Stem Cell Biology and Regenerative Medicine

Donald G. Phinney
Editor

Adult Stem Cells

Biology and Methods of Analysis

Stem Cell Biology and Regenerative Medicine

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Adult Stem Cells

Biology and Methods of Analysis

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Preface

Recent advances in stem cell research include the discovery of stem/progenitor cell populations resident in many adult tissues and organs. However, efforts to validate the stem cell-like nature of these populations have been hampered by the fact that many archetypal assays established to study hematopoietic stem cells, such as competitive repopulation and serial transplantation assays, are not applicable beyond the hematopoietic system. This has mandated the development of surrogate assays to discriminate stem from progenitor and somatic cells. These include quantifying the replicative life span of cells cultured in vitro, their capacity to grow at clonal density, exclude specific DNA binding dyes, express telomerase and/or other pluripotency-related genes, and undergo multilineage differentiation. However, because the mechanisms regulating cell proliferation, life span, self-renewal, and senescence are inextricably intertwined, distinguishing between stem, progenitor, and somatic cells based on their in vitro characteristics is often more difficult than anticipated. Therefore, the goal of this volume is to provide a current overview of adult stem cell biology, describe and critically evaluate methods used to assess stem cell self-renewal, and distinguish this process from other aspects of cell survival, such as regulation of life span, senescence, and immortalization at a molecular level. By providing a comprehensive and critical overview of the biology and methods used to characterize adult stem cells, the volume will serve as a valuable resource for both established scientists and those just entering the field of stem cell biology.

Jupiter, FL　　　　　　　　　　　　　　　　　　　　　　Donald G. Phinney

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Part I
Basic Biology of Adult Stem Cells

Chapter 1
Molecular Mechanisms Regulating Adult Stem Cell Self-Renewal

David M. Panchision

Abstract Stem cells have defining properties that are suited for tissue homeostasis and repair, but a central issue is how these cells generate mature cell types without exhausting their capacity for self-renewal. This chapter focuses on the central nervous system (CNS) to exemplify the regulation of adult stem cell self-renewal in the service of organ function. Topics will include conventional and novel methods used to distinguish stem cells from other proliferating cell types and the pitfalls in attempting to precisely characterize stem cell function. These methods reveal that stem cells interpret multiple signals to control the balance of self-renewal and quiescence. Among the emerging themes is that stem cell self-renewal is dynamically regulated throughout life, is integrally connected with lineage specification, and reflects a conservation of many intrinsic and extrinsic mechanisms from fetal development.

Keywords Neural stem cell • Intermediate progenitor cell/Transit amplifying cell • Neuroblast • Self-renewal • Multipotency • Clonal analysis • Cell cycle • Mitogen • Morphogen • Symmetric division • Asymmetric division • Lateral inhibition • Selection • Instruction • Neurogenesis

Abbreviations

AraC	Cytosine arabinoside
BMP	Bone morphogenetic proteins
BrdU	5-Bromo-2-deoxyuridine
EGF	Epidermal growth factor
FGF2	Fibroblast growth factor 2
Fox	Forkhead winged helix family proteins

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GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
IPC	Intermediate progenitor cell
NB	Neuroblast
NSC	Neural stem cell
SVZ	Subventricular zone
SGZ	Subgranular zone
Shh	Sonic hedgehog
Wnt	Wingless/Int homolog proteins

1.1 Tissue Homeostasis and the Stem Cell Concept

Cell turnover is a major feature of tissues throughout the life of organisms; replenishment of lost cells is critical for the proper function of organs. While the concept of a primitive replenishing cell has existed for more than a century, experimental evidence came from studies done in the wake of the atomic bombings of Hiroshima and Nagasaki, where many survivors of the bombing eventually succumbed to radiation sickness. Subsequent experiments in the mid-1950s showed that radiation damage to the immune systems of mice and humans was reversed by grafting bone marrow from a donor (Little and Storb 2002). In the early 1960s, studies by James Till, Ernest McCulloch and colleagues (McCulloch and Till 2005) led to the positive identification of the reconstituting cell type.

Termed a stem cell, it was defined by the capability to make copies of itself (called “self-renewal”) throughout life, to generate multiple daughter cell types (“multipotency”), and to regenerate the organ system in which it resides (“reconstitution”). These three characteristics have become benchmarks in distinguishing stem cells from other types of proliferating cells. In subsequent decades, other organs were found to maintain their own specific stem cells (Ohlstein et al. 2004). Importantly, the reconstitution capacity of stem cells is fully testable only when the organism can survive the complete loss of an organ (e.g., the immune system). For most organs, partial reconstitution of ablated regions is used as a surrogate assay.

Adult tissue stem cells are called somatic stem cells to distinguish them from the germline stem cells of the gonads that generate haploid egg and sperm, the totipotent stem cells of the early morula stage embryo, and the pluripotent “embryonic” stem cells of the pregastrulation blastocyst (Surani et al. 2008). While stem cells have defining properties that are suited for tissue homeostasis and repair, a central issue is how these cells generate mature cell types without exhausting their capacity for self-renewal. In this chapter, we will focus on the central nervous system (CNS) to exemplify the regulation of adult stem cell self-renewal in the service of organ function. Topics will include conventional and novel methods used to distinguish stem cells from other proliferating cell types and the pitfalls in attempting to precisely characterize stem cell function. These methods reveal that stem cells interpret multiple signals to control the balance of self-renewal and quiescence.

Among the emerging themes is that stem cell self-renewal is dynamically regulated throughout life, is integrally connected with lineage specification, and reflects a conservation of many intrinsic and extrinsic mechanisms from fetal development.

1.2 Methods for Analysis of Stem Cells and Derivatives

The combination of in vitro and in vivo analysis of stem cells and their derivatives is indispensable for studying the mechanisms controlling cell fate in the nervous system (Panchision and McKay 2002). In vitro analysis of isolated cells has the advantage of easier manipulation, higher throughput, and more detailed biochemical and molecular analysis. The drawback is that removing cells from their endogenous environment may artifactually alter their properties, calling into question the relevance of results to the normal function of the organ (Anderson 2001). In vivo analysis minimizes these potential artifacts but is more labor-/resource-intensive and, though methods are improving, generally not as amenable to detailed molecular analysis such as for intracellular signaling. Despite the potential for artifacts, in vitro culture remains in widespread use particularly for studying human cell biology and disease, targeting cell dysfunction in tumors (Singh et al. 2004), or for therapeutically replacing cells that are lost due to degeneration (Lindvall et al. 2004).

1.2.1 Clonal Analysis

Stem cell properties are most rigorously measured by clonal analysis, a family of methods by which the mitotic descendents of a single cell (i.e., a clone) are traced over time. The techniques date back to at least the 1920s and are fundamental to studying lineage relationships between cells during development (see Chap. 6). Two requirements are a *lineage marker*, which is an autonomous distinguishing feature that is propagated to all descendents of the original marked cell, and *limiting dilution*, where initially marked cells are sufficiently isolated from each other to ensure that all progeny are clonally related and there is no mixing of colonies. In vitro, this can be done by growing cells in culture at limiting dilution; for neural stem cells, self-renewal is measured by the ability to form neurosphere or monolayer clones in serial passage, while multipotency is operationally defined as the ability to generate neurons, astrocytes, and oligodendrocytes (see Chap. 7). Cells that can form clones initially but not after serial passage are not operationally considered stem cells, even though they may be multipotent.

In vivo, clonal analysis can be performed by generating chimeras of grafted cells that utilize observable differences between donor and host cells (e.g., distinctive nuclei of quail cells visualized in chick host), or by generating mosaics using dyes or genetic markers introduced by low titer infection. Additionally, genetic labeling methods such as mosaic analysis with a repressible cell marker (MARCM) are used

to randomly lineage-mark small numbers of cells in invertebrates, while the mosaic analysis with double markers (MADM) method is used to allow simultaneous lineage and gene knockout analysis in mouse (Luo 2007). Another potential lineage analysis tool is the Brainbow technique, whereby multiple fluorescently labeled proteins are expressed in a random and combinatorial pattern under a neural cell-type specific promoter (Livet et al. 2007). In contrast to clonal analysis, in vitro analysis of proliferating cells in high density mass culture can provide insight on the properties of enriched precursor cell populations, but does not allow stem cells to be definitively distinguished from other proliferating cells.

1.2.2 Prospective Isolation

Cell-type markers can be used for prospective isolation of subclasses of CNS and neural crest cells (Morrison et al. 1999; Mujtaba et al. 1999; Rietze et al. 2001), whereby the selectable property of the cell closely predicts cell function. The physical sorting of cells typically occurs by flow cytometry or magnetic separation using antibodies against cell surface antigens. Reporters of gene expression can also be used, such as green fluorescent protein (GFP) driven by a cell-type specific gene promoter. Many useful CNS cell surface antigens were originally identified in hematopoietic cells based on cluster of differentiation (CD) experiments (Robin et al. 2003). The markers CD133 (prominin), CD15 (3-fucosyl-*N*-acetyl-lactosamine, Lewis X, SSEA-1), and CD24 (heat-stable antigen) have all been used to characterize and select different subsets of CNS cells. For example, cells from both the human and rodent adult periventricular region expressing high levels of CD133 ($CD133^{+/hi}$), with low or no CD24 ($CD24^{-/lo}$), have the highest frequency of initiating neurospheres (Coskun et al. 2008; Uchida et al. 2000). The polysialylated form of NCAM (PSA-NCAM) has been used to identify neuronal-restricted precursors (Carpenter et al. 2001; Weickert et al. 2000), while A2B5 has been used to identify bipotent glial-restricted precursors (Roy et al. 1999; Windrem et al. 2002). In addition to antigen/antibody-based sorting, other cell properties such as transmembrane transporter activity can be used (see Chap. 8). The combination of cell sorting with clonal analysis is a rapid and powerful method for distinguishing stem cells from other cell types.

1.2.3 Additional Techniques

A wide variety of techniques can be coupled with clonal lineage tracing to characterize the functional properties of stem and progenitor cells. Immunocytochemistry is commonly used to measure markers indicative of particular cell types. Antibodies to the cell surface antigens described above can be used histochemically, provided signal differences are strong enough to detect by light microscopy. Among the most commonly used cell type markers is the intermediate filament Nestin, which is

expressed in neural precursor cells but not their postmitotic progeny. However, Nestin does not distinguish CNS stem cells from more committed progenitors and it is also expressed in precursors from non-CNS tissues, so caution must be used when studying samples that may contain non-CNS cell types. The transcription factor Sox2 and the orphan nuclear receptor tailless (Tlx) are strongly implicated in maintaining adult NSCs and have a restricted, but not necessarily exclusive, expression to NSCs. Other intermediate filaments such as glial fibrillary acidic protein (GFAP) and the middle molecular weight subunit of neurofilament (NF160) are used to identify astrocytes and neurons, respectively. In the adult brain, however, NSCs share many markers with astrocytes, including the astrocyte-specific glutamate transporter (GLAST), brain lipid binding protein (BLBP) and, in some species, GFAP. This exemplifies how no single marker can unambiguously identify any cell type; multiple markers must be used simultaneously to distinguish cell identities (Kim et al. 2003; Kriegstein and Alvarez-Buylla 2009; Schwartz et al. 2008). There are also related but more dynamic methods that have so far been used primarily to analyze fetal development, including time lapse imaging of dissociated cells in culture and multiphoton confocal microscopic imaging using fluorescent reporters in slices; these allow real-time analysis of cytokinesis, migration and other cell behaviors linked to self-renewal and fate choice (Haydar 2005; Ravin et al. 2008; Sun et al. 2005).

Conditional and inducible gene targeting is increasingly used to study the function of specific genes in stem cells in a spatially and temporally controlled manner. There are numerous such genetic methods; one example uses cell-type-specific promoters driving Cre-Lox mediated recombination that mutates a target gene to an altered or nonfunctional variant. Additional temporal control can be achieved by adding an inducible regulatory domain (e.g., estrogen receptor) to Cre so that recombinase activity is regulated by addition or subtraction of an exogenous factor (e.g., tamoxifen) (Gama Sosa et al. 2010). Targeted ablation of rapidly proliferating cells allows determination of the functional requirement for these cells compared with slowly dividing or nondividing cells. Pharmacological ablation can be achieved using antimitotic drugs like cytosine arabinoside (c); pharmaco-genetic ablation can be achieved by expressing the "suicide gene" thymidine kinase (TK) under cell-specific promoters; treatment with ganciclovir then yields toxic metabolites that kill actively proliferating cells (Singer et al. 2009). This is often done in conjunction with labeling of newly synthesized DNA in dividing cells using 5-bromo-2-deoxyuridine (BrdU) treatment. Thus, BrdU labeling can identify recently divided precursors as well as "newly born" neurons.

1.3 Determining Location, Origin, and Function of Adult Stem Cells: The Brain as an Example

The discovery of neural stem cells (NSCs) and neurogenesis in the adult brain is one of the more remarkable paradigm shifts in biology. Since the initial histological characterization of the brain in the 1800s, it was long thought that the complex

architecture of the adult brain was incompatible with cellular regeneration and that no new nerve cells were born after development was completed. This dogma was slowly overturned in the latter half of the twentieth century with the finding that new neurons were continuously generated in discrete brain locations (Chojnacki et al. 2009; Colucci-D'Amato et al. 2006). This discovery raised three important questions: Where are stem cells located in the adult brain? How do they arise? What function do they serve?

1.3.1 Location and Identity

While studies have indicated proliferative cells in many regions of the adult CNS, the best-established locations of adult neurogenesis are: (1) the periventricular zone adjacent to the striatum and (2) the dentate gyrus of the hippocampal formation (Fig. 1.1). Neuronal progenitors are generated from the forebrain periventricular area and migrate, via the rostral migratory stream (RMS), to the olfactory bulb, where they mature into neurons. In the hippocampus, however, neuronal progenitors do not migrate extensively and integrate into the granule neuron layer of the dentate gyrus (Zhao et al. 2008). The late-developing cerebellum continues neurogenesis until after birth; while in vitro clonal analysis has identified stem cells from early postnatal cerebellum (Lee et al. 2005), it is not clear that cerebellar neurogenesis persists into adulthood. BrdU incorporation studies have identified proliferating cells in other regions of the adult brain such as the cortex, but the question of whether these are stem cells or even neurogenic is in dispute (Breunig et al. 2007a; Gould 2007). Nonetheless, the continual production of new neurons in the adult brain led to efforts to identify whether a self-renewing, multipotent cell type was the source of these new neurons.

There has been detailed anatomical mapping of the ventricular lining of the postnatal mammalian brain (Alvarez-Buylla et al. 2008). Ciliated ependymal cells line the ventricle, while the subventricular zone (SVZ) is populated by heterogeneous cell types. Extensive in vitro and in vivo clonal analysis has identified a lineage progression, whereby type B cells (multipotent NSCs) give rise to type C cells (intermediate progenitor cells, IPCs), which give rise to type A cells (neuronal progenitors), which then migrate tangentially and differentiate into neurons in the olfactory bulb. Type B cells can generate both neurons and oligodendrocytes in vivo; their quiescent state is phenotypically similar to astrocytes and they may directly differentiate into a postmitotic astrocyte without a committed IPC stage. The type B cells nearly always maintain contact with ependymal cells and with the basal lamina of endothelial cells, a component of the surrounding blood vasculature. Additionally, many type B cells contain a ciliated process that maintains contact with the cerebrospinal fluid (CSF) of the ventricle, a potential source of many secreted factors. While the anatomy is best characterized in rodents, a similar organization of progenitor cells and surrounding architecture is found in the postnatal human brain (Quinones-Hinojosa et al. 2006; Sanai et al. 2004).

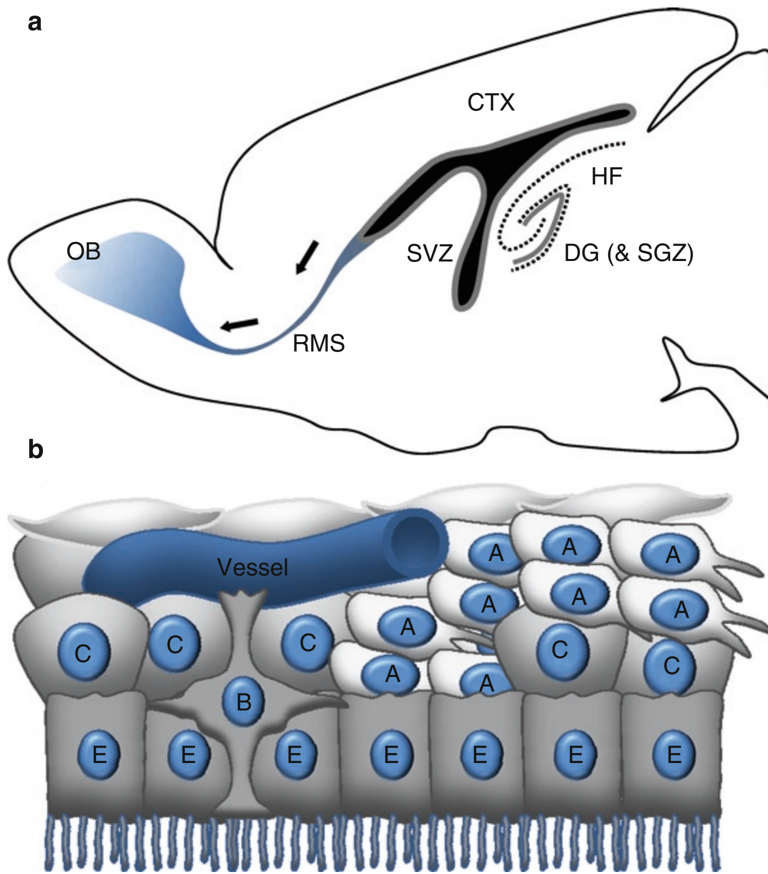

Fig. 1.1 Location of adult brain germinal zones. (**a**) Sagittal section of adult rodent brain (anterior on the *left*, dorsal on the *top*) illustrating two known sites of adult neurogenesis. The lateral ventricles of the brain (*black*) beneath the cortex (CTX) are surrounded by a wall of cells including the subventricular zone (SVZ, in *gray*). Dividing SVZ cells ultimately migrate through the rostral migratory stream (RMS, in blue) to the olfactory bulb (OB). A second region, the hippocampal formation (HF, *dotted lines*), contains a V-shaped lamina of granule neurons within the dentate gyrus (DG, *dotted line*). Enclosed in this lamina is the subgranular zone (SGZ, in *gray*), a second site of cell division. Neurons generated from this site migrate a short distance to integrate into the DG. (**b**) Magnified schematic representation of the cellular lining of the lateral ventricles constituting the SVZ niche, showing cells implicated in regulating proliferation and neurogenesis; the ventricular lumen is at bottom. The dentate gyrus SGZ niche (not shown) has some analogous features. *A* type A neuroblast cell; *B* type B neural stem cell; *C* type C intermediate progenitor (transit amplifying) cell; *E* ciliated ependymal cell

This anatomical colocalization suggests that endothelial cells, ependymal cells, and the choroid plexus (the source of CSF) are sources of signals that may regulate postnatal and adult NSCs. An ongoing and as yet unresolved debate is whether ependymal cells also are capable of multipotentiality in vivo, or whether they are strictly a terminally differentiated derivative of radial glia. Part of the controversy

may reflect differences in the periventricular subregions and phenotyping methods used to choose cells for analysis (Chojnacki et al. 2009).

In the hippocampus, neurogenesis has been characterized in the subgranular zone (SGZ) of the dentate gyrus within the hippocampal formation. Progenitor cells are identified in part by morphology and immunohistochemistry; type 1 progenitors appear as radial astrocytes and express Nestin, GFAP, and Sox2, while type 2 progenitors are smaller with shorter processes and lack GFAP expression. Based on their proliferation kinetics and unique combinatorial expression of Tbr2 and other transcription factors, type 2 progenitors are postulated to be functionally analogous to IPCs present earlier in forebrain development (Hodge et al. 2008). All SGZ progenitor cells are adjacent to the layer of granule neurons in the dentate gyrus and are also surrounded by astrocytes and oligodendrocytes, which are all potential sources of regulatory signals (Zhao et al. 2008). Based on prior evidence that Sox2 functions in pluripotent and multipotent stem cells, lineage analysis using a Sox2-GFP reporter line has shown that type 2 cells self-renew and generate both neurons and astrocytes, thus fulfilling most of the operational definition of a NSC; the type 1 cells show no evidence of division, but this may reflect slow division kinetics relative to the type 2 cells (Suh et al. 2007). As a tool for studying SGZ NSCs, this reporter line is critical since in vitro expansion of precursors from the hippocampus has been problematic until recently (Bonaguidi et al. 2008). The differentiation and maturation of neurons from SGZ NSCs is reviewed in Zhou and Gage (Zhao et al. 2008).

Adult stem cells have several unusual features that distinguish them from other proliferating cell types. They survive in mitotic cell ablation assays because they divide only infrequently, a feature that is called quiescence (Doetsch et al. 1999; Morshead et al. 1994). A corollary to this quiescence is that adult NSCs express certain markers of differentiated astrocytes such as GFAP as well as precursor markers like Nestin. In contrast, the majority of cell division occurs by the immediate daughters of stem cells, the IPCs, or transit amplifying cells (Diaz-Flores et al. 2006). But all progeny are part of a clone, meaning they are the descendants of that original stem cell. They reside in a very specialized region, called a niche, which consists of more mature cells presenting cues that control the stem cell division rate and the types of daughter cells generated (see Chap. 2). They also have mechanisms to resist external cues, including surface proteins called ABC transporters that pump out a wide variety of chemicals that would otherwise cause the stem cell to differentiate or die (see Chap. 8).

1.3.2 Spatial/Temporal Origins and Clonal Diversity

As adult stem cells are a relatively late stage in the developmental history of tissues, alternative models can be invoked to explain their origin. In principle, there could be multiple clonal pools of stem cells that are specified in distinct times and locations and which contribute to different stages of development. This type of mechanism is utilized with hematopoiesis in the developing immune system, where primitive

hematopoiesis originates with stem cells in the yolk sac, whereas subsequent definitive hematopoiesis originates with stem cells in the aorta-gonad-mesonephros region, which migrate to the liver and ultimately become adult stem cells of the bone marrow (Robin et al. 2003). An alternative mechanism could involve a single clone pool that is generated early, contributes to all stages of development, and gives rise to successive generations of stem cells and their progeny throughout the lifespan (Fig. 1.2).

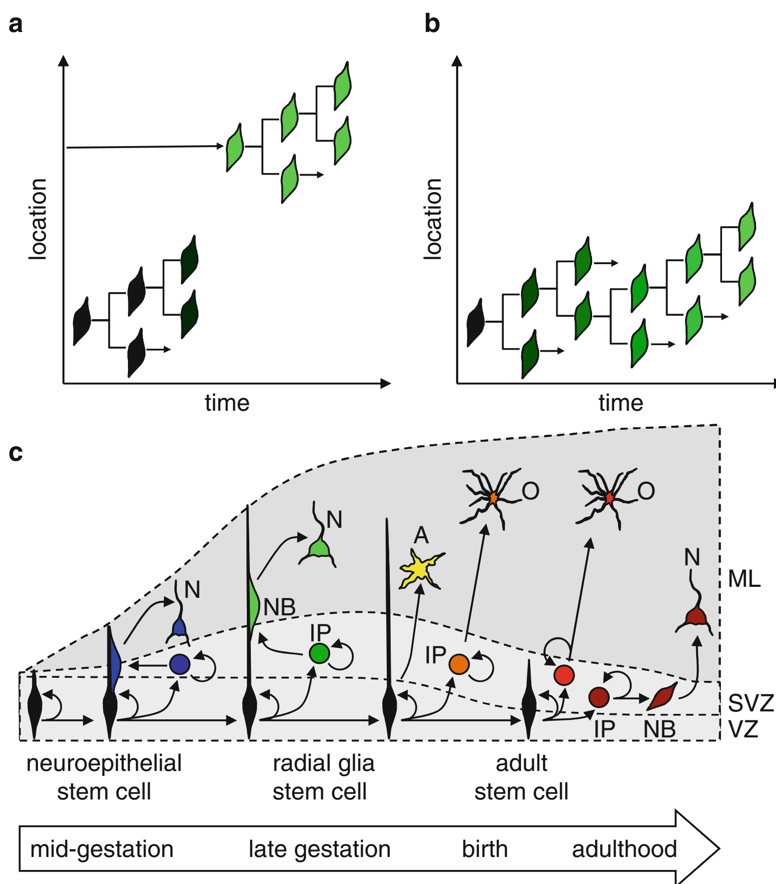

Fig. 1.2 Alternative models for the developmental origin of adult stem cells. (**a**) Partitioning of distinct stem cell pools early in development, which are preferentially selected at different times during development and into adulthood. (**b**) Stem cells are all lineally related, but the properties of each succeeding generation may change such that adult stem cell self-renewal and potency may differ from fetal stem cells. (**c**) A current working model of adult neural stem cell origins from fetal development based on lineage tracing experiments. NSCs generate IPCs and their progeny throughout development, but their properties change over time (represented by *color* changes). In the adult SVZ, NSCs are type B cells, IPCs are type C cells, NBs are type A cells. A similar origin is hypothesized for the adult dentate gyrus SGZ (not shown). *NSC* neural stem cell (in *black*); *IP* intermediate progenitor (or IPC); *NB* neuroblast; *N* neuron; *A* astrocyte; *O* oligodendrocyte; *VZ* ventricular zone; *SVZ* subventricular zone; *ML* mantle layer

This second mechanism is incorporated into the increasingly accepted working model for the CNS, whereby adult NSCs are the direct progeny of NSCs that generate the nervous system during development, while the anatomical regions in which they reside are the reduced remnants of proliferative ventricular zone (VZ) and SVZ in the embryonic neural tube. This working model comes from several lines of evidence. Lineage tracing techniques (detailed in Chap. 6) have been used to establish radial glia as multipotent NSCs in the embryonic forebrain; these same types of lineage tracing studies showed that a subset of radial glia in the ganglionic eminence give rise to stem cells in the postnatal SVZ (Merkle et al. 2004). The specification of radial glia from the earlier stage of neuroepithelial NSCs is delayed in the absence of FGF10 signaling, suggesting a regulated transition between these two cell types (Sahara and O'Leary 2009). These and other findings support a progression whereby early gestation neuroepithelial NSCs give rise to radial glia NSCs, which in turn give rise to postnatal and then adult NSCs (Kriegstein and Alvarez-Buylla 2009).

A related concept is that of *clonal diversity*, which refers to heterogeneity in the proliferative capacity and the proportions of daughter fates generated between stem cells (Fig. 1.3). These stem cells may originate at the same time and same anatomical compartment but exhibit differences in behavior. Clonal diversity is commonly found in the hematopoietic system and is measured in part as stereotypical differences in clone expansion kinetics during serial passaging (Muller-Sieburg and Sieburg 2006). Clonal diversity can hypothetically originate from a single stem cell whose daughter stem cells become progressively more diverse over time due to extrinsic or intrinsic mechanisms. Some clonal diversity may result from stochastic processes. Lineage tracing of the adult hippocampus revealed that downregulation of Sox2, a NSC promoting transcription factor, could regulate the random insertion of LINE-1 mobile transposable elements during the early stages of neuronal lineage specification, leading to a diverse clonal composition of neurons (Muotri et al. 2005). While this particular transposition occurred during early neuronal specification of NSCs, similar transposition could occur during NSC self-renewal divisions, yielding heterogeneity in proliferation kinetics and daughter fates while still meeting the operational definition for stem cells.

An alternative clonal diversity model could reflect multiple originating clones representing distinct classes of stem cells that exhibit fixed behavior over time (Viswanathan and Zandstra 2003). This latter model could be embodied by the retention in adult germinal regions of rostrocaudal and dorsoventral specification that occurred in the VZ and SVZ during brain development. Just as discrete embryonic domains of transcription factor expression control subsequent forebrain organizational diversity (O'Leary and Sahara 2008), the adult SVZ shows evidence of a retained pattern. Sp8 is a transcription factor that contributes to specifying rostral/ventral forebrain, including the septum, striatum, and olfactory system; later conditional loss of Sp8 function still leads to mis-specification of new neuronal progenitors leading to the olfactory bulb. Although the conditional deletion was in a neurogenic progenitor population and occurred during fetal development, the study also found expression of Sp8 in the postnatal SVZ and RMS, suggesting that Sp8

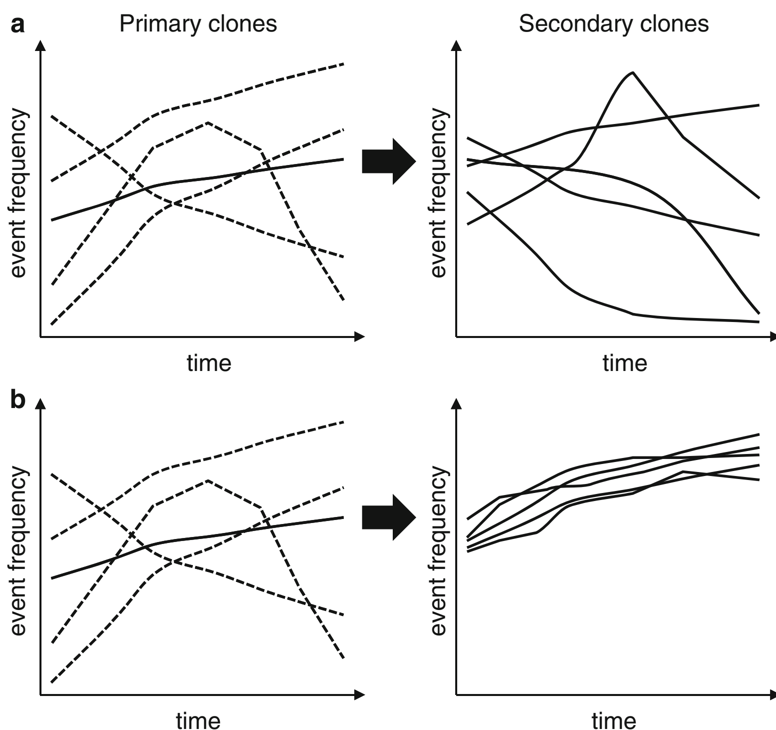

Fig. 1.3 Models explaining clonal diversity. (**a**) Heterogeneous behavior of different stem cell clones may continuously evolve over time from a single homogeneous mother stem cell pool; isolating one clone (*solid line*) out of many (*dotted lines*) yields subclones with continuing heterogeneous behavior, since the properties of the stem cells are not fixed. (**b**) Heterogeneous behavior may be the product of heterogeneous mother stem cell pools; isolating one clone yields subclones with behavior similar to the parent clone, since the properties and trajectory of that clone were fixed at an earlier time in development. Event frequency (*vertical axis*) can refer to any behavior such as cell division, cell death, or commitment to a particular lineage

functions might be required in progenitors even in the adult (Waclaw et al. 2006). This was addressed even more directly using Cre-lox fate mapping driven by promoters of transcription factors important for distinct regions of forebrain development. Young et al (2007) found that Emx1$^+$ derived adult cortex germinal zones gives rise to most calretinin expressing interneurons, while Gsh2$^+$ derived adult striatal germinal zones gives rise to all calbindin expressing interneurons. Furthermore, distinct SVZ subregions generate neurospheres with differing frequencies.

While these studies could not distinguish whether this retained transcriptional code resides in the NSCs or their committed progeny, a separate study used injected virus to target dorsal versus ventral radial glia (NSCs) of neonates or GFAP expressing cells in juveniles; analysis of the anatomically distant olfactory bulb showed regionally distinct types of labeled neuronal progeny and validated that the GFAP expressing cells were adult primary progenitors (likely NSCs). In vitro

analysis also showed that this regional distinction persisted in culture, suggesting at least some intrinsic component to clonal diversity (Merkle et al. 2007). These results are consistent with the maintenance of regional NSC identity throughout the lifespan, which is reflected in diverse NSC clone behavior. It should be noted that these different adult germinal zones are in close proximity and can easily be cross-contaminated when dissecting SVZ tissue, thereby explaining clonal heterogeneity found during clonal analysis in vitro. Thus, there are experimental examples in which clonal diversity can be generated by early developmental patterning and by later deterministic or stochastic changes. These results suggest that molecular analysis of self-renewal and fate choice that does not account for clonal heterogeneity may yield spurious results and misleading conclusions. However, this clonal diversity occurs in the context of a unified lineage mechanism whereby the earliest postgastrulation, VZ-localized neuroepithelial NSCs are the parents of all subsequent NSCs into adulthood.

1.3.3 Function

Adult NSCs are inseparably linked to the process of adult neurogenesis. The functional role of newly born neurons in the adult brain remains uncertain but is a focus of intense research. This topic cannot be covered in detail in this book and is reviewed elsewhere (Zhao et al. 2008). Briefly, neurons generated from progenitors of the forebrain periventricular integrate into the olfactory bulb; genetic ablation and behavioral studies link these newly born neurons to olfactory discrimination and memory. Similar experimental studies provide a functional association between newly born neurons of the adult dentate gyrus to pattern separation, contextual memory, and spatial memory (Clelland et al. 2009; Imayoshi et al. 2008). Neurogenesis increases in response to a wide variety of environmental stimuli, including enriched environments, learning, exercise/activity, and stressors involving the hypothalamic-pituitary-adrenal axis. The immune system and pathological influences such as seizures and radiation also regulate neurogenesis. Antidepressants are known to increase neurogenesis, suggesting an association, as yet unproven, between aberrant control of neurogenesis and the etiology of depression. Despite progress on the functional properties of newly born adult neurons, it is still unclear why certain functions of the brain might require highly localized but continuous neurogenesis.

1.4 Cellular Properties and Determinants of Self-Renewal and Potency

One of the defining characteristics of stem cells is the capacity to self-renew. This is a very specific type of cell division, which must be operationally distinguished from other forms of proliferation by the capacity of the daughter cell to retain the

same generative potency of the parent cell. Thus, markers of proliferation (e.g., BrdU, cell cycle antigens like Ki67) are useful but not sufficient for validating self-renewal. Clonal analysis must be used to determine the number and fates of progeny, which retrospectively identifies the degree of potency of the starting cell. Totipotency is the ability to generate all cell types (embryo plus extraembryonic tissue), pluripotency is the ability to generate all cell types of the embryo proper, multipotency is the ability to generate many cell types (generally organ-specific), and bipotency is the ability to generate two cell types. In some organs, the ability to self-renew plus generate one other cell type has been sufficient qualification for a stem cell.

Cell division yields two daughter cells, either of which may retain stem cell properties. *Symmetric division* is the mode by which both or neither daughter cell retains the stem cell properties of the parent cell. These types of divisions are thus either self-renewing or differentiative. *Asymmetric division* is the mode by which one daughter cell retains stem cell properties and the other does not (Fig. 1.4). The daughter cell that does not retain stem cell properties is fated to differentiate or die. Some investigators measure numbers of differentiated cell types as a percentage of total clone size to estimate whether division is symmetric or asymmetric. However, this is subject to error depending on how many divisions occur between the initiation of the experiment and analysis; the mode of division may intrinsically change with each division, external instructive or selective cues can change during the experiment, or cell death may skew the results if the dead cells cannot be counted. Time lapse imaging has addressed many of these issues, since division, death, and morphological differentiation can be followed in real time (Haydar 2005; Ravin et al. 2008; Sun et al. 2005). Fluorescent reporters can also be incorporated to follow fate changes as they happen. Orientation of division (e.g., plane of cleavage relative the ventricular surface) can be correlated with mode of division. This has been used for studying the developing brain but has yet to be applied to the study of adult NSCs, possibly because of their slow kinetics of proliferation.

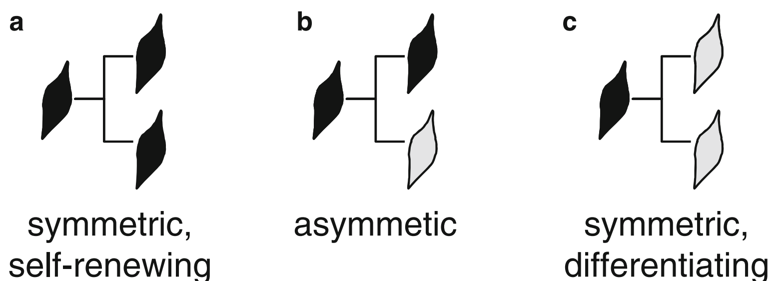

Fig. 1.4 Symmetric and asymmetric division. (**a**) Stem cells (*black*) may numerically expand by undergoing symmetric self-renewing divisions. (**b**) Stem cells may maintain numbers by undergoing asymmetric divisions to yield one committed daughter (*gray*). (**c**) Stem cells may decrease numbers by undergoing symmetric differentiating divisions. This distinction is based on tracking the fate of the daughters and does not presume any molecular mechanism

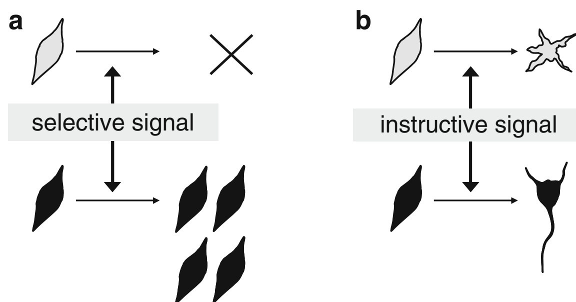

Fig. 1.5 Selective versus instructive actions on stem cells. (**a**) Selection acts to differentially affect numbers of a particular cell type, such that the same signal may cause one cell to die (*gray*) and another to numerically expand (*black*). (**b**) Instruction acts on a cell to assume a particular property or fate, such that the same signal can cause different cells to assume distinct fates

The process by which fate decisions are made is called determination or specification. At its earliest stages, it is integrally connected to stem cell self-renewal and potency. Determinants of cell fate can be either *extrinsic* to the cell (e.g., secreted mitogens, morphogens, cell adhesion molecules or diffusible gases) or *intrinsic* to the cell (e.g., receptors, second messengers, transcription factors). By definition, extrinsic determinants come from outside the cell and act upon stem cells or their derivatives to regulate cell cycle and fate decisions; thus, they are closely tied to the cell types and architecture of the stem cell niche (see Chap. 2).

The effects of these cues on cells can be either *selective* or *instructive* (Fig. 1.5). Selection occurs when external cues differentially act upon a diverse population of cells, resulting in the favored survival or proliferative expansion of one cell type over another. Instruction occurs when external cues direct cells to assume a fate or carry out an action. In vitro and in vivo gain/loss-of-function studies have identified multiple factors that regulate stem cell self-renewal and fate.

1.4.1 Mitogens and Morphogens

Mitogens are classically thought of as selective factors that promote the proliferative expansion of particular precursor cell types, but they often have fate-directing activities as well. Basic fibroblast growth factor (bFGF, FGF2) and epidermal growth factor/transforming growth factor alpha (EGF/TGFα) are central to the study of NSCs, in part because historically they are the only known ligands that alone or in combination can indefinitely drive neural precursor proliferation in vitro. FGF2 promotes in vitro expansion of neural precursors from all ages of development, whereas EGF-responsiveness does not emerge until midgestation; some evidence suggests that the mitogens selectively expand different populations of cells during embryogenesis (Panchision and McKay 2002). It is possible that the

factors may act similarly on NSC self-renewal but differently on the asymmetric production of neurogenic versus gliogenic progenitors.

In the adult, both FGF and EGF promote SVZ proliferation, but while FGF2 promotes neurogenesis in the olfactory bulb, EGF does not (Kuhn et al. 1997). Consistent with this, loss of the FGF receptor *fgfr1* decreases SGZ neurogenesis (Jin et al. 2003; Zhao et al. 2007). In contrast, EGF exposure in vitro can convert type C IPCs into type B NSCs and inhibit their differentiation into neuroblasts (Doetsch et al. 2002). Overexpression of the EGF receptor ErbB2 promotes a radial glia phenotype in astrocytes (Ghashghaei et al. 2007), suggesting that EGF signaling drives cells into an NSC-like state. Furthermore, infusion of EGF into the adult brain promotes proliferation of type B NSCs that yield predominantly oligodendrocytes, which are normally generated in far fewer numbers than neuroblasts (Aguirre et al. 2007; Gonzalez-Perez et al. 2009). This stem cell and glial-promoting, anti-neurogenic effect of EGF may be explained by a dose effect whereby increasing levels of EGF receptor activation bias toward a gliogenic response (Burrows et al. 1997; Viti et al. 2003). Indeed, such a mechanism might help explain the progressively greater glia-like properties of NSCs during development and the switch from predominantly neurogenesis to gliogenesis during development (Kriegstein and Alvarez-Buylla 2009).

Morphogens are extrinsic factors that act from a distance to instruct cell identity and tissue shape in a dose-dependent manner during development. In addition to instructive (fate-directing) actions, they also can exert selective (proliferative or survival) control, so it is often difficult to distinguish factors that are strictly mitogenic or antimitogenic from those that have morphogenic actions. Numerous morphogens involved in early neural patterning are also involved in controlling self-renewal of adult stem cells and proliferation of their progeny. Among these are sonic hedgehog (Shh), Wingless/Int proteins such as Wnt1, 3, 3a, and 7 and Bone Morphogenetic Proteins such as BMP2, 4, and 7, whose actions during brain development are reviewed in (Panchision and McKay 2002).

Shh is a morphogen that is secreted from ventral signaling centers to specify ventral identity in the developing neural tube; resulting cell types include motor neurons of the spinal cord, inhibitory neurons of the basal ganglia and oligodendrocytes at many locations along the rostrocaudal axis. Shh also has mitogenic actions on neural precursor cells which continue into adulthood. In studies of the postnatal cerebellum, Lee et al. (2005) prospectively isolated cells based on their combinatorial expression of CD133 and a GFP-expressing reporter for Asc1 (Mash1), a basic-helix-loop-helix (bHLH) transcription factor that promotes neurogenic differentiation. They found that $CD133^{+}/Asc1^{-}$ NSCs generate neurospheres only in response to FGF2, while the more mature neurogenic $Asc1^{+}$ cells proliferated in response to Shh but did not have the long-term neurosphere expansion capacity as the bFGF-responding cells. This suggests that Shh is a mitogen not for NSCs but for progenitors with neuronal specification (analogous to either IPCs or neuroblasts). In the adult, in vivo administration of the Shh inhibitor cyclopamine reduces proliferation in the SVZ; while Shh alone cannot promote neurosphere formation in vitro, it synergizes with low doses of EGF to promote neurosphere

generation (Palma et al. 2005); this would be consistent with an action on either NSCs or IPCs. Ahn and Joyner (2005) addressed this using conditional, inducible lineage marking of Shh-responding cells. They showed that, in the adult SVZ, Shh-responding cells coexpress GFAP (marking both astrocytes and adult NSCs) that proliferate in vivo. Many of these cells were relatively quiescent, since ablation of rapidly proliferating cells still left small numbers of lineage-marked cells that then expanded over time and generated cells expressing markers of neurons, astrocytes and oligodendrocytes, thus supporting their identity as stem cells. In a separate study, administration of a Shh agonist could partially rescue the loss of hippocampal NSCs and neurogenesis in Sox2 null mutant mice, suggesting that Shh acts downstream of this NSC-regulating transcription factor (Favaro et al. 2009). While this does not prove that Shh is required for NSC self-renewal, it strongly suggests that Shh can be a cofactor in NSC self-renewal and has a more specific role in the expansion of transit amplifying IPCs and neuroblasts.

Wnt proteins can have morphogenic, proliferative, or antiproliferative actions depending on the context. In the canonical pathway of Wnt signaling, Wnt ligand binding to the frizzled family of receptors causes a stabilization of cytoplasmic β-catenin, which accumulates and translocates to the nucleus; β-catenin then interacts with TCF/LEF family transcription factors to promote gene expression. In the adult hippocampus, Wnt3 is expressed strongly in astrocytes while Wnt receptors and intracellular signaling components are expressed in progenitors; coculture of astrocytes increased β-catenin activity. Overexpression of Wnt3 in vitro or in vivo promoted the generation of new neurons, while blocking Wnt signaling in vitro or in vivo prevented the generation of new neurons, as measured by cells coexpressing BrdU and neuron markers (Lie et al. 2005). Since total proliferation was not significantly changed and oligodendrocyte numbers were unchanged, it is possible that the proliferative effect was specific for neuroblasts. This was supported by findings that Wnt signaling activates the bHLH transcription factor NeuroD1, which is required for the differentiation of neuroblasts to neurons. Both the NeuroD1 promoter and LINE-1 mobile transposable elements involved in neuron differentiation have dual overlapping binding sites for potential activation by Wnt or repression by Sox2, a positive regulator of stem cell multipotency and pluripotency. Critically, using a Sox2-Cre-GFP construct to mark the NSC lineage, it was found that the loss of β-catenin impaired the lineage specification and survival of neuronal progenitors from NSCs, while Sox2^{+} NSCs cells remained constant; furthermore, the proportion of Sox2-GFP^{+} cells expressing GFAP increased, suggesting that the loss of Wnt signaling shifted NSCs to quiescence (Kuwabara et al. 2009). However, studies of mice lacking the orphan nuclear receptor Tlx, which is required for stem cell self-renewal, uncovered a role for Wnt7/β-catenin signaling downstream of Tlx in promoting serial clone expansion in bFGF/EGF-containing cultures (Qu et al. 2010). One interpretation of these combined studies is that Wnt signaling may activate quiescent NSCs to self-renew, but specifically for asymmetric divisions in the service of neurogenesis rather than symmetric divisions for NSC expansion. Like Shh, Wnt actions may be modified by other signals driving either mitogenesis (e.g., bFGF, EGF) or differentiation.

BMPs are pleiotropic morphogens that are secreted by dorsal signaling centers to specify dorsal identities in the developing neural tube; additionally they may indirectly promote proliferation via Wnt signaling or differentiation through direct block of the cell cycle. The most commonly studied ligands in the brain are BMP2, 4, and 7; they bind a receptor complex that activates a canonical heterohexameric transduction complex, small/mothers against decapentaplegic (Smad) homologs 1/5/8 plus the obligate partner Smad4, which then translocates to the nucleus to regulate gene transcription. In the adult, BMPs exert uniformly antiproliferative and gliogenic actions (reviewed in Chen and Panchision 2007). BMPs are expressed at high levels in the choroid plexus and may be secreted into the CSF, thereby accessing NSCs communicating with the ventricle. Ependymal cells lining the ventricle express the secreted factor Noggin, a competitive antagonist of BMP ligand-receptor binding which promotes greater neurogenesis by activating type B NSCs (Lim et al. 2000). Quiescent NSCs and surrounding astrocytes may themselves express BMPs. Hippocampal SGZ progenitors express BMPs at higher levels than SVZ progenitors and, unlike SVZ NSCs, putative SGZ NSCs require continual Noggin exposure in order to maintain clonal expansion in vitro. Noggin infusion into the hippocampus increases the numbers of long-term BrdU label-retaining cells that are identified as the quiescent NSCs (Bonaguidi et al. 2008). Thus, while the balance of BMP signaling regulates NSC self-renewal in both CNS germinal zones, BMP signaling exerts greater inhibitory influence in the hippocampal SGZ than in the SVZ.

With the increased focus on the stem cell niche concept, more effort has been placed on identifying novel self-renewal signals based specifically on their localization on cells surrounding NSCs. For example, proliferation in the adult CNS is associated with astrocytes surrounding capillaries (Palmer et al. 2000); this effect could be the result of secreted factors or on integral membrane proteins such as cell adhesion molecules. Additionally, adult NSC self-renewal is promoted both by direct adhesion with and by soluble factors released from endothelial cells (Shen et al. 2004, 2008); a candidate screen identified pigment epithelium-derived factor (PEDF) as one of these self-renewal promoting factors (Ramirez-Castillejo et al. 2006). Furthermore, PEDF appears to regulate lateral inhibition and symmetric self-renewal divisions. The function of Notch signaling in this process is considered next.

