysis of pancreatic endocrine ontogeny. We would suggest, however, that it does beg the question of whether totally differentiated cells can be reprogrammed backwards, to a pre-organ-commitment naïve state, and then pushed into the desired organ fates by instructive and permissive cuing. Certainly, a more directed analysis would be necessary to define the true nodal or execution points in such programs, at which fundamental gene-regulatory switches act to control progenitor flux down specific differentiation tracks (see Fig. 15.2). Of particular note, a single region of the primordial gut tube containing the ventral pancreatic progenitors, as shown by various mutations in the mouse, has early-stage competence to enter three organ programs: pancreas, liver (hepatobiliary) or intestine. When deficient in the exquisitely pancreas-specific transcription factor Ptf1a, many cells, although they still experience and respond to the signals to become pancreas allocated (that is, there is regional activation of the *Ptf1a* locus), instead are diverted to an intestinal or biliary fate (Kawaguchi et al. 2002). In Hhex mutants, the ventral foregut endoderm does not move out of proximity to the cardiac anlage, FGF signaling from the cardiac mesoderm persists, which blocks pancreas specification, and progenitors turn to a hepatic

fate. If ventral foregut endoderm is dissected before exposure to cardiac signaling and cultured in vitro, cells "default" to the pancreas fate and do not adopt the liver fate (Bort et al. 2004). Not only pancreatic progenitors are competent to transfer between organ fates, but various mutants demonstrate a similar transformation to pancreas from other endodermal organ progenitors. These observations could lead to the perhaps overly simplistic conclusion that the pancreas represents some kind of default endodermal state. Hes1 mutations cause focal ectopic pancreas in the bile duct, stomach and duodenum (Fukuda et al. 2006), and Sox17 inactivation leads to ectopic pancreas at the expense of biliary tissue (Spence et al. 2009). The general picture here is that, developmentally at least, we are gaining an understanding of the transcriptional control triggers that cause major shifts of organ progenitors within the foregut between specific organ programs. How such information could be applied with respect to partial cellular reprogramming in the adult organ, or forward programming in vitro from ES or iPS cells, remains an open but invigorating question.

To some degree, the adult liver has many desirable qualities when pondering organ-to-organ reprogramming. As noted above, liver and ventral pancreas develop from an endodermal region of potentially common progenitor competence (organ allocation being dictated by regional signaling from adjacent organ primordia), suggesting a relatively close epigenomic relationship between adult cell types of both organs (Chung et al. 2008; Deutsch et al. 2001; Wandzioch and Zaret 2009). Unlike the adult pancreas, the adult liver can undergo remarkable compensatory growth of the remaining tissue after surgical removal of major parts of the total organ. For example, resection of three quarters of the liver causes the remaining quarter to grow back to the entire organ's original size. Because removal of parts of the liver is tolerated well, liver cells could be attractive for reprogramming to pancreatic cell types, via ex vivo reprogramming and in vitro culture.

Copper deficiency, pancreas transplantation in rats and growth factor (KGF, FGF7) mis-expression in islets can all cause hepatocytes to appear in the midst of the pancreas (Rao et al. 1986, 1988; Dabeva et al. 1997; Krakowski et al. 1999). Rat pancreatic tumor cell lines with characteristics of pancreatic endocrine and exocrine cells can initiate transdifferentiation towards hepatocytes when stimulated by the glucocorticoid dexamethasone (Shen et al. 2000). Despite considerable evidence for pancreas to liver transdifferentiation, evidence for liver to pancreas is less striking. The ability of the biliary tree to give rise to pancreatic endocrine cells demonstrates that "hepatic" cells can at least access the pancreatic program (Dutton et al. 2007; Eberhard et al. 2008). To study this transdifferentiation further, various studies ectopically expressed pancreatic transcription factors in the adult liver. Ectopic expression of a superactive form of Pdx1 in the differentiating liver (at a stage not yet "fully differentiated") leads to exocrine/endocrine pancreatic differentiation in *Xenopus* (Horb et al. 2003). Adenoviral delivery of Pdx1 increased insulin gene transcription in the mouse liver, which seemed to ameliorate hyperglycemia in an induced diabetic condition (Ferber et al. 2000; Ber et al. 2003; Sapir et al. 2005; Wang et al. 2007). This effect may be aided by supplying Ngn3 or NeuroD1 along with Pdx1 (Kaneto et al. 2005). Given the current state of the art, with better delivery methods and better ways of identifying

lineal relationships between source and final cells, as well as ways of assessing the completeness of the transition, we might now anticipate seeing deeper and more rigorously analysed studies of in vitro transformation from hepatic cell lines. We should expect the same in mouse models undergoing controlled expression of multiple factors, all with the goal of more comprehensively elucidating whether the liver is a viable intervention site for functional reprogramming to offset or cure diabetes. Other endodermal organs, including the stomach and small intestine, have endocrine cell types similar in many ways to those of the pancreas, and parts of these organs might be built and maintained by intercellular programs with directly analogous relationships to those in the pancreas. Because the caudal stomach, rostral duodenum and pancreas all emerge from within the same Pdx1-expressing domain, both could be suitable epigenomically related tissues for a secondary source of authentic β-cells.

15.7 Discussion and Future Directions

Very much reflecting the fact that the field of pancreas molecular developmental biology has had essentially only 15 years or so of activity, this article has been dominated by discussions of the mutant phenotypes and reprogramming potential related to transcription factors. It is of course highly likely that other molecules play instructive or permissive roles in the process, and that we will see information arising on the influence of extracellular matrix molecules, intercellular signaling molecules, cytokines, and so on. Generally, we would like to know much more about the micro and macro-environments that are conducive to reprogramming, how reprogramming is induced and temporally regulated, and how extrinsic cell biological information interacts with cellular competencies and predispositions to direct the acquisition of specific fates. Adding to these ideas, it might be very pertinent to test if there are any aging-related severe reductions in the responsiveness to reprogramming triggers.

One of course should also be fascinated over whether or not there is a basal level of cellular inter-conversion in any mature organ. It may be true that, as suggested for the pancreas, cell-replication is the major contributor to tissue homeostasis, but we need to be ready to detect situations when facultative progenitors feed the formation of physiologically critical cells such as the β-cell. We might expect to see data emerging on the role of adjacent tissues in the response to injury and induced regeneration, as such studies might provide clues as to the intercellular signals that can induce or enhance plasticity, and the reprogramming-competent state. Regarding the pancreatic "regeneration" and reprogramming caused by PDL and β-cell destruction, and other techniques, we propose that it will be important to learn about the role of inflammation-associated factors and signals, the tissue responses in terms of the endogenous intercellular signals that might be evoked, as well as the differences between general vs. specific "call" signals to produce certain cell types. These questions would include how the selective loss of β-cells is recognized, and whether the tissue response is local or, in fact, much broader and even extending into the portion not overtly affected by the injury.

As we have already mentioned, chemical library screening is potentially providing a parallel discovery platform for determining the most efficient ways of coaxing cells along a forward differentiation program, and there could well be simple, clean methods that can be developed to relax the resistance to reprogramming in non-β-cells. It is even tenable to suggest that the intercellular signals and epigenomic transition inducers discussed in this review could be completely replaced by mixtures of small molecules that are functionally selected from the various chemical libraries. Cell-type-specific methods of relaxing mitotic quiescence, directed at possibly transiently increasing the β-cell replication rate, could be considered as another therapeutic angle that overlaps with the cell-to-cell reprogramming discussed above (see, for example Furuya et al. 2010; Fang et al. 2010). We clearly need to keep in mind the potential for tissue-reprogramming sources that are extra-pancreatic, and also that stop-gap positions using partially reprogrammed β-like cells could be envisaged. The latter might provide useful restorative stepping stones on the road to a successful full-scale therapy achieved by reprogramming and cellular replacement.

With these ideas in mind, we certainly should expect new screens to identify new relaxers and reprogrammers, at a genetic and molecular/chemical level, and to learn perhaps substantially from careful studies in several model organisms, including the zebrafish. There may be additional improvements in the reprogramming and growth of cells gained by applying new knowledge on how cells respond to artificial extracellular matrix concoctions, and on how certain biopolymers can support or enhance reprogramming and/or the directed differentiation of ES cells. Nevertheless, the human pancreas is definitely the most relevant, and while mouse and human tissues may have equivalent responses, we foresee a future transitional period in which there is a progressive drift away from our intense focus on rodent models. We could perhaps turn to mature β-like or α-like human cell lines (and their ilk from other organs), and we might gain access to viable human tissue for differentiation and reprogramming studies. Depending upon the ethical landscape, however, a complementary viewpoint could be to look towards great improvements in ES/iPS-cell forward differentiation protocols, which might allow detailed cell biological, genetic and epigenomic discoveries related to precisely identified transitional intermediates and mature cell types. Furthermore, the generation of directed differentiation protocols to generate all the cell types that might play a role in the type I diabetes disease state – not just β-cells, but reconstructing the patient's immunity system in vitro – could allow new insight into whether the root cause of type I diabetes is in fact a fault in the β-cells themselves, rather than a rogue immune attack on normal β-cells.

It is suitable to close this chapter with the proposition, based upon recent publications on nonpancreatic cells (Kim et al. 2010; Polo et al. 2010; Zwaka 2010), that iPS cells derived from any organ, including the pancreas may retain, under certain conditions and perhaps for a limited period, a cryptic "closeness" to their cell of origin. The idea here is that there exists, even in what looks like a naïve-induced pluripotent state, a residual chromotypic imprint that could facilitate, or even predispose, highly proliferative cells towards β-cell-directed forward programming cues.

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Chapter 16
Phenotype and Developmental Potential of Cardiomyocytes from Induced Pluripotent Stem Cells and Human Embryonic Stem Cells

Christopher Rao, Nadire N. Ali, Thanos Athanasiou, Cesare Terracciano, and Sian Harding

Abstract The adult human heart has limited regenerative capacity. Loss of myocardium, most commonly through ischemic injury, results in the clinical syndrome of heart failure. Current therapies are largely palliative and the only treatment for end-stage heart failure with established long-term efficacy is transplantation. Consequently, there has been significant interest in developing novel regenerative strategies. Use of human embryonic stem cell (hESC) therapy is complicated by ethical and technical problems including possible host immune rejection of transplanted cells. Induced pluripotent stem cells (iPSCs) share many properties with hESC but do not require the ethically problematic destruction of embryos. Furthermore, as iPSC can be generated from somatic cells of specific patients, it is theoretically possible to perform allogenic cell transplantation or to create patient-specific in vitro disease models using iPSC-derived cardiac myocytes. In this chapter, we discuss the developmental potential of hESC and iPSC for cardiomyogenesis, including existing methods for differentiation and maturation.

16.1 Introduction

The adult human heart has limited regenerative capacity (Bergmann et al. 2009). Loss of myocardium, most commonly through ischemic injury, results in a failure of the heart to adequately perfuse metabolically active tissue (McMurray and Stewart 2000). This results in a clinical syndrome of heart failure characterized by fatigue, edema and dyspnea. The long-term prognosis is poor and death often results from fatal arrhythmia in the pathological myocardium (Bonneux et al. 1994;

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McMurray and Stewart 2000). Current therapies for heart failure are largely palliative (Yamada et al. 2008). The only treatment for end-stage heart failure with established long-term efficacy is transplantation (Taylor et al. 2007); however, the increasing prevalence of heart failure, and existing shortage of donor organs, makes this unsustainable (Gridelli and Remuzzi 2000). Consequently, there has been significant interest in developing novel regenerative strategies. Stem cells are undifferentiated cells that can renew themselves through mitotic cell division, and have the ability to differentiate into specialized cell types. Stem cell therapy is perhaps one of the most promising strategies and the therapeutic potential of stem cells from several different sources has been investigated (Lee and Terracciano 2010).

There is evidence of native stem or progenitor cell niches within the adult myocardium (Beltrami et al. 2003; Moretti et al. 2006). Whilst the proliferative potential of cardiac stem/progenitor cells is high (Bearzi et al. 2007), they are few in numbers and are not thought to be important in adaptive mechanisms in cardiomyopathies. This population of cells, however, has been efficiently isolated and expanded in vitro (Messina et al. 2004) and phase I/II clinical trials are currently underway (NCT00474461, NCT00893360) (Novak 2010; Wollert and Drexler 2010).

Bone marrow-derived stem cells (BMSC) are far more readily available than native cardiac stem/progenitor cells, and there has also been considerable interest in the therapeutic potential of BMSC since it was first suggested that they had the potential to differentiate into cardiac myocytes (Orlic et al. 2001). This has culminated in several animal studies and clinical trials (Abdel-Latif et al. 2007). The results of these studies have, however, been disappointing, with limited improvements in function tending to be clinically insignificant and transient (Gersh et al. 2009). Furthermore, it remains to be demonstrated whether BMSC can differentiate into cardiac myocytes at any significant frequency (Murry et al. 2004). However, several clinical trials utilizing BMSC are still ongoing (Wollert and Drexler 2010). Similarly, skeletal myoblasts are not thought to be able to differentiate into cardiac myocytes in vivo or in normal culture conditions (Lee and Terracciano 2010). Furthermore, clinical trials raised concerns about the arrhythmogenicity of skeletal myoblasts (Abraham et al. 2005; Menasche et al. 2001), potentially caused by the inability of myocytes derived from the donor stem cells to integrate and form a functional syncytium with the host myocardium (Rubart et al. 2004).

Human embryonic stem cells (hESC), by contrast, are pluripotent and can differentiate into all cell types in the body, unlike multipotent adult stem cells which only have the ability to differentiate into a limited number of cell types (Thomson et al. 1998). Additionally, in specific conditions, they can be propagated in vitro for many months without loss of normal karyotype (Ying et al. 2003). However, as they are derived from the inner cell mass of preimplantation embryos (Thomson et al. 1998), they are associated with both ethical (Lo and Parham 2009; Passier et al. 2008) and technical problems such as possible host immune rejection of transplanted cells when used in cell therapy (Boheler et al. 2002; Saric et al. 2008). Induced pluripotent stem cells (iPSCs), a novel type of stem cell first described by Takahashi and Yamanaka (2006), share many properties with hESC (Takahashi and Yamanaka 2006). iPSC are reprogrammed pluripotent cells that are created by forced expression of embryonic transcription factors in nonpluripotent cells, such as

adult somatic cells (Takahashi et al. 2007; Yu et al. 2007). In addition to being pluripotent, like hESC, they can be readily cultured and transfected. iPSC, however, do not require the ethically problematic destruction of embryos (Lo and Parham 2009; Passier et al. 2008), and as allogeneic transplantation is feasible host immune rejection can potentially be avoided in cell therapy (Boheler et al. 2002; Saric et al. 2008). Furthermore, as iPSC can be generated from somatic cells of patients with specific disease phenotypes, it is theoretically possible to create in vitro disease models using iPSC-derived cardiac myocytes (iPSC-CM) (Carvajal-Vergara et al. 2010; Caspi et al. 2009; Moretti et al. 2010).

In this chapter, we discuss the developmental potential of hESC and iPSC for cardiomyogenesis, including existing methods for differentiation and maturation. We discuss gene expression in hESC-derived cardiomyocytes (hESC-CM) and iPSC-CM. Finally, we will compare the structural and functional phenotypes of cardiomyocytes derived from hESC-CM and iPSC-CM.

16.2 Differentiation and Developmental Potential of hESC and iPSC

16.2.1 The Embryoid Body Method

The most commonly reported method in the literature of differentiating hESC and iPSC into cardiomyocytes remains the embryoid body (EB) system. First applied to hESC in 2001 by Kehat et al. (2001), the EB method requires hESC or iPSC colonies to be dissociated into multi-cellular three-dimensional aggregates, termed EBs, and cultured initially in suspension (4–10 days) in serum-supplemented differentiation medium. Within a few days of EB formation, the cells differentiate into all three germ layers and their descendant lineages (Itskovitz-Eldor et al. 2000; Kehat et al. 2001). Spontaneously contracting areas appear in the EB outgrowths (Habib et al. 2008; Kehat et al. 2001; Xu et al. 2002) and subsequent analysis suggests that these areas have a number of cardiomyocyte characteristics (Ameen et al. 2008), including the expression of cardiac markers.

The EB method for cardiac differentiation has several limitations. Its efficacy is highly variable due to the heterogeneous size and shape of aggregates which can differ significantly in size and morphology (Vidarsson et al. 2010). Forced-aggregation methods have been developed to standardize the size and shape of EB by centrifuging a defined number of hESC or iPSC in low-adherence 96-well plates (Burridge et al. 2007). The requirement for serum not only introduces further heterogeneity to the efficacy of the EB method but makes future therapeutic application of hESC and iPSC technology problematic (Habib et al. 2008; Ludwig and Thomson 2007; Vidarsson et al. 2010). Consequently, there is considerable interest in developing defined, animal product-free differentiation protocols (Habib et al. 2008; Vidarsson et al. 2010).

One of the most significant limitations of the EB method, however, remains the relatively low cardiomyocyte yield (Habib et al. 2008; Vidarsson et al. 2010) and

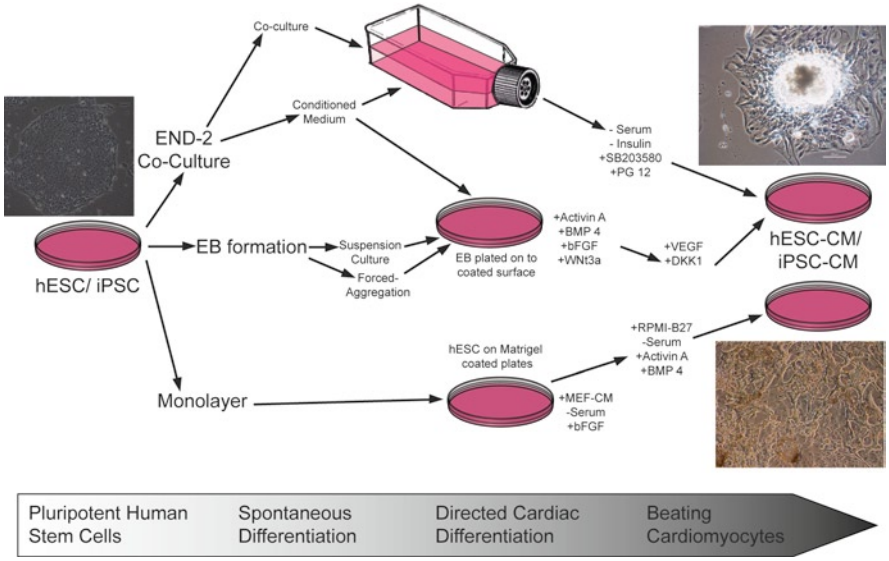

Fig. 16.1 Summary of methods for differentiating pluripotent stem cells into cardiomyocytes. Figure reproduced with permission from Vidarsson et al. (2010)

several directed differentiation protocols have been developed to improve the efficiency of iPSC and hESC cardiomyocyte differentiation either with or without EB formation (Tran et al. 2009; Yang et al. 2008) (Fig. 16.1).

16.2.2 Directed Differentiation Techniques

As differentiation of hESC and iPSC into cardiomyocytes parallels many of the molecular and structural events seen during early in vivo cardiogenesis (Lev et al. 2005), lessons from developmental biology are readily applicable in stem cell research (Habib et al. 2008). For example, the endoderm that lies directly posterior to the embryonic cardiac crescent is considered essential to initiate cardiac differentiation (Graichen et al. 2008; Olson 2001). In vitro experiments confirm that cardiac differentiation requires extracellular signals from the surrounding endoderm (Olson 2004; Zaffran and Frasch 2002) and that co-culturing of undifferentiated ESC with a murine visceral endoderm-like cell line (END-2) has been shown to induce cardiac differentiation (Mummery et al. 2003). Even hESC lines, which ordinarily do not spontaneously differentiate into cardiomyocytes, have been shown to differentiate into cardiomyocytes when co-cultured with END-2 cells (Mummery et al. 2003). The co-culture differentiation system has since been further optimized by the removal of serum and insulin and the addition of prostaglandin I2 to the culture medium (Graichen et al. 2008; Passier et al. 2005; Xu et al. 2008). Although the END-2 culture improves efficiency of differentiation compared to the EB method, and results in more

homogeneous cells, with up to 85% of the hESC-CM displaying ventricular phenotype (Mummery et al. 2003), reliance on murine feeder cells limits the therapeutic applications of the method and represents an additional source of heterogeneity.

By focusing on factors expressed in the early endoderm known to regulate cardiac development, and on factors known to induce endoderm, mesoderm and specification of cardiac mesoderm, several protocols have been developed to direct differentiation of hESC into cardiac myocytes. These protocols use defined factors from the Wnt family or transforming growth factor β (TGF-β) superfamily, such as TGF-β itself, activin and the bone morphogenic proteins (BMP) (Filipczyk et al. 2007; Yang et al. 2008; Laflamme et al. 2007).

TGF-β and the BMP have been shown to promote cardiomyogenesis in all species (Olson 2001; Schuldiner et al. 2000) and are thought to be important in promoting mesoderm formation and myocardial lineage commitment during hESC differentiation (Laflamme et al. 2007; Nostro et al. 2008; Pal and Khanna 2007; Schuldiner et al. 2000; Yang et al. 2008; Yao et al. 2006; Zhang et al. 2008). Activin A has also been shown to promote hESC cardiac differentiation (Laflamme et al. 2007; Yang et al. 2008; Yao et al. 2006), and a differentiation system which combines Activin A and BMP4 has been reported to improve the efficiency of hESC cardiac differentiation to over 30% cardiomyocytes using serum-free defined medium (Laflamme et al. 2007).

Unlike BMP and activin pathways, the canonical wnt signaling pathway, however, both promotes and inhibits cardiomyogenesis depending on the stage of cardiac differentiation. Prior to differentiation activation of the canonical wnt pathway maintains pluripotency (Sato 2004). In early differentiation and prior to gastrulation, it leads to enhanced cardiomyogenesis, probably by promoting formation of mesoderm, but inhibits subsequent cardiac specification of mesoderm (Ueno et al. 2007; Yang et al. 2008). Finally, whilst it has been shown to be important in promoting the formation of cardiac progenitor cells, it negatively affects differentiation of these cells into cardiomyocytes (Cohen et al. 2007; Qyang et al. 2007). The noncanonical wnt signaling (Wnt11), on the other hand, has been shown to promote cardiac differentiation (Flaherty et al. 2008).

Several other compounds, such as specific p38 MAP kinase inhibitors, have been found to promote cardiac differentiation in vitro (Graichen et al. 2008) and research is currently underway using novel high-throughput screening technology and bioinformatic approaches to identify novel molecules that could further promote cardiac differentiation of hESC and iPSC (Takahashi et al. 2003; Willems et al. 2009; Wu et al. 2004; Yoshida and Yamanaka 2010).

16.2.3 Identification of Cardiac Progenitor Cells Derived from hESC and iPSC

Despite improvements in the efficiency of cardiomyocyte formation from hESC and iPSC using current directed differentiation protocols, only a small and heterogeneous population of cardiomyocytes are produced (Passier et al. 2008). This is

inadequate for currently envisaged therapeutic applications and has limited utility in disease models. This has resulted in both considerable interest in developing methods to purifying cardiac myocytes without genetic modification (which may compromise their utility) (Hattori et al. 2010), and interest in identifying a lineage-committed cardiac progenitor population that could be expanded in vitro and differentiated efficiently into a homogeneous population of cardiomyocytes.

It is increasingly understood that cardiac tissue, containing cardiomyocytes, smooth muscle and endothelium, develops from a common cardiovascular progenitor cell (Yang et al. 2008). Consequently, there is considerable interest in identifying these cells (Vidarsson et al. 2010). In cells derived from murine ESC lines, Flk-1^{+} (kinase insert domain protein receptor, also known as Kdr)/Isl1^{+} cell populations have been identified which differentiate into cardiomyocytes, endothelial and smooth muscle cell lineages (Kattman et al. 2006; Moretti et al. 2006). Similarly, using cells derived from murine ESC with a GFP reporter targeting the brachyury T (Bry) gene, a population of Flk1^{+}/GFP-Bry^{+} cells which differentiate into cardiomyocytes, endothelial and smooth muscle cells have been identified (Kattman et al. 2006). More recently, it has been demonstrated that murine ESC-derived cKit^{+}/Nkx2.5^{+} cell populations can be expanded and differentiated into cardiomyocytes and smooth muscle cells (Wu et al. 2006) (Fig. 16.2).

Similar cardiovascular progenitor cell populations have been identified in cell populations derived from hESC (Yang et al. 2008). Using a novel stage-specific differentiation protocol involving the combination of Activin A, BMP4, bFGF, VEGF and DKK1 in serum-free chemically defined medium and in a hypoxic (5% O_2) atmosphere, a population of Kdr (Flk-1)^{low}/c-kit^{-ve} expressing cells were generated from hESC. Over 50% of these cells subsequently differentiated into cardiac myocytes (Yang et al. 2008). The location on cell membrane of Kdr and c-kit enables isolation of cells by FACS.

16.2.4 Differences in Gene Expression and Differentiation Potential of iPSC and hESC

During early cardiogenesis, it is known that there is an initial reduction in the expression of pluripotency genes such as OCT4 (Niwa et al. 2000) and NANOG (Chambers et al. 2003; Mitsui et al. 2003) following the initiation of differentiation, with a concomitant increase in the levels of mesodermal markers such as brachyury T (Marcellini et al. 2003). This is followed by expression of the cardiac transcription factors such as Nkx2.5 (Durocher et al. 1997; Garg et al. 2003), MEF2c (Bi et al. 1999) and GATA4 (Durocher et al. 1997; Garg et al. 2003). Finally, genes encoding cardiac-specific structural proteins are expressed. These include cardiac troponin T (TNNT2) (Townsend et al. 1994), α-myosin heavy chain (MYH6) (Brand et al. 1991), α-actinin (ACTN2) (Eldstrom et al. 2003), myosin light chain

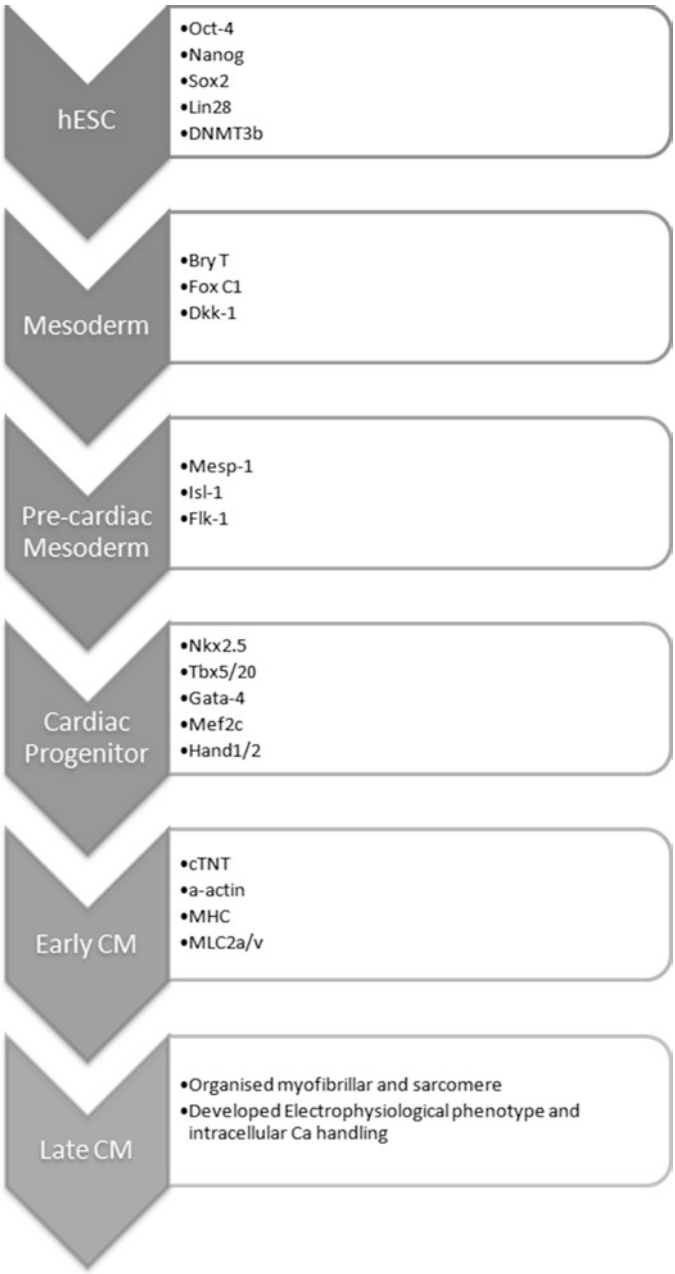

Fig. 16.2 Summary of gene expression during differentiation from pluripotent stem cells into cardiomyocytes. Figure reproduced with permission from Vidarsson et al. (2010)

2 atrial isoform (MYL7) (Kubalak et al. 1994) and myosin light chain 2 ventricular isoform (MYL2) (Macera et al. 1992); atrial natriuretic factor (HPPA) (Saito et al. 1989); and phospholamban (PLN) (Tada et al. 1974). Whilst this is broadly consistent with gene expression patterns in the differentiation of hESC and iPSC into cardiac myocytes (Habib et al. 2008; He et al. 2003; Kehat et al. 2001; Mummery et al. 2003; Pekkanen-Mattila et al. 2009, 2010b; Xu et al. 2002; Xue et al. 2005; Zhang et al. 2009; Zwi et al. 2009), there is evidence that there are also differences between gene expression patterns (Chin et al. 2009) and DNA methylation patterns in hESC and iPSC (Deng et al. 2009). Furthermore, evidence has recently emerged that ESC may differentiate into specific lineages more efficiently than iPSC (Deng et al. 2009; Hu et al. 2010).

Differences between iPSC and hESC in the expression of pluripotency genes have been reported, because of persistent transgene expression in iPSC during cardiogenesis (Zhang et al. 2009), however, it is not clear if this affects either the potential of iPSC to differentiate into cardiomyocytes or the phenotype of the resulting iPSC-CM. Trends towards more efficient cardiac differentiation in hESC compared to iPSC have been reported (Zhang et al. 2009). However, there is considerable variation between the efficiency with which different hESC lines differentiate into cardiomyocytes and variation between iPSC and hESC is consistent with the variation between different hESC lines (Adewumi et al. 2007; Moore et al. 2008; Osafune et al. 2008; Pekkanen-Mattila et al. 2009). Whilst some studies suggest that there is no difference in the time taken for iPSC and hESC to differentiate into cardiomyocytes (Narazaki et al. 2008; Zhang et al. 2009), others report that iPSC do take longer to differentiate into cardiomyocytes than hESC (Mauritz et al. 2008). It has been proposed by some authors that variation in the site of transgene integration may account for these differences (Zhang et al. 2009). While this is not consistent with reports that differences in gene expression between iPSC and hESC are independent of the method by which pluripotency is induced (Chin et al. 2009), there is considerable interest in developing and refining methods to induce pluripotency without genetic manipulation (De Miguel et al. 2010; Selvaraj et al. 2010).

16.2.5 Direct Trans-Differentiation of Somatic Cells into Cardiomyocytes

Evidence that murine fibroblasts can be trans-differentiated into neuronal cells (Vierbuchen et al. 2010) and cardiomyocytes (Ieda et al. 2010) has resulted in considerable interest in developing techniques to trans-differentiate human noncardiac somatic cells into cardiomyocytes. Direct differentiation has been shown to be considerably quicker and more efficient than generation of iPSC-CM from fibroblasts in murine cells (Ieda et al. 2010), and has the theoretical advantages of avoiding the heterogeneity that is introduced in reprogramming. Despite the potential of induced cardiomyocytes (iCM), however, full functional characterization and comparison with iPSC-CM, hESC-CM and adult cardiomyocytes is still required.

16.3 Structure and Functional Phenotype of hESC and iPSC-CM

The study of structure and function in cardiomyocytes derived from pluripotent stem cells is complicated by evidence that differentiation method (Pekkanen-Mattila et al. 2010a) and culture conditions (Otsuji et al. 2010) may strongly influence phenotype. It is also uncertain whether hESC-CM and iPSC-CM have different phenotypes.

16.3.1 Structural Properties

Structural and functional properties of cardiomyocytes are intimately related. Ultrastructural analysis shows that hESC-CM develop in vitro from spherical cells to elongated cells with a more organized sarcomeric pattern (Snir et al. 2003) (Fig. 16.3). Transmission electron microscopy of the hESC-CM at varying developmental stages shows progressive ultrastructural maturation from an irregular myofilament distribution with parallel nascent z-bands containing myofibrils to a more mature sarcomeric organization containing well-defined sarcomeres with recognizable A, I and M-bands in older hESC-CM (Baharvand et al. 2006; Otsuji et al. 2010; Snir et al. 2003). It has also been suggested by some authors that maturation of the ultrastructure is associated with withdrawal from the cell cycle in hESC-CM (Snir et al. 2003). iPSC-CM also have functional, albeit immature, sarcomeric structures (Zwi et al. 2009) and comparative studies between hESC-CM and iPSC-CM have not shown any difference in ultrastructural phenotype (Zhang et al. 2009).