1.4.2 Lateral Inhibition and Stem Versus Not-Stem Cell Fate Choice

One means by which NSCs can choose self-renewal versus lineage specification is by amplifying initially small differences between daughter cells to yield large effects, so that a cell that commits to a particular fate (e.g., neuron) inhibits adjacent cells from assuming that fate. This mechanism is called *lateral inhibition* and, from a lineage tracking standpoint, can appear as an asymmetric division, even though it

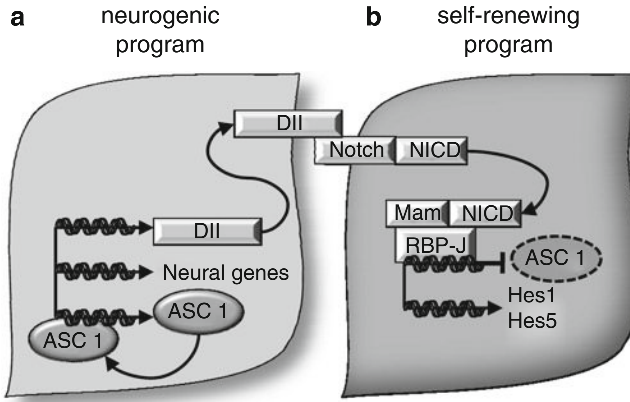

Fig. 1.6 Lineage commitment by mode of division or lateral inhibition. Lateral inhibition through extrinsic signaling can amplify initially small phenotypic differences to yield asymmetric daughter fates without necessarily involving asymmetric inheritance of intrinsic determinants. (**a**) Asc1 promotes the expression of proneural genes along with ligands (e.g., Dll family) to the Notch receptor. (**b**) Binding to the Notch receptor promotes its proteolytic cleavage to generate the active NICD, which binds to an Mm/RBP-J transcriptional complex to inhibit transcription of neurogenic genes (e.g., Asc1) and activate transcription of genes promoting self-renewal. Gene abbreviations defined in text

has a major extrinsic component. Notch signaling is a highly conserved lateral inhibition pathway across vertebrates and invertebrates (Fig. 1.6). There are four mammalian Notch receptors (notch 1–4) that bind to the Jagged and Delta family of ligands. Binding activates the enzymatic cleavage of the Notch receptor to yield an activated Notch intracellular domain (NICD) that translocates to the nucleus and interacts with a complex containing mastermind (Mam) and recombining binding protein suppressor of hairless (RBP-J, also known as CBF-1). The activated complex promotes transcription of lineage repressor genes, such as the hairy/enhancer of Split (Hes) homologs Hes1 and Hes5, while inhibiting transcription of neurogenic/oligodendrogenic genes like Ascl1/Mash1. However, Ascl1 itself promotes the expression of Notch ligands, so that cells expressing high levels of neurogenic genes also secrete high levels of Notch ligands, which repress neurogenic programs in their immediate neighbors. This feedback loop is thought to amplify initially small transcriptional differences to yield one cell with a strong lineage specification program and an adjacent cell with a strong stem cell self-renewal program (reviewed in (Corbin et al. 2008)).

While the role of Notch signaling in self-renewal and neural specification is heavily based on invertebrate lineage studies, mammalian studies are increasingly using improved lineage tracing techniques to characterize the role of Notch in NSCs and other progenitor cell types. Differential Notch activity is itself a consequence of asymmetric division of cell polarity determinants; gain- or loss-of-function for the cell polarity determinant Par3 leads to disrupted asymmetric

division of radial glia during embryogenesis, including localization of Notch signaling components (Bultje et al. 2009). Conditional Nestin-Cre inactivation of mind bomb 1 (mib1), an E3 ubiquitin ligase, indicated that its expression and function are localized to IPCs, which then promote Notch activation in radial glia NSCs, resulting in self-renewing asymmetric divisions. One of the functions of mib1 is endocytic trafficking of Jagged and Dll ligands (Yoon et al. 2008). While the functions of these genes have not yet been tested for adult NSCs, the ability to pinpoint molecular mechanisms of lateral inhibition between mammalian NSCs and their IPC progeny is a significant advance.

The general role of Notch signaling in lateral inhibition appears to be conserved in germinal zones later in development. Induced loss of Notch function (driven by hGFAP-CreER) in postnatal dentate gyrus resulted in a reduction of NSCs and an increase in neuronal committed progenitors. In contrast, conditional gain of Notch function (hGFAP-CreER × flox-stop-NICD) resulted in greater numbers of NSCs and fewer neuronal committed progenitors (Breunig et al. 2007b). While ablation was not conditionally induced later in adult cells, this finding provides insight into the mechanisms by which Notch signaling controls self-renewal in the postnatal brain germinal regions. In the adult dentate gyrus, overexpression of Asc1 leads to large numbers of oligodendrocytes, consistent with a role in lineage specification at the expense of self-renewal. In contrast, Asc1 overexpression in the SVZ leads to large numbers of neurons, suggesting that extrinsic signals or intrinsic progenitor differences modify the lineage specification response between neurogenesis and oligodendrogenesis (Jessberger et al. 2008).

Further evidence of the importance of niche signals in regulating self-renewal and fate is in the finding of PEDF and Notch interactions (Andreu-Agullo et al. 2009). In the SVZ, detectable Notch signaling is restricted mainly to Sox2^{+}GLAST^{+} cells, consistent with an NSC identity; nearly all BrdU label-retaining cells have detectable Notch activity, consistent with quiescent NSCs. Among these cells, neurospheres are readily generated from cells with high Notch but not from those with low Notch activity. PEDF promotes transcriptional activity downstream of Notch signaling (e.g., RPB-J reporter and Hes1 promoter activity) and neurosphere generation specifically in cells with low Notch activity. This is because PEDF signaling removes the nuclear corepressor (NCoR), a binding partner of RBP-J and repressor of Notch-mediated transcriptional activity. Furthermore, analysis of newly divided NSC daughter pairs showed that asymmetric localization of the EGF receptor correlates with high levels of the activated NICD. PEDF stimulation of these daughter pairs leads to symmetrically high EGF receptor expression in daughters regardless of NICD levels. This remarkable finding indicates that PEDF and Notch signals converge on transcriptional targets mediating NSC self-renewal, such that PEDF can override Notch-mediated lateral inhibition. Thus, PEDF can regulate the balance between symmetric and asymmetric divisions, thereby controlling NSC pool size and lineage specification.

While Notch is involved in the maintenance of the NSC population, it is also involved in the progressive acquisition of more glia-like characteristics during the

transition from neuroepithelium to radial glia to adult stem cells. This may explain why exposure to differentiation factors like BMPs promotes neurogenesis from early stage precursors but gliogenesis from later stage precursors (Chen and Panchision 2007; Corbin et al. 2008). Ependymal cells are postmitotic derivatives of radial glia NSCs that express a number of glial markers, but they can exhibit self-renewal and multipotency under some experimental conditions. Inactivation of Notch signaling in ependymal cells (by a conditional Foxj1-Cre driven RBP-J null mutation) causes them to proliferate and generate granule and periglomerular neurons that integrate into the olfactory bulb (Carlen et al. 2009). There are several implications of these findings. First, this suggests that sufficient levels of Notch signaling may drive proliferating cells to quiescence rather than self-renewal. Second, it suggests that ependymal cells may be remobilized to assume NSC-like properties by modulating Notch signaling in the stem cell niche. Third, this suggests that the essential features of lateral inhibition lend themselves to reiterative use, such that Notch signaling may be utilized for making binary decisions in different intermediate progenitor types or in later stages of cell maturation and plasticity (Corbin et al. 2008).

1.4.3 Coordination of Cell Cycle, Potency and Self-Renewal

The cell division cycle is a highly regulated process to ensure proper DNA replication and segregation of cellular components among daughter cells during cytokinesis. An increasing number of molecules are known to coordinate the cell cycle and NSC self-renewal (Fig. 1.7). Since many of these molecules are common across tissue types, they may constitute a core self-renewal program, independent of the factors that regulate tissue-specific potency. Additionally, since cell cycle control is intimately linked to the mechanisms underlying cancer, many factors involved in stem cell self-renewal are also involved in cancer, in support of the "cancer stem cell" hypothesis (see Chap. 11).

Multiple protein complexes act to positively or negatively regulate the cell cycle. The cyclin-dependent kinases (CDK) are activated upon association with a cyclin protein and, through phosphorylation of serine/threonine residues on target proteins, promote transcription of genes involved in cell cycle progression (Sanchez and Dynlacht 2005). They in turn are regulated through CDK inhibitors (CDK-i) of the INK4 or Cip/Kip family, such as $p16^{Ink4a}$, $p21^{cip1}$, $p27^{kip1}$ and $p57^{kip2}$, and alternative reading frame protein products such as $p19^{Arf}$, which in general promote cell cycle arrest (Besson et al. 2008). The relative strength of these opposing signals regulates the balance between cell cycle progression and mitotic arrest leading to terminal differentiation or apoptosis. CDK2 acts in the transition through the G1 and S phases through its binding to cyclin E and cyclin A. Analysis of CDK2 deficient mice showed that CDK2 is required for overall proliferation and self-renewal

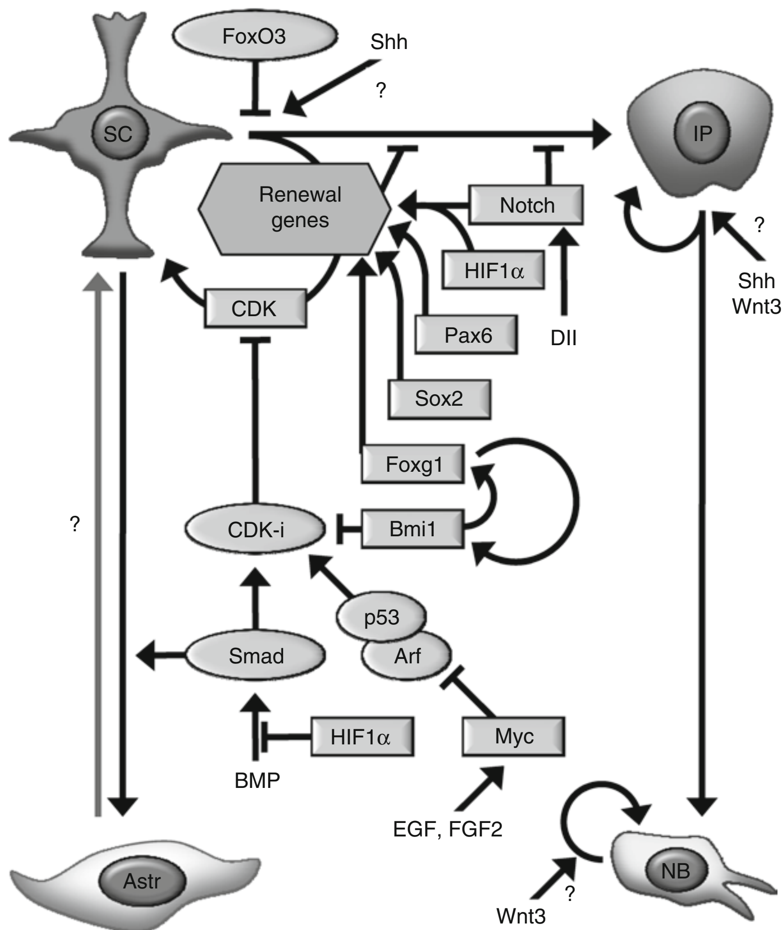

Fig. 1.7 Molecular control of self-renewal versus quiescence. Schematic diagram illustrating factors controlling cell cycle progression or expression of genes promoting self-renewal, multipotency, and/or repression of lineage commitment. Factors in *rectangular boxes* promote stem cell (SC) self-renewal, while factors in ovals inhibit self-renewal (e.g., CDK inhibitors) or maintain either quiescence or asymmetric self-renewal to preserve the stem cell pool (e.g., FoxO3). Extrinsic factors are italicized; some (e.g., Shh or Wnt3) may act as mitogens at several progenitor steps or may promote neurogenic asymmetric divisions. Stem cells (SC) can respond by self-renewing (*circular arrows*) or committing to intermediate progenitor (IP) cells, which in turn can self-renew or commit to neuroblasts (NB); these cells ultimately differentiate into neurons. Reduction in the stem cell pool can occur directly through astrocyte (Astr) differentiation; it is possible that this is reversible (*gray arrow*) in some astrocytes, indicating that this state is a form of quiescence. Functional relationships between factors are examples and not meant to be exhaustive; they usually involve intermediary steps rather than direct physical interactions. Gene abbreviations are defined in text

by the neurosphere assay in adult SVZ stem cells, but not proliferation in the SGZ (Jablonska et al. 2007; Vandenbosch et al. 2007), suggesting that another cell cycle regulator may have redundant functions in the SGZ; the viability of CDK2 null mice overall suggests the same (Sanchez and Dynlacht 2005).

There is evidence that c-Myb and the Myc family transcription factors and protooncogenes play roles in adult NSC self-renewal. This is consistent with their general role in promoting proliferation; for example, Myc is activated by mitogens such as EGF, Shh, and Wnts and promotes cyclin upregulation and CDK-i repression (Knoepfler and Kenney 2006). c-Myb is expressed at higher levels in the SVZ than in nonproliferative regions of the brain and is coexpressed with markers of all cell types in this region. Conditional Nestin-Cre ablation of c-Myb in proliferating precursors results in a decrease in both proliferation and neurogenesis in vivo, as well as decreased neurosphere formation in vitro (Malaterre et al. 2008). There is also a decrease in Sox2 and Pax6 expression in these neurospheres, both of which are implicated in regulating NSC identity (Osumi et al. 2008; Suh et al. 2007). Overexpression of c-Myc and N-Myc results in enhanced frequency of neurosphere formation; using a more rigorous approach of a serial neurosphere-forming assay, it was shown that Myc overexpressing cells retain enhanced self-renewal even under the strong differentiating condition of serum exposure. A separate experiment used a mutant form of c-Myc that failed to bind Miz-1, a repressor of INK4 and Cip/Kip family CDK inhibitors; this mutant Myc still yields increased proliferation but not enhanced serial neurosphere-forming activity, suggesting that c-Myc action on NSC self-renewal may be mediated by a distinct pathway from proliferation of non-NSCs (Kerosuo et al. 2008). Part of the actions of Myc is to repress the $p19^{Arf}$ and p53 tumor suppressor pathway (Nagao et al. 2008), which is known to block adult NSC self-renewal (Meletis et al. 2006).

1.4.4 Transcription Factors Regulating Self-Renewal in Development and Throughout the Lifespan

Some transcription factors originally studied for their functions during embryonic development have conserved functions in adult NSCs. Typically, embryonic studies have identified functions in neural precursors based on the effects of transgenic mis-expression or null mutations on nestin-expressing cells, but have not used clonal analysis to determine if the these functions are unique to stem cells or common to all neural precursors. However, clonal analysis is now being applied more frequently in these studies due to better characterization of distinct precursor states. The transcription factor Sox2 is expressed in NSCs and/or germinal regions throughout embryonic development and in the adult (Ellis et al. 2004) and is a key factor for inducing pluripotency during adult cell reprogramming (Hochedlinger and Plath 2009), suggesting it may be part of a core program regulating self-renewal and potency. Conditional ablation of Sox2 showed that it is required for NSC maintenance in the hippocampus through its regulation of Shh in late embryonic and postnatal NSCs, although its function in adult NSCs was not directly assayed

(Favaro et al. 2009). However, a separate clonal analysis of Sox2-GFP reporter cells in the adult hippocampal SGZ clearly indicated that Sox2 expressing cells are extensively self-renewing and multipotent (Suh et al. 2007). This suggests that Sox2 positively regulates the NSC program throughout the lifespan.

Hypoxia-inducible factor (HIF)-1α is a transcription factor that regulates a broad set of genes involved in metabolism and cell fate. HIF-1α is rapidly degraded at high oxygen tensions and is most active in low physiological oxygen tensions. Fetal and postnatal NSC and precursor expansion is enhanced by lowered oxygen tensions (Chen et al. 2007; Pistollato et al. 2007); since oxygen may be transported by transmembrane channels rather than passive diffusion, this suggests that astrocyte and endothelial niche cells may play an important role in oxygen availability (Panchision 2009). HIF-1α interacts with signal transduction pathways controlling NSC self-renewal. The activated NICD, which is generated by Delta ligand binding to the Notch receptor, binds to HIF-1α and is required for repressing neuronal differentiation in low oxygen (Gustafsson et al. 2005). The canonical SMAD signal transduction pathway downstream of BMPs is negatively regulated by HIF-1α at low oxygen tensions, while degradation of HIF-1α at higher oxygen tensions relieves the block in SMAD activation. While adult studies have only been performed on tumor-derived neural precursors, evidence supports the interaction of oxygen response signals with pathways regulating both stem cell self-renewal and differentiation (Panchision 2009).

Some transcription factors acting downstream from morphogens to control regional pattern specification (e.g., rostrocaudal, dorsoventral) are also involved in self-renewal decisions. Pax6, a member of the paired box family of transcription factors, is expressed in precursor cells from the earliest stages of neural development. It becomes regionally restricted to portions of the dorsal telencephalon, diencephalon, and ventral spinal cord; within these domains it is expressed most strongly in radial glia (midgestation NSCs). In embryonic cortex, loss of Pax6 leads to a depletion of the progenitor pool and subsequent production of neurons. In the postnatal brain, Pax6 is also expressed in the two germinal areas, the SVZ and SGZ, as well as some discrete postmitotic locations. Adult mice haploinsufficient for Pax6 show reduced proliferation in the SGZ and neuron production in the granule layer. In both embryos and adult, gene expression analysis indicates that Pax6 represses $p16^{Ink4a}$, $p19^{Arf}$, $p21^{cip1}$, $p27^{kip1}$; and $p57^{kip2}$; conversely, it upregulates a number of progenitor regulators. Additionally, there is circumstantial evidence that the balance self-renewal versus neurogenesis may depend on the levels of Pax6 expression (Osumi et al. 2008; Sansom et al. 2009). Although clonal analysis has not yet been performed on Pax6-deficient cells in vivo, these results suggest that Pax6 controls a key step in self-renewal versus neurogenesis.

1.4.5 Adult Functions of Self-Renewal Factors and the Balance of Quiescence and Exhaustion

As mentioned above, adult NSCs appear to differ from their fetal counterparts in having a very slow rate of cell division. Stem cell quiescence may have several

protective roles. One may be to limit the accumulation of mutations leading to cancer (see Chap. 11). Another may be to prevent exhaustion of the stem cell pool, which would reflect an intrinsic limit to the number of self-renewing divisions among stem cells.

The forkhead (Fox) winged helix family of transcription factors have diverse roles as transcriptional regulators in the development of many tissues (Kaestner et al. 2000). Members of the FoxO subfamily (FoxO1, 3, 4) are required for postnatal maintenance of NSCs; mice with conditional or tissue-specific deletions of FoxO3 alone or FoxO1/3/4 show an initial increase in proliferation shortly after birth, resulting in an increased brain size (Paik et al. 2009; Renault et al. 2009). Surprisingly, this is followed by decreased numbers of BrdU label-retaining cells, in indicator of relatively quiescent stem cells, as well as decreased neurosphere forming capacity in vitro. Since FoxO proteins are inactivated by the PI3 kinase – Akt pathway (Salih and Brunet 2008) that acts downstream of many mitogens, this supports the idea that FoxO genes regulate the balance between self-renewal and quiescence in order to prevent premature exhaustion of the stem cell pool. Furthermore, analysis of these mutants indicates that FoxO transcription factors regulate a large number of genes involved in metabolism, quiescence, hypoxia-response, and aging, suggesting that this pathway integrates a wide variety of environmental inputs into self-renewal decisions. While it is not clear whether FoxO proteins are required for self-renewal in developing as well as adult stem cells, it is known to be required for greater longevity of insulin mutant invertebrates (Kenyon 2005), suggesting that it has specific functions regulating tissue maintenance and lifespan.

An important transcriptional regulator with a unique requirement in adult stem cells is Bmi-1, a member of the polycomb (PcG) group genes that act as epigenetic gene silencers, thereby preserving cell identity (Buszczak and Spradling 2006; Lund and van Lohuizen 2004). Bmi-1 is active in the embryonic CNS (Fasano et al. 2007), but not required for the normal embryonic development of the brain or for the generation of normal stem cell numbers at birth. However, the absence of Bmi-1 leads to the depletion of stem cells and ultimately of new neurons (Molofsky et al. 2005). Clonal analysis indicates that the loss of Bmi-1 does not affect the number of neuron-only or glia-only clones, supporting the idea that it is required specifically for self-renewal of stem cells and not committed progenitors. The effect of Bmi-1 is mediated by its regulation of the cell cycle machinery. Loss of Bmi-1 leads to the upregulation of the cell cycle inhibitors $p16^{Ink4a}$ and $p19^{Arf}$ (Bruggeman et al. 2005; Molofsky et al. 2005) as well as $p21^{cip1}$ and the retinoblastoma (Rb) protein (Fasano et al. 2007). These results are consistent with for role of Bmi-1 as a repressor of cell cycle inhibitors. Additionally, Bmi-1 acts cooperatively with FoxG1, a winged helix transcription factor required for the development of the forebrain. Bmi-1 induces expression of Foxg1 in adult SVZ cells, which in turn promotes stem cell survival; downregulation of Foxg1 causes a decrease in Bmi-1 and an increase in $p21^{cip1}$, suggesting a feedback loop, whereby the two factors reinforce each other's expression (Fasano et al. 2009).

There is a link between adult-specific functions of self-renewal factors and the mechanisms of aging (Levi and Morrison 2008). Increased expression of $p16^{Ink4a}$ and $p19^{Arf}$ appear to play a role in adult stem cell aging (Molofsky et al. 2006). The expression of $p19^{Arf}$ is higher in adult NSCs than those from earlier ages and appears to play a role in shifting NSCs from self-renewal to a gliogenic program (Nagao et al. 2008). While Bmi-1 is also required for repressing these factors in adult stem cells, it is dispensable in stem cells at earlier ages (Molofsky et al. 2003), suggesting that another factor has a redundant role at earlier ages. One such factor is the transcriptional regulator Hmga2, which represses both $p16^{Ink4a}$ and $p19^{Arf}$ and promotes stem cell renewal in neonates and young adults; however, it decreases with age due to the increasing expression of the microRNA let-7b, which negatively regulates Hmga2 expression (Nishino et al. 2008). This may account for the progressive loss of self-renewal capacity with aging, a topic that will be covered in greater detail in Chap. 9.

1.5 Summary and Perspectives

While the focus of this chapter has been on NSCs, many mechanisms are known to be conserved in adult stem cells from other tissues. In particular, the mechanisms regulating the balance of quiescence and self-renewal are remarkably similar in the hematopoietic system, including conserved roles of FoxO transcription factors, Bmi-1 and many of the cell cycle regulators (Levi and Morrison 2008). The increasing availability of tools for tissue-specific conditional gene deletion will permit the characterization of these genes in regulating stem cell self-renewal and quiescence in a wide variety of tissues.

Furthermore, large scale technologies are allowing the molecular phenotyping of cells to identify their epigenetic state (epigenome), transcriptional state (transcriptome), protein composition and modifications (proteome), and intermediary metabolism (metabolome). This is being done extensively in embryonic stem cells to identify candidate molecules and gene regulatory networks controlling “stemness,” a core program underlying self-renewal and potency. Other efforts are comparing embryonic and tissue-specific adult stem cells in order to distinguish global stem cell programs from tissue-specific stem cell programs (Muller et al. 2008). The combination of global phenotyping and functional characterization of individual genes will yield a more integrative picture of the cellular properties of self-renewal and potency and the environmental signals that regulate them (Chambers and Tomlinson 2009; Chen et al. 2008). Results from these studies have already blurred the distinction between stemness as a lineage entity and stemness as a phenotypic state, since an increasing number of ways exist to reprogram differentiated somatic cells to a stem-like state (Hochedlinger and Plath 2009; Leychkis et al. 2009).

The usefulness of the data from these phenotyping strategies is dependent on functionally validating the cell types being screened. Clonal analysis, using a lineage marker with limiting dilution, remains the definitive tool for identifying

lineage relationships between cells and measuring stem cell properties. While immunocytochemistry for cell-type specific markers is a common and indispensable tool for phenotyping cells, it alone is insufficient to identify a cell as a stem cell. Likewise, prospective isolation to enrich specific cell types is always dependent on clonal analysis for functional validation. Studies that ascribe a functional role for genes/factors in stem cell self-renewal of fate in the absence of clonal analysis are actually suggestive rather than conclusive.

The complementary use of in vivo and in vitro clonal analysis has yielded important insights into the mechanisms of stem cell self-renewal and fate. Many studies combine these approaches, such that the strengths of one approach compensate for the weaknesses of the other. There is evidence that the potency of cells in vitro is much greater than in vivo due to the loss of constraints on fate specification (Anderson 2001). However, this exact property has been exploited to study the underlying molecular mechanisms of fate specification and to reprogram cells for therapeutic purposes.

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Chapter 2
Maintenance of Adult Stem Cells: Role of the Stem Cell Niche

Yoshiko Matsumoto, Hiroko Iwasaki, and Toshio Suda

Abstract Self-renewing stem cells are maintained in functionally specific microenvironments that are termed niches. Optimum combination of intrinsic programs and extrinsic stimuli from niches is essential for maintaining stem cell quiescence and for proper mobilization and differentiation. The most advanced imaging technologies allow the clearer real-time visualization of the behavior of hematopoietic stem cells in living animal. Functions of niche factors, such as adhesion molecules and cytokines, are well studied. Concentration levels of local oxygen and reactive oxygen species also affect the fate of stem cells in their niches. Analysis of niche regulation will lead to a better understanding of stem cell behavior. Moreover, altering the cancer stem cell fate through modification of the niche regulation may be one of the candidates for new therapeutic strategies for diseases such as chemoresistant leukemia.

Keywords Hematopoietic stem cells • Endosteal niche • Perivascular niche • Osteoblasts • Self-renewal • Symmetric division • Asymmetric division • Cell cycle • Reactive oxygen species

Abbreviations

Atm	Ataxia telangiectasia mutated gene
BMT	Bone marrow transplantation
BrdU	5-Bromo-2-deoxyuridine
CAR	CXCL12-abundant reticular cells
Casr	Calcium-sensing receptor
CDK	Cyclin-dependent kinase
ECM	Extracellular matrix

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Flk1	Fetal liver kinase1
FoxOs	Forkhead box Os
G-CSF	Granulocyte colony-stimulating factor
Gfi1	Growth factor independence-1
GFP	Green fluorescent protein
GSCs	Germ line stem cells
HA	Hyaluronic acid
HSC	Hematopoietic stem cell
ICAM-1	Intercellular adhesion molecule 1
JAK	Janus kinase
Lgr5	Leucine-rich repeat-containing G-protein-coupled receptor 5
LSC	Leukemic stem cells
MCAM	Melanoma-associated cell adhesion molecule
MMP-9	Matrix metalloproteinase-9
Mpl	Myeloproliferative leukemia virus oncogene protein
MSC	Mesenchymal stem cells
NAC	N-acetyl-L-cysteine
NE	Norepinephrine
Opn	Osteopontin
PPRs	Parathyroid hormone/parathyroid hormone-related peptide (PTH/PTHrP) receptors
Pten	Phosphatase and tensin homolog
ROS	Reactive oxygen species
SDF-1	Stromal cell-derived factor 1
SLAM	Signaling lymphocyte activation molecule
Sr	Strontium
STAT	Signal transducer and activator of transcription
THPO	Thrombopoietin
Tie2	Endothelium-specific receptor tyrosine kinase 2
UPD	Unpaired
VCAM-1	Vascular cell adhesion molecule 1

2.1 Introduction

Stem cells are the cells with two unique characteristics: self-renewal and differentiation into multiple lineages. They exist virtually in every organ and supply an adequate number of differentiated cells to maintain the functions of the organ. The existence of stem cells was first demonstrated in 1961 by Till and McCulloch in a study on hematopoiesis (Till and McCulloch 1961). The establishment of the concept of hematopoietic stem cells (HSCs) was followed by the discovery of tissue stem cells in other organs in mammals, e.g., epithelial stem cells, neural stem cells, and intestinal stem cells (Fuchs and Segre 2000).

Stem cells can be found in specific areas of each organ termed "niches" where they maintain stem cell function. In 1978, Schofield proposed the concept of "niche" as the functionally specific microenvironment of the stem cells (Schofield 1978). This niche function was substantiated by the study of germ line stem cells (GSCs) in *Drosophila*, where the existence of niches in vivo was demonstrated for the first time (Xie and Spradling 2000). In female *Drosophila*, GSCs are found within the germarium, which is a generative region located at the tip of ovariole. Cap cells located at the tip of the germarium and adjacent to GSCs were first defined by Xie and Spradling as the key niche cells that support GSCs. Further studies revealed that GSCs are mainly and strictly governed by bone morphogenetic protein (BMP) signaling in the niche (Chen and McKearin 2003; Song et al. 2004). In contrast, apical hub cells act as a cellular niche in fly testes (Fuller and Spradling 2007).

In mammals, hematopoiesis has taken the lead in niche studies, while the variety of cells in organs and the complexity of tissues in mammals have hampered investigations into niche function.

2.2 Quiescence and Self-Renewal

In the mammalian hematopoietic system two types of stem cells have been identified: quiescent HSCs and cycling HSCs (Arai and Suda 2007). The quiescent state is necessary to maintain the self-renewal capacity of HSCs and to prevent stem cell exhaustion. Loss of p21, which is a G1 checkpoint regulator and a cyclin-dependent kinase (CDK) inhibitor, allows the escape of HSCs from quiescence and promotes their entrance into the cell cycle (Cheng et al. 2000). As a result, $p21^{-/-}$ mice exhibit HSC proliferation and a consequent increase in the total number of HSCs, as well as an enhancement in their sensitivity to cell cycle-specific myelotoxic injury because of hematopoietic cell depletion. The self-renewal capacity of primitive cells was also impaired in serial bone marrow transplantation (BMT) of HSCs derived from $p21^{-/-}$ mice. HSCs lacking expression of growth factor independence-1 (*Gfi1*), phosphatase and tensin homolog (*Pten*), or forkhead box Os (*FoxO*s) also exhibit activation of cell cycling and similar phenotypes (Hock et al. 2004a; Yilmaz et al. 2006; Zhang et al. 2006; Miyamoto et al. 2007; Tothova et al. 2007; Orford and Scadden 2008; Yalcin et al. 2008).

The cell cycle state is monitored mainly by detecting the retention of 5-bromo-2-deoxyuridine (BrdU) in chromosomes. Bradford et al. reported that the BrdU is retained in primitive HSCs (Bradford et al. 1997). The Fuchs group developed a method using a histone H2B/green fluorescent protein (H2B-GFP), with which GFP-labeled, nondividing or slow-cycling cells can be sorted and collected (Tumbar et al. 2004). Using the BrdU and the H2B-GFP fusion protein, Trumpp and his colleague showed that the most primitive stem cells ($Lin^{-}Sca1^{+}cKit^{+}CD48^{-}CD150^{hi}CD34^{-}$HSCs) actually comprise two subsets of cells: a dormant HSC population, which most probably divides only every 145–193 days, and an activated HSC population,

which divides every 28–36 days (Wilson et al. 2008). They proposed that the dormant population is activated to enter the cell cycle under conditions of hematopoietic stress to produce mature cells and then returns to quiescence after reestablishment of homeostasis, where they act as a silent reservoir during the normal state. Another group also used the H2B-GFP fusion protein to show that the $Lin^{-}Sca1^{+}cKit^{+}$ $CD150^{+}$ $CD48^{-}$ fraction, which is highly enriched for HSCs, represents a heterogeneous population in which less than 20% of cells divide at a slower rate (Foudi et al. 2009).

The maintenance of quiescent HSCs is the main role of the HSC niche in bone marrow (BM). Cytokines, growth factors, local environmental factors, and adhesion molecules that anchor HSCs to the endosteum play a key role in maintaining quiescence. The concept of coexistence of different types of HSCs in niches – quiescent and cycling stem cells – is now accepted and proved for other types of stem cells. For example, the Clevers group demonstrated that the leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5)-positive cells in the pyloric gland are actively cycling pyloric stem cells, whereas $villin^{+}$ stem cells are quiescent (Qiao et al. 2007; Barker et al. 2010).

The ability to self-renew is the most important characteristic of stem cells. However, it is not recapitulated well *in vivo* and *in vitro*. The process of stem cell self-renewal differs from the maintenance of quiescence and has long been attributed to the capacity of stem cells to undergo symmetric and asymmetric cell division. The asymmetric division of a stem cell gives rise to both an exact copy of itself and a daughter cell that will differentiate into mature cells of the tissue. Asymmetric division of stem cells is supported by observation of GSCs in female *Drosophilia* (Deng and Lin 1997). When cell division occurs such that the mitotic spindle is oriented perpendicular to the niche, one daughter cell maintains its exposure to the niche and retains its stem cell properties while the other is displaced from it and undergoes differentiation (Yamashita et al. 2003). In this way, the commitment to differentiation of fly GSCs seems to be an “all-or-nothing” event. In contrast, this commitment process seems to be more complex in mammalian HSCs, in which both symmetric and asymmetric divisions are proposed to be present in the multistep models.

Stem cell fate is regulated by both intrinsic programs and extrinsic stimuli. For example, cells derived from a single progenitor and exposed to the same growth-promoting environment often develop into different kinds of colonies, indicating that fate decisions were controlled by intrinsic or stochastic mechanisms (Suda et al. 1984). Differential responsiveness of stem cells to extrinsic signaling rather than environmental variability (time and concentration) of stimuli also supports the intrinsic programming of stem cells. In contrast, the Schroeder group used time-lapse microscopy to show that the differentiation of progenitors of neutrophils and macrophages is regulated by external stimuli (Rieger et al. 2009).

Interestingly, HSCs change their living space during ontology. In the midgestation embryo, the first definitive HSCs arise from the hemogenic endothelium on the ventral side of the aorta, enter the circulation, seed the placenta, and continue to expand and mature in the fetal liver. Before birth, cycling HSCs migrate from the liver to the developing BM (Orkin and Zon 2008). At the end of the preadult phase, organ growth slows down and eventually stops. Stem cell quiescence coincides with the switch from developmental expansion to homeostatic maintenance. Thus, it is important to distinguish adult hematopoiesis from the hematopoiesis that is associated with stem cell expansion in fetal liver and infantile BM. Quiescence is a characteristic feature of adult HSCs. In particular, slow cell cycling or quiescence confers stress resistance to adult stem cells (Fig. 2.1).

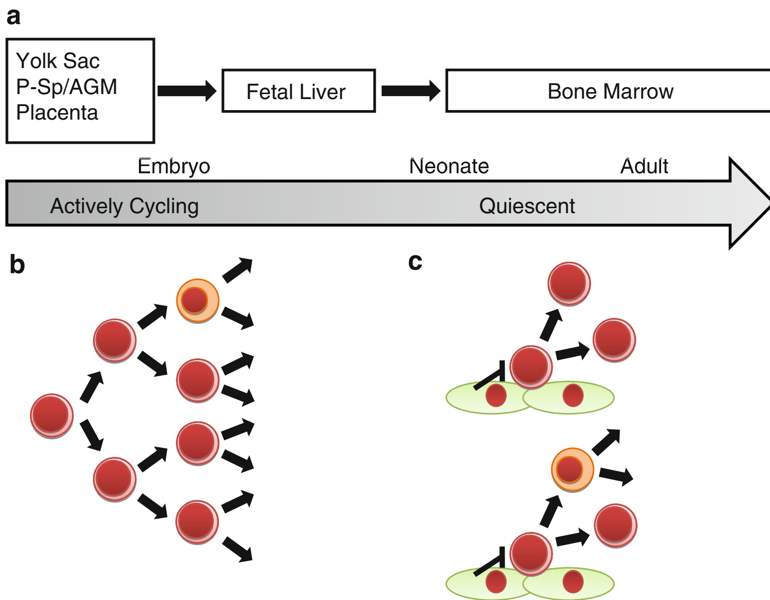

Fig. 2.1 A schema of mammalian hematopoiesis. (**a**) Primitive hematopoiesis occurs in the yolk sac where erythrocytes are mainly produced. The first definitive HSCs arise from the hemogenic endothelium on the ventral side of the aorta in the midgestation embryo, enter the circulation, seed the placenta, and continue to expand and mature in the fetal liver. Before birth, cycling HSCs migrate from the liver to the developing BM. At the end of the preadult stage, organ growth slows down and eventually stops. Stem cell quiescence coincides with the switch from developmental expansion to homeostatic maintenance. *P-Sp* para-aortic splanchnopleural mesoderm; *AGM* aorta-gonad-mesonephros. (**b**) HSCs actively cycle and expand during development. (**c**) HSCs are kept quiescent in the adult BM niche. HSCs divide symmetrically or asymmetrically. Differentiated daughter cells continue to differentiate into mature cells, whereas the copy of the parent cell is kept in the niche. In the presence of stress, the niche environment may be changed to activate HSC division

2.3 The Hematopoietic Stem Cell Niche

2.3.1 Niche Cell Components

2.3.1.1 Two Types of Niches

The pathways via which two different types of HSCs, quiescent and cycling, are maintained in the HSC niche in the BM are of great interest. A dynamic model of hematopoiesis has been proposed, (Arai and Suda 2007; Iwasaki and Suda 2009) wherein quiescent HSCs are located and maintained on the surface of the endosteum during homeostasis. Occasionally, these cells move to the perivascular niche when needed and become activated by extrinsic signals to enter the cell cycle. Subsequently, cycling HSCs divide and provide peripheral blood with mature cells. When the supply of mature cells is no longer needed, activated HSCs located in the perivascular niche return to the osteoblastic niche on the surface of endosteum, where they fall back to the quiescent state and are maintained (Fig. 2.2a).

Osteoblasts are bone-forming cells that are derived from mesenchymal stem cells (MSCs), which are the main population of cells covering the endosteal surface. Osteoblasts are prime candidates for HSC niche cells based on several studies:

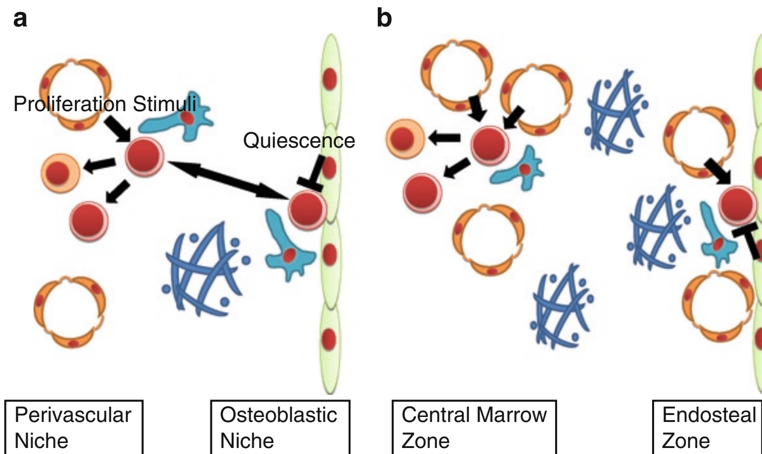

Fig. 2.2 Niche structure models. (**a**) The "two types of niche model" involves the osteoblastic niche and the perivascular niche. Quiescent HSCs are maintained in the osteoblastic niche. When needed HSCs exit from the endosteal area and migrate into the perivascular niche where their cell cycle is activated. As a result, HSC proliferation and differentiation occur to produce mature cells. HSCs shuttle between the two types of niche. (**b**) Recent investigations revealed the presence of close interactions between the osteoblastic and endothelial niches. Microvessels exist near the endosteum. HSCs receive inhibitory signals that block the cell cycle from the cells located on the endosteal surface and activation signals from the endothelial cells located at the endosteal zone. In contrast, HSCs are exposed mainly to the activation signals from the endothelial cells located at the central marrow zone

osteoblasts secrete cytokines that modulate stem cell function, stimulate the growth of hematopoietic cells in in vitro culture, and promote BM engraftment of HSCs in vivo when cotransplanted (El-Badri et al. 1998; Taichman 2005). The groups of Li and Scadden demonstrated that osteoblasts are a HSC niche in in vivo studies (Calvi et al. 2003; Zhang et al. 2003). They showed that the number of HSCs is controlled by the number of osteoblasts, i.e., niche size. The Li group demonstrated that the BMP signaling through the BMP receptor type IA in osteoblasts controls the number of osteoblasts that express N-cadherin, which correlates with the number of HSCs. These authors also showed via histological analysis that a subset of osteoblasts expressing high levels of N-cadherin function as a key component of the osteoblastic niche. Scadden and his colleague stimulated osteoblasts using activated parathyroid hormone/parathyroid hormone-related peptide (PTH/PTHrP) receptors (PPRs). The stimulated osteoblasts increased in number and produced high levels of the Notch ligand jagged 1, which led to an increase in the number of HSCs. Visnjic et al. confirmed these results in a transgenic mouse model wherein expression of the herpes virus thymidine kinase gene is under the control of the collagen α 1-type promoter. In this model, hematopoiesis was severely suppressed following conditional ablation of osteoblasts by exposing mice to ganciclovir (Visnjic et al. 2004).

The osteoblastic niche on the endosteal surface comprises not only mature osteoblasts, but also multiple subsets of endosteal cell populations, which may act synergistically to form a complex that supports the HSCs located at the endosteum. Osteoblasts are a heterogeneous population that includes immature and mature cells (Kiel and Morrison 2008). We performed a precise investigation of the osteoblasts that cover the BM endosteum, and identified an osteoblastic population that most effectively enhances the long-term repopulation activity of HSCs. Gene expression profiling also revealed the heterogeneity of endosteal cell populations (Nakamura et al. 2010).

The perivascular niche is composed mainly of endothelial cells of the sinusoid. Hematopoietic and endothelial cells are derived from a common progenitor, the hemangioblast (Choi et al. 1998). Both HSCs and endothelial cells express CD31, CD34, CD133 (Prominin-A), fetal liver kinase1 (Flk1), and endothelium-specific receptor tyrosine kinase 2 (Tie2) (Takakura et al. 1998; Rafii et al. 2002). During embryonic development, HSCs and endothelial cells are tightly linked, and studies of this stage have taken the lead in the area of perivascular niche research. It has been shown that primary endothelial cells in the adult liver are able to support hematopoietic progenitors in culture (Cardier and Barbera-Guillem 1997). Heissig et al. proposed that after BM ablation, hematopoietic progenitors are translocated to the perivascular niche, which is initiated by soluble Kit ligand cleaved by matrix metalloproteinase-9 (MMP-9) (Heissig et al. 2002).

The Morrison group conducted a study using antibodies against the signaling lymphocyte activation molecule (SLAM) family of receptors, to distinguish HSCs from progenitor cells in the BM, and found that HSCs are highly concentrated in the $CD150^{+}CD244^{-}CD48^{-}$ population (Kiel et al. 2005). The ability to purify HSCs based on a simple combination of SLAM receptors allowed the relatively easy identification of HSCs in tissue sections. In their histological study, the majority of

$CD150^{+}CD244^{-}CD48^{-}$ cells were found near the sinusoidal endothelium in the spleen and BM, although some were associated with the endosteum. Cells located near sinusoids are exposed to many factors streaming in the peripheral blood, such as hormones, growth factors, oxygen, and nutrients. Therefore, HSCs located in the perivascular niche can sensitively perceive signal changes according to homeostatic need and be activated for proliferation and differentiation to produce mature blood cells.

Cells other than osteoblasts and endothelial cells exist in the BM. Recent work suggests that the other populations also participate in the regulation of HSCs. Sugiyama et al. found that most HSCs were in contact with reticular cells expressing a high amount of chemokine (C-X-C motif) ligand 12 (CXCL12, also known as stromal cell-derived factor-1 [SDF-1]), the ligand for chemokine (C-X-C motif) receptor 4 (CXCR4) (Sugiyama et al. 2006). These cells are called CXCL12-abundant reticular (CAR) cells. Severe reduction in number of HSC and higher sensitivity to myelotoxic injury were observed in the absence of CXCL12-CXCR4 signaling. CAR cells are present both in vascular and endosteal niches, although the origin of CAR cells remains unknown.

Katayama et al. demonstrated that the sympathetic nervous system, which secretes norepinephrine (NE), regulates the attraction of stem cells to their niche by controlling the BM niche (Katayama et al. 2006). In UDP-galactose ceramide galactosyltransferase-deficient ($Cgt^{-/-}$) mice, which exhibit abnormal nerve conduction, no hematopoietic stem and progenitor cells egress from the BM after induction of migration. Further studies indicated that NE mediates the suppression of osteoblasts and the downregulation of bone CXCL12 that are induced by granulocyte colony-stimulating factor (G-CSF). These modifications result in changes in hematopoietic stem and progenitor cell mobilization.

Osteoclasts and adipocytes may also take part in the regulation of HSCs, although the function of these cells in the BM niche is not well understood. Strontium (Sr) is a bone anabolic agent that increases bone and osteoblast formation and inhibits osteoclast activity. BMT into Sr-treated mice led to a delay in hematopoietic recovery despite the increase in osteoblastic activity (Lymperi et al. 2008). In another study, Kollet et al. stimulated osteoclasts using several stresses and demonstrated that activation of osteoclasts increases the recruitment of hematopoietic progenitors into the circulation (Kollet et al. 2006). Adipocytes are enriched in the BM, especially in the elderly. Naveiras et al. focused on the difference in adipocyte content between the vertebrae of the tail and the thorax and demonstrated that adipocytes were enriched in vertebrae of the tail where HSCs and short-term progenitors were reduced (Naveiras et al. 2009). In A-ZIP/F1 mice, which are fatless, marrow engraftment was accelerated compared with wild-type animals. These data indicate that adipocytes may act as negative regulators of the HSC niche while adiponectin, which is secreted mainly from adipocyte, reportedly functions as a positive regulator of HSCs (DiMascio et al. 2007).

The identification of MSCs, the progeny of which constitutes the BM niche, has led to further progress in investigation of the HSC niche compartment. The concurrent formation of the hematopoietic environment and of heterotopic bone via

in vivo transplantation of BM fragments or BM stromal cells into an ectopic site suggests that HSCs and MSCs coexist in the BM. Sacchetti et al. showed that melanoma-associated cell adhesion molecule (MCAM) (also known as CD146)-expressing subendothelial cells in human BM can organize an ectopic hematopoietic microenvironment within a miniature bone organ (Sacchetti et al. 2007). These subendothelial cells maintain the self-renewal capacity in the ectopic hematopoietic region. This result suggests the subendothelial cells that express MCAM may be MSCs. Another group led by Weissman sorted cells collected from fetal bones (at 15.5 days *postcoitum*) and identified a population of progenitor cells that expressed the surface markers $CD45^{-}Tie2^{-}\alpha_{V}^{+}CD105^{+}Thy1.1^{-}$ ($CD105^{+}Thy1.1^{-}$) and produced donor-derived ectopic bones when transplanted under the adult mouse kidney capsule (Chan et al. 2009). The ectopic bone was generated through a cartilage intermediate, and a marrow cavity populated by host-derived long-term HSCs was also generated within this structure. Transplantation of $CD105^{+}Thy1.1^{-}$ cells with concomitant suppression of the expression of factors involved in endochondral ossification in the host mice resulted in the absence of niche formation. This result indicates the necessity of endochondral ossification for the formation of the BM niche.

2.3.1.2 Close Interaction Between the Endosteal and Perivascular Zones

As described above, there are many types of HSC-supporting cells in the BM. Therefore, it is difficult to define two distinct BM niches according to the types of the cells involved in the formation of niche. Close interaction between the osteoblastic and perivascular niches has been identified using new imaging technologies. The Scadden group observed HSCs at the single-cell level using a combinational method of high-resolution confocal microscopy and two-photon video imaging, and found that osteoblasts were enmeshed in microvessels and formed a complex tissue (Lo Celso et al. 2009). After transplantation into irradiated or c-Kit receptor deficient mice, HSCs and progenitors localized near endosteum, while more differentiated cells stayed at distant locations from endosteum. Under physiological challenge of BMT, which demands engraftment or expansion of HSCs, HSCs and progenitors moved into positions near endosteum and osteoblasts. The Li group traced the migration of GFP^{+} HSCs using a newly developed real-time ex vivo imaging technology (Xie et al. 2009). They demonstrated that HSCs tended to migrate to the endosteum when transplanted to irradiated mice and a fraction of these cells divided actively. In contrast, HSCs were located randomly in BM when transplanted in nonirradiated mice. In this study, the authors observed the presence of well-vascularized endosteum and vasculatures near N-cadherin^{+} preosteoblasts. These results indicate that HSCs located in the endosteal zone interact with both endosteal and endothelial cells and are prompted to expand in the endosteum zone in response to BM damage, which means that the osteoblastic and perivascular niches cannot be distinguished clearly. Therefore, Li and his colleagues proposed an alternate concept for two types of BM niches, i.e., the endosteal zone and the

central marrow zone. They suggest that, although the endosteal zone normally sends inhibitory signals for cell cycling to maintain HSC quiescence, it transiently turns into a stimulatory environment when the BM is damaged (Fig. 2.2b).

2.3.2 Niche Factors

Given the complexity of the niche structure and its cellular components, it is not only important to analyze niche functions based on its physical structure but also by the factors that are produced by the niche components themselves. Each type of niche cell exhibits a specific gene expression pattern, which may lead to secretion of a specific combination of potential niche factors for HSC maintenance. The BM also is comprised of extracellular matrix (ECM) and each ECM component may also contribute to the niche. Therefore, to fully understand the characteristics of the niche, research should focus on adhesion molecules, cytokines, growth factors, and some local environmental factors (Fig. 2.3).

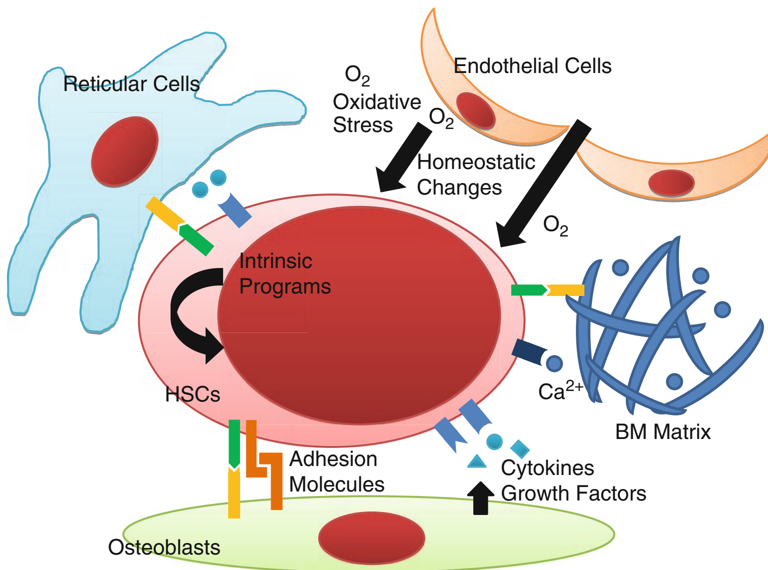

Fig. 2.3 Components of the hematopoietic niche in BM. HSCs are maintained by intrinsic and extrinsic stimuli. HSCs attach to niche cells and to the BM matrix via adhesion molecules, some of which also activate signals for HSC maintenance. BM cells secrete cytokines and growth factors, such as Wnt, MPL, and CXCL12. Oxygen concentration is higher around the sinusoid. Signals from peripheral blood reflect homeostatic changes. Ca^{2+} is abundant in the BM and regulates HSCs. Intrinsic programs, especially those associated with transcription factors such as *Hox* and *Evi-1*, also regulate HSC fate

2.3.2.1 Adhesion Molecules

One of the primary functions of the niche is to physically interact with stem cells. HSCs and their niche cells attach to each other through adhesion molecules, which are also important for lodging and engraftment. Niche cells express various adhesion molecules including N-cadherin, which is a hemophilic adhesion molecules and binds to N-cadherin expressed on HSCs, intercellular adhesion molecule 1 (ICAM-1), which binds to integrin alpha-L (LFA-1A) expressed by HSCs, vascular cell adhesion molecule 1 (VCAM-1) that binds to integrin alpha-4 (VLA-4) expressed by osteoblasts, and VCAM-1 that binds with VLA-4 on endothelial cells (Wilson and Trumpp 2006).

The Li group, which had identified the osteoblastic niche (composed of N-cadherin$^+$ osteoblasts), observed the presence of N-cadherin/β catenin complexes between HSCs and osteoblasts (Zhang et al. 2003). This result suggests that N-cadherin$^+$ osteoblasts support HSCs via this adhesive interaction. N-cadherin is a representative adhesion molecule in the osteoblastic niche and is the only member of the cadherin family that is expressed in both quiescent HSCs and osteoblasts (Arai et al. 2004).

Integrins are also important adhesion molecules in the HSC niche. In particular, integrin-superfamily heterodimers, VLA-4 and integrin $\alpha5\beta$ 1 (VLA-5), are expressed in HSCs, which bind to fibronectin and osteopontin (Opn) in the BM ECM, and to VCAM-1 on the niche cell surface (Whetton and Graham 1999; Wilson and Trumpp 2006). Binding to fibronectin regulates the expression of specific transcription factors that have roles in stem cell maintenance (Dao and Nolta 2007). For example, human CD34$^+$ cells cultured on a fibronectin peptide containing the binding site for VLA-4 and VLA-5 expressed higher levels of the protooncogene protein c-Myb and endothelial transcription factor GATA-2 in vitro, which are known to be abundant in primitive cells, as compared to cells grown in suspension culture. The cells also expressed lower levels of p21 and the transcription factor PU.1, which are upregulated during monocyte and myeloid differentiation, respectively. The interaction between VLA-4 and VCAM-1 is also important for lodging and engraftment at the endothelial niche (Papayannopoulou et al. 1995). We demonstrated that the interaction between Tie2, which is expressed in HSCs, and angiopoietin-1 (Ang1), which is produced by the osteoblasts, activates β1-integrin and N-cadherin and that this enhanced adhesion mediates the maintenance of stem cell quiescence in the osteoblastic niche (Arai et al. 2004).

The osteoblastic niche sends inhibitory signals to HSCs to prevent their proliferation and maintain their quiescence. In some cases, adhesion molecules themselves send inhibitory signals to HSCs. For example, N-cadherin inhibits the activity of β-catenin, which is the key intercellular molecule in the Wnt signaling pathway (Wilson and Trumpp 2006).

Opn, a matrix glycoprotein that is exclusively produced by osteoblasts on the endosteal bone surface, is a key adhesion component of the osteoblastic niche (Nilsson et al. 2005; Stier et al. 2005; Adams and Scadden 2006). Opn binds to VLA-4, VLA-5, and CD44 expressed in HSCs, which supports the adhesion of

HSCs to the osteoblastic niche. In addition, Opn acts as a negative regulator of HSC proliferation. Markedly enhanced cycling of HSCs was observed in *Opn*$^{-/-}$ mice and the proliferation of hematopoietic stem/progenitor cells was suppressed by exogenous Opn in vitro (Nilsson et al. 2005). The Scadden group stimulated the BM niche in *Opn*$^{-/-}$ mice using parathyroid hormone and observed an abnormal increase in the number of HSCs (Stier et al. 2005; Adams and Scadden 2006). Therefore, when the osteoblastic niche is activated Opn may function to restrict excessive stem cell expansion.

Finally, CD44 is a cell surface receptor expressed on HSCs that binds to the glycosaminoglycan hyaluronic acid (HA), which is a major component of the BM ECM. The CD44/HA interaction has also been shown to play important roles in modulating the migration and anchorage of HSCs in the BM niche (Avigdor et al. 2004; Adams and Scadden 2006).

2.3.2.2 Cytokines and Growth Factors

Molecular signaling pathways that regulate stem cell function in the niche of the GSCs of *Drosophila* may be simpler compared with those observed in mammal niches. In the fly ovariole, GSCs are mainly and strictly governed by BMP signaling (Chen and McKearin 2003; Song et al. 2004). In contrast, in *Drosophila* testis hub cells support the self-renew and maintenance of GSCs by expressing unpaired (UPD) , which activates the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway (Kiger et al. 2001; Tulina and Matunis 2001).

In contrast with what is observed in the GSCs of *Drosophila*, HSCs in mammals are maintained by multiple regulators expressed in the niche. Extrinsic signaling pathways, such as the Wnt and Notch pathways play fundamental roles in the niche-mediated regulation of HSCs (Reya et al. 2003; Duncan et al. 2005). These signals are also important for development. Overreaching signals, however, are detrimental. For example, although Wnt signaling was first reported to expand HSCs while maintaining their function (Reya et al. 2003) further investigation revealed that an overabundance of Wnt signaling leads to exhaustion of the long-term HSC pool (Kirstetter et al. 2006; Scheller et al. 2006). In contrast, an in vivo study demonstrated that the simultaneous deficiency of β-catenin, an essential factor for Wnt signaling, and γ-catenin, a close homolog of β-catenin, in conditional knockout (KO) mice did not impair HSC function (Koch et al. 2008). Similarly, adult HSCs can be maintained even under inactivation of Notch1 and its receptor Jagged1 (Mancini et al. 2005). In mammals, maintenance of stem cells and, ultimately, the organs in which they reside may not totally depend upon any single signal and different signals may compensate for each other in the case of functional impairment. Additionally, some signaling pathways impinge specifically on niche cells. For example, Zhang et al. showed that BMP signaling in osteoblasts controls the number of osteoblasts themselves, which leads to the regulation of the number of HSCs (Zhang et al. 2003).

Several cytokines and growth factors that are expressed by niche cells act on HSCs. Thrombopoietin (THPO), which is the ligand of the myeloproliferative

leukemia virus oncogene protein (Mpl), is a representative factor in the HSC niche. It also regulates megakaryogenesis. Jacobsen and our groups demonstrated that THPO/Mpl signaling is an important pathway in the regulation of HSC maintenance in the osteoblastic niche (Qian et al. 2007; Yoshihara et al. 2007). We showed that long-term HSCs that express Mpl are in close contact with THPO-producing osteoblastic cells, which lead to the maintenance of HSC quiescence. β1-integrin and the CDK inhibitor *p57*^{Kip2} are also upregulated in HSCs by THPO/Mpl signaling. The Jacobsen group found a 150-fold decrease in the number of HSCs in adult *Thpo*$^{-/-}$ mice. Moreover, HSCs in these mice were induced to enter the cell cycle and mRNA expression levels of the CDK inhibitors *p57*^{Kip2}, *p19*^{INK4D} as well as multiple *Hox* transcription factors were downregulated. They also demonstrated that fetal HSC expansion does not require THPO/Mpl signaling.

CXCL12/CXCR4 signaling plays a critical role in hematopoiesis during ontology (Ara et al. 2003). *Cxcl12*$^{-/-}$ mouse embryos exhibit a severe impairment in the homing of the HSCs into BM after their expansion in fetal liver and of the myeloid cells. As described by Sugiyama et al., CXCL12/CXCR4 signaling plays an essential role in the maintenance of HSCs in the adult BM (Sugiyama et al. 2006). CXCL12/CXCR4 signaling also regulates trafficking and mobilization of HSCs. Furthermore, this signaling has a role not only in hematopoiesis but also in metastasis and engraftment mechanism of malignant cells in BM (Zhang and Li 2008). Thus, CXCL12/CXCR4 signaling has multiple functions and is active in many types of cells, and as such deserves a greater level of attention and investigation.

2.3.2.3 Local Environmental Factors

Local environmental factors are also important niche factors. One of the major local components of the niche is oxygen, as the concentration of this molecule directly affects the production level of reactive oxygen species (ROS) in HSCs. The superoxide anion radical, hydrogen peroxide, and the hydroxyl radical are important ROS. These molecules can act as free radicals and cause DNA damage (Naka et al. 2008). Quiescent HSCs are kept in a hypoxic environment and protected from ROS-mediated oxidative damage. Increased ROS levels in multipotent HSCs in *Drosophila* cause precocious differentiation into mature red blood cells (Owusu-Ansah and Banerjee 2009).

Jang and Sharkis performed serial transplantations to demonstrate that HSCs with lower intercellular ROS level have a higher self-renewal potential (Jang and Sharkis 2007). In contrast, HSCs of the ROS^{high} population were exhausted during serial transplantation, which was rescued by treatment with the antioxidation agent N-acetyl-L-cysteine (NAC). This suggests that intracellular ROS levels play a crucial role in the control of the long-term self-renewal ability of HSCs.

The ataxia telangiectasia mutated (*Atm*) gene plays a role in genomic stability. *Atm* activates a key cell cycle checkpoint in response to DNA damage, telomeric instability, and oxidative stress. In *Atm*$^{-/-}$ mice, a smaller proportion of cells are found in the stem cell fractions. Colony formation capacity after long-term culture and long-term BM reconstitution capacities were also impaired in hematopoietic cells from *Atm*$^{-/-}$ mice

(Ito et al. 2004). Progressive BM failure following the defective maintenance of the adult HSC pool was observed in $Atm^{-/-}$ mice older than 24 weeks. This was associated with elevated ROS levels, and treatment with NAC recovered the function of $Atm^{-/-}$ HSCs and rescued the mice from BM failure. Further investigation revealed that the defect in the maintenance of HSC quiescence in $Atm^{-/-}$ mice resulted from phosphorylation of the p38 mitogen-activated protein kinase (MAPK) in HSCs, which was induced by elevation of ROS levels (Ito et al. 2006).

FoxOs regulate a variety of processes in cells, such as induction of cell cycle arrest, stress resistance, and apoptosis. To assess the role of FoxO family members in hematopoiesis, Tothova et al. deleted three *FoxOs* (*FoxO1*, *FoxO3*, and *FoxO4*) in mice in a conditional manner (Tothova et al. 2007). The deletion of these *FoxO*s led to the expansion of myeloid lineages and to the decrease in the number of hematopoietic stem/progenitor cells. Defective long-term repopulation activity with activated cell cycling, apoptosis, increased accumulation of ROS, and changes in the expression of genes that regulate ROS were also observed in HSCs in *FoxO*-null mice. These phenotypes were rescued by treatment with NAC, which suggests that FoxO proteins prevent the accumulation of oxidative stress (or ROS) in HSCs and thereby mediate the quiescence and maintenance of HSCs. Among all, *FoxO3* is the most highly expressed *FoxO* in the hematopoietic cells in BM. Detailed analysis of $FoxO3^{-/-}$ mice revealed that HSCs from these mice had a decreased ability to support long-term reconstitution of hematopoiesis (Miyamoto et al. 2007). $FoxO3^{-/-}$ HSCs did not remain in quiescence, were highly sensitive to cell cycle-dependent myelotoxic injury, and exhibited elevation of ROS levels. Furthermore, the defects in the activities of $FoxO3^{-/-}$ HSCs were rescued by NAC treatment (Yalcin et al. 2008). These studies collectively suggest that *FoxO3* is a key factor for maintaining HSC quiescence. The endosteal surface may be more hypoxic than the perivascular zone, and therefore, the endosteal zone seems to be better suited for maintaining quiescence of HSCs.

Signals received from the simple mineral content of the niche also regulate the behavior of HSCs. Calcium-sensing receptor (*Casr*) expressed on HSCs recognizes concentrations of calcium ions on the endosteal surface. In $Casr^{-/-}$ mice, HSCs in fetal liver show normal characteristics, and migrate and home to the BM (Adams et al. 2006). However, $Casr^{-/-}$ antenatal mice have few primitive hematopoietic cells in the BM because of defects in the ability of HSC to localize to the osteoblastic niche and to adhere to the ECM protein collagen I.

2.4 Intrinsic Programs in HSCs

The autonomous maintenance activity is essential for sustaining healthy HSCs. HSCs exhibit some characteristic expression profile of genes; for example, transcription factors which are expressed at a high level uniquely in HSCs play

important roles in determining their biological characteristics. These transcription factors may be divided into two categories; those that are required for self-renewal and maintenance of HSCs and those necessary for lineage-specific differentiation. Although the strict grouping of transcription factors into these two categories is difficult, runt related transcription factor 1 (*Runx1*) (Growney et al. 2005), ets variant gene 6 (*Etv6*, also known as *Tel*) (Hock et al. 2004b), T-cell acute lymphocytic leukemia 1 (*Tal1*, also known as *Scl*) (Endoh et al. 2002), homeobox B4 (*Hoxb4*) (Kyba et al. 2002), and Ecotropic viral integration site-1 (*Evi1*) (Goyama et al. 2008) have been shown to be critical for HSC self-renewal, maintenance, or both.

Homeobox transcription factors are important regulators of primitive hematopoietic cell proliferation and differentiation. In particular, *Hoxb4* is a strong positive regulator of HSC self-renewal. Antonchuk et al. demonstrated that over expression of *Hoxb4* in HSCs induces cell expansion in vitro while retaining the repopulating capacity (Antonchuk et al. 2002).

Evi1 is a member of the SET/PR domain family of zinc-finger transcription factors. The number of HSCs was severely reduced in *Evi1*$^{-/-}$ mouse embryos, which was accompanied by defects in the proliferative and repopulating capacities of these cells (Goyama et al. 2008). As a result, hematopoiesis was not maintained in *Evi1*$^{-/-}$ adult mice.

Bmi1 polycomb ring finger oncogene (*Bmi1*) also plays an important role in transcription. Its product, the polycomb complex protein Bmi-1, is essential for the self-renewal of hematopoietic and neural stem cells and its autonomous activity has been extensively studied. *Bmi1*$^{-/-}$ postnatal mice exhibited a remarkable reduction in the number of HSCs (Park et al. 2003). Moreover, there was a complete lack of HSC self-renewal capacity in *Bmi1*$^{-/-}$ adult mice. In addition, the expression pattern of genes associated with stem cell survival, transcription, or proliferation was altered in *Bmi1*$^{-/-}$ BM mononuclear cells.

Expressions of a certain transcription factors is not only differentiation stage-specific but often also developmental stage-specific. For example, the transcription factor *Sox17* is expressed in HSCs of fetal liver and in neonatal BM but downregulated in adult BM (Kim et al. 2007). In contrast, other transcription factors, such as *Gfi-1* (Hock et al. 2004a) and *Tel/Etv6* (Hock et al. 2004b), are important for the self-renewal and maintenance of adult HSCs but not during fetal development. It may be important to segregate the supportive molecular mechanisms that influence the capacities of HSCs, according to the physiological needs in each developmental stage.

In some tissue stem cell systems, the intrinsic program is sufficient for stem cell maintenance and differentiation. The anatomy of the intestinal crypt is uniquely suited for the study of tissue stem cells in their niche. Crypt base columnar cells which express Lgr5 are not quiescent stem cells; rather, they are stem cells dividing everyday (Barker et al. 2007). The Clevers group showed that single Lgr5^{+} build crypt – villus structures in vitro in the absence of the niche (Sato et al. 2009a).

2.5 Mobilization of Normal and Leukemic Stem Cells

Stem cells are mobilized from the niche. This process is not purely stochastic. Rather, it is well regulated by extrinsic signals that are derived from the surrounding cells. The decrease in the number of blood cells following the administration of cytotoxic drugs, especially the decrease of progenitor cells, initiate the exit of quiescent HSCs from the niche and their proliferation to fulfill the necessary mature cells.

Treatment of mice with G-CSF induces the efficient mobilization of HSCs to the peripheral blood. This mobilization includes serial events: exit of HSCs from the niche, egression from the BM, and shift to the marginal pool in the blood vessel. G-CSF has been shown to induce the release of proteolytic enzymes that cleave adhesion molecules, such as VCAM1, VLA-4, and CXCL12 (Petit et al. 2002). ROS may also be involved in the release of stem cells from the niche via the downregulation of adhesion molecules in HSCs (Hosokawa et al. 2007). Thus, stem cell mobilization is triggered by altering the niche microenvironment. Therefore, understanding the regulation of the niche will be important to improve BMT methodology or for developing cancer treatments that target quiescent cancer stem cells (CSCs).

Cancer tissues comprise stem cell-like cells (Bonnet and Dick 1997), which mimic the phenotypes and behavior of normal stem cells. Dick et al. termed these cells as CSCs or cancer-initiating cells (see Chap. 10). Small numbers of human CSCs can give rise to tumors in immunodeficient mice, whereas other progenitor-like cancer cells do not. CSCs exhibit niche dependency and slow cell cycling, similar to the normal stem cells (Ishikawa et al. 2007). These CSCs are chemoresistant and considered as the cause of relapse and metastasis.

Chronic myeloid leukemia (CML) is a chronic myeloproliferative disorder caused by the transformation of HSCs. Ito et al. investigated whether the treatment outcome of CML could be improved by forcing the quiescent leukemic stem cells (LSCs) into a cycling state using arsenic trioxide, which is a strong ROS generator (Ito et al. 2008). The number of quiescent LSCs decreased significantly after the arsenic treatment. More LSCs entered the cell cycle compared with normal stem cells after treatment with arsenic trioxide, which resulted in good effectiveness of chemotherapy in the mouse model. Similarly, interferon α (IFNα) signaling induces HSCs to enter the cell cycle (Essers et al. 2009; Sato et al. 2009b). Experiments performed in mice suggest that 5-FU-resistant normal HSCs can be killed efficiently by a 1–3 day pretreatment with IFNα. These data suggest that IFNα may also activate and sensitize LSCs for subsequent elimination by tyrosine kinase inhibitors, such as Imatinib. Exiting the niche leads to active cell cycling, and therefore, the combination of chemotherapy or radiation with the artificial induction of HSC mobilization may improve the therapeutic outcome in treatment of leukemia. However, the first pilot studies using G-CSF for LSC cycling did not show the effectiveness of this strategy (Drummond et al. 2009), and further optimization and improvement in protocols may be necessary (Fig. 2.4).

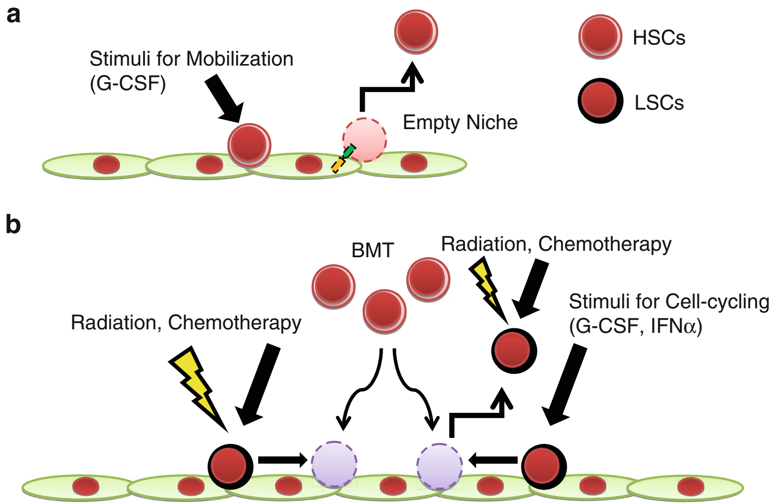

Fig. 2.4 A schema of HSC mobilization. (**a**) Mobilization stimuli (e.g., G-CSF) lead to the migration of HSCs from their BM niche, which results in empty niches. This characteristic can be applied to the therapy of leukemia. (**b**) LSCs mimic HSCs and reside in the BM niche to maintain the quiescent state. Radiation therapy or chemotherapy alone will not completely purge the cancer cells as some quiescent LSCs may survive and remain quiescent in the BM niche. These surviving LSCs may cause relapse of the disease. It may be possible to target these LSCs more effectively if they come out of niche and their cell cycle is activated. Development of efficient and safe methods to manipulate LSC maintenance by the niche is desired

2.6 Concluding Remarks

The niche is a cradle for stem cells and supplies both a special microenvironment and soluble factors that regulate stem cell function. In addition, under conditions that limit the supply of mature cells, niche cells and the factors they secrete can be modulated to activate stem cells and accelerate their differentiation.

The importance of niche regulation has been demonstrated in a disease model. Retinoic acid receptor γ (*RAR*γ)-deficient niche may lead to myeloproliferative syndromes, without any intrinsic defects in hematopoietic cells (Walkley et al. 2007). It has been reported that regulations by microenvironments are also involved in the development, maintenance, proliferation, and even metastasis of CSCs (Iwasaki and Suda 2009). These ongoing investigations suggest that the niche could be an important target for cancer therapies. From a biological and clinical perspective, the study of stem cell niches and their intrinsic programs is a promising field for the understanding of the nature of adult stem cells.

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Chapter 3
The Emerging Role of microRNAs in Adult Stem Cells

Jessica M. Shookhoff and G. Ian Gallicano

Abstract Since their discovery nearly two decades ago, microRNAs (miRNAs) have been found in a variety of organisms including plants, fish, and mammals and been shown to have an enormous impact on development and disease. This chapter provides a comprehensive overview of the biogenesis of miRNAs, their mechanisms of action, and their specific role in regulating self-renewal and lineage specification of embryonic and adult stem cells. In the latter case, emphasis is placed on the miRNA regulatory networks that mediate cellular differentiation. Their role in regulating cellular responses to stress and in various neurologic diseases, autoimmunity, and cancer are also described. Finally, a brief overview of methods used to analyze the function of miRNAs and identify their targets in vivo is provided.

Keywords miRNAs • siRNA • Embryonic stem cells • Adult stem cells • Neurogenesis • Osteogenesis • Hematopoiesis • Neural stem cells • Hematopoietic stem cells • Mesenchymal stem cells

Abbreviations

AGO	Argonaute
aOSCs	Adult osteogenic stem cells
ASCs	Adult stem cells
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
DHFR	Dihydrofolate reductase
Dnmts	DNA methyl transferases
EBs	Embryoid bodies
EpSCs	Epithelial stem cells

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ER	Estrogen receptor
ESC	Embryonic stem cells
GBM	Glioblastoma multiforme
HD	Huntington's disease
hESCs	Human embryonic stem cells
HSCs	Hematopoietic stem cells
Htt	Huntingtin protein
mESCs	Mouse ESCs
miRISC	miRNA-induced silencing complex
MSCs	Mesenchymal stem cells
MTX	Methotrexate
NB	Neuroblastoma
NSCs	Neural stem cells
nt	Nucleotides
NTD	Neural tube defects
piRNAs	PIWI RNAs
PIWI	P-element-induced wimpy testis
Pre-mi	Precursor miRNA
Pri-mi	Primary miRNA
PTBP1	Polypyramidine tract-binding protein 1
RA	Retinoic acid
REST	*Repressor* element 1 silencing *transcription* factor
RNAi	RNA interference
SCI	Spinal cord injury
siRNA	Silencing-RNAs
SOCS	Suppressor of cytokine signaling 3 SRF, serum response factor
SVZ	Subventricular zone
TRBP	HIV-1 transactivating RNA-binding protein

3.1 Introduction

In 1993, a seminal discovery by the laboratories of Victor Ambros and Gary Ruvkun revealed the existence of a small, noncoding stretch of RNA in *Caenorhabditis elegans*, dubbed *lin-4*, which regulated the translation of *lin-14* mRNA into protein via an antisense RNA–RNA interaction (Lee et al. 1993). In 2000, Gary Ruvkun's laboratory discovered another of these small, noncoding RNAs, *let-7*, that regulated the translation of *lin-41* in a similar manner (Reinhart et al. 2000). Both of these papers showed that loss of either *lin-4* or *let-7* caused retention of early cell types, establishing a role for these RNAs in developmental timing and the generation of mature cell lineages. In 2008, both Victor Ambros and Gary Ruvkun, along with David Baulcombe, won the Lasker award for their contribution to the understanding of the RNA interference (RNAi) pathway (McGreevey 2008). An interview of these researchers discussing their discoveries can be viewed at the

Lasker Foundation website: http://www.laskerfoundation.org/awards/2008_b_interview_ambros.htm

Only 18–22 nucleotides (nt) long, the cumulative research of the past 17 years has shown that these tiny RNAs, christened microRNAs (miRNAs), have an enormous impact on development, disease, and even drug sensitivity (Lee et al. 1993; Reinhart et al. 2000; Bilen et al. 2006; Ding and Han 2007; Mishra et al. 2007). They have been found in a variety of organisms including plants, worms, fish, and mammals (Ding and Han 2007). While the majority of research so far has focused on embryonic stem cells (ESCs) and early developmental time points, more research is beginning to explore the role of miRNAs in pluripotent cell populations. An understanding of miRNAs and their diametric functions in the maintenance of pluripotency and in differentiation should lead to a better explanation of how adult stem cell (ASC) populations are able to persist in a fully mature organism. An exciting potential of miRNAs is the possible means of stimulating and differentiating a patient's own ASC population into cell types that can repair or replace cells lost to traumatic injuries, disease, or genetic defects.

3.2 microRNA Biogenesis

To date, 721 experimentally validated miRNAs are known to exist within the human genome (Griffiths-Jones 2004; Griffiths-Jones et al. 2006, 2008), and they are predicted to regulate translation of at least 30% of all protein-coding mRNAs (Mishra et al. 2007; Ding and Han 2007; Grimson et al. 2007) and perhaps even up to 90% of human mRNAs (Ding and Han 2007). miRNAs can exist in the genome singly, under the control of its own promoter, or they can be located within clusters that are transcribed polycistronically (Sonntag et al. 2005). They have been found to be present in introns, exons, and the noncoding regions of the genome (Gangaraju and Lin 2009; Lau et al. 2008) and are subject to regulation via methylation by an order of magnitude more than protein-coding genes, implying that their expression is under tight control (Weber et al. 2007; Lujambio et al. 2007; Han et al. 2007).

The biogenesis of many miRNAs begins the same way it does for protein-coding genes: the formation of a transcription complex involving RNA Polymerase II (RNA Pol II) (Hammond and Sharpless 2008). In fact, many miRNAs are under direct regulation of well-known transcription factors, such as serum response factor (SRF), c-Myc, and E2F (Hammond and Sharpless 2008). However, up to 20% of miRNAs may be transcribed by RNA Pol III (Daskalova et al. 2007; Borchert et al. 2006), which also transcribes tRNA, 5S rRNA, 7SL RNA, and other small, noncoding RNAs (Daskalova et al. 2007). These miRNAs may be regulated by less traditional or well-known transcription factors.

Whether transcribed by RNA Pol II or RNA Pol III, the miRNA transcription product is hundreds or thousands of nucleotides long and is known as primary miRNA (pri-miRNA or pri-mi), which folds into a long double-strand stem-loop structure (Hammond and Sharpless 2008; Suh et al. 2004). Pri-mis are posttranscriptionally modified in a similar manner to protein-coding mRNAs in that they

undergo splicing and polyadenylation (Gangaraju and Lin 2009). They are then processed in the nucleus into hairpin precursors (pre-miRNA or pre-mi) by the collaboration of at least two enzymes: DGCR8, a double-strand RNA-binding protein that is specific to miRNA processing (Gangaraju and Lin 2009; Wang et al. 2007), and an RNaseIII enzyme, Drosha, that selectively cleaves a large terminal loop of about 10 nt on the pri-miRNA two helical turns into the stem (Suh et al. 2004; Wang et al. 2007; Kosik 2006). The cleavage product is approximately 70–100 nt long and also folds into a stem-loop structure with a 2 nt overhang (Gangaraju and Lin 2009; Suh et al. 2004; Kosik 2006). The pre-mi is then exported to the cytoplasm by the exportin 5:Ran-GTP complex where it undergoes further processing by another enzyme pair (Gangaraju and Lin 2009; Kosik 2006) and Dicer (Suh et al. 2004; Kosik 2006; Bernstein et al. 2003). Pre-mis may also arise from the introns of protein-coding mRNAs via splicing at the spliceosome complex in the nucleus (Gangaraju and Lin 2009). These pre-mis, generated without processing by Drosha, are referred to as mitrons (Gangaraju and Lin 2009).

Similar to the nuclear pri-mi processing complex, HIV-1 transactivating response RNA-binding protein (TRBP), a double-strand RNA-binding protein (Ding and Han 2007; Wu and Belasco 2005), and Dicer, another RNaseIII enzyme, cleave the loop end of the pre-mi, creating an asymmetric RNA duplex consisting of a guide strand and a passenger strand (Ding and Han 2007; Kosik 2006; Wu and Belasco 2005). The passenger strand is usually degraded and only found at low levels in the cytoplasm, while the guide strand is retained and mediates miRNA:mRNA-regulation (Kosik 2006). Human Dicer is known to be a general component of the RNAi program, processing both silencing-RNAs (siRNA) as well as miRNA (Bilen et al. 2006; Bartel 2004).

While siRNA and miRNA are both small RNAs that perform similar biochemical functions, there are a few important distinctions. First, siRNAs are derived from long, completely double-strand RNA or extended stem-loop transcripts, while miRNAs are processed from a partially double-strand pri-mi that forms local stem-loop structures (Bartel 2004). Second, multiple siRNA duplexes can be processed from precursor siRNAs (pre-siRNA or pre-si), while only a single miRNA:miRNA* duplex is processed from the pre-mi (where * denotes the passenger strand that is usually degraded) (Bartel 2004). Third, endogenous siRNA does not seem to show any conservation between related organisms, while a subgroup of miRNAs is highly conserved between related organisms and even over a wide range of species. For example, miR-124 is completely conserved from worm to human (Bartel 2004; Visvanathan et al. 2007). Fourth, siRNAs are involved in "auto-silencing" where they often target the locus from which they were derived, whereas miRNAs are involved in "hetero-silencing" where they target different genes than the ones with which they were coexpressed (Bartel 2004).

3.3 microRNA Mechanisms of Action

miRNA is generally thought of as a posttranscriptional silencing mechanism that downregulates the translation of target mRNAs, although it has been shown in at least one instance that miR-10a can upregulate translation of specific ribosomal

proteins known as 5′ TOP proteins (Ørom et al. 2008). In order to mediate down-regulation, they must first be loaded into the miRNA-induced silencing complex (miRISC) (Ding and Han 2007). The miRISC contains an Argonaute (AGO) family member, which in humans can be AGO1-4, with AGO2 capable of mediating both siRNA and miRNA repression of mRNA. AGO1-4 contain a PAZ (polyubiquitin-associated zinc finger) domain and a PIWI (P-element induced wimpy testis) domain that serves to bind RNA (Ding and Han 2007; Kosik 2006). TRBP, originally identified in the processing of the HIV transactivating response RNA, has been shown to form a complex with Dicer, stabilizing it as well as recruiting AGO2 (Ding and Han 2007; Wu and Belasco 2005). Other proteins shown to be involved in miRISC formation include the fragile-X-related protein, the mammalian Gemin helicase family (Dostie et al. 2003), Rck (p54) (Ding and Han 2007; Kosik 2006), and GW182 (Ding and Han 2007).

Once the miRISC complex has been formed, the miRNA is directed to its mRNA target for binding (Ding and Han 2007; Kosik 2006; Cao et al. 2007). Binding between the miRNA and its target on the 3′ (UTR) of the mRNA is mediated by a region at the 5′ end of the miRNA known as the seed region, which usually resides between nucleotides 2–8 on the miRNA (Ding and Han 2007; Grimson et al. 2007; Cao et al. 2007). miRNAs with similar seed regions are usually grouped into families and often miRNA genes that are clustered together in the genome have similar seed regions (Foshay and Gallicano 2009). Binding at the seed region usually must have complete complimentarity between the miRNA and its target for efficient, thermodynamically stable interaction (Cao et al. 2007; Foshay and Gallicano 2009). The binding between the rest of the miRNA and the mRNA can either be completely complimentary (as is often the case in plants) (Ding and Han 2007; Grimson et al. 2007; Cao et al. 2007) or can include a loop of 2–3 nt long with variable pairing between the rest of the miRNA 3′ end and its target (Cao et al. 2007).

While a great emphasis has been placed on the interaction between the target mRNA and the miRNA seed region, other factors have recently been shown to be just as important in mediating binding (Grimson et al. 2007; Cao et al. 2007). These factors will be discussed in more detail in the section on using miRNA target prediction algorithms.

The degree of complimentarity between a miRNA and its target determines the mechanism of action. Complete complimentarity between a miRNA and its target mRNA will result in the cleavage of the target. In humans, this cleavage can only be carried out by AGO2 since only AGO2 has a functional PIWI domain (Ding and Han 2007; Kosik 2006). Functional PIWI domains have nuclease activity that can cleave the target mRNA between the positions that correspond to nucleotides 10 and 11 on the miRNA (Ding and Han 2007; Cao et al. 2007). This is fairly rare in animals, and instead, is seen more often in plants (Ding and Han 2007). In animals, the presence of a loop between nucleotides 9–11 of the miRNA thwarts this cleavage because it prevents formation of the miRNA:mRNA duplex into an A-form double-strand helix (Cao et al. 2007). Instead, other mechanisms of repression must occur to halt translation of the target mRNA (Ding and Han 2007; Cheung et al. 2009).

In order for these other mechanisms to be carried out, the miRISC must first localize to cytoplasmic RNA-processing bodies (P bodies) (Ding and Han 2007; Kosik 2006); this localization is dependent upon the miRISC protein GW182, without which miRISCs can neither localize to P bodies, nor repress target mRNA translation. Once at the P body, mRNAs can be repressed by interruption of the assembly of the ribosomal complex prior to initiation (Ding and Han 2007; Cheung et al. 2009). This may occur through AGO2 which has a cap-binding domain capable of binding the 5′ cap of the target mRNA, preventing its loading onto the ribosomal complex (Kiriakidou et al. 2007). Other forms of repression include disruption of elongation, degradation of the newly formed protein, polyadenylation of the poly(A) tail, promoting mRNA degradation, or degradation through the general mRNA decay machinery (Ding and Han 2007; Cheung et al. 2009).

Almost all of the current research focuses heavily on miRNA targeting the 3′ UTR of the mRNA; however, recently, it was shown that this convention is too narrow in scope. miR-10a was discovered to bind to the 5′ UTR of a group of ribosomal proteins called 5′ TOP proteins due to a specific motif in the 5′ UTR that renders them sensitive to cellular stress and a decrease in available nutrients (Ørom et al. 2008). The usual response of ribosomal protein mRNAs to nutrient deprivation is disassociation from active polysomes to inactive ribonucleoprotein complexes, but in the presence of miR-10a these mRNAs remained actively translated, illustrating that miR-10a has the capacity to affect global protein synthesis during periods of cellular stress (Ørom et al. 2008). Indeed, repression of miR-10a via an inhibitor led to a 40% loss of total protein, while transfection of cells with an exogenous miR-10a mimic led to a 30% increase in total protein. Interestingly, the authors of this study also showed that binding of miR-10a to the 5′ UTR did not involve complete seed region base-pairing, revealing evidence of nonconventional miRNA binding.

Further evidence for nonconventional miRNA binding came from a recent study that examined synchronized binding in both the 3′ and 5′ UTRs forming miRNA:mRNA duplexes designated miBridges (Lee et al. 2009). While this research did not clearly delineate whether one miRNA was simultaneously binding the 3′ and 5′ UTRs, or whether multiple copies of the same miRNA were binding both UTRs, it did show that binding of both UTRs acted synergistically to enhance repression. Another fascinating point not expounded on was the possibility of miRNAs to bind more than one target mRNA at a time. This research showed that miR-34a had validated binding sites in both the 3′ and 5′ UTRs of AXIN2, a mediator of the Wnt signaling pathway (Lee et al. 2009). The research further showed that Wnt1 had a nonconventional miR-34a binding site in its 5′ UTR. This raises an interesting question? If indeed miRNAs can simultaneously bind to the 3′ and 5′ UTRs of an mRNA, forming a miBridge, then perhaps a single miRNA can simultaneously bind two targets at a time, forming a triple RNA helical structure. More research will need to be conducted to validate the existence of miBridges and explore the possibility of miRNAs targeting two mRNAs concurrently.

3.4 The Role of miRNAs in Embryonic Stem Cells

As noted in Chen et al. (2004), “stem cells exhibit a ‘promiscuous beginning’ a so-called priming state in which many lineage-specific genes required for subsequent unique lineages are coexpressed. Selective gene silencing might be a key even during subsequent . . . lineage differentiation events.”

ESCs are capable of differentiating into more than 200 specific cell types, making them an invaluable tool for ascertaining the process of differentiation and the acquisition of specific cell fates (Cao et al. 2008; Gu et al. 2008). The central mystery of ESCs is twofold: how do they maintain their undifferentiated state and how are they capable of proliferating far more rapidly than other cell types?

Crucial to the increased proliferation capacities of ESCs is a shortened G1 phase that precedes DNA synthesis and cell division (Wang et al. 2008; Kim 2008). This shortened cycle is critical to developing organisms because it allows them to attain the proper cell density to form all the various tissues and organs before the onset of differentiation (Wang et al. 2008). One of the best-studied models for maintenance of pluripotency, differentiation, and the role of miRNAs in ESCs is murine (mouse) ESCs (mESCs). In mESCs it has been shown that the cyclinE-Cdk2 complex facilitates the G1–S transition, and its inhibition by Cdkn1a (p21) prolongs cells in G1 and delays the shift to S-phase (Wang et al. 2008; Kim 2008). Maintenance of stem cell plasticity depends, in part, on down-regulation of p21 by the mESC-specific miR-290 cluster (Wang et al. 2008; Kim 2008; Sinkkonen et al. 2008) (Fig. 3.1).

The miR-290 cluster begins expression in the zygote, increasing during preimplantation, peaking in the blastocyst, and remaining high in undifferentiated mESCs (Kim 2008; Sinkkonen et al. 2008). Another important target of this cluster in mESC is the Rbl2 protein, another inhibitor of the cyclin E-Cdk2 complex (Han et al. 2007; Wang et al. 2008; Kim 2008) (Fig. 3.1). Interestingly, two separate studies concluded that Rbl2 also inhibited expression of three DNA methyltransferase genes, Dnmt3a, Dnmt3b, and Dnmt3l (Sinkkonen et al. 2008; Benetti et al. 2008). Both Dnmt3a and Dnmt3b act by adding methyl groups (CH_3) to cytosines that

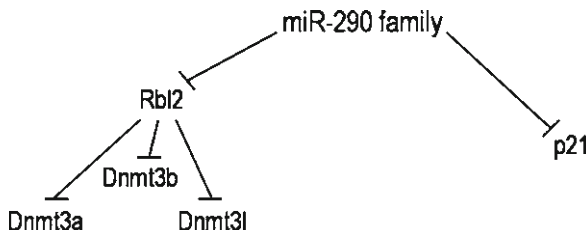

Fig. 3.1 miRNAs expressed in ES cells. The mESC-specific microRNA family, miR-290, targets p21, a cell cycle progression inhibitor as well as Rbl2. Rbl2 targets the DNA methyltransferases Dnmt3a, Dnmt3b, and Dnmt3l and suppresses their actions, thereby repressing de novo genomic methylation

exist in CpG dinucleotides, which is thought to play a role in decreasing transcription of the methylated area, with Dnmt3l facilitating the formation of Dnmt3a–Dnmt3b complexes in the nucleus. The ability to methylate DNA is crucial to ESCs in general because it allows the cells to initiate the silencing of genes that maintain pluripotency, such as *Oct4*, *Sox2*, *Nanog*, and *Tcf3*, to name a few, and prepare for the onset of differentiation (Kim 2008; Sinkkonen et al. 2008). Methylation, via Rbl2-suppression by the miR-290 family, also helps maintain the proper organization of the telomeres, the cellular clocks that control the overall life of cells (Benetti et al. 2008). Conversely, it is this methylation that lays the foundation for initiation of differentiation, as eventually even the pluripotency genes, such as *Oct4*, become methylated and silenced (Gangaraju and Lin 2009). Since mESCs in vitro maintain their pluripotency even with high miR-290 family expression, some other factor must prevent the methylation of pluripotency genes until a specific differentiation signal is encountered. The comprehensive elucidation of this dual role has yet to be completed.

Another interesting fact is the high expression of predicted miR-290 mRNA targets in the oocyte, but not the blastocyst or more differentiated cells (Sinkkonen et al. 2008). An overall role of the miR-290 cluster may be to restrict the embryonic expression of genes that are already highly maternally expressed in the oocyte, avoiding overexpression of these genes which could be deleterious to the zygote. Other miRNA families are expressed in an ESC-specific manner, such as the miR-17/20/93/106 cluster and the miR-29 family, but they do not appear to be as important to regulation of methylation in ESCs as is miR-290 (Sinkkonen et al. 2008).

Maintenance of pluripotency is another important factor in the relationship between the transcriptional repressor, *repressor* element 1 silencing *transcription* factor (REST), and miR-21 (Singh et al. 2008). REST mediates suppression via binding to a conserved repressor element (RE1) present in the regulatory elements of target genes (Visvanathan et al. 2007; Wu and Xie 2006). REST contains a DNA-binding domain as well as two domains that interact with its corepressors, CoREST and mSin3a, which in turn recruit the methyl DNA-binding protein, MeCP2 (as well as other silencing cofactors that alter chromatin structure), into an inactive form (Wu and Xie 2006). Initially thought to be expressed only in neural stem cells (NSCs), REST has now been shown to play a role in cardiogenesis, hematogenesis, oncogenesis, tumor suppression, and self-renewal in mESCs (Visvanathan et al. 2007; Singh et al. 2008; Wu and Xie 2006). It is highly expressed in the inner cell mass (ICM) of blastocysts and is predicted to take part in the ESC-specific transcription factor Oct4-Sox2-Nanog network (Wu and Xie 2006). These same transcription factors have been found at the promoters of ESC-specific miRNAs, indicating a positive feedback loop between ESC-specific transcription factors and ESC-specific miRNAs (Kim 2008). An important target of REST is the *miR-21* gene, which is expressed at low levels in ESCs, but is highly upregulated upon differentiation and predicted to target both *Sox2* and *Nanog* mRNA 3′ UTRs (Singh et al. 2008; Krichevsky and Gabriely 2009). In an unprecedented study, Singh et al. (2008) showed that not only did addition of exogenous REST protein maintain pluripotency in mESCs when grown under differentiating conditions, but also that

inhibition of miR-21 alone was sufficient to prevent differentiation. These results, however, are not without controversy, and the work of Singh et al. (2008) is hotly contested by Jørgensen et al. (2009) as well as Buckley et al. (2009) who claim that their labs found no evidence of regulatory relationship between REST and miR-21. While this debate has yet to be resolved conclusively, the research by Singh et al. (2008) has been convincing enough to be cited by many papers including noted microRNA researcher Anna Kirchevsky (Krichevsky and Gabriely 2009). The debate between Jørgensen et al. (2009), Buckley et al. (2009), and Singh et al. (2008) can be accessed via the following web pages.

http://0-www.nature.com.library.lausys.georgetown.edu/nature/journal/v457/n7233/pdf/nature07783.pdf
http://0-www.nature.com.library.lausys.georgetown.edu/nature/journal/v457/n7233/pdf/nature07784.pdf
http://0-www.nature.com.library.lausys.georgetown.edu/nature/journal/v457/n7233/full/nature07785.html

Human embryonic stem cells (hESCs) also express specific miRNAs and over 100 have been shown to be differentially expressed between hESCs and embryoid bodies (EBs; EBs represent the first step in differentiation of ES cells) (Xu et al. 2007). While the miRNA expression profile of hESCs is well known, their downstream mRNA targets are not.

The miR-371 family from chromosome 19 (miR-371, -372, -373, and -373*) are the human homologs of the mESC miR-290 family (Suh et al. 2004; Blakaj and Lin 2008; Laurent et al. 2008). Another commonly expressed miRNA family is comprised of miR-430 (in telosts, such as zebrafish), miR-427 (in amphibian, such as xenopus), and miR-302 (in mammals, such as mice and human), which all share the same 5v seed sequence and similarity in their 3′ region (Rosa et al. 2009). While each is required for full development, they differ in the impact their loss has on the embryo. For instance, loss of miR-430 in zebrafish does not manifest defects until late in development, near organogenesis, while in xenopus miR-427 is necessary for proper endoderm and mesoderm differentiation, organizer formation, and body axis specification (Rosa et al. 2009). Interestingly, miR-302 was shown to have a dual effect by maintaining the expression of *Oct4* and *Nanog,* while also promoting proper mesoderm and endoderm formation. This seemingly diametric function of miR-302 may be a similar theme in ESC-specific miRNAs, where embryonic conditions lead them to facilitate pluripotency, while differentiating conditions cause them to take on the role of promoting lineage-specific cell fates. A dual role for cellular regulators is not uncommon and is often found in transcription factors.

Another important miRNA/protein relationship in the maintenance of stem cell pluripotency is that between LIN28 and the miRNA, let-7. Lin28 along with Oct4, Sox2, c-Myc, and Klf4 are some of the few factors that can provoke somatic cells to dedifferentiate into induced pluripotent stem (iPS) cells, which exhibit remarkably similar properties to ESCs (Gangaraju and Lin 2009; Xu et al. 2009; Takahashi and Yamanaka 2006; Heo et al. 2009). Lin28 promotes pluripotency in part by recruiting TUT4 to pre-let-7, which adds a poly(U)-tail to its 3′ end, blocking

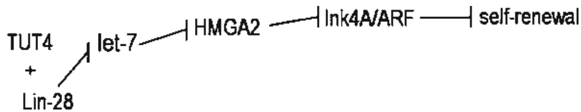

Fig. 3.2 Role of miRNAs in self-renewal. Lin28 represses let-7 function via recruitment of TUT4 which uridylates pre-let-7 on its 3′ end, blocking processing by Dicer. HMGA2, which facilitates maintenance of pluripotency in stem cell populations by repressing Ink4 and ARF expression, is a target of let-7. Thus, by suppression of HMGA2 translation, let-7 is able to promote differentiation by derepressing Ink4 and ARF, potent inhibitors of self-renewal

processing of pre-let-7 into a mature miRNA by Dicer (Gangaraju and Lin 2009; Heo et al. 2009). Lin28 is also able to induce polyuridylation to at least three other miRNAs: miR-107, -143, and -200c (Heo et al. 2009). Down-regulation of let-7 has important stem cell renewal implications, since let-7 targets the chromatin-associated protein, HMGA2, which is shown to be an important regulator of self-renewal in both embryonic and ASC populations (Hammond and Sharpless 2008). Expression of HMGA2 declines as cells age and coincides with loss of proliferative capacity (Fig. 3.2).

Does this mean, then, that miRNAs are necessary for maintenance of pluripotency in stem cells? Surprisingly, the answer is no. This startling conclusion comes from a series of experiments that knocked out either Dicer or DGCR8 in ESCs (Wang et al. 2007, 2008; Gu et al. 2008; Kim 2008; Sinkkonen et al. 2008; Benetti et al. 2008; Singh et al. 2008; Blakaj and Lin 2008; Ivey et al. 2008). The main defect observed in these studies was actually an inability to differentiate, as well as prolonged population doubling times. What then is the purpose of miRNA expression in ESCs? As stated earlier, they appear to mainly contribute at the embryonic stage by facilitating bypass of the G1–S checkpoint, allowing for rapid proliferation. miRNAs also appear to repress expression of genes in the zygote that are already highly expressed in the oocyte. In addition, they may prepare the zygote for differentiation by widespread, nonspecific genomic methylation, which is later removed only in the regions of lineage-specific genes, conferring mature cell and tissue identity while maintaining repression of nontissue genes (Fouse et al. 2008).