In adult human ventricular cardiomyocytes, the cell membrane has regular invaginations called t-tubules which are considered critical for normal function (Brette and Orchard 2003). Contraction is caused by a net flux of Ca^{2+} ions into the cardiomyocyte through L-type calcium channels in t-tubules. The increase in cytoplasmic [Ca^{2+}] causes the ryanodine receptors (RyR) in the membrane of the sarcoplasmic reticulum (SR) to release stored Ca^{2+} into the cytoplasm. Ca^{2+}-induced Ca^{2+} release (CICR) (Bers 2002) accounts for between 70 and 90% of the rise in cytoplasmic [Ca^{2+}] during contraction of the adult cardiomyocyte (Bers et al. 2006). Cytoplasmic calcium binds to Troponin C, displacing tropomyosin and initiating contraction of the sarcomere. Cytoplasmic Ca^{2+} is taken up by the sarco/endoplasmic reticulum Ca^{2+}-ATPase pump (SERCA) into the SR, or is ejected from the cell by the sodium-calcium exchanger (NCX). Cytoplasmic [Ca^{2+}] drops and tropomyosin complex return over the active site of the actin filament, ending contraction. This beat-to-beat rise and fall in [Ca^{2+}] is known as a calcium transient (Bers 2002) (Fig. 16.4). T-tubules ensure that the L-type calcium channels are in close proximity to the RyR, minimizing the diffusion distance between the two ion transporters (Brette and Orchard 2003, 2007). This ensures that Ca^{2+} is released synchronously from RyR across the cell (Brette and Orchard 2003) and that there is no delay in release of Ca^{2+} from RyR in the center of the cell. In pathological cells, detubulation occurs, resulting in a time delay in release of Ca^{2+} from the SR in the center of the cell (Brette and Orchard 2003, 2007; Song et al. 2005). Some studies have been unable to detect t-tubules in hESC-CM (Lieu et al. 2009) while others find that they

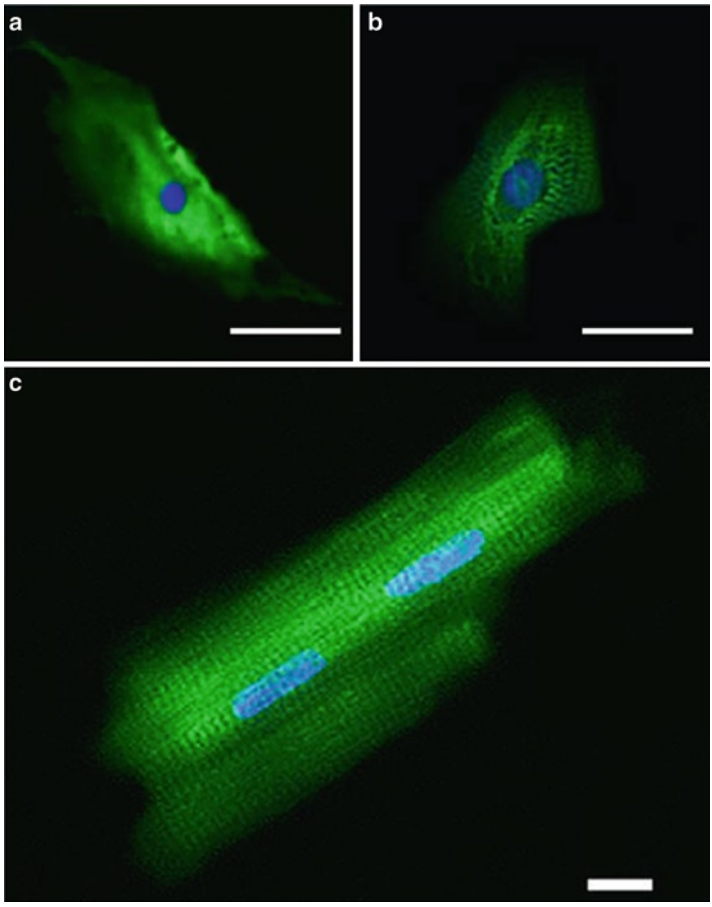

Fig. 16.3 Myosin heavy chain (MHC, *green*) and nuclear (DAPI, *blue*) staining of hESC-CM without (**a**) and with (**b**) characteristic sarcomeric striation patterns, compared with adult rat ventricular myocyte. (**c**) Scale bar is 20 μm. With thanks to Dr. Mirna Chahine

are present (Baharvand et al. 2006; Satin et al. 2008) but clearly less abundant and less organized than in adult ventricular cardiomyocytes. This may explain why the calcium handling of hESC-CM resembles that of failing or atrial myocytes which have fewer and less-organized t-tubules (Satin et al. 2008).

16.3.2 Functional Properties

hESC-CM and iPSC-CM form functional syncytia-like adult myocardial tissue (Caspi et al. 2009; Kong et al. 2010; Zhang et al. 2009), with evidence that cells which beat together in clusters are connected by gap junctions (Caspi et al. 2009;

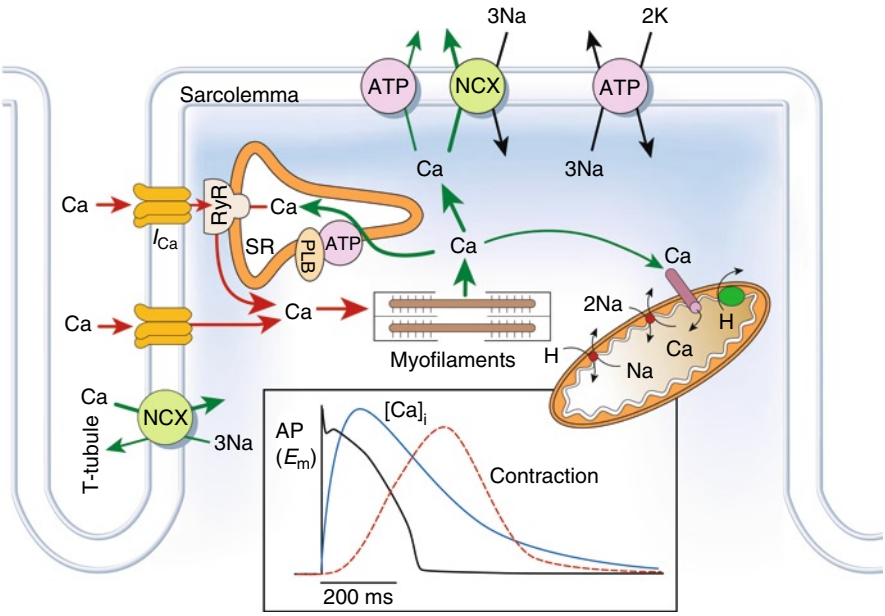

Fig. 16.4 Excitation-contraction coupling. The action potential (AP) triggers L-type calcium channels to open during the plateau phase of the cardiac AP, causing an influx of calcium ions (Ca^{2+}) into the cardiomyocyte. The increase in intracellular Ca^{2+} causes the ryanodine receptors to release Ca^{2+} stored in sarcoplasmic reticulum (SR) into the cytoplasm. The cytoplasmic calcium binds to Troponin C, initiating contraction of the sarcomere. Intracellular Ca^{2+} is taken up by the sarco/endoplasmic reticulum Ca^{2+}-ATPase pump into the SR, or ejected from the cell by the sodium-calcium exchanger (NCX). Intracellular Ca^{2+} concentration drops and the tropomyosin complex returns over the active site of the actin filament, ending contraction. Figure reproduced with permission from Bers (2002)

Pekkanen-Mattila et al. 2010a). Action potential (AP) can be recorded either from clusters of beating cells using sharp pipettes (Zhang et al. 2009) or enzymatically isolated myocytes (Pekkanen-Mattila et al. 2010a; Sartiani et al. 2007). It is suggested that even enzymatically isolated cells from the same cluster have similar AP characteristics (Zhang et al. 2009).

hESC-CM and iPSC-CM are more heterogeneous than adult cardiomyocytes and have key functional differences (Kong et al. 2010; Pekkanen-Mattila et al. 2010a; Zhang et al. 2009). With the exception of a small number of cells within the pacemaker and conduction system (nodal cells), adult cardiomyocytes normally maintain a stable negative membrane potential and are electrically inactive until they are depolarized (Kleber and Rudy 2004). This usually occurs by the passage of ions from adjacent myocytes through connecting gap junctions (Desplantez et al. 2007). When cardiomyocytes are sufficiently depolarized, an AP is initiated (Bers and Despa 2009; Kleber and Rudy 2004) (Fig. 16.5). This is the result of an initial influx of Na^+ that further depolarizes the cell, followed by an influx of Ca^{2+} and efflux of K^+ that maintain the cell in a depolarized state. The influx of intracellular Ca^{2+}

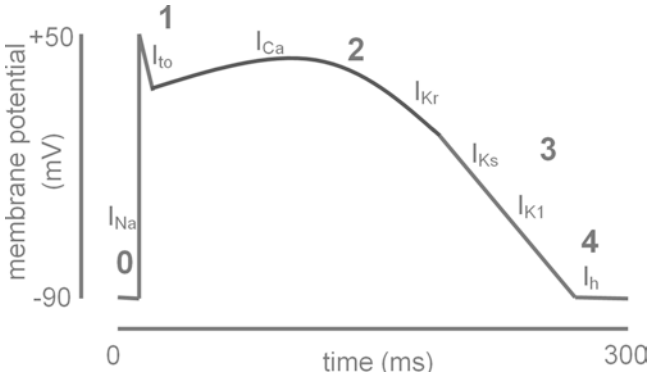

Fig. 16.5 Diagram of an idealized adult human ventricular AP. The phases of the AP are labeled (phase 0–4). The predominant cardiac ion currents at each point in the AP are labeled (I_{Na} = sodium current; I_{to} = transient outward potassium current; I_{Ca} = calcium current; I_{Kr} = rapidly activating delayed rectifier potassium current; I_{Ks} = slowly activating delayed rectifier potassium current; I_{K1} = inward rectifier potassium current)

causes SR to release stored Ca^{2+} and the cytoplasmic Ca^{2+} initiates contraction of the sarcomere (Bers 2002) (Fig. 16.4). Finally, an efflux of K^+ predominates which repolarizes the cell.

A key difference between adult cardiomyocytes and hESC-CM or iPSC-CM is that although some are electrically inactive until stimulated, many exhibit a high degree of automaticity with spontaneous AP (Kong et al. 2010; Pekkanen-Mattila et al. 2010a; Zhang et al. 2009), unlike adult myocardium in which this is only seen in pacemaker cells (DiFrancesco 2010). The mechanisms that underlie the automaticity in hESC-CM and iPSC-CM probably differ from the mechanisms that underlie automaticity in pacemaker cells. Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels allow the passage of Na^+/K^+ into specialized pacemaker cells in the adult myocardium when the cell is repolarized in diastole. This slowly activating, inward "funny" current (I_f) (DiFrancesco 2010) may play a role in the automaticity of hESC-CM; however, other mechanisms are probably more important (Satin et al. 2004). hESC-CM express a large sodium current density and their action potential duration (APD) is shortened by L-type calcium channel blockade (Habib et al. 2008; Satin et al. 2004). It has been suggested that whilst a diverse set of potassium channels are expressed in older hESC-CM compared to early-differentiated hESC-CM (Sartiani et al. 2007), the expression of the inward rectifier potassium current (I_{K1}) remains low (Kong et al. 2010; Satin et al. 2004). Consequently, the presence of comparatively large inward sodium (I_{Na}) and relatively small I_{K1} probably explains the increased automaticity of hESC-CM (Satin et al. 2004). Fluctuations in intracellular Ca^{2+} play a critical part in the automaticity of developing murine myocardium (Viatchenko-Karpinski et al. 1999). Whilst they may also contribute to the automaticity of hESC-CM, it is not thought that they have a dominant role (Satin et al. 2004). This is supported by studies which show that co-culture of HEK-293 cells expressing I_{K1} electrically coupled to rat neonatal cardiomyocytes

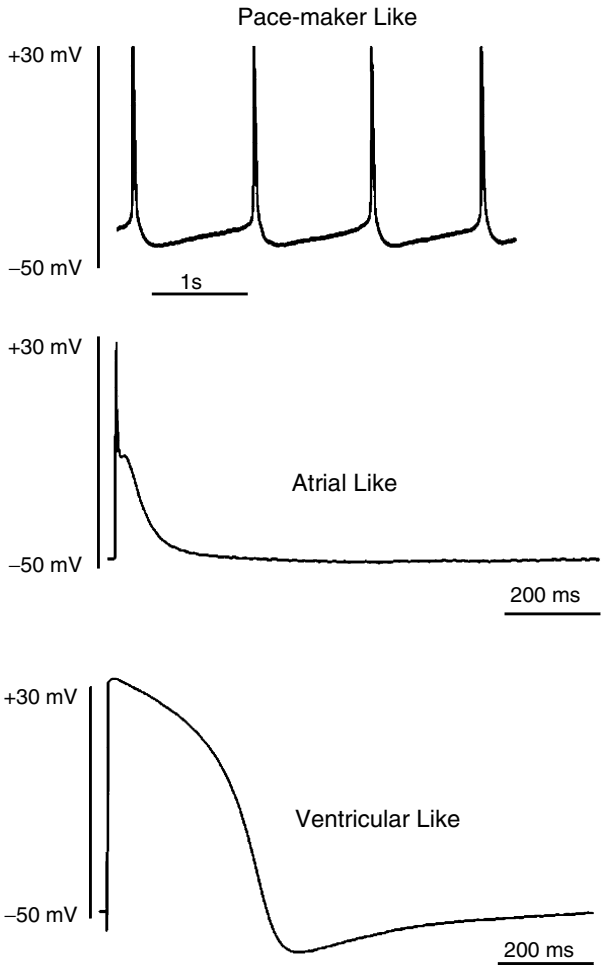

Fig. 16.6 Different AP phenotypes recorded from hESC-CM (our unpublished data)

reversibly inhibits their automaticity (de Boer et al. 2006), and emerging evidence which suggests that forced expression of the Kir2.1 gene in hESC-CM which encodes the I_{K1} channel results in a more mature electrophysiological phenotype (Kong et al. 2010).

The AP measured in hESC-CM and iPSC-CM are often categorized as "ventricular," like "atrial" like or "nodal" like (pacemaker) based on their resemblance to adult AP. "Ventricular" cells are said to be characterized by a fast and large upstroke, and plateau phase. "Atrial" like cells are said to have more triangular AP. "Nodal" like cells are said to have a slower and smaller upstroke, prominent phase four depolarization and to be relatively depolarized in diastole (Fig. 16.6). In some cases, these phenotypes are also quantified using parameters such as APD at 90% repolarization (APD90), maximum rise of the AP upstroke (dV/dt_{max}), action potential

amplitude (APA) and maximum diastolic potential (MDP) (Cao et al. 2008; He et al. 2003; Mummery et al. 2003; Pekkanen-Mattila et al. 2010a; Sartiani et al. 2007; Satin et al. 2004; Yoshida and Yamanaka 2010; Zhang et al. 2009). Whether "ventricular," like "atrial" like and "nodal" like cells represent distinct populations of cells or a continuous range of AP characteristics within one population is yet to be shown. Some studies suggest there is considerable heterogeneity in AP characteristics within these groups and considerable overlap in the characteristics between groups (Pekkanen-Mattila et al. 2010a). It is also suggested that "atrial" like cells have a higher rate of spontaneous contraction (Zhang et al. 2009); however, this is not supported by all studies (Pekkanen-Mattila et al. 2010a). In addition to using the electrophysiological characteristics, attempts have been made to employ specific reporters to delineate subtypes of hESC-CM (Kolossov et al. 2005; Kuratomi et al. 2009; Muller et al. 2000). Neither direct nor indirect comparison of the electrophysiological properties of hESC-CM and iPSC-CM suggests that there is any difference in phenotype, with both cell types exhibiting similar AP morphology and response to pacing at different rates (Zhang et al. 2009). Finally, a number of studies have used either isolated cells (Caspi et al. 2009; Moretti et al. 2010; Otsuji et al. 2010; Pekkanen-Mattila et al. 2010a) or beating clusters plated onto multi-electrode arrays (Caspi et al. 2009; Kehat et al. 2001; Otsuji et al. 2010; Tanaka et al. 2009; Zwi et al. 2009) to demonstrate that hESC-CM and iPSC-CM have an appropriate chronotropic response and change in AP morphology in response to several pharmacological agents including adrenergic and cholinergic agents (Kehat et al. 2001; Moretti et al. 2010; Mummery et al. 2003; Pekkanen-Mattila et al. 2010a; Tanaka et al. 2009; Xu et al. 2002; Zwi et al. 2009), I_{Kr} channel blockade (Caspi et al. 2009; Moretti et al. 2010; Otsuji et al. 2010; Pekkanen-Mattila et al. 2010a; Tanaka et al. 2009; Zwi et al. 2009), I_{Ks} channel blockade (Zwi et al. 2009) and calcium channel blockade (Tanaka et al. 2009; Zwi et al. 2009). These similarities to an authentic cardiac phenotype support the feasibility of using hESC-CM and iPSC-CM as in vitro model systems (Caspi et al. 2009; Moretti et al. 2010).

hESC-CM have intracellular calcium transients that correspond to their beating activity (Kehat et al. 2001; Mummery et al. 2003; Satin et al. 2008). Whilst experiments with murine ESC-CM demonstrated that a functioning SR was essential for regulating contraction (Fu et al. 2006), early experiments with hESC-CM suggested that they did not respond to caffeine, which depletes SR calcium stores, or ryanodine which blocks the RyR receptor, suggesting that hESC-CM did not have functional SR and that their contractions result from influx of extracellular Ca^{2+} into the cell (Dolnikov et al. 2006). A subsequent study, however, suggested that 40% of hESC-CM were responsive to caffeine, ryanodine and the SERCA inhibitor thapsigargin (Liu et al. 2007). Furthermore, in contrast to earlier work (Dolnikov et al. 2006), as in adult cardiomyocytes, a positive force-frequency relationship was demonstrated in this population of cells (Liu et al. 2007). Other studies suggest that not only is a functional SR present in hESC-CM, as demonstrated by caffeine and ryanodine sensitivity, but (like murine cells, Fu et al. 2006) this is present from as early as 2 days following the commencement of beating (Satin et al. 2008).

Finally, protein expression and functional data suggest that NCX expression is higher in hESC-CM compared to adult cardiomyocytes (Fu et al. 2010); by contrast expression of regulatory proteins such as junctin, triadin and calsequestrin which are usually expressed in adult cardiomyocytes are completely absent in hESC-CM. Calsequestrin, in particular, plays a critical role in excitation contraction coupling, acting as a critically important Ca^{2+} buffer in some intracellular compartments (Beard et al. 2004) and regulating CICR (Bassani et al. 1995; Lukyanenko et al. 1996; Shannon et al. 2000).

There is limited evidence to suggest that differentiation protocols may affect the electrophysiology of hESC-CM. In a study comparing the EB with the END-2 differentiation system, EB-derived hESC-CM were found to be less likely to express an atrial phenotype, more likely to beat continuously, and to have a significantly more hyperpolarized MDP (Pekkanen-Mattila et al. 2010a). The effect of time in culture on the electrophysiological phenotype has not yet been fully characterized. Whilst it has been suggested that the AP maturity increases with time in culture (Sartiani et al. 2007), which is consistent with evidence from murine models (Hescheler et al. 1997), other studies show time in culture does not significantly affect the maturation of hESC-CM (Pekkanen-Mattila et al. 2010a). Whether or not hESC-CM and iPSC-CM mature in culture, there is considerable interest in accelerating and homogenizing the maturation of cells derived from hESC and iPSC not only to facilitate representative disease models but also to ensure the safety of cell therapy. Promising techniques range from cyclical replanting and suspension of beating clusters in culture (Otsuji et al. 2010) to forced expression of the genes which encode the I_{Kr} channel or calsequestrin in order to improve the maturity of the AP or intracellular calcium handling, respectively (Kong et al. 2010).

16.4 Current Uncertainties and Future Directions

There is considerable interest in the scientific and therapeutic applications of pluripotent stem cells as a result of significant improvements in the understanding of the molecular processes that underlie early differentiation of human pluripotent stem cells, better understanding of the phenotype of the cardiomyocytes derived from pluripotent stem cells in vitro, and the simultaneous discovery of techniques that induce pluripotency in human somatic cells. The possible applications of pluripotent stem cells are essentially twofold. Firstly, that cardiomyocytes derived from pluripotent stem cells may one day be used to replace cardiomyocytes lost from the adult myocardium due to aging or pathological processes such as ischemia. Secondly, that cardiomyocytes derived from iPSCs may be used as in vitro models to study cardiac development, primary diseases of the myocardium, to develop novel pharmacotherapy, or to test the efficacy and toxicity of novel pharmacotherapy.

The therapeutic potential of iPSC in particular is unquestionably very exciting and early experimental data are promising (Nelson et al. 2009). Research to address the technical problems relating to the delivery and retention of stem cells

transplanted into the host myocardium is already at a stage that would allow rapid transition into the clinic (Gersh et al. 2009). There are, however, several areas of uncertainty that must be addressed if iPSC and hESC are to be used in cell therapy. Technology such as left ventricular assist devices (LVAD) is available which could both provide a "bridge to transplantation" (Clegg et al. 2006), during which iPSC-CM could be generated and multi-cellular constructs created (Chen et al. 2010). It is also possible that these devices may make the physiology of the myocardium more amenable to cell transplantation by reducing left ventricular pressure, oxygen consumption and improving coronary blood flow (Pantalos et al. 1988; Tansley et al. 2004). The presence of the LVAD supports heart function against deleterious consequences of iPSC-CM implantation, such as arrhythmias, while the anticipated transplant would deliver the explanted heart for investigation of the efficiency of cell integration.

Similarly, whilst the concept of in vitro disease models has been proven (Carvajal-Vergara et al. 2010; Caspi et al. 2009; Moretti et al. 2010), the practical application of this technology has not been fully realized. The feasibility of human pluripotent cell-derived cardiomyocyte disease models was demonstrated when hESC-CM were used to investigate potential mechanisms for the arrhythmogenic effect of bile acids on the fetal heart (Abdul Kadir et al. 2009). Older hESC-CM were shown to be more resistant than early hESC-CM to bile acid-induced disruption of rhythm, depression of contraction and desynchronization of cell coupling, paralleling the difference between fetal and maternal susceptibilities (Abdul Kadir et al. 2009). The feasibility of pluripotent stem cells to investigate adult arrhythmias was more recently demonstrated when the prolonged APD seen in patients with the inherited long-QT syndrome was replicated in vitro using iPSC-CM (Moretti et al. 2010). Significantly, the susceptibility to catecholamine-induced tachyarrhythmia, which can be fatal in patients, was also replicated in vitro (Moretti et al. 2010). Not only have disease phenotypes been replicated using iPSC-CM (Carvajal-Vergara et al. 2010; Moretti et al. 2010) but there is also emerging evidence to suggest that iPSC may have a critical role in elucidating the molecular mechanisms that underlie these disease phenotypes (Carvajal-Vergara et al. 2010).

Interestingly, the problems that prevent the application of hESC and iPSC in both these areas are similar. Discounting the considerable but technical problems of the cost and time-consuming nature of this technology, future application is critically dependent on improving the homogeneity of differentiated cardiomyocytes in order to ensure safe cell therapy and reproducible, representative disease models. This will either require directed differentiation protocols or identification of progenitor cells to be improved and the factors which determine in vitro maturation of cardiomyocytes to be better understood.

Finally, comparative characterization of the phenotype of hESC-CM and iPSC-CM is currently limited. Given the emerging, albeit limited, evidence that there are differences between hESC and iPSC, this must be further evaluated, especially given the dynamic nature of iPSC technology and the rapid evolution of reprogramming techniques.

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Chapter 17
The Generation of Disease-Specific Cell Lines and Their Use for Developing Drug Therapies

Stefanie Gögel and Stephen L. Minger

Abstract Regenerative medicine has become an increasingly important field of research in contemporary bioscience. The importance of this research area is based on demographic changes in our aging societies and driven by the establishment of human embryonic stem cell lines. Disease-specific cell lines can be derived by various strategies, all aiming to enhance the understanding of disease at a cellular level and opening doors for drug discovery and development. The long-term goal of stem cells could lie in the potential to replace tissue and organs susceptible to age-related degeneration or traumatic injury.

17.1 Introduction

The exciting field of regenerative medicine and stem cell research has become increasingly dynamic since the establishment of the first human embryonic stem (ES) cell lines harvested from the inner cell mass of human blastocysts in 1998 (Thomson et al. 1998). This area of research will be increasingly important with respect to the huge potential in clinical applications of stem cells. Not only can ES cells continuously divide and remain undifferentiated, they also have the capacity to differentiate into all of the approximately 220 different cell types of the human body. The necessity for regenerative medicine is stimulated by the rising incidence of degenerative diseases based on demographic changes in an aging society.

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Numerous ethical, scientific, and legislative hurdles still need to be overcome before stem cell therapies for patients become a reality, but the long term therapeutic goal lies in the potential to produce replacement tissue of specific cells that would make the transplantation of donor organs, that are accompanied by high costs of long term aggravating drug therapy, a second choice of treatment.

In the near term, disease-specific cell lines may serve a vital role as fundamental research tools to better understand degenerative conditions caused by the death or dysfunction of one or few cell types, as well as novel sets of cells that may lead to new therapeutic advances. They thus have the potential to be used for identifying the underlying mechanisms that cause the development of disease, for fundamental drug discovery, and for predictive toxicological testing. The differentiation of stem cells can provide a model system to study early developmental pathways, and therefore may shed light into important patho-physiological processes like infertility, and onset of degenerative diseases.

There are currently four major methods of generating disease-specific human ES cell lines from individuals or embryos with known genetic disorders, but these may also be derived through genetic manipulation of genetically normal human ES cell lines (see Fig. 17.1).

17.2 Generation of Disease-Specific Cell Lines via:

17.2.1 Preimplantation Genetic Diagnosis (PGD)

Preimplantation genetic diagnosis (PGD) is used for screening the developing embryo for genetic inherited diseases. It is offered to couples who are aware of a genetic predisposition to a known inherited disease. During PGD, a single cell is isolated from a cleavage stage embryo, and subsequent polymerase chain reaction (PCR) using disease-specific primers will elucidate the occurrence of a mutation in simple monogenic disorders. The remaining embryos found to be unaffected by the genetic mutation will be transferred into the uterus and developed further. Embryos, which have been diagnosed with a genetic disorder following PGD, are generally discarded, but may be donated for human ES cell derivation under the appropriate regulations. The process of cell line derivation is well described in the review by Stephenson et al. (2009).

17.2.2 Homologous Recombination

The technique of gene targeting using ES cells derived from the preimplantation blastocyst of a mouse is a powerful tool for studying the exact role of genes of

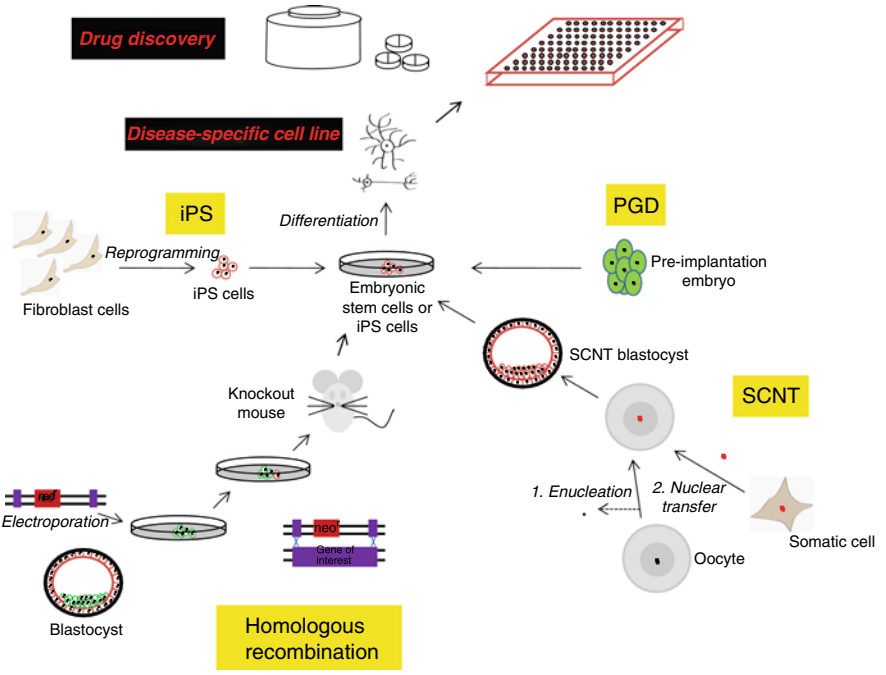

Fig. 17.1 Schematic diagram illustrating the different strategies to develop disease-specific cell lines for drug discovery. Couples who undergo in vitro fertilization can donate embryos for research, which have inherited a genetic disease previously discovered by preimplantation genetic diagnosis (PGD). During somatic cell nuclear transfer (SCNT) a nucleus of a somatic cell is transferred into an enucleated oocyte, which induces the transformation of a somatic transcriptional program to an embryonic transcriptional program. The blastocyst can be implanted into a surrogate mother (Dolly the sheep) or can be used to derive stem cell lines. Homologous recombination has been widely used to generate knockout mice to study genes of interest. From these, mouse stem cell lines can be derived. Viral or plasmid transduction of fully differentiated cells leads to reprogramming of the cell. These induced pluripotent stem (iPS) cells are highly similar to stem cells and can also be differentiated into disease-specific cell lines for subsequent drug discovery

interest. Via homologous recombination these genes can be "knocked-out" or site-mutated at a specific codon, and this is often used to generate knock-out mice. This method can also generate cell lines with mutant genes in their normal physiological and genetic context.

Exogenous DNA to generate disease-specific cell lines might also be introduced via electroporation or chemical transfection. However, the transfection efficiency and survival rate of transfected hES cells is very low, and successful targeting occurs in approximately 1 in 10 million cells (Zwaka and Thomson 2003). Using homologous recombination, mutant and normal cell lines differ only in the gene of interest but share the same genetic background. This is clearly advantageous over viral or plasmid transfection where the exact integration site of exogenous DNA cannot be controlled.

17.2.3 Somatic Cell Nuclear Transfer (SCNT) or Therapeutic Cloning

Somatic cell nuclear transfer (SCNT) describes the process of the transfer of an interphase nucleus or intact cell into an unfertilized enucleated oocyte, which induces the transformation of a somatic transcriptional program to an embryonic transcriptional program. It is often also referred as therapeutic cloning in the case where a genetically identical ES cell line to the donor nucleus is created. The first experiments were done in 1952, when Briggs and King transplanted nuclei from blastula stage *rana pipiens* embryos into enucleated oocyte, which further developed into normal tadpoles and frogs (Briggs and King 1952). This oocyte-based reprogramming was repeated in 1962 by Gurdon and colleagues, who used the nuclei of a differentiated intestinal epithelial cell and transferred them into an enucleated egg of *xenopus laevis* (Gurdon 1962; Gurdon and Uehlinger 1966). The cloned embryo developed into an adult frog. These outstanding experiments have clearly proven that cytoplasmic factors within an oocyte can reprogramme the somatic cell nucleus into a nucleus with the transcriptional profile of an undifferentiated pluripotent cell, which is referred to as nuclear transfer embryonic stem cells (ntES).

SCNT is more difficult in mammals, and first positive results were only obtained in 1975 when Bromhall reported the development of a morula stage embryo after transferring rabbit morula cells into enucleated rabbit eggs (Bromhall 1975). A major breakthrough came about in 1997 when Ian Wilmut and Keith Campbell reported the successful creation of a cloned mammal by SCNT using nuclei from follicular cells of an adult sheep, which were transferred into an enucleated oocyte, which ultimately lead to the birth of "Dolly" (Wilmut et al. 1997).

A large number of cloned mammals and pluripotent stem cells have been generated using different species like rabbit, cow, mouse, and others, but the cloning of human embryos and resultant cloned human ES cells has been unsuccessful to date, and previously published results from Korea were proven to be fraudulent. There has been speculation that the UV light that is used during the procedure of enucleation might damage the oocyte, and therefore more elaborate methods using polarized light instead of Hoechst dye and UV to visualize the nucleus during the procedure of enucleation have reportedly increased the success of generating pluripotent stem cells by SCNT (Mitalipov et al. 2007). Byrne et al. published the first successful derivation of primate ES cells following SCNT using fibroblasts from an adult rhesus male macaque, although with very low efficiency. The authors generated only two pluripotent stem cell lines out of 304 primate oocytes, and there is significant evidence that the nucleus was not completely reprogrammed in these cells (Byrne et al. 2007).

SCNT with human cells not only has been unsuccessful to date, the research in this direction is highly restricted due to the profound shortage of high quality human oocytes and strong ethical concerns. Groups attempting to derive SCNT-derived human ES cell lines have generally had to rely on donated oocytes that have failed

to fertilize which are of generally poor quality for nuclear transfer (Stojkovic et al. 2005). However, in a complex study, Qi Zhou's group assessed the relationship between oocyte morphology and embryo development. They established four oocyte grades based on maturation signs, including oocyte morphology, first polar body, cytoplasmic granula distribution, and perivitelline space. Since the mature cytoplasm seems to hold the key role in reprogramming the donor cell genome, only very high quality human eggs are capable of getting to the blastocyst stage (Yu et al. 2009). To overcome these issues, one can either use nonhuman eggs as recipient for the nuclear transfer, or ideally analyze the underlying biology of the epigenetic reprogramming capacity of oocyte and apply it to somatic cells.