The major role that miRNAs appear to play is that of guiding cues for initiation of differentiation, the acquisition of lineage-specific cell fate, and the transition from stem cell population to pluripotent population to progenitor population to mature cell type. We will now examine how undifferentiated cells attain cell specificity via miRNAs and the role they may play in the self-renewal capabilities in ASC populations.

3.5 The Role of miRNAs in Differentiation of Adult Stem Cells

The role of miRNAs in ESCs is more clear than that of ASCs in part because most distinct ASCs are not well-characterized in relation to surrounding progenitor cells, and also because obtaining pure ASC populations in which to conduct miRNA

expression studies is extremely difficult (Hammond and Sharpless 2008). At present, the best information upon which to base further inquiry for the role of miRNAs in ASCs are the roles that have been defined for them during development, as progenitor and mature cell types emerge.

ESC and ASC self-renewal and subsequent differentiation is mediated, in part, by conserved clusters of miRNAs that are expressed in a lineage-specific manner (Gangaraju and Lin 2009). miRNAs form a fluid regulatory network whereby their expression is induced or suppressed by transcription factors, many of which are themselves targets of miRNAs. Many individual miRNAs are expressed in a variety of tissues and cell types, calling into question a purported role in the specification of various cell types (Harris et al. 2008). However, it may be the particular combinations of miRNAs expressed, either concurrently or consecutively, that endow specificity, not any particular individual miRNA (Harris et al. 2008; Li et al. 2008).

3.5.1 ES to EB Transition: The Beginnings of ASCs

The first step in maturation in vitro is from undefined ESCs to EBs, which, to a degree, recaptures in vivo embryonic development (Ivey et al. 2008). miRNA expression dramatically changes as cells begin to lose their embryonic plasticity starting with the down-regulation of the miR-290 family and the concomitant increase of other differentiation-associated miRNAs (Sinkkonen et al. 2008). The miR-17 family is one of the most conserved families and is thought to have evolved along with vertebrates and is perhaps best known for its abnormal expression in cancer cells (Foshay and Gallicano 2009; Hudder and Novak 2008). This family, which is activated by c-Myc (an effector in pluripotency in ESC as well as an oncogene), is expressed at low levels in mESCs, but increases significantly upon the differentiation of gastrulating cells into mesoderm and mesendoderm (Foshay and Gallicano 2009; Hudder and Novak 2008). This family targets a 3′ UTR of a downstream transcription factor of the JAK/STAT signaling pathway, STAT3, which is important in early development as well as mesoderm specification. STAT3 acts as a transcriptional activator upon phosphorylation and, while its activation is not required for maintenance of hESC populations, it is still highly expressed; therefore its down-regulation is a necessary component in the initiation of differentiation, not only in ESCs but hematopoietic stem cells (HSCs) and NSCs (Foshay and Gallicano 2009). Mouse embryos that do not express STAT3 die upon initiation of gastrulation at embryonic day (E) 7.0, indicating that STAT3 plays dual roles in the maintenance and down-regulation of pluripotency (Foshay and Gallicano 2009).

miR-145 also plays an important role in the initiation of differentiation in hESC by targeting the 3′ UTRs of Oct4 and Klf4, fully downregulating them, as well as partially downregulating Sox2 (Xu et al. 2009). miR-145, whose seed sequence is conserved from zebrafish to humans, is involved in a reciprocal relationship of repression with Oct4, which binds to its promoter preventing its expression

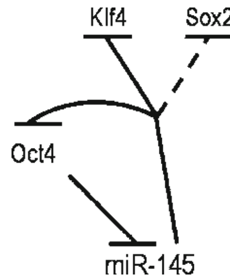

Fig. 3.3 Regulation of pluripotency genes by miRNAs. miR-145 helps ESCs transition to EBs by downregulating Oct4 and Klf4, important pluripotency genes, and also partially downregulates Sox2. miR-145 is repressed by the binding of Oct4 to its promoter elucidating one means by which Oct4 helps to maintain pluripotency in stem cells

(Fig. 3.3). miR-145 transitions ESCs to EBs by shortening the prolonged S-phase of hESCs and promotes acquisition of early ectoderm and mesoderm lineages. Most interestingly, addition of miR-145 alone is enough to promote differentiation in hESC even in hESC promoting growth conditions, and conversely, its loss significantly reduces differentiation (Xu et al. 2009).

3.5.2 Cardio- and Myogenesis

As cells differentiate, they progressively mature and become more specialized. One of the first cell types to arise in EBs is cardiac cells, visibly beating by day 7 of differentiation from mESCs (Ivey et al. 2008). Both miR-1-1 and miR-1-2 contribute to the cardiac phenotype and are expressed by E8.5 in the mouse in a proximal manner, with miR-1-1 specific to the looping heart tube and atria while miR-1-2 is specific to the ventricles (Zhao et al. 2005). These miRNAs, which are highly conserved from flies to humans, are cooperatively induced by SRF and myocardin, and both appear to target a central transcription factor, Hand2 (Gangaraju and Lin 2009; Ivey et al. 2008; Zhao et al. 2005). These two miRNAs are also highly expressed in the somites that flank the developing neural tube, with expression peaking around E11.5 (Zhao et al. 2005) (Fig. 3.4).

miR-1 also plays a role in skeletal muscle differentiation along with miR-133, with which it is clustered together on both mouse chromosomes 2 and 18 (Kwon et al. 2005). Although they are transcribed together, miR-1 and miR-133 have different roles in developing skeletal cells. miR-1 pushes C2C12 mouse myoblast cells toward terminal differentiation, while miR-133 actually retains myoblast proliferation (Kwon et al. 2005). miR-1 guides myogenesis by repressing HDAC4, an inhibitor of the muscle-specific transcription factor Mef2c (Kwon et al. 2005). The 3′ UTR of HDAC4 has two putative miR-1-binding sites that are conserved across vertebrates (Gangaraju and Lin 2009). miR-133, on the other hand, targets the 3′ UTR of SRF, revealing a negative regulatory feedback loop which may help to

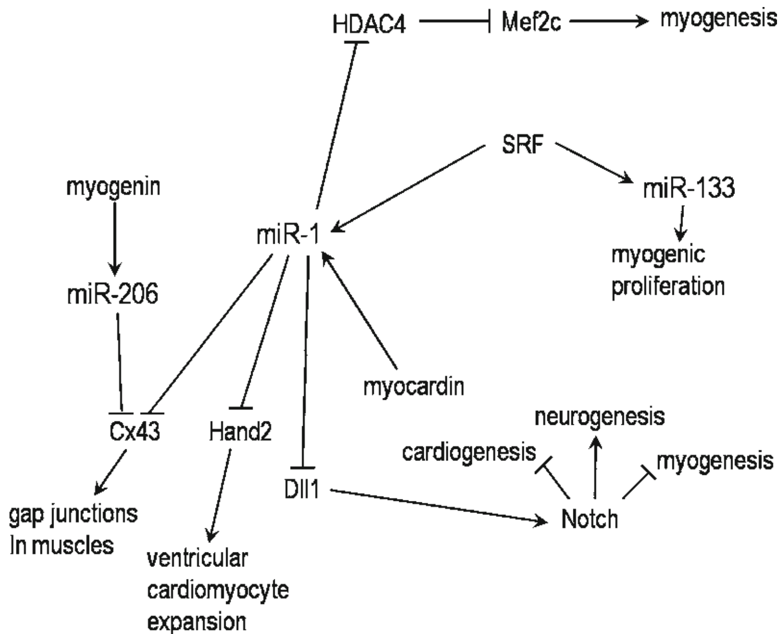

Fig. 3.4 Role of miRNAs in myogenesis. miR-1 and miR-133 play a role in acquisition of skeletal muscle identity. Both miR-1 and miR-133 are induced by SRF. miR-133 holds cells in myogenic progenitor state by means of an unknown target, while miR-1 promotes terminal myogenic differentiation by repression of HDAC4, an inhibitor of Mef2c, a muscle-specific transcription factor that promotes myogenesis. miR-1 is also induced by MyoD and myocardin and also facilitates cardiogenesis by repression of Dll1, a Notch ligand. Notch promotes neurogenesis at the expense of myogenesis and cardiogenesis. miR-1 may also promote atrial cardiomyocyte expansion by repression of Hand2, which induces ventricular cardiomyocyte expansion. miR-206 is stimulated by myogenin and cooperatively suppresses Cx43 with miR-1. Cx43 aids in gap junction formation that is essential in early muscle development, but which later become detrimental as terminal differentiation occurs

maintain the proliferative qualities of myoblasts until other factors emerge to induce terminal myogenesis (Kwon et al. 2005). miR-1 and miR-133 work together to suppress endoderm and neural differentiation in mESCs, with miR-1 specifically targeting Dll1, a Notch ligand that promotes neurogenesis (Ivey et al. 2008). The regulatory roles of miR-1 and miR-133 were shown to be similar in hESCs (Ivey et al. 2008), as well as in *Drosophilia*, where miR-1 also targets the *Drosophilia* homolog to Dll1, Dll (Gangaraju and Lin 2009), revealing further conservation of miR-1 function (Kwon et al. 2005).

Another important miRNA is miR-27b, which acts earlier in skeletal muscle specification (Crist et al. 2009). Skeletal muscle progenitors are defined by expression of Pax3 and Pax7, in both the developing embryo as well as the adult progenitors that repair muscle damage, and miR-27b mediates the shift from myogenic progenitors to mature muscle cell in both embryonic myoblasts and adult satellite cells by targeting and decreasing translation of Pax3 (Crist et al. 2009).

Crist et al. (2009) represent one of the few studies that specifically looked at the actions of a miRNA on an ASC population. They also looked at how expression of miR-27b affected the ability of satellite cells to repair muscle injuries in mice. Inhibition of miR-27b increased the Pax3+/Pax7+ cells (e.g., progenitor cells) by 30%, while decreasing muscle fiber size, indicative of a delay in repair. This study demonstrates continuity in miRNA expression and function between embryonic and adult progenitor populations.

miR-1 also plays an important role in myogenesis by interacting with miR-206 to repress translation of Cx43, which forms gap junctions essential for normal development early in muscle formation, but must be downregulated for terminal differentiation (Gangaraju and Lin 2009). Two other miRNAs involved in myogenesis are miR-26a and miR-24 (Gangaraju and Lin 2009). miR-26a inhibits a histone methyltransferase that silences the muscle-specific gene EZH2, while miR-24 is known to play a general role in myogenesis and can be suppressed by the binding of SMAD3 and SMAD4 to its promoter in a TGFβ-dependent manner.

3.5.3 Neurogenesis

A well-studied area regarding miRNAs is neurogenesis and, while there is much information of changes in miRNA expression and some information of miRNA interactions with targets during neurogenesis, an overall comprehensive understanding has yet to be established.

Neural differentiation is miRNA-dependent as shown by Dicer ablation during embryogenesis in the mouse telencephalon (De Pietri Tonelli et al. 2008). Dicer ablation in the mouse telencephalon led to formation of a smaller, massively disorganized hippocampus as well as a hypertrophic cortex. Loss of Dicer, and therefore miRNAs, in various subpopulations of neurons impairs differentiation and causes degeneration and apoptosis in the ventricular zone (VZ) and the subventricular zone (SVZ), the birthplaces of neurons. Surprisingly, neuroepithelial cells were mostly unaffected by Dicer ablation, while the neural progenitors and neurons were dramatically affected, revealing that when cells alter their state they increase their dependence on miRNAs (De Pietri Tonelli et al. 2008).

mESCs can be strongly induced to differentiate to multiple neural cell types by addition of *all-trans* retinoic acid (RA) which upregulates many miRNAs including let-7a, let-7c, let-7e, miR-9/9*, miR-22, miR-23, miR-124, miR-125, and miR-128, to list just a few (Krichevsky et al. 2006; Smirnova et al. 2005). miR-9 and miR-124 have been shown to decrease the phosphorylation of STAT3, most likely by binding of upstream factors, and their addition to differentiating neural precursor cells shifts cell fate from astrocytes to neurons (Krichevsky et al. 2006). miR-23 is specific to astrocytes in the mouse brain, while miR-124, miR-125, and miR-128 were expressed in neurons. miR-143 was also upregulated during the transition from neural precursors to fully differentiated neural cells, with expression maintained postdifferentiation (Krichevsky et al. 2006). This list of miRNAs is typical of the

majority of current information in this area: miRNA profiles of neural cells as they differentiate with only a few, validated targets and little understanding of how changes in miRNA expression bring about various neuronal subtypes.

In the developing CNS, neural progenitor proliferation occurs in the VZ, the cell layers next to the ventricles which house the cerebrospinal fluid (Visvanathan et al. 2007). NSCs mature as they migrate outward from the VZ, becoming postmitotic once they have settled in the outermost layer (Visvanathan et al. 2007). In the developing brain, proper size and patterning are achieved through selective neuronal and glial apoptosis with anywhere from 20 to 80% of terminally differentiated cells lost, depending on the area of the brain (Kosik 2006).

The SVZ is one of two areas in the adult mammalian brain capable of neurogenesis, the other being located in the dentate gyrus of the hippocampal formation (Cheng et al. 2009). The SVZ-NSCs mostly generate olfactory bulb interneurons, but a few develop into oligodendrocytes. In these adult neural stem cells (aNSCs), miR-124 is upregulated as they transition to neuroblasts, increasing further as they exit the cell cycle (Cheng et al. 2009). Interestingly, inhibition of miR-124 maintains the aNSC phenotype while overexpression induces premature differentiation. Inhibition of miR-124 in aNSC in vivo after injury causes hyperplasia (abnormally high proliferation of normal cells) and delays but does not prevent neuronal regeneration (Cheng et al. 2009). Overexpression of miR-124 in vivo after injury reduces the number of astrocyte-like cells, but does not affect Olig2+ oligodendrocyte lineage cells. miR-124 also targets Dlx2, a transcription factor involved in interneuron formation, and Jag1, a Notch ligand important for self-renewal (Cheng et al. 2009) (Fig. 3.5).

miR-125 expression increases in the brain as it develops and targets Lin28, an enhancer of pluripotency (Wu and Belasco 2005), while miR-9 targets the peripheral myelin protein, PMP22 (Lau et al. 2008). Both oligodendrocytes of the CNS and Schwann cells of the PNS synthesize myelin; oligodendrocytes express miR-9 but not PMP22, while Schwann cells do not express miR-9 but do express PMP22 (Lau et al. 2008) (Fig. 3.5).

Neural-specific miRNAs continue to be highly expressed in adult neurons and may play a role in local translational control of plasticity (Kosik 2006). Functional RISCs have been found at the site of developing axonal outgrowths along with Ago3, Ago4, Dicer, and FMRP (Kosik 2006). miR-134 is a brain-specific miRNA that, via its repression of LimK1, a stimulator of dendritic outgrowth by promotion of actin polymerization, decreases spine size in neurons (Kosik 2006; Bak et al. 2008). This is contrasted by the actions of miR-132 which increases total neurite length as well as primary neurite number by inhibition of p250GAP, a member of the Rho/Rac family that is highly enriched in the CNS and functions as a repressor of neurite outgrowth (Bak et al. 2008). miR-132 is enriched in the hippocampus, cortex, and brainstem (Bak et al. 2008) (Fig. 3.5).

An interesting regulatory relationship based on computational methods advanced by Wu and Xie (2006) has proposed the up-regulation of neural-specific miRNAs via CREB by binding to upstream-conserved CREB response elements (CRE sites), such as miR-9/9*, miR-124, and miR-132. CREB, which plays an important role in

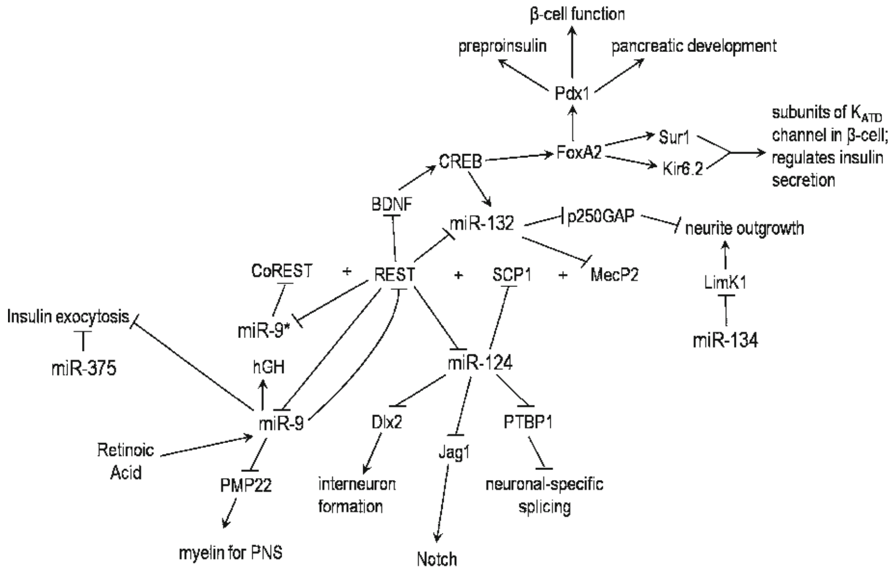

Fig. 3.5 Role of miRNAs in neurogenesis. A complex, interdependent relationship exists between members of the REST complex and neural-specific miRNAs such as miR-9/9*, miR-132, and miR-124. miR-124 is a target of REST and also targets SCP1, a corepressor that facilitates REST binding to RE1 sites in the promoters of REST-repressed genes. miR-124 also targets Dlx2, thereby excluding interneuron fate for developing neurons. miR-124 also repressed Jag1, a Notch mediator that, by activating Notch, prolongs the neural progenitor state. A decrease in Notch activation facilitates the transition from progenitor to mature neural cell types. miR-124 also suppresses PTBP1, which prevents neuronal-specific splicing of mRNA in the nucleus. miR-124 also modulates β-cells by either direct or indirect repression of FoxA2, which increases β-cell function and pancreatic development and activates preproinsulin. FoxA2 also induces Sur1 and Kir6.2, subunits of the KATD channel that regulates insulin secretion. Another target of REST is miR-9*, which downregulates CoREST. miR-9 is also a target of REST and, in turn, represses REST itself. miR-9 also facilitates myelination in the CNS by oligodendrocytes via repression of PMP22, a protein expressed by Schwann cells that specifically myelinates the PNS. miR-9 also suppresses insulin exocytosis in conjunction with miR-375. REST is regulated by RA, most likely through miR-9, which is induced by RA. REST also targets both miR-132 and BDNF. BDNF indirectly induces miR-132 by stimulation of CREB, an activator of miR-132. miR-132 acts in concert with miR-9/9* and miR-124 to downregulate the REST complex by targeting MecP2, a methyl-binding protein that targets methylated cytosines and recruits silencing machinery. miR-132 also increases neurite extensions from neurons by inhibiting p250GAP, a repressor of neurite outgrowth. Neurite outgrowth can be checked, however, by miR-134 which targets LimK1, an inducer of neurite lengthening. *CNS* central nervous system; *PNS* peripheral nervous system; *RA* retinoic acid

memory formation, behavioral adaptation, and developmental plasticity in response to BDNF, binds to a consensus CRE site in the miR-132 gene and induces its expression (Vo et al. 2005). BDNF and miR-132 as well as miR-9/9* and miR-124 are repressed by the REST complex, consisting of CoREST, MecP2, and SCP1 (Johnson et al. 2008) (Fig. 3.5).

As discussed earlier, REST expression is high in ESC, then gradually declines as cells differentiate. REST is also expressed in NSC and its expression, as expected, decreases as they transition to progenitors with fully mature neurons only expressing very low levels (Visvanathan et al. 2007). The expression of REST is regulated by RA, which is a potent inducer of the neuro-ectodermal lineage and is secreted by the somites that flank the neural tube during embryogenesis (Visvanathan et al. 2007; Wu and Xie 2006). REST represses expression of neural-specific miRNAs including miR-9, miR-124, and miR-132, which are all predicted to target members of the REST complex (Wu and Xie 2006; Johnson et al. 2008; Packer et al. 2008). miR-124, which is expressed in the VZ of the developing CNS including the neural tube and the dorsal root ganglia, targets SCP1, a corepressor of REST, which recruits REST to RE1 sites (Visvanathan et al. 2007). miR-124 also targets the polypyramidine tract-binding protein 1 (PTBP1), a repressor of neuronal-specific splicing (Cheng et al. 2009). miR-132 targets MecP2 (Wu and Xie 2006; Johnson et al. 2008), while miR-9* represses CoREST, and miR-9 inhibits REST itself (Packer et al. 2008) (Fig. 3.5). These intertwining relationships reveal a complex regulatory system that may be necessary to generate the many neuronal subtypes present in fully developed brains.

Both miR-9 and miR-124 also have roles outside of neurogenesis in the pancreas. miR-124, in an unknown manner, modifies the development of β-cells through either direct or indirect repression of FoxA2, possibly by targeting and downregulating CREB (Baroukh et al. 2007). Downstream targets of FoxA2 in the pancreas include Pdx1, Sur1, and Kir6.2 (Baroukh et al. 2007). Pdx1 is a homeobox gene involved in pancreatic development and maintenance of β-cell function; it also activates preproinsulin. Sur1 and Kir6.2 are subunits of the KATP channel in β-cells that help regulate insulin secretion. miR-9, as well as miR-375, decreases insulin exocytosis in insulin-secreting cells and miR-9 also increases human growth hormone release in rat insulinoma cells (Baroukh et al. 2007) (Fig. 3.5).

3.5.4 Hematopoiesis

Hematopoiesis is another well-studied area involving examination of miRNA expression and cell subtype specification. As in neurogenesis, only a limited number of validated targets are known. Hematopoiesis represents a constant process of differentiation from a well-known pool of ASCs, HSCs, which can mature into at least eight different cell types. This process must simultaneously coordinate proliferation, commitment, terminal differentiation, and apoptosis, making it a natural focus for study of miRNA regulation (Chen et al. 2004). HSC populations can be obtained from bone marrow, mobilized peripheral blood, and umbilical cord blood where they are especially rich (Jin et al. 2008).

Many of the miRNAs expressed during hematopoiesis appear to function as a group to specify cell type, rather than an individual miRNA being specific to only one cell type during the differentiation process. For instance, miR-25, miR-125b,

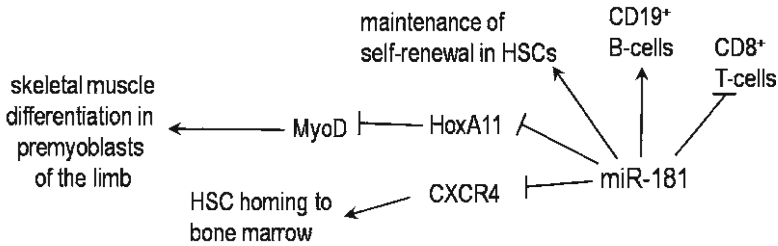

Fig. 3.6 Role of miRNAs in hematopoiesis. miR-181 plays an important role in hematopoiesis by maintaining self-renewal in HSCs. miR-181 allows for mobilization of HSCs into peripheral blood by repression of CXCR4, a chemokine receptor that holds HSCs in the bone marrow. miR-181 also promotes acquisition of the CD19+ B-cell phenotype at the expense of Thy-1.2+ T-lymphoid cells, especially CD8+ T-cells. miR-181 also promotes terminal skeletal muscle differentiation in the limb by down-regulation of HoxA11 in premyoblasts. HoxA11 suppresses differentiation by down-regulation of MyoD. *HSC* hematopoietic stem cells

miR-130a/b, and miR-191 are highly expressed in HSCs (Garzon and Croce 2008), while miR-10a/b (Garzon and Croce 2008), miR-17, miR-24, miR-128, miR-146 (Georgantas et al. 2007), miR-155 (Garzon and Croce 2008; Georgantas et al. 2007), and miR-181 (Gangaraju and Lin 2009; Jin et al. 2008; Georgantas et al. 2007) are all upregulated in HSCs and are believed to hold early HSCs at the stem cell stage by blocking differentiation to progenitor cells. However, miR-181 is also highly upregulated in B-lymphocytes and increases the number of CD19+ B-cells in vivo at the expense of Thy-1.2+ T-lymphoid cells, particularly CD8+ T-cells (Tili et al. 2008) (Fig. 3.6). It also increases sensitivity of the T-lymphocyte receptor to peptide antigens by reducing the signaling threshold via accumulation of phosphorylated intermediates through down-regulation of target phosphatases (Garzon and Croce 2008). miR-181 was also detected in human B-cells, T-cells, monocytes, and granulocytes (Ramkissoon et al. 2006).

Once HSCs differentiate into multipotent progenitors (MPs), they can either become a common lymphoid progenitor (CLP) or a common myeloid progenitor (CMP) (Gangaraju and Lin 2009; Garzon and Croce 2008). miR-146 blocks the transition from MPs to CLPs; miR-181 and miR-223 expression shifts cell fate towards that of CLPs, while miR-17, miR-24, and miR-155 appear to prevent transition to that of CMPs (Gangaraju and Lin 2009; Georgantas et al. 2007). miR-24 also works further down the pathway in prevention of the differentiation from megakaryocytic–erythroid progenitors to erythroid progenitors by repression of ALK4 which promotes erythropoiesis (Gangaraju and Lin 2009).

3.5.5 Osteogenesis

Adult osteogenic stem cells (aOSCs) are multipotent mesenchymal stem cells (MSCs) that are induced to differentiate into osteoblasts via BMP signaling (Gangaraju and Lin 2009). So far, most of the miRNAs characterized in osteogenesis

appear to maintain the MSC-state (Gangaraju and Lin 2009), which is lost upon BMP2 signaling (Li et al. 2008). BMP2 signaling appears to release osteo-specific mRNAs from miRNA inhibition, upregulating only a small number of miRNAs early in differentiation (~12%) and downregulating the vast majority (~88%) (Li et al. 2008).

There is some overlap of miRNAs from the cardiogenesis, myogenesis, neurogenesis, and hematopoiesis pathways. miR-133 and miR-135 act in concert to regulate osteoblastogenesis in MSCs. miR-133 functions by targeting p27 (a cell cycle regulator), Hox10a and RUNX2, which induces expression of alkaline phosphatase and osteocalcin, and miR-135 targets Smad5, which activates expression of early osteoblast differentiation markers (Li et al. 2008) (Fig. 3.7). miR-125b is also expressed and prevents differentiation by an unknown mechanism that decreases proliferation (Gangaraju and Lin 2009). p27 is upregulated in response to BMP2, which downregulates miR-133 and miR-135, and Smad5 together with C/EBPβ can bind to RUNX2 and increase its transcriptional activity (Inose et al. 2009). RUNX2, along with osteocalcin and β-catenin, is essential for osteoblast formation (Gangaraju and Lin 2009).

Another inhibitor of osteogenesis is miR-206 (also involved in myogenesis), which is highly expressed in the osteoprogenitor cells of the perichondrium and is significantly downregulated on initiation of osteogenesis. Inose et al. (2009) showed that knockdown of miR-206 in primary mouse osteoblasts was sufficient to induce differentiation, while overexpression of miR-206 inhibited differentiation and affected bone formation rate, leading to decreased bone mass independent of a shift from osteogenesis to myogenesis.

As osteogenesis proceeds, more miRNAs are upregulated, with maximal expression peaking during the mineralization stage when noncollagen proteins that facilitate the last step in terminal differentiation are secreted (Li et al. 2009). miR-29b inhibits

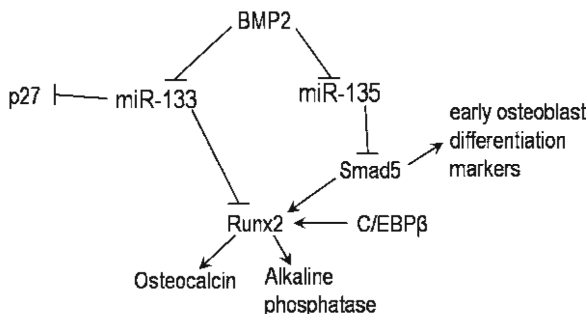

Fig. 3.7 Role of miRNAs in early osteogenesis. BMP2 induces differentiation of aOSCs by down-regulation of miR-133 and miR-135. miR-133 helps maintain the osteogenic state by repression of p27, a cell cycle regulator. miR-135 helps maintain the osteogenic state by repression of Smad5, a transcription factor that activates early osteoblast differentiation markers. miR-133 also targets Runx2, which is activated by both Smad5 and C/EBPβ. Runx2 induces osteocalcin, which aids in bone mineralization, and alkaline phosphatase, a marker of osteoblast differentiation. *aOSC* adult osteogenic stem cells

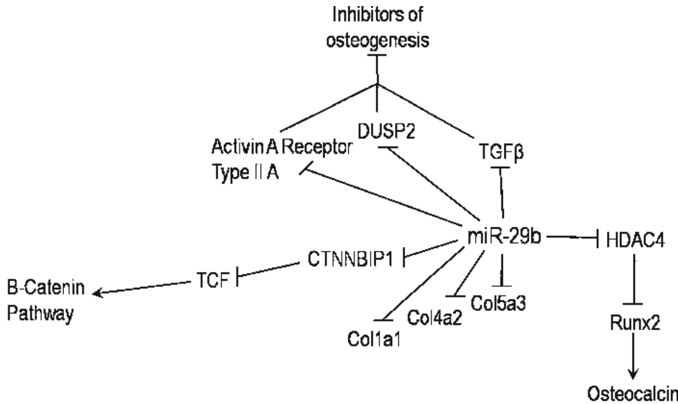

Fig. 3.8 Role of miRNAs in late stages of osteogenesis. miR-29b promotes later osteogenesis by repressing inhibitors of osteogenesis, such as Activin A Receptor Type II A, DUSP2, CTNNBIP1, and TGFβ. miR-29b also targets HDAC4, an inhibitor of Runx2, which upregulates Osteocalcin. CTNNBIP1 is a repressor of TCF, an activator of the β-catenin pathway, which is essential for osteoblast formation. miR-29b aids in the final steps of terminal osteogenic differentiation by repressing the collagen genes Col1a1, Col4a2, and Col5a3 at the initiation of mineralization

the inhibitors of osteogenesis, such as TGFβ, activin A receptor type IIA, CTNNBIP1, and DUSP2. Contrary to expectation, Li et al. (2009) found that miR-29b targets a cohort of collagen genes: Col1a1, Col4a2, and Col5a3. Since collagen type I is necessary to promote osteogenesis, these results seemed in contradiction. However, miR-29b expression does not appear until day 6 in primary rat osteoblasts differentiation and is briefly downregulated starting at day 8. Repression peaked at day 12, then came on strongly at day 14, the same day that mineralization begins, and collagen protein levels drop dramatically. miR-29b also targets HDAC4, a validated target of miR-1 in myogenesis, which decreases RUNX2 expression via deacetylation (Li et al. 2009) (Fig. 3.8).

3.5.6 *Epitheliopoiesis*

The epidermis is constantly regenerated by proliferation of epithelial stem cells (EpSCs) in the basal layer that undergo epitheliopoiesis as they begin to migrate upwardly (Gangaraju and Lin 2009; Yi et al. 2008). About 95% of holoclone keratinocytes demonstrate stem-like qualities when cultured in vitro, while meroclone keratinocytes possess only moderate proliferative abilities (Yi et al. 2008; Aberdam et al. 2008). The holoclone keratinocytes are considered to be adult slow-cycling stem cells and make up about only 0.01% of mouse basal cells.

In embryonic mice, miR-203 becomes highly expressed between E13.5 and E15.5 in the epidermis and hair follicles upon induction of stratification in epidermal progenitors (Yi et al. 2008; Aberdam et al. 2008). Adult mice also express

miR-203 in the upper basal layers, but not lower basal layers exactly inverse to p63, a transcription factor necessary for maintenance of proliferation in EpSCs and formation of the epidermis. p63 is downregulated by miR-203 to promote holoclone keratinocyte differentiation; however, it cannot promote differentiation on its own (Yi et al. 2008; Aberdam et al. 2008) (Fig. 3.11). What other miRNAs contribute and how they function in acquisition of mature epithelial phenotype is unknown at this time.

3.5.7 Germ Cells

Another important stem cell population is germline stem cells (GSCs) which originate from primordial germ cells after migration to the embryonic gonad (Gangaraju and Lin 2009). In mammalian males, GSCs are found in the testicular cords. GSCs are enriched not only in miRNAs, but also piRNAs, so named because they target PIWI subfamily proteins which are necessary for germline development, GSC self-renewal, and gametogenesis (Gangaraju and Lin 2009). The role of piRNAs is still being explored, although they do represent a distinct class of small, noncoding RNAs. While the precise role of miRNAs in GSCs has yet to be elucidated, they are required for PGC formation in early embryos as shown by depletion of maternal Dicer1 (Gangaraju and Lin 2009).

3.5.8 Loss of Self-Renewal

ASC populations do not retain their proliferative abilities indefinitely and, as organisms age, their stem cell populations slowly lose the ability to self-renew. This is due in part to HMGA2, a protein that mediates self-renewal by indirect suppression of the INK4A/ARF locus, which suppresses self-renewal (Hammond and Sharpless 2008) (Fig. 3.2). HMGA2, like REST, is highly expressed in ESC, becomes moderately expressed in stem cell populations (like HSCs and NSCs), and is lowest in mature cells. In mouse NSCs, HMGA2 was specifically shown to affect stem cell renewal and not proliferation (Hammond and Sharpless 2008). HMGA2 is not necessary for generation of NSCs during embryogenesis, only their maintenance from infancy through adulthood. INK4A and ARF expression increases with age in a manner reciprocal to HMGA2 expression (Hammond and Sharpless 2008).

HMGA2 expression is maintained by Lin28 which suppresses let-7 via uridylation (Hammond and Sharpless 2008; Ivey et al. 2008). Let-7 also is inversely correlated with stemness and increases as cells become more differentiated. For example, normal mammary progenitor cells have lower expression of let-7, and overexpression of let-7 reduces their numbers (Hammond and Sharpless 2008). HMGA2 expression may explain the retention of progenitor cells throughout the life of an organism, and its down-regulation may explain the loss of these populations during aging. Temporary inhibition of let-7 in stem cell populations may be

useful to increase the number of progenitor cells available for repair of tissues damaged in disease or trauma, although much more research needs to be done.

3.6 miRNAs in Cellular Stress Disease, and Trauma

The role of miRNAs continues in cells even after terminal differentiation has been reached. This next section will explore how miRNAs respond and contribute to pathological cellular conditions.

3.6.1 Cellular Stress and Apoptosis

In 2006, a group from the Harvard School of Public Health published a study that examined the miRNA response of a human cell line (TK-6 lymphoblasts) to several forms of cellular stress: folate deficiency, exposure to sodium arsenite, and treatment with γ-irradiation (Marsit et al. 2006). While γ-irradiation produced no significant perturbances in miRNA expression, folate deficiency and exposure to sodium arseniate produced a profound global increase in miRNA expression. One miRNA, miR-222, increased ~2-fold in TK-6 cells under conditions of folate deficiency and following sodium arsenite exposure and was also shown to be moderately upregulated in six patients with extremely low dietary folate relative to five patients with extremely high dietary folate. The series of experiments showed that alterations in miRNA expression in vitro may be indicative of in vivo responses, although this research is still too preliminary to definitively make such a claim. Alterations in miRNA expression may have been due to loss of genomic methylation since both folate deficiency and arsenic exposure can cause hypomethylation of DNA. However, Marsit et al. (2006) did not find any evidence of global methylation reductions. Since miRNA genes are methylated approximately an order more than protein-coding genes, these results may indicate that even small disturbances in methylation can significantly alter miRNA expression (Weber et al. 2007).

In healthy H9c2 rat embryonic ventricular cells, miR-1 alone was sufficient to induce apoptosis (Xu et al. 2007). Exposure to H_2O_2, an oxidative stressor that mimics pathological cardiac conditions, induces a concentration-dependent apoptosis which was dramatically increased upon overexpression of miR-1. Confirming the apoptotic-promoting properties of miR-1, H9c2 cells exposed only to H_2O_2 increased miR-1 expression by threefold. Conversely, overexpression of miR-133 significantly decreased apoptosis and increased cell viability (Xu et al. 2007). Even more intriguing was the finding that coexpression of miR-1 and miR-133 canceled their respective effects on apoptosis (Xu et al. 2007).

miR-1 was shown to target the heatshock proteins HSP60 and HSP70, which inhibit the mitochondrial apoptosis pathway, while miR-133 targets caspase-9, an important mediator of apoptosis (Xu et al. 2007) (Fig. 3.9). Caspase-9 complexes with cytochrome c and Apaf-1 to induce caspase-3, resulting in initiation of apoptosis.

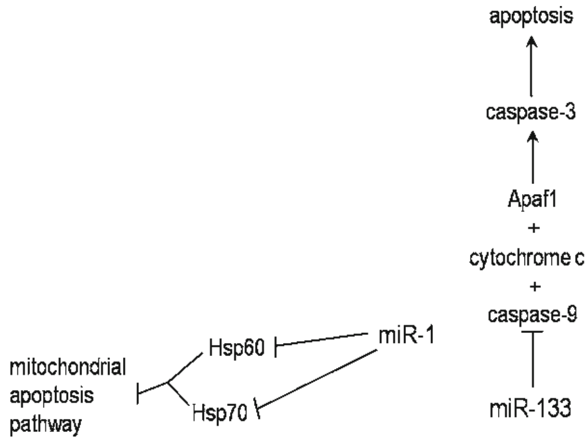

Fig. 3.9 Role of miRNAs in apoptosis. H_2O_2 exposure induces a concentration-dependent apoptosis mediated by an increase in miR-1 expression. miR-1 pushes cells towards apoptosis by targeting the heatshock proteins Hsp60 and Hsp70, which inhibit the mitochondrial apoptosis pathway. miR-133 acts in a diametric fashion to oppose apoptosis by repression of caspase-9. Caspase-9 works in concert with cytochrome c and Apaf1 to induce caspase-3 expression which then initiates apoptosis

This study reveals not only how miR-1 and miR-133 can control cell fate, but raises future clinical implications for ameliorating cell death during ischemia by increasing miR-133 and inhibition of miR-1.

3.6.2 Cancer

Cancer cell lines represent an intriguing medium in which to study miRNA expression and function as it relates to stemness since cancer cells recapitulate many of the features of stem cell proliferation and self-renewal. Comparison of normal tissues to tumors has revealed altered miRNA expression profiles. These include let-7 and HMGA2, which have been shown to regulate expression of critical targets including the RAS oncogene (Hammond and Sharpless 2008). These altered miRNA expression profiles may reflect the altered genomic methylation found in tumors. Indeed, Saito et al. (2006) found that exposure to 5′-aza-2-deoxycytidine, a competitive cytodine analog that irreversibly binds DNA methyl transferases (Dnmts) resulting in global genomic hypomethylation, and 4-phenylbutryic acid (PBA), a histone deacetylase, increased 17 miRNAs by more than threefold in T24 human bladder cancer cells. One of the most highly upregulated was miR-127, which is strongly silenced in many cancer cell lines, but expressed as part of the normal miRNome. miR-127 targets the proto-oncogene BCL6, which suppresses p53 expression. Therefore, strong suppression of miR-127 may facilitate transition to a cancer phenotype.

Estrogen receptor (ER)α amplification and protein overproduction are common in breast cancer, with high ERα expression correlated to a favorable response to endocrine therapy (Kondo et al. 2008; Adams et al. 2007). miR-206 targets ERα and is strongly downregulated in ERα^{+ve} breast cancers and, conversely, strongly upregulated in ERα^{-ve} breast cancers. Overexpression of miR-206 inhibits cell growth of ERα^{+ve} tumor cells, even in the presence of estrogen (Kondo et al. 2008; Adams et al. 2007). Interestingly, although miR-1 and miR-206 share a similar sequence, miR-1 could not decrease ERα or decrease cell growth despite its apoptotic enhancement of oxidatively stressed cardiomyocytes (Xu et al. 2007; Kondo et al. 2008) Estrogen inhibits miR-206 expression, which may explain why hormone replacement therapy increases the risk of breast cancer in a small number of women. Loss of miR-206 may also contribute to breast cancer because it increases cell death, which would halt the runaway proliferation present in progressive breast cancer (Adams et al. 2007).

Another cancer-related miRNA is miR-34a, which is decreased in neuroblastomas (NBs) (Welch et al. 2007). Overexpression of miR-34a in three separate NB cell lines diminished cell proliferation and viability while simultaneously increasing caspase-3 and caspase-7, illustrating that these reductions were via the caspase-dependent apoptotic pathway. miR-34a, which can be induced by RA, suppresses E2F3 expression, a transcription factor of cell cycle progression, which may explain how its downregulation contributes to cancer pathogenesis (Welch et al. 2007) (Fig. 3.11).

Increased expression of the miR-17 family has been shown to promote tumorogenesis (Silber et al. 2008). Glioblastoma multiforme (GBM) is a particularly unrelenting cancer that is unresponsive to surgery, radiation, and chemotherapy. Silber et al. (2008) showed that miR-124 and miR-137 are strongly downregulated in GBM cells due to hypermethylation and deacetylation of their genes. Overexpression of miR-124 and miR-137, which are also upregulated upon differentiation of SVZ-NSC, results in a marked shift towards more mature neuronal cells as evidenced by increased Tuj1+ expression, a marker of mature neurons. miR-124 and miR-137 also shift the cell cycle from the S-phase to the G0/G1-phase, indicating that they decreased proliferation without increasing apoptosis. miR-124 and miR-137 were also downregulated in human oligodendrogliomas and astroblastomas (Silber et al. 2008).

Another important miRNA implicated in cancer is miR-24. Loss of the miR-24-binding site in the 3′ UTR of dihydrofolate reductase (DHFR) has been shown to induce expression of this enzyme leading to subsequent methotrexate (MTX) resistance (Mishra et al. 2007). DHFR is one of the first enzymes that processes folic acid for methyl donors in the methylation pathway and it is the target of MTX, a chemotherapeutic agent widely used in several malignancies. Loss of the miR-24-binding site not only eliminates posttranslational regulation, but also results in a more stable mRNA folding pattern that increases the half-life of DHFR by twofold (Mishra et al. 2007). This increased stability negates MTX treatment and promotes a means of distinguishing which patients should or should not receive the drug. It also suggests that cotreatment of an miR-24 mimic with MTX could be beneficial to patients with normal DHFR.

3.6.3 Spinal Cord Injury

Another emerging role of miRNAs is in the pathophysiology of traumatic injuries. Recent studies have examined changes in the miRNome occurring after spinal cord injury (SCI). A study by Liu et al. (2009) showed that of 269 miRNAs examined in rats, 97 showed significant changes in expression following SCI, with 14 miRNAs showing a complex pattern of initial up-regulation 4 h post-SCI, then becoming strongly decreased compared to control at days 1 and 7 post-SCI. miR-124 was initially increased 4 h post-SCI, but became rapidly downregulated day 1 post-SCI, which continued through day 7 post-SCI. miR-137 was shown to be downregulated in the injured area, which is interesting because post-SCI sites undergo rapid cellular proliferation for several days (Wu et al. 2010). This observation is consistent with the role of miR-137 in shifting the cell cycle profile from S-phase to G0G1-phase (Silber et al. 2008).

A study by Nakanishi et al. (2010) in mice showed some overlap with the Liu et al. (2009) study, although they examined most miRNAs only 12 h post-SCI, thereby missing the complex regulatory pattern observed in rats. Two more closely examined miRNAs were miR-223 (increased ~2.4-fold) and miR-124 (decreased about ~1.5-fold) and these results were roughly correlated by the work of Liu et al. (2009). miR-223, as previously discussed, represses expression of NF1-A, which inhibits terminal granulocyte differentiation (Georgantas et al. 2007). Therefore, miR-223 may play a role in the large inflammatory response at the SCI site (Wu et al. 2010). Nakanishi et al. (2010) also showed that miR-124 is slightly increased at 6 h post-SCI and then is progressively downregulated. This loss of miR-124 may reflect a repair paradigm that focuses on early cell proliferation at the SCI site at the expense of later specification of proper cell types to replace motor neurons, sensory neurons, and interneurons. miR-124 is also highly expressed in mature neurons, therefore its decline suggests loss of this cell population secondary to the original SCI.

After SCI, NG2^{+ve} cells, which are purported to be spinal cord stem cells, increase dramatically at the site of injury, eventually compensating for lost cells in numbers, but not in type (Wu et al. 2010). After the initial injury, a spreading wave of glial and oligodendrocyte cell death occurs, with 50% or greater loss of oligodendrocytes by 2 days post-SCI. However, NG2^{+ve} cell proliferation continues for at least a month, eventually replacing myelinating oligodendrocytes in the area surrounding the SCI. This is in contrast to the central lesion in which no oligodendrocytic or axon myelination is found. Rather, empty cavities are dispersed among microglia and macrophages that contribute to the massive inflammation at the injury site, peak in numbers between 3 and 7 days post-SCI, and mediate chronic inflammation (Wu et al. 2010).

Failure of the developing neural tube to close properly is termed neural tube defects (NTDs) and can be considered another form of SCI. Severity of NTDs can range from closed spinal bifida to holoprosencephaly. While primarily due to folic acid deficiency, recapitulation of this phenotype in fetal rats can be achieved by

administration of RA to the developing spinal cord. RA dramatically decreases expression of the CNS-specific miRNAs miR-9/miR-9*, miR-124, and miR-125 at the site of the spinal cord lesion (Zhao et al. 2008). Disruption of these miRNAs during fetal development may help to explain folate- and inositol-resistant NTDs.

3.6.4 Ethanol Exposure in the Fetal Brain

Chronic ethanol exposure via maternal ingestion can cause fetal alcohol spectrum disorders that appear to initially induce proliferation that depletes stem cell populations by premature transition from stem to blast cells during neurogenesis. Sathyan et al. (2007) showed that in fetal rat cerebral cortex-derived progenitor cells, not only did high levels of ethanol (sufficient to mimic chronic high alcohol intake) alter miRNA expression (decreased miR-9, miR-21, miR-153, miR-335), but even at lower ethanol levels (sufficient to mimic social drinking) miRNA expression was perturbed (increased miR-335). Inhibition of miR-21 alone in these cells resulted in apoptosis; however, dual inhibition of miR-21 and miR-335 alleviated apoptosis, similar to what was observed following overexpression of both miR-1 and miR-133 in oxidatively stressed cardiomyocytes (Xu et al. 2007). The collective actions of miR-21, miR-153, and miR-335 normally act to suppress Jag1, which promotes HSC and NSC proliferation, and their down-regulation via ethanol increases Jag1 expression promoting proliferation observed in the rat cerebral cortex-derived progenitor cells (Fig. 3.11). In contrast, miR-9, miR-153, and miR-335 collectively downregulate ELAV2, which facilitates mature neuronal differentiation by suppressing nonneural mRNA, thus their decrease results in increased ELAV2 expression, which is responsible for the premature differentiation observed (Sathyan et al. 2007).

While this study is highly informative, it is limited by its in vitro model. An in vivo model was used by Wang et al. (2009) to study the effects of ethanol exposure on miRNAs expressed in developing embryonic mouse brains. While this research confirmed ethanol exposure-altered miRNA expression, none of the same miRNAs were altered, except for miR-9, which was found to be increased. These differences most likely arise from the fact that two different animal models were used (rat vs. mouse), assayed were performed using cell-specific neuronal population vs. whole brain RNA extraction, and different levels of ethanol exposure were employed. Intriguingly, not only were brain defects observed by Wang et al. (2009), but NTDs were also present, starting at the lowest dose of ethanol exposure, which were rescued by folic acid administration. Concomitant with folic acid administration was a decrease by ~60% in the overexpression of miR-10a due to ethanol. miR-10a targets HoxA1 and Hox genes play a role in the vertebrate establishment of anterior–posterior axis polarity via positional cues expressed along precise molecular gradients. Like folic acid deficiency, ethanol can perturb normal genomic methylation patterns (Sathyan et al. 2007). Therefore, these data reveal an important mechanism wherein NTD progression is mediated by miRNA misexpression due to altered genomic methylation.

3.6.5 Polyglutamine-Induced Neurogenesis

A specific subset of neurodegenerative diseases, called PolyQ diseases, is due to the expansion of a CAG repeat encoding the amino acid glutamine within the open reading frame of the affected genes (Bilen et al. 2006). The resulting transcription and translation of those altered genes results in pathogenic protein accumulation to toxic levels that end in neurodegeneration. In *Drosophilia* and murine postmitotic Purkinje cells as well as human HeLa cells, loss of miRNAs resulted in an increase of Ataxin-3 expression, a protein involved in spinocerebellar ataxia, a polyQ disease (Bilen et al. 2006; Bak et al. 2008). These studies showed that loss of miRNA expression exacerbated the neurotoxic effects of pathogenic polyQ proteins.