As an alternative to human oocytes, which will always be of low abundance, there has been interest in using animal oocytes from animals that are killed for food supply like cows, pigs, and rabbits to reprogramme human somatic cells to overcome the shortage of human oocytes. Enucleated rabbit oocytes have been used as hosts for human fibroblasts (cybrids), and large numbers of cloned blastocysts have been obtained from which several hESC lines were generated (Chen et al. 2003). Despite numerous attempts to repeat these experiments (Jingjuan et al. 2005; Vogel 2006), this work has not been replicated. The Lanza group compared, in an extensive study, the reprogramming of human somatic nuclei using human and animal oocytes (Chung et al. 2009). They observed, despite an obvious similar morphological development until the morula stage, striking differences in the pattern of reprogramming when using animal donor eggs. Only human-human embryos showed extensive reprogramming of the nucleus monitored by the upregulation of genes important for pluripotency like Oct4, Sox2 and Nanog, which puts the use of animal eggs as donor for SCNT to produce patient-specific stem cells into question. However, Sheng's group has also recently shown that bovine oocytes are also very competent in reprogramming human somatic cells to the blastocyst stage with an efficiency of 0.87% (Li et al. 2008) and that unlike the data reported in the Lanza paper, pluripotency genes are subsequently activated at the morula and blastocyst stage.

Since the genetic material in the derived hESCs is not exclusively in the nucleus, one might speculate if these embryos are indeed truly human. The mitochondrial DNA (mtDNA) lies within the cytoplasm, and cloned embryos can possess mtDNA exclusively from the host oocyte (homoplasmy), or a mixture of oocytic and donor mtDNA (heteroplasmy). There are around 100,000 mitochondria in a mouse oocyte, which reduces itself to about 1,000 copies in each cell of a blastocyst stage embryo, and is further reduced to about ten per primordial germ cell (Shoubridge and Wai 2007). The number of mitochondria in somatic cells correlates with the amount of oxygen that is required in the differentiated cell. In humans, zygotic transcription begins at about the four-cell stage and many maternal products are sequentially degraded. Therefore, as development proceeds, the amount of nonhuman DNA in the cybrid cells would become insignificant. Beside this ethical issue, an important scientific question needs to be addressed: due to evolutionary distance between the species, the mitochondrial function might be compromised and therefore success rate very low.

17.2.4 Induced Pluripotency Stem Cell (iPS)

Not only does the fusion of somatic cells with an enucleated oocyte lead to the reprogramming of the somatic nucleus, but also the fusion with ES cells reprogrammes the somatic cell, which leads to the conclusion that factors that are contained in the ES cell induce pluripotency (Cowan et al. 2005; Tada et al. 2001). However for practical reasons (low efficiency; inability to determine the extent of reprogramming) this is not a viable route to reprogramming, especially with the advent of newer more straightforward methods that may yield cloned human cells which have all or most of the attributes of pluripotent cells from human IVF embryos.

The first groundbreaking paper using the method of induced reprogramming was published by Takahashi and Yamanaka (2006). The authors originally chose 24 factors with a potential role in the induction of pluripotency in different combinations, and analyzed them in regard to their capability of reprogramming differentiated cells. The expression of four specific transcription factors via retrovirus transduction reprogrammed the developmental state of fully developed mouse fibroblast cells into cells that are highly similar to ES cells, commonly referred to as "induced pluripotent stem (iPS) cells" (Takahashi and Yamanaka 2006). The fibroblasts had a drug resistance cassette inserted into the *fbx15* locus, a gene exclusively expressed in ES cells. The antibiotic resistance gene would be expressed only upon reprogramming into an ES-cell like state. By this elegant method the authors could screen for G418 resistant colonies.

In November 2007 this new technique that reverses developmental processes of cells and induces pluripotency was applied to human adult somatic cells. The Yamanaka group used the original four factors used in mES cells (c-myc, KLF4, Oct4, Sox2) (Takahashi et al. 2007), whereas the Thomson lab used Oct4, Sox2, Nanog, and Lin28 (Yu et al. 2007). With both sets of genes, these iPS cells were able to produce teratomas after transplantation into SCID mice, which suggested that they were indeed pluripotent. iPS cells are functionally, molecularly, and morphologically highly similar to ES cells with regard to gene expression, epigenetic status and in vitro differentiation, but they are not identical to ES cells. DNA microarray data showed a significant difference in gene expression of 1,267 genes. Furthermore, differences in the methylation state of promoters of pluripotency genes like Nanog and Oct4 have been observed (Takahashi et al. 2007).

Since the original publication a plethora of follow-up papers have been published, where fine-tuning of the reprogramming method has been reported, for example, the Hochedlinger group used nonintegrating adenovirus (adeno-iPS) for the generation of iPS that transiently expressed exogenous Oct4, Sox2, Klf4, and c-Myc (Stadtfeld et al. 2008). The Yamanaka group has subsequently used plasmid transfection for reprogramming embryonic fibroblasts introducing Oct4, Klf4, and Sox2 from a polycistronic vector and c-myc from a separate plasmid (Okita et al. 2008). Finally, it has also been demonstrated that one or more of the reprogramming factors can be replaced by small molecules, enabling the generation of hES with only two factors

(Oct4 and Sox2) in combination with the histone deacetylase inhibitor, valproic acid (Huangfu et al. 2008).

This exciting technique allows the derivation of induced pluripotent cells from patient's skin cells, and subsequent differentiation into potentially any cell type of the body without the need of using human oocytes at all. These tailor-made patient stem cells could then be used for drug screening and basic developmental biology and disease research or even for cellular transplantation in the future. Since iPS cells retain the genetic identity of the donor, no immunoreaction in the recipient is expected, which would reduce or even eliminate the costs for drugs that suppress the patient's immune system.

To date, the clinical application of iPS is still open. Before clinical application can be considered a number of questions need to be addressed. For example, the mechanisms that control epigenetic reprogramming are still unknown. Many inducing factors from the early papers are now regarded as irrelevant or nonessential. They are oncogenes by definition, therefore the concern is that the cell may produce cancers in patients following implantation. The fact that the oncogene *c-myc* is dispensible for reprogramming is a significant step forward to the clinical application (Wernig et al. 2007; Nakagawa et al. 2008).

The use of purely chemical means that activate reprogramming proteins or their translation would be the ultimate goal for reprogramming to make the process safer and more practical. Furthermore, not all human differentiated cells appear to be capable of being reprogrammed. There seems to be a reverse correlation between the degree of differentiation and the capability of being reprogrammed. For example, the Schöler group could show successful reprogramming of mouse adult neural stem cells with only one single factor, Oct4 (Kim et al. 2009). Reprogramming of human fibroblasts is even less efficient and takes much longer to achieve in comparison to mouse cells due to the lower mitotic index. But recently other somatic cell types have been successfully reprogrammed including liver and stomach cells (Aoi et al. 2008) and mature B-lymphocytes (Hanna et al. 2008).

The use of retroviral vectors in the original publications to introduce the reprogramming cocktail of factors could potentially induce oncogenic transformation by integrating into the genome and disrupting or disturbing gene expression. Therefore the use of adenoviruses might prove to be advantageous although the reprogramming frequency is lower than retrovirus induced pluripotency (adeno iPS). This might be due to the fact that these factors are expressed only for a short period of time (Stadtfeld et al. 2008). A still outstanding question is if human adeno iPS cells are indeed as potent as human ES cells, are differentiated cells we make from iPS exactly the same as those derived from the inner cell mass of the developing blastocyst and do they function in the same way. Since stem cell scientists worldwide are trying to improve the iPS method, research papers that aim to reprogramme human somatic cells without the use of viruses are to date in the spotlight. Kaji et al. used a single multiprotein expression vector, comprising *c-myc*, *klf4*, *oct4* and *sox2* to reprogramme mouse and human fibroblasts and succeeded in establishing reprogrammed human cell lines from embryonic fibroblasts when using a *piggyback* transposon (Kaji et al. 2009; Woltjen et al. 2009).

The iPS technique has now been further improved by using recombinant proteins to induce pluripotency (Zhou et al. 2009). Mouse embryonic fibroblasts underwent four cycles of transfection to assert sustained activity of reprogramming proteins (Oct4, Sox2, Klf4, and c-Myc). These protein-induced pluripotent stem cells (piPSC) show all characteristics of conventional mESCs. If this approach can be applied to human cells, this can be considered as a huge step forward towards clinical application.

Nevertheless, a comparable research between hESC and iPS is still essential. One potential means of analyzing iPS and hES cells from the same donor genome would be to use the same patient-specific somatic cells, and reprogramme using human oocytes and iPS induction and then rigorously compare the resultant cell lines containing the same genome side-by-side.

Despite the fact that the mechanisms underlying reprogramming of somatic cells are not yet completely understood, the advantages of iPS to SCNT are convincing: it is a rather simple method that does not require a high level of skill, it is inexpensive and it is expected to move the field forward rapidly. It also solves many of the ethical issues associated with the use of human embryos for research since there is no need to destroy a human embryo in order to derive a hESC line, and has the potential to have a huge impact on scientific and political aspects of ES cell research worldwide. Nevertheless, SCNT derived stem cells would appear to be biologically closer to hES cells derived from the inner cell mass than iPS cells, they are isogenic to the donor, fully pluripotent and not genetically modified whatsoever.

17.3 Examples of Disease-Specific Cell Lines

Monogenetic diseases that result in the death or malfunction of one single cell type are ideal examples to be examined in disease-specific cell line model systems. They could provide a better model for complex human diseases that are difficult to reproduce in animal models.

For example, the molecular mechanisms underlying the neuronal loss caused by CAG/polyglutamine (polyQ) disorders like Huntington's disease or a mutation in the hypoxanthine phosphoribosyltransferase (*Hprt*) gene remain elusive. A gene-targeted mouse ES cell line containing expanded CAG repeats in the *Hprt* gene exhibit aberrant neuronal differentiation and CAG-length-dependent decreased neuronal survival which is caused by persistent Oct4 and repressor element-1 transcription factor/neuron restrictive silencing factor (REST/NRSF) expression (Lorincz et al. 2004). This is one of the first studies to show that modified ES cells can be used as a reliable in vitro system for inherited disorders.

The first example that clearly proves the therapeutic potential of iPS cells derived from adult fibroblasts has been shown for the mouse model of sickle cell anemia (Hanna et al. 2007). The authors could show that mice carrying the mutation for sickle cell anemia can be rescued by transplantation of hematopoietic progenitors

obtained in vitro from autologous iPS cells after correction by gene-specific targeting/homologous recombination.

iPS cells have also been developed from patients with genetic diseases like amyotrophic lateral sclerosis (ALS), CAG/polyglutamine (polyQ) repeat expansion diseases like Huntington disease (HD), juvenile-onset, type 1 Diabetes Mellitus (JDM), Parkinson disease, to mention a few (Dimos et al. 2008; Park et al. 2008).

The Eggan group established iPS cell lines from an 82-year-old patient with hereditary ALS by introducing *klf4*, *sox2*, oct4, and *c-myc* into fibroblasts (Dimos et al. 2008). ALS can be caused by a mutation in the superoxide dismutase (*sod1*) gene and leads to the progressive loss of motor neurons in the spinal cord and subsequent paralysis and death. These iPS cells have been differentiated into motor neurons and glia implicated in this disease to study the mechanism underlying the cell loss. In this proof-of-principle experiment they demonstrated that iPS cells can be generated from an elderly patient whose cells have been exposed to disease-causing agents for a lifetime.

Spinal muscular atrophy (SMA) is a neurological disease in which a single mutation in the *smn1* gene causes the death of motor neurons (Lefebvre et al. 1995). Current SMA treatment options are very limited, and there is no cure. The Svendsen lab generated iPS cells from a young type 1 SMA patient and successfully differentiated these into motor neurons, showing that a *smn1* mutation affects motor neuron survival. Additionally, iPS-SMA cells have been responsive to drug treatment (Ebert et al. 2009).

Parkinson's disease is a progressive neurodegenerative disorder caused by the loss of dopaminergic neurons in the *substantia nigra*. Recently ES cells for in vitro analysis for Parkinson disease have been established by SCNT using tail tip fibroblasts from the *Aphakia* mouse model, that show a selective loss of dopaminergic neurons due to a deletion in the *pitx3* gene. These cell lines were used to address the mechanisms of dopamine neuron degeneration and the role of Pitx3 in the generation and maturation of DA neurons (Amano et al. 2009).

It has been shown that transplantation of embryonic nigral transplants can restore dopamine function in human patients (Piccini et al. 1999). Due to the shortage of donor material, logistical and ethical problems, the transplantation of fetal brain tissue cannot be regarded as a universal treatment for Parkinson's disease. In 2008 the Jaenisch group derived neuronal progenitor cells from reprogrammed fibroblasts that differentiated into neurons and glia cells in culture. After transplantation into fetal rat brains they migrated into different areas and integrated into the host brain. In a rat model of Parkinson's disease they significantly improved behavioral function after transplantation (Wernig et al. 2008).

17.4 Conclusions

Animal models including *Drosophila melanogaster*, *Xenopus levis*, *Caenorhabditis elegans*, and *Mus musculus* served and still serve as excellent tools for the molecular analysis of a plethora of inherited diseases. Since molecular pathways are

often conserved among species, the resulting outcome of animal research is well transferable to the human condition. Nevertheless, modeling a disease in a dish using human cells can be advantageous in terms of achieving research goals within given temporal and financial limits.

Since the groundbreaking experiments by Briggs and King in the 1950s, it has been clear that adult cells can revert back to their embryonic state. The recent discovery of the iPS technique has the potential to revolutionize basic research with pluripotent cells and perhaps to develop new therapeutic approaches. Although not applicable in the clinic at the moment, this is the only feasible technique allowing the production of disease-specific cell lines for immediate use in the analysis of underlying mechanisms, search for new therapies, and drug discovery, without the use of a human embryo at all.

For clinical application of ES cell-based therapies stable in vitro differentiation protocols need to be developed, since ES cells can give rise to tumors and immune rejection response after transplantation. Since the costs for individual patient specific cell lines can be immense, banking of MHC-compatible hESC lines might prove to be necessary. Taylor et al. for example, reported that with only around 150 cell lines approximately 85% of the UK population can be covered for a reasonable HLA match (Taylor et al. 2005).

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Chapter 18
Advances in the Culture of Human Embryonic Stem Cells

Takamichi Miyazaki and Hirofumi Suemori

Abstract Over the last ten years, more than 1,000 hESC lines have been established throughout the world, and a wealth of knowledge of how to sustain undifferentiated growth of hESCs in culture has been accumulated. In the meantime, scientists have realized that there are some differences in the individual potential of different hESC lines, even though they are routinely established from identical sources. To comprehend the potential of individual hESCs, it is necessary to provide a common arena maintaining uniform cellular status for comparison. This issue in hESC research will be applicable to all other pluripotent stem cells that are currently being produced, such as the induced pluripotent stem (iPS) cell and the germline stem cell. This chapter will focus on the differences of hESC characteristics identified so far, and the current methods to support hESC culture, for further understanding of pluripotent stem cells.

18.1 Introduction

Human embryonic stem cell (hESC) research has continued for over 10 years since Thomson et al. reported the derivation of the first hESC line in 1998 (Thomson et al. 1998). During this decade, over 1,000 lines of hESC have been established throughout the world, and a wealth of knowledge of how to sustain undifferentiated growth of hESCs in culture has been accumulated. In the meantime, scientists have realized

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that there are some differences in the individual potential of different hESC lines, even though the hESCs have routinely been established from identical sources, surplus in vitro fertilized embryos. To comprehend the potential of individual hESCs, it is necessary to provide a common arena maintaining uniform cellular status for comparison. This issue in hESC research will be applicable to all other pluripotent stem cells that are currently being produced, such as the induced pluripotent stem (iPS) cell (Takahashi et al. 2007; Yu et al. 2007) and the germline stem cell (Conrad et al. 2008). This chapter will focus on the differences of hESC characteristics identified so far, and the current methods to support hESC culture, to aid further understanding of pluripotent stem cells.

18.2 Derivation and Individual Characteristics of hESCs

A key question is what actually causes variation in hESC potential? The hESC is generally derived from the inner cell mass (ICM) of a blastocyst. The derivation procedure usually begins with the isolation of ICM. The first derivation by Thomson et al. in 1998 used immunosurgery for ICM isolation, whereas other investigators employed mechanical methods. The ICM, regardless of isolation protocol, is plated onto a feeder layer and generally forms a dissimilar morphology to established hESCs during the initial phase of derivation culture. They initially maintain a silent state but start to expand after approximately 2 days (Fig. 18.1a). The initial colony from the ICM contains robust proliferating cells and various differentiated cells (Fig. 18.1b). After a few passages, cells exhibit stable proliferation, at which point the enlarged colonies are ready to passage for further expansion (Fig. 18.1c). Since each hESC line is derived from a different single embryo, they have genetically distinct backgrounds. Indeed, many hESC scientists note that individual hESCs vary in behavior. The hESCs generally show slight differences in morphology and have differential efficiency in their proliferation rate, plating, or tendency toward

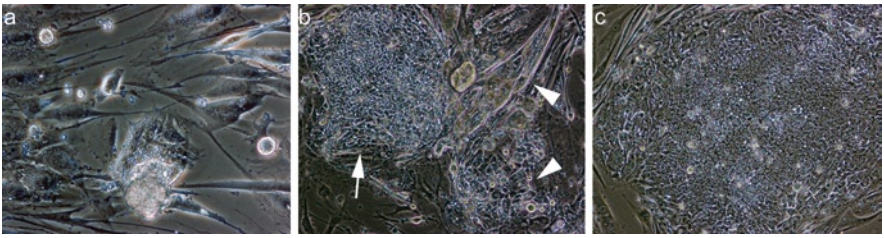

Fig. 18.1 The derivation process of hESC. During culture on feeder layers, the ICM changes to a cell population that proliferates stably and actively. (**a**) Day 2 – a part of the cell mass starts to proliferate. The cells in the center of the panel show an initial growth on MEF feeder cells. (**b**) Day 11 – the colony contains various cell populations. *Arrow* indicates a cell population that resembles hESCs. *Arrowheads* indicate differentiated cells. (**c**) A colony mainly composed of compact cells, which tightly adhere to each other. These cells exhibit active proliferation

differentiation. Ware et al. reported a general comparison of their efficiencies using sixteen hESC lines registered at NIH, acquired from UCSF (HSF-6), WiCell (H1, H7, H9, H13, H14), ESI (HES-1 to 4, -5), MizMedi (Miz-hES1), Bresagen (BG01–03) (Ware et al. 2006). According to their report, the hESCs had a range of doubling times from 31 h (HSF-6 and BG03) to 57 h (H13), cloning efficiency from 0.8% (BG02) to 9.2% (H13), and stable transfection rates following electroporation from 0% (BG02) to 53% (BG03). For data preparation, the hESCs were carefully cultured by one experienced technician to avoid the influence of culture technique, because personal skill can have profound effects on cellular status. They also noted a different handiness of individual lines; H1, H7, and H14 appear relatively stable but H9 and H13 have a tendency to develop problems, even though those lines were established at the same institute. With respect to specific differentiation characteristics, Osafune et al. assessed the spontaneous and inducible differentiation potential of hESCs (Osafune et al. 2008). They used 17 hESC lines (HUES 1–17, from the Harvard Stem Cell Institute), and reported that hESCs showed greater than 100-fold differences in lineage specific gene expression, indicating that there are some hESCs that have a tendency to differentiate into specific cell lines. Thus, the hESCs can be regarded as having a variety of characteristics in terms of specificity and proliferation.

If some hESCs have a tendency for specific cellular differentiation, they can be used as the situation demands, e.g., for cell transplantation or for research into drug discovery related to specific tissues. For progress in such medical studies, it is necessary to accumulate hESC information from a variety of laboratories, since a single institute has limitations in bulk derivation. However, given the present state of affairs, it is impossible to liberally share such detailed information on the characteristics of numerous lines, because the flux of hESCs between laboratories is mordantly controlled in some countries. To gain an understanding of characteristics and a comparison of hESCs generated worldwide, the International Stem Cell Initiative (ISCI) gathered hESC information and compared the features of hESCs (Adewumi et al. 2007). ISCI collected the cellular information of 59 hESC lines provided from 17 laboratories in 11 countries, and analyzed 17 cell-surface antigens and expression of 93 genes. The standard hESCs commonly express several cell-surface antigens, including SSEA3, SSEA4, TRA-1-60, TRA-1-81, and show a high degree of gene expression correlation for *NANOG, POU5F1/OCT4, TDGF, DNMT3B, GABRB3, GDF3*. Undifferentiated hESCs do not largely differ in the expression of such key markers. On the other hand, it was found that expression of some other genes such as *IGF2*, which is related to cell growth, differed markedly. Interestingly, X chromosomes, which are typically active in undifferentiated female hESCs, showed variable degrees of inactivation, thus indicating different levels of cellular differentiation. The early embryos at the time of hESC establishment are in the process of epigenetic reprogramming (Monk et al. 1987). That means that hESCs are susceptible to epigenetic modification during the period of derivation. However, not only modification prior to derivation controls the differential characteristics of hESCs, the methods of maintenance post derivation may also have a significant impact.

18.3 The Standard Culture of hESCs

Culture conditions strongly affect the undifferentiated status of hESCs. To screen the range of hESC potential, it is necessary to adapt the same procedure to routinely culture the lines. Table 18.1 shows a list of media and components widely used in hESC culture on feeder cells, summarized from the information submitted to the ISCI hESC comparison study. Many institutes use hESC culture medium of similar composition, but there are some local methods that include specific supplements (Table 18.1). The hESC culture medium is generally composed of DMEM/F12 (or Knockout-DMEM), Knockout Serum Replacement (KSR), β-mercaptoethanol, the non-essential amino acid, L-glutamine, and the addition of bFGF (Adewumi et al. 2007). In the initial stage, the maintenance of hESCs was carried out using fetal bovine serum (FBS), and a few laboratories continue to use FBS. Nowadays, hESCs are generally maintained in medium containing KSR instead of FBS. FBS is known to slightly increase the expression of genes associated with differentiation (Skottman et al. 2006), whereas KSR increases the growth rate of hESCs in comparison to FBS (Koivisto et al. 2004). This difference correlates with the tendency of hESCs to undergo differentiation in FBS. In addition, FBS often varies dependent on the sample so hESC culture differs based on the optimal product lot. Although KSR reduces the plating efficiency of hESCs in comparison to FBS, it is more important to maintain a high efficiency of sustaining the undifferentiated state. bFGF, which is a key factor for cellular growth in hESC medium, is used in the range of 4–10 ng/mL, but it is not known whether there are any significant differences among these bFGF concentrations. Some laboratories still add hLIF, but this might be dispensable because it is reported that hESCs do not require the hLIF signal for cellular growth and sustaining the undifferentiated state (Daheron et al. 2004). Although some laboratories use antibiotics, many hESCs are susceptible to toxic substances.

A mouse embryonic fibroblast (MEF) line is commonly used as a feeder layer. The density of the feeder cells and the source of cells are key parameters for successful culture of hESCs (Heng et al. 2004). However, the preparation of feeder layers differs between laboratories (Adewumi et al. 2007). Each laboratory has a different protocol for feeder preparation, with regard to various mouse strains (CF-1, MF-1, CD-1, 129sv, FVB) and seeding density (1.0×10^4–7.2×10^6cells per cm^2). This issue is also relevant to the use of feeders derived from human sources.

The most significant differences between laboratories are associated with passaging of hESCs. During the expansion of hESCs, each laboratory uses different protease enzymes, such as trypsin, collagenase IV, and dispase, in addition to mechanical dissociation (Table 18.2). Denning et al. reported that some hESCs, which are accustomed to exposure to a specific enzyme, have difficulty adapting to other enzymatic treatments (Denning et al. 2006). They showed that some protease treatments altered the differentiation potential of hESCs otherwise cultured under the same conditions. The bulk passage without regard to the kind of enzymatic or mechanical dissociation leads to changes in karyotype instability and various differences in gene expression (Mitalipova et al. 2005), thus the continuous monitoring of genetic stability is crucial.

Table 18.1 The various standard culture media for hESCs

DMEM/F12	KSR	NEAA (%)	L-glutamine	β-mercaptoethanol (mM)	bFGF	Additional component	Cultured hESCs (institute)
80%	20%	1	1 mM	0.1	4 ng/mL		Principle
KO-DMEM (80%)	20%	1	1 mM	0.1	4 ng/mL		Principle
80%	20%	1	1 mM	0.1	5 ng/mL		KhES-1,-2,-3 (KYOU)
85%	15%	[a]	[a]	[a]	4 ng/mL	Antibiotics	CCTL-9,-12,-14 (CAOS)
80%	20%	1	1 mM	[a]	[a]	[a]	H1, H7, H9, H13, H14 (WCEL)
80%	20%	1	L-glutamine (1%)	0.1	6 ng/mL	ITS (1%) antibiotics	FES21,22,29,30 (UHEL)
80%	KSR (10%) Plasmanate (10%)	1	GlutaMAX (2 mM)	0.055	[a]		CCTL-9,12,14 (CAOS), HUES-1 to -17 (CSCB)
DMEM (80%)	FBS (20%)	1	2 mM	0.1	Unknown	ITS (1%) antibiotics	HES-1,3,4,5 local (MIRD), MEL-1,2 local (SCSA)
DMEM (80%)	FBS (20%)	1	2 mM	0.1	Unknown	ITS (1%) antibiotics	HESC-NL1 local (HLAB)
KO-DMEM (84%)	KSR (8%) Plasmanate (8%)	1	GlutaMAX (2 mM)	0.055	bFGF (10 ng/mL) hLIF (10 ng/mL)		CA1,2 local (SLRI)

(continued)

Table 18.1 (continued)

DMEM/F12	KSR	NEAA (%)	L-glutamine	β-mercaptoethanol (mM)	bFGF	Additional component	Cultured hESCs (institute)
HES medium	2 parts BRL media, 3 parts BRL-CM, 1,000 U/mL hLIF, antibiotics BRL medium: DMEM, 20% ESFCS, 1% NEAA, 1% L-glutamine 0.1 mM β-mercapto-ethanol, sodium pyruvate						WT3, WT4, CF1 (WKCL)

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CAOS Department of Biology, Faculty of Medicine, Masaryk University, Brno, Czech Republic, and Department of Molecular Embryology, Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, Brno, Czech Republic

WCEL WiCell Research Institute, USA

UHEL Biomedicum Stem Cell Center, Biomedicum Helsinki, University of Helsinki, Finland

CSCB Center for Stem Cell Biology, Department of Molecular Biology and Biotechnology, The University of Sheffield, UK

MIRD Center for Reproduction and Development, Monash Institute of Medical Research, Monash University & the Australian Stem Cell Center, Australia

SCSA Center for Stem Cell and Regenerative Medicine, Keck School of Medicine, University of Southern California, USA

WKCL Wolfson Center for Age-Related Diseases, King's College London, UK

HLAB Hubrecht Laboratory, The Netherlands

SLRI Samuel Lunenfeld Research Institute, University Avenue, Toronto, Ontario, Canada

UCSF The University of California, San Francisco, USA

NIHN NIH Stem Cell Unit, National Institutes of Health, USA

CELL Stem Cell Center, Lund University, Sweden

UNEW Institute of Human Genetics, International Center for Life, University of Newcastle, UK

NCEL Novocell, USA

[a]No detailed information

Table 18.2 The various dissociation protocols for general passage of hESCs

Dissociation method	Handling cell lines (institute)
Trypsin	HUES-1 to17 (CSCB), CA1,2, CA1,2 local (SLRI)
Collagenase IV	HSF1–14 (UCSF), CCTL-9,12,14 (CAOS), H1,7,9,13,14 (WCEL), FES21,22,29,30 (UHEL), H1,UC06 (NIHN)
Mechanical	SA001,002 (CELL), hES-NCL-1 (UNEW), MEL-1,2 (SCSA), MEL-1,-2 local (SCSA), BG01,02,03 (NCEL), (NIHN)
Dispase	HESC-NL1, HESC-NL1 local (HLAB)
Trypsin/collagenaseIV	KhES-1,-2,-3 (KYOU)
Mechanical/dispase	HES-1,2,3,4,5,9,10,11,12,13 (MIRD)
Mechanical/collagenase	SHEFF-1,2,3 (CSCB)

Note: the lists on Table 18.1 and Table 18.2 are adapted from Supplemental Table 6 of an article that appeared in *Nature Biotechnology*. 2007; 25(7) (Adewumi et al. 2007). This list does not indicate the current method used in each laboratory

Therefore, even though a standard culture protocol has been organized, there are still variations between individual laboratories. This results in huge variability between hESCs, and makes the development of hESC study difficult. Particularly with regard to the varied use of feeder layers, the total quality of the feeders is difficult to standardize among the institutes in comparison to the hESC culture medium. In contrast, the defined medium or feeder-free culture, which is described in the following section, is easy to standardize for potential comparisons among laboratories.

18.4 The Advanced Culture of Human ES Cells: The Role of Soluble Substances

In comparison to the standard hESC culture system on a feeder layer, the use of defined media may allow better comparison of the characteristics of hESCs from different laboratories, because the components are well defined and readily available to all. Defined media were originally developed to construct a system of xeno-, pathogen-, unknown factor-free culture for the prospective medical applications of hESCs. To construct a defined medium, one must first identify what factors actually support the proliferation of hESCs in an undifferentiated state. Many investigators have sought to identify the key factors and constructed defined media based on their results. Jamie et al. systematically identified the receptors specifically expressed on hESCs by a comparison of cDNA microarrays among hESCs, human germ cell tumor cell lines and tumor samples, somatic cell lines and testicular tissue samples (Sperger et al. 2003). They found that the hESCs commonly expressed receptors for several signaling factors such as Wnt, GABA, FGF, IGF, ActivinA, and BMP. Ludwig et al. explored these soluble factors for the construction of novel medium and determined that some of them could work as key factors for the undifferentiated growth of hESCs (Ludwig et al. 2006a). Their medium, TeSR1, includes bFGF, and

LiCl, GABA (gamma-aminobutyric acid), pipecolic acid and TGF-β (transforming growth factor-beta) as key components. mTeSR™1 (StemCell Technologies Inc), which is modified TeSR1 using animal components in place of the human components, was subsequently developed as a medium for research purposes (Ludwig et al. 2006b).

Unlike the traditional basal medium, Genbacev et al. reported successful culture by using X-VIVO 10 with a high dose (80 ng/mL) of bFGF (Genbacev et al. 2005). X-VIVO 10 was originally developed for the culture of hematopoietic stem cells and is composed solely of human derived and recombinant proteins. Li et al. also reported that the addition of Flt3 ligand, which is a stimulating factor for hematopoietic cells, can decrease the requirement for a high dose of bFGF in X-VIVO 10 medium (Li et al. 2005). On the other hand, Liu et al. utilized the supplements N2 and B27, which support the growth and expression of post-mitotic neurons and tumor cells with a neuronal phenotype, for the maintenance of hESCs with addition of high dose (100 ng/mL) bFGF (Liu et al. 2006). Using N2 and B27 supplement, Yao et al. also succeeded in hESC culture, reducing the bFGF dose by adding BSA (Yao et al. 2006).

Some researchers have noted that a high dose of bFGF could solely support the undifferentiated growth of hESCs in various defined media. Interestingly, Levenstein et al. reported that 100 ng/mL bFGF is sufficient to solely support the undifferentiated culture of hESCs in non-conditioned medium similar to MEF conditioned medium for up to 164 population doublings using H1 and H9 cells (Levenstein et al. 2006).

As noted above, the soluble factors and the medium used for culture of hESCs have been widely developed. In contrast, the matrix used for culture substrate has not been rigorously addressed. Most defined medium is based on the use of mouse tumor-derived Matrigel™ as a basic scaffold. But, there have been recent improvements in the matrix for hESC culture.