Perhaps, the best known polyQ disease is Huntington's disease (HD) which, like all polyQ diseases, is dominantly inherited (Bilen et al. 2006; Johnson et al. 2008; Packer et al. 2008). HD symptoms usually occur well into adulthood with sufferers displaying increasingly severe cognitive defects and motor control impairment, which lead to premature death. HD pathophysiology is caused by the accumulation of the toxic mutant Huntingtin protein (Htt) coupled with the loss of normal Htt, which provides a neuro-protective effect to cortical and striatal neurons (Johnson et al. 2008; Packer et al. 2008). The molecular pathway of Htt includes REST, which it normally sequesters in the cytoplasm of neurons, promoting neuronal identity. The Htt mutant loses the ability to bind to REST, allowing it to translocate to the nucleus and mediate suppression of neuronal genes including BDNF (Johnson et al. 2008). As discussed earlier, miR-124 and miR-132 are targets of REST and are decreased in HD. The down-regulation of miR-124 may perturb mature, terminal differentiated neuronal identity, while decrease of miR-132 may shorten neurite outgrowth and disturb neuronal connectivity.

Htt is ubiquitously expressed, but HD symptoms are primarily CNS-based (Packer et al. 2008). miR-9/9*, which are decreased early in HD, target REST and CoREST, respectively, and are also both suppressed by REST (Fig. 3.5). Early loss of miR-9/9* may facilitate an increase in nuclear REST levels, amplifying the effects of HD.

Another neurologic disorder where miRNAs may play a role is in schizophrenia, where altered miRNA profiles have been shown in the prefrontal cortex of patients (Bak et al. 2008). They may also play a role in fragile X syndrome, which is due to a 5′ UTR CCG repeat expansion in the FMR1 gene which codes for FMRP, a protein known to associate with the RISC complex as well as miRNAs themselves (Kosik 2006).

3.6.6 Autoimmunity

A role for miRNAs in the pathophysiology of autoimmunity was first discovered in 2002 in the autoimmune serum for P bodies (also called GW bodies, GWB for short) from a patient with motor and sensory neuropathy (Pauley et al. 2009). Sera that is reactive against GWBs has been found in patients with a wide range of

autoimmune disorders such as rheumatoid arthritis and systemic lupus erythrematosis and implicates autoimmunity against the miRNA/RNAi as a significant contributing factor or the basis for autoimmune diseases (Tili et al. 2008; Pauley et al. 2009). For instance, in regulatory T-cells (Treg), which abrogate autoimmune responses, Dicer ablation eliminated their suppressor activity in vivo, leading mice to develop a systemic and fatal autoimmune disease (Pauley et al. 2009).

miR-155 expression in activated macrophages, B-cells, and T-cells is essential for their normal functioning; its loss impairs adaptive immunity via nonfunctional B- and T-cells and prevents normal antigen presentation (Pauley et al. 2009). Since miR-155 targets PU.1, which suppresses the generation of high-affinity IgG1 producing B-cells, its loss also causes impairment in the generation of IgG1 antibodies. Another miRNA, miR-181b, mediates class switch recombination in B-cells, a critical step in increasing specificity of activated B-cells (Pauley et al. 2009).

TNFα and IL-1β, mediators of innate immunity, both increase miR-146a expression in an NF-κB-dependent manner (Pauley et al. 2009). miR-146a appears to downregulate at least two proinflammatory cytokines, IL-8 and RANTES, but it is not known if these genes are downstream or direct targets of miR-146a. miR-125b, which targets TNFα, is decreased in murine macrophages following exposure to lipopolysaccharide (LPS), indicating that its down-regulation is required for LPS-dependent activation of TNFα (Pauley et al. 2009) (Fig. 3.10).

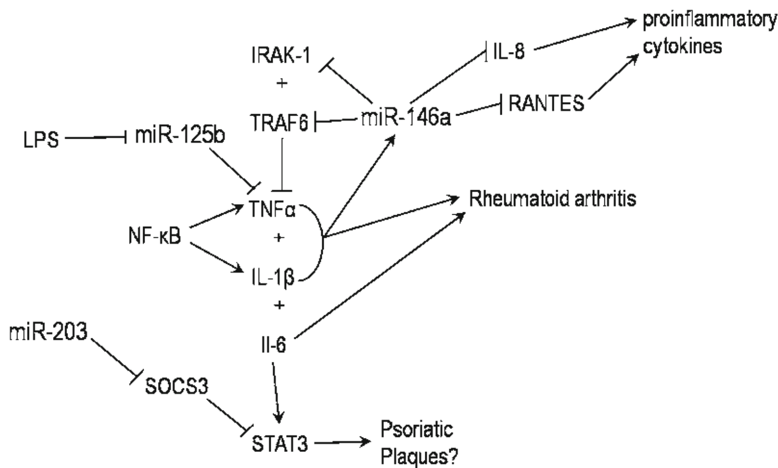

Fig. 3.10 Role of miRNAs in autoimmunity. miR-146a is increased by TNFα and Il-1β via a NF-κB-dependent mechanism. miR-146a either directly or indirectly represses expression of proinflammatory cytokines, indicating it is an important mediator of immune and autoimmune responses. TNFα is a downstream target of miR-125b; this repression is alleviated by LPS. miR-146a may contribute to rheumatoid arthritis if it loses its ability to bind and downregulate IRAK-1 and TRAF6, prolonging TNFα expression. TNFα, IL-1β, and Il-6 are all implicated in playing a role in the development of rheumatoid arthritis. In psoriasis, loss of miR-125b may be partially responsible for the increase in TNFα that is observed. Overexpression of miR-203 may also contribute to psoriasis by downregulating SOCS3, which allows for constitutive activation of STAT3 by IL6. *LPS* lipopolysaccharide

Peripheral blood mononuclear cells of patients with rheumatoid arthritis exhibit increased expression of miR-16, miR-132, miR-146a, and miR-155, and coexpression of miR-16 with miR-146a is a known indicator of active disease (Tili et al. 2008). Pauley et al. (2009) speculated that rheumatoid pathogenesis may be due to the inability of upregulated miR-146a to inhibit two of its targets, TRAF6 and IRAK-1, which results in prolonged TNFα production that in turn promotes a constitutive increase in miR-146a as part of a dysfunctional feedback loop. Both TRAF6 and IRAK-1 are regulators of the TNFα signaling pathway (Sonkoly et al. 2007) (Fig. 3.10).

Psoriasis is a chronic inflammatory disorder characterized by the formation of plaques consisting of rough, dry skin that may cover only small areas to extreme cases where most of the body is affected. While the exact cellular basis for psoriasis is unknown, it is believed to involve cross talk between keratinocytes, fibroblasts, and infiltrating immune cells (T-cells, mast cells, and monocytes). Keratinocytes affected by psoriasis are aberrant in several distinct ways: cell-signaling, proliferation, differentiation, and the recruitment and activation of immune cells (Sonkoly et al. 2007). Interestingly, abnormally high miR-146a is also observed in psoriatic skin plaques (Sonkoly et al. 2007), indicating that it may serve as a general mediator of autoimmunity, with other miRNAs conferring the specificity of the affected areas. TNFα is also increased in psoriatic lesions and this may be due, in part, to a decrease in miR-125b, which inhibits TNFα translation (Pauley et al. 2009) (Fig. 3.10).

Another miRNA increased in psoriasis is miR-203. Sonkoly et al. (2007) showed circumstantial evidence of miR-203 targeting suppressor of cytokine signaling 3 (SOCS3), a downregulator of STAT3, which when constitutively activated in mice leads to spontaneous development of psoriatic plaques. IL-6, a cytokine highly expressed in psoriatic affected keratinocytes, is able to constitutively activate STAT3 upon loss of SOCS3 (Fig. 3.10).

A similar, but not exact, miRNA profile was observed between psoriasis and eczema, another chronic inflammatory skin disease (Griffiths-Jones et al. 2008). Upregulated miRNAs include members of the miR-17 family, miR-17-5p, miR-20a, miR-106a (psoriasis), and miR-106b (eczema), as well as miR-21 and miR-146a (Sonkoly et al. 2007). miR-17-5p, miR-20a, and miR-146a were upregulated about twice as much in psoriasis. miR-203 was not upregulated in eczema, nor was miR-125b downregulated, making the changes in these miRNAs specific to psoriasis. It is puzzling to note that increased expression of the miR-17 family should lead to a decrease in STAT3. As in rheumatoid arthritis, loss of 3′ UTR-binding sites may explain the point of cellular deregulation that leads to a pathogenic phenotype and is an area of future research necessary for fully elucidating the nature of this disorder.

Tumor necrosis factor-α (TNFα) is a modulator of the immune system that has autoinhibitory properties located in the 3′ UTR of its mRNA. Autoinhibition is relieved upon stimulation via LPS (Tili et al. 2008). Surprisingly, cotransfection of miR-155 and a plasmid containing the green fluorescent protein (GFP) followed by the TNF 3′ UTR actually increases GFP expression instead of inhibiting it, indicating that miR-155 (which is also activated by LPS) increases translation of TNFα.

3.7 Miscellaneous miRNA-Related Disorders

Texel sheep are characterized by pronounced muscular hypertrophy which is caused by a mutation that creates a binding site for miR-1 and miR-206 in the mRNA of myostatin, which usually restricts muscle growth (Kosik 2006). Alteration of the 3′ UTR of HMGA2 is also associated with growth in regards to height in both mouse and humans (Hammond and Sharpless 2008). Pygmy mice carry a spontaneous mutation in the 3′ UTR of HMGA2 that leads to a decrease in adult size, while 3′ UTR truncation in humans and mice might lead to increased height and possibly even gigantism.

miRNA genes are not only found in a wide range of animals, but are also encoded by various viral genomes, some of which not only control expression of their own transcripts, but are also able to control expression of their host's immune response. For example, the human cytomegalovirus expresses miR-UL112-1, which targets the MHCI-related chain B preventing NK cells from targeting and removing infected cells (Tili et al. 2008). Another interesting viral miRNA, miR-K12-1, found in the Kaposi's-sarcoma-associated herpes virus is an ortholog of miR-155 and therefore induces an effect similar to miR-155 overexpression including an increase in B-cell tumors (Tili et al. 2008). This also introduces the intriguing idea that viral miRNAs may have contributed to the acquisition of new miRNAs or miRNA families within animal genomes.

An overview of all the pathways discussed herein is presented in Fig. 3.11, which is comprised of the previous figures and allows for visualization of how the miRNA pathways discussed are interconnected. Figure 3.11 illustrates how small shifts in miRNA expression can alter terminal differentiation paradigms and contribute to or cause disease states.

3.8 Brief Description of miRNA Protocols

Established protocols for determining miRNA targeting and function include a wide range of assays from Northern, qRT-PCR, microarrays, ChIP, and the LUC 3′ UTR reporter assay, which is especially important for confirming in vitro binding and action of the miRNA of interest. microRNA mimics, inhibitors, probes, qRT-PCR kits, microarrays, and isolation kits are commercially available from many companies including Invitrogen, Qiagen, Ambion, Exiquon, and Dharmacon.

Obviously, miRNA research begins with a comprehensive literature search. Once this is completed and a pathway, mRNA, or miRNA of interest has been determined, the next vital step requires use of a miRNA target prediction algorithm. Although many algorithms exist, they are not all equal. Many early algorithms particularly emphasized the $-\Delta G$ energy for thermodynamic stability, degree of binding between seed region of miRNA and target, and conservation across species. However, it has been shown that $-\Delta G$ energy is not especially predictive, binding

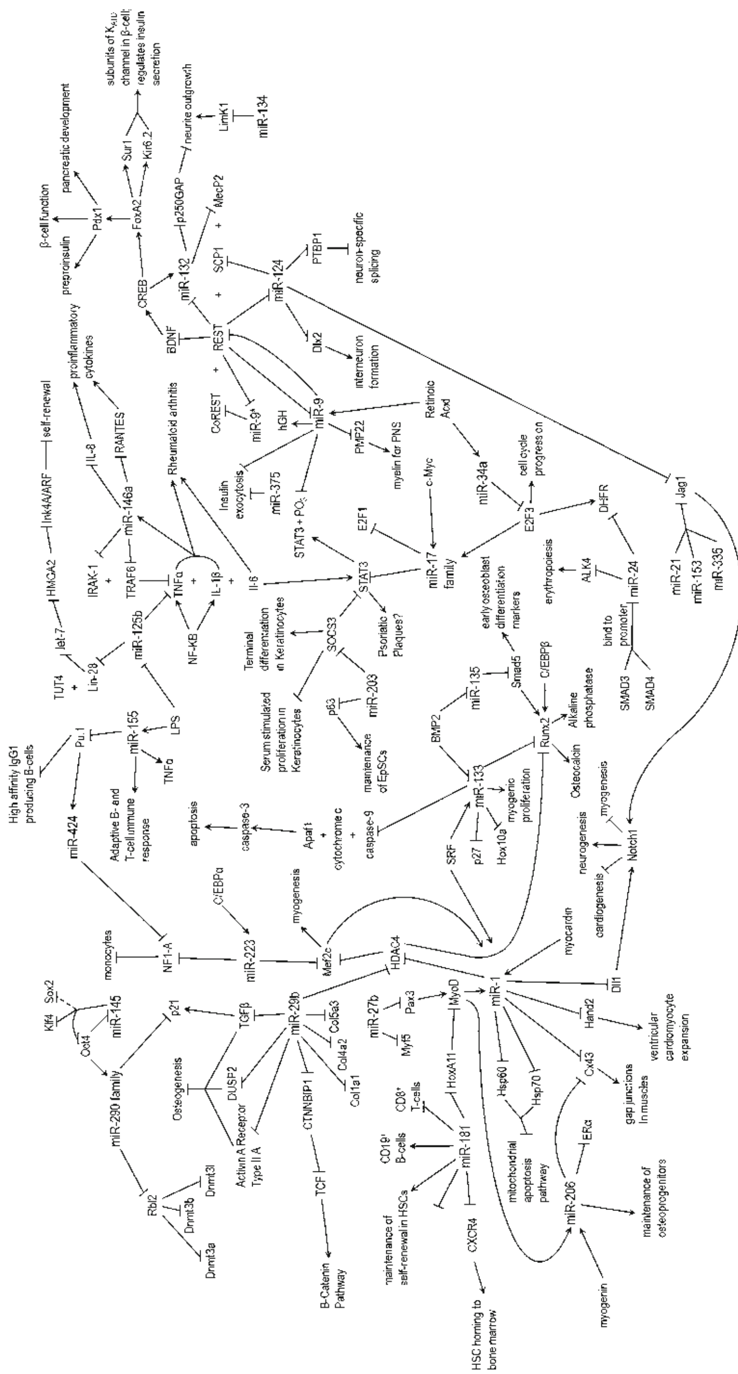

Fig. 3.11 Overview of miRNA pathways discussed

at sites other than the seed region plays an important role that needs to be considered, and results based on conservation miss many miRNAs that are nonconserved and species-specific (Ye et al. 2008). While some algorithms are easier to use than others, their high false positive rate can return far too many results (in the hundreds) to properly assess. Currently, the best prediction algorithms are the ones that consider, in addition to seed region complementarity, the presence of multiple, cooperatively and closely spaced miRNA binding sites in the target 3′ UTR, base-pairing at the 12–17 nt of the miRNA, binding sites located in AU-rich regions of the target 3′ UTR and at least 15 nt from the stop codon, binding sites near the ends but not the center of a long target 3′ UTR, and the presence of a central loop from 9 to 11 nt in the miRNA (Grimson et al. 2007; Ye et al. 2008).

Several miRNA prediction algorithms incorporate many of these features, such as TargetScan, FindTar, and RNA22. FindTar and RNA22 also allow for manipulation of score parameters, and RNA22 can also scan genomic sequences for the presence of miRNA genes. For a more detailed discussion of these parameters, see Grimson et al. (2007) and Ye et al. (2008). miRNA expression or expression of its target mRNA can be assessed via RT-CPR or qRT-PCR (RNA), Northerns (RNA), or Westerns (protein). To test if a miRNA of interest actually blocks translation of a target protein, a Western can be performed. miRNA mimics allow for testing of cellular and phenotypic alterations when an miRNA of interest is overexpressed, while miRNA inhibitors allow for observations of changes due to a single miRNA. Transfection of mimics or inhibitors is a convenient and easy way to test miRNA functions compared to knocking out or knocking in a gene. However, validating actual targets may be difficult due to the large number of targets a single miRNA may have and to the high false positive rate of algorithms used to narrow the search. This is why a comprehensive literature search is a critical first step.

The gold standard for validating miRNA:mRNA interactions is the luciferase assay. This involves inserting the 3′ UTR of the putative mRNA target downstream of the luciferase gene (known as the reporter plasmid) and cotransfecting either a mimic and/or inhibitor of the miRNA of interest. Cotransfection of a mimic with the reporter plasmid should significantly decrease fluorescence of the luciferase protein if the miRNA of interest truly targets the mRNA. Conversely, cotransfection of both a mimic and inhibitor to the miRNA of interest along with the reporter plasmid should rescue the fluorescence of the luciferase gene, as the inhibitor will block all interactions of the miRNA of interest.

A particularly interesting assay involves creation of a locked nucleic acid (LNA) single-stranded competitive oligonucleotide that binds to the miRNA-binding site on the 3′ UTR of the reporter plasmid and blocks any binding of the miRNA of interest (Packer et al. 2008). This allows for absolute certainty that the miRNA of interest is the cause of any down-regulation without causing any confounding effects by inhibiting all of the miRNAs interactions.

Effects of endogenous levels of a particular miRNA on a purported target can be observed through RT-PCR, qRT-PCR, and a Western. Both RT-PCR (or qRT-PCR) and a Western should be performed as miRNA binding may affect both protein levels, but not always mRNA levels of targets. Specificity can be confirmed by

mutating 1 or 2 nt in the miRNA-binding site of the 3′ UTR, which should disrupt the down-regulation and can be observed once again through RT-PCR (or qRT-PCR) and a Western.

Fluorescent in situ hybridization can be done on whole mount, paraffin embedded, or frozen tissue sections and allows visualization of miRNA expression in specific cells and tissue types in developmental, disease, or trauma models. The technique is particularly good for easy visualization of both a miRNA and cell type via a fluorescent mRNA probe of a different color.

Microarrays can be utilized to analyze large changes in miRNA expression that is cell- and tissue-specific, as well as in response to changes in development, disease, or trauma. The data can be used to develop miRNA profiles (miRNomes which represent the actively expressed miRNAs) specific to cell types or determine biomarkers indicative of disease. Changes between normal and pathological cell states can also be used to explain the cellular and molecular pathophysiology of disease states and may also offer insights to development of clinical treatments.

These assays offer just a brief overview of miRNA-focused experiments. Many of the companies listed earlier offer not only miRNA-specific kits and reagents, but detailed experimental protocols. miRNA research articles also discuss protocols used in the Materials and Methods section.

miRNA changes can also be observed in vivo by the injection of mimics or inhibitors directly to an animal model. However, this may lead to perturbation of the endogenous miRNA biogenesis pathway as described by Khan et al. (2009). Khan et al. (2009) analyzed 151 transfection experiments in seven different human cell types and concluded that saturating levels of the transfected mimics overwhelm and outcompete endogenous miRNAs, effectively inhibiting their processing and upregulating endogenous miRNA targets. Krützfeldt et al. (2005), on the other hand, found no problems with injection of miRNA inhibitors (antagomirs) into mice and a study by McLaughlin et al. (2007) even showed that injection of miRNA mimics targeting Bcr-Abl inserted into a lentiviral vector was capable of prolonging life in a mouse model of lymphoid leukemia. A possible remedy to disturbances in miRNA biogenesis is enhancement of miRNA processing by phosphorylating TRBP, which stabilizes and increases the processing capacity of the RISC complex as was observed by Paroo et al. (2009).

A major challenge to any miRNA therapy involving the CNS is the blood–brain barrier, although several strategies have been proposed to circumvent it, which include intranasal delivery, lipid encapsulation and targeted delivery, and direct administration via convection-enhanced delivery (Silber et al. 2008).

3.9 Conclusions

The field of miRNAs, though existing for almost two decades, remains in its infancy as the role of miRNAs is still being elucidated. The enormous potential of miRNAs lies in clinical applications, as well as in the understanding of cellular

transformations during development, disease, and trauma. miRNAs may also provide insight into the evolutionary changes that gave rise to the complex physical organization present in many species. There is a significant difference in complexity present between vertebrates and invertebrates, despite having a similar number of coding genes (Inose et al. 2009). It is believed that complexity arises through regulation of expression, not simply possession of more coding genes (Paroo et al. 2009), and miRNAs may possess a regulatory potential that exceeds the regulatory network of transcription factors (Hammond and Sharpless 2008; Inose et al. 2009).

With 98% of all human genomic output that of small, noncoding RNAs (Inose et al. 2009), there is no doubt that they play an enormously important role in many cellular and developmental processes. Increasing our knowledge about the biology of miRNAs and their role in ASCs will require first an exact definition of the population being studied. This population must then be isolated and its miRNome determined, as well as its response to alterations in miRNA expression under different conditions, both in vitro and in vivo. This broader understanding of miRNAs in ASCs can then, hopefully, lead to clinical applications for disease and trauma.

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Chapter 4
Expression and Function of Pluripotency Genes in Adult Stem Cells*

Antonio Lo Nigro, Philip Roelandt, and Catherine M. Verfaillie

Abstract In this chapter we will discuss the data published regarding expression of two key ESC transcription factors, *Oct4* and *Nanog*, in adult stem cells. In an introduction, we will discuss what is known regarding these two genes during embryonic development, as well as the evidence existing for the presence of pseudogenes as well as alternative spliced forms of these two genes. We will also address the tools available (or not) to discriminate between these different forms of *Oct4* and *Nanog*, and how these might help in elucidating in the future; whether adult somatic stem cells express the *Oct4* and *Nanog* genes that are known to play a role in the self-renewal and pluripotency characteristics of ESC, and whether such expression plays a role in the potency of adult tissue derived stem cells.

Keywords Adult stem cells • Embryonic stem cells • *Nanog* • *Oct4* • Pluripotency • Pseudogenes • Somatic tissue-derived stem cells

Abbreviations

AFSs	Amniotic fluid-derived stem cells
BM	Bone marrow
E	Embryonic day
ESC	Embryonic stem cells
haGSCs	Human adult germ line stem cells
HSC	Hematopoietic stem cell
ICM	Inner cell mass
KO	Knockout

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LIF	Leukemia inhibitory factor
maGSCs	Multipotent adult germ line stem cells
MAPC	Multipotent adult progenitor cells
MASCs	Multipotent adult spermatogonial-derived stem cells
MSC	Mesenchymal stem cells
NTD	N-terminal domain
PGC	Primordial germ cells
SPCs	Spermatogonial progenitor cells
SSCs	Spermatogonial stem cells
TF	Transcription factor
VSEL	Very small embryonic like cells

4.1 POU5F1 (Also Known as OCT3, OCT4, OTF3 or OTF4)

4.1.1 Introduction

The *Pou5f1* (also known as *Oct3, Oct4, Otf3* or *Otf4*) gene encodes for a transcription factor (TF) that belongs to the POU DNA binding domain family, which bind the conserved motive ATTTGCAT (Scholer et al. 1989; Pesce and Scholer 2001; Okamoto et al. 1990; Scholer et al. 1990; Rosner et al. 1990). Oct4 plays a crucial role in embryo development and in maintenance of both self-renewal ability and pluripotency of embryonic stem cells (ESCs). It is one of the ESC-specific TF who's levels requires tight control, as 50% loss of expression drives ESCs to trophectoderm and 50% greater levels of expression induces primitive endoderm or mesoderm as shown by Niwa et al. (2000). Knockout (KO) experiments in mouse demonstrated failure of ESC colony formation in vitro and embryonic lethality at the preimplantation stage in vivo (Nichols et al. 1998).

Oct4 expression is maternally derived in fertilized oocytes until the two-cell stage; from the four-cell stage, zygotic *Oct4* expression starts and is finely regulated to allow the correct formation of embryonic and extraembryonic tissues. Decreased expression of *Oct4* at embryonic day (E) 3.5 stage in some of the cells of the morula leads to the formation of *Cdx2*$^{+}$ trophectoderm (Palmieri et al. 1994). On E4.5, some of cells in the inner cell mass (ICM) express transiently higher levels of *Oct4*, which allows development towards hypoblast (primitive endoderm and extraembryonic endoderm), whereas cells with stable *Oct4* expression become the epiblast that will give rise to embryonic ectoderm (Niwa et al. 2000). During gastrulation, the Oct4 promoter becomes progressively silenced by repressive epigenetic modifications from the DNA methyltransferase DNMT3a/b and the histone methyltransferase, G9a (Feldman et al. 2006). By E7.5, *Oct4* is exclusively present in the newly formed primordial germ cells (PGCs) (Yeom et al. 1996). In the adult, spermatogonia and gonocytes are the only cells where *Oct4* expression can be detected (Pesce et al. 1998; Tadokoro et al. 2002).

4.1.2 Oct4 Pseudogenes

Pseudogenes are genomic regions that show high sequence homology to a parental gene. However, they do usually not give rise to a functional protein. Pseudogenes can be classified in two groups, depending on the mechanism via which they are generated: (1) processed pseudogene (reverse transcription from a normal mRNA; absence of introns and 5′ promoter) or (2) nonprocessed pseudogene (gene duplication due to chromosomal rearrangements) (Harrison et al. 2005; Mighell et al. 2000). Pseudogenes are commonly considered to be "evolutionary junk," as they are in general nonfunctional due to mutations. Nevertheless, there is quite good evidence that at least some pseudogenes may play a role in cell behavior (Hirotsune et al. 2003; Korneev et al. 1999).

Pain et al. (2005) found that multiple pseudogenes for pluripotent-specific genes are present in the human genome, at a frequency significantly higher than the classical somatic genes. Six pseudogenes for OCT4A have been identified (five processed and one nonprocessed (Table 4.1)), ten for Nanog, and sixteen for Stella. Using primers that amplify the *OCT4* gene and all six pseudogenes, Suo et al. (2005) detected expression of the pseudogenes *OCT4-PG1* and *OCT4-PG5* either alone or together in cancer cell lines (HeLa, HepG2 and MCF-7) and different primary cancer tissues (uterine cervix, urinary bladder, colon, kidney, breast, thyroid, esophagus and lung) but not in normal skin or muscle biopsies. They were also not expressed in embryonal carcinoma cell lines (NTERA-2 or PA-1). This might suggest that *OCT4-PG1* and *OCT4-PG5* may play a role in carcinogenesis. However, as there is no evidence that the genes are translated into proteins, direct molecular proof for a role in cancer development is not yet available.

These studies do however demonstrate that expression of *Oct4* in adult tissues may be artifactual, due to amplification of certain pseudogenes. Indeed processed and nonprocessed pseudogenes can be detected by RT or qRT-PCR, for instance in case of incomplete Dnase digestion before the cDNA synthesis. Hence, more effort should be put into the design of specific primers able to exclude at least the known pseudogenes.

Table 4.1 *Oct4* pseudogenes

Pseudogenes	Identity (%)	Chromosome	Type of pseudogene
Pg1	97.5	8	Processed
Pg2	97.8	12	Processed
Pg3	97.5	1	Processed
Pg4	93.7	10	Processed
Pg5	90.4	3	Nonprocessed
Pg6	84.7	8	Processed

4.1.3 Oct4 Gene and Isoforms

The human *POU5F1* gene is located on chromosome 6 and it spans a region of ~7 Kb containing five exons. By alternative splicing, the gene can generate two different isoforms; *OCT4A* and *OCT4B* (Fig. 4.1) (Takeda et al. 1992; Lee et al. 2006). The OCT4A protein contains 360 amino acids (aa) and has 87% homology with the murine Oct4 protein. It is comprised of exon 1a (E1a), the 3′-half of exon 1b (E1b) and exons 2 (E2), 3 (E3), and 4 (E4), using the internal acceptor site of exon 1b. The OCT4B protein contains 260aa and is generated from the complete E1b and the E2, E3, and E4.

The two isoforms have an identical C-terminal domain (CTD). The POU DNA binding domain of OCT4A (POU^A) consists of 156aa while POU DNA binding domain of OCT4B (POU^B) consists of 154aa. The N-terminal domain (NTD) of the two variants is isoform-specific; the NTD^A consists of 133aa while the NTD^B of just 44aa.

The function of these two different splice variants was investigated by Lee et al. (2006). The authors used a complementation assay in ZHBTc4 cells, a murine ESC line harboring a tetracycline-repressible *Oct4* (Niwa et al. 2005). After repression of endogenous *Oct4*, the human OCT4A but not OCT4B isoform was able to rescue the ESC phenotype, i.e., inhibition of differentiation and maintenance of self-renewal. Using EGF-tagged OCT4A and OCT4B, the authors demonstrated a

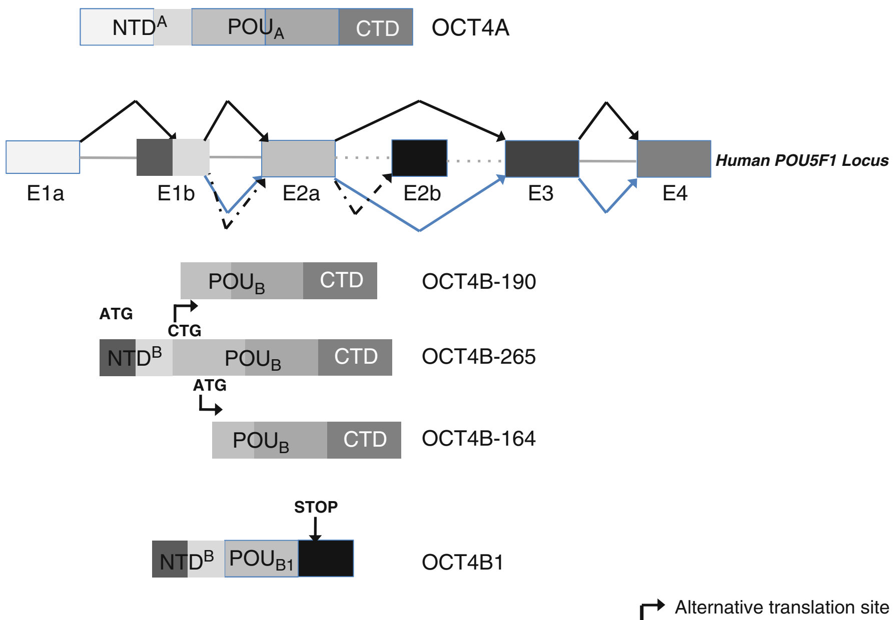

Fig. 4.1 Human *Oct4* locus and its isoforms. *Arrows* show the different splicing producing the mRNA isoforms. *Black* OCT4A, *gray* OCT4B, *red* OCT4B1

nuclear signal for human OCT4A while the human OCT4B remains cytoplasmatic. Furthermore, using an electromobility shift assay they found that the NTD of OCT4B inhibits the binding of OCT4 to the DNA and inhibits activate transcription of OCT4-responsive genes. During human preimplantation development, the OCT4A isoform is highly concentrated in the nucleus of cells in the morula, whereas OCT4B is found in the cytoplasm from the four-cell stage onwards (Cauffman et al. 2005, 2006). These studies strongly suggest that OCT4A is responsible for the maintenance of stemness and keeping ESC in an undifferentiated state.

Wang et al. (2009) demonstrated that OCT4B transcripts contain a putative internal ribosome entry site (IRES) that results in three possible isoforms generated from alternative translation initiation sites: OCT4B-265 (starting from the ATG at position 102), OCT4B-190 (starting from the CTG in E2), and OCT4B-164 (starting from the ATG in E2). Mutation in these alternative translation sites respectively abolished the equivalent isoforms. Of note, OCT4B-190 was upregulated under stress condition and may have an antiapoptotic function in response to stress.

Recently, a fifth and novel OCT4 spliced variant, termed OCT4B1, was described by Atlasi et al. (2008). OCT4B1 is generated by E1b, E2, and the whole 225 bp of the intron 2 region, as a putative novel exon, termed exon 2b. This new variant results in an open reading frame of 348 nucleotides and in a predicted peptide of 115aa for the presence of an in frame TGA-stop codon in E2b. Amino acids 1–80, containing the NTD^{B} and part of the N-terminal part of POU^{B} are identical with the OCT4B-265 isoform. However, OCT4B1 lacks the C-terminal part of the POU domain and COOH-terminal transactivation domain. Interestingly, OCT4B1 is expressed in human ESC and EC cells and is downregulated following their differentiation. Whether OCT4B1 plays a role in maintaining stemness cannot yet be determined, as no protein expression has been shown yet.

4.1.4 Oct4 in Nonembryonic Stem Cells

4.1.4.1 Testis-Derived Stem Cells

Oct4 was described for the first time by Scholer et al. (1989) in the mouse embryo and in the germ line cells. $Oct4^{+}$ cell populations are also freshly isolated from mouse adult testis. Seandel et al. (2007, 2008) isolated highly proliferative spermatogonial progenitor cells (SPCs) from the testis of adult mice. If maintained on mitotically inactivated mouse embryonic feeders in a long-term culture, SPCs give rise to GPR125 positive multipotent adult spermatogonial-derived stem cells (MASCs). $GPR125^{+}$ MASC can differentiate in cells of the three germ layers in vitro and give rise to partial chimeras when injected in the blastocyst.

Spermatogonial stem cells (SSCs) are another example of $Oct4^{+}$ cells. As shown by Guan et al. (2006, 2007), these cells can be isolated using a genetic approach and acquire ESC features in response to ESC culture conditions. Like ESC, the

cells obtained after this “transdifferentiation,” named multipotent adult germ line stem cells (maGSCs), are able to differentiate into derivatives of the three somatic lineages in vitro, make teratomas in vivo, and generate germ line competent chimeras.

Dedifferentiated SSCs similar to populations of maGSC and MASC have also been isolated from human testis biopsies (Conrad et al. 2008; Kossack et al. 2009). Human adult germ line stem cells (haGSCs) are derived from SSCs isolated using magnetic-activated cell separation for CD49f, CD90 (GDNFR-α1), or CD133. Following culture of these SSCs with leukemia inhibitory factor (LIF) and/or glial-derived neurotrophic factor, dedifferentiation to cells with ESC features was observed in 14 days; haGSCs produced teratomas if transplanted in immunodeficient mice and differentiated into different somatic types of all three germ layers if cultured under condition used for differentiation of human ESCs.

These studies demonstrate that under culture conditions similar to that used for ESCs, cells expressing Oct4 can acquire greater potency than the single lineage they are destined to make. In view of the studies by Yamanaka and others (Takahashi and Yamanaka 2006; Takahashi et al. 2007; Kim et al. 2009) demonstrating that forced expression in terminally differentiated somatic cells of specific transcription factors alone or in combination with epigenetic modifying small molecules (Shi et al. 2008) can induce a pluripotent state close to that of ICM cells or epiblast cells, the finding that reprogramming of cells that already express Oct4 to (near) pluripotency by exposure to specific culture conditions is perhaps not that surprising.

4.1.4.2 Somatic Tissue-Derived Stem Cells

A large number of publications have described *Oct4* expression in stem cells isolated from different somatic tissues (bone marrow, peripheral blood, hair follicle, epidermis, thyroid, liver, kidney, pancreas, breast, brain, muscle and endometrium) as well as amniotic fluid and umbilical cord blood (Table 4.2). Detection of *Oct4* was usually used to substantiate a greater potency of populations of stem cells compared to classic adult stem cells.

Among these publications, only three studies described Oct4 expression in freshly isolated cells. A nonhematopoietic SSEA1$^+$ subpopulation of murine bone marrow (BM), termed Pre-MSC (Anjos-Afonso and Bonnet 2007) was isolated after depletion of the Lin/CD45/CD31$^+$ cells. Pre-MSCs express *Oct4, Nanog,* and *Rex1* at both the mRNA and protein level. Expression levels of *Oct4, Nanog,* and *Rex1* increased when pre-MSCs were subsequently cultured under multipotent adult progenitor cell (MAPC) culture conditions. Such clonally expanded cells were shown to differentiate into cells of the three germ layers in vitro and to mesodermal derivatives in vivo (chondroblasts, osteoblasts, endothelial and hematopoietic cells).

VSEL (very small embryonic like) cells are isolated from mouse BM and human umbilical cord blood using cell sorting to obtain a population enriched for c-KIT, CXCR4, and SSEA4 (human)/Ssea1 (mouse) (Kucia et al. 2006, 2007, 2008;

Table 4.2 Adult stem cell populations expressing Oct4 and/or Nanog

Tissue of isolation	Species	Oct4	Nanog	Name of the cell population	References
Adipose tissue	Rh	Yes	No	rASC (Rhesus adipose stem cells)	Izadpanah et al. (2006)
Amniotic fluid	H, M	Yes	Yes	AFS (amniotic fluid-derived stem cell)	De Coppi et al. (2007a)
Amniotic fluid	H	Yes	ND	Amniocytes	Kim et al. (2007)
Amniotic fluid	H	Yes	Yes	AFC (amniotic fluid cell)	Bossolasco et al. (2006)
Amniotic fluid	H	Yes	ND	AFMSC (amniotic fluid mesenchymal stem cell)	Tsai et al. (2006)
BM	M	Yes	No	MAPC (multipotent adult progenitor cell)	Jiang et al. (2002)
BM	H	Yes	Yes	MIAMI (marrow isolated adult inducible cell)	D'Ippolito et al. (2004, 2006a, b)
BM	M	Yes	Yes	Pre-MSC (premesenchymal stem cell)	Anjos-Afonso and Bonnet (2007)
BM	H, M	Yes	Yes	VSEL (very small embryonic like cell)	Kucia et al. (2006, 2008)
BM	M	Yes	ND	MSC (mesenchymal stem cell)	Lamoury et al. (2006)
BM	H	Yes	ND	hMSC (human mesenchymal stem cell)	Moriscot et al. (2005)
BM	H	Yes	ND	MSC (mesenchymal stem cell)	Grayson et al. (2007)
BM	M	Yes	ND	$CD34^{+}$ HSCs	Goolsby et al. (2003)
BM	H	Yes	ND	Stromal cell subpopulation	Pochampally et al. (2004)
BM	M	Yes	ND	Putative germ cells	Johnson et al. (2005)
BM	M	Yes	ND	Bone marrow-derived germ cell	Nayernia et al. (2006)
BM	M	Yes	Yes	Cardiomyocytes progenitor cell	Pallante et al. (2007)
BM	M	Yes	ND	Marrow stromal cell	Ren et al. (2006)
Brain	M	Yes	ND	Neurospheres	Okuda et al. (2004)
Brain	Rh	Yes	ND	NSPC (neural stem and progenitor cells)	Davis et al. (2006)
Breast	H	Yes	ND	HBEC (human breast epithelial cell)	Tai et al. (2005)
Endometrium	H	Yes	ND	Primary cell	Matthai et al. (2006)
Endometrium	M	Yes	ND	Endometrial label retaining cell	Cervello et al. (2007)
Epidermis	M	Yes	Yes	Keratinocyte side population	Redvers et al. (2006)
Fetus	M	Yes	ND	FSSC (fetal somatic stem cells)	Kues et al. (2005)
Hair follicle	H	Yes	Yes	Hair follicle progenitor cell and follicular bulge	Yu et al. (2006)
Heart, BM, and liver	H	Yes	Yes	MASC (multipotent adult stem cell)	Beltrami et al. (2007)

(continued)

Table 4.2 (continued)

Tissue of isolation	Species	Oct4	Nanog	Name of the cell population	References
Kidney	R	Yes	ND	MRPC (multipotent renal progenitor cell)	Gupta et al. (2006)
Kidney	R	Yes	ND	Parietal epithelial stem cell	Sagrinati et al. (2006)
Liver	H	Yes	ND	Hepatic epithelial colonies	Selden et al. (2003)
Lung	M	Yes	ND	Pulmonary cells	Ling et al. (2006)
Lung	H	Yes	ND	Lung-derived fibroblasts	Rieske et al. (2005)
Muscle	R	Yes	ND	PPSC (pluripotent stem cell)	Romero-Ramos et al. (2002)
Pancreas	R	Yes	ND	Epithelial cells from islets	Wang et al. (2004)
Pancreas	R	Yes	ND	Pancreatic stem cells (PSC)	Danner et al. (2007)
Periodontal ligament	H	Yes	Yes	PDL-derived stem cells	Huang et al. (2009)
Peripheral blood	H	Yes	ND	MSC	Tondreau et al. (2005)
Peripheral blood	H	Yes	Yes	$CD14^{+}CD34^{low}$ EPC (endothelial progenitor cell)	Romagnani et al. (2005)
Peripheral blood	M	Yes	ND	Putative germ cells	Johnson et al. (2005)
Placenta	H	Yes	ND	PDMSC (placenta-derived multipotent stem cell)	Chang et al. (2006)
Skin	P	Yes	ND	PSOS (porcine skin-originated stem cell)	Dyce et al. (2006)
Testis	M	Yes	Yes	Neonatal testis cells	Kanatsu-Shinohara et al. (2004)
Testis	H	Yes	Yes	Adult human testis cells	Mizrak et al. (2010)
Testis	M	Yes	Yes	MASCs (multipotent adult spermatogonial-derived stem cells)	Seandel et al. (2007, 2008)
Testis	M	Yes	Yes	maGSC (multipotent adult germ line stem cells)	Guan et al. (2006, 2007)
Testis	H	Yes	Yes	haGSC (human adult germ line stem cell)	Conrad et al. (2008)
Testis	H	Yes	Yes	hMGSC (human multipotent germ line stem cell)	Kossack et al. (2009)
Thyroid	H	Yes	ND	Thyroid cell	Thomas et al. (2006)
Umbilical cord	H	Yes	ND	MSC	Tondreau et al. (2005)
Umbilical cord	H	Yes	ND	Cord blood stem cell	Sun et al. (2007)
Umbilical cord	H	Yes	Yes	CB-SC (cord blood stem cell)	Zhao et al. (2006)
Umbilical cord	H	Yes	ND	USSC (unrestricted somatic stem cell)	Kogler et al. (2006)
Umbilical cord	R	Yes	ND	RUCMC (rat umbilical cord matrix cell)	Jomura et al. (2007)

Human (H), mouse (M), pig (P), rat (R), and Rhesus monkey (Rh) tissues. *ND* not determined

Wojakowski et al. 2009). VSELs express the pluripotency genes OCT4, REX1, and NANOG as well markers of committed adult stem cells, including GFAP and MYOD. Their differentiation potential in vitro and in vivo is not fully clear.

Amniotic fluid-derived stem cells (AFSs) are isolated from both rodent and human amniotic fluid based on the expression of c-KIT. AFS can be expanded for more than 250 population doublings without telomere shortening while maintaining a normal karyotype (De Coppi et al. 2007a; Chiavegato et al. 2007). AFSs express OCT4 and SSEA4 but are negative for other ESC cell surface antigens such as TRA-1-81, TRA-1-60, and SSEA3, even though it is not fully clear whether expression of the ESC transcription factors was detected in uncultured cells. In vitro, AFSs give rise to neuronal, hepatic, endothelial, and mesenchymal cell types. The coculture of AFSs with rat cardiomyocytes induces differentiation into smooth muscle and cardiomyocytes. In vivo, however, AFSs did not contribute to the myocardium of normal, immunodeficient, or immunosuppressed rats (Chiavegato et al. 2007; De Coppi et al. 2007b).

In the many other papers (Table 4.2) *Oct4* expression (and in some instances *Nanog* and *Sox2*) was only detected in cell populations following in vitro culture. This could represent dedifferentiation from a somatic cell to cells that reexpress some of the pluripotency genes. As suggested above, and will be discussed, these data need to be interpreted cautiously as they may be based on the detection of *OCT4B* or pseudogenes if primers and antibodies used are not carefully chosen to exclude these possibilities (Kogler et al. 2006).

Lengner et al. (2007) demonstrated that *Oct4* expression is dispensable for mouse somatic stem cell self-renewal. Using conditional knockout mice the *Oct4* gene was specifically deleted in a number of somatic tissues including liver, hair follicles, intestinal epithelium, liver, brain, and bone marrow (MSC and HSC). However, *Oct4* deletion did not negatively impact the regeneration of the intestinal epithelium, liver, or hair follicle, the ability of HSC to engraft in myeloablated hosts, or the proliferation of MSCs and their osteogenic and chondrogenic capacity. In addition, loss of *Oct4* in the brain did not lead to behavioral defects or abnormalities in brain morphology (by Ki67 staining). In a second approach, wherein a knock-in-add-on *Oct4-EGFP* reporter mouse was used, no EGFP positive cells were found by FACS in the bone marrow and no EGFP signal was detected using antibodies raised against EGFP from sections obtained from stomach, skeletal muscle, lungs, skin, heart, thymus, bladder, spleen, prostate, and kidney. These studies demonstrate that if Oct4 positive cells exist outside of the male germ line in mice, they are extremely rare, and their function in tissue repair is negligible.

4.1.5 Oct4 in Somatic Tumors and Transformed Cell Lines

As would be expected, *OCT4* is expressed in many germ cell neoplasias, such as embryonic carcinoma (EC), dysgerminoma, germinoma, and seminoma (Tai et al. 2005; Lau and Chang 2006). When force expressed postnatally in mice, *Oct4* blocks

progenitor differentiation leading to dysplasia in epithelial tissues (Hochedlinger et al. 2005). Although many studies have suggested that *OCT4* is expressed in adult tumors (bladder cancer, breast cancer, pancreatic cancer, chondro- and osteosarcoma) and tumor-derived cell lines (HeLa, Mcf-7 and several derived from breast, pancreatic, kidney, gastric and liver tumor-tissues) (Tai et al. 2005; Iki and Pour 2006; Atlasi et al. 2007; Ezeh et al. 2005; Trosko 2006; Ponti et al. 2005; Gibbs et al. 2005), the same cautions used in evaluating *OCT4* expression in normal human stem cells must be applied to studies of tumors and tumor cell lines. In fact, Cantz et al. (2008) demonstrated using proper positive and negative controls that *OCT4A* is not expressed in HeLa and MCF7 cells, and that the previously described expression of *OCT4* in these cells could be ascribed to expression of the *OCT4B* isoform located in the cytoplasm. That *OCT4A* is not expressed was further ascertained by demonstrating that methylation of the *OCT4* promoter is >90% in HeLa and MCF7 cells while almost completely unmethylated in ESCs.

4.2 NANOG

4.2.1 Introduction

In 2003, a new homeobox transcription factor in ESCs was described and named *Nanog* after *Tír na nÓg*, the Land of Ever-Young in the old Irish mythology. (Mitsui et al. 2003; Chambers et al. 2003). *Nanog* is expressed in the late morula and the ICM of the blastocyst, as well as ESC and germ cell lines (Cavaleri and Schöler 2003; Hyslop et al. 2005; Hatano et al. 2005). In the ICM, *Nanog* is lost from cells that will give rise to parietal and visceral endoderm (Hamazaki et al. 2004), whereas cells destined to become the epiblast retain *Nanog* expression. *Nanog* then becomes asymmetrically expressed in the proximal epiblast (assumed primitive streak) and during gastrulation cells that migrate through the primitive streak and give rise to future mesoderm quickly downregulate *Nanog* (Hart et al. 2004). Nearly all fetal testicular cells remain *Nanog*$^{+}$ and rare *Nanog*$^{+}$ cells can be found up to 2–3 months postnatally, while *Nanog* is no longer detected at older age except in the case of germ cell tumors (Hart et al. 2004; Hoei-Hansen et al. 2005).

Nanog appears to be crucial during the second embryonic cell fate decision (between early and late blastocyst) as in *Nanog*$^{-/-}$ mice cells in the ICM fail to mature into pluripotent epiblast cells (Mitsui et al. 2003; Chambers et al. 2003; Silva et al. 2009). *Nanog*$^{-/-}$ cells are blocked in a transitional prepluripotent stage and ultimately will undergo apoptosis or develop into trophoblast. A similar effect is seen in somatic reprogramming where transfection with *Oct4*, *Sox2,* and *Klf4* drives somatic cells to a *Nanog*$^{-}$ prepluripotent stage and acquisition of *Nanog* is mandatory to gain full reprogramming to pluripotent cells (Silva et al. 2009).

On the other hand, Hyslop et al. (2005) demonstrated that *Nanog* is important for self-renewal and maintenance of the undifferentiated state of ESCs as shown by spontaneous differentiation of *Nanog*$^{-/-}$ ESCs into trophectoderm and extraembryonic (primitive) endoderm. Activation of *Stat3* is necessary for the self-renewal

capacity of murine ESCs and its activation can be induced by Leukemia Inhibitory Factor (LIF). Forced expression of *Nanog* but not *Oct4* in ESC results in LIF-independent proliferation, feeder-free growth, and increased colony formation, further demonstrating the importance of *Nanog* in maintaining pluripotency (Mitsui et al. 2003; Chambers et al. 2003; Darr et al. 2006; Pan and Thomson 2007).