18.5 The Advanced Culture of hESCs for Future Usage: The Role of Coating Substances

The report from Xu et al. (2001) was groundbreaking for feeder cell-free culture. They used Matrigel™ as a substrate for cellular adhesion. Matrigel™ is a basement membrane component derived from a mouse EHS tumor, primarily consisting of laminin, collagen IV, nidogen, and heparan sulfate proteoglycan. The combination of Matrigel™ with conditioned medium derived from MEFs (MEF-CM) robustly supports the undifferentiated growth of hESCs, similar to that on mouse feeder cells; and has become the method of choice for providing simple feeder-free conditions. As shown in Table 18.3, Matrigel™ has a good track record in combination with a range of defined media. Nearly all current defined media, TeSR1 series, N2B27 supplement medium, and X-VIVO 10 basal, can sustain hESCs on a Matrigel coated surface. It would be preferable if this product could

Table 18.3 The key substrates successfully added to feeder-free medium

Substrate	Source	Culture medium	Tested hES (hiPS) line	Reference
Matrigel™	Mouse, EHS tumor	MEF-CM	H1, H7, H9, H14	Xu et al. (2001)
		Feeder derived from hES – CM	hES-NLC1, H1	Stojkovic et al. (2005)
		DMEM/F12, 20%KSR, AlbuMAX, 40 ng/mL bFGF, 500 ng/mL Noggin	H1, H9, H14	Xu et al. (2005)
		X-VIVO, 80 ng/mL bFGF, or 40 ng/mL bFGF+ 15 ng/mL Flt3 ligand	H1	Li et al. (2005)
		TeSR1	H1, H7, H9, H14	Ludwig et al. (2006b)
		mTeSR™1	H14 and others	Ludwig et al. (2006a)
		mTeSR™1	HUES1	Braam et al. (2008)
		mTeSR™1	HS401, Regea06/015	Hakala et al. (2009)
		DMEM/F12, N2B27, 0.5 mg/mL BSA fractionV	H1, HSF6	Yao et al. (2006)
		DMEM/F12, N2B27, 100 ng/mL bFGF	H1, H9	Liu et al. (2006)
		DMEM/F12, 2% BSA fraction V, 1×trace elements A,B&C, 10 ng/mL HRG1β, 10 ng/mL ActivinA, 200 ng/mL LR^3-IGF1, 8 ng/mL bFGF	H1, BG01, BG02, BG03, CyT49	Wang et al. (2007)
Fibronectin	Human, cellular or plasma	MEF-CM, 4 ng/mL bFGF, 1,000 U/mL LIF, 120 pg/mL TGF-β1	I-6, I-3, H9	Amit et al. (2004)
	Bovine	MEF-CM, 4 ng/mL bFGF, 1,000 U/mL LIF, 120 pg/mL TGF-β1	I-6, I-3, H9	Amit et al. (2004)
	Human	DMEM/F12, N2B27, 100 ng/mL bFGF	H1, H9	Liu et al. (2006)
Laminin	Mouse, EHS tumor	MEF-CM	H1, H7, H9, H14	Xu et al. (2001)
		DMEM, SR, 50 ng/mL ActivinA, 50 ng/mL KGF, 10 mM nicotinamide	HSF6	Beattie et al. (2005)
	Human, placenta	X-VIVO, 80 ng/mL bFGF	UCSF-1	Genbacev et al. (2005)
		X-VIVO, 80 ng/mL bFGF or 40 ng/mL bFGF+15 ng/mL Flt3 ligand	H1	Li et al. (2005)
	Human, recombinant	MEF-CM	KhES-1, KhES-3	Miyazaki et al. (2008)
	Human, recombinant	O3(variant of mTeSR1), H3(TeSR2 equivalent)	HS420,HS207,HS401 (BJ#12, LDS1.4)	Rodin et al. (2010)

(continued)

Table 18.3 (continued)

Substrate	Source	Culture medium	Tested hES (hiPS) line	Reference
Collagen I	Porcine	hESF9	HUES-1, Shef1	Furue et al. (2008)
Vitronectin	Human, plasma	mTeSR™1	HUES1	Braam et al. (2008)
		MEF-CM	HUES1	Braam et al. (2008)
	Human, recombinant	mTeSR™1	HUES1, HES2, HESC-NL3	Braam et al. (2008)
Mixture of collagenIV, fibronectin, laminin, vitronectin	Human	TeSR1	H1, H7, H9, H14	Ludwig et al. (2006b)
E-Cad-Fc	Human, recombinant	mTeSR1, MEF-CM	H1, H9, (hiPSC2a,hiPSC3a, hiPSC6a)	Nagaoka et al. (2010)
sIPN	Hydrogel, polymer	MEF-CM	HSF-6	Li et al. (2006)
PMEDSAH	Synthetic polymer	StemPro hESC SFM, human cell-CM, MEF-CM	H9, BG01	Villa-Diaz et al. (2010)
BSP-PAS VN-PAS	Synthetic peptide -acrylate surfaces	X-VIVO 10, 80 ng/mL hrbFGF, 0.5 ng/mL hrTGF-β1	H1, H9	Melkoumian et al. (2010)

be shifted to one derived from human sources, but there is no similar basement component derived from human sources.

Fibronectin is also commonly used in combination with a range of defined media. Fibronectin is relatively easy to prepare from human plasma, and could therefore be a central substrate for a defined culture system. The only concern is that the hESCs cultured on fibronectin with MEF-CM for a long period of time exhibit reduced *OCT4* and *hTERT* expression, in comparison to when they are grown on other types of matrix, such as Matrigel, laminin, collagen, or MEF feeders (Xu et al. 2001).

Laminin, derived from mice, is also a good substrate to be used in defined medium. This protein is a major component (approximately 60–80%) of Matrigel™ and many articles report similar results as with Matrigel but with lower efficiency due to weaker attachment of the cells to the matrix. On the other hand, human laminin from the placenta or plasma have been successfully used in supporting a defined medium. Interestingly, only one medium, hESF9, basically uses collagen I as its culture scaffold (Furue et al. 2008), whereas this major matrix is generally reported to have a poor efficiency.

From the perspective of a custom matrix, Braam et al. and Miyazaki et al. reported a method to determine the optimal substrate for hESC culture. Both groups observed that integrins are expressed on hESCs. Integrin is a major receptor for cellular adhesion expressed on the cell surface; there are various isoforms, based on their specificity for RGD-, laminin-, and collagen-binding (Braam et al. 2008; Miyazaki et al. 2008). Furthermore, both groups employed a recombinant protein, considering the future need for bulk production, and essential requirement for avoidance of contamination with pathogens. Braam et al. concluded that recombinant vitronectin was suitable for culture of hESC, whereas Miyazaki et al. showed that recombinant laminin could support hESC culture. To date, it has been reported that there are fifteen kinds of laminin isoforms and there are specific combinations between laminin isoforms and integrin isoforms. According to the report from Miyazaki et al., the integrin α6β1 is largely expressed on hESCs. The integrin α6β1 is known to interact with laminin-511(521), -332, -111. The authors therefore showed that these laminin isoforms could work as a substrate for hESC culture. Laminin-111 does not have a stronger affinity than laminin-332 or laminin-511 for the integrin α6β1. The Matrigel and mouse laminin, purified from Matrigel, is solely composed of laminin-111; the other laminin isoforms are accordingly expected to support robust adhesion, even where mouse laminin failed for cellular adhesion. Rodin et al. also succeeded in long-term culture on recombinant laminin-511 in combination with a complete xeno-free medium. It is noteworthy that laminin-511 showed higher affinity to hESC than Matrigel (Rodin et al. 2010). Artificial substrate would be valuable for the scalable culture of hESC, because such materials are expected to be more suitable for large scale preparation compared to recombinant ECM proteins, from the view points of cost and handling as well as for the elimination of animal derived contaminants. In recent years, synthesized substrate or peptide-conjugated surface have been reported to sustain the undifferentiated growth of hESC (Li et al. 2006; Melkoumian et al. 2010; Nagaoka et al. 2010; Villa-Diaz et al. 2010). Although it is neccessary to further examine the efficacy of

these attachment matrices on the maintenance of hESC using more cell lines and various defined media, these materials may be promising for establishing a culture system compliant with clinical application.

In summary, in spite of the progress in finding soluble factors to be added to defined media, the matrix substrate for undifferentiated growth of hESCs is still developing. To construct high quality defined media, it is also necessary to further improve the culture substrate.

18.6 Perspectives for hESC Culture

The hESC is a pluripotent stem cell, which has now been under investigation for over a decade. Whilst the culture methods for hESCs are steadily converging, they still require further improvement. hESC researchers are attempting to develop a better culture system, thus no standard protocol for the maintenance of hESCs has yet been established. hESCs show a variety of characteristics due to not only genetic differences of the individual embryos and the method of derivation but also due to differences in their general maintenance. These variations make it difficult to develop and standardize the culture conditions. In the immediate future, many pluripotent stem cells will be produced, in addition to iPS cells, and germline stem cells. These pluripotent stem cells will also have an inherent range of characteristics and optimal protocols should be developed for their maintenance. To estimate the potential of pluripotent stem cells, either by comparing single cell types or comparing to other stem cells, a preferable standardized culture system is therefore necessary to provide a uniform cellular environment. The improvement in hESC culture will assist those studies.

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Chapter 19
Culture Adaptation of Pluripotent Stem Cells: Challenges and Opportunities

Neil J. Harrison, Duncan Baker, and Peter W. Andrews

Abstract Embryonic stem (ES) cells may acquire genetic and epigenetic changes upon prolonged passage in culture, which can confer on them more robust growth characteristics. The genetic changes are often manifest cytogenetically as nonrandom gains of chromosomal regions that are also typically amplified in embryonal carcinoma (EC) cells, the malignant counterpart of ES cells. Although this raises some concerns for the future use of ES or induced pluripotent stem (iPS) cells in regenerative medicine, or in vitro screening applications, these concerns remain largely hypothetical. It may be that potential problems can be substantially mitigated when we understand more about the underlying causes and mechanisms of culture adaptation. At the same time, this phenomenon also provides a tool that can help dissect the mechanisms controlling stem cell behavior, while potentially providing more robust cells for use in some applications.

19.1 Introduction

It is a fundamental tenet of modern biology that all living organisms are subject to continual mutation, and that the resulting variants are subject to selection for those best adapted to reproduce in the environment in which they find themselves. This applies as much to individual cells as to the whole organisms that they comprise. Indeed, to maintain their integrity, much of the genome of multicellular organisms is dedicated to mechanisms that limit the occurrence of genetic variation in their component cells, and any selective growth advantages that may arise in the event of

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somatic mutations. Cancer is the most obvious manifestation of defects in those mechanisms. In this connection, stem cells present a special problem. Those stem cells that may exist to maintain tissues that continually turn over during the life of an organism must proliferate in such a way that exactly 50% of their progeny retain a stem cell phenotype (self-renewal), and exactly 50% die, or commit to differentiate, ultimately to the functional cells of the tissue. If less than 50% retain a stem cell phenotype, then there would be a slow loss of capacity to replace the tissue, with eventual disastrous consequences for the organism. It is at least plausible that such a failure could contribute to aging. On the other hand, if more than 50% of the progeny of the stem cell retain a stem cell phenotype, and remain viable, then there will be a continual and unlimited expansion of the stem cell pool. This provides a possible mechanism for the formation of an incipient cancer, and it has been suggested that one reason for the evolution of a tissue architecture with only a small stem cell pool is that it limits the target size for mutations that could lead to tumor development (Cairns 1975).

Embryonic stem (ES) cells, established in culture by the explantation of pluripotent cells from the early blastocyst stage of mouse (Evans and Kaufman 1981; Martin 1981) or human (Thomson et al. 1998) embryos, must be subject to the same rules of mutation and natural selection as whole organisms. Arguably, the pluripotent cells from which they are derived, the inner cell mass cells, are not "stem cells" in the sense that the term was widely used for adult stem cells, since cells with these pluripotent characteristics disappear from the developing embryo after gastrulation. Rather they might be better regarded as progenitor cells. Nevertheless, once maintained in culture, away from any regulatory influences of the embryo, ES cells are apparently capable of indefinite expansion, implying that considerably more than 50% of their progeny retain a pluripotent stem cell phenotype at each round of replication. However, a persistent feature of ES cell culture is that these cells continually tend to differentiate, and those who work with them must seek culture conditions that limit their differentiation and promote self renewal. In the case of mouse ES cells, our detailed understanding of the molecular mechanisms that control their behavior has allowed the development of defined media and culture conditions that do allow efficient maintenance of the undifferentiated cells (Ying et al. 2008). Although significant progress has also been made with human ES cells, and defined media are available, it still remains difficult to maintain cultures with little or no spontaneous differentiation (Akopian et al. 2010).

A notable feature of human ES cells is that cultures may become easier to maintain with progressive passaging (Draper et al. 2004a). Also, unlike mouse ES cells, human ES cells typically show very low plating efficiencies when seeded at low cell densities, but their clonogenicity often increases substantially in later passages. The improved growth capacity of these cells may result from the acquisition of mutations, which are most easily seen as karyotypic changes (Baker et al. 2007; Draper et al. 2004b). We have referred to these changes as culture adaptation (Enver et al. 2005). As a working hypothesis, it seems plausible that culture adaptation reflects the selection of variant stem cells that are more able to undergo self renewal than the alternative fates of differentiation or cell death.

The genetic changes associated with culture adaptation of human ES cells clearly raise concerns about the possible consequences for the safety and efficacy of applications of ES cells in regenerative medicine. They might also influence their utility in other applications, such as drug screening and toxicology, which are also dependent upon producing specific derivative cells with particular functions. Such concerns are equally applicable to induced pluripotent stem (iPS) cells. So understanding the causes and nature of culture adaptation is crucial to the exploitation of the many opportunities offered by these pluripotent stem cells. Nevertheless, these concerns remain hypothetical and largely untested. It is not necessarily the case that mutations that provide a selective advantage in the stem cells will have any influence upon their differentiated derivatives. For example, a mutation leading to an increased expression of NANOG might be advantageous for an ES cell, since it would be expected to limit its propensity for differentiation (Chambers et al. 2007). On the other hand, once such a cell has differentiated, the mutation might have no effect since NANOG is not expressed at all in most somatic cells.

Culture adaptation can also present us with opportunities. Since we presume that mutations providing a selective advantage affect genes that control the balance between self-renewal, differentiation, and survival, such mutations may provide insights into these control mechanisms. Further, since variant cells may be cultured more easily, they could provide more robust tools for in vitro screening systems used for drug discovery or toxicology, provided that the mutations that they carry do not influence the specific end points required. Separately, if the hypothesis is correct that cancer involves defects in stem cell regulation, then culture adaptation of stem cells may provide an in vitro paradigm for studying cancer development and progression.

19.2 Genetic and Epigenetic Change in Cultured ES Cells

Karyotypic changes occur in both mouse and human ES cells after prolonged passage in culture (Baker et al. 2007; Longo et al. 1997). Most commonly these changes involve gain of chromosomes, although losses are also sometimes observed. Further, the gains appear to be nonrandom. In the mouse, gains of chromosome 8 and chromosome 11 are the most commonly observed (Eggan et al. 2002; Guo et al. 2005; Liu et al. 1997), whereas in humans the changes most often involve gains of chromosomes 17 and 12 (Baker et al. 2007; Brimble et al. 2004; Cowan et al. 2004; Draper et al. 2004b; Mitalipova et al. 2005), although gains of other chromosomes, notably chromosomes 1, 20 and the X chromosome have also been seen on several occasions (Baker et al. 2007; Brimble et al. 2004; Inzunza et al. 2004; Ludwig et al. 2006; Lefort et al. 2008; Spits et al. 2008) (Fig. 19.1). The nonrandom nature of the chromosomal acquisitions suggests strongly that these chromosomes carry a gene, or genes that provide the ES cells with a population growth advantage when over-expressed. Certainly, the extra gene dosage does lead to over expression of many of the affected genes, so that expression array data can be used to indicate

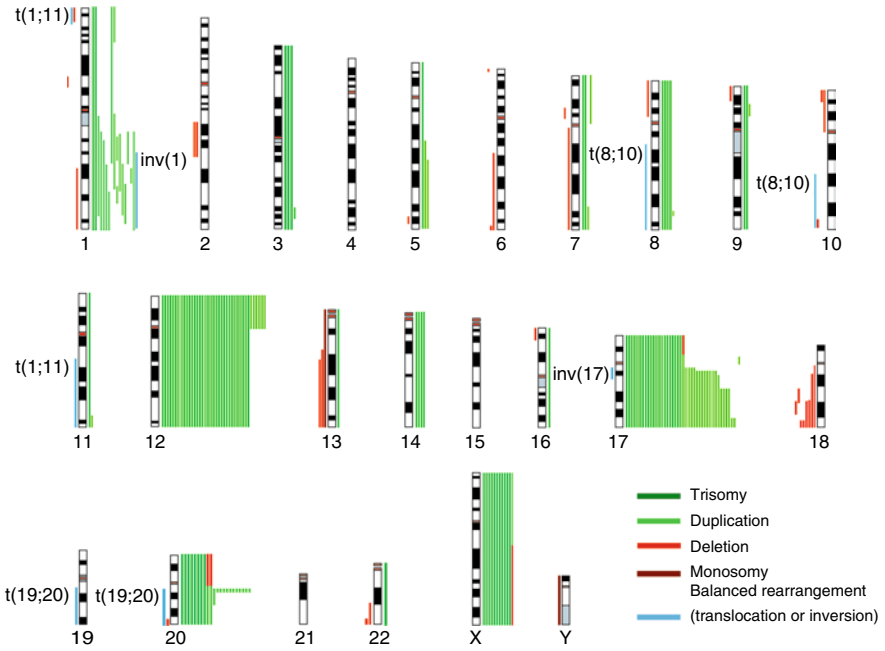

Fig. 19.1 Karyotypic abnormalities observed in human ES cells. Chromosomal gains are shown in *green* to the *right* of the corresponding region, and chromosomal losses shown in *red* to the *left*. Balanced rearrangements are shown in *blue*, with details of the change indicated. The data is taken from our laboratory in Sheffield, and reports in the literature

karyotypic change (Enver et al. 2005). Most strikingly, human embryonal carcinoma (EC) cells, the stem cells of teratocarcinomas, and the malignant counterparts of ES cells, always possess extra copies of at least part of the short arm of chromosome 12, and commonly extra copies of chromosome 17, although they typically have many other changes as well (Baker et al. 2007; Skotheim et al. 2006). This suggests that any selective growth advantage is common to both ES cells in vitro and EC cells in tumors, and so arises from an intrinsic aspect of the properties of these pluripotent cells rather than a response to their growth conditions, which are evidently vastly different in vitro and in vivo. Obvious candidate genes that might offer such growth advantages are *NANOG* on chromosome 12, and *SURVIVIN* on chromosome 17. High expression of *NANOG* would be expected to inhibit or retard differentiation (Chambers et al. 2007; Darr et al. 2006), whereas *SURVIVIN* would enhance stem cell survival (Blum et al. 2009). Either of these situations could provide an intrinsic growth advantage to affected pluripotent stem cell populations. It is notable that mouse chromosome 11 is syntenic to much of human chromosome 17, suggesting a commonality of mechanism between both species.

In many cases, the karyotypic changes reported involve the gain of whole chromosomes, suggesting that they result from chromosomal nondysjunction at mitosis. However, in a number of cases the changes do involve unbalanced chromosomal

rearrangements. Analysis of these has helped to narrow down some of the regions in which genes offering selective advantage may lie to two regions on the short arm of human chromosome 12 (12p11.2-12, 12p13.31), the terminal half of the long arm of human chromosome 17, and a small region near the centromere of the short arm of chromosome 17 (17p11.2) (Baker et al. 2007). *NANOG* is located in one of the regions of chromosome 12p, whereas *SURVIVIN* is located in the terminal half of chromosome 17q, though direct evidence that these genes are the principal drivers of growth advantage is lacking, and obvious candidates in 17p11.2 are still to be identified. More recently, detailed searches, using SNP or CGH arrays, for genetic change at levels below that detectable by standard cytogenetics have also revealed amplification of small regions of the genome in cells that appear otherwise diploid (Narva et al. 2010; Werbowetski-Ogilvie et al. 2009). So far these studies have not pinpointed specific genes, though a small region on chromosome 20 has recently emerged from these studies as another region subject to common amplification (Lefort et al. 2008; Spits et al. 2008). Curiously, there is no strong evidence for nonrandom loss of genes during culture adaptation of human ES cells, though in the mouse, XO EC cells are not uncommon (Silver et al. 1983). By contrast, human ES and EC cells often show gains of the X chromosome (Baker et al. 2007; Wang et al. 1980).

If abnormal expression of particular genes can provide a selective growth advantage for stem cells, such a result could also be achieved by aberrant epigenetic alterations on prolonged passage. Certainly marked changes in the DNA methylation profiles of late passage human ES cells have been reported (Allegrucci and Young 2007). There has been little study of whether any of these changes are nonrandom or whether they affect chromosomal regions subject to common genomic amplification. However, we found that in the case of culture adaptation of the H7 human ES cell line, the X-chromosome is inactive in early passage, permanently loses inactivation in late passage, and is not reinactivated upon differentiation (Enver et al. 2005). Further, loss of X-inactivation as a product of prolonged culture has also been reported in a separate study (Silva et al. 2008). In the study by Enver et al. (Enver et al. 2005), another locus that we found to be strongly up regulated in adapted H7 cells is *DLK1*. This is a gene encoding a putative ligand for the NOTCH receptor, which we have shown is involved in promoting the survival of human ES cells (Fox et al. 2008). However, *DLK1* is encoded on chromosome 14, which was not subject to amplification in these cells, so an aberrant epigenetic mechanism driving increased expression of *DLK1* seems likely. Indeed, DLK1 is a gene subject to imprinting, which may be abnormal in certain human clinical conditions (Astuti et al. 2005; Temple et al. 2007). On the other hand, studies of many other imprinted loci have found that imprinting often appears to be rather stable in human ES cells compared to mouse ES cells (Adewumi et al. 2007; Rugg-Gunn et al. 2005).

Although karyotypic change in human ES cells has been widely reported, many groups also report that such changes are rare or do not occur in their culture conditions (Amit et al. 2000; Caisander et al. 2006). It has been further proposed that the generation of these changes is promoted by sub-optimal culture conditions, and particularly by the use of enzymes e.g. trypsin, to disperse cells in “mass-passaging” techniques

(Mitalipova et al. 2005). It was suggested that passaging colonies of ES cells manually by the so-called "cut-and-paste" method could avoid these genetic changes. However, there are several key points to consider. First, when karyotypic changes occur in human ES cell cultures, two independent mechanisms must be involved – mutation, followed by selection of advantageous variants. Second, mutation is stochastic and the probability of a particular mutation occurring in a group of cells is dependent upon population size. Finally, the commonality of amplification of chromosomes 12 and 17 in ES and EC cells argues that at least the selective advantage of these amplifications is not contingent upon growth conditions, although of course these might affect mutation rate.

To look at this further we carried out a Monte Carlo simulation to assess how the key parameters of mutation rate, population size, and selective advantage may influence culture adaptation and mutant spread in stem cell cultures (Olariu et al. 2010). Certainly, a marked effect of each of these parameters on the predicted rate at which cultures are expected to accumulate advantageous genetic changes was readily seen. However, one aspect that was a surprise was the extent to which maintaining cells in small cultures retarded the rate of appearance of abnormal cells, even when the same total number of cells was maintained in multiple small cultures compared to being maintained in one large culture.

The results of the Monte Carlo simulation were also compared to data about the observed rate at which karyotypically abnormal cells take over ES cell cultures, either in experiments where a small number of culture adapted cells are deliberately mixed with "normal" cells, or in situations in which a small proportion of abnormal cells are first observed and the time taken for them to expand and constitute 100% of the cells is tracked. In either case, the rate at which the proportion of abnormal cells rose from a few percent to 100% was surprisingly rapid, in many cases within 5–10 passages, implying a strong selective advantage for the common variant cells containing extra copies of chromosomes 12 or 17. Assuming that chromosomal nondysjunction is random and that the common acquisition of chromosomes 12 or 17 represents about 10% of all variants arising by nondysjunction (i.e., 2 out of 23 chromosomes), we estimated that their observed selective advantage is consistent with an overall rate of nondysjunction in human ES cells of 10^{-4}–10^{-6}, given the typical culture sizes of ES cells that are commonly maintained. This compares with estimates of 10^{-3}–10^{-5} per mitosis as the rate of general chromosomal rearrangements in other diploid cells (Holliday 1989; Petersson and Mitelman 1985). However, direct measurement of the rate at which karyotypical abnormalities arise in ES cell cultures is very difficult and to date has not been reported.

19.3 Adaptation, Nullipotency and Cancer Progression

The concept that cancer development involves aberrations in the mechanisms that control the differentiation of tissue stem cells, and that many tumors contain small populations of cancer stem cells, or cancer initiating cells, analogous to, or possibly derived from, tissue stem cells is an old one (Dick 2008). Many of these ideas have

been developed from the original studies of Till and McCullough demonstrating the existence of hematopoietic stem cells (Till et al. 1964). But perhaps the first experimental evidence of a cancer stem cell came from the work of Pierce and his colleague who showed that a single EC cell transplanted to a host mouse is capable of generating a teratocarcinoma which contains multiple tissues (Kleinsmith and Pierce 1964). A further facet of this is that it became clear, as mouse EC cells and teratocarcinomas became better understood, that the differentiated derivatives of mouse EC cells are largely incapable of regenerating a tumor, and that the malignancy of teratocarcinomas resides in the EC cells (Pierce et al. 1960). The situation is not quite so clear cut in human germ cell tumors in which some non-EC elements may possess malignant tumorigenic characteristics in their own right (Mostofi and Sesterhenn 1985). Nevertheless, it is only the EC cells that give rise to the somatic elements of teratocarcinomas.

Typically EC cells from teratocarcinomas are pluripotent and capable of differentiating into somatic cells of all three germ layers. However, tumor cells, that are phenotypically identical to pluripotent EC cells, but with only a limited, or no capacity for differentiation, may sometimes also be derived from murine teratocarcinomas. These have been called "nullipotential" or "nullipotent" EC cells (Berstine et al. 1973; Martin and Evans 1975). Such nullipotency is much more common among the EC cells derived from human teratocarcinomas (Andrews et al. 1980; Mostofi and Sesterhenn 1985). Although a nullipotent stem cell may seem to be an oxymoron, their existence could be easily explained by the selection of variant stem cells that progressively lose the capacity of differentiation. As we have argued for culture adaptation of ES cells, the reduction in any tendency to differentiate should offer the variant cells a significant growth advantage over those that retain a tendency to differentiate. Indeed, given time, one might reasonably expect that any stem cell would tend to progress to a nullipotent state. Much of the discussion of whether the cancer stem cell hypothesis has any general validity often involves an assumption that, if true, then only a small proportion of a tumor will be composed of cancer initiating cells (Dick 2003; Schatton et al. 2008). However, given the hypothesis that any stem cell system will tend to progress towards nullipotency, one might expect that, over time, the proportion of stem cells in a stem cell based tumor should progressively increase until it is composed entirely of stem cells. To date, we have not seen the evolution of fully nullipotent culture adapted ES cells, but that may be a consequence of the period of time over which such changes have been observed. Nevertheless, we have seen changes in patterns of differentiation as a consequence of adaptation. However, progression of tumors from a histologically complex state, providing a caricature of their tissue of origin (Harris 2004) to a more uniform, anaplastic state, is not uncommon.

19.4 Effects of Change on Cell Growth and Differentiation

The selection of mutant ES cells during culture adaptation demonstrates that these variants have an increased growth capacity compared to their normal counterparts. In the case of stem cells, this increased growth must be the product of an increased

propensity for self-renewal over differentiation or death. This improved growth is typically measured as higher cloning efficiency and/or decreased doubling time (Enver et al. 2005; Werbowetski-Ogilvie et al. 2009), yet such assays read-out a combination of behaviors, and do not reveal specific changes in cell biology. Indeed, this is a difficult issue to address, since there are a number of pathways capable of impacting on ES cell fate, any of which may be affected during adaptation. However, a number of research groups have attempted to define what separates the abnormal cells from their normal sisters.

One study (Herszfeld et al. 2006) suggested that culture adapted cells are protected from apoptosis through expression of CD30, working through NF-κβ. However, neither the expression of CD30 nor decreased apoptosis was a universal feature of abnormal cells (Harrison et al. 2009). Another common event in culture adaptation may be retarded differentiation, which has been suggested by various data. For example, when the abnormal human cells are induced to form teratomas in immune-compromised mice, the tumors have been reported to exhibit fewer differentiated features than those from "normal" cells (Werbowetski-Ogilvie et al. 2009), and also to contain cells with an ES cell-like phenotype (Andrews et al. 2005; Blum and Benvenisty 2009). These ES-like cells can be transplanted into culture and grown as ES cells, demonstrating that the abnormal cells share malignant EC cell characteristics. This "trapping" of cells in an undifferentiated state was also inferred in one of our studies (Enver et al. 2005), in which ES cell characteristics were assessed based on surface antigen expression. Here, it was found that variant cells, unlike their karyotypically normal sister cells, could lose expression of SSEA3, a marker for undifferentiated cells, yet retain clonogenic capacity. A concurrent transcriptional analysis also showed that the transcriptome of SSEA3(−) adapted cells clustered with that of SSEA3(+) normal and adapted cells, whereas the SSEA3(−) normal cells were separated from these 3 populations. These results from both types of assay were consistent with the view that the SSEA3(−) adapted cells were still retained in the undifferentiated stem cell compartment, whereas the SSEA3(−) normal cells were outside that compartment and committed to differentiation.

When assessing the effect of adaptation one must remember that a number of facets of cell behavior may be affected, and it is unlikely that the examples discussed above will be relevant in all cases. In addition, the evolutionary nature of this process should also be considered, and seemingly characterized abnormal lines may undergo further adaptation. It seems that in the assessment of culture adapted cells, a set of standardized assays would be valuable, particularly tests which could specifically quantify self-renewal or differentiation potential.

19.5 Conclusion

Human ES cells maintained in vitro are inevitably subject to evolutionary pressures, and so are likely to acquire epi/genetic changes that increase their growth capacity. Accepting this, one must then consider whether these changes are detrimental to the future usage of these cells, or if they present a research opportunity.

That the karyotypic changes observed in abnormal human ES cells are similar to those observed in EC cells, the malignant counterpart of ES cells, raises concerns about the safety of the abnormal cells for therapy. However, it is unclear whether in differentiated cells, which would be used for transplantation, these changes would have a similar impact on cell growth. A further concern relates to the impact that adaptation may have on differentiation capacity, since evidence suggests that creating cells of a particular lineage e.g., endoderm, may be difficult using abnormal cells. Here, regular functional monitoring would be necessary to determine any changes in potency. Bias towards, or against, certain lineages has also been reported between normal cell lines (Osafune et al. 2008), and so dealing with adapted cell lines may be considered no more problematic than handling the cell lines already available.

Even considering any issues raised for the use of the variant cells in therapy, these cells still provide a valuable tool to study stem cell biology. That the adapted cells must display increased self-renewal capacity affords insight into the factors that control stem cell fate, both in ES and EC cells. Indeed, by using the adaptation of human ES cells as a paradigm for cancer stem cell generation, one may begin to understand more about tumor generation and maintenance. At present, the culture adaptation of human ES cells is an accepted phenomenon, yet the underlying molecular events are by no means fully elucidated, particularly considering the number of manners through which adaptation can be achieved. The next challenge then, is to reveal how these epi/genetic changes can control pluripotent cell fate, at which point we will be better equipped to harness the enormous potential that these cells offer.

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Chapter 20
Epilogue

Takashi Tada, Justin Ainscough, and Shinya Yamanaka

Recent fascinating developments in the interwoven areas of molecular biology, biochemistry, and biomedicine are starting to provide explanations for many complicated "phenomena," enabling them to be broken down into more readily understandable pockets of information that can be defined using words such as "molecules" and "factors". Conversion of phenomenon into molecules and factors is a key initial step for facilitating the feedback of significant research findings to the general population. Importantly, the conversion process is also an essential prerequisite to translating basic research findings into "bench to bedside" applications. The time is now upon us where the once mysterious phenomenon of nuclear reprogramming, first demonstrated over half a century ago using elegant nuclear transfer techniques, is being broken down and understood; translated into molecules and factors. Key molecules involved in the reprogramming process are Oct4, Sox2 and Klf4. We were very surprised indeed to find that this simple cocktail of transcription factors is all that is needed to jump start the process of direct conversion of a somatic cell into a pluripotent stem cell; in essence to revert the process of differentiation and change a cell that had a specific function into one with a renewed ability to become multifunctional. Because of the manner in which they are generated these new types of cells are now commonly referred to as "*induced* pluripotent stem" (iPS) cells. In the short time since iPS cells were first generated it has become increasingly clear that a new avenue has been opened, providing a glimmer of hope that some, perhaps most, diseases may one day be curable, using genetically identical pluripotent stem cells that have been differentiated into "healthy" cell types of multiple different tissues ready for direct clinical application.

To realize regenerative medicine, using not only iPS cells but also other pluri- or multipotent stem cells including embryonic stem cells and somatic stem cells,

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continued and dedicated technological development is essential to overcome a number of roadblocks that remain in the way of practical application at the bedside. These include (but are not limited to): good manufacturing practice for improved tissue collection and stem cell development; ability to generate genetic modification-free iPS cells; improving culture conditions such that the key problem of genomic instability is kept to a minimum, or indeed eliminated; ability to efficiently differentiate stem cells into stable and fully functional specialized cell types; the identification and purification of suitably reprogrammed cells; improvement in transplantation technology; and not least the avoidance of tumor generation. All of these, and many other facets, require critical evaluation and significant improvement before the path to clinical application can be fully realized. In addition to technological innovation and medical validation, social, ethical and religious issues have to be carefully considered that differentially apply to each country, each hospital, and each person. There are still many roadblocks. We, however, believe that we will one day soon be able to overcome these roadblocks and realize the ultimate goal of efficient clinical application derived directly from nuclear reprogramming technology. This will require the continued effort and collaboration of researchers around the world. There is no doubt in our mind that the tremendous findings of recent years will contribute to improved human health and, we hope, happiness.

Appendix

The following articles are reprinted with permission from 1. The Company of Biologists, 2. Nature Publishing Group, 3. and 4. Elsevier:

1. Gurdon J (1962) The developmental capacity of nuclei taken from intestinal epithelial cells of feeding tadpoles. J Embryol Exp Morphol 10:622–640
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The Developmental Capacity of Nuclei taken from Intestinal Epithelium Cells of Feeding Tadpoles

by J. B. GURDON[1]

From the Embryology Laboratory, Department of Zoology, Oxford

WITH ONE PLATE

INTRODUCTION

AN important problem in embryology is whether the differentiation of cells depends upon a stable restriction of the genetic information contained in their nuclei. The technique of nuclear transplantation has shown to what extent the nuclei of differentiating cells can promote the formation of different cell types (e.g. King & Briggs, 1956; Gurdon, 1960*c*). Yet no experiments have so far been published on the transplantation of nuclei from fully differentiated normal cells. This is partly because it is difficult to obtain meaningful results from such experiments. The small amount of cytoplasm in differentiated cells renders their nuclei susceptible to damage through exposure to the saline medium, and this makes it difficult to assess the significance of the abnormalities resulting from their transplantation. It is, however, very desirable to know the developmental capacity of such nuclei, since any nuclear changes which are necessarily involved in cellular differentiation must have already taken place in cells of this kind.

The experiments described below are some attempts to transplant nuclei from fully differentiated cells. Many of these nuclei gave abnormal results after transplantation, and several different kinds of experiments have been carried out to determine the cause and significance of these abnormalities.

The donor cells used for these experiments were intestinal epithelium cells of feeding tadpoles. This is the final stage of differentiation of many of the endoderm cells whose nuclei have already been studied by means of nuclear transplantation experiments in *Xenopus*. The results to be described here may therefore be regarded as an extension of those previously obtained from differentiating endoderm cells (Gurdon, 1960*c*).

MATERIAL AND METHODS

The animals used for these experiments belong to the subspecies *Xenopus laevis laevis*. The transplantation technique has been carried out as described previously (Elsdale *et al.*, 1960), except that the donor tissue was exposed to

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the dissociating Versene solution (5×10^{-4} M) for 30–40 minutes. The *Xenopus* nuclear marker was used (Elsdale *et al.*, 1960), and marked donor nuclei were transplanted into unmarked recipient eggs. Among the transplant-embryos described below, all those which developed beyond the blastula stage contained marked nuclei, thus proving that they were derived from the transplanted nucleus and not from the egg nucleus. The nuclear marker can only be seen in embryos which have passed the blastula stage.

Donor cells

The differentiated cells used to provide donor nuclei were intestinal epithelium cells from the mid-intestine of feeding tadpoles (stages 46–48 of Nieuwkoop & Faber, 1956). These cells (plate) have the following features characteristic of their differentiated state: a tall columnar shape with basally situated nuclei; pigment granules inside the surface exposed to the gut lumen; and, most important, the striated border typical of cells having an absorptive function. Some of these cells still contain a few yolk platelets, but these are rapidly absorbed at about this stage. All the epithelium cells in the part of the intestine used for these experiments are of this kind except for less than 1 per cent. which are typical gland cells; there are no undifferentiated cells in the epithelium at this stage. The epithelium cells are larger than the other cell types present in the mid-intestine, and so can be easily recognized in the dissociated cell preparations.

Controls

Owing to the variable quality of the *Xenopus* recipient eggs laid in the laboratory (Gurdon, 1960*b*), the transplantation of intestinal epithelium cell nuclei has been accompanied by control transplantations of blastula or gastrula nuclei. Since no change in developmental capacity has been detected in *Xenopus* nuclei until after the late gastrula stage, either blastula or gastrula nuclei have been used as controls according to convenience.

RESULTS

Six experiments involving the transplantation of intestinal epithelium cell nuclei (referred to as intestine nuclei) gave similar results, and these have been combined in Table 1. In each experiment control transfers from blastulae or gastrulae (referred to as embryonic nuclei) were interspersed with transfers of intestine nuclei.

Normal tadpoles

Altogether 10 normal feeding tadpoles have been obtained from the transplantation of intestinal epithelium cell nuclei. These tadpoles have diploid nuclei carrying the nuclear marker referred to above. They therefore provide a clear demonstration that at least a few differentiated intestine cells contain nuclei

TABLE 1

The development resulting from the transplantation of nuclei from differentiated and embryonic cells of Xenopus laevis

Donor stage (Nieuwkoop & Faber, 1956)	*Total transfers*	*No cleavage*	*Total transfers resulting in cleavage*	*Development resulting from transplanted nuclei*								
				Abortive cleavage	*Partial cleavage*	*Complete blastulae*	*Arrested blastulae*	*Abnormal gastrulae*	*Abnormal post-neuralae*	*Stunted tadpoles*	*Died as swimming tadpoles*	*Normal feeding tadpoles*
Intestinal epithelium cell nuclei (stage 46–48)	726	347	379	175	156	48	18	8	5	6	1	10
	100%	48%	52%	24%	21·5%	6·5%	—	—	—	—	—	1·5%
Blastula or gastrula endoderm nuclei (stage 8–12)	279	66	213	8	32	173	4	17	19	27	6	100
	100%	24%	76%	3%	11%	62%	—	—	—	—	—	36%

which are capable of giving rise to all the cell types necessary for the formation of a feeding tadpole.

These normal tadpoles constitute only $1\frac{1}{2}$ per cent. of the 726 transplanted intestine nuclei, and all the remaining transfers resulted in various degrees of abnormality ranging from a complete lack of cleavage to nearly normal tadpoles (Table 1). Some experiments have been carried out in order to determine the significance of these abnormalities and, in particular, whether the abnormalities are due to a limited developmental capacity of the transplanted nuclei or to other factors such as variation in technique. The two methods used were first, the cytological analysis of eggs fixed soon after they had been injected with nuclei, and secondly, the serial transplantation of nuclei from abnormal transplant-embryos.

The cytological analysis of eggs fixed soon after receiving transplanted nuclei

The procedure followed in this analysis was to transplant nuclei from one donor embryo into eggs laid by one frog. Soon after transplantation some of the eggs were taken at random and fixed while the remainder were allowed to develop as far as they were able. The fixed eggs were then serially sectioned and stained. Subsequent microscopic examination of the sections often revealed abnormalities of the transplanted nucleus and achromatic apparatus. The eggs which were not fixed served as exact controls since they were laid by the same frog as the fixed eggs and contained transplanted nuclei from the same donor embryo. These showed how the sectioned eggs would have developed if they had not been fixed. In this way a certain cytological abnormality could be associated with a particular developmental abnormality, thus indicating the cause of the latter. This analysis was carried out on eggs with transplanted intestine nuclei as well as on those with transplanted embryonic nuclei.

The total lack of cleavage following nuclear transfer

Forty-eight per cent. of the intestine nuclei and 24 per cent. of the embryonic nuclei failed to promote cleavage of any kind after transplantation (Table 1). The following results show that this can be attributed to a failure in the technique such that the transplanted nucleus was not effectively exposed to the recipient egg cytoplasm. The eggs fixed about 40 minutes after receiving transplanted nuclei fell into two distinct categories. First there were those with distinct regions of cytoplasm; these had an almost yolk-free area in the animal half of the egg, containing the developing transplanted nucleus, and close to it the dying irradiated egg nucleus (Gurdon, 1960*a*, fig. 1). This is the typical condition of irradiated recipient eggs which have been fertilized or in which a successful nuclear transfer has been made. The other fixed eggs revealed an entirely different situation. These had a relatively homogeneous cytoplasm just as in newly laid unfertilized eggs, and the irradiated egg nucleus was found in the vegetal half.

There was no yolk-free area in the animal half of the egg and the transplanted nucleus was either entirely absent or else could be seen inside an intact donor cell.

The total absence of the transplanted nucleus from a recipient egg is probably due to the donor cell sticking to the injection pipette and so being withdrawn from the egg with the pipette. This would not be observed in the course of an experiment unless looked for carefully. The presence of a whole donor cell in the egg clearly results from a failure to break the wall of the donor cell when it is sucked into the injection pipette. The successful breaking of the cell wall depends upon the extent to which the cell is distorted in the pipette. A very close correlation has been found between the degree of donor cell distortion and the proportion of transfers which result in normal cleavage (Gurdon, 1960*b*). It was found that if the cell wall is very little distorted, the great majority of transfers fail to cleave, while strong distortion results in many developmental abnormalities probably through exposure of the nucleus to the saline medium. It is very difficult to distort intestine cells to an ideal degree, and in order to avoid damage to the nuclei these cells were distorted rather little. Transplanted intestine nuclei would therefore be expected to result in a total lack of cleavage much more often than the nuclei of the larger embryonic cells.

TABLE 2

The cytological analysis of eggs fixed 60–80 *minutes after transplantation*

	Number of eggs fixed	*Eggs with no developing nucleus*	*Chromosomes clumped at first mitosis*	3–4 *polar spindle at first mitosis*	*Normal at first mitosis*
(*a*) Tadpole nuclei from intestinal epithelium cells	70	22 out of 70 31·5%	3 out of 11* 27%	4 out of 11* 36%	—
(*b*) Nuclei from blastulae and gastrulae	59	8 out of 59 13·5%	0 out of 30* 0%	4 out of 30* 13%	20 out of 30* 67%

The cleavage of control transfers which were allowed to develop as far as they were able

	Number of transfers	*Uncleaved*	*Abortive cleavage*	*Partial blastulae*	*Complete blastulae*
(*c*) Tadpole nuclei from intestinal epithelium cells	60	18 30%	16 26·5%	21 35%	5 8·5%
(*d*) Nuclei from blastulae and gastrulae	95	12 12·5%	2 2%	14 15%	67 70·5%

* Only these eggs were fixed at the time of the first nuclear division.

The results of the cytological analysis of fixed eggs are compared with the development of their controls in Table 2. There is a very close correspondence between the proportion of developing eggs which failed to cleave and the

proportion of fixed eggs in which the transplanted nucleus was either lacking or was present in an intact donor cell. This applies to the results of transplanting intestine as well as embryonic nuclei, and justifies the conclusion that the total lack of cleavage following nuclear transplantation can be attributed to the technical difficulty described above. The developmental capacity of nuclei which fail to promote any cleavage at all after transplantation has not therefore been tested.

Abortive Cleavage

This term refers to eggs which consist only of abnormal blastomeres and uncleaved regions of cytoplasm. The blastomeres are of irregular size and shape, and contain no normal nuclei though sometimes asters and chromatin can be seen. Such eggs usually die after a few irregular cleavages. Many of the eggs which receive intestine nuclei develop in this way, but very few of those with embryonic nuclei do so (Table 1). Useful information regarding the cause of this abnormality is provided by the cytological examination of eggs fixed during metaphase of the first division of the transplanted nucleus. Only 11 eggs with intestine nuclei were found to have been fixed at exactly this time, and in 3 of these the chromosomes were clumped and pycnotic. In some cases the spindle also seemed abnormal. It is clear that these eggs could not have cleaved normally; the chromosomes would probably have broken up into pycnotic lumps and have been distributed to abnormal blastomeres. As shown in Table 2, the percentage of fixed eggs with clumped chromosomes was very close to the percentage of control transfers which resulted in abortive cleavage. It can be concluded that abortive cleavage results from the incapacity of the transplanted nucleus to divide normally at its first division.

It is not known why some nuclei divide abnormally after transplantation, but it is possibly because their chromosomes have not replicated by the time they enter mitosis. The nuclei of intestinal epithelium cells divide infrequently and have a relatively long interphase period between mitoses. Since chromosome replication takes place during interphase, some nuclei would by chance be transplanted when at the beginning of interphase and so would have unreplicated chromosomes. The time at which a transplanted nucleus enters division is determined by the egg cytoplasm, and except in nuclei which become tetraploid, this division takes place at about 80 minutes after transplantation. Thus the situation may arise in which an intestine nucleus is forced to enter mitosis even though its chromosomes are unreplicated. This would be expected to lead to the abnormal chromosome condition described above. Embryonic nuclei, on the other hand, divide at frequent intervals during cleavage. The interphase period in which their chromosomes are unreplicated is short, and embryonic nuclei are therefore generally transplanted with already replicated chromosomes. This would explain why intestine nuclei give abortive cleavage much more often than embryonic nuclei. An hypothesis of this general kind has also been

suggested by Briggs, King, & Di Berardino (1960) to account for abnormal cleavage in their experiments.

It can be concluded that transplanted nuclei which promote abortive cleavage do so through their inability to divide normally. This prevents them showing the range of cell types that they are genetically capable of giving rise to.

Partial cleavage

A blastula is described as partially cleaved when part of it consists of normal blastomeres, and the rest is uncleaved or abortively cleaved. Blastulae of this kind usually die before gastrulation commences, but if the uncleaved portion is very small they may form abnormal gastrulae. Transplanted intestine nuclei result in partial cleavage more commonly than embryonic nuclei (Table 1). Eleven eggs with transplanted intestine nuclei were fixed during the first nuclear division, and 4 of these had apparently normal chromosomes but an abnormal achromatic apparatus with 3- or 4-polar spindles (Table 2). At least some chromosomes were present on each of the three or four metaphase plates. It is possible that a normal set of chromosomes might be distributed to one of the poles of such a spindle, leaving an aneuploid number of chromosomes at the other poles. In this way a partial blastula could be formed with the aneuploid blastomeres giving rise to the abnormally cleaved part of the egg. Whatever the cause of this condition, there is agreement between the proportion of fixed eggs with an abnormal mitotic apparatus but apparently normal chromosomes, and the proportion of the developing controls which became partial blastulae (Table 2). The significance of partial cleavage has been directly determined by the serial transplantation experiments described below.

Developmental abnormalities following the transplantation of nuclei from a foreign genus

Experiments involving the transfer of nuclei from different genera to eggs of the same species show that genetically very different nuclei may give rise to the same percentages of abnormal cleavage. These experiments therefore show that the frequency of cleavage abnormalities does not necessarily represent the degree of genetic difference between transplanted nuclei. Blastula nuclei from *Hymenochirus curtipes* and *X. laevis* were transplanted to recipient eggs of *X. laevis*, and the results are given in Table 3. The genetic difference between *Hymenochirus* and *Xenopus* is demonstrated by the early arrest in development of all *Xenopus* eggs which received *Hymenochirus* nuclei in contrast to the normal development of many of the *Xenopus* to *Xenopus* transfers. However, in spite of this, the percentage of transfers which resulted in partial, abortive, or entirely deficient cleavage was the same in both cases. These results show that the post-blastula development of transplant-embryos can indicate a genetic difference between the nuclear and cytoplasmic species, while this is not necessarily so of cleavage. Since genetically very different nuclei give the same frequency and

severity of abnormal cleavage, this provides an additional reason for believing that the abnormal cleavage resulting from transplanted intestine nuclei does not indicate any genetic difference between the nuclei of intestine and embryonic cells.

TABLE 3

The transplantation of nuclei from Hymenochirus *and* Xenopus *into recipient eggs of* Xenopus

Donor nuclei	*Total number of trans-plantations*	*Uncleaved*	*Abortive cleavage*	*Partial blastulae*	*Complete blastulae*	*Neural folds*	*Normal swimming tadpoles*
Hymenochirus curtipes early gastrula	169 100%	62 37%	1 1%	22 12%	84 50%	0 —	0 —
Xenopus laevis early gastrula	78 100%	22 28%	3 4%	11 14%	42 54%	35 —	20 —

The transplantation of nuclei from abnormal nuclear transplant-embryos

Information on the cause and significance of partial blastulae and of abnormal post-blastulae has been obtained by means of serial nuclear transfers. The basic design of the experiments was as follows. Nuclear transfers were made using original intestine or embryonic donor cells. When the resulting 'first-transfer' embryos had differentiated as far as they were able, some of their endoderm nuclei were used for serial transfers, giving rise to the 'first serial-transfer' generation. As a result of experience, the best differentiation that will be achieved by an abnormal transplant-embryo can be judged to within narrow limits, before developmental arrest takes place and cell death sets in. For instance, partial blastulae in which an appreciable part of the surface area is uncleaved, never develop beyond the late blastula or very early gastrula stage. Similarly, it has been found that embryos in which part of the yolk-plug protrudes during gastrulation will never form normal late gastrulae or neurulae, and that embryos which do not elongate properly will remain as stunted post-neurulae with a belly oedema. The furthest differentiation to be expected can with practice be judged to within much narrower limits than these. By this type of experiment the differentiation of the most normal serial-transfer embryos can be directly compared with that of the first-transfer embryo from whose nuclei they were derived.

Original donor nuclei were taken from 31 abnormal first-transfer embryos (11 from original gastrula nuclei and 20 from feeding tadpole intestine nuclei). These abnormal embryos were selected arbitrarily and are a random sample of the partial blastulae and abnormal post-blastulae included in Table 1. The nuclei from each first-transfer embryo gave rise to a wide range of abnormal embryos, and sometimes to normal tadpoles, as shown in Table 4. The differentiation of each first-transfer embryo is compared with that of the serial-transfer embryos derived from its nuclei in Text-figs. 1 and 2. In these diagrams the

stage of differentiation attained by each first-transfer embryo is shown by a solid line; the dotted continuation of this line represents the most normal differentiation achieved by any of the resulting serial-transfer embryos. It can be seen

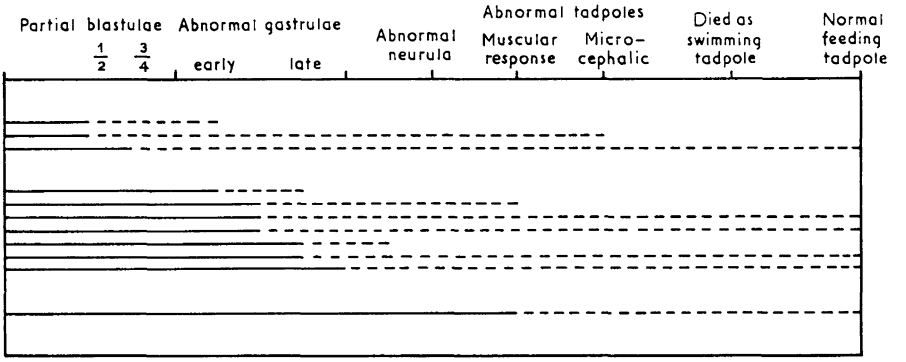

FIG. 1. Serial nuclear transfers from abnormal first-transfer embryos. Original gastrula donor nuclei (embryonic nuclei). Furthest differentiation attained by each first-transfer embryo (solid line) and by the most normal of the serial-transfer embryos derived from its nuclei (dotted line).

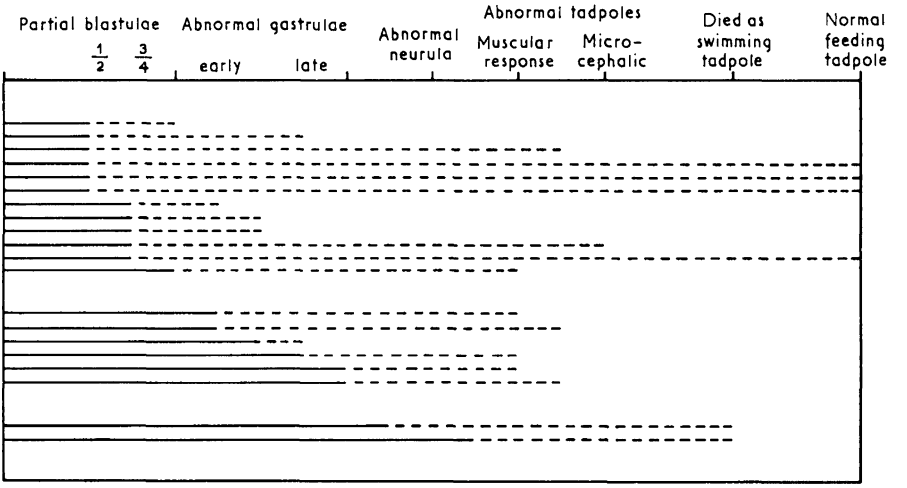

FIG. 2. Serial nuclear transfers from abnormal first-transfer embryos. Original tadpole intestine nuclei. Furthest differentiation attained by each first-transfer embryo (solid line) and by the most normal of the serial-transfer embryos derived from its nuclei (dotted line).

that in all 31 cases some of the serial-transfer embryos differentiated more normally than the first-transfer embryo from whose nuclei they were derived. It is interesting that the nuclei of partial blastulae can sometimes promote the

development of a normal or nearly normal tadpole. This is of some importance since a large proportion of transplanted intestine nuclei result in partial blastulae.

TABLE 4

Some typical examples of the development promoted by nuclei from abnormal nuclear transplant-embryos

Original donor	*Abnormal transplant-embryos used as donors*	*Total transfers*	*Partial and complete blastulae*	*Complete blastulae*	*Post-neurulae*	*Heartbeat tadpoles*	*Normal feeding tadpoles*
Gastrula endoderm cells; stages 11–13 of Nieuwkoop & Faber, 1956	½ cleaved blastula	36	8	2 (died as gastrulae)	—	—	—
	½ cleaved blastula	36	13	11	7	4	—
	¾ cleaved blastula	36	13	6	5	4	4
	Total exogastrula	35	—	9 (3 developed further than the donor)	—	—	—
	Abnormal mid gastrula	26	—	24	18	8	4
	Abnormal late gastrula	32	—	12	8	3	3
	CONTROL: Normal gastrula	57	—	14	4	3	3
Intestinal cells of feeding tadpoles; stages 46–48 of Nieuwkoop & Faber, 1956	½ cleaved blastula	36	4	4	3	3	—
	½ cleaved blastula	36	10	10	4	4	2
	¾ cleaved blastula	36	15	6 (died as gastrulae)	—	—	—
	Abnormal mid gastrula	48	—	17	4	—	—
	Abnormal late gastrula	54	—	17	4	1	—
	Abnormal early neurula	72	—	20	12	9	4
	CONTROL: Normal neurula	58	—	14	4	4	3

There are two possible explanations for these results. First, the developmental capacity of a nucleus might increase as a result of nuclear transplantation so that serial-transfer embryos are more normal than first-transfer embryos. Second, the abnormality of the first-transfer embryo might be due to some non-genetic cause such as poor egg quality. In the latter case the developmental capacity of the transplanted nucleus would not increase, but would not always be fully expressed owing to the effect of factors such as poor egg quality.

Evidence has already been obtained from different kinds of experiments that

the developmental capacity of a nucleus does not increase as a result of transplantation (Gurdon, 1960*c*). Confirmation of this has been obtained in the present experiments by making further serial-transfers in the same way as described above. Nuclei from an abnormal first-transfer embryo gave rise to the embryos of the first serial-transfer generation; the most normal of these embryos was then used to provide nuclei for a further transfer generation. In each case the most normal embryo of one transfer generation was used to provide nuclei for the next. If the developmental capacity of nuclei increases as a result of transplantation, this kind of selective serial-transfer experiment should lead to more normal development in each successive transfer generation. About ten such experiments have been carried out, and in some of these, four serial-transfer generations were made from the nuclei of one abnormal first-transfer embryo. In every experiment, the development of the embryos in each serial-transfer generation was about the same, and the later transfer generations did not contain more normal embryos than the first serial-transfer generation. These results show that the developmental capacity of a nucleus does not increase as a result of serial transplantation. It can therefore be concluded from these experiments that the minimum developmental capacity of a nucleus is shown by the most normal transplant-embryo to which it gives rise. Thus the presence of a feeding tadpole among any of the transplant-embryos derived from a nucleus shows that the nucleus had the genetic information required for this before transplantation, even though the first-transfer embryo as well as most of the serial-transfer embryos may have been abnormal.

The more normal development of the first serial-transfer embryos compared to the first-transfer embryos can be satisfactorily explained by attributing the abnormalities of the first-transfer embryos to a non-genetic cause. It is known that the quality of eggs laid by *Xenopus* in the laboratory is variable, and that poor egg quality sometimes causes abnormal development of transplant-embryos (Gurdon, 1960*b*). If the quality of some recipient eggs is variable, only a certain proportion of the transplant-embryos resulting from these eggs will develop as normally as the developmental capacity of their nuclei will allow. The effect of making serial transfers is to transplant the mitotic products of a nucleus into several different recipient eggs, so that at least some of these are of good quality and therefore demonstrate the real developmental capacity of the original somatic nucleus. So long as a sufficient number of transfers are made in the first serial-transfer generation for at least some eggs to be of good quality, the later serial-transfer generations would not be expected to contain more normal embryos than the first-transfer generation. As pointed out above, the most normal embryo that can be obtained from an original donor nucleus is generally contained in the first serial-transfer generation. The effects of poor egg quality therefore account for all the results reported here.

It has now been shown that the developmental capacity of a nucleus and of its mitotic products does not increase from one transfer generation to the next,

but that the serial-transfer embryos may differentiate more normally than the first-transfer embryo derived from the same original donor nucleus. These two observations together show that the abnormality of many transplant-embryos must be due to non-genetic factors such as poor egg quality. This is true of all abnormal transplant-embryos which contain some nuclei capable of giving rise to more normal differentiation than they showed themselves, and this includes all 31 first-transfer embryos used in these experiments.

The developmental capacity of original donor nuclei from which abnormal first-transfer embryos were obtained

It has been established above that the developmental capacity of a somatic nucleus is generally shown by the most normal embryos of the first serial-transfer generation. The right-hand end of each dotted line in Text-figs. 1 and 2 therefore represents the developmental capacity of one original somatic nucleus. The developmental capacity of the original intestine nuclei used in these experiments can now be compared with that of the original embryonic nuclei. The capacity of embryonic and intestine nuclei to give rise to normal feeding tadpoles is shown by the number of dotted lines which reach the right-hand extremity of Text-figs. 1 and 2. It can be seen that 6 out of 11 (55 per cent.) of the original embryonic nuclei and 4 out of 20 (20 per cent.) of the original intestine nuclei were able to promote the differentiation of a normal feeding tadpole.

The following conclusions can be drawn for these results: (i) Of the original intestine nuclei which gave rise to partial blastulae and abnormal post-blastulae after first-transfer, 20 per cent. contained the genetic information required for the formation of feeding tadpoles. The equivalent figure for original gastrula nuclei was 55 per cent. The numbers on which these figures are based are too small to show whether they indicate a significant difference in developmental capacity between intestine and embryonic nuclei. (ii) Of the original intestine nuclei which gave rise to partial blastulae and abnormal gastrulae after first-transfer, 12 out of 18 (67 per cent.) were capable of giving rise to *muscular-response stage tadpoles* with functional nerve and muscle cells. The comparable figure for original gastrula nuclei was 7 out of 10 (70 per cent.). Both of these values are obtained from the results given in Text-figs. 1 and 2.

DISCUSSION

The genetic information contained in the nuclei of differentiated cells

The genetic information contained in a nucleus before transplantation is shown by the range of cell types that its mitotic products can form after its transplantation so long as the genetic information carried by a nucleus does not change as a result of transplantation itself. It will be assumed in this discussion that the developmental capacity of a nucleus and of its daughter nuclei

does not increase as a result of transplantation. There is no evidence that any increase does take place, and the evidence that it does not comes from the serial transplantation of nuclei between different species and subspecies (Gurdon, 1962*b*) and of nuclei from abnormal transplant-embryos within the same subspecies (p. 632). Thus, if a transplanted nucleus supports the development of a feeding tadpole, this is regarded as showing that it possessed the genetic information required for this when it was present in the donor cell, and did not acquire this information only after transplantation. This applies to the results of serial transfers just as much as to the results of first transfers, since apart from variation in donor stage, exactly the same procedure is involved in both kinds of experiment. Thus the genetic information contained in a nucleus is regarded as equal to or greater than that required to form the most normal of the transplant-embryos derived from it, whether by first or serial transfer. This is so even when the first-transfer embryo is abnormal and when only a few of the serial-transfers form feeding tadpoles.

The minimum genetic information contained in the nuclei of intestinal epithelium cells can be determined by combining the results of first transfers and serial transfers. The first transfers showed that $1\frac{1}{2}$ per cent. of the intestine nuclei can give rise to feeding tadpoles. Serial transfers have shown that a further $5\frac{1}{2}$ per cent. of the intestine nuclei also have this capacity. This figure of $5\frac{1}{2}$ per cent. represents the proportion of intestine nuclei which gave partial blastulae and abnormal post-blastulae after first-transfer, but which could give rise to some feeding tadpoles after serial transfer. This figure is calculated as follows. It was found that 27 per cent. of the original intestine nuclei formed partial blastulae and abnormal embryos after first transfer (Table 1). About 20 per cent. of these, or $5\frac{1}{2}$ per cent. of the original intestine nuclei, gave rise to feeding tadpoles after serial transfer (p. 633). Combining the results of first and serial transfers, at least 7 per cent. of intestinal epithelium cell nuclei contain the genetic information required to form all cell types of a feeding tadpole, except perhaps for the germ cells whose presence has not yet been looked for.

This figure of 7 per cent. is expressed in terms of the total number of transplanted intestine nuclei. However, the developmental capacity of many of these nuclei was not in effect tested, and the percentage of intestine nuclei capable of promoting the formation of feeding tadpoles is increased if this is taken into account. The cytological examination of fixed eggs showed that the total lack of cleavage after transplantation can be wholly attributed to technical difficulties. The transfers which result in no cleavage are a random sample of the total transfers made and can be discounted from the results. The intestine nuclei capable of promoting the formation of feeding tadpoles then constitute 13 per cent. of the remaining successful transfers (Table 5).

It can be inferred from the cytological analysis of fixed eggs that abortive cleavage results from nuclei which happen to have been taken for transplantation at an unsuitable stage in their mitotic cycle. If this is so, then the nuclei

which give rise to abortive cleavage are a random sample of those transplanted, and can be excluded from the total number of transfers. The remaining nuclei are those which were transplanted successfully as well as at a suitable stage in their mitotic cycle for their developmental capacity to be tested; the intestine nuclei capable of supporting the development of feeding tadpoles then constitute 24 per cent. of these (Table 5).

TABLE 5

Summary of conclusions reached regarding the developmental capacity of nuclei taken from differentiated and embryonic cells of Xenopus laevis

Donor nuclei	*Developmental capacity of nuclei. Stages of Nieuwkoop & Faber, 1956*	*Results of first-transfers only as percentage of total transfers*	*Combined results of first and serial transfers**		
			As percentage of total transfers	*As percentage of total transfers, less those resulting in no cleavage*	*As percentage of total transfers, less those resulting in no cleavage or abortive cleavage*
Intestinal epithelium cell nuclei	Capable of forming feeding tadpoles; stage 50	1·5% (10)	7% (49)	13% (49)	24% (49)
Blastula or gastrula cell nuclei		36% (100)	57% (158)	74% (158)	77% (158)
Intestinal epithelium cell nuclei	Capable of forming muscular response tadpoles; stage 26	2·3% (17)	20% (142)	37% (142)	70% (142)
Blastula or gastrula cell nuclei		48% (133)	65% (181)	85% (181)	88% (181)

The figures in brackets represent the number of individuals.
* The figures for serial transfers were calculated as described on p. 634.

The main conclusions from these results are the following, though the evidence for each is not equally strong.

(i) It has been clearly shown that about 7 per cent. of the total number of transplanted intestine nuclei have the genetic information required to form normal feeding tadpoles. This figure represents the combined results of first and serial transfers expressed as a percentage of total transfers.

(ii) Thirteen per cent. of the eggs receiving *successfully* transplanted intestine nuclei can give rise to feeding tadpoles. This figure represents the combined results of first and serial transfers expressed as a percentage of those transfers which resulted in some kind of cleavage. There is good evidence that the transfers which result in no cleavage do so for technical reasons and are a random sample of the total transfers.

(iii) The formation of normal feeding tadpoles can be promoted by 24 per cent. of the intestine nuclei which were transplanted successfully as well as at

a suitable stage in their mitotic cycle. This figure is the combined results of first and serial transfers expressed as a percentage of the total transfers excluding those which resulted in no cleavage or abortive cleavage.

If the capacity of a transplanted nucleus to give rise to muscular response tadpoles with functional nerve- and muscle-cells is considered, then a greater percentage of intestine nuclei fall into the three categories above. These percentages as well as the equivalent figures for embryonic nuclei are given in Table 5.

The differentiation of cells and the developmental capacity of their nuclei

The results so far obtained from nuclear transplantation experiments in Amphibia have contributed in two ways to the question of whether stable nuclear changes are causally connected with cellular differentiation. Some experiments have shown that different kinds of cells may have unchanged nuclei, while others have demonstrated a stable restriction of developmental capacity in the nuclei of differentiating cells.

The following results have demonstrated the wide range of genetic information contained in the nuclei of cells which are approaching, or which have actually attained, the differentiated state. The experiments described above are of this kind; they show that at least 7 per cent. of the nuclei of intestinal epithelium cells can promote the formation of normal feeding tadpoles, and that at least 20 per cent. can promote the formation of muscular response tadpoles with functional muscle- and nerve-cells (Table 5). Evidence of this kind has also been described by King & McKinnell (1960). From 142 eggs of *Rana pipiens* injected with 10–20 adenocarcinoma cell nuclei they obtained one post-neurula embryo showing a certain degree of tissue differentiation.

It can be argued that some cells may become differentiated under the influence of nuclei from neighbouring cells, and hence that a few cells in a differentiated tissue may have nuclei capable of forming normal tadpoles while the majority of cells have nuclei which do not possess the capacity to form other cell types. Such an argument seems to be excluded by the experiments with intestine nuclei in *Xenopus*, since it was found that at least 20 per cent., and probably 70 per cent. (Table 5), of these nuclei could give rise to muscle- and nerve-cells after transplantation. These experiments therefore show that a nucleus can be responsible for the formation of an intestinal epithelium cell and at the same time possess the capacity to form other kinds of differentiated cells.

Other experiments have clearly shown that some of the nuclei derived from somatic cells have undergone a stable change restricting their developmental capacity. The clearest evidence for these changes comes from serial nuclear transplantation experiments in *R. pipiens* (King & Briggs, 1956) and in *Xenopus* (Gurdon, 1960*c*). These experiments have not shown that the nuclear changes took place during the normal development of the donor embryos from which the nuclei were taken. If this should prove to be the case, it still remains to be

determined whether these nuclear changes are necessary for cellular differentiation to take place, or whether they are only a result of this.

Until the significance of stable nuclear changes is known, the results of nuclear transplantation experiments seem to be consistent with the view that stable changes restricting the developmental capacity of nuclei are not essential for cellular differentiation to take place. This conclusion can now be related to different theories of differentiation.