The Nanog protein has a dimerization domain and a single DNA binding homeodomain but the exact sequence is still controversial (TAATGG vs. CATT) (Pan and Thomson 2007; Chambers and Tomlinson 2009). Liu et al. (2009) revealed that Nanog interacts with Smad1 (signal transducer in BMP pathway), Sall4, Nr0b1, Nac1, Esrrb, Zfp81, Hdac2, and Sp1. *Nanog* also upregulates the matrix extracellular genes *Mmp3, Mmp11, Mfap3, Gpc3, Spp1, Timp3,* and *Emilin2* and the proliferation genes *Cxcl2, Tacstd2, Prl, Tgfb2, Kitlg, Pdcd1lg1, IL-6, Mapre2,* and *Fzd3* (Liu et al. 2009). *Oct4* and *Sox2* bind upstream of the *Nanog* promoter making them important for *Nanog* regulation. *FoxD3* is also an activator of *Nanog* transcription while *Tcf3* and *p53* have a negative influence (Pan and Thomson 2007).

Nanog acts as an activator of the *Oct4* promoter and several *Id* (inhibitor of differentiation) genes (Hatano et al. 2005; Pei 2009). *Gata4* and *Gata6*, genes that promote primitive endoderm differentiation, are inhibited by *Nanog* in the ICM (Mitsui et al. 2003; Chambers et al. 2003; Hyslop et al. 2005). To demonstrate this hypothesis, Darr et al. (2006) overexpressed Nanog leading to acquisition of a primitive ectoderm phenotype (*Rex1*$^{-}$ *Gbx2*$^{-}$ *Fgf5*$^{+}$). In addition Chambers et al. (2007) found that *Nanog* is essential for germ cell formation, as Nanog$^{-/-}$ mESC lack germ line development.

4.2.2 Nanog Pseudogenes and Alternative Splice Forms

The human gene for NANOG, also known as *ENK* (early embryo specific expression NK family), is located on chromosome 12p13.31 and consists of four exons (Hoei-Hansen et al. 2005; Booth and Holland 2004). The N-terminal region of Nanog protein is rich in serine, threonine, and acidic residues, while the C-terminal region contains a tryptophan repeat (WR), transactivation domain (CD2), and a linker (CD1) between the WR and the homeodomain (Pan and Pei 2005).

As for *OCT4*, many (eleven) human *NANOG* pseudogenes have been identified by Booth and Holland (2004) with a high orthology rate compared to the chimpanzee genome (Fairbanks and Maughan 2006), probably reflecting the crucial position of *NANOG* in mammalian evolution. The first pseudogene was estimated to be mutated over 150 million years ago (Booth and Holland 2004). The pseudogene *NANOGP8* was found to have the highest similarity to *NANOG*, while *NANOGP3, 6,* and *11* had the lowest degree of homology (Table 4.3) (Pain et al. 2005).

Aside from pseudogenes, alternative slice variants have been described. Kim et al. (2005) demonstrated that the splice variant *NANOG-DELTA 48* has similar binding capacity as *NANOG* and also inhibits the transactivation of the *Gata4*-promotor. However, *NANOG-DELTA 48* is not expressed in ESCs. Interestingly, the ratio of *NANOG-DELTA 48* to full length *NANOG* transcript is higher in adult progenitor cells, which might suggest a possible role in differentiation (Kim et al. 2005).

Table 4.3 *Nanog* pseudogenes. Identity has been calculated by comparing nucleotide sequences using Megablast

Pseudogenes	Identity (%)	Chromosome	Type of pseudogene
Nanogp1	97	12	Nonprocessed
Nanogp2	90.7	2	Processed
Nanogp3	86.2	6	Processed
Nanogp4	93.7	7	Processed
Nanogp5	88.1	9	Processed
Nanogp6	75.2	10	Processed
Nanogp7	93.3	14	Processed
Nanogp8	99.3	15	Processed
Nanogp9	90.1	X	Processed
Nanogp10	89.9	X	Processed
Nanogp11	89.4	6	Processed

4.2.3 Nanog Expression in Adult Cells

In contrast to *Oct4* and *OCT4A, Nanog* is not expressed in late postnatal germ line stem cells. As for *Oct4* and *OCT4A* expression*, Nanog* has been reported in numerous articles in adult somatic stem cells (Table 4.2). *Nanog* was found in primary isolated pre-MSCs (Anjos-Afonso and Bonnet 2007), VSELs (Kucia et al. 2006) and AFS cells (De Coppi et al. 2007a), as well as in a number of studies following culture of MSC (Riekstina et al. 2009) in vitro even though many other studies did not detect *Nanog* (Jiang et al. 2002; Izadpanah et al. 2006). Both Go et al. (2008) and Liu et al. (2009) demonstrated that forced expression of *NANOG* in MSCs increased their proliferation and osteogenic and chondrogenic capacity but reduced their reduced their adipogenic differentiation.

As no good tools are yet available to discriminate between the *Nanog* gene and protein itself and the pseudogenes, or to discriminate between the *Nanog* isoforms, it is unclear whether the detection of *Nanog* in some adult stem cell populations and not in others represents true differences between these cell populations or results from artifactual detection of *Nanog*. Promoter methylation studies as have been described for the *OCT4* promoter could help clarify whether *Nanog* is expressed in adult somatic cells.

4.3 Conclusion

Oct4 and *Nanog* expression in adult stem cells and tumors remains controversial. The only adult stem cell population retaining *Oct4* expression is the SSC (Kehler et al. 2004; Boiani et al. 2004), whereas in adult life these no longer express Nanog (Hart et al. 2004; Hoei-Hansen et al. 2005). Genetic studies have shown that if Oct4 positive cells exist outside of the male germ line in mice they are extremely rare, and their function in tissue repair is negligible (Lau and Chang 2006). No such studies are available yet for *Nanog*.

Based on the studies demonstrating that pluripotency can be regained from unipotent male germ line stem cells (Seandel et al. 2007; Guan et al. 2006; Kossack et al. 2009; Conrad et al. 2008) and the fact that somatic terminally differentiated cells can be reprogrammed to an ES-like state using forced expression of some transcription factors and small molecules that can alter the methylation and acetylation state of histones and DNA (Takahashi and Yamanaka 2006; Takahashi et al. 2007; Kim et al. 2009; Shi et al. 2008), it remains possible that in some studies wherein *Oct4* and/or Nanog was detected in cultured adult somatic stem cells spontaneous epigenetic alterations accounted for reexpression of these pluripotency genes.

However, all these studies should be assessed with the knowledge that both human *OCT4* and *NANOG* have multiple isoforms and that only *OCT4A* and *NANOG* have been proven to maintain stemness. Nevertheless, many pseudogenes also exist that can be amplified by RT-PCR. Hence, primers should be used that discriminate between these various forms of *OCT4* and *NANOG* (Pain et al. 2005; Suo et al. 2005; Kogler et al. 2006) and antibodies that identify the unique N-terminal portion specific to *OCT4A* that is localized to the nucleus and not cytoplasm (Cauffman et al. 2005, 2006). Such *Nanog*-specific antibodies and studies evaluating the expression of *Nanog* in the nucleus or cytoplasm of cells are still missing. As demonstrated by Cantz et al. (2008), the use of bisulphite sequencing to assess the methylation status of either the *OCT4* or *NANOG* promoter would provide definitive proof that OCT4A and NANOG can be expressed, and hence may be of importance for the perceived greater potency of adult somatic stem cells that appear to express these stemness transcription factors.

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Chapter 5
Adult Stem Cell Plasticity Revisited

Eva Mezey

Abstract Cell biologists have long realized that most cells do not live as long as the organisms they comprise; thus, cells in almost every tissue need to be renewed/replaced during the natural lifespan of the organism. Depending on the turnover rate of cells in any given organ, this process can be very frequent or very rare. Epithelial cells in the mouth and the GI tract are exposed to a variety of insults (such as heat, cold, extreme changes in pH, strong spices, etc.) and have a very fast turnover rate; nerve cells get wired during embryonal development and either do not turn over or have a very low turnover rate. The rest of the tissues are somewhere in between. This kind of tissue regeneration relies on undifferentiated tissue-specific stem cells (also known as somatic stem cells) that are found in all adult animals and humans and multiply by cell division. They replenish cells that die from old age and regenerate those that have been damaged.

Scientific interest in adult stem cells has centered on their ability to divide or *self-renew* indefinitely and generate all the cell types of the organ from which they originate, potentially regenerating the entire organ from a few cells. Unlike embryonic stem cells, the use of adult stem cells in research and therapy is not considered to be controversial as they are derived from adult tissue samples rather than human embryos. They have mainly been studied in humans and model organisms such as mice and rats.

Adult stem cells can potentially be used (1) to help us understand basic biological mechanisms (2) to regenerate aged or damaged tissues, and (3) to improve the health of organs by releasing agents that promote growth or differentiation of cells. Below, I will try to summarize the present knowledge of adult stem cells with regard to their (trans)differentiation abilities. Due to the vast amount of literature available, I will focus on human data when they are available and refer the reader to reviews for more details than I can provide in the space available.

Keywords Adult stem cells • Bone marrow-derived stem cells • Chimerism • Neurogenesis • Tissue-specific stem cells • Transdifferentiation

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Abbreviations

BM	Bone marrow
BMSC	Bone marrow-derived mesenchymal stem cell
DMD	Duchenne's muscular dystrophy
EGFP	Enhanced green fluorescent protein
FISH	Fluorescent in situ hybridization
GVHD	Graft versus host disease
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell

5.1 Introduction

The first adult stem cells that were recognized and used in clinical therapy were bone marrow (BM) stem cells. The history of BM transplantation started with the recognition that bone marrow from identical siblings can successfully reconstitute the entire hematopoietic system when it is destroyed by either radiation or chemicals. The more general therapeutic use of BM transplantation for patients suffering from leukemia or aplasia started after the major histocompatibility antigens were discovered and their role was better understood (Perry and Linch 1996; Thomas 2005). Due to the success of appropriately matched transplants, a large network of BM banks has been established. Marrow is now routinely matched to recipients who have no close family donors available.

In addition to the BM-derived stem cells which include hematopoietic stem cells (HSCs) and bone marrow stromal or mesenchymal stem cells (BMSCs) (which are the precursors of bone, fat, and cartilage), many other types of tissue-specific stem cells have now been identified and studied in detail – stem cells from endothelium, skin, fat, GI tract, liver, lung, CNS, a variety of endocrine organs, heart, and skeletal muscle (Barrilleaux et al. 2006; Mimeault and Batra 2006, 2008; Mimeault et al. 2007; Sell 2005; Verstappen et al. 2009; Walker et al. 2009). Tissue regeneration is usually prompted by environmental changes in specific tissue domains and is regulated by a variety of cytokines, chemokines, and growth factors released by injured cells and immune cells recruited to the site, as well as stem/progenitor cells themselves (Gnecchi et al. 2008; Mimeault and Batra 2007). While there is a consensus about tissue-specific stem cells that regenerate injured or aged tissue, there is a hot debate in the literature about the exclusive commitment of these cells towards the tissue where they reside. According to classical developmental biological dogma, stem cells can only contribute to the tissue that they live in, and this "lineage commitment" cannot be overridden under any circumstances. During development, this may be true, but during the last decade, studies in humans and in animals have suggested that "tissue specific" stem cells may be much more flexible than previously envisioned. This flexible behavior of stem cells has been referred to as "transdifferentiation" because the cells appear to be ignorant of the laws we have made for

them and sometimes cross lineage “barriers.” The idea that transdifferentiation may occur remains controversial. The current state of the literature examining this phenomenon is summarized below.

5.2 Is There Transdifferentiation?

Excellent reviews are available on the topic of transdifferentiation (Gruh and Martin 2009; Leri et al. 2005; Phinney and Prockop 2007; Rovo and Gratwohl 2008; Vieyra et al. 2005) and therefore emphasis will be placed on discussing a few key points related to this phenomenon. First and foremost, what criteria must be met to convincingly demonstrate transdifferentiation? To prove that cells can cross dermal lineages, one must be absolutely certain that the starting cell and the cells derived from it can be reliably characterized. The original cell must also be able to renew itself (to make an identical stem or progenitor cell) and produce differentiated daughter cells. Finally, the resulting cells must function normally in the tissue where they reside and should contain only one set of chromosomes, thereby demonstrating reprogramming as opposed to being the product of fusion between the stem cell and a cell resident within the tissue of interest. While HSCs have been proven to replenish virtually all circulating hematopoietic cell lineages, they are not believed to contribute to other nonhematopoietic lineages.

Many of the controversies in the transdifferentiation literature can be attributed to the fact that there is no consistency among studies. The cells used are frequently poorly characterized; they are cultured in a variety of ways before they are used; they are labeled in a number of ways; and their final morphology and function is not always rigorously established. Below is a critical overview of studies suggesting that transdifferentiation might exist and can have meaningful consequences. Due to the vast literature, attention will be focused on BM-derived adult stem cells.

5.3 Contribution of Bone Marrow-Derived Stem Cells to Cells of Other Organs

5.3.1 Bone Marrow Differentiation into Organs of Ectodermal Origin

5.3.1.1 CNS

Until about 20 years ago, it was generally accepted that, after the developmental period ends, neurons in the brain could not be regenerated. This principle was overturned when neuronal stem cells were first discovered and a whole new field of neurobiology was born (Reynolds and Weiss 1992). Many studies have been published on adult neurogenesis and several outstanding reviews have appeared on this

subject (Gage 2002; Taupin and Gage 2002). These stem cells are present in the embryonal brain and develop together with the brain itself, keeping the potential of producing additional neurons postnatally during physiological processes, such as learning and exercise (van Praag 2009). Even more surprising were data suggesting that circulating cells from the blood might be able to enter the rodent brain and develop into new neural cells including microglia, macroglia, and even neurons (Brazelton et al. 2000; Eglitis and Mezey 1997; Mezey et al. 2000). These findings were met initially with a lot of skepticism due to the possible technical problems that could have been responsible for the results. Many groups continued to study the possible regenerative and/or neuroprotective effect of BM transplantation or injection of different populations of BM cells into the CNS following a variety of injuries including, but not restricted to, stroke, trauma, and degenerative diseases (Hess and Borlongan 2008; Li and Chopp 2009; Mezey 2007; Phinney and Isakova 2005; Sadan et al. 2009) and most workers concluded that BM-derived cells help the healing process. The most widely accepted mechanism of this healing is that the BM cells enter the CNS, migrate to the injury site, and release neuroprotective and survival factors as well as anti-inflammatory agents. Interestingly, studies of post-mortem human samples confirmed the rodent results and showed that female patients who received gender-mismatched BM transplants had Y chromosome containing neural cells (including neurons) present in their brains (Cogle et al. 2004; Crain et al. 2005; Mezey et al. 2003; Weimann et al. 2003). With the exception of Purkinje cells (Weimann et al. 2003), these cells were diploid suggesting that they were not the products of cell fusion (Cogle et al. 2004). These human data suggest that transdifferentiation is a real, naturally occurring phenomenon, even if it is very rare. In any event, because BM cells are able to enter the brain and travel to areas of need, it has been suggested that they might be used as vectors for missing factors (e.g., enzymes) and/or agents that could selectively kill cancer cells (Li and Chopp 2009; Rath et al. 2009; Sadan et al. 2009; Vaquero and Zurita 2009). These exciting possibilities deserve to be explored in greater detail (Table 5.1).

5.3.1.2 Skin/Hair Follicles/Fingernails

Skin epithelia constantly renew themselves physiologically and very frequently need repair due to everyday demand on the body surface and injuries. To satisfy this need, there is a large reservoir of stem cells in the skin that has been the focus of basic biologists as well as dermatologists. These stem cells are well described in several recent reviews (Abbas and Mahalingam 2009; Blanpain and Fuchs 2009; Fuchs 2009). Due to the continuous need for skin regeneration, one wonders if the pool of skin stem cells itself requires renewal, and if so, whether circulating cells contribute to this pool. Interestingly, the hematopoietic progenitor marker CD34 proved to be a specific marker of bulge cell keratinocytes in murine cutaneous epithelium (Trempus et al. 2003). In the human, however, CD34 is absent from the bulge area, but is present in stem cells of the outer root sheath (Hoang et al. 2009; Kloepper et al. 2008; Poblet et al. 2006). Since these cells lack CK15, a type I keratin,

Table 5.1 A summary of data studying differentiation of bone marrow-derived stem cells with special focus on human data where available

Organ studied	Species	Source of Stem cell	Contribution to organ	Method(s) to recognize cells	Reference
Ectoderm					
CNS	Rat	BM	Astrocyte, microglia	Y/viral DNA	Eglitis and Mezey (1997)
	Ms	BM	Neuron	GFP	Brazelton et al. (2000)
	Ms	BM	Neuron, microglia, astrocyte	GFP&Y	Mezey et al. (2000)
	Hu	BM	Purkinje – fusion	Y	Weimann et al. (2003)
	Hu	BM	Neuron, microglia	Y	Crain et al. (2005); Mezey et al. (2003)
	Hu	BM/PBSC	Neuron, no fusion	Y	Cogle et al. (2004)
Skin/Hair	Ms	single CD34+	Epithelial cells in lung, liver, GI tract, and skin	Y	Krause et al. (2001)
	Hu	BM/PBSC	2–7% Keratinocyte chimerism	Y or DNA analysis	Korbling et al. (2002)
	Hu	PBSC	1.6–7.8% Keratinocyte chimerism	Y	Murata et al. (2007)
Fingernail	Hu	PBSC	9–73% Chimerism	STR	Imanishi et al. (2007)
	Hu	PBSC	14–58% Chimerism 8 patients more if GVHD	STR	Pearce et al. (2008)
Epidermis/ microvessel	Hu	PBSC	Aorta and periph arteries 1 patient 25% of aorta endothelium is donor origin	Y	Suzuki et al. (2003)
Vasc endothel	Hu	PBSC	18 Patients keratinocytes and endothelial cells only when GVHD	Y	Murata et al. (2007)
Endoderm					
Oral mucosa	Hu	BM	High percentage of donor genotype in mixed blood/epithelial samples; 2 of 10 patients only donor	STR	Endler et al. (1999)
	Hu	BM/PBSC	Average 21% donor cell in buccal swabs	STR	Thiede et al. (2000)
	Hu	BM/PBSC	0.8–12.7% Chimerism, 0.01% fusion	Y	Tran et al. (2003)
Uterine mucosa	Hu	BM	0.2–48% of epithelium	HLA typing	Taylor (2004)
	Ms	cd45+	Up to 80% chimerism after pregnancy in endometrium	CD45-Cre/GFP-lox	Bratincsak et al. (2007)

(continued)

Table 5.1 (continued)

Organ studied	Species	Source of Stem cell	Contribution to organ	Method(s) to recognize cells	Reference
GI tract	Hu	BM/liver transplant	4–40% Hepatocytes, cholangiocytes	Y	Theise et al. (2000)
	Hu, Ms	BM	Donor-derived myofibroblasts and epithelium in small intestine and colon	Y	Brittan et al. (2002)
	Hu	BM	Following ulcer and GVHD, lots of epithelial cells are Y +	Y	Okamoto et al. (2002)
	Hu	BM	No epithelial cells	Y	Meignin et al. (2004)
	Hu	PBSC	Real epithelial chimerism 0.2% of cells no fusion	Y	Spyridonidis et al. (2004)
Liver	Rat	BM/liver transplant	There are hepatocytes from donor	Y	Petersen et al. (1999)
	Hu	BM/liver transplant	4–40% Hepatocytes, cholangiocytes	Y	Theise et al. (2000)
	Hu	BM/PBSC	0–7% of hepatocytes; also skin and GI epithelium	Y or DNA analysis	Korbling et al. (2002)
	Ms	BM	Hepatocytes form when pressured; very few in healthy mice	Y	Mallet et al. (2002)
	Ms	BM	Corrects an enzyme deficiency by fusion	Y	Vassilopoulos et al. (2003)
Pancreas	Ms	splenocytes	Diabetes is corrected; Y + islet and ductal cells	Y	Kodama et al. (2003)
	Ms	BM	BM-derived islet cells are functional. No fusion	Cre-LoxP/EGFP-insulin system	Ianus et al. (2003)
Lung	Ms	single CD34+	Epithelial cells in lung, liver, GI tract, and skin	Y	Krause et al. (2001)
	Hu	PBSC	2–8% epithelial 37–42% endothelial chimerism	Y	Suratt et al. (2003)
	Hu	PBSC	Surfactant positive cells are t II pneumocytes	Y	Mattsson et al. (2004)
	Hu	BM/lung transplant	t II Pneumocytes, endothelial cells, and macrophages; not fusion	Y	Albera et al. (2005)
	Hu	PBSC	t II Pneumocytes, tracheal, and bronchial epithelium	Y	Krause (2008)
	Ms	BM	Cytokine-induced G(1)/S interface of the cell cycle transdifferentiation	EGFP	Dooner et al. (2008)

Kidney	Hu	renal transplant	0.6–6.8% Y+ tubular epithelium	Y	Poulsom et al. (2001)
	Ms	BM	Glomeruli mesangial cells	GFP	Imasawa et al. (2001)
	Rat	BM	Glomeruli mesangial cells	GFP	Ito et al. (2001)
	Hu	renal transplant	1% Y+ tubular epithelium	Y	Gupta et al. (2002)
	Ms	BM	6.2% per glomerulus mesangial cells	LacZ	Sugimoto et al. (2006)
Mesoderm					
Skeletal muscle	Ms	BM	mdx Mouse: dystrophin+ myocytes	Y	Bittner et al. (1999); Ferrari et al. (1998)
	Ms	HSC	mdx Mouse functional recovery	distrophin	Gussoni et al. (1999)
	Hu	BM	Duchenne MD – muscle cells 0.5–0.9%	distrophin	Gussoni et al. (2002)
	Ms	single HSC	Muscle injury; myocytes	CD45.1–2; lacZ/GFP	Camargo et al. (2003); Corbel et al. (2003)
	Ms	BM	GFP+ myocytes mostly after injury	GFP	Abedi et al. (2007)
Cardiac muscle	Ms	lin–, c-kit+	68% of infarct is GFP+	GFP	Orlic et al. (2001b)
	Hu	Heart transplants	0.04% of cardiomyocytes are Y+	Y	Laflamme et al. (2002)
	Hu	Heart transplants	Chimerism (14–16%)	Y	Quaini et al. (2002)
	Hu	BM/PBSC	Myocardium and vessels	Y	Thiele et al. (2004b)
	Hu	BM/PBSC	2% Endothelial cells in skin and gut; no fusion	Y	Jiang et al. (2004)
	Ms	BM	Myocardium and vessels	DNA tag	Rota et al. (2007)
	Ms	BM	“Nitric oxide” deficiency – no chimerism	GFP	Perry et al. (2009)

Hu human; *Ms* mouse; *BM* bone marrow; *PBSC* peripheral blood stem cells; *STR* short tandem repeat; *GFP/EGFP* green fluorescent protein; *Y* Y chromosome; *FISH* fluorescent in situ hybridization; *t II* type II

they were suggested to be transit-amplifying cells, precursors of the bulge stem cells (Hoang et al. 2009; Poblet et al. 2006). In a groundbreaking study, Krause et al (2001) used female mice transplanted with a single male HSC and showed the presence of male cells in the skin at a frequency of 1.2–2.7% using fluorescent in situ hybridization (FISH) analysis. Korbling et al. (2002) obtained tissues from female recipients transplanted with male HSCs and also identified male donor cells in the skin using a combination of immunochemistry for specific epithelial antigens and FISH; 2–7% of the cells in their biopsy samples were Y chromosome positive. In female patients with acute graft versus host disease (GVHD) who received HSC transplants from male donors, Murata et al. (2007) demonstrated that 1.6–7.8% of keratinocytes and 2.2–9.4% of endothelial cells were donor-derived, and similar outcomes were shown in a human patient by Suzuki et al. (2003). Deng et al. (2005) transplanted fluorescently labeled BMSCs from white male mice into lethally irradiated black female mice and observed the recipient mice grew white hair all over their bodies. The authors concluded that BM-derived cells give rise to functional skin cells in an injury setting. Two other groups demonstrated a high level of chimerism (9–73%) in fingernail shavings from gender-mismatched transplant patients using short tandem repeat PCR (Imanishi et al. 2007; Pearce et al. 2008). The above data suggest that – especially in injury settings – hematopoietic progenitors are able to participate in skin regeneration in both mice and humans. On the other hand, Hematti et al. (2002) could not detect any donor-derived cells in the skin of female transplant recipients of male BM. The reason for this is unclear.

5.3.2 BM Differentiation into Organs of Endodermal Origin

5.3.2.1 Oral Mucosa

PCR analysis of short tandem repeats from blood and mouthwash samples was performed to determine the genotypes of recipient vs. donor cells in 17 BM transplant patients (Endler et al. 1999). To the surprise of the researchers, donor DNA was detected even after all blood cells were removed from mouthwash samples and they concluded that this must be due to technical problems. In a large number of patients using the same technique, another group found 5–63% chimerism (cells of donor origin) in buccal swab samples. Tran et al. (2003) used buccal scrapings on microscope slides and Y chromosomal FISH combined with cytokeratin as an epithelial marker to look for donor-derived cells in gender-mismatched transplant patients. The patients received either BM or HSC transplants. Since all the samples had chimeric cell populations (0.8–12.7% donor-derived), they further analyzed the cells for fusion and found only two potentially fused cells (0.01%). Finally, they used microsatellite markers to see if the buccal cell Y chromosome is identical to the donor or to the son of one patient. The Y chromosome showed a 100% identity to the donor, and none with the son, confirming that the cells must be of donor BM or HSC origin.

5.3.2.2 Uterine Endometrium

The uterine lining must fully regenerate after every menstrual cycle, and Taylor (2004) wondered whether circulating cells contributed to the process in humans. He used tissue samples from four human leukocyte antigen (HLA)-mismatched transplant recipients to look for donor HLA markers by means of RT-PCR. All four women had donor HLA in their epithelial (0.2–48%) and stromal (0.3–52%) cell populations (Taylor 2004) and Taylor concluded that nonuterine cells can contribute to endometrial regeneration. Similar results in rodents were published by Bratincsak et al. (2007) who constructed a mouse in which Cre recombinase is driven by the CD45 promoter and bred it to a mouse with enhanced green fluorescent protein (EGFP) preceded by a strong promoter and a floxed transcriptional stop. These mice expressed EGFP in all CD45 lineage cells. The uterine epithelium had patches of green fluorescent epithelia, suggesting that the cells were clonal in origin, and the number of these patches seemed to increase with the age of the animal, i.e., the number of estrus cycles it had gone through. Following pregnancy, about 80% of the uterine epithelial cells expressed the marker suggesting a very major contribution to this population by circulating cells that had once been CD45 positive.

5.3.2.3 Gastrointestinal (GI) Tract

Due to the relatively rapid turnover of GI epithelial cells, several groups searched for a contribution to this cell population by exogenous cells. Following irradiation and BM transplantation, donor-derived myofibroblasts and epithelium in the small intestine and colon were found in both humans and mice (Brittan et al. 2002; Okamoto et al. 2002) using Y chromosome FISH. The number of donor-derived cells seemed to increase if ulcer or inflammation (GVHD) was present (Okamoto et al. 2002). Contrary to these observations, Meignin could not demonstrate any epithelial cells in the duodenum of BM-transplanted patients who developed GVHD (Meignin et al. 2004). In a very thorough study using a rigorous three-dimensional analysis on single sections of colon biopsies triple stained with donor-specific, epithelial-specific, and hematopoietic-specific markers, Spyridonidis et al. (2004) demonstrated that chimerism of colon epithelium occurs after human hematopoietic cell transplantation. The authors excluded cell fusion as the possible underlying mechanism of the findings. They observed that tissue damage enhances engraftment by donor-derived epithelial cells.

5.3.2.4 Liver

Petersen et al. (1999) identified BM as a source of hepatic cells in female rats following transplantation with male cells using the Y chromosome as a marker of BM derivatives and dipeptidyl peptidase IV enzyme, and L21-6 antigen to identify liver cells. Using archived specimens of gender-mismatched BM or liver transplanted humans, Theise et al. (2000) showed that 4–40% of the liver hepatocytes arose from

exogenous cells confirming the animal data. A year later, another group reported that intravenous injection of adult BM cells rescued the Fah (−/−) mouse, an animal model of tyrosinemia, and showed that only purified HSCs gave rise to hepatic regeneration (Lagasse et al. 2000). Using the same system and the Fah (−/−) mice, Vassilopoulos et al. (2003) demonstrated that the new hepatocytes are generated by fusion of hematopoietic and liver cells and following two more publications with similar conclusions (Terada et al. 2002; Ying et al. 2002), the possibility of transdifferentiation was ignored and fusion favored in all instances. It is worthwhile to take a closer look at the problem in the light of data known about liver regeneration.

Guidotti et al. (2003) showed that cultured hepatocytes commonly replicate their genomes in the absence of cell division. The resulting cells are tetraploid, and if the above process repeats itself, their children or grandchildren can even be octaploid. The *Fah*$^{-/-}$ mice have a neonatal lethal phenotype resulting from hepatic dysfunction and require treatment with 2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3 cyclo -hexamedione to survive. Drug treatment was suspended after the mice were transplanted, and surely this was stressful to the *Fah*$^{-/-}$ hepatocytes (Vassilopoulos et al. 2003; Wang et al. 2003). To understand the importance of this stress, we would have to know why, among all cells of the body, the liver cells are polyploid. Hepatocytes are the body's first line of defense against harmful agents in the diet, and polyploidy may permit them to manufacture detoxifying enzymes at higher levels. Furthermore, it may also protect them from loss of recessive oncogenes and malignant transformation; the more copies of a gene you have, the less likely it is that all of these will be lost. Given these facts, stress could induce liver cells to fuse as part of an organ-specific defense mechanism. In any case, fusion may be much less common in cells that are prone to remain diploid throughout their lives, and the suggestion that the fusion of stem cells to differentiated cells could account for all of the evidence presented in favor of transdifferentiation is very likely to be incorrect. The issue has been discussed in several reviews (Austin and Lagasse 2003; Dorrell and Grompe 2005; Grompe 2003; Popp et al. 2006) and it seems likely that both mechanisms (i.e., transdifferentiation and fusion) may play a role in tissue regeneration by exogenous cells.

5.3.2.5 Pancreas

The regeneration of pancreatic beta cells has been a hot topic for decades due to the ever-increasing prevalence of diabetes and the need for better treatments. Thus, Kodama et al. (2003) excited both interest and controversy when they published a study showing successful treatment of NOD mice with end-stage diabetes by injection of donor splenocytes and complete Freund's adjuvant. This treatment appeared to eliminate the autoimmune response and permanently restore normoglycemia. This was accompanied by the reappearance of pancreatic beta cells. Kodama et al. (2003) demonstrated that donor male or labeled splenocytes rapidly differentiated into islet and ductal epithelial cells within the pancreas in diabetic NOD females. While others have successfully reversed diabetes using Kodama's protocol (Chong et al. 2006;

Nishio et al. 2006; Suri et al. 2006), the mechanism of the effect is still debated. Ianus et al. (2003) reached similar conclusion to Kodama using BM cells from male mice that express EGFP if the insulin gene is actively transcribed. When these cells were transplanted into lethally irradiated recipient female mice, Y chromosome and EGFP double-positive cells were seen in the pancreatic islets of recipients. EGFP could not be detected in either BM cells or circulating peripheral nucleated cells. The EGFP-positive islet cells were shown to express insulin, glucose transporter 2, and transcription factors typically found in pancreatic beta cells. These results suggest that there are cells in the BM (and in the spleen) with the capacity to differentiate into functionally competent pancreatic endocrine beta cells and they might represent a potential for cell-based treatment of diabetes mellitus (Ianus et al. 2003).

5.3.2.6 Lung

In her seminal paper, Krause et al. (2001) reported that one single CD34+ cell is capable of repopulating the BM and giving rise to a variety of tissue cells including lung epithelium in mice. Three groups later studied the relevance of the above finding in human biopsied or archived material from female patients who received gender-mismatched BM or lung transplants using the Y chromosome as a marker. All groups concluded that, indeed, there is chimerism in both epithelium and endothelium. One group found 2.5–8% epithelial and 37.5–42.3% endothelial donor contributions (Suratt et al. 2003). Similarly, Mattsson et al. (2004) found 2–6% Y positive and surfactant positive lung epithelial cells following nonmyeloablative HSC transplantations. In lungs of male patients who had received female lung transplants, Y chromosome positive, surfactant positive type II pneumocytes were found. Using concomitant X and Y chromosome hybridization, the authors excluded fusion as a possible explanation for the finding (Albera et al. 2005). More recently, Krause (2008) examined the effect of radiation damage to the lung on the level of BM-derived cell engraftment. She concluded that lung injury stimulates both hematopoietic and mesenchymal BM cells to contribute to the lung epithelium.

5.3.2.7 Kidney

The kidney is a very complex organ and contains more than 20 different cell types. These cells also differ in their proliferation rate and more than one type of tissue resident stem cell exists to help maintain the balance of proliferation in health and in injury. It was almost 40 years ago when Sinclair published his observations on the endothelial cells of 40 human kidneys transplanted to recipients of the opposite sex and showed that the donor endothelium had persisted. However, in three poorly functioning damaged grafts, a high proportion of the endothelial cells in capillaries and veins seemed to derive from the host. He suggested that the repopulated endothelium possibly derived from circulating cells (Sinclair 1972). With the technical development and the increased knowledge about stem cell biology, many groups

continued to study the contribution of circulating cells to the kidney in patients who received gender-mismatched organ transplants. Several groups have reported the presence of Y chromosome containing renal tubular cells ranging from 0.6 to 6.8% in one study (Poulsom et al. 2001) and about 1% in another (Gupta et al. 2002) in kidneys of male patients who received kidneys from female recipient. These studies also suggested that cells outside of the kidney are able to be recruited and replenish tubular epithelium and they conclude that kidney injury seems to increase the number of cells that are recruited from the circulation. Rodent studies using GFP+ BM transplanted into lethally irradiated mice and rats also demonstrated that circulating BM-derived cells could differentiate into glomeruli mesangial cells (Imasawa et al. 2001; Ito et al. 2001). In an interesting study, BM-derived cells were also examined for their ability to repair a genetic collagen defect in mice. The authors used a model of Alport syndrome, when genes encoding the type IV collagen have mutations that result in structural basement membrane defects leading to proteinuria and eventually renal failure. They demonstrated that in a mouse with similar defects the transplanted beta-galactosidase (β-Gal) positive and gender-mismatched BM gives rise to cells that can replace podocytes and mesangial cells and can partially restore glomerular function (Sugimoto et al. 2006). They found 6.2% β-Gal positive cells per glomerulus at 12 weeks of age and the number seemed to be increasing, reaching over 10% by week 20. The authors did not rule out fusion as a mechanism and suggest that kidney injury needs to be present for the BM cells to be recruited. The possible sources and ways of how the different compartments of the kidneys can regenerate are nicely summarized in recent reviews (Gupta and Rosenberg 2008; Krause and Cantley 2005). Among these possibilities, it seems very likely that BMSCs contribute to kidney regeneration through the release of growth factors, anti-inflammatory cytokines, and antiapoptotic factors (Baer and Geiger 2010; Camussi et al. 2010).

5.3.3 BM Differentiation into Organs of Mesodermal Origin

5.3.3.1 Skeletal Muscle

Ferrari et al. (1998) reported that BM cells can contribute to myogenesis in genetically diseased muscle of mice with muscular dystrophy. They did not define the nature of the cells that differentiated into myocytes, but their work spawned studies aimed to do just that. Gussoni et al. (1999) first reported in an animal model of Duchenne's muscular dystrophy (DMD) that the intravenous injection of either normal HSCs or a novel population of muscle-derived stem cells into irradiated animals resulted in the incorporation of donor-derived nuclei into muscle and the partial restoration of dystrophin expression in the affected muscle. He later published an interesting case report showing that a person who received gender-mismatched BM as a child and later developed DMD presented donor nuclei in muscle biopsies within a small number of muscle myofibers (0.5–0.9%). These data documented the occurrence of BM cells fusing with skeletal muscle cells and maintaining the fused DNA for over

a decade (Gussoni et al. 2002). Corbel et al. (2003) transplanted isolated GFP+ HSCs into GFP-recipients and studied their possible contribution to healthy as well as diseased muscle. Their results showed that in a portion of the healthy animals a few GFP+ myocytes could be found, but when a toxin was used to damage the anterior tibial muscle, all animals seemed to incorporate the donor HSCs into their regenerating muscle. The authors did not rule out fusion as a possible explanation of the findings. In a similar experiment, Abedi et al. (2007) transplanted animals with different populations of GFP+ BM-derived cells and looked for GFP+ myocytes in toxin-injured *tibialis anterior* muscles. The authors concluded that HSCs rather than mesenchymal cells or more differentiated hematopoietic cells preferentially participated in skeletal muscle regeneration.

5.3.3.2 Cardiac Muscle

Heart attacks are the leading cause of death and kill close to half a million people in the US every year. New treatments that might help victims survive and achieve a good quality of life receive attention from the public and research communities alike. Thus in 2001, when Orlic et al. (2001a) reported that Lin(−) c-kit(+) BMCs from syngeneic mice injected into the hearts of animals had acute infarcts differentiated into myocytes and vascular structures, many groups started to work on cellular repair in heart disease. In a subsequent article the same group reported that cytokine-mobilized BM cells caused a significant drop in mortality rate and improvement of function following myocardial infarctions. They observed large numbers of new myocytes and capillaries in the damaged heart (Orlic et al. 2001b). Nygren et al. (2004) has demonstrated that BM-derived cells fuse with cardiomyocytes in infarcted mouse hearts. BMSCs, on the other hand, were shown very early to be able to differentiate into cardiomyocytes in vitro and in vivo (Makino et al. 1999; Tomita et al. 1999). Based on testing different fractions of hematopoietic cells, Doyonnas et al. (2004) concluded that the c-kit-positive immature myelo-monocytic precursors are responsible for the newly generated myocytes following local intra-muscular injection of the cells. A few years ago in a very elegant study, Rota et al. (2007) injected BMSCs in the border of the infarct and used a variety of techniques to conclude that BMSCs engraft into the injured heart and become functional (as shown by electrical activity and mechanical function of the heart) cardiomyocytes in the mouse. After initial enthusiasm based on the above rodent data, there were disappointing results in human trials, e.g., CD34+ cells did not appear to proliferate or transdifferentiate into cardiomyocytes (Balsam et al. 2004; Murry et al. 2004). Thiele et al. (2004b) examined gender-mismatched transplant patients and found a lower incidence (1.6%) of chimeric cardiomyocytes after allografting with peripheral blood stem cells than after full BM transplants (5.3% of donor-derived cells). Assmus et al. (2007) showed that in patients the intracoronary infusion of BMSCs results in a significant reduction of postinfarction heart failure. At this time, there are clinical data on over 1,000 patients with heart disease who received stem cell therapy and the data have been carefully analyzed (Gersh et al. 2009). This includes

a variety of cell types (whole BM; HSC, BMSC) and routes of administration. The consensus is that the procedure seems to be relatively safe and that cardiac function shows a modest improvement on average. Although there is still a debate about the mechanism of action (transdifferentiation, fusion, paracrine effects, or a combination of any of the above), based on safety it is surely worthwhile to learn much more about the biology of these stem cells and to optimize their efficiency in healing the injured heart. Irrespective of mechanism of action, it is the final interest of patients to pursue and understand the process that can potentially help them.

5.4 Summary

Many questions remain about the plasticity of adult stem cells. In spite of this, we have learned a lot about these cells in the last decade:

1. All organs appear to harbor some kind of stem/progenitor cell that is capable of regenerating most of the cells that comprise it.
2. Circulating cells seem to contribute to the regeneration of all organs studied to date. These cells originate in the BM and may be either HSCs, BMSCs, or organ-specific progenitors. The existence of a circulating endothelial progenitor is generally accepted.
3. HSCs can replenish all blood cell lineages and most likely also endothelial cells.
4. BMSCs are quite plastic with respect to their differentiation potential. These cells are known to be heterogeneous at the population level (Crigler et al. 2006; Phinney 2007), but have been shown to secrete a variety of growth factors, chemokines and cytokines. They differentiate into a variety of cell types and/or help healing and regeneration by blocking apoptosis and secreting survival and growth factors.

As described above, the available data support the suggestion that transdifferentiation occurs. If the environmental need arises, there are cells that are able to switch fate and help regenerate injured tissues. The magnitude of this phenomenon seems to be rather low normally, and its physiological importance could be debated, but it might be possible to stimulate the process so that it has therapeutic value. For some time, workers in the field have debated the roles of hematopoietic vs. stromal stem cells in tissue regeneration. This may not be useful. The two stem cell pools may have a common progenitor or may be able to recommit themselves. The recent observation that single hematopoietic cells may give rise to fibroblasts in mice (Ebihara et al. 2006) as well as humans (Shirai et al. 2009) is very important in this regard. Although one group could not reproduce these results (Koide et al. 2007), the discrepancy is most likely due to technical problems (Ogawa and LaRue 2007). If the colony forming unit fibroblasts cultured from BM were indeed derived from HSCs at some point during development (or even during postnatal life), then the two populations of BM stem cells might indeed have many overlapping function and the seemingly contradictory data in the literature will need to be reevaluated (Ogawa et al. 2006).

The most important message is that we have to keep an open mind and focus not only on what happens physiologically, but also on what is possible and never forget that nature is usually much more flexible than our dogmas.

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Part II
Characterization of Adult Stem Cells

Chapter 6
Lineage Tracing of Tissue-Specific Stem Cells In Vivo

Kurtis T. Sobush, Keitaro Matsumoto, Huaiyong Chen, and Barry R. Stripp

Abstract Tissue-specific stem cells are characterized by their ability to undergo unlimited self-renewal and generate transit-amplifying progeny that yields all specialized cell types of a tissue. Both identification of stem cells and characterization of their properties have been possible through the use of a range of methods to track cell fate in vivo or in vitro. In vivo systems for lineage tracing offer the advantage of keeping stem cells within their local microenvironment affording them exposure to signaling molecules that help govern their tissue-specific behavior; this will serve as the focus of discussion within this chapter. Multiple methods of lineage tracing in vivo will be described. These methods account for difficulties of lineage tracing within complex organ systems such as analysis of rare numbers of cells, large variability in the type and number of differentiated cell types, impaired visualization of cell type-specific markers in solid organs, and differences between organs in the rate of cell turnover. Methods discussed will include classical methods such as the incorporation of DNA nucleoside analogs or chromatin manipulation. This will be followed by discussion of more contemporary methods focused on the incorporation of genetic reporters through mosaic or chimera models and the implantation of ectopic stem cells. Although these approaches are described individually in the following sections, they are often applied in combination to overcome specific limitations of individual methods and to more rigorously define stem cell behavior in vivo.

Keywords Adult stem cells • Asymmetric cell division • Cre-lox • DNA replication • DNA nucleoside analogs • Green fluorescent protein • Label retention • Light-emitting reporters • Lineage tracing • Self-renewal

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Abbreviations

3T3	3-day transfer, inoculum 3×10^5
4-OHT	4-Hydroxy-tamoxifen
BADJ	Bronchioalveolar duct junction
BrdU	Bromodeoxyuridine
CCSP	Clara cell secretory protein
CFP	Cyan fluorescent protein
CldU	Chlorodeoxyuridine
Cre-*lox*	Cyclization recombination protein-locus of X over P1
DBA	Dolichos biflorus agglutinin
Dox	Doxycycline
EdU	Ethynyl deoxyuridine
ER	Estrogen receptor
FGFR	Fibroblast growth factor receptor
GFP	Green fluorescent protein
GnRH-1	Gonadotropin-releasing hormone-1
HSC	Hematopoietic stem cell
IdU	Iododeoxyuridine
MLL	Mixed lineage leukemia
MMP	Matrix metalloproteinase
NEB	Neuroepithelial bodies
RFP	Red fluorescent protein
rtTA	Reverse tet-transactivator
TetO	Tetracycline operator
TetR	Tetracycline repressor
tTA	Tet-transactivator
YFP	Yellow fluorescent protein
β-gal	Beta-galactosidase

6.1 Incorporation of DNA Nucleoside Analogs and Chromatin Manipulation

Basic tenets involving stem cell and progenitor cell characterization include cellular self-renewal capacity and the capacity to participate in multilineage differentiation. DNA replication is fundamental in providing the genetic template to progeny cells that possess functional characteristics of a respective organ system. As chromosomes enter into the S phase of the cell cycle, DNA base-pair binding sites become accessible for experimentally produced nucleoside analogs. These methods have far-reaching applications since analogs can be traced in an in vivo setting over time and provide data that outlines cell life span and proliferative capacity. Since stem cells and progenitor cells can be distinguished by an unlimited or finite capacity for cell renewal, respectively, DNA analog incorporation can provide important

cellular information regarding stem-like characteristics in a variety of solid tissues.

6.1.1 Tritiated Thymidine

Tritiated thymidine (H3-thymidine) is a thymidine analog that has been utilized in both human and animal studies as a label of proliferating cells. The chemical isotopic structure provides a useful means to evaluate cell proliferation through X-ray uptake and subsequent radiographic imaging techniques. The low molecular weight of additional hydrogen ions also keeps the experimental analog relatively small. The small size allows analog incorporation without significant disruption of the DNA double helical structure. Granular opacities (also referred to as grain counts) become apparent in chromosomal structures of mitotic cells following H3-thymidine administration suggesting DNA incorporation of the nucleoside analog. H3-thymidine incorporation also can be seen in daughter cells as they transition from mitosis to interphase. The timeframe for DNA incorporation is relatively short for in vivo studies due to the inherent unstable nature of the isotope. Estimates suggest that H3-thymidine is rapidly catabolized in approximately 20 minutes following intravenous injection in man (Rubini et al. 1960).

Highly proliferative cells, as defined by increased relative uptake of H3-thymidine, have certain characteristics of stem or progenitor cells. This includes more frequent reentry into the cell cycle relative to postmitotic cells. Like many methods that have developed to evaluate stem cell behavior, the early studies with H3-thymidine assessed the hematopoietic system (Clarkson et al. 1967). More recent studies have incorporated such methods to assess stem cell behavior in solid organ systems. These studies using H3-thymidine have included the basilar papilla of the chick following gentamycin toxicity, neuronal populations in the ventricular zone and subventricular zone of embryonic rodents, bronchiolar-alveolar epithelial and interstitial cells in rats exposed to asbestos, and elements of the cardiac conduction system in embryonic chickens (Brody and Overby 1989; Reznikov et al. 1997; Cheng et al. 1999; Stone et al. 1999).

Despite the utility observed in studies incorporating H3-thymidine, this nucleoside analog has multiple limiting factors. First, elevated levels of H3-thymidine have been shown to disturb the mitotic cycle duration. Not only does an altered mitotic cycle change in vivo quantitative proliferative indices, but also adds carcinogenic potential. A very narrow dosing range is therefore required to assure adequate labeling while not interfering with in vivo proliferative behavior. Second, high degrees of catabolic activity result in a reduction of radiographic signal in a percentage of mitotic cells along with subsequent daughter cells in periods of interphase. Reduced signal contributes to less accurate morphometric analysis needed for this technique. Lastly, extensive technical expertise and long durations of dedicated technical time are necessary for data collection. Radiographs require sample fixation and processing with multiple steps that could potentially introduce technical error. Exposure times often require days to weeks for adequate signal detection.

6.1.2 Chlorodeoxyuridine, Bromodeoxyuridine, and Iododeoxyuridine

The halogenated deoxyuridine nucleoside analogs provide an effective method to label proliferating cells. Similar to [^{3}H]-thymidine, chlorodeoxyuridine (CldU), bromodeoxyuridine (BrdU), and iododeoxyuridine (IdU) have the capacity to become incorporated into DNA during the S phase of the cell cycle. This method has limitations in in vivo human studies due to carcinogenic potential; however, it has been used extensively in nonhuman studies in vivo as a means to explore stem cell behavior in complex organ systems.

Use of halogenated deoxyuridine nucleoside analogs requires both the administration of the labeling molecule along with efficient detection via multiple approaches. CldU, BrdU, and IdU can be delivered to animals via intravenous or intraperitoneal injection in studies designed to require a short labeling period, or it can be delivered by adding it to drinking water for studies that benefit from long periods of labeling duration at a relatively constant rate. The halogen carrying side group adds a negligible contribution to the overall molecular weight relative to endogenous nucleosides. This allows CldU, BrdU, or IdU to be easily incorporated into the DNA helical structure without significant disruption of DNA architecture in vivo. Furthermore, the efficient incorporation allows for label retention to be detected in the nuclei of the daughter cells of an originally proliferating parent cell. Detection of this cell marker, however, requires the use of a relatively large monoclonal antibody directed at CldU, BrdU, or IdU and DNA denaturation techniques are required for effective cellular labeling. Monoclonal antibodies with incorporated fluorophores can subsequently be detected for morphometric analysis by immunocytochemical fluorescence. Van Furth and Van Zwet (1988) reported a method used to denature DNA that allowed for demonstrable BrdU detection with minimal untoward effects on the morphology of individual cell types. Additional protocols have subsequently been generated for proliferative cell labeling (Yokochi and Gilbert 2007; Kimoto et al. 2008; Frum et al. 2009; Leuner et al. 2009).