Cellular differentiation is most probably initiated by the effect of the cytoplasmic environment on a nucleus, so that the nucleus provides specific genetic information which promotes the formation of a particular cell type (recent discussion by Fischberg & Blackler, 1961). Three possible ways in which this could happen are the following. First, nuclei might undergo a progressive loss of genetic material, so that cellular differentiation would result from the genetic material that is retained in different nuclei. Secondly, an inactivation of certain parts of the genetic material might take place, so that specific genetic information would be provided by the non-inactivated parts of a genome. This kind of inactivation would be stable under the normal conditions of cell mitosis. A theory of differentiation along these lines is suggested by various reports of stable nuclear changes in somatic cells (e.g. Brink, 1960). The third possibility is that the genetic information provided by a nucleus is entirely dependent on its cytoplasmic environment at any one time; in this case a nucleus would never undergo any stable changes having a qualitative effect on its function. This kind of system is suggested by the reversible appearance of puffs in the polytene chromosomes of insects (e.g. Breuer & Pavan, 1955) and by cases of metaplasia (e.g. Reyer, 1954). The first of these three possibilities is rendered very improbable by the results of the experiments reported in this article; these have shown that a nucleus may be responsible for the differentiation of one cell type while still possessing the capacity to form all other types of somatic cell in a feeding tadpole. It has previously been found that most of the normal feeding tadpoles resulting from transplanted nuclei of *Xenopus* will eventually form adult frogs (Gurdon, 1962*a*). However, the possibility still exists that intestine nuclei may have undergone stable changes restricting their capacity to form adult frogs and normal germ cells, since intestine nuclei have not yet been tested in these respects. These results are therefore consistent with any theory of cell differentiation which does not require that the nucleus of a differentiated cell has lost the genetic information required for the formation of other differentiated somatic cell types.

SUMMARY

1. Nuclei from differentiated intestinal epithelium cells of feeding tadpoles and from control blastulae of *Xenopus* have been transplanted into enucleated recipient eggs. The differentiated state of the intestinal epithelium cells was shown by their possession of a striated border.

2. The cleavage and embryonic development resulting from the intestinal epithelium cell nuclei was much more abnormal than that resulting from control blastula transfers.

3. 1½ per cent. (10 out of 726) of the first transfers of intestine nuclei resulted in normal feeding tadpoles.

4. The serial transplantation of nuclei and of their mitotic products showed that some of the intestine nuclei which promoted abnormal development after first transfer could nevertheless promote the formation of normal feeding tadpoles after serial transfer. The combined results of first transfers and of serial transfers demonstrated that at least 7 per cent. of the intestine nuclei possessed the genetic information required for the formation of normal feeding tadpoles.

5. The cytological examination of eggs fixed soon after receiving transplanted nuclei indicated that the lack of cleavage and abortive cleavage following transplantation result from nuclei which were not effectively exposed to the recipient egg cytoplasm or which were transplanted at an unsuitable stage in their mitotic cycle. If these cases are excluded from the results, the intestine nuclei capable of promoting the formation of feeding tadpoles then constitute 24 per cent. of the remaining successful transfers.

6. A similar interpretation of the experimental results shows that 70 per cent. of the successfully transplanted intestine nuclei have the genetic information required to form muscular response stage tadpoles with functional muscle- and nerve-cells.

7. These results show that a nucleus can promote the formation of a differentiated intestine cell and at the same time contain the genetic information necessary for the formation of all other types of differentiated somatic cell in a normal feeding tadpole. It is concluded that the differentiation of a cell cannot be dependent upon the incapacity of its nucleus to give rise to other types of differentiated cell.

RÉSUMÉ

Potentialités de développement de noyaux issus de cellules de l'épithélium intestinal de têtards se nourrissant

1. Des noyaux de cellules différenciées de l'épithélium intestinal et de blastulas témoins ont été transplantés dans des œufs énucléés chez le *Xénope*. On reconnaît l'état différencié des cellules de l'épithélium intestinal à la présence d'une bordure striée.

2. La segmentation et le développement embryonnaire obtenus à partir de noyaux de cellules de l'épithélium intestinal sont beaucoup plus anormaux que ceux obtenus à partir de noyaux de blastulas.

3. 1½ pour cent (10 sur 726) des transplantations simples ont donné des têtards normaux se nourrissant.

4. La transplantation en série de noyaux et de leurs descendants a montré que des noyaux d'intestin ne fournissant qu'un développement anormal lors

de la première transplantation sont néanmoins capables de réaliser le développement d'un têtard normal à la prise de nourriture après une série de transplantations. Les résultats combinés des transplantations simples et des transplantations en série ont montré qu'au moins 7 pour cent des noyaux de l'intestin possèdent l'information génétique nécessaire au développement d'un têtard normal se nourrissant.

5. L'étude histologique d'œufs fixés sitôt après la transplantation d'un noyau a montré que l'absence de segmentation et l'apparition de segmentation abortive après transplantation de noyau provenaient de cas où le noyau ne s'était pas vraiment trouvé au sein du cytoplasme de l'œuf hôte, ou bien de noyaux ayant été transplantés au cours d'une phase défavorable du cycle mitotique. En tenant compte de ces cas dans les résultats, les noyaux d'intestin capables d'édifier un têtard normal se nourrissant représentent 24 pour cent des cas où la transplantation a été effectuée avec succès.

6. Une interprétation semblable des résultats expérimentaux montre que 70 pour cent des noyaux d'intestin transplantés avec succès possèdent l'information génétique permettant d'obtenir un embryon au stade de la réponse musculaire, possédant des cellules musculaires et nerveuses fonctionnelles.

7. Ces résultats prouvent qu'un noyau est capable de participer à la formation d'une cellule intestinale différenciée, tout en possédant cependant l'information génétique nécessaire à l'édification d'un têtard normal. On peut en conclure que la différenciation d'une cellule n'est pas pour son noyau l'inaptitude à fournir tout autre type de cellule différenciée.

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NUCLEI FROM DIFFERENTIATED CELLS

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EXPLANATION OF PLATE

Sections of the mid-intestine of a feeding tadpole of *X. laevis*. Owing to the coiling of the intestine the sections are only transversely cut in some places. By serial sections it could be seen that some part of each cell reaches the gut lumen and constitutes part of the striated border. The striated border and underlying pigment granules can be most clearly seen in fig. C.

Figs. A and B. Stage 46 of Nieuwkoop & Faber (1956).

Fig. C. Stage 47. Most of the donor cells used in these experiments were taken from tadpoles at this stage.

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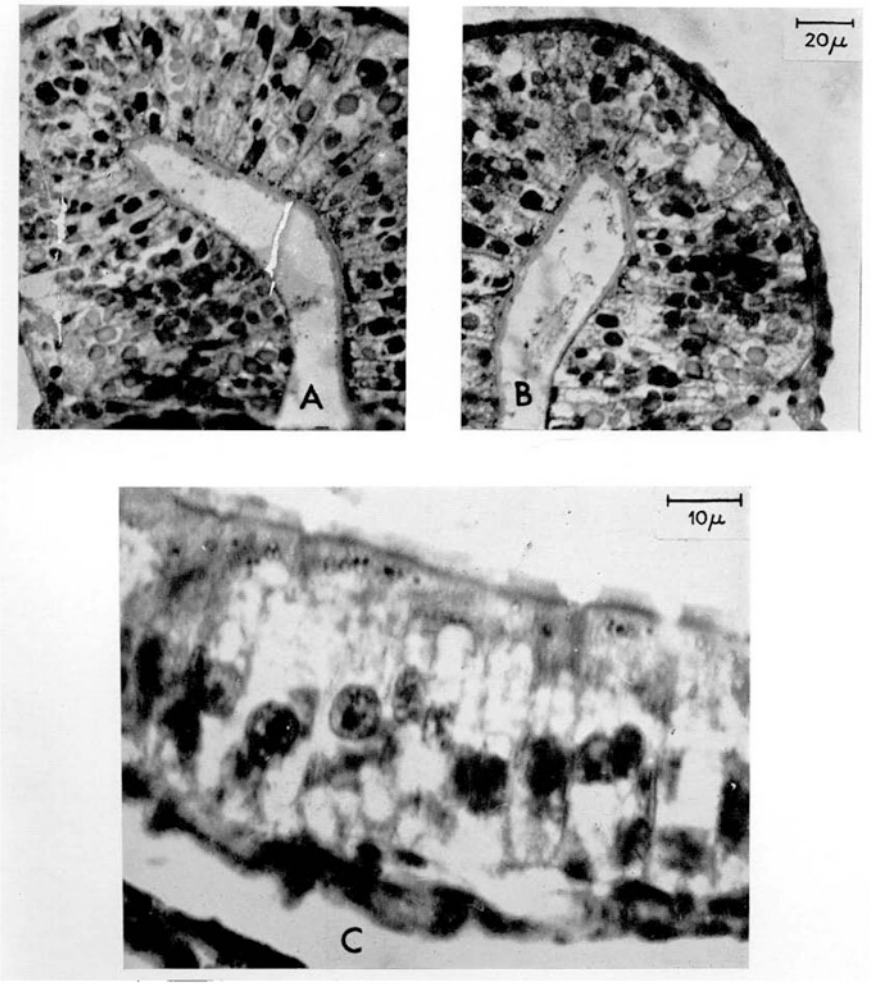

J. B. GURDON

Viable offspring derived from fetal and adult mammalian cells

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Fertilization of mammalian eggs is followed by successive cell divisions and progressive differentiation, first into the early embryo and subsequently into all of the cell types that make up the adult animal. Transfer of a single nucleus at a specific stage of development, to an enucleated unfertilized egg, provided an opportunity to investigate whether cellular differentiation to that stage involved irreversible genetic modification. The first offspring to develop from a differentiated cell were born after nuclear transfer from an embryo-derived cell line that had been induced to become quiescent[1]. Using the same procedure, we now report the birth of live lambs from three new cell populations established from adult mammary gland, fetus and embryo. The fact that a lamb was derived from an adult cell confirms that differentiation of that cell did not involve the irreversible modification of genetic material required for development to term. The birth of lambs from differentiated fetal and adult cells also reinforces previous speculation[1,2] that by inducing donor cells to become quiescent it will be possible to obtain normal development from a wide variety of differentiated cells.

It has long been known that in amphibians, nuclei transferred from adult keratinocytes established in culture support development to the juvenile, tadpole stage[3]. Although this involves differentiation into complex tissues and organs, no development to the adult stage was reported, leaving open the question of whether a differentiated adult nucleus can be fully reprogrammed. Previously we reported the birth of live lambs after nuclear transfer from cultured embryonic cells that had been induced into quiescence. We suggested that inducing the donor cell to exit the growth phase

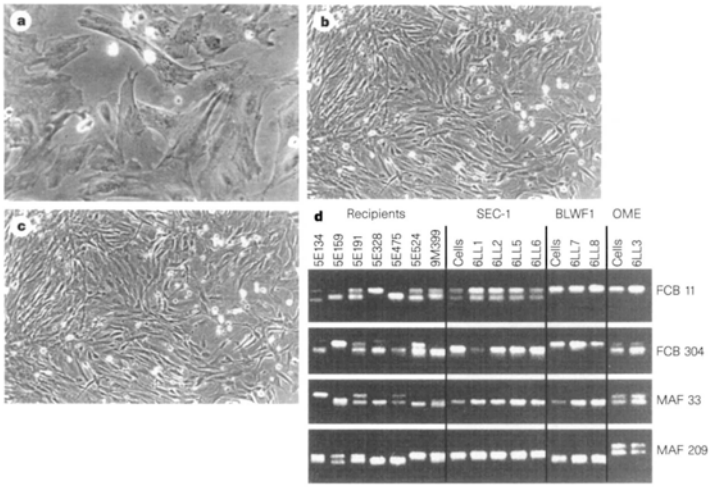

Figure 1 Phase-contrast photomicrograph of donor-cell populations: **a**, Embryo-derived cells (SEC1); **b**, fetal fibroblasts (BLWF1); **c**, mammary-derived cells (OME). **d**, Microsatellite analysis of recipient ewes, nuclear donor cells and lambs using four polymorphic ovine markers[22]. The ewes are arranged from left to right in the same order as the lambs. Cell populations are embryo-derived (SEC1), fetal-derived (BLW1), and mammary-derived (OME), respectively. Lambs have the same genotype as the donor cells and differ from their recipient mothers.

causes changes in chromatin structure that facilitate reprogramming of gene expression and that development would be normal if nuclei are used from a variety of differentiated donor cells in similar regimes. Here we investigate whether normal development to term is possible when donor cells derived from fetal or adult tissue are induced to exit the growth cycle and enter the G0 phase of the cell cycle before nuclear transfer.

Three new populations of cells were derived from (1) a day-9 embryo, (2) a day-26 fetus and (3) mammary gland of a 6-year-old ewe in the last trimester of pregnancy. Morphology of the embryo-derived cells (Fig. 1) is unlike both mouse embryonic stem (ES) cells and the embryo-derived cells used in our previous study. Nuclear transfer was carried out according to one of our established protocols[1] and reconstructed embryos transferred into recipient ewes. Ultrasound scanning detected 21 single fetuses on day 50–60 after oestrus (Table 1). On subsequent scanning at ~14-day intervals, fewer fetuses were observed, suggesting either mis-diagnosis or fetal loss. In total, 62% of fetuses were lost, a significantly greater proportion than the estimate of 6% after natural mating[4]. Increased prenatal loss has been reported after embryo manipulation or culture of unreconstructed embryos[5]. At about day 110 of pregnancy, four fetuses were dead, all from embryo-derived cells, and post-mortem analysis was possible after killing the ewes. Two fetuses had abnormal liver development, but no other abnormalities were detected and there was no evidence of infection.

Eight ewes gave birth to live lambs (Table 1, Fig. 2). All three cell populations were represented. One weak lamb, derived from the fetal fibroblasts, weighed 3.1 kg and died within a few minutes of birth, although post-mortem analysis failed to find any abnormality or infection. At 12.5%, perinatal loss was not dissimilar to that occurring in a large study of commercial sheep, when 8% of lambs died within 24 h of birth[6]. In all cases the lambs displayed the morphological characteristics of the breed used to derive the nucleus donors and not that of the oocyte donor (Table 2). This

Table 1 Development of embryos reconstructed with three different cell types

Cell type	No. of fused couplets (%)*	No. recovered from oviduct (%)	No. cultured	No. of morula/ blastocyst (%)	No. of morula or blastocysts transferred†	No. of pregnancies/ no. of recipients (%)	No. of live lambs (%)‡
Mammary epithelium	277 (63.8)[a]	247 (89.2)	-	29 (11.7)[a]	29	1/13 (7.7)	1 (3.4%)
Fetal fibroblast	172 (84.7)[b]	124 (86.7)	- 24	34 (27.4)[b] 13 (54.2)[b]	34 6	4/10 (40.0) 1/6 (16.6)	2 (5.9%) 1 (16.6%)§
Embryo-derived	385 (82.8)[b]	231 (85.3)	- 92	90 (39.0)[b] 36 (39.0)[b]	72 15	14/27 (51.8) 1/5 (20.0)	4 (5.6%) 0

* As assessed 1 h after fusion by examination on a dissecting microscope. Superscripts a or b within a column indicate a significant difference between donor cell types in the efficiency of fusion ($P < 0.001$) or the proportion of embryos that developed to morula or blastocyst ($P < 0.001$).
† It was not practicable to transfer all morulae/blastocysts.
‡ As a proportion of morulae or blastocysts transferred. Not all recipients were perfectly synchronized.
§ This lamb died within a few minutes of birth.

Figure 2 Lamb number 6LL3 derived from the mammary gland of a Finn Dorset ewe with the Scottish Blackface ewe which was the recipient.

Table 2 Delivery of lambs developing from embryos derived by nuclear transfer from three different donor cells types, showing gestation length and birth weight

Cell type	Breed of lamb	Lamb identity	Duration of pregnancy (days)*	Birth weight (kg)
Mammary epithelium	Finn Dorset	6LL3	148	6.6
Fetal fibroblast	Black Welsh	6LL7	152	5.6
	Black Welsh	6LL8	149	2.8
	Black Welsh	6LL9†	156	3.1
Embryo-derived	Poll Dorset	6LL1	149	6.5
	Poll Dorset	6LL2‡	152	6.2
	Poll Dorset	6LL5	148	4.2
	Poll Dorset	6LL6‡	152	5.3

* Breed averages are 143, 147 and 145 days, respectively for the three genotypes Finn Dorset, Black Welsh Mountain and Poll Dorset.
† This lamb died within a few minutes of birth.
‡These lambs were delivered by caesarian section. Overall the nature of the assistance provided by the veterinary surgeon was similar to that expected in a commercial flock.

alone indicates that the lambs could not have been born after inadvertent mating of either the oocyte donor or recipient ewes. In addition, DNA microsatellite analysis of the cell populations and the lambs at four polymorphic loci confirmed that each lamb was derived from the cell population used as nuclear donor (Fig. 1). Duration of gestation is determined by fetal genotype[7], and in all cases gestation was longer than the breed mean (Table 2). By contrast, birth weight is influenced by both maternal and fetal genotype[8]. The birth weight of all lambs was within the range for single lambs born to Blackface ewes on our farm (up to 6.6 kg) and in most cases was within the range for the breed of the nuclear donor. There are no strict control observations for birth weight after embryo transfer between breeds, but the range in weight of lambs born to their own breed on our farms is 1.2–5.0 kg, 2–4.9 kg and 3–9 kg for the Finn Dorset, Welsh Mountain and Poll Dorset genotypes, respectively. The attainment of sexual maturity in the lambs is being monitored.

Development of embryos produced by nuclear transfer depends upon the maintenance of normal ploidy and creating the conditions for developmental regulation of gene expression. These responses are both influenced by the cell-cycle stage of donor and recipient cells and the interaction between them (reviewed in ref. 9). A comparison of development of mouse and cattle embryos produced by nuclear transfer to oocytes[10,11] or enucleated zygotes[12,13] suggests that a greater proportion develop if the recipient is an oocyte. This may be because factors that bring about reprogramming of gene expression in a transferred nucleus are required for early development and are taken up by the pronuclei during development of the zygote.

If the recipient cytoplasm is prepared by enucleation of an oocyte at metaphase II, it is only possible to avoid chromosomal damage and maintain normal ploidy by transfer of diploid nuclei[14,15], but further experiments are required to define the optimum cell-cycle stage. Our studies with cultured cells suggest that there is an advantage if cells are quiescent (ref. 1, and this work). In earlier studies, donor cells were embryonic blastomeres that had not been induced into quiescence. Comparisons of the phases of the growth cycle showed that development was greater if donor cells were in mitosis[16] or in the G1 (ref. 10) phase of the cycle, rather than in S or G2 phases. Increased development using donor cells in G0, G1 or mitosis may reflect greater access for reprogramming factors present in the oocyte cycoplasm, but a direct comparison of these phases in the same cell population is required for a clearer understanding of the underlying mechanisms.

Together these results indicate that nuclei from a wide range of cell types should prove to be totipotent after enhancing opportunities for reprogramming by using appropriate combinations of these cell-cycle stages. In turn, the dissemination of the genetic improvement obtained within elite selection herds will be enhanced by limited replication of animals with proven performance by nuclear transfer from cells derived from adult animals. In addition, gene targeting in livestock should now be feasible by nuclear transfer from modified cell populations and will offer new opportunities in biotechnology. The techniques described also offer an opportunity to study the possible persistence and impact of epigenetic changes, such as imprinting and telomere shortening, which are known to occur in somatic cells during development and senescence, respectively.

The lamb born after nuclear transfer from a mammary gland cell is, to our knowledge, the first mammal to develop from a cell derived from an adult tissue. The phenotype of the donor cell is unknown. The primary culture contains mainly mammary epithelial (over 90%) as well as other differentiated cell types, including myoepithelial cells and fibroblasts. We cannot exclude the possibility that there is a small proportion of relatively undifferentiated stem cells able to support regeneration of the mammary gland during pregnancy. Birth of the lamb shows that during the development of that mammary cell there was no irreversible modification of genetic information required for development to term. This is consistent with the generally accepted view that mammalian differentiation is almost all achieved by systematic, sequential changes in gene expression brought about by interactions between the nucleus and the changing cytoplasmic environment[17]. □

Methods

Embryo-derived cells were obtained from embryonic disc of a day-9 embryo from a Poll Dorset ewe cultured as described[3], with the following modifications. Stem-cell medium was supplemented with bovine DIA/LIF. After 8 days, the explanted disc was disaggregated by enzymatic digestion and cells replated onto fresh feeders. After a further 7 days, a single colony of large flattened cells was isolated and grown further in the absence of feeder cells. At passage 8, the modal chromosome number was 54. These cells were used as nuclear donors at passages 7–9. Fetal-derived cells were obtained from an eviscerated Black Welsh Mountain fetus recovered at autopsy on day 26 of pregnancy. The head was removed before tissues were cut into small pieces and the cells dispersed by exposure to trypsin. Culture was in BHK 21 (Glasgow MEM; Gibco Life Sciences) supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM) and 10% fetal calf serum. At 90% confluency, the cells were passaged with a 1:2

division. At passage 4, these fibroblast-like cells (Fig. 1) had modal chromosome number of 54. Fetal cells were used as nuclear donors at passages 4–6. Cells from mammary gland were obtained from a 6-year-old Finn Dorset ewe in the last trimester of pregnancy[18]. At passages 3 and 6, the modal chromosome number was 54 and these cells were used as nuclear donors at passage numbers 3–6.

Nuclear transfer was done according to a previous protocol[1]. Oocytes were recovered from Scottish Blackface ewes between 28 and 33 h after injection of gonadotropin-releasing hormone (GnRH), and enucleated as soon as possible. They were recovered in calcium- and magnesium-free PBS containing 1% FCS and transferred to calcium-free M2 medium[19] containing 10% FCS at 37 °C. Quiescent, diploid donor cells were produced by reducing the concentration of serum in the medium from 10 to 0.5% for 5 days, causing the cells to exit the growth cycle and arrest in G0. Confirmation that cells had left the cycle was obtained by staining with antiPCNA/cyclin antibody (Immuno Concepts), revealed by a second antibody conjugated with rhodamine (Dakopatts).

Fusion of the donor cell to the enucleated oocyte and activation of the oocyte were induced by the same electrical pulses, between 34 and 36 h after GnRH injection to donor ewes. The majority of reconstructed embryos were cultured in ligated oviducts of sheep as before, but some embryos produced by transfer from embryo-derived cells or fetal fibroblasts were cultured in a chemically defined medium[20]. Most embryos that developed to morula or blastocyst after 6 days of culture were transferred to recipients and allowed to develop to term (Table 1). One, two or three embryos were transferred to each ewe depending upon the availability of embryos. The effect of cell type upon fusion and development to morula or blastocyst was analysed using the marginal model of Breslow and Clayton[21]. No comparison was possible of development to term as it was not practicable to transfer all embryos developing to a suitable stage for transfer. When too many embryos were available, those having better morphology were selected.

Ultrasound scan was used for pregnancy diagnosis at around day 60 after oestrus and to monitor fetal development thereafter at 2-week intervals. Pregnant recipient ewes were monitored for nutritional status, body condition and signs of EAE, Q fever, border disease, louping ill and toxoplasmosis. As lambing approached, they were under constant observation and a veterinary surgeon called at the onset of parturition. Microsatellite analysis was carried out on DNA from the lambs and recipient ewes using four polymorphic ovine markers[22].

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Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells

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The resetting of a somatic epigenotype to a totipotential state has been demonstrated by successful animal cloning, via transplantation of somatic nuclei into enucleated oocytes. We have established an experimental system, which reproduces the nuclear reprogramming of somatic cells in vitro by fusing adult thymocytes with embryonic stem (ES) cells. Analysis of the lymphoid-cell-specific V-(D)-J DNA rearrangement of the T cell receptor and immunoglobin genes shows that the ES cells have hybridized with differentiated cells. In these ES cell hybrids, the inactivated X chromosome derived from a female thymocyte adopts some characteristics of an active X chromosome, including early replication timing and unstable *Xist* transcription. We also found that an *Oct4-GFP* transgene, which is normally repressed in thymocytes, is reactivated 48 hr after cell fusion. The pluripotency of the ES-thymocyte hybrid cells is shown in vivo, since they contribute to all three primary germ layers of chimeric embryos. The somatic DNA methylation pattern of the imprinted *H19* and *Igf2r* genes is maintained in these hybrids, unlike hybrids between ES and EG (embryonic germ) cells in which the differential methylation is erased. Thus, ES cells have the capacity to reset certain aspects of the epigenotype of somatic cells to those of ES cells.

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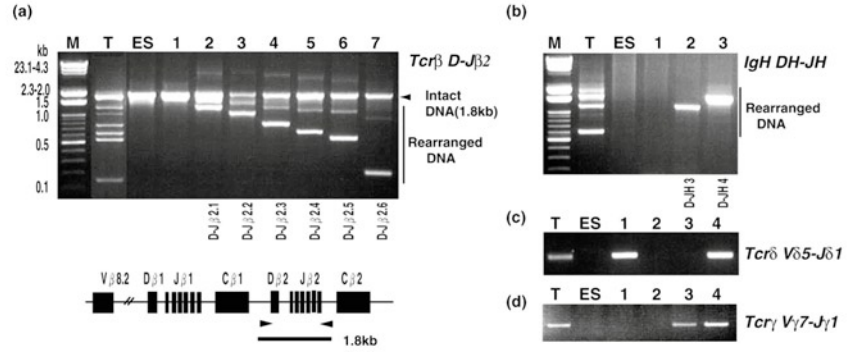
Results and discussion

The capacity for reprogramming somatic nuclei has now been demonstrated by nuclear transplantation to enucleated oocytes in many mammalian species [1]. To reproduce nuclear reprogramming in vitro, we hybridized adult thymocytes with embryonic stem (ES) cells. To ensure that ES cells hybridized with differentiated cells, PCR amplification with four primer sets specific to the D-J region of T cell receptor (*Tcr*)β, the D-J region of immunoglobulin (*Ig*) H, and the V-J region of *Tcr*δ and *Tcr*γ was performed on DNA extracted from ES hybrid cells with thymocytes. DNA rearrangement is one of the clear signs that thymocytes have differentiated into lymphoid cells [2]. In 45% of the hybrid clones, rearrangements specific to *Tcr*β D-Jβ 2.1, -2.2, -2.3, -2.4, -2.5, or -2.6 were found (Figure 1a). Similarly, rearrangements of the *IgH* D-J region (Figure 1b) and the V-J region of *Tcr*δ (Figure 1c) and *Tcr*γ (Figure 1d) were found in some clones. In total, out of 31 ES hybrid clones, 17 (55%) carried at least one of the rearrangements under investigation. Thus, in these cases, the ES cells had hybridized with thymocyte nuclei after differentiation to lymphoid cells.

To analyze X chromosome activity, we studied the well-characterized phenomenon of X chromosome replication asynchrony using a replication-banding technique (Figure 2a) [3]. After continuous incorporation of BrdU through the second half of S phase and acridine orange staining, the active X chromosome and the autosomes are seen as banded red and green elements. The inactive X chromosome is uniformly dull red in a female somatic cell (Figure 2b), due to delayed replication. In 6 clones of hybrid cells between XY male ES cells and XX female thymocytes, all 32 cells karyotyped (4n = 80) carried 3 synchronously replicating X chromosomes (Figure 2c). Consistent with this, *Xist* (inactive X-specific transcript) RNA was unstably accumulated (spotted) on three X chromosomes in two ES hybrid cell lines examined by RNA FISH (fluorescent in situ hybridization) (Figure 2d). *Xist* RNA accumulation was also unstable on the active X chromosome of a male ES cell, but was stable on the inactive X chromosome of a female thymocyte (painting signal). Thus, the inactive X chromosome derived from the somatic nucleus adopts several characteristics of an active X chromosome after hybridization, namely, replication and a pattern of *Xist* expression found in undifferentiated cells.

To visualize reprogramming of the somatic cell nuclei, we used a mouse strain carrying an *Oct4-GFP* transgene

Figure 1



DNA rearrangement of *Tcr*β, *Tcr*δ, *Tcr*γ, and *IgH* genes derived from thymocytes in ES hybrid cells. **(a)** D-J region of *Tcr*β, **(b)** D-J region of *IgH*, **(c)** V-J region of *Tcr*δ, and **(d)** V-J region of *Tcr*γ. PCR analysis detects intact and rearranged DNA of Dβ2-Jβ2 of *Tcr*β in (a). The intact DNA is visualized as a 1.8-kb band by PCR amplification with primers (shown between arrowheads in the genomic map). T, thymocytes from (Rosa26 × Oct4-GFP)F1 mice; ES, ES cells; M, marker mixture of λ/HindIII DNA and 100-bp ladder DNA.

Figure 2

Reactivation of an X chromosome derived from thymocytes in ES hybrid cells. **(a)** R-banding analysis of X chromosome replication timing in ES hybrid cells. In ES hybrid cells, three X chromosomes (two X chromosomes from a female thymocyte and one X chromosome from a male ES cell) are detected as red and green elements, showing that they are active. Three synchronously replicating X chromosomes in (a) are magnified in **(c)** (arrowheads). An X chromosome in a female somatic cell (arrow in **[b]**) and a Y chromosome (in [a]) are stained uniformly red, indicating that they are inactive. **(d)** *Xist* RNA is detected as a spotted red signal on an active X chromosome of a male ES cell, whereas *Xist* RNA is coating an inactivated X chromosome of a female thymocyte, as shown by a large red signal. Three spotted red signals per nucleus are detected in two ES hybrid cell lines (ES × T1 and ES × T2) examined.

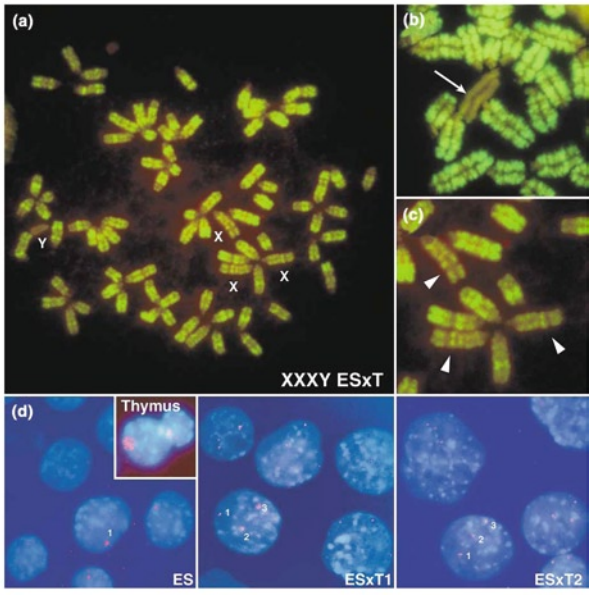
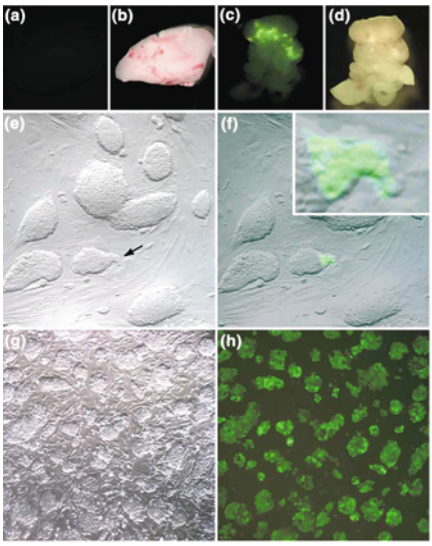


Figure 3



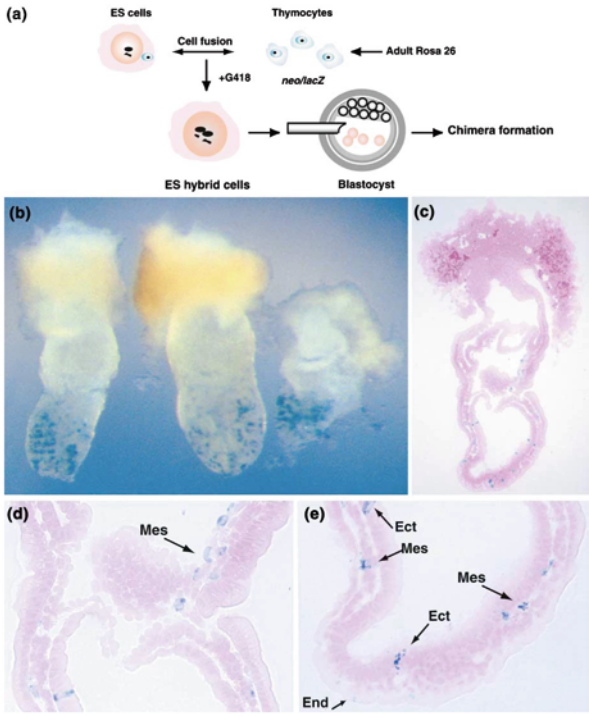
Reactivation of *Oct4-GFP* in ES hybrid cells. GFP-fluorescent images and bright field images of the **(a,b)** thymus the **(c,d)** ovary from (Rosa26 × Oct4-GFP)F1 mice used for the production of ES hybrid cells. **(e)** A bright field image of **(f)** (the arrow shows the GFP-positive colony). (f) A small GFP-positive colony alongside nonexpressing ES cell colonies on the second day after fusion. The positive colony is shown under higher magnification in the inset. **(g)** A bright field image of **(h)**. (h) GFP-positive cells expanded from a colony after G418 selection.