Use of halogenated deoxyuridine nucleoside analogs can define stem cell behavior in two different ways. First, stem cells and progenitor cells possess a higher capacity for proliferative events relative to other cells within their microenvironment. When exposed to prolonged periods (multiple rounds of a particular cell cycle) of CldU, BrdU, or IdU, these progenitors label multiple times due to frequent reentry into S phase. Daughter cells that are not contributing to the progenitor pool will only take up label after a single proliferative event. These cells will have a slow, constant dilution of immunofluorescent label over time. Therefore, stem and progenitor cells can be defined by having relative "label retention" relative to the postmitotic daughter cells.

Second, double labeling with CldU, BrdU, or IdU has been demonstrated as a means to define spatial and temporal analysis of cell proliferation. Distinct commercial primary antibodies that have been raised in different species specifically label either CldU or IdU. When used in conjunction with highly purified secondary antibodies with different excitation wavelengths, researchers have shown that

simultaneous detection of both CldU and IdU is possible within the same cell nucleus (Bakker et al. 1991; Aten et al. 1992).

Teta et al. (2007) utilized the double labeling technique to further understand the reconstitution of mouse gut epithelium and if certain specialized progenitor cells played a role in generating epithelial progeny. Duodenal villi and crypts were evaluated after sequential DNA labeling of CldU and IdU. Via this technique, specialized progenitors were sought as defined by their capacity to undergo multiple rounds of division and subsequently colabel with CldU and IdU within the nucleus. CldU was administered for 1 day followed by IdU administration for 1 day. Spatial resolution was noted as colabeled cells were prominent in the intestinal crypt with postmitotic progeny (as defined by single-labeled CldU positive cells) populating the middle of the villi. This strongly suggested that rapidly dividing progenitors reside in the intestinal crypt and reconstitute the intestinal villi with postmitotic cells supporting previous work by Potten et al. (1997). In contrast, the progenitor–progeny behavior of insulin-secreting beta cells of the adult mouse pancreas did not reveal a specific progenitor subset. The results of this study reflected that colabeled cells were infrequent in the adult beta cell population suggesting that beta cell mass expansion typically occurs through generalized self-renewal as opposed to arising from a specialized progenitor cell compartment.

6.1.3 Ethynyl Deoxyuridine

Ethynyl deoxyuridine (EdU) is a newer thymidine analog alternative that can be delivered to organisms in vivo through intravenous or intraperitoneal administration. Much like the other labeled DNA precursors, EdU becomes incorporated into the DNA helical structure during the S phase of the cell cycle (Chehrehasa et al. 2009). The detection of EdU requires the use of a chemical method called the click reaction (Rostovtsev et al. 2002; Tornoe et al. 2002). In the click reaction, an ethynyl side group reacts with a fluorescent azide in the presence of a copper catalyst. This cycloaddition reaction incorporates small reaction components allowing for easy inclusion into the DNA double helix without a need for denaturing conditions. The small molecules of the click reaction are estimated to be approximately 0.6 kDa in comparison to a larger anti-BrdU labeling antibody that is around 150 kDa (Buck et al. 2008).

The EdU detection method has been compared to the classic DNA precursor labeling strategies that employ either BrdU or H3-thymidine. Salic and Mitchison (2008) initially evaluated the relative detection of EdU and BrdU in cultured 3T3 cells. All BrdU-labeled cells were found to also incorporate EdU label. Furthermore, EdU labeling was more intense in vitro suggesting that EdU is an efficacious method to detect cell proliferation. Chehrehasa et al. (2009) also validated EdU labeling efficiency after detecting proliferating cells in vivo in the neurogenic zones of the brain in mouse pups.

Mouse small intestine has been evaluated in vivo using explanted tissue at different time points after EdU intraperitoneal injection. Labeled cells were notable in

the intestinal crypt 24 h postinjection and subsequently in the villus tip at the 96 h time point. This demonstrates the dynamic migration of proliferative cells from the crypt up the villus, suggesting that gut progenitors reside in the crypt and daughter cells migrate toward the intestinal lumen (Salic and Mitchison 2008).

EdU has also been used in defining progenitor cell behavior through a double labeling strategy assessing avian cochlear cells. Kaiser et al. (2009) exposed cochlear cells to damaging doses of gentamycin. Regenerating supporting cochlear cells labeled strongly with EdU. Furthermore, labeling efficiency was comparable to the established BrdU labeling technique. EdU was also found to colabel with *Sox2* along with myosins VI and VIIa in a time-dependent fashion, thus providing insight into the expression of developmental markers with cochlear cell proliferation following injury.

6.1.4 Tagged Histone Proteins

The use of fusion proteins allows for direct incorporation of a lineage tag into chromatin. These strategies are conceptually similar to DNA analog methods in that they provide means to track mitotic events and can elucidate cell differentiation fates in an in vivo environment. One method utilizing chromatin localization involves the human histone H2B and this method, like many eukaryotic biological approaches, originated in prokaryotic systems. Flach et al. (1994) were the first to note that a fusion protein including green fluorescent protein (GFP) and yeast H2B localized into yeast nuclei suggesting that this method could be translated into living mammalian cells (more complete discussion of GFP in Sect. 6.2.3.1). Kanda et al. (1998) were able to demonstrate that a fusion protein between GFP and human histone H2B could be effectively incorporated into the nucleosome without disrupting the cell cycle in vitro. These studies were the hallmark of an effective method that used GFP tagging of histone proteins to track stem cell behavior of tissue-specific stem cells in vivo.

GFP-H2B expression systems provide a means to label certain cells that maintain “label retention” emphasizing the role of cellular self-renewal or infrequent division – key descriptive features that help delineate stem cells from more differentiated cells. Zhang et al. (2009) exploited a GFP-H2B expression system along with an inducible Cre system using tamoxifen to track cell divisions in the bulge of murine hair follicles (more complete discussion of Cre technology in Sect. 6.2.2). Through these methods, proliferative behavior was effectively described as bulge cells migrated out of the bulge niche during quiescent periods while also contributing to self-renewal in the adult organism. In another example, Falkowska-Hansen et al. (2010) incorporated an inducible tetracycline Cre system crossed with a GFP-H2B construct in an effort to mimic in vivo skin and pancreatic features in specialized culture environments. With this approach, “label retaining” cells were able to be isolated in an in vivo-like environment and exhibited the possible roles of this type of system for pursuing stem cell behavior characteristics in future studies.

6.2 Cellular Marking Through Genetic Reporter Strategies

The rapid evolution of sophisticated techniques that allows for genetic manipulation of the mouse germline and/or soma, as well as greater understanding of mouse embryology, created new opportunities for ectopic expression, inactivation, and modification of various genes. Genetically modified animal models provide critical tools for analysis of in vivo function at the level of the gene, cell, tissue, and whole organism. Commonly used approaches allow for ectopic expression of reporter genes, transcription factors, and DNA-modifying enzymes, allowing for either transient or permanent tracing of cell lineages. Two general classes of methods have been used for genetic tagging of cells and assignment of lineage relationships. The first involves the genetic manipulation of somatic cells for the creation of mosaic models. The other involves the creation of chimeras through either embryonic manipulation or transplanted delivery of cells to adult organisms. The following sections will describe these methods and their application towards understanding cell lineage relationships and progenitor cell biology.

6.2.1 Mosaics Generated Through Retroviral Tagging

One of the first genetic lineage tracing approaches employed to tag somatic cells in vivo involved the use of recombinant retroviral vectors for stable expression of histochemically detectable reporter genes within single cells and their progeny. Retroviral vectors were considered to be favorable for lineage tracing due to their capacity for stable integration of their proviral genome into host cell DNA, thus leading to subsequent transmission to progeny cells. Furthermore, retroviruses are relatively innocuous and do not spread into other cells that are not of experimental interest. Many heterologous genes can be packaged with the retroviral vectors, allowing tagged cells to be distinguished from their uninfected counterparts. Typically, a low multiplicity of infection is used to ensure that proviral integration occurs in rare cells. When used in combination with histochemically detectable markers in developing embryos, subsequent development allows for retrospective evaluation of stem cells in vivo (Cepko et al. 1984; Stern and Fraser 2001). The low frequency of lineage tagging achieved using this approach increases the probability that clusters of marked cells are clonally related, thus allowing assignment of capacity for clonal expansion and differentiation potential. Price et al. (1987) were the first to employ a retroviral cell lineage marking system in vertebrates. They used *Escherichia coli* β-galactosidase (β-gal) as their histochemical marker – a reporter gene that had previously been used with success in lower eukaryotic species (Garabedian et al. 1986). In these experiments, rats were anesthetized at birth and underwent surgery allowing infection of retinal epithelial cells with a β-gal expressing retroviral vector. Rats were sacrificed between 1 and 9 weeks of age and retinas were subsequently analyzed as whole mounts or frozen sections. Histochemical staining for the β-gal reporter revealed that

a small number of retinal cells were infected and served to allow assignment of lineage relationships through analysis of tagged clones. They found that postmitotic cell types, such as ganglion cells, horizontal cells, and cone photoreceptors, were never observed among the β-gal positive population. In contrast, rod photoreceptors expressed β-gal and expanded to form clusters. This confirmed their proliferative potential and allowed analysis of their differentiation potential through the evaluation of cell types present within the tagged clones. Furthermore, tagged cells appeared in a radial distribution with most clusters located at the periphery of the retina. Importantly, this gene transfer method did not appear to alter normal retinal development and served as a basis for lineage tracing in vivo.

A number of other studies have been generated using retroviral vectors to further explore stem and progenitor cells in the nervous system. As one example, Smith and Luskin (1998) used a β-gal retrovirus vector encoding the *lacZ* gene that was injected into postnatal murine brain tissue. As the retroviral lineage was placed into the rostral migratory pathway, it was shown that proliferating progenitor cells gave rise to daughter cells that contributed to the olfactory bulb. Zhou et al. (2006) constructed a retroviral vector that expressed a dominant negative fibroblast growth factor receptor (FGFR) as a technique to analyze the role of fibroblast growth factor 2 on murine oligodendrocyte progenitor differentiation in vivo. These methods have added insight into stem cell and progenitor cell behavior. However, there are some limitations to account for with the retroviral technique.

One limitation of retroviral tagging studies is the inability to retrospectively define the phenotype of initially tagged cells due to the stochastic nature of retroviral infection and the capacity for integration of proviral DNA into any proliferative cell. An added difficulty in unambiguously assigning clonality is based upon the presence of "adjacent"-labeled cells within a cell cluster. Two possibilities arise in this scenario. The first is that a progenitor does give rise to an "adjacent" daughter cell as reflected by a standard lineage tag. However, due to the random occurrence of infection events, it is always possible that adjacent cells have taken up label independently and do not reflect a progenitor–progeny relationship. This concern has been addressed through utilization of mixed populations of retrovirus that either express distinct reporters or carry distinguishing sequence tags (Golden et al. 1995; Li et al. 1997; Cepko et al. 1998; Liu et al. 2009a). Using this approach, the possibility of clonal misassignment cannot be eliminated but it can be quantified, thus providing a tool for method optimization and data interpretation. These limitations continue to be the focus of method refinement in retroviral studies.

6.2.2 Mosaicism Through Cre Recombinase-Mediated Site-Specific Recombination

Cre (Cyclization Recombination Protein)-*lox* (Locus of X over P1) systems provide effective lineage tracing of tissue-specific stem cells in vivo. The Cre-*lox* system developed as a genetic tool out of two key studies. First, Sternberg (1981) analyzed a

recombination reaction occurring between two specific components in bacteriophage P1-infected *Escherichia coli*. These two components are the well-characterized 38 kDa Cre recombinase protein and the 34 base-pair long (an 8 base-pair core sequence and two flanking 13 base-pair inverted repeats) *lox*P sequence that has Cre-specific recognition. Both components were required for the packaging of single viral genome equivalents from a linear array of genomes generated by the "rolling circle" method of viral DNA replication. They described effective DNA recombination through multiple *lox* sites in a variety of orientations. Second, Sauer and Henderson (1988) used the Cre-*lox* system to induce DNA recombination in mammalian cells that were engineered to express Cre recombinase and harbor a substrate DNA sequence containing adjacent *lox*P elements. Through this work, it was recognized that Cre recombinase serves as the catalytic enzyme that mediates *lox*P site-specific cleavage and ligation of DNA. Similar findings were made for the FLP recombinase system that mediates homologous recombination between *FRT* sequences within the 2μ plasmid of *Saccaromycetes cerevisiae* (Schwartz and Sadowski 1990; Radhakrishnan and Srivastava 2005). Application of these systems for enzymatically catalyzed, site-specific, homologous recombination within cells of higher eukaryotic organisms has formed the basis for advanced techniques to genetically alter the soma. When coupled with transgenesis and site-specific genetic modification of the mouse germline, use of Cre-*lox* systems provides a means to introduce genetic tags in a cell type-specific manner for analysis of both lineage relationships and capacity for self-renewal. Depending on the types of Cre or *lox*P strains that are used, various strategies can be employed in a variety of research applications (Jaisser 2000; Nagy 2000 (Fig. 6.1). These strategies generally rely upon manipulation of signaling pathways that impact the behavior of progenitor cells within a tissue, but are not well-suited for lineage tracing per se, due to the constitutive activity of Cre recombinase.

With classical conditional transgenesis, practical research problems limit its utility. The Cre-*lox* system, when used in this fashion, allows no control of timing of either transgenic or Cre expression. When applied to mouse models, site-specific expression may be seen but expression during certain stages of development may confer a lethal phenotype or cause spatial alterations in a particular organ functionally changing in vivo environmental factors. Inducible Cre strains were developed to help circumvent these limitations. With an inducible Cre-*lox* system, a particular inducing agent can be administered at a specific time point to promote Cre expression and/or Cre activity. This highlights two basic approaches that have been employed to achieve temporal regulation of Cre recombinase: first is the use of ligand-dependent systems to control expression of Cre and second is the use of ligand-dependent systems to control the activity/subcellular distribution of Cre.

6.2.2.1 Transgenic Systems for Drug-Regulated Expression of Cre

Regulation of a constitutively active Cre was originally achieved through the use of the tetracycline-based system initially developed by Gossen and Bujard (1992). This was developed by engineering components of the *Escherichia coli Tet* operon

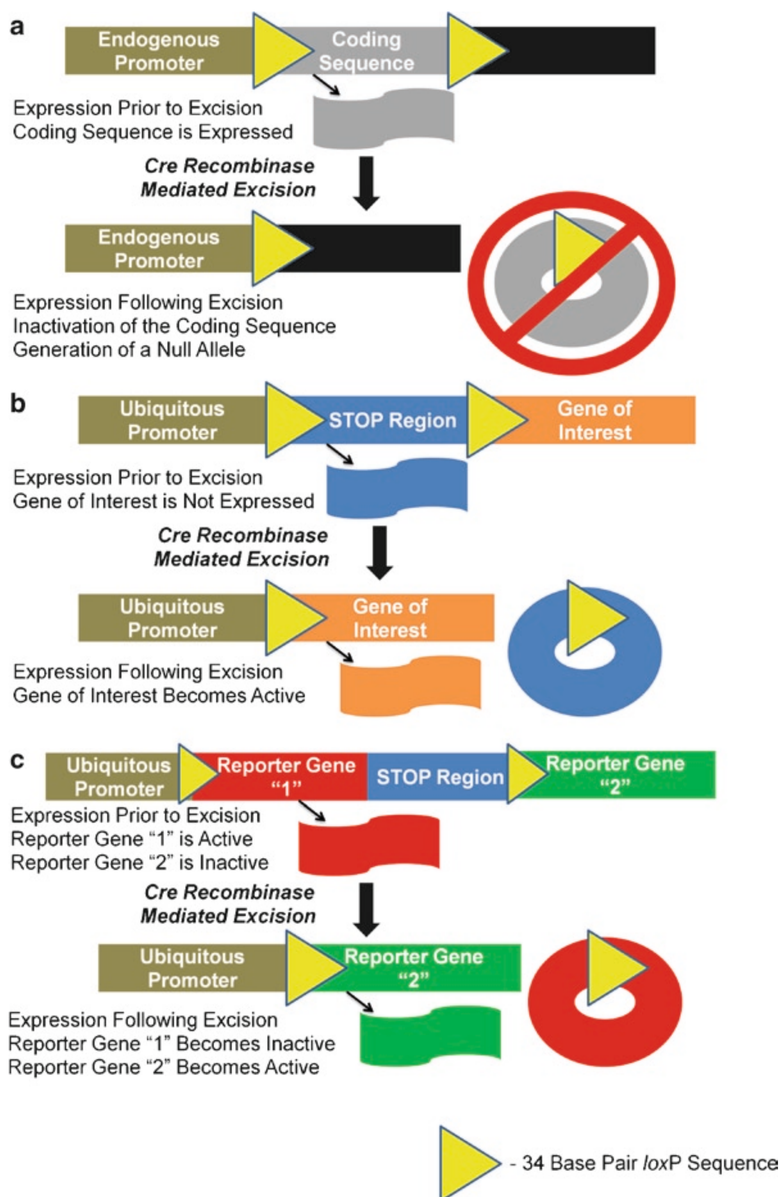

Fig. 6.1 Applications of Cre-*lox* systems for gene regulation and lineage tracing. (**a**) An endogenous gene coding sequence is flanked by two similarly oriented *lox*P sites ("Floxed"). Preexcision conditions allow for normal expression of the particular gene. Following Cre recombinase-mediated recombination, the specific coding sequence is lost producing a functionally null allele. (**b**) A ubiquitous (or tissue-specific) promoter is upstream of a STOP region that is flanked by two similarly oriented *lox*P sites ("Floxed Stop Region"). In cellular environments that are devoid of Cre recombinase-mediated recombination, the STOP codon is transcriptionally active and the gene of interest remains unexpressed. Alternatively, the presence of Cre recombinase aids in excision

for the creation of hybrid promoter elements to control expression of target genes. The system is dependent upon multiple components including tetracycline (or tetracycline derivatives such as doxycycline [Dox])-based modulation of DNA binding activity of mutant forms of a tetracycline repressor (*tetR*), a DNA protein that normally binds to cis-acting DNA elements (referred to as the *tet*-operator, or *tetO*, sequences), and transcriptional control elements of the *Tet* operon. In the presence of Dox, the *tetR* DNA binding domain dissociates from *tetO* elements. For experimental utility, a mutant form of this *tetR* was also generated that allowed for the converse, the Dox-dependent DNA binding to *tetO* elements (referred to as the reverse *tet* system or rt). In each case, *tetR* and reverse *tetR* were fused to the transactivation domain from herpes simplex virus VP16 protein, leading to the generation of, what is commonly referred to as, the *tet*-transactivator (tTA) or the reverse *tet*-transactivator (rtTA), respectively. When a hybrid promoter containing multimerized *tetO* sequences is linked to a Cre expression cassette and introduced into the mouse germline in the form of a transgene, Cre expression is placed under tight control of either tTA or rtTA. Either of these transactivators is typically placed under the regulation of cell type-specific promoter elements to confer specificity to Dox-regulated Cre expression (Furth et al. 1994; Passman and Fishman 1994; Gossen et al. 1995; St-Onge et al. 1996; Utomo et al. 1999).

Multiple studies demonstrate the utility of tetracycline-induced Cre-*lox* technology as it pertains to somatic stem cells. Yu et al. (2005) developed a model using an rtTA along with the nestin promoter/nestin second intron combination to temporally regulate expression of neural cell precursors. In this study, nestin was expressed throughout murine neural tubes during periods of development. Furthermore, adult expression was observed only in known areas of neurogenesis in the subventricular zone and the dentate gyrus. Another study that utilized a doxycycline-induced Cre-*lox* system evaluated the role of a tumor suppressor gene, *Pten*, on bronchioalveolar stem cell behavior as it pertains to the development of lung adenocarcinomas (Yanagi et al. 2007). An rtTA was utilized to generate *Pten*-deficient mice following induction with Dox. Postnatal mice that were administered Dox developed increased numbers of bronchoalveolar stem cells along with increased frequencies of lung adenocarcinomas. Loss of *Pten* has been associated with activation of *Akt*, increased expression of *c-Myc* and *Shh*, and reduced expression of *p27*; all these gene alterations have been shown to induce stem cell renewal. Through this system, the role of bronchoalveolar stem cells in tumorigenesis was further clarified in vivo.

Fig 6.1 (continued) of the STOP region and allows for expression of the gene of interest. Examples of these approaches include the targeted ROSA allele or transgenes driven under the control of a ubiquitous promoter such as CAG. The gene of interest may or may not be followed by an IRES-reporter to identify cells that have excised the STOP codon. (**c**) Multiple transgenic reporter genes are placed downstream of a ubiquitous promoter. Reporter gene expression is conferred by the promoter driving Cre expression. In the absence of Cre recombinase-mediated recombination, the first reporter gene is expressed and the downstream STOP codon prevents expression of the second reporter gene. When Cre is expressed by the specific promoter, both the first reporter gene and the STOP region are excised and the second reporter gene becomes transcriptionally active

A separate study of mouse lung epithelial cells utilized a Dox inducible system in which rat clara cell secretory protein (CCSP) and human surfactant protein C gene promoters were used to express the rtTA during early mouse development. Perl et al. (2005) administered doxycycline to transgenic to allow expression of Cre and activation of alkaline phosphatase or GFP. Labeling patterns provided insight into lineage tracing in distinct subset of cells in the trachea, bronchi, and alveoli. Both ciliated and nonciliated cells became labeled, suggesting a common progenitor during lung development.

An alternative method that has been utilized to regulate the expression of a constitutively active Cre is by placing it under the transcriptional control of inducible promoters such as the Cyp1A1 promoter. Promoter elements of the Cyp1A1 gene, including the aryl hydrocarbon response element, allow regulated transgene expression following activation by systemic exposure to β-naphthoflavone. Sansom et al. (2004) used β-naphthoflavone/Cyp1A1-regulated expression of Cre to both generate a conditional null of the *APC* gene and investigate the consequences of Wnt pathway activation within the small intestine. Highly efficient recombination of the floxed *APC* allele was observed which led to expansion of the crypt progenitor domain at the expense of specialized cell types of the villus epithelium. In the same study, a histochemically detectable Cre reporter allele was used to demonstrate the cell type-specificity of Cre-mediated recombination. A modified allele of the ROSA26 locus was used in which a *lox*P-STOP-*lox*P element had been engineered between the ubiquitous ROSA26 promoter and a *lacZ* reporter. In a comparable study of murine small intestine, Shorning et al. (2009) helped define the role of the *LKB1* gene that also has been implicated in tumor suppression. Again, high levels of recombination events were observed. Ultimately, through this system, goblet cell and Paneth cell differentiation were altered through the modification of Notch signaling components.

6.2.2.2 Ligand-Regulation of Cre Activity

A number of mutant forms of Cre recombinase have been generated so that Cre activity is under drug regulation. The most widely used system involves fusion of Cre to portions of the mutant human estrogen receptor (CreER). These portions include the DNA binding domain and domains mediating interaction with heatshock proteins and 4-hydroxy-tamoxifen (4-OHT). Expression of the CreER fusion protein within eukaryotic cells leads to its sequestration in the cytoplasm in the absence of 4-OHT. In contrast, the addition of 4-OHT as an inducing agent promotes nuclear translocation (Danielian et al. 1993; Littlewood et al. 1995). Transgenic mouse lines have been developed that ubiquitously express CreER for regulated recombination of floxed genomic sequences within the mouse genome (Feil et al. 1996, 1997; Metzger and Chambon 2001; Hayashi and McMahon 2002; Leone et al. 2003). However, when coupled with the use of cell type-specific promoters to regulate the expression of CreER, both temporal and site-specific regulation can be achieved. As with Dox-regulated Cre systems, coupling of appropriate Cre reporter alleles to

the 4-OHT/CreER system can be used to trace cell lineage relationships in vivo (Vasioukhin et al. 1999; Hong et al. 2004; Barker et al. 2008, 2009).

Hong et al. (2004) generated a mouse line that incorporated a CreER system that traced the fate of K14-expressing basal cells in the tracheobronchial epithelium. Lineage tracing was performed following chemical ablation of abundant airway secretory cell progenitors which led to activation of basal cell progenitors to subsequently effecting epithelial repair. In this study, K14-expressing basal cells were shown to self-renew and generate ciliated and nonciliated secretory cells. Similar studies involving the use of a secretory cell-specific CreER line have revealed that Scgb1a1-expressing cells in the bronchiolar airways have the capacity for long-term self-renewal. On the other hand, those cells in the tracheobronchial airways are replaced over time with the presumption that these are the progeny of basal cell progenitors (Rawlins et al. 2009).

Neural stem cell behavior has also been assessed through the use of a CreER transgenic mouse system (Young et al. 2010). Fibroblast growth factor receptor 3 (FGFR3) was used as a marker of radial glial cells in the embryonic neural tube and along with astrocytes in the mature central nervous system. Through cross-breeding, an Fgfr3-CreER^{T2} mouse allowed for inducible expression following tamoxifen administration. FGFR3-positive cells were noted in the adult mouse subventricular zone and possessed stem cell characteristics. Mice were followed up to 6 months posttamoxifen administration. Through this method of lineage tracing, it was determined that subventricular stem cells contribute to adult neurogenesis of multiple interneuron subclasses in the olfactory bulb.

A final inducing agent, RU 486, has also been used in an inducible Cre-*lox* system for the assessment of stem cell behavior. RU 486 is a synthetic steroid that has binding activity at a human progesterone receptor. Kellendonk et al. (1996) originally constructed and optimized a temporally regulated mouse line that combined an inducible Cre-*lox* system to a targeted mutant human progesterone receptor that binds preferentially to RU 486. A similar approach was used to evaluate the roles of follicular stem cells and *Stat3* in skin carcinogenesis. Kim et al. (2009) induced targeted mutations in the keratinocyte stem cells localized to the follicular bulge region. Mice were cross-bred with K15.CrePR1 and *Stat3*$^{fl/fl}$ strains to allow for targeted disruption of the *Stat3* gene following RU 486 administration (Sano et al. 1999; Morris et al. 2004). Following RU 486 administration, skin carcinogenesis was experimentally induced with topical treatment of DMBA and TPA as chemical tumor promoters. *Stat3* expression and tumorigenesis were noted to be altered following RU 486 induction of the cross-bred mice. Evidence was shown that keratinocyte stem cells in the follicular bulge required *Stat3* expression for the initiation of skin tumors and protective apoptotic events were minimized.

6.2.2.3 Limitations of Cre-*lox* Systems

With increasing use of Cre-*lox* technology, a variety of limitations have become apparent due to particular untoward experimental effects from certain components of the Cre-*lox* system. For instance, Dox is a known inhibitor of matrix metalloproteinases

(MMP) and this effect can impact both cellular remodeling and repair. Kadar et al. (2009) has shown that ocular injuries sustained following sulfur mustard exposure increase MMP activity and facilitate a regional limbal epithelial stem cell deficiency. Chronic administration of Dox effectively delays the onset of injury and attenuates the reduction of limbal epithelial stem cells. The impact of Dox on MMP inhibition appears to have potential direct effects on stem cell behavior.

Since CreER systems require tamoxifen administration, various limitations exist due to the direct systemic effects of this drug as it pertains to cellular behavior. Agca et al. (2008) evaluated the effects of ER modulation on neurogenesis during sheep development. Gonadotropin-releasing hormone-1 (GnRH-1) helps stimulate early phases of neuron development and is influenced by the ER. GnRH-1 neurons were cultured in vitro and treated with tamoxifen. In the tamoxifen-treated neurons, it was apparent that this ER ligand contributed to deleterious effects on neurogenic precursors and highlights the important role of steroids on central nervous system development.

Sisson et al. (2006) helped to recognize potential toxic manifestations of an rtTA in a study designed to look at the impact of an rtTA on mouse lung phenotypes. In this study, a double transgenic mouse model was used that incorporated a rat CCSP promoter that controlled the rtTA gene along with a *tetO* sequence controlling the urokinase plasminogen activator. A control group of the double transgenic mice was followed for up to 30 weeks without any exposure to Dox. The control group exhibited an emphysematous phenotype, suggesting that rtTA expression alone can contribute to pathogenesis and altered cell behavior. Possible toxic effects of either tTA or rtTA should be considered a potential consequence in related experiments utilizing these systems (Whitsett and Perl 2006; Perl et al. 2009).

The previously mentioned examples do not encompass all of the unique impacts that components of the Cre-*lox* system may have on regulation of cellular behavior. Nevertheless, ongoing studies will further delineate and modify aspects of this technology to gain a clearer understanding of stem cell behavior in vivo.

6.2.3 Light-Emitting Reporters

A small number of proteins exhibit fluorescent properties and emit light under certain conditions in both prokaryotic and eukaryotic cells. This section will focus on the incorporation of fluorescent protein genes into animals as a means to mark certain cells. In vivo tracking of these incorporated markers allows for effective assessment of stem cell behavior in a variety of animals and organ systems.

6.2.3.1 Green Fluorescent Protein

One of the well-applied and understood fluorescent protein cell markers is GFP. Historically, specific bioluminescent properties were originally evaluated by Shimomura et al. (1962) to explain how the Pacific Northwest jellyfish, *Aequorea*

victoria, would emit bright green fluorescence when exposed to blue light. The bioluminescent phenomena were defined as an energy transfer between two closely associated proteins, aequorin and GFP, in the presence of calcium ions at room temperature. GFP was further isolated in the jellyfish and both the chemical and physical properties of aequorin and GFP were described in greater detail (Prendergast and Mann 1978; Prasher et al. 1992). Crystal structures of GFP were later described by Ormo et al. (1996) to help describe how the native GFP protein folding allows for light emission.

Chalfie et al. (1994) were the first to publish data that complementary DNA for GFP could be utilized to monitor gene expression in other living organisms such as *Escherichia coli* and *Caenorhabditis elegans*. One of the major factors in the usefulness of GFP is effective expression without the need of exogenous substrates or cofactors that may alter the in vivo environment. Successful expression of GFP was seen in a small number of neurons under the control of a *mec-7* gene promoter. Furthermore, GFP expression was rather stable, resisted photobleaching, did not appear to alter cell growth, and remained strong despite exposure to fixation preparations like formaldehyde. GFP was found to have significant utility as a stable cellular marker that could be used in more complex organisms. When combined with improved genetic engineering and targeted mutations, spectral characteristics were optimized and mammalian stem cells could be assessed through lineage tracing studies in vivo through this method (see Tsien 1998).

Multiple organ systems have been evaluated using transgenic incorporation of GFP to assess stem cell behavior. For example, Buckingham (2007) targeted the mouse *Pax3* gene with a GFP reporter to trace skeletal muscle satellite cells. Purified satellite cells were grafted into *mdx nude* mice that were devoid of dystrophin and under a continuous muscular repair state. Through this approach, it was noted that GFP-positive satellite cells contribute to the reconstitution of the progenitor cell population. Suh et al. (2007) generated *Sox2*-GFP transgenic mice to evaluate the role of neural stem cells in the adult mouse hippocampus. In this experiment, GFP-positive cells within the subgranular zone were shown to represent dividing undifferentiated cell populations. Furthermore, cells expressing *Sox2* in the subgranular zone were noted to give rise to neurons, astrocytes, and other *Sox2*-expressing cells through lineage tracing with GFP. Zhang et al. (2009) evaluated bulge cells in the hair follicle in a histone H2B-GFP mouse driven by an epithelial Keratin 5 promoter. These mice were cross-bred into a Dox repressible conditional system to allow precise timing of cellular division events. When followed in vivo, it was noted that bulge cells leave their niche prior to division and differentiation. Furthermore, certain bulge cells undergo self-renewal as determined by temporal or spatial phenomena.

6.2.3.2 Fluorescent Protein Variants

GFP has multiple attractive features as an in vivo stem cell lineage tracing method; however, there are a variety of limitations that prompted the development of other fluorescent protein markers (Taghizadeh and Sherley 2008). For example, excitation

of the protein for extended periods of time in vitro has been shown to contribute to cell death through production of free radicals (Liu et al. 1999). Newer clones of GFP proteins tend to be tetrameric and form oligomers. This can result in aggregation and will impair localization to target cells (Zacharias et al. 2002). Furthermore, DNA methylation effects may reduce GFP expression over time as the presence of a methyl transferase inhibitor aids in retention of GFP expression (Hong et al. 2001). To address this, variants of GFP have been designed that are similar in crystal structure with a mild change to confer a different excitation-emission spectrum, limit oligomer formation, and remain stable for longer periods of time. Variants that are both commercially available and have been reported for in vivo studies include the cyan fluorescent protein (CFP), the yellow fluorescent protein (YFP), and the red fluorescent protein (RFP) (Chudakov et al. 2005).

Taghizadeh and Sherley (2008) performed a side-by-side experiment evaluating the usefulness of CFP and YFP in comparison to GFP in rat hepatic stem cells in vitro. Through their studies, CFP and YFP maintained greater fluorescence after following the clonal population through 120 doublings. Furthermore, cells did not have significant alteration in morphology or kinetics. With this type of effectiveness seen in vitro in long-term label retention, studies have developed to look at slowly renewing stem cells in vivo with fluorescent variants. An example of this was reported by Van Keymeulen et al. (2009) using YFP in a pulse fashion to label Merkel cells in adult mice. During periods of homeostasis, it was shown that epidermal progenitor cells coexpressing K15 and YFP in the whisker give rise to new Merkel cells and help maintain their population in the steady-state.

6.2.3.3 Combined Fluorescent Protein Expression (*Brainbow* Mice)

Greater numbers of fluorescent proteins have become available for cellular marking with variable color spectrums. These advances in technology provide the opportunity to utilize a strategy that incorporates a multitude of different color fluorescent proteins. Similar to a television monitor that uses variable combinations of three different colored hues to display a wider spectrum of color, the incorporation of variably expressed fluorescent proteins can generate an extensive array of visually distinguishable color markers within individual cells. Through unique color expression, individual cells can be followed over time in vivo and provide a strategy to assess stem cell behavior in complex tissue systems.

Livet et al. (2007) designed genetic strategies to express multiple fluorescent proteins in a stochastic fashion that also incorporated a Cre-*lox* recombination system. Two types of transgenic mouse strategies were generated and referred to as *Brainbow-1* and *Brainbow-2*. *Brainbow-1* utilized Cre-mediated methods that allowed for mutually exclusive recombination events through excision between pairs of incompatible *loxP* sites. *Brainbow-2* made use of Cre-mediated inversion of DNA segments within *loxP* sites to generate different recombination outcomes. With both mouse strategies, limited recombination events helped generate over 90 distinguishable, uniquely colorful cellular markers within the nervous system

that could be traced in vivo. Through these methods, both neuronal and glial cellular fates were followed over time showing the potential for this method for in vivo assessment of stem cell behavior.

6.2.3.4 Luciferase

Luciferase enzymes broadly refer to a class of oxidative enzymes that produce bioluminescence. The firefly luciferase enzyme has been widely used in biology since cloning of the gene and subsequently used as a molecular tool since light emission can occur in normal physiologic conditions (de Wet et al. 1987; Gould and Subramani 1988; Contag et al. 1998). Luciferase has been utilized as a reporter gene after transfection into organisms of interest to assess transcriptionally active cells. In contrast to fluorescent reporter proteins (such as GFP) that require an external source of light for excitation, luciferase self-emits light in the presence of ATP, magnesium, oxygen, and the substrate D-luciferin. Due to these principles, there is reduced background autofluorescence in the luciferase system adding to its utility in molecular imaging (Massoud and Gambhir 2003).

Nishijo et al. (2009) generated an example of a luciferase biomarker system to evaluate the dynamics of muscle stem cell turnover in vivo during various growth phases. A reporter mouse line was created that incorporated an inducible Cre-*lox* system with a pairing of the firefly luciferase gene along with the human placental secreted alkaline phosphatase gene. With this dual reporter approach, myogenic lineages were able to be traced. Furthermore, cell division events could be quantified based on luciferase expression during injury and noninjury investigations. In another example of luciferase use in stem cell lineage tracing, Sher et al. (2009) utilized an implantation assay that followed the fate of purified luciferase-labeled neural stem cells after engraftment into a demyelinated mouse corpus callosum. Bioluminescent imaging revealed that the implanted neural stem cells differentiated into an oligodendrocytic lineage and helped contribute to the remyelination of axons.

6.2.4 Chimera Models

A chimera is an animal from Greek mythology that is comprised of the head of a lion, the body of a goat, and the tail of a serpent. This mythological concept offers a descriptive role as it pertains to biological research in chimera modeling. A chimera animal model is defined as a single organism that develops and incorporates two or more genetically distinct cellular groups that originate from different zygotes. Ultimately, these distinct cellular groups will reside in their tissue-specific domains and take on the normal functional and anatomical characteristics within a chimeric organism.

Chimerism may occur as a result of both natural and experimental phenomena. Naturally occurring chimerism has been described as a result of perturbations of

embryogenic cell division and growth. In particular, multipotent parent cells (two nonidentical zygotes or a zygote combined with a haploid sperm/ovum) will fuse to generate one chimeric animal. The resulting animal will possess cell lines derived from all the parent cells that fused during embryogenesis. This section will focus on various experimental chimera studies, contrasting embryonic generation and delivery of cells to adult organisms, and how these systems can be maximized to assess in vivo stem cell behavior.

6.2.4.1 Hematopoietic Stem Cell Origin of Chimeric Models

Experimentally induced chimeras have been extensively applied and researched in hematopoietic stem cells (HSCs). To examine the role of genes of interest in HSC functions, bone marrow chimera can be made by cotransplanting wild-type HSC and HSC with a known mutation in these genes. Such mutations can be tracked and differentiated from wild-type genes within HSCs by tagging mutated HSCs with fluorescent proteins or by targeting antigens exhibiting different allotypes. One example would include the leukocyte common antigen, CD45, since it exists in CD45.1/Ly5.1 and CD45.2/Ly5.2 allelic forms. These allotypes have been traced after being administered intravenously into a lethally irradiated recipient mouse that is devoid of hematopoietic cells. Such an experiment creates a bone marrow chimera with Ly5.2$^+$ wild-type HSCs mixed with Ly5.1$^+$ HSCs that carry a mutation in the mixed lineage leukemia (MLL) gene. As subsequent hematopoietic cell reconstitution occurs, the functional roles of these antigens help define characteristics of HSCs for this mouse model. Using this method, Jude et al. (2007) observed that Ly5.1$^+$ HSCs carrying the MLL gene and their progeny were subsequently lost in an irradiated recipient. This loss helped demonstrate that HSCs require MLL for self-renewal.

6.2.4.2 Embryonic Models of Chimerism

Compared to experiments involving HSC, chimeric experimental models involving stem cell function in solid organs or tissues have been studied less frequently. These studies are more difficult (if accomplished postnatally) due to engraftment concerns (Johnson et al. 2010). Furthermore, tissues that harbor stem cells with poorly defined characteristics (pancreas, lung) complicate experiments that require valid methods to follow these cells in vivo. Some of these difficulties have been overcome with the generation of chimeras through manipulation of embryos. An embryo aggregation chimera was developed to answer questions about stem cell function in solid organs. Ponder et al. (1985) utilized the embryo aggregation model and stained mouse sections of chimera intestinal epithelium with Dolichos biflorus agglutinin (DBA). Cell progeny remained labeled with DBA and multiple patches of labeled cells were observed. Based on the large size and focal distribution of patches, it was determined that individual intestinal crypts are consistently

composed of epithelial cells derived from only one parental type consistent with a single progenitor cell.

Intestinal epithelial stem cell lineages were also studied using an embryonic chimera model by Hermiston et al. (1993). With this approach, embryonic stem cells are transfected with recombinant DNA consisting of a reporter of interest. Once embryonic stem cells have undergone transfection, they are incorporated into C57BL/6 host blastocysts generating a chimeric system. After mice undergo gut morphogenesis and enter into the adult state, two separate gut epithelial populations become apparent in similar positions along the crypt/villus or duodenal/colonic axes and become useful in gain-of-function or loss-of-function gene experiments to help define progenitor behavior.

More recently, Giangreco et al. (2009) generated wild-type:GFP chimeric mice utilizing 8- to 16-cell embryo aggregation methods that incorporated both wild-type and GFP transgenic mouse strains. This allowed for evaluation of airway stem cell contribution to epithelial maintenance during steady-state and following naphthalene injury. Results showed that in both steady-state airways and moderately injured airways, chimeric patches were relatively small. Furthermore, GFP-positive patches were spatially distributed in a random fashion and not confined to classical airway stem cell niches (the bronchioalveolar duct junction [BADJ] or branch point associated neuroepithelial bodies [NEB]). Single, randomly distributed progenitor cells were therefore suggested to help maintain normal airway epithelial homeostasis. In contrast, airway repair following severe naphthalene lung injury resulted in the generation of rare, large clonally derived cell patches that were spatially associated with BADJ or NEB, confirming that NEB- and BADJ-associated stem cells are involved with more significant repair.

6.2.4.3 Chimerism Via Delivery of Hematopoietic Stem Cells to Adult Organisms

Researchers have developed chimera models that are based on the delivery of purified HSCs into irradiated host organisms. The delivered multipotent cells take on tissue-specific function through engraftment or cell fusion mechanisms depending on the organ system. The native in vivo environment can be exploited through this method by both providing means to determine cellular fate while also providing possible opportunities for cellular-based therapy. Through these models, markers are introduced via various approaches to define self-renewal properties along with differentiation properties in tissues like the lung epithelium and hepatocytes (Lagasse et al. 2000; Krause et al. 2001; Krause 2008).

6.2.4.4 Lineage Tracing in Human Mitochondrial DNA

Both ethical and practical limitations exist in performing lineage tracing studies in human subjects due to a variety of experimental risks. Nevertheless, recent techniques

have been developed to help detect clonal populations of cells and provide a means for fate mapping in human tissues that have been surgically resected as a standard of care for cancer treatment. Even though this may not represent true in vivo environments, dynamic cell kinetics can be evaluated in an in vivo-like niche and provide added insight into stem cell behavior as it relates to oncogenesis.

Clonal proliferative units can be followed by tracing mitochondrial DNA mutations that translate into a detectable biochemical deficiency. Mitochondria serve as an effective means to track stem cells due to their propensity towards nonpathogenic mutations. Acquisition of these mutations takes prolonged periods of time before producing an experimentally detectable cellular phenotype. Since stem cells have expanded life spans relative to other more differentiated cell types, they reach a level of a detectable biochemical deficiency and can be followed in subsequent cell progeny. This has been demonstrated in human gastrointestinal and liver tissue through variable expression of cytochrome *c* oxidase. Patches of monoclonally derived units helped define the spatial location of the stem cell niche and provided insight into cellular origins of various cancers (Fellous et al. 2009a, b).

6.2.5 Phylogenetic Fate Mapping

Certain organisms that have a transparent appearance allow researchers to evaluate the fate of cells in vivo by direct observation or with the addition of cellular markers. With careful tracking, cell divisions and cell death can be followed at every stage of development in a simple organism without remarkable size or organ system hierarchies. In the case of the nematode, *Caenorhabditis elegans*, Sulston et al. (1983) tracked the fate of each cell as the embryo developed from 1 to 558 cells via direct observation. Generation of a fate map for simple organisms has provided significant framework for the behavior of cells during development. Following the effective use of this method as a template, multiple studies have focused on newer methods that allow for the more complicated mapping of cellular fates in more complex organisms and this has been largely through various cell markers described earlier. These methods account for the opaque nature of certain organ systems in which cells divide, the longer duration of the cell cycle, and the significantly increased number of total cells. Phylogenetic fate mapping is a different approach that monitors the accumulation of cellular mitotic mutations over time. Careful evaluation of these mutations allows for delineation of original progenitor cells and their subsequent daughter cells and provides an opportunity to follow stem cell like behavior in complex organisms with equally complex organ systems.

The most recent phylogenetic fate mapping studies employ the use of high-powered statistical methods to retrospectively trace the origins of a cell at any point during the lifecycle of an organism. With careful evaluation of the statistical outcomes, the probability of a particular cell fate can be estimated and compared to classic cell marker lineage tracing techniques. This has been assessed in mouse fibroblasts, somatic cells harvested from the adult mouse heart, lung, kidney,

spleen, and skeletal muscle (Salipante and Horwitz 2007; Salipante et al. 2008). Ultimately, more data comparing these high-powered statistical techniques to established methods of lineage tracing will need to be collected to further validate advanced phylogenetic approaches.

6.3 Ectopic Stem Cell Implantation

Key characteristics of stem cells include potential for both self-renewal and multi-lineage differentiation. Specifically, somatic stem cells that adopt the classic stem cell hierarchy should be able to be differentiated into several types of specific cells or be able to make tissue structures similar to the original organ. The majority of data collected to prove these functions have come from in vitro culture systems (Kim et al. 2005; Xin et al. 2007). However, these systems often lack key components of the cellular microenvironment of an in vivo system. In moderate contrast to transplantation of stem cells into multipotent blastocysts in chimera models, other methods have been developed to incorporate stem cell implantation into tissue-specific organ systems that have progressed through typical developmental stages. Stem cell implantation assays have been developed to allow for in vivo lineage tracing of cells that have been preprocessed and classified by in vitro methods.

Tissues and certain cell types such as the epidermis, myocytes, eye, hepatocytes, neurons, and pancreatic islet cells have been previously utilized as effective systems to incorporate implanted stem cells (Bharat et al. 2005; Simpson et al. 2007; Majo et al. 2008; Jensen et al. 2009; Madhavan et al. 2009; Tateno et al. 2010). These systems have had proven success with implantation due to their relatively simple structure at the cellular or basic organ level. In contrast, certain tissues possess increasingly complex structures with numerous cell types and highly variable three-dimensional organization. This complexity contributes to increased difficulty in stem cell implantation, making this a marginal approach to delineate stem cell function and fate in complex tissues like the lung, gastrointestinal tract, prostate, and kidney. To solve this issue, ectopic implantation assays that functionally mimic in vivo cellular milieu have been developed to evaluate stem cell behavior found in complex organs.

6.3.1 Renal Capsular Assays

Renal capsular assays are the most widely used ectopic implantation model. Cells are mixed with collagen gels and then implanted into the subrenal space to take advantage of abundant vascularization. Adequate vascularization is a key component for successful implantation. Approximately 4–12 weeks after implantation, cells are then harvested for ongoing assessment (Eirew et al. 2008; Leong et al. 2008; Konuma et al. 2009). This assay has proven better than subcutaneous implantation (Vu et al. 2003). Despite being relatively effective, there are limitations to the renal

capsular assay. Vascularization of the ectopic model is still relatively less than in situ autoimplantation (see Lau et al. 2009). Additionally, the surgical procedure required for capsular implanting is technically challenging and requires significant technical expertise.

6.3.2 Implantation Models Using Biomatrices

A separate implantation assay involves the biomatrix Matrigel (product description by BD Biosciences), a useful tool that has been used in vitro to manipulate stem cells (Kim et al. 2005; Xin et al. 2007; McQualter et al. 2010). Cells in this assay are immobilized in Matrigel, a heterogeneous semisolid matrix that contains high levels of laminin, collagen, and other factors found in extracellular environments. Certain studies have used Matrigel as an implantation assay in vivo by first injecting stem cells into the semisolid matrix and then subsequently into a subcutaneous region (Lawson et al. 2007; Xin et al. 2007). However, the vascularization of a Matrigel subcutaneous plug remains controversial. To illustrate this controversy, Liu et al. (2009b) showed that Matrigel subcutaneous plugs devoid of supplements will have reduced vascularization. Conversely, Bharat et al. (2005) described comparable vascularization between renal capsule and subcutaneous implantation with pancreatic islet stem cells. Furthermore, when angiogenic factors (transforming growth factor-receptor inhibitor, basic fibroblast growth factor, and vascular endothelial growth factor) are added to a Matrigel plug, angiogenesis is induced within the plug and effectively supports ectopic subcutaneous stem cell implantation (Liu et al. 2009b). Ultimately, additional studies are needed to optimize conditions that will allow subcutaneous stem cell implantation so that experiments can be performed efficiently without the need for technical experience required for a complicated surgical procedure.

6.3.3 Improving Ectopic Implantation Methods

In situ autoimplantation assays are ideal to confirm the fate of stem cells as these assays allow cells to function in their native environment. As mentioned before, complex organ systems remain difficult to study. To move forward with improved implantation methods, certain studies are ongoing to define the barriers to engraftment and function of stem cells. Lungs, for example, are among the most difficult organ systems to implant stem cells for two primary reasons. First, cellular structure and spatial distribution are relatively complex. Second, ongoing experiments are further evaluating the role of pulmonary resident macrophages as an immunologic interference that limits effective engraftment. Hegab et al. (2010) showed that lung stem cells could be successfully implanted in the injured lung, but still be functionally inefficient. Further development of this method will be necessary for improved implantation assays to prove the functions of stem cells in these organs.