(Figure 3). *Oct4* expression is unique to germ cells, preimplantation embryos, and the epiblast of early postimplantation embryos. Therefore, the activity of *Oct4* provides an ideal marker for the identification of toti- and/or pluripotent cells. The *Oct4-GFP* expression pattern is comparable to the endogenous *Oct4* expression pattern [4]. To ensure this, transgenic thymuses and ovaries were observed. GFP was not detected in the thymocytes, but was detected in the growing oocytes (Figure 3a–d). Thymocytes from the *Oct4-GFP* transgenic mice were hybridized with ES cells, cultured without selection, and observed every 12 hr for GFP expression. A GFP-positive colony, consisting of 16 cells located at the edge of a larger nonexpressing colony, was first detected after 48 hr (Figure 3e,f). Subsequently, several additional GFP-positive colonies were observed on the same culture plate, prior to confluence. No GFP-positive cells were found in unfused thymocytes cultured under the same conditions. To address whether reprogramming of the somatic nuclei occurred in all ES hybrid cells, thymocytes from (Rosa26 × Oct4-GFP)F1 mice, which are resistant to G418 selection, were used. Following selection, 36 out of 37 (97%) clones that were obtained expressed GFP. This expression was stably maintained through several passages of subculture (Figure 3g,h), demonstrating reprogramming of the thymocyte nuclei in the majority of ES hybrid cells.

To investigate developmental competence, the hybrid cells were microinjected into normal diploid blastocysts (Figure 4a). We used one hybrid clone with a (differentiated) thymocyte from (Rosa26 × Oct4-GFP)F1 mice and one with a thymocyte from Rosa26 mice. Staining for β-galactosidase activity showed the relative contribution of hybrid cells in each chimera, which was expected to be poor due to severe loss of tetraploid cells in chimeras of diploid and tetraploid embryos [5]. Eight out of 20 E7.5 embryos were positive, showing restricted contribution of the hybrid cells (Figure 4b,c). Detailed analysis revealed the presence of hybrid cell derivatives in the embryonic ectoderm, embryonic mesoderm, and visceral endoderm (Figure 4d,e). Thus, the ES hybrid cells possess the developmental potential to differentiate into the three primary germ layers of early postimplantation embryos, although we were not able to determine whether tissue-specific markers are also appropriately expressed from the thymocyte genome.

We next investigated whether nuclear reprogramming of thymocytes also affected methylation at imprinted loci. The maternally expressed *H19* locus contains a paternally methylated region upstream of the gene, thought to hold the primary methylation imprint [6]. Following digestion with BamHI and the methylation-sensitive restriction enzyme HhaI, a 3.8-kb SacI probe detected paternally methylated 10-kb and 2.7-kb fragments and maternally unmethylated 7.0-kb and 1.8-kb fragments in DNA from both thymocytes and ES cells. The same pattern was seen in the hybrid clones, with no difference in relative intensity (RI) between the methylated (RI = 0.60) and unmethylated (RI = 0.40) bands (Figure 5a). Similar results were obtained using the BamHI probe, which identified a paternally methylated fragment at 2.7 kb and maternally unmethylated fragments at 1.8 and 0.8 kb. The methylated (RI = 0.55) and unmethylated (RI = 0.45) bands are similarly detected in all samples (Figure 5a). Another well-characterized primary methylation imprint is an intronic CpG island of the maternally expressed *Igf2r* gene. This region, however, is only methylated on the expressed allele [7]. Following digestion with PvuII and the methylation-sensitive restriction enzyme MluI, a 330-bp *Igf2r* CpG island probe detected a 2.9-kb maternally derived methylated fragment and a 2.0-kb paternally derived unmethylated fragment in DNA from both thymocytes and ES cells (Figure 5b). The same pattern

Figure 4



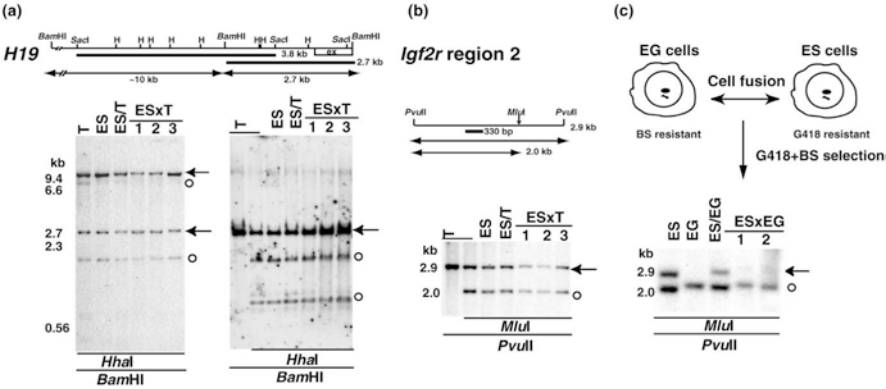
The developmental potential of ES hybrid cells in vivo. **(a)** An experimental scheme for generating ES hybrid cells and making chimeric embryos. **(b)** E7.5 chimeric embryos with ES hybrid cells. The contribution of hybrid cells is shown as blue cells with X-gal staining. **(c)** A representative longitudinal thin section of an E7.5 chimeric embryo. **(d,e)** Thin sections of chimeric embryos at higher magnification. Ect, ectoderm; Mes, mesoderm; End, endoderm.

was seen in DNA from the hybrid clones, with no difference between the RI of the methylated (RI = 0.55) and unmethylated (RI = 0.45) bands. These findings demonstrate that the primary methylation of both the *H19* upstream region and the *Igf2r* intronic region in the thymocyte genome is not affected after hybridization with ES cells.

The finding described above differs from our previous observations in hybrid clones between thymocytes and EG cells derived from gonadal PGCs of E12.5 mouse embryos [8], in which the maternal-specific methylation of *Igf2r* was erased. Maintenance of the somatic methylation pattern in ES hybrid cells suggests that the regulatory mechanism controlling DNA methylation of imprinted genes differs between ES and EG cells. To investigate this, we produced hybrids between ES and EG cells (Figure 5c). Maternal allele-specific methylation of *Igf2r* was detected in ES cells, but not in EG cells, and was detected at a ratio of approximately 1:3 (methylated/RI = 0.27, unmethylated/RI = 0.73) in a 1:1 control mixture of ES and EG cell DNA. In ES × EG hybrids, the methylated band was lost, demonstrating that the demethylation activity present in EG cells is dominant to the methylation imprint maintenance seen in ES cells.

We demonstrate an in vitro system for reprogramming somatic nuclei by generating ES × somatic cell hybrids. We used these hybrids to examine X chromosome replication timing, *Xist* RNA accumulation, exogenous *Oct4* gene expression, developmental potential, and DNA methylation of the imprinted *H19* and *Igf2r* genes. In female somatic cells, one of two X chromosomes is randomly inactivated for dosage compensation of X-linked genes. X chromosome inactivation occurs during early cell differentiation and induces epigenetic modifications, including

Figure 5



DNA methylation of the *H19* and *Igf2r* genes in ES hybrid and ES × EG hybrid cells. **(a)** *H19*; DNA was digested with BamHI and the methylation-sensitive restriction enzyme HhaI and was hybridized with 3.8-kb SacI and 2.7-kb BamHI probes (black bars in the genomic map). Three ES hybrid clones between ES cells and Rosa26 thymocytes, and their parental cells, were analyzed. **(b)** *Igf2r*; DNA was digested with PvuII and the methylation-sensitive restriction enzyme MluI and was hybridized with a 330-bp *Igf2r* intronic CpG island-specific probe (black bar in the genomic map). Three ES hybrid clones between ES cells and Rosa26 thymocytes, and their parental cells, were analyzed. **(c)** *Igf2r*; DNA methylation of the same MluI site was analyzed in two ES × EG hybrid clones. Arrows indicate methylated DNA fragments, and open circles mark unmethylated DNA fragments. The experimental scheme is summarized. T, thymocytes; ES/T, 1:1 mixture of ES and thymocyte DNA; ES/EG, 1:1 mixture of ES and EG DNA.

the delay of DNA replication to late S phase, DNA hypermethylation, and histone H4 hypoacetylation. In cloned embryos made by nuclear transplantation of somatic nuclei into oocytes, the inactive X chromosome of a female somatic cell is reactivated [9]. Thus, both X chromosomes become active, providing an indicator for the occurrence of nuclear reprogramming. Changes in replication timing and *Xist* RNA accumulation on a somatic X chromosome in ES hybrid cells indicated that somatic nuclei are reprogrammed after cell fusion. This was supported by our analysis of *Oct4-GFP* expression in the hybrids. The *Oct4-GFP* transgene, which was repressed in thymocytes prior to cell fusion, was reactivated in the ES hybrid cells. The third, and most conclusive, piece of evidence that the somatic nuclei were reprogrammed in the ES hybrids came from their ability to contribute to the formation of the endoderm, mesoderm, and ectoderm during early development of chimeric embryos. We conclude that ES cells have the capacity to reprogram at least some aspects of somatic nuclei in vitro, providing an experimental system that can be manipulated to investigate the molecular mechanisms involved.

In contrast to the reprogramming activities discussed above, the somatic differential methylation pattern of *H19* and *Igf2r* remained unchanged after thymocyte fusion with ES cells. A similar situation was found in EC (embryonic carcinoma)-thymocyte hybrid cells [10]. However, this allele-specific methylation represents a primary imprint, which is normally retained through postfertilization development, but not through germ cell development [6, 7]. Indeed, in EG-thymocyte hybrid cells, the somatic methylation pattern of several imprinted genes, including *Igf2r*, was disrupted, and both alleles became undermethylated [8]. Taken together, both ES and EG cells appear to retain similar cellular factors that are able to reprogram the epigenetic status of a somatic nucleus, making it competent for embryonic development. However, unlike EG cells, ES cells are not able to reprogram parental imprints. Methylation analysis of *Igf2r* in ES × EG hybrid cells indicates that EG cells may carry an additional dominant factor involved in more extensive epigenetic reprogramming. Indeed, the properties of ES and EG cells appear to reflect those of their cellular origins. Thus, both ES and EG cells should provide useful materials for identifying factors involved in epigenetic reprogramming and demethylation in early embryonic cells and in gonadal PGCs.

Considering the cloning of animals from somatic nuclei, it is clear that the proportion of clones that survive to adulthood is extremely low. Loss of embryos before implantation may be in part due to the failure of nucleo-

cytoplasmic interactions [11, 12]. Furthermore, many cloned embryos are lost during midgestation and soon after birth. One possible reason for this developmental failure is a lack of sufficient reprogramming of the somatic nuclei. If this were a common phenomenon, we may have predicted that somatic *Oct4-GFP* would not be expressed in 100% of the ES hybrid clones generated in this study, because *Oct4* expression is required for the maintenance of developmental potential [13]. However, we observed stable GFP expression in the majority of ES hybrid clones examined, suggesting that, in this system, successful nuclear reprogramming was the norm. For the *H19* and *Igf2r* genes, the primary methylation imprint from somatic cells was maintained in the ES hybrids, indicating that the epigenetic profile of some genes is not affected by cell fusion. This is supported by the finding that an inactive X chromosome from a somatic cell "remembers" its origin and is nonrandomly chosen for inactivation in the trophectoderm cells of cloned embryos [9]. It is, however, possible that the epigenetic profiles of some key genes responsible for normal development are aberrantly reprogrammed during the cloning process.

The mechanisms involved in epigenetic reprogramming of somatic nuclei, leading to competence for normal embryonic development, remain largely unexplored. It has been shown recently that mutations in the *ATRX* gene, which is a member of the SWI2/SNF2 helicase/ATPase family, give rise to alterations in methylation profiles of highly repeated sequences in mammals [14]. Thus, demethylation may occur as a consequence of chromatin remodeling. It has been suggested that the maternal activity of nucleosome-dependent ATPase ISWI may function as a chromatin remodeler in the process of nuclear reprogramming of cloned somatic cells in frogs [15]. Nuclei of the *Xenopus* XTC-2 epitheial cells incubated briefly in *Xenopus* egg extract are remodeled and lose TBP as a key component of the basal transcriptional complex. The reprogramming activity of ES cells may, therefore, facilitate the formation of loose chromatin, leading to the loss of somatic cell epigenetic memory.

Epigenetic instability of some imprinted genes in murine ES cells suggests that the epigenetic status of human ES cells must be assessed before clinical applications [16]. If the host ES cell chromosomes could be eliminated successfully, ES hybrid cells would provide useful therapeutic tools. Ultimately, epigenetic engineering may be enabled through the use of reprogramming factors, once they have been identified. We could then envisage the production of clonal or tissue-specific stem cells, from adult somatic cells, without the need for a contribution from mammalian embryos. Such technology would have important implications in the production of donor cells for numerous clinical applications involving cell or tissue transplantation.

Supplementary material

Supplementary material including full Materials and methods is available at http://images.cellpress.com/supmat/supmatin.htm.

Acknowledgements

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Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors

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SUMMARY

Differentiated cells can be reprogrammed to an embryonic-like state by transfer of nuclear contents into oocytes or by fusion with embryonic stem (ES) cells. Little is known about factors that induce this reprogramming. Here, we demonstrate induction of pluripotent stem cells from mouse embryonic or adult fibroblasts by introducing four factors, Oct3/4, Sox2, c-Myc, and Klf4, under ES cell culture conditions. Unexpectedly, Nanog was dispensable. These cells, which we designated iPS (induced pluripotent stem) cells, exhibit the morphology and growth properties of ES cells and express ES cell marker genes. Subcutaneous transplantation of iPS cells into nude mice resulted in tumors containing a variety of tissues from all three germ layers. Following injection into blastocysts, iPS cells contributed to mouse embryonic development. These data demonstrate that pluripotent stem cells can be directly generated from fibroblast cultures by the addition of only a few defined factors.

INTRODUCTION

Embryonic stem (ES) cells, which are derived from the inner cell mass of mammalian blastocysts, have the ability to grow indefinitely while maintaining pluripotency and the ability to differentiate into cells of all three germ layers (Evans and Kaufman, 1981; Martin, 1981). Human ES cells might be used to treat a host of diseases, such as Parkinson's disease, spinal cord injury, and diabetes (Thomson et al., 1998). However, there are ethical difficulties regarding the use of human embryos, as well as the problem of tissue rejection following transplantation in patients. One way to circumvent these issues is the generation of pluripotent cells directly from the patients' own cells.

Somatic cells can be reprogrammed by transferring their nuclear contents into oocytes (Wilmut et al., 1997) or by fusion with ES cells (Cowan et al., 2005; Tada et al., 2001), indicating that unfertilized eggs and ES cells contain factors that can confer totipotency or pluripotency to somatic cells. We hypothesized that the factors that play important roles in the maintenance of ES cell identity also play pivotal roles in the induction of pluripotency in somatic cells.

Several transcription factors, including Oct3/4 (Nichols et al., 1998; Niwa et al., 2000), Sox2 (Avilion et al., 2003), and Nanog (Chambers et al., 2003; Mitsui et al., 2003), function in the maintenance of pluripotency in both early embryos and ES cells. Several genes that are frequently upregulated in tumors, such as *Stat3* (Matsuda et al., 1999; Niwa et al., 1998), *E-Ras* (Takahashi et al., 2003), c-*myc* (Cartwright et al., 2005), *Klf4* (Li et al., 2005), and β-*catenin* (Kielman et al., 2002; Sato et al., 2004), have been shown to contribute to the long-term maintenance of the ES cell phenotype and the rapid proliferation of ES cells in culture. In addition, we have identified several other genes that are specifically expressed in ES cells (Maruyama et al., 2005; Mitsui et al., 2003).

In this study, we examined whether these factors could induce pluripotency in somatic cells. By combining four selected factors, we were able to generate pluripotent cells, which we call induced pluripotent stem (iPS) cells, directly from mouse embryonic or adult fibroblast cultures.

RESULTS

We selected 24 genes as candidates for factors that induce pluripotency in somatic cells, based on our hypothesis that such factors also play pivotal roles in the maintenance of ES cell identity (see Table S1 in the Supplemental Data available with this article online). For β-catenin, c-Myc, and Stat3, we used active forms, S33Y-β-catenin (Sadot et al., 2002), T58A-c-Myc (Chang et al., 2000), and Stat3-C (Bromberg et al., 1999), respectively. Because of the reported negative effect of Grb2 on pluripotency (Burdon et al., 1999; Cheng et al., 1998), we included its dominant-negative mutant Grb2ΔSH2 (Miyamoto et al., 2004) as 1 of the 24 candidates.

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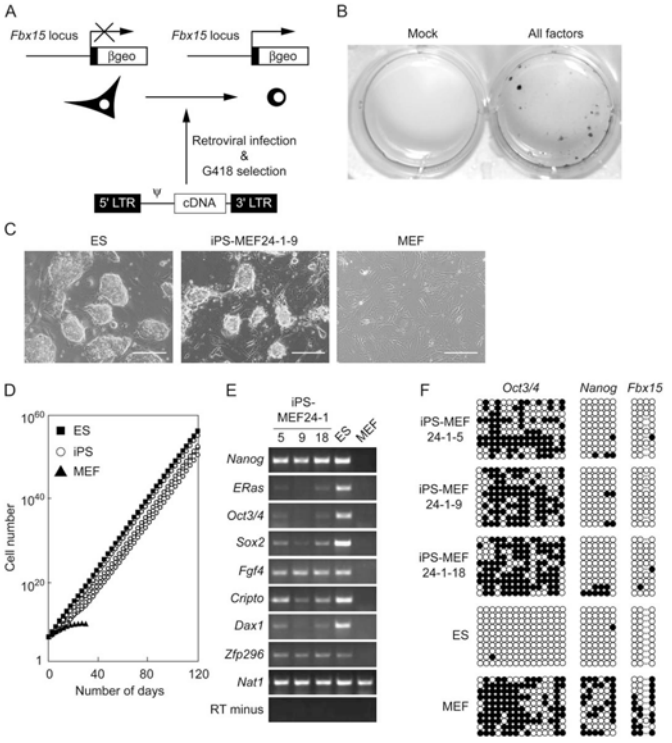

Figure 1. Generation of iPS Cells from MEF Cultures via 24 Factors

(A) Strategy to test candidate factors.

(B) G418-resistant colonies were observed 16 days after transduction with a combination of 24 factors. Cells were stained with crystal violet.

(C) Morphology of ES cells, iPS cells (iPS-MEF24, clone 1-9), and MEFs. Scale bars = 200 μm.

(D) Growth curves of ES cells, iPS cells (iPS-MEF24, clones 2-1–4), and MEFs. 3×10^5 cells were passaged every 3 days into each well of six-well plates.

(E) RT-PCR analysis of ES cell marker genes in iPS cells (iPS-MEF24, clones 1-5, 1-9, and 1-18), ES cells, and MEFs. *Nat1* was used as a loading control.

(F) Bisulfite genomic sequencing of the promoter regions of *Oct3/4*, *Nanog*, and *Fbx15* in iPS cells (iPS-MEF24, clones 1-5, 1-9, and 1-18), ES cells, and MEFs. Open circles indicate unmethylated CpG dinucleotides, while closed circles indicate methylated CpGs.

To evaluate these 24 candidate genes, we developed an assay system in which the induction of the pluripotent state could be detected as resistance to G418 (Figure 1A). We inserted a *βgeo* cassette (a fusion of the β-galactosidase and neomycin resistance genes) into the mouse *Fbx15* gene by homologous recombination (Tokuzawa et al., 2003). Although specifically expressed in mouse ES cells and early embryos, Fbx15 is dispensable for the maintenance of pluripotency and mouse development. ES cells homozygous for the βgeo knockin construct ($Fbx15^{\beta geo/\beta geo}$) were resistant to extremely high concentrations of G418 (up to 12 mg/ml), whereas somatic cells derived from $Fbx15^{\beta geo/\beta geo}$ mice were sensitive to a normal concentration of G418 (0.3 mg/ml). We expected that even partial activation of the *Fbx15* locus would result in resistance to normal concentrations of G418.

We introduced each of the 24 candidate genes into mouse embryonic fibroblasts (MEFs) from $Fbx15^{\beta geo/\beta geo}$

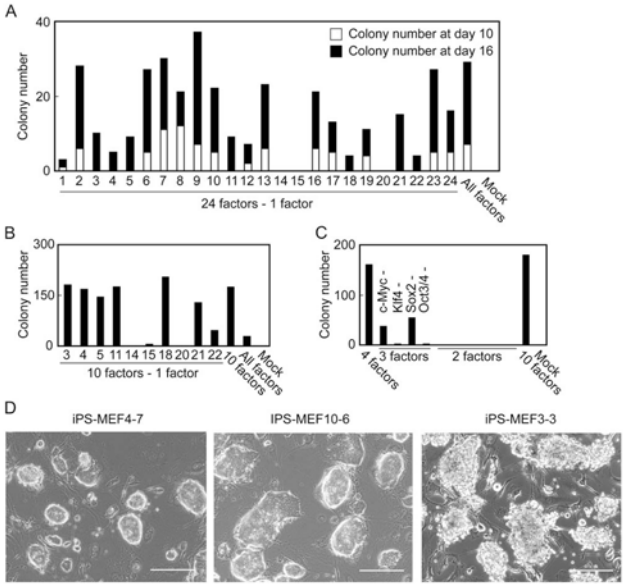

Figure 2. Narrowing down the Candidate Factors

(A) Effect of the removal of individual factors from the pool of 24 transduced factors on the formation of G418-resistant colonies. *Fbx15*$^{\beta geo/\beta geo}$ MEFs were transduced with the indicated factors and selected with G418 for 10 days (white columns) or 16 days (black columns).

(B) Effect of the removal of individual factors from the selected 10 factors on the formation of G418-resistant colonies 16 days after transduction.

(C) Effect of the transduction of pools of four, three, and two factors on the formation of G418-resistant colonies 16 days after transduction.

(D) Morphologies of iPS-MEF4 (clone 7), iPS-MEF10 (clone 6), and iPS-MEF3 (clone 3). Scale bars = 200 μm.

embryos by retroviral transduction (Morita et al., 2000). Transduced cells were then cultured on STO feeder cells in ES cell medium containing G418 (0.3 mg/ml). We did not, however, obtain drug-resistant colonies with any single factor, indicating that no single candidate gene was sufficient to activate the *Fbx15* locus (Figure 1B; see also Table S2, which summarizes all of the transduction experiments in this study).

In contrast, transduction of all 24 candidates together generated 22 G418-resistant colonies (Figure 1B). Of the 12 clones for which we continued cultivating under selection, 5 clones exhibited morphology similar to ES cells, including a round shape, large nucleoli, and scant cytoplasm (Figure 1C). We repeated the experiments and observed 29 G418-resistant colonies, from which we picked 6 colonies. Four of these clones possessed ES cell-like morphology and proliferation properties (Figure 1D). The doubling time of these cells (19.4, 17.5, 18.7, and 18.6 hr) was equivalent to that of ES cells (17.0 hr). We designated these cells iPS-MEF24 for "pluripotent stem cells induced from MEFs by 24 factors." Reverse transcription PCR (RT-PCR) analysis revealed that the iPS-MEF24 clones expressed ES cell markers, including *Oct3/4*, *Nanog*, *E-Ras*, *Cripto*, *Dax1*, and *Zfp296* (Mitsui et al., 2003) and *Fgf4* (Yuan et al., 1995) (Figure 1E). Bisulfite genomic sequencing demonstrated that the promoters of *Fbx15* and *Nanog* were demethylated in iPS cells (Figure 1F). By contrast, the *Oct3/4* promoter remained methylated in these cells. These data indicate that some combination of these 24 candidate factors induced the expression of ES cell marker genes in MEF culture.

Next, to determine which of the 24 candidates were critical, we examined the effect of withdrawal of individual factors from the pool of transduced candidate genes on the formation of G418-resistant colonies (Figure 2A). We identified 10 factors (3, 4, 5, 11, 14, 15, 18, 20, 21, and 22) whose individual withdrawal from the bulk transduction pool resulted in no colony formation 10 days after

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transduction and fewer colonies 16 days after transduction. Combination of these 10 genes alone produced more ES cell-like colonies than transduction of all 24 genes did (Figure 2B).

We next examined the formation of colonies after withdrawal of individual factors from the 10-factor pool transduced into MEFs (Figure 2B). G418-resistant colonies did not form when either Oct3/4 (factor 14) or Klf4 (factor 20) was removed. Removal of Sox2 (factor 15) resulted in only a few G418-resistant colonies. When we removed c-Myc (factor 22), G418-resistant colonies did emerge, but these had a flatter, non-ES-cell-like morphology. Removal of the remaining factors did not significantly affect colony numbers. These results indicate that Oct3/4, Klf4, Sox2, and c-Myc play important roles in the generation of iPS cells from MEFs.

Combination of the four genes produced a number of G418-resistant colonies similar to that observed with the pool of 10 genes (Figure 2C). We continued cultivation of 12 clones for each transduction and were able to establish 4 iPS-MEF4 and 5 iPS-MEF10 clones. In addition, we could generate iPS cells (iPS-MEF4wt) with wild-type c-Myc instead of the T58A mutant (Table S2). These data demonstrate that iPS cells can be induced from MEF culture by the introduction of four transcription factors, Oct3/4, Sox2, c-Myc, and Klf4.

No combination of two factors could induce the formation of G418-resistant colonies (Figure 2C). Two combinations of three factors—Oct3/4, Sox2, and c-Myc (minus Klf4) or Klf4, Sox2, and c-Myc (minus Oct3/4)—generated a single, small colony in each case, but these could not be maintained in culture. With the combination of Oct3/4, Klf4, and Sox2 (minus c-Myc), we observed the formation of 36 G418-resistant colonies, which, however, exhibited a flat, non-ES-cell-like morphology. With the combination of Oct3/4, Klf4, and c-Myc (minus Sox2), we observed the formation of 54 G418-resistant colonies, of which we picked 6. Although all 6 clones could be maintained over several passages, the morphology of these cells (iPS-MEF3) differed from that of iPS-MEF4 and iPS-MEF10 cells, with iPS-MEF3 colonies exhibiting rough surfaces (Figure 2D). These data indicate that the combination of Oct3/4, c-Myc, and Klf4 can activate the *Fbx15* locus, but the change induced by these three factors alone is different from that seen in iPS-MEF4 or iPS-MEF10 cells.

We performed RT-PCR to examine whether ES cell marker genes were expressed in iPS cells (Figure 3A). We used primers that would amplify transcripts of the endogenous gene but not transcripts of the transgene. iPS-MEF10 and iPS-MEF4 clones expressed the majority of marker genes, with the exception of *Ecat1* (Mitsui et al., 2003). The expression of several marker genes, including *Oct3/4*, was higher in iPS-MEF4-7, iPS-MEF10-6, and iPS-MEF10-7 clones than in the remaining clones. *Sox2* was only expressed in iPS-MEF10-6. The iPS-MEF4wt clone also expressed many of the ES cell marker genes (Figure S1). Chromatin immunoprecipitation analyses showed that the promoters of *Oct3/4* and *Nanog* had increased acetylation of histone H3 and decreased dimethylation of lysine 9 of histone H3 (Figure 3B). CpG dinucleotides in these promoters remained partially methylated in iPS cells (Figure 3C). iPS-MEF4 and iPS-MEF10 cells were positive for alkaline phosphatase and SSEA-1 (Figure 3D) and showed high telomerase activity (Figure S2). These results demonstrate that iPS-MEF4 and iPS-MEF10 cells are similar, but not identical, to ES cells.

In iPS-MEF3 clones, *Ecat1*, *Esg1*, and *Sox2* were not activated (Figure 3A). *Nanog* was induced, but to a lesser extent than in iPS-MEF4 and iPS-MEF10 clones. *Oct3/4* was weakly activated in iPS-MEF3-3, -5, and -6 but was not activated in the remaining clones. By contrast, *E-Ras* and *Fgf4* were activated more efficiently in iPS-MEF3 than in iPS-MEF10 or iPS-MEF4. These data confirm that iPS-MEF3 cells are substantially different from iPS-MEF10 and iPS-MEF4 cells.

We compared the global gene-expression profiles of ES cells, iPS cells, and *Fbx15*$^{lgeo/lgeo}$ MEFs using DNA microarrays (Figure 4A). In addition, we examined *Fbx15*$^{lgeo/lgeo}$ MEFs in which the four factors had been introduced without G418 selection, immortalized MEFs expressing K-RasV12, and NIH 3T3 cells transformed with H-RasV12. Pearson correlation analysis revealed that iPS cells are clustered closely with ES cells but separately from fibroblasts and their derivatives (Figure 4A). The microarray analyses identified genes that were commonly upregulated in ES cells and iPS cells, including *Myb*, *Kit*, *Gdf3*, and *Zic3* (group I, Figure 4B and Table S3). Other genes were upregulated more efficiently in ES cells, iPS-MEF4, and iPS-MEF10 than in iPS-MEF3 clones, including *Dppa3*, *Dppa4*, *Dppa5*, *Nanog*, *Sox2*, *Esrrb*, and *Rex1* (group II). Lower expression of these genes may account for the lack of pluripotency in iPS-MEF3 cells. In addition, we found genes that were upregulated more prominently in ES cells than in iPS cells, including *Dnmt3a*, *Dnmt3b*, *Dnmt3l*, *Utf1*, *Tcl1*, and the *LIF receptor* gene (group III). These data confirm that iPS cells are similar, but not identical, to ES cells.

We examined the pluripotency of iPS cells by teratoma formation (Figure 5A; Table S6 and Figure S3). We obtained tumors with 5 iPS-MEF10 clones, 3 iPS-MEF4 clones, 1 iPS-MEF4wt clone, and 6 iPS-MEF3 clones after subcutaneous injection into nude mice. Histological examination revealed that 2 iPS-MEF10 clones (3 and 6), 2 iPS-MEF4 clones (2 and 7), and the iPS-MEF4wt-4 clone differentiated into all three germ layers, including neural tissues, cartilage, and columnar epithelium. iPS-MEF10-6 could give rise to all three germ layers even after 30 passages (Table S6 and Figure S3). We confirmed differentiation into neural and muscle tissues by immunostaining (Figure 5B) and RT-PCR (Figure S4). By contrast, these teratomas did not express the trophoblast marker *Cdx2* (Figure S4). iPS-MEF10-1 tumors differentiated into ectoderm and endoderm, but not mesoderm, and no signs of differentiation were observed in tumors derived from the remaining iPS-MEF10 (7 and 10) or from iPS-MEF4-10.

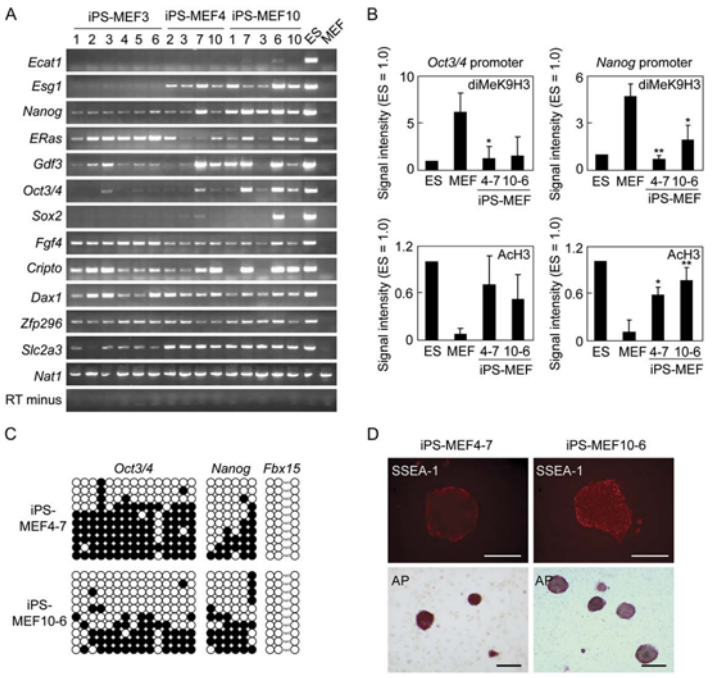

Figure 3. Gene-Expression Profiles of iPS Cells

(A) RT-PCR analysis of ES marker genes in iPS cells, ES cells, and MEFs. We used primer sets that amplified endogenous but not transgenic transcripts.

(B) The promoters of *Oct3/4* and *Nanog* were analyzed by ChIP for dimethylation status of lysine 9 of histone H3 and acetylation status of histone H3 in ES cells, MEFs, and iPS cells (MEF4-7 and MEF10-6). Data were quantified by real-time PCR. Shown are the averages and standard deviations of relative values compared to ES cells ($n = 3$). *$p < 0.05$; **$p < 0.01$ compared to MEFs.

(C) The promoters of *Oct3/4*, *Nanog*, and *Fbx15* were analyzed with bisulfite genomic sequencing for DNA methylation status in iPS-MEF4-7 and iPS-MEF10-6. The DNA methylation status of these promoters in ES cells and MEFs is shown in Figure 1F.

(D) iPS-MEF4-7 and iPS-MEF10-6 clones were stained with a mouse monoclonal antibody against SSEA-1 (480, Santa Cruz) or with an alkaline phosphatase kit (Sigma). Scale bars = 500 μm (SSEA1) and 1 mm (AP).

These data demonstrate that the majority of, but not all, iPS-MEF10 and iPS-MEF4 clones exhibit pluripotency.

In contrast, all tumors derived from iPS-MEF3 clones were composed entirely of undifferentiated cells (Table S6 and Figure S3). Thus, although the three factors (Oct3/4, c-Myc, and Klf4) could induce the expression of some ES cell marker genes, they were not able to induce pluripotency.

iPS-MEF10, iPS-MEF4, and iPS-MEF3 cells formed embryoid bodies in noncoated plastic dishes (Figure 5C). When grown in tissue culture dishes, the embryoid bodies from iPS-MEF10 and iPS-MEF4 cells attached to the dish bottom and initiated differentiation. After 3 days, immunostaining detected cells positive for α-smooth muscle actin (mesoderm marker), α-fetoprotein (endoderm marker), and βIII tubulin (ectoderm marker) (Figure 5D). By contrast, embryoid bodies from iPS-MEF3 cells remained undifferentiated even when cultured in gelatin-coated dishes (Figure 5C). These data confirmed pluripotency of iPS-MEF10 and iPS-MEF4 and nullipotency of iPS-MEF3 in vitro.

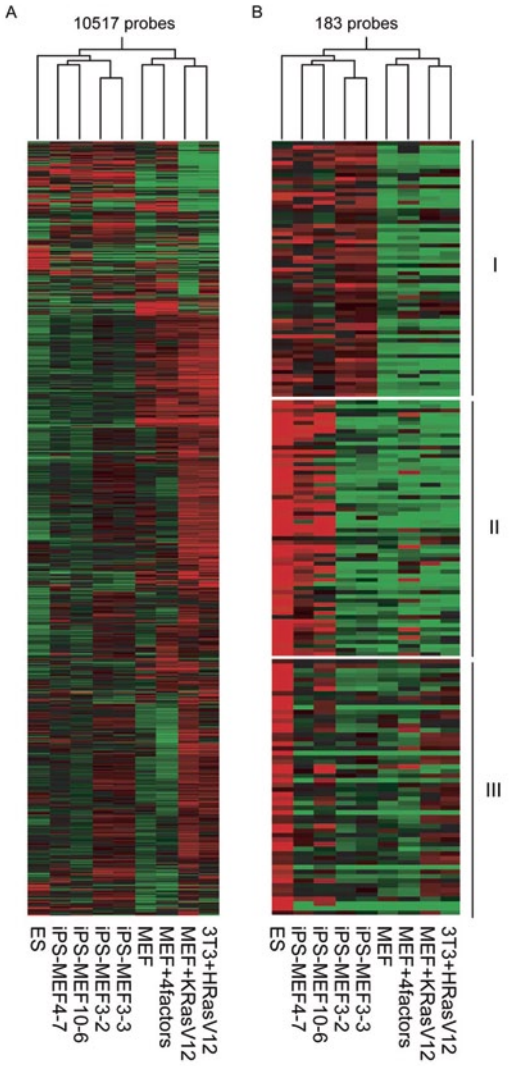

Figure 4. Global Gene-Expression Analyses by DNA Microarrays

(A) Pearson correlation analysis of 10,517 probes was performed to cluster ES cells, iPS cells (MEF4- 7, MEF10-6, MEF3-2, and MEF3-3), MEFs, MEFs expressing the four factors, immortalized MEFs expressing K-RasV12, and NIH 3T3 cells transformed by H-RasV12. Red indicates increased expression compared to median levels of the eight samples, whereas green means decreased expression.

(B) Genes upregulated in ES and/or iPS cells. Genes in group I are genes upregulated in ES cells and iPS cells. Genes in group II are upregulated more in ES cells, iPS-MEF4-7, and iPS-MEF10-6 than in iPS-MEF3 cells. Genes in group III are upregulated more in ES cells than in iPS cells. Lists of genes are shown in Tables S3–S5.

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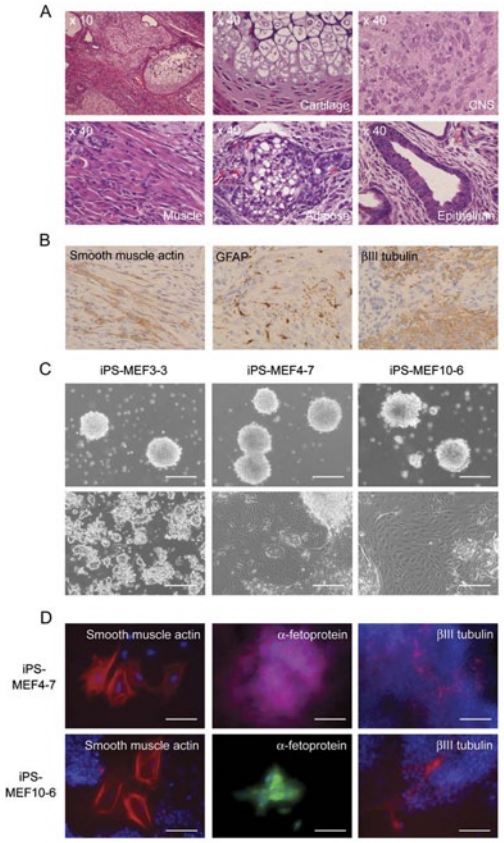

Figure 5. Pluripotency of iPS Cells Derived from MEFs

(A) Various tissues present in teratomas derived from iPS-MEF4-7 cells. Histology of other teratomas is shown in Figure S3 and Table S6.

(B) Immunostaining confirming differentiation into neural tissues and muscles in teratomas derived from iPS-MEF4-7.

(C) In vitro embryoid body formation (upper row) and differentiation (lower row). Scale bars = 200 μm.

(D) Immunostaining confirming in vitro differentiation into all three germ layers. Scale bars = 100 μm. Secondary antibodies were labeled with Cy3 (red), except for α-fetoprotein in iPS-MEF10-6, with which Alexa 488 (green) was used.

We next introduced the four selected factors into tail-tip fibroblasts (TTFs) of four 7-week-old male *Fbx15*$^{\beta geo/\beta geo}$ mice on a C57/BL6-129 hybrid background. We obtained 3 G418-resistant colonies, from each of which we could establish iPS cells (iPS-TTF4). We also introduced the four factors into TTFs from a 12-week-old female *Fbx15*$^{\beta geo/\beta geo}$ mouse, which also constitutively expressed green fluorescent protein (GFP) from the CAG promoter and had a C57/BL6-129-ICR hybrid background. Of the 13 G418-resistant colonies obtained, we isolated 6 clones from which we could establish iPS cells (iPS-TTFgfp4, clones 1–6). In addition, we established another iPS-TTFgfp4 (clone 7), in which the cDNA for each of the four factors was flanked with two loxP sites in the transgene. These cells were morphologically indistinguishable from ES cells (Figure 6A). RT-PCR showed that clones 3 and 7 of iPS-TTFgfp4 expressed the majority of ES cell marker genes at high levels and the others at lower levels (Figure 6B). In another attempt, we used either the T58A mutant or the wild-type c-Myc for transduction and established 5 iPS-TTFgfp4 clones (clones 8–12) and 3 iPS-TTFgfp4wt clones (clones 1–3) (Figure S5). RT-PCR

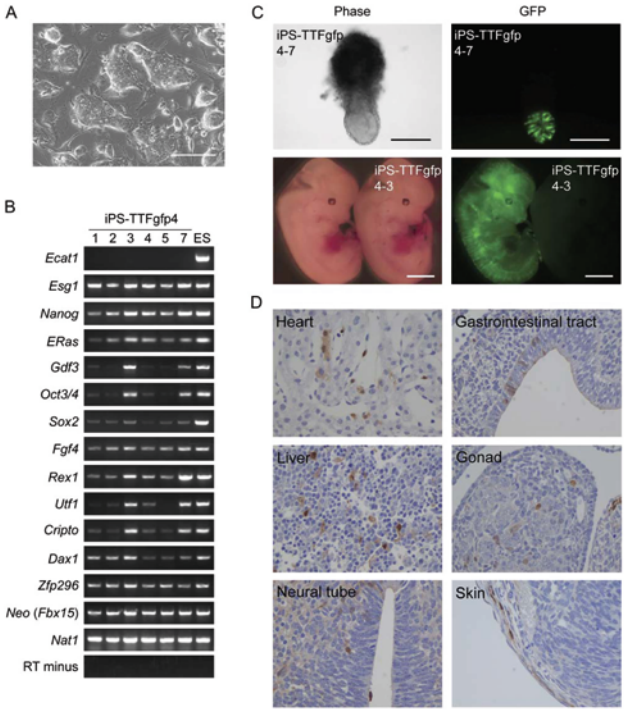

Figure 6. Characterization of iPS Cells Derived from Adult Mouse Tail-Tip Fibroblasts

(A) Morphology of iPS-TTFgfp4-3 on STO feeder cells.

(B) RT-PCR analysis of ES marker gene expression in iPS-TTFgfp4 cells (clones 1–5 and 7). We used primer sets that amplified endogenous but not transgenic transcripts.

(C) Contribution of iPS-TTFgfp4-7 and iPS-TTFgfp4-3 cells to mouse embryonic development. iPS cells were microinjected into C57/BL6 blastocysts. Embryos were analyzed with a fluorescence microscope at E7.5 (upper panels, iPS-TTFgfp4-7) or E13.5 (lower panels, iPS-TTFgfp4-3). Scale bars = 200 μm (upper panels) and 2 mm (lower panels).

(D) The E13.5 chimeric embryo was sectioned and stained with anti-GFP antibody (brown). Cells were counterstained with eosin (blue).

showed that iPS-TTFgfp4wt cells also expressed most of the ES cell marker genes (Figure S6).

We transplanted 2 iPS-TTF4 and 6 iPS-TTFgfp4 clones into nude mice, all of which produced tumors containing tissues of all three germ layers (Table S6 and Figure S3). We then introduced 2 clones of iPS-TTFgfp4 cells (clones 3 and 7) into C57/BL6 blastocysts by microinjection. With iPS-TTFgfp4-3, we obtained 18 embryos at E13.5, 2 of which showed contribution of GFP-positive iPS cells (Figure 6C). Histological analyses confirmed that iPS cells contributed to all three germ layers (Figure 6D). We observed GFP-positive cells in the gonad but could not determine whether they were germ cells or somatic cells. With iPS-TTFgfp4-7, we obtained 22 embryos at E7.5, 3 of which were positive for GFP. With the 2 clones, we had 27 pups born, but none of them were chimeric mice. In addition, iPS-TTFgfp4 cells could differentiate into all three germ layers in vitro (Figure S7). These data demonstrate that the four selected factors could induce pluripotent cells from adult mouse fibroblast cultures.

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We further characterized the expression of the four factors and others in iPS cells. Real-time PCR confirmed that endogenous expression of *Oct3/4* and *Sox2* was lower in iPS cells than in ES cells (Figure S8). However, the total amount of the four factors from the endogenous genes and the transgenes exceeded the normal expression levels in ES cells. In contrast, Western blot analyses showed that the total protein amounts of the four factors in iPS cells were comparable to those in ES cells (Figure 7A; Figure S8). We could detect Nanog and E-Ras proteins in iPS cells, but at lower levels than those in ES cells (Figures 7A and 7B; Figure S8). The p53 levels in iPS cells were lower than those in MEFs and equivalent to those in ES cells (Figure 7A; Figure S9). The p21 levels in iPS cells varied in each clone and were between those in ES cells and MEFs (Figure S9). Upon differentiation in vitro, the total mRNA expression levels of *Oct3/4* and *Sox2* decreased but remained much higher than in ES cells. In contrast, their protein levels decreased to comparable levels in iPS cells and ES cells (Figure 7B).

Southern blot analyses showed that each iPS clone has a unique transgene integration pattern (Figure 7C). Karyotyping analyses of the iPS-TTFgfp4 (clones 1, 2, 3, 7, and 11) and iPS-TTFgfp4wt (clones 1–3) demonstrated that 2 iPS-TTFgfp4 clones and all of the iPS-TTFgfp4wt clones showed a normal karyotype of 40XX (Figure 7D), while the other 3 iPS-TTFgfp4 clones were 39XO, 40XO +10, and 40Xi(X). Analyses of PCR-based simple sequence length polymorphisms (SSLPs) demonstrated that iPS-MEF clones have a mixed background of C57/BL6 and 129 (Table S7), whereas iPS-TTFgfp clones have a mixed background of ICR, C57/BL6, and 129 (Table S8). Finally, we found that iPS cells could not remain undifferentiated when cultured in the absence of feeder cells, even with the presence of LIF (Figure 7E). These results, together with the different gene-expression patterns, exclude the possibility that iPS cells are merely contamination of preexisting ES cells. Finally, subclones of iPS cells were positive for alkaline phosphatase and could differentiate into all three germ layers in vitro (Figure S10), confirming their clonal nature.

DISCUSSION

Oct3/4, Sox2, and Nanog have been shown to function as core transcription factors in maintaining pluripotency (Boyer et al., 2005; Loh et al., 2006). Among the three, we found that Oct3/4 and Sox2 are essential for the generation of iPS cells. Surprisingly, Nanog is dispensable. In addition, we identified c-Myc and Klf4 as essential factors. These two tumor-related factors could not be replaced by other oncogenes including E-Ras, Tcl1, β-catenin, and Stat3 (Figures 2A and 2B).

The c-Myc protein has many downstream targets that enhance proliferation and transformation (Adhikary and Eilers, 2005), many of which may have roles in the generation of iPS cells. Of note, c-Myc associates with histone acetyltransferase (HAT) complexes, including TRRAP, which is a core subunit of the TIP60 and GCN5 HAT complexes (McMahon et al., 1998), CREB binding protein (CBP), and p300 (Vervoorts et al., 2003). Within the mammalian genome, there may be up to 25,000 c-Myc binding sites (Cawley et al., 2004), many more than the predicted number of Oct3/4 and Sox2 binding sites (Boyer et al., 2005; Loh et al., 2006). c-Myc protein may induce global histone acetylation (Fernandez et al., 2003), thus allowing Oct3/4 and Sox2 to bind to their specific target loci.

Klf4 has been shown to repress *p53* directly (Rowland et al., 2005), and p53 protein has been shown to suppress *Nanog* during ES cell differentiation (Lin et al., 2004). We found that iPS cells showed levels of p53 protein lower than those in MEFs (Figure 7A). Thus, Klf4 might contribute to activation of *Nanog* and other ES cell-specific genes through *p53* repression. Alternatively, Klf4 might function as an inhibitor of Myc-induced apoptosis through the repression of *p53* in our system (Zindy et al., 1998). On the other hand, Klf4 activates $p21^{CIP1}$, thereby suppressing cell proliferation (Zhang et al., 2000). This antiproliferation function of Klf4 might be inhibited by c-Myc, which suppresses the expression of $p21^{CIP1}$ (Seoane et al., 2002). The balance between c-Myc and Klf4 may be important for the generation of iPS cells.

One question that remains concerns the origin of our iPS cells. With our retroviral expression system, we estimated that only a small portion of cells expressing the four factors became iPS cells (Figure S11). The low frequency suggests that rare tissue stem/progenitor cells that coexisted in the fibroblast cultures might have given rise to the iPS cells. Indeed, multipotent stem cells have been isolated from skin (Dyce et al., 2004; Toma et al., 2001, 2005). These studies showed that ~0.067% of mouse skin cells are stem cells. One explanation for the low frequency of iPS cell derivation is that the four factors transform tissue stem cells. However, we found that the four factors induced iPS cells with comparably low efficiency even from bone marrow stroma, which should be more enriched in mesenchymal stem cells and other multipotent cells (Tables S2 and S6). Furthermore, cells induced by the three factors were nullipotent (Table S6 and Figure S3). DNA microarray analyses suggested that iPS-MEF4 cells and iPS-MEF3 cells have the same origin (Figure 4). These results do not favor multipotent tissue stem cells as the origin of iPS cells.

There are several other possibilities for the low frequency of iPS cell derivation. First, the levels of the four factors required for generation of pluripotent cells may have narrow ranges, and only a small portion of cells expressing all four of the factors at the right levels can acquire ES cell-like properties. Consistent with this idea, a mere 50% increase or decrease in Oct3/4 proteins induces differentiation of ES cells (Niwa et al., 2000). iPS clones overexpressed the four factors when RNA levels were analyzed, but their protein levels were comparable to those in ES cells (Figures 7A and 7B; Figure S8), suggesting that the iPS clones possess a mechanism (or mechanisms) that

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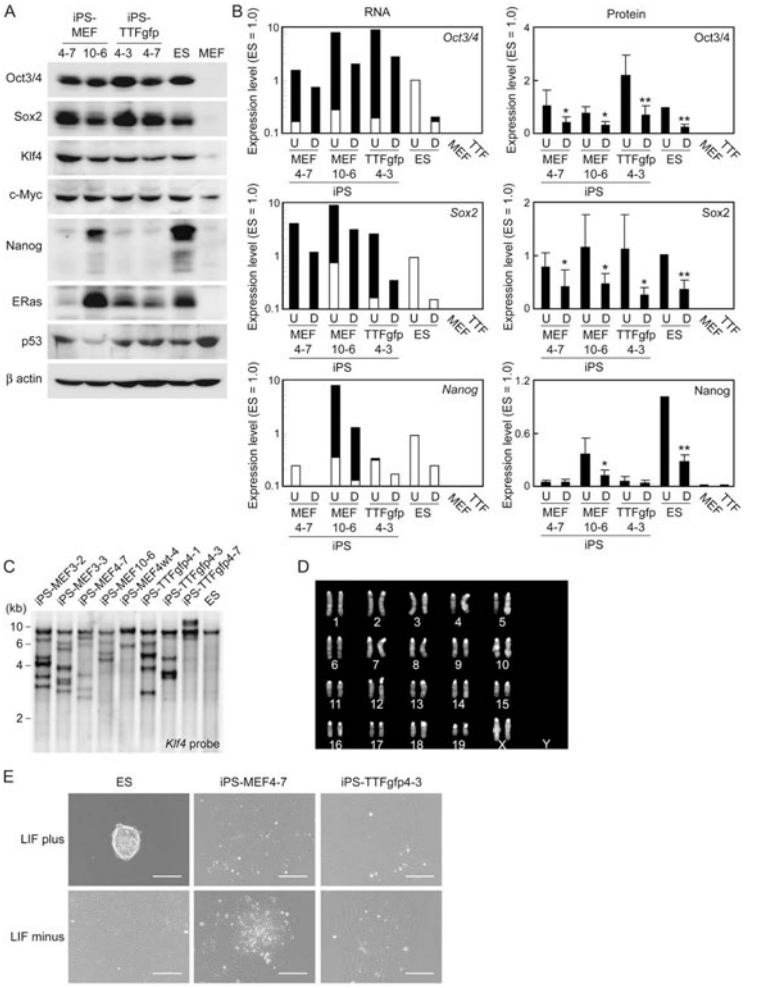

Figure 7. Biochemical and Genetic Analyses of iPS Cells

(A) Western blot analyses of the four factors and other proteins in iPS cells (MEF4-7, MEF10-6, TTFgfp4-3, and TTFgfp4-7), ES cells, and MEFs.

(B) Changes in RNA (left) and protein (right) levels of Oct3/4, Sox2, and Nanog in iPS cells and ES cells that were undifferentiated on STO feeder cells (U) or induced to differentiated in vitro through embryoid body formation (D). Shown are relative expression levels compared to undifferentiated ES cells. Data of MEFs and TTFs are also shown. RNA levels were determined with real-time PCR using primers specific for endogenous transcripts

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tightly regulates the protein levels of the four factors. We speculate that high amounts of the four factors are required in the initial stage of iPS cell generation, but, once they acquire ES cell-like status, too much of the factors are detrimental for self-renewal. Only a small portion of transduced cells show such appropriate transgene expression. Second, generation of pluripotent cells may require additional chromosomal alterations, which take place spontaneously during culture or are induced by some of the four factors. Although the iPS-TTFgfp4 clones had largely normal karyotypes (Figure 7D), we cannot rule out the existence of minor chromosomal alterations. Site-specific retroviral insertion may also play a role. Southern blot analyses showed that each iPS clone has ~20 retroviral integrations (Figure 7C). Some of these may have caused silencing or fusion with endogenous genes. Further studies will be required to determine the origin of iPS cells.

Another unsolved question is whether the four factors we identified play roles in reprogramming induced by fusion with ES cells or nuclear transfer into oocytes. Since the four factors are expressed in ES cells at high levels, it is reasonable to speculate that they are involved in the reprogramming machinery that exists in ES cells. Our result is also consistent with the finding that the reprogramming activity resides in the nucleus, but not in the cytoplasm, of ES cells (Do and Scholer, 2004). However, iPS cells were not identical to ES cells, as shown by the global gene-expression patterns and DNA methylation status. It is possible that we have missed additional important factors. One such candidate is ECAT1, although its forced expression in iPS cells did not consistently upregulate ES cell marker genes (Figure S12).

More obscure are the roles of the four factors, especially Klf4 and c-Myc, in the reprogramming observed in oocytes. Both Klf4 and c-Myc are dispensable for preimplantation mouse development (Baudino et al., 2002; Katz et al., 2002). Furthermore, c-*myc* is not detected in oocytes (Domashenko et al., 1997). In contrast, L-*myc* is expressed maternally in oocytes. *Klf17* and *Klf7*, but not *Klf4*, are found in expressed sequence-tag libraries derived from unfertilized mouse eggs. Klf4 and c-Myc might be compensated by these related proteins. It is highly likely that other factors are also required to induce complete reprogramming and totipotency in oocytes.

It is likely that the four factors from the transgenes are required for maintaining the iPS cells since the expression of Oct3/4 and Sox2 from the endogenous genes remained low (Figure 7B; Figure S8). We intended to prove this by using transgenes flanked by two loxP sites and obtained an iPS clone (TTF4gfp4-7). However, we noticed that these cells contain multiple loxP sites on multiple chromosomes, and, thus, the Cre-mediated recombination would cause not only deletion of the transgenes but also inter- and intrachromosomal rearrangements. Studies with conditional expression systems, such as the tetracycline-mediated system, are required to answer this question.

We showed that the iPS cells can differentiate in vitro and in vivo even with the presence of the retroviral vectors containing the four factors. We found that Oct3/4 and Sox2 proteins decreased significantly during in vitro differentiation (Figure 7B). Retroviral expression has been shown to be suppressed in ES cells and further silenced upon differentiation by epigenetic modifications, such as DNA methylation (Yao et al., 2004). The same mechanisms are likely to play roles in transgene repression in iPS cells since they express *Dnmt3a*, *3b*, and *3l*, albeit at lower levels than ES cells do (Table S5). In addition, we found that iPS cells possess a mechanism (or mechanisms) that lowers protein levels of the transgenes and Nanog (Figure 7B; Figure S8). The same mechanism may be enhanced during differentiation. However, silencing of *Oct3/4* in iPS-TTFgfp4-3 cells was not complete, which may explain our inability to obtain live chimeric mice after blastocyst microinjection of iPS cells.

An unexpected finding in this study was the efficient activation of *Fgf4* and *Fbx15* by the combination of the three factors devoid of Sox2 since these two genes have been shown to be regulated synergistically by Oct3/4 and Sox2 (Tokuzawa et al., 2003; Yuan et al., 1995). It is also surprising that Nanog is dispensable for induction and maintenance of iPS cells. More detailed analyses of iPS cells will enhance our understanding of transcriptional regulation in pluripotent stem cells.

Our findings may have wider applications, as we have found that transgene reporters with other ES cell marker genes, such as *Nanog*, can replace the *Fbx15* knockin during selection (K. Okita and S.Y., unpublished data). However, we still do not know whether the four factors can generate pluripotent cells from human somatic cells. Use of c-Myc may not be suitable for clinical applications, and the process may require specific culture environments. Nevertheless, the finding is an important step in controlling pluripotency, which may eventually allow the creation of pluripotent cells directly from somatic cells of patients.

EXPERIMENTAL PROCEDURES

Mice

Fbx15$^{\beta geo/\beta geo}$ mice were generated with 129SvJae-derived RF8 ES cells as described previously (Tokuzawa et al., 2003) and were

(white columns) or those common for both endogenous and transgenic transcripts (white and black columns). RNA expression levels are shown on logarithmic axes. Protein levels were determined by Western blot normalized with β-actin. Protein levels are shown as the averages and standard deviations on linear axes (n = 4). *$p < 0.05$ compared to undifferentiated cells.

(C) Southern blot analyses showing the integration of transgenes. Genomic DNA isolated from iPS cells and ES cells was digested with EcoRI and BamHI, separated on agarose gel, transferred to a nylon membrane, and hybridized with a *Klf4* cDNA probe.

(D) Normal karyotype of iPS-TTFgfp4-2 clone.

(E) Morphology of ES cells and iPS cells cultured without feeder cells. One thousand cells were cultured on gelatin-coated six-well plates for 5 days, with or without LIF. Scale bars = 200 μm.

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backcrossed to the C57/BL6 strain for at least five generations. These mice were used for primary mouse embryonic fibroblast (MEF) and tail-tip fibroblast (TTF) preparations. To generate *Fbx15*$^{\beta geo/\beta geo}$ mice with constitutive expression of GFP, an *Fbx15*$^{\beta geo/\beta geo}$ mouse (C57/BL6-129 background) was mated with an ICR mouse with the *GFP* transgene driven by the constitutive CAG promoter (Niwa et al., 1991). The resulting *Fbx15*$^{\beta geo/+,GFP/+}$ mice were intercrossed to generate *Fbx15*$^{\beta geo/\beta geo,GFP/GFP}$ mice. Nude mice (BALB/Jcl-nu) were purchased from CLEA.

Cell Culture

RF8 ES cells and iPS cells were maintained on feeder layers of mitomycin C-treated STO cells as previously described (Meiner et al., 1996). As a source of leukemia inhibitory factor (LIF), we used conditioned medium (1:10,000 dilution) from Plat-E cell cultures that had been transduced with a LIF-encoding vector. ES and iPS cells were passaged every 3 days. Plat-E packaging cells (Morita et al., 2000), which were also used to produce retroviruses, were maintained in DMEM containing 10% FBS, 50 units/50 μg/ml penicillin/streptomycin, 1 μg/ml puromycin (Sigma), and 100 μg/ml of blasticidin S (Funakoshi).

For MEF isolation, uteri isolated from 13.5-day-pregnant mice were washed with phosphate-buffered saline (PBS). The head and visceral tissues were removed from isolated embryos. The remaining bodies were washed in fresh PBS, minced using a pair of scissors, transferred into a 0.1 mM trypsin/1 mM EDTA solution (3 ml per embryo), and incubated at 37°C for 20 min. After incubation, an additional 3 ml per embryo of 0.1 mM trypsin/1 mM EDTA solution was added, and the mixture was incubated at 37°C for 20 min. After trypsinization, an equal amount of medium (6 ml per embryo DMEM containing 10% FBS) was added and pipetted up and down a few times to help with tissue dissociation. After incubation of the tissue/medium mixture for 5 min at room temperature, the supernatant was transferred into a new tube. Cells were collected by centrifugation (200 × g for 5 min at 4°C) and resuspended in fresh medium. 1 × 10^6 cells (passage 1) were cultured on 100 mm dishes at 37°C with 5% CO_2. In this study, we used MEFs within three passages to avoid replicative senescence.

To establish TTFs, the tails from adult mice were peeled, minced into 1 cm pieces, placed on culture dishes, and incubated in MF-start medium (Toyobo) for 5 days. Cells that migrated out of the graft pieces were transferred to new plates (passage 2) and maintained in DMEM containing 10% FBS. We used TTFs at passage 3 for iPS cell induction.

Retroviral Infection

The day before transduction, Plat-E cells (Morita et al., 2000) were seeded at 8 × 10^6 cells per 100 mm dish. On the next day, pMXs-based retroviral vectors were introduced into Plat-E cells using Fugene 6 transfection reagent (Roche) according to the manufacturer's recommendations. Twenty-seven microliters of Fugene 6 transfection reagent was diluted in 300 μl DMEM and incubated for 5 min at room temperature. Nine micrograms of plasmid DNA was added to the mixture, which was incubated for another 15 min at room temperature. After incubation, the DNA/Fugene 6 mixture was added drop by drop onto Plat-E cells. Cells were then incubated overnight at 37°C with 5% CO_2.

Twenty-four hours after transduction, the medium was replaced. MEFs or TTFs were seeded at 8 × 10^5 cells per 100 mm dish on mitomycin C-treated STO feeders. After 24 hr, virus-containing supernatants derived from these Plat-E cultures were filtered through a 0.45 μm cellulose acetate filter (Schleicher & Schuell) and supplemented with 4 μg/ml polybrene (Nacalai Tesque). Target cells were incubated in the virus/polybrene-containing supernatants for 4 hr to overnight. After infection, the cells were replated in 10 ml fresh medium. Three days after infection, we added G418 at a final concentration of 0.3 mg/ml. Clones were selected for 2 to 3 weeks.

Plasmid Construction

To generate pMXs-gw, we introduced a Gateway cassette rfA (Invitrogen) into the EcoRI/XhoI site of the pMXs plasmid. Primers used are listed in Table S9. Mutations in β-*catenin*, *c-myc*, and *Stat3* were introduced by PCR-based site-directed mutagenesis. For forced expression, we amplified the coding regions of candidate genes by RT-PCR, cloned these sequences into pDONR201 or pENTR-D-TOPO (Invitrogen), and recombined the resulting plasmids with pMXs-gw by LR reaction (Invitrogen).

Teratoma Formation and Histological Analysis

ES cells or iPS cells were suspended at 1 × 10^7 cells/ml in DMEM containing 10% FBS. Nude mice were anesthetized with diethyl ether. We injected 100 μl of the cell suspension (1 × 10^6 cells) subcutaneously into the dorsal flank. Four weeks after the injection, tumors were surgically dissected from the mice. Samples were weighed, fixed in PBS containing 4% formaldehyde, and embedded in paraffin. Sections were stained with hematoxylin and eosin.

Bisulfite Genomic Sequencing

Bisulfite treatment was performed using the CpGenome modification kit (Chemicon) according to the manufacturer's recommendations. PCR primers are listed in Table S9. Amplified products were cloned into pCR2.1-TOPO (Invitrogen). Ten randomly selected clones were sequenced with the M13 forward and M13 reverse primers for each gene.

Determination of Karyotypes and SSLP by PCR

Karyotypes were determined with quinacrine-Hoechst staining at the International Council for Laboratory Animal Science (ICLAS) Monitoring Center (Japan). We obtained PCR primer sequences for SSLP from the Mouse Genome Informatics website (The Jackson Laboratory, http://www.informatics.jax.org). Allele sizes were approximated on the basis of the known allele sizes in various inbred strains.

Western Blot Analyses

Western blot was performed as previously described (Takahashi et al., 2003). The primary antibodies used were anti-Oct3/4 monoclonal antibody (C-10, Santa Cruz), anti-Sox2 antiserum (Maruyama et al., 2005), anti-Klf4 polyclonal antibody (H-180, Santa Cruz), anti-c-Myc polyclonal antibody (A-14, Santa Cruz), anti-Nanog antiserum (Mitsui et al., 2003), anti-E-Ras antiserum (Takahashi et al., 2003), anti-p53 polyclonal antibody (FL-393, Santa Cruz), and anti-β-actin monoclonal antibody (A5441, Sigma).

RT-PCR for Marker Genes

We performed reverse transcription reactions using ReverTra Ace -α- (Toyobo) and the oligo dT_{20} primer. PCR was done with ExTaq (Takara). Real-time PCR was performed with Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) according to manufacturer's instructions. Signals were detected with an ABI7300 Real-Time PCR System (Applied Biosystems). Primer sequences are listed in Table S9.

DNA Microarray

Total RNA from ES cells, iPS cells, or MEFs were labeled with Cy3. Samples were hybridized to a Mouse Oligo Microarray (G4121B, Agilent) according to the manufacturer's protocol. Arrays were scanned with a G2565BA Microarray Scanner System (Agilent). Data were analyzed using GeneSpring GX software (Agilent).

In Vitro Differentiation of iPS Cells

Cells were harvested by trypsinization and transferred to bacterial culture dishes in the ES medium without G418 or LIF. After 3 days, aggregated cells were plated onto gelatin-coated tissue culture dishes and incubated for another 3 days. The cells were stained with anti-α-smooth muscle actin monoclonal antibody (N1584, Dako), anti-α-fetoprotein polyclonal antibody (N1501, Dako) or anti-βIII tubulin monoclonal antibody (CBL412, Abcam) along with 4′-6-diamidino-2-phenylindole (Sigma). Total RNA derived from plated embryoid bodies on day 6 was used for RT-PCR analysis.

Cell

Chromatin Immunoprecipitation Assay

We performed chromatin immunoprecipitation (ChIP) as previously described (Maruyama et al., 2005). Antibodies used in this experiment were anti-dimethyl K9 H3 rabbit polyclonal antibody (ab7312-100, Abcam) and anti-acetyl H3 rabbit polyclonal antibody (06-599, Upstate). PCR primers are listed in Table S9.

Statistical Analyses

Data are shown as averages and standard deviations. We used Student's t test for protein-level analyses and one-factor ANOVA with Scheffe's post hoc test for ChIP analyses. All statistical analyses were done with Excel 2003 (Microsoft) with the Statcel2 add-on (OMS).

Supplemental Data

Supplemental Data include 12 figures and 9 tables and can be found with this article online at http://www.cell.com/cgi/content/full/126/4/663/DC1/.

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Accession Numbers

Microarray data are available in GEO (Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/projects/geo/index.cgi) with the accession number GSE5259.

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