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Chapter 7
Surrogate Measures of Adult Stem Cell Self-Renewal: The Neural Stem Cell Paradigm

Loic P. Deleyrolle, Brent A. Reynolds, and Florian A. Siebzehnrubl

Abstract Since the discovery that neural stem cells (NSCs) reside within specific neurogenic regions of the adult brain, much effort has been devoted to delineating their unique biology and evaluating their therapeutic potential to treat injury and disease. This chapter provides an overview of NSC biology and examines in detail methods used to study the biology and function of these cells. Emphasis is placed on in vitro assays, such as the neurosphere and neural colony-forming assays developed to propagate and enumerate NSCs. The physical and metaphysical limitations of these assays with respect to their capacity to delineate between stem, progenitor, and nonstem cells are discussed together with obstacles associated with developing in vivo assays to measure stem cell function and potency.

Keywords Neural stem cell • In vitro assay • In vivo repopulation assay • Quantum stem cell mechanics

Abbreviations

EGF	Epidermal growth factor
HSC	Hematopoietic stem cell
NCFCA	Neural colony-forming cell assay
NSA	Neurosphere assay
NSC	Neural stem cell
RMS	Rostral migratory stream
SGZ	Subgranular zone
SVZ	Subventricular zone

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7.1 Introduction

Adult brain stem cells have been discovered comparably recently (Reynolds and Weiss 1992) after their existence had been debated for decades (Ramon y Cajal 1991; Altman 1962; Rakic 1985; Nottebohm 1985). Postnatal neurogenesis is now a well-established phenomenon and, despite the existence of adult neural stem cells (NSCs) being widely acknowledged, they possess some unique characteristics. For instance, NSCs and their progeny can be cultured in suspension, where they form globular cell clusters called "neurospheres" – a culture paradigm that has translated into other fields of stem cell research as well (Vescovi et al. 2006). Furthermore, stem cells exist only in two distinct locations of the brain, the hippocampus and the areas lining the ventricular system. Moreover, the birth of new neurons and their addition to existing neural circuits is a tightly regulated process occurring only in two very specialized structures, the dentate gyrus and the olfactory bulb (van Praag et al. 2002; Rochefort et al. 2002; Lledo et al. 2006). Evidence for neurogenesis in other brain areas is scarce and controversial; it should be noted, however, that in vitro multipotent progenitor cells can be isolated from a great number of brain regions (Palmer et al. 1999; Lie et al. 2002). Due to the high complexity of the brain, a stunning number of highly specialized cell types exist in this organ. However, adult stem cells form only certain types of neurons, depending on the neurogenic region and context and only a very limited number of glia under normal conditions in vivo (Zhao et al. 2008). It has been shown that stem cells have some potential for regeneration following various injuries including cortical lesions, inflammatory demyelination, seizures, strokes, and excitotoxic striatal lesions (Szele and Chesselet 1996; Calza et al. 1998; Zhang et al. 2001; Parent et al. 2002a, b; Arvidsson et al. 2002; Tattersfield et al. 2004; Ohab et al. 2006), although nonneurogenic brain areas seem to be too inhibitory for stem cell proliferation, migration, and differentiation for effective regeneration. However, these discoveries provide hopes for the field of regenerative medicine and the idea of a stem cell therapy for many CNS disorders.

Based on the pioneering work from Fernando Nottebohm's lab showing the functional relevance of adult neurogenesis in songbirds (Nottebohm 2004), a flurry of studies (reviewed in Lie et al. 2004) followed the initial isolation and culturing of NSCs from the adult mouse brain (Reynolds and Weiss 1992). Adult neurogenesis has now been demonstrated in the rodent, tree shrew (Gould et al. 1997), monkey (Gould et al. 1998), and adult human brain (Eriksson et al. 1998; Curtis et al. 2007), persisting in discrete CNS regions. Within these regions, adult NSCs continue to generate new neurons throughout life, whose integration into preexisting neural circuitry is thought to be critical for the maintenance of brain integrity and optimal brain function (van Praag et al. 2002; Rochefort et al. 2002). The largest neurogenic region is the subventricular zone (SVZ) of the forebrain, located between the lateral ventricle and the parenchyma of the medial striatum, together with its rostral extension into the olfactory bulb (the rostral migratory stream, or RMS), and its target area, the cortex of the olfactory bulb. Despite ongoing controversy regarding their actual identity and nature, a subset of the SVZ cells expressing the astroglial marker

glial fibrillary acidic protein (GFAP) has been identified as a putative adult NSC population in this region (Doetsch et al. 1999). Relatively quiescent, with a proposed cycling time of 28 days, these astrocyte-like adult stem cells are referred to as type B cells and represent a minor subset of the total astrocyte population in the SVZ (Doetsch et al. 1999). In vivo, NSCs generate fast cycling progenitor cells with a cell cycle time of about 12 h (Morshead et al. 1998). These rapidly cycling type C cells retain multipotentiality and give rise to more mature lineage-restricted progenitors, type A cells, that migrate in chains through the RMS into the olfactory bulb, where they functionally integrate as new interneurons in the cortical layers. Growing evidences support a role of the newly generated neurons in olfactory memory and discrimination (Rochefort et al. 2002; Gheusi et al. 2000; Petreanu and Alvarez-Buylla 2002; Enwere et al. 2004) and, while the fine details still need to be delineated, the SVZ is also present in the human brain (Curtis et al. 2007; Sanai et al. 2004).

A similar hierarchal neurogenic system is operating within the subgranular zone (SGZ) of the rodent (Seri et al. 2001, 2004) and human hippocampus (Eriksson et al. 1998; Roy et al. 2000). Neurogenesis occurs in close association with blood vessels, wherein astrocyte-like cells generate new interneurons through intermediate progenitors that correspond to the type C cells of the SVZ (Seri et al. 2001; Zhao et al. 2008). Newly generated neurons in the hippocampus do not migrate long distances as in the SVZ. Several studies have established that about 9,000 new cells are generated on a daily basis in the young adult rodent dentate gyrus and that a fraction of these cells integrate into the neuronal network by receiving synaptic input and sending axonal projections to appropriate targets (van Praag et al. 2002; Cameron and McKay 2001; Markakis and Gage 1999; Carlen et al. 2002; Stanfield and Trice 1988; Hastings and Gould 1999). Inhibition of cell genesis in the hippocampus modifies the formation of different types of memory, suggesting that dentate gyrus neurogenesis regulates hippocampus-dependent learning and memory mechanisms (Shors et al. 2001, 2002; Shors 2008).

One of the most challenging quests in the field of adult stem cell research has been the task of identifying the "actual" stem cell, a cell that is at the topmost level of the cellular hierarchy, giving rise to all cell lineages beneath it. Because the CNS does not have a physiologically high dynamic turnover like the hematopoietic system, a way to functionally identify adult NSCs in vivo (comparable to the bone marrow repopulation studies of the hematopoietic field) is so far missing. Thus, great effort has been put into the search for markers identifying such a stem cell in vivo and into in vitro assays to measure and quantify stem activity. Therefore, based on in vitro assays and on ultrastructural, phenotypical, and immunohistochemical observations made in vivo, a stem cell hierarchy has been established, with multipotent, self-renewing stem cells at the top, giving rise to transit-amplifying cells (with more restricted capacity for self-renewal) that in turn produce lineage-committed functional progenitors. Due to the difficulty in functionally identifying adult NSCs in vivo, multiple in vitro assays are necessary to separate stem cells from nonstem cells. However, all of these in vitro approaches have their limitations and it is prudent to keep these in mind when working on adult stem cells.

7.2 Measuring NSC Activity In Vitro

7.2.1 The Neurosphere Assay, a Gold Standard Methodology

In a series of experiments published from 1961 to 1965, Till and coworkers provided the first definitive evidence for the existence of adult stem cells (Till and McCulloch 1961; Siminovitch et al. 1963; McCulloch et al. 1965). This group used an in vivo spleen colony-forming cell assay involving the implantation of donor bone marrow cells into receiver animals that had been irradiated, followed by the analysis of clonally derived colonies in the recipient's spleen. Based on their analysis, they hypothesized that the colony-forming cells, representing only a rare subpopulation, had no distinguishing criteria other than giving rise to spleen colonies. From their results, they concluded that spleen forming stem cells properties could be deduced by studying the capability to generate colonies and that stem cells are retrospectively analyzed based on a functional definition. This seminal finding formed the foundation for the study of all adult stem cells and has defined the key elements of the functional features of a stem cell: (a) proliferation, (b) self-maintenance, (c) production of a large number of differentiated functional progeny, (d) regeneration of tissue after injury, and (e) flexibility in the use of these options (Potten and Loeffler 1990). Based on these tenets, stem cells have been extensively studied in other tissues. Up until the early 1990s, adult stem cells were thought to be present only in tissues that demonstrated a substantial degree of cell turnover, such as the blood, skin, and intestines. Consequently, one never expected to find such stem cells in the brain, which was thought to be the most static tissue of the body.

To detect and expand a putative stem cell from the adult brain, Reynolds and Weiss (1992) employed a serum-free culture system supplemented with epidermal growth factor (EGF), whereby a small population (<0.1%) of growth factor-responsive cells dissected from the periventricular region entered a period of active proliferation forming a cluster of undifferentiated cells referred to as a neurosphere that in turn could be: (a) dissociated to form numerous secondary spheres, or (b) induced to differentiate, generating the three main cell types of the CNS (Fig. 7.1). Although under these minimalistic culture conditions (i.e., serum-free) the majority of primary differentiated CNS cells harvested did not survive, the stem cell population could be passed, theoretically indefinitely with maintenance of a stable profile with regard to karyotype, molecular pathway activation, self-renewal, differentiation potential, and most importantly, without oncogenic transformation (Foroni et al. 2007). Reynolds and Weiss (1992) had therefore determined a methodology enabling isolation of cells exhibiting the critical stem cell attributes of proliferation, self-renewal, and the ability to give rise to a number of differentiated functional progeny (Potten and Loeffler 1990; Hall and Watt 1989). Hence, following a well-defined protocol (Deleyrolle and Reynolds 2009b), renewable sources of undifferentiated CNS precursors can be produced, portions of which are stem cells. These precursors could be expanded as neurospheres or reliably differentiated into defined proportions of neurons, astrocytes,

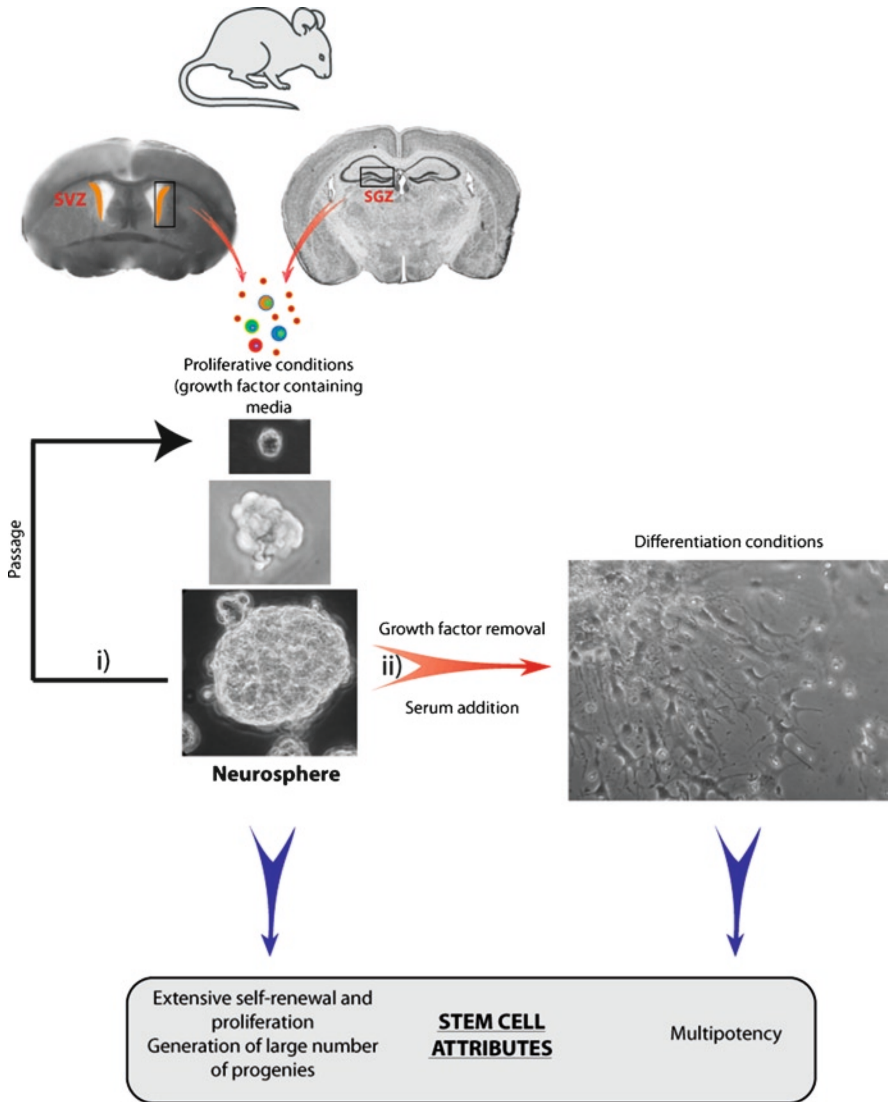

Fig. 7.1 Neurosphere Assay. Cells from the adult subventricular zone (SVZ) or subgranular zone (SGZ) of the hippocampus are dissociated into a single-cell suspension and cultured in serum-free conditions supplemented with growth factors where the majority of cells do not survive, except for the cells that divide in response to the mitogenic stimulation. A small population of growth factor-responsive cells proliferate to form clusters of undifferentiated cells referred to as neurospheres that in turn can be: (**i**) dissociated to form numerous secondary spheres or (**ii**) induced to differentiate, generating the three major cell types of the CNS; neurons, astrocytes, and oligodendrocytes

and oligodendrocytes (Reynolds and Weiss 1992; Gritti et al. 1995; Weiss et al. 1996). Because of its reproducible nature and its simplicity of utilization, the neurosphere assay (NSA) is the main methodology used to isolate, expand, and study NSCs of embryonic and adult sources. Confirming the existence of stem cells

in the mammalian central nervous system, the NSA contributed to the demise of the "no new neuron" dogma that has dominated developmental and regenerative neurobiology for the majority of the past century (Gross 2000).

7.2.2 Limitations of the Neurosphere Assay

The NSA has been used to identify factors and molecular pathways controlling stem cell properties and to determine a stemness signature with several genes commonly expressed in different stem cell populations (Ivanova et al. 2002; Ramalho-Santos et al. 2002). Although evidence supports the maintenance of regional specification and intrinsic differences in neurospheres cultured from different compartments of the brain (Hitoshi et al. 2002; Parmar et al. 2002; Ostenfeld et al. 2002), several studies suggest deregulation of the spatial identity and differentiation potential of stem cells grown in this culture system (Machon et al. 2005; Santa-Olalla et al. 2003).

Due to its robustness, the NSA has been extensively used as a sphere-forming assay to establish the existence of stem cells in diverse regions of the CNS at distinct developmental stages and to estimate the dynamic changes in NSC activity after diverse treatments (Reynolds and Weiss 1992; Morshead et al. 1994; Gritti et al. 1995; Weiss et al. 1996; Hitoshi et al. 2002; Maslov et al. 2004; Lu and Wong 2005; Marshall et al. 2005; Yang and Levison 2006). However, in light of the observations that neurospheres are not only generated by stem cells, but mainly from precursor cells with limited capacity for self-renewal (Reynolds and Weiss 1992; Louis et al. 2008; Deleyrolle and Reynolds 2009a), it is now clear that the one-to-one association between NSCs and neurospheres is mistaken (Reynolds and Rietze 2005). Growing evidences suggest that neurosphere formation is not fundamentally associated to the existence of NSCs (Bull and Bartlett 2005; Marshall et al. 2006; Seaberg and van der Kooy 2002). Bull and Bartlett (2005) demonstrated the lack of long-term self-renewal of adult mouse hippocampus-derived multipotent neurospheres. Moreover, Seaberg and van der Kooy (2002) isolated two different lineage-restricted neurosphere clones from the adult dentate gyrus, which were incapable of self-renewal and able to generate only glial-restricted progeny or neuronal-restricted progeny, respectively. Together, these results suggest an absence of actual stem cells in the adult hippocampus.

As a result, neurosphere generation does not provide sufficient evidence of a NSC, nor can the enumeration of neurospheres be used as a means to accurately detect changes in NSC numbers. Consequently, studies that have used the NSA as a readout for purification and enrichment strategies of NSCs need to be revised. Innovative assays are clearly required to meaningfully and quantitatively detect specific changes in NSC activity.

7.2.3 The Neural Colony-Forming Cell Assay to Enumerate Actual Neural Stem Cells

Louis et al. (2008) developed the neural colony- forming cell assay (NCFCA) to overcome the lack of an accurate assay to distinctively detect and quantify specific changes in the NSC compartment. This in vitro methodology is built on the observation that progenitor cells display restricted proliferative potential when compared to stem cells, thereby indicating that both cell populations can be distinguished based on the size (diameter) of the colony they can generate, directly reflecting their proliferative potential. Unlike the NSA, the NCFCA (using a defined serum-free medium) ensures clonality of the colonies due to the use of a semisolid collagen matrix. Both assays demonstrate a similar sphere/colony frequency indicating that the NCFCA growth conditions do not inhibit the proliferation of the neural stem/precursor cells. However, in contrast to the NSA, which only allows the cells to grow under healthy conditions for 5–7 days between passages, the NCFCA experimental conditions are appropriate to allow stem and precursor cells to proliferate and form colonies for more than 3 weeks without any passing or dissociation step, enabling the maximal proliferative capacity of the cells to be manifested over time. As a result, NSC and neural progenitor cell frequency can be established with regard to colony size. Four categories of colonies were measured based on diameter (0.5, 0.5–1, 1–2, and >2 mm), thereby illustrating the difference in the proliferative potential of the original colony-forming cell (Fig. 7.2). Using this colony size

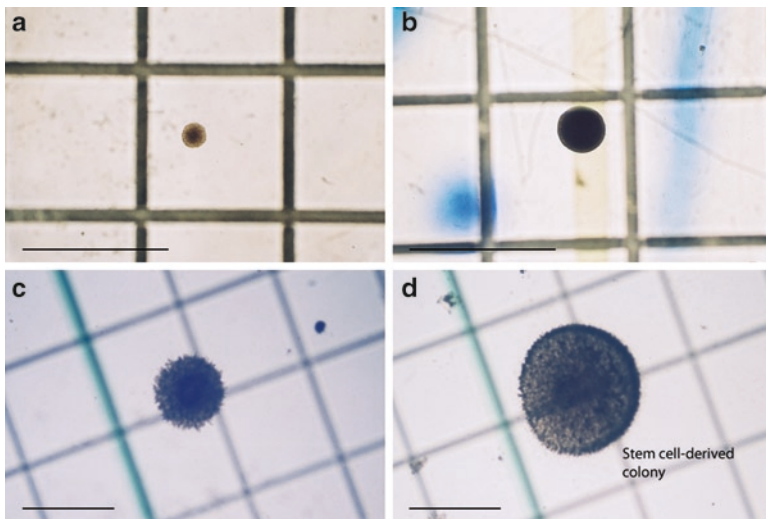

Fig. 7.2 Neural colony-forming cell assay. Four categories of colony size can be classified based on their diameter: (**a**) <0.5 mm, (**b**) 0.5–1 mm, (**c**) 1–2 mm, and (**d**) >2 mm. Scale bars represent 2 mm. Photos reproduced with permission from STEMCELL Technologies Inc.

classification, only large colonies (those >2 mm) presented all the cardinal stem cell features (extensive self-renewal, generation of a large number of progeny, and multilineage differentiation potential), while smaller-ranked colonies did not (restricted self-renewal capacity and limited differentiation capability). Using these conditions, Louis et al. (2008) determined that the majority of the colonies (>90%) are derived from a nonstem cell. This research group demonstrated the cogency of the NCFCA and its capacity to distinguish between neural stem and progenitor cells from embryonic- and adult-derived neural precursor cells. They also used the assay to reevaluate several research reports that have relied on the NSA as a measure of stem cell frequency and demonstrated that stem cell frequency is regularly and generally overestimated. Hence, the NCFCA represents a meaningful method to study NSC biology and constitutes an essential tool to study neural development and regeneration.

7.3 Stem Cell Niche Repopulation In Vivo

Since the identification of a hematopoietic stem cell (HSC) in the 1960s (Till and McCulloch 1961; McCulloch and Till 1964), long-term repopulation of the bone marrow after transplantation into lethally irradiated mice has evolved as the method of choice to prove stemness of HSCs (Purton and Scadden 2007). Functional in vitro assays for stemness exist for HSCs, such as colony-forming assays (Coulombel 2004). However, “stem cells” identified in such in vitro assays do not necessarily reflect long-term repopulating stem cells in vivo (Coulombel 2004) and thus may lead to misconceptions about the actual number of stem cells present in any given sample. Importantly, for the past 40 years, functional in vivo assays (i.e., repopulation of the bone marrow) have been the only way to identify true HSCs. The importance of the last two sentences cannot be overstated: the term “hematopoietic *stem* cell” is based upon this functional definition in vivo. Even when homogenous cell populations able to fulfill this criterion were identified (Matsuzaki et al. 2004), this way of defining a HSC by its most important functional feature has retained its power.

Similar misinterpretations about the nature of presumed stem cells may also affect functional in vitro assays employed for testing NSCs. The lack of functional in vivo assays for NSCs is a major obstacle for the understanding and identification of true adult stem cells in the CNS. That such an assay has not yet been developed is likely the result of several noteworthy differences between the neural and hematopoietic system, which render depletion and reconstitution of CNS neurogenic zones much more difficult. First, effective ablation of the stem cell niche in the CNS most likely requires more than single doses of radiation or chemotherapy (Louis et al. 2008). Second, even though presumed NSCs can be readily isolated from animals, the actual frequency of stem cells in neurosphere cultures is rather low (Fig. 7.3) (Reynolds and Rietze 2005; Louis et al. 2008), making delivery of cultured neurospheres a poor choice as a donor stem cell population. Finally, the readout of an in vivo assay for the CNS is much more complicated than in the bone marrow, where nonreconstituted, lethally irradiated animals simply die. Successful demonstration of repopulation of

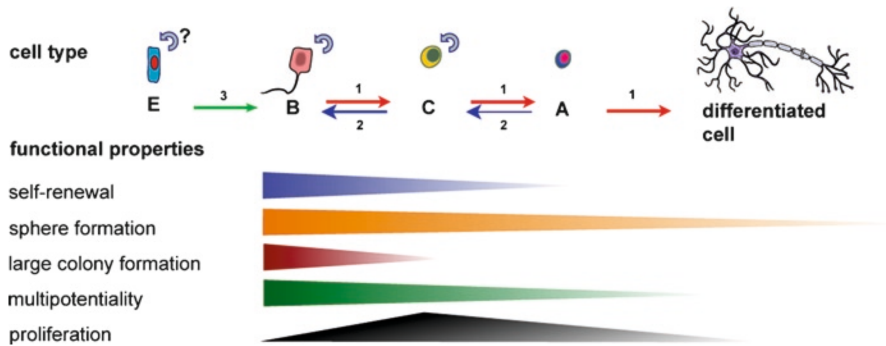

Fig. 7.3 Functional properties of stem and progenitor cells. The classical way of viewing stemness is unidirectional (**1**), from the neural stem cell (**B**) to transient-amplifying progenitor cells (**C**) to lineage-committed progenitor cells (**A**) to differentiated cells. Functional stemness properties (as testable in vitro) are gradually declining towards the differentiated end of the spectrum. According to the Uncertainty Principle, these properties cannot be tested simultaneously with arbitrary precision. In cell culture conditions, the stemness state of other cells (**C, A**) might be reversible (**2**). These cells then act as stem-like cells, which are indistinguishable from "genuine" stem cells in vitro. During injury, ependymal cells (**E**) may gain a state of stemness (**3**), which allows them to generate neuronal progenies

the SVZ needs to rely on histochemical characterization of the repopulated niche. So far, it is unclear if ablation of the SVZ stem cell population has a behavioral correlate, which could be used for a behavioral readout, rendering the assessment of *functional* reconstitution of the stem cell niche challenging, if not impossible.

Major challenges for the identification of *bona fide* adult stem cells in both the hematopoietic system and the CNS are the lack of a reliable correlation between cellular phenotype and function, the heterogeneity of cells identified in single assays, and potential ontogeny-related differences in stem cell self-renewal and proliferation (i.e., different physiology of human and mouse cells) (Coulombel 2004). While the latter cannot be addressed by studying animal stem cells, both the former obstacles can be overcome by in vivo functional assays. Concentrating on the functional definition of a stem cell eclipses phenotypic similarities between different cell types. Research experience in the hematopoietic system shows that cellular heterogeneity can be dissected by rigorous testing in vivo, i.e., multiple rounds of repopulation.

One way of functionally defining adult stem cells is based on quiescence. The cell cycle length differs dramatically between type B stem cells (estimated 15–28 days) and type C progenitor cells (12 h) (Morshead et al. 1998). Quiescence of adult NSCs so far has been only an assumption, even though a very intuitive one. Recent studies in the hematopoietic field, however, have shown that HSCs indeed are extraordinarily quiescent in vivo, with dormant stem cells dividing only 5 times during the lifespan of a mouse (Wilson et al. 2008). Proliferative quiescence has been suggested as mechanism to protect the stem cell pool from depletion and individual stem cells from acquiring mutations that might result in tumor formation (Orford and Scadden 2008).

Previous studies have attempted reconstitution of the brain neurogenic niche with varying degrees of success (Marshall et al. 2006; Neumeister et al. 2009). As discussed previously, it is our opinion that two very important factors contribute to the success (or failure) of repopulation of the SVZ: (a) substantial ablation of the stem cell niche, as partial depletion may challenge efficient engraftment of transplanted NSCs into their appropriate compartment and (b) purity of the grafted stem cell population. The frequency of actual stem cells in neurospheres is approximately 1 in 3,000 cells (Louis et al. 2008; Reynolds and Rietze 2005). In one previous study, a total of 20,000 neurosphere-derived cells were transplanted per adult animal, a figure far too small if only stem cells have repopulation ability (Marshall et al. 2006). A more successful study found that adult stem cells have the ability to repopulate the SVZ when transplanted into the embryonic brain and do so multiple times (Neumeister et al. 2009). Unfortunately, this study lacks quantitative data on the number of successfully engrafted type B cells in the SVZ.

A general problem with in vivo repopulation studies in the adult CNS is the low frequency of stem cells and the slow turnover in the niche. This renders the quality of the readout for a repopulation experiment questionable, if not doubtful. The question thus becomes whether it is at all possible to put the tag “stem cell” on any given cell, if there are no in vivo functional assays to test for stemness?

7.4 “Quantum” Stem Cell Mechanics

While it would be preferable to have an in vivo functional readout for stemness, to date stem cells can only be identified by more or less circumstantial characteristics (location, morphology, markers, and in vitro assays). It is a general problem of any such assays that stem cells will always escape attempts to exactly identify them because of the experimental design inherent to such assays. In other words, it is impossible to measure with accuracy two or more defining properties of stem cells (e.g., location and fate) and thus it is impossible to determine the stemness of a given cell with arbitrary precision. This has also been called the “Heisenberg principle” of stem cell research (Potten and Loeffler 1990). Ironically, this principle also applies to the hematopoietic field, where functional in vivo assays for stemness have been developed.

The Uncertainty Principle formulated by Heisenberg in 1927 states that any two conjugated variables cannot be measured with arbitrary precision (Heisenberg 1927). In physics, the most often cited example for this effect is the position and momentum of an electron. When observing both properties of an electron, more precision in position measurement causes an unavoidable disturbance in momentum, leading to a more imprecise measurability of momentum (Heisenberg 1927). There is, however, a notable difference between the Uncertainty Principle and the *observer effect* (the phenomenon of one measurement affecting the outcome of another) even though both are related. The *observer effect* in human sciences (also called Hawthorne effect) describes that human subjects modify their behavior not in reactivity to any particular experimental manipulation, but solely in response to

being experimentally studied (Mayo 1933; Roethlisberger and Dickson 1939; Franke and Kaul 1978). In physics, the *observer effect* refers to the impact of making an observation on a physical system and infers that a different experimental paradigm or more precise instruments may overcome the problem, while the Uncertainty Principle is a general phenomenon comparable to a natural law. Furthermore, according to the Uncertainty Principle, also *failure* to measure the particle produces the disturbance. Heisenberg discovered that this uncertainty in observing conjugated pairs of variables is a natural law deeply etched into the universe. Thus, the Heisenberg Principle in general applies to all natural sciences governed by the laws of physics. Put in more general terms, it is impossible to determine with precision two (or more) properties of any component of a system that are codependent, but not related.

More specifically applied to biology, a consequence of the Heisenberg Principle is that we have to accept that certain parameters can be measured not absolutely, but only in probabilities. Even though it may seem trivial, the importance of the last sentence cannot be overstated. In a stricter sense, and when applied to stem cell biology, one cannot accurately measure more than one defining property of a stem cell simultaneously. The generally accepted characteristics of stem cells are their proliferative capacity, long-term ability to self-renew (which is in some ways tied to the proliferative capacity), relative quiescence, and potential to give rise to all lineages of an organ. Since these properties are quite antipodal to each other, it is impossible to determine all of them accurately at the same time. For instance, adult NSCs reside in the lining of the lateral ventricles and can give rise to transit-amplifying progenitors, which will develop into more mature cells of either the neuronal or the glial lineage. There are several markers known that are expressed by stem cells, but none of them are exclusive to this population. Both stem cells and their immediate progeny can self-renew (although progenitor cells less so than stem cells) and are multipotent. When observing a cell in the right place (adjoining the ventricle) and at the right time (adulthood), even though this cell may look morphologically and phenotypically like a stem cell, we cannot conclude that it actually *is* a stem cell, until the cell in question also gives rise to neuronal and glial progeny. So, even if we are able to identify stemness based on in vivo repopulation, we are unable to conclude which of the transplanted cells the repopulating cell is (up to the point of single-cell repopulation studies – which will grossly underestimate the actual stem cell frequency).

Since we cannot identify stem cells on a single-cell level, we have to use other means for the identification of stem cells. We may conclude from existing experimental data that a cell population contains a certain number of stem cells, or that any given cell within this population has a certain probability of being a stem cell (number of stem cells divided by overall number of cells). This presents, if not a solution, at least a work-around for our problem. By assigning probabilities of stemness to cell populations and working with these, we can still arrive at the necessary functional conclusions. Again, this might seem trivial, but is important to keep in mind. Thus, we could define a continuum over our population ranging from an absolute stem cell to a perfectly terminally differentiated cell. Any cell in this population will be on a

certain point along this continuum, but since our methodology is limited, we can determine its position in this continuum only with a finite amount of (un-)certainty. Thus, stemness is no longer an exclusive property of stem cells, but rather a state almost any cell can be in (in vitro and under certain circumstances, such as injury, also in vivo). This is also illustrated by the fact that only a small number of transcription factors can turn any cell into an embryonic stem-like, induced pluripotent (iPS) cell. With this in mind, it is evident that in vitro culture conditions can easily induce some cells to revert into a stem-like state. Are these cells stem cells? For all purposes of the in vitro experiment, yes. Since at this point we cannot determine if these cells would act as stem cells in vivo (i.e., successfully repopulate the niche), we would have to conclude that stem-like cells are stem cells.

The core of quantum mechanics is the philosophical notion that an experimental system is not deterministic, but uncertain. We cannot make predictions on the behavior of the systems components, even though we might know all possible variables. The events in such a system occur impartially at random. Going back to stem cell biology, of course stem cells in our population are not randomly assigned. These cells are stem cells a priori and will be stem cells until they differentiate or die. However, since we are essentially blind as to what cell is a stem cell in our population, it seems as if this characteristic was distributed at random. We cannot make predictions which cell will be a stem cell – even when we look at the expression of all known markers of stemness, there will always be a population expressing these markers without having stemness characteristics and a stem cell-containing population negative for these markers.

Since we cannot distinguish between discrete stemness states (the "gray area" between a stem cell and a multipotent progenitor cell), we might conclude that transition between these states is possible – a stem cell can give rise to a progenitor cell, but a progenitor cell may become a functional stem cell as well (Fig. 7.3). A similar observation has been made in the mouse testis, where transit-amplifying progenitor cells would revert to stem cells upon injury (Nakagawa et al. 2007), and such processes may also be the basis for the previous controversies of ependymal cells acting as stem cells (Johansson et al. 1999; Carlen et al. 2009). A comparable process was also observed in the small intestine where stem cells, residing around the fourth cell position from the bottom of the crypt of Lieberkuhn, produce transit-amplifying progenitors that differentiate as they migrate toward the intestinal lumen; however, in the initial transit divisions, these amplifying cells retain the potential to revert into fully active stem cells.

Given the current tools of adult NSC research, we are unable to identify different states along the stemness continuum in the cell cultures and animals we study. It is possible that we never will be able to make a distinction between a stem cell (type B) and a multipotent progenitor (type C) in an in vitro culture. The distinction between type B cell and type C cell in vitro is academic at this time. Stem cells can be discerned from progenitor cells in vivo by virtue of different location, morphology, and phenotype, but as soon as these cells are isolated and placed in an in vitro setting, the differences between those cells blur and both cell types act similarly.

7.5 Summary

Adult CNS stem cells are a population of great interest to the research community. Due to the lack of definitive markers for these cells and the lack of functional in vivo assays for testing stemness, many studies utilize in vitro experiments to estimate stem cell function. However, in vitro assays are inherently limited in their ability to identify stem cells – both physically and metaphysically. Until functional in vivo assays (comparable to the hematopoietic repopulation work) for adult brain stem cells are developed, these in vitro tests will be the only way of addressing stemness of isolated potential stem cells. At the same time, this retards the identification of stem cell markers.

The main problem in developing in vivo assays seems that the stem cell niche in the brain is harder to target than the blood niche. Since the grafted putative stem cell population has to outcompete the existing stem cells for repopulating the niche, successful donor integration is much more likely if the host niche is fully ablated. This is not trivial, as cell turnover in the brain is much slower than in the hematopoietic system. Moreover, any attempt to ablate NSCs will also affect other cell types including endothelial cells, microglia, and possibly hematopoietic cells, all of which potentially interact with NSCs and the absence of which might affect the outcome of the repopulation. Another factor determining the success or failure of repopulation attempts is the number of grafted cells. In the hematopoietic system, putative stem cells are infused into the circulation and there is almost no limit to the number of grafted cells. This has certainly helped for the initial repopulation studies of the 1960s. If the donor cells are to be grafted directly into the brain, however, the volume of the graft is limited to 2–3 μl or about 200,000 cells. Given that the frequency of stem cells in a neurosphere culture is 1 in 3,000, this would result in transplanting less than 70 stem cells. Hence, the putative stem cell population must either be enriched (by relying on prospective markers), or injected into the vascular system (and somehow home to the brain).

Overall, the current system of combining in vitro stemness assays that address individual stem cell parameters has worked well so far, although such in vitro approaches are reaching their limits when being used to isolate stem cells on a single-cell level. As outlined above, in vivo assays that follow the lines of the hematopoietic field may be difficult to implement. Future work should continue to improve the existing in vitro assays and develop practicable in vivo approaches for testing stemness, especially in the CNS.

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Chapter 8
ABC Transporters, Aldehyde Dehydrogenase, and Adult Stem Cells

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Abstract Despite many years of intensive effort, there is surprisingly little consensus on the most suitable markers with which to locate and isolate stem cells from adult tissues. So-called markers of stem cells have been varied but can be broadly categorized into molecular determinants that govern self-renewal, clonogenicity, multipotentiality, adherence to the niche and longevity. This chapter describes two specific attributes of many stem cells that appear to be the main determinants of stem cell survival, namely either an ability to detoxify many potentially cytotoxic molecules by virtue of high aldehyde dehydrogenase (ALDH) activity or an ability to actively efflux a wide variety of cytotoxic agents by virtue of the presence of one or more ATP-binding cassette transporters (ABC transporters), and indeed many stem cells may be endowed with both properties. Antibodies are available against the ALDH enzyme family and ABC transporters, but the vast majority of studies have used cell sorting techniques to enrich for cells expressing these molecules. Live cells expressing high ALDH activity are usually identified by the ALDEFLUOR kit and sorted by fluorescence-activated cell sorting (FACS), the latter technique is also being used to isolate cells with high ABC transporter activity after incubation with a fluorescent dye (usually Hoechst 33342) that is actively effluxed from these cells giving rise to a fluorescent dull population known as the "side population". Since both ALDH and ABC transporter activities are cytoprotective strategies, it is not surprising that many cancer stem cells have these mechanisms working robustly, but they are also present in normal adult stem cells. This chapter critically reviews ALDH activity and the SP as markers of normal adult stem cells.

Keywords ABC transporters • Adult stem cells • Aldehyde dehydrogenase • Side population

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Abbreviations

ABC	ATP-binding cassette
ALDH	Aldehyde dehydrogenase
BCRP1	Breast cancer resistance protein
DAPI	Diaminophenylindole
DEAB	Diethylaminobenzaldehyde
FACS	Fluorescence-activated cell sorting
Ho	Hoechst
HSC	Hematopoietic stem cell
hUCB	Human umbilical cord blood
MDR	Multidrug resistance
SLAM	Signalling lymphocytic activation molecule

8.1 Introduction

Adult stem cells are classically defined as cells with the capacity for limitless self-renewal and the ability to produce differentiated progeny (Alison and Islam 2009). To demonstrate such stem cell-derived clones, the gold standard in mice involves lineage tracing, commonly employing hormone-dependent Cre recombinase expression. In this widely adopted methodology, a putative stem cell-specific promoter expresses Cre, achieving temporal control by fusion to an oestrogen receptor. This necessitates low-dose tamoxifen to bring about translocation of Cre to the nucleus where it "floxes" out a stop sequence, allowing reporter gene (e.g. *lacZ*) expression in the stem cell and all its progeny thereafter. The phenotyping of such genetically marked clones thus allows us to enquire as to whether the proposed stem cell has multipotentiality. Alternative methods of stem cell identification can be less robust; stem cells are supposed to have inherent properties such as being slow cycling, enabling DNA label retention after a pulse of the likes of BrdU, but label retention may be because of imminent cell cycle exit, moreover a slow cycling nature has not been found in all stem cells. The cell surface phenotype is often used to identify putative adult stem cells, but without subsequent lineage tracing the unique attributes of stem cells cannot be demonstrated. Moreover, many of these so-called markers of adult stem cells lack specificity. For example in the crypts of the colon Musashi1, CD44 and CD133 have all been proposed as suitable stem cell markers, but expression can also be seen outside the stem cell zone extending to the transit amplifying cells higher up the crypt (Nishimura et al. 2003; Huang et al. 2009). Other proposed markers of stem cells include particularly high levels of cell adhesion molecules, especially in the basal layers of squamous epithelia, and also the ability to evade cytotoxic insults. These cytoprotective strategies come in two forms: firstly, low expression of certain cytochrome P450 enzymes, a superfamily of hemoproteins involved in oxidative (phase I) metabolism; in the

pulmonary airways this is the situation in the so-called pollutant-resistant stem cells (Reynolds et al. 2000), and in the liver, stem/progenitor cells clonally expand after hepatocyte loss in the face of toxic insult also by virtue of low cytochrome P450 enzymes (Vig et al. 2006); and secondly high expression of either efficient enzyme-based detoxification systems or proteins with the ability to rapidly export potentially harmful xenobiotics.

This chapter describes the latter two strategies, one based upon the high expression of aldehyde dehydrogenase (ALDH) activity and the other based upon the ability to efflux a fluorescent dye, usually Hoechst 33342, described as the "side population", first reported by Goodell et al. (1996) for the isolation of murine haematopoietic stem cells (HSCs) with long-term multilineage repopulating potential. Although in this chapter we describe the utility of these assays for identifying normal adult somatic stem cells, both methods and in particular the activity of ALDH have come to the fore in identification of the so-called "cancer stem cells" (Alison et al. 2010). Since ALDH is an effective detoxifying enzyme, high expression of ALDH can be detrimental to tumour eradication. High expression of ALDH can also provide a route for tumours to resist chemotherapy (Bunting and Townsend 1996; Moreb et al. 1998; Canuto et al. 2001). For example, cyclophosphamide treatment of human colonic xenografts enriches for $CD44^{+}ALDH^{+}$ cells, and these double positive cells are more tumorigenic than cells selected solely on the basis of CD44-positivity (Dylla et al. 2008).

8.2 Aldehyde Dehydrogenases

ALDHs are a family of $NAD(P)^{+}$-dependent enzymes involved in detoxifying a wide variety of aldehydes to their corresponding weak carboxylic acids (Sladek 2003). They serve to detoxify both xenobiotic aldehydes (e.g. cyclophosphamide) and many other intracellular aldehydes, e.g. ethanol and vitamin A (Vasiliou et al. 2004; Vasiliou and Nebert 2005). There are 20 *Aldh* genes in the mouse and 19 *ALDH* genes in humans, the latter being organized into 11 groups (1–9, 16 and 18), the largest being group 1 with six members comprising three subfamilies (A, B and L), thus ALDH1A1 is the first *ALDH* gene in group 1, subfamily A (see www.aldh.org and www.genenames.org/genefamily/aldh.php). Most isoforms are widely distributed in the body, though highest expression is seen in the kidney and liver (Douville et al. 2009). In the liver, ALDH1 mainly functions as a retinoic acid (RA) biosynthetic enzyme, catalyzing the conversion of vitamin A (retinol) to RA. In HSCs where the utility of ALDH as a stem cell marker was first recognized (Storms et al. 1999), RA signalling is believed to be crucial for cell fate determination and inhibiting ALDH activity leads to an expansion of HSC numbers (Chute et al. 2006; Purton et al. 2006; Purton 2007; Muramoto et al. 2010).

Cells expressing ALDH1 or other ALDH family members may be identified by immunohistochemistry using specific antibodies, but functional activity of all ALDHs may be accurately assessed in living cells using the commercial reagent

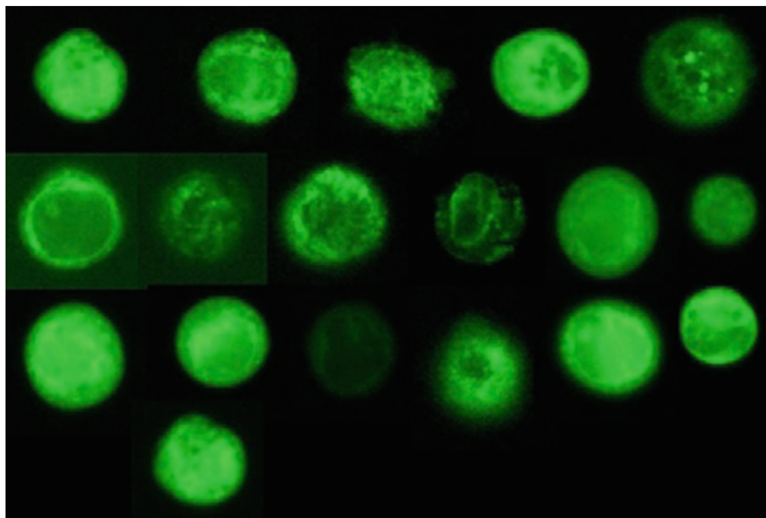

Fig. 8.1 Range of fluorescence exhibited by Panc-1 cells (a pancreatic cancer cell line) stained with Aldefluor. Composite image showing cells from images captured with identical exposure times. Original magnification 600×

ALDEFLUOR (Stem Cell Technologies Inc.) (Fig. 8.1). The Aldefluor substrate, BODIPY® aminoacetaldehyde (BAAA), is taken up by live cells by passive diffusion, whereupon it is converted in the cytoplasm into a fluorescent molecule (the negatively charged BODIPY®; BAA) under the action of ALDH (Storms et al. 1999). This fluorescent product accumulates in cells, partly due to the presence of ABC transporter inhibitors in the assay buffer, which prevent active efflux, allowing cells with high ALDH activity to be identified by their bright green fluorescence (see Figs. 8.2 and 8.3) and subsequently isolated by flow cytometry. ALDH activity can be inhibited by an addition of diethylaminobenzaldehyde (DEAB), allowing differentiation of cells with high ALDH activity from those with low or no activity. Pertinently, targeted deletion of *Aldh1a1* (a member of the retinaldehyde dehydrogenase family) has been shown not to affect the stem cell status, nor the degree of Aldefluor fluorescence of murine haematopoietic or neural stem cells (Levi et al. 2009), indicating not only functional overlap between ALDH family members but also that evaluation of pan-ALDH activity via Aldefluor fluorescence may correlate more accurately with ALDH-mediated regulation of SC phenotype.

8.2.1 ALDH Activity in Haematopoietic Cells

Among normal adult cell populations, ALDH activity has by far the greatest utility in sorting for stem/progenitor cells within the various haematopoietic systems. In conjunction with other markers, high levels of ALDH activity have been shown

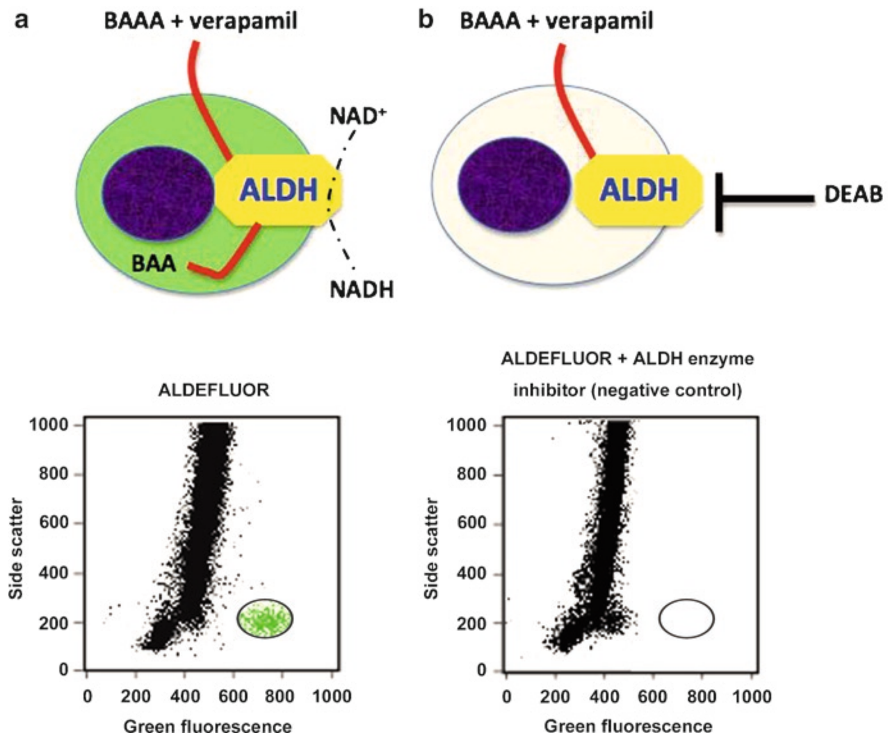

Fig. 8.2 The basis of the ALDEFLUOR reaction (see Storms et al. 1999 for further details). Cells are incubated with BODIPY® aminoacetaldehyde (BAAA) in the presence of verapamil to inhibit multidrug resistance. (**a**) Cells with high ALDH activity convert BAAA in the cytoplasm into a fluorescent molecule (the negatively charged BODIPY® aminoacetate; BAA); these cells appear as a distinct cohort of cells exhibiting green fluorescence and low side scatter (SSC) – *lower panel*. (**b**) In the presence of diethylaminobenzaldehyde (DEAB), ALDH activity is abolished and no highly green fluorescent subpopulation can be detected. Both plots are theoretical FACS histograms

to characterize highly clonogenic, undifferentiated multipotential stem/progenitor cells of mouse bone marrow (Armstrong et al. 2004; Hess et al. 2006), human bone marrow (Mirabelli et al. 2008) and human cord blood (Pearce et al. 2005; Christ et al. 2007; Hess et al. 2008). Although high ALDH activity has been advocated for the selection of progenitors among the mouse Lin^- bone marrow population (Armstrong et al. 2004), other studies (Pearce and Bonnet 2007) have suggested that ALDH activity via the Aldefluor kit is not suitable for the detection of murine $Lin^-Sca\text{-}1^+$ HSCs, and rather that the SP represents a huge enrichment in murine HSCs. Within human Lin^- bone marrow mononuclear cells, $Lin^-CD34^+CD38^{low/-}$ can be found throughout the ALDH activity spectrum, but are especially enriched within the combined SP/$ALDH^{bri}$ subpopulation (Pierre-Louis et al. 2009). Within human umbilical cord blood (hUCB), elevated ALDH activity markedly enriches for HSCs with repopulating ability in immunodeficient mice, and in fact there is a declining gradient of ALDH activity as haematopoietic cells differentiate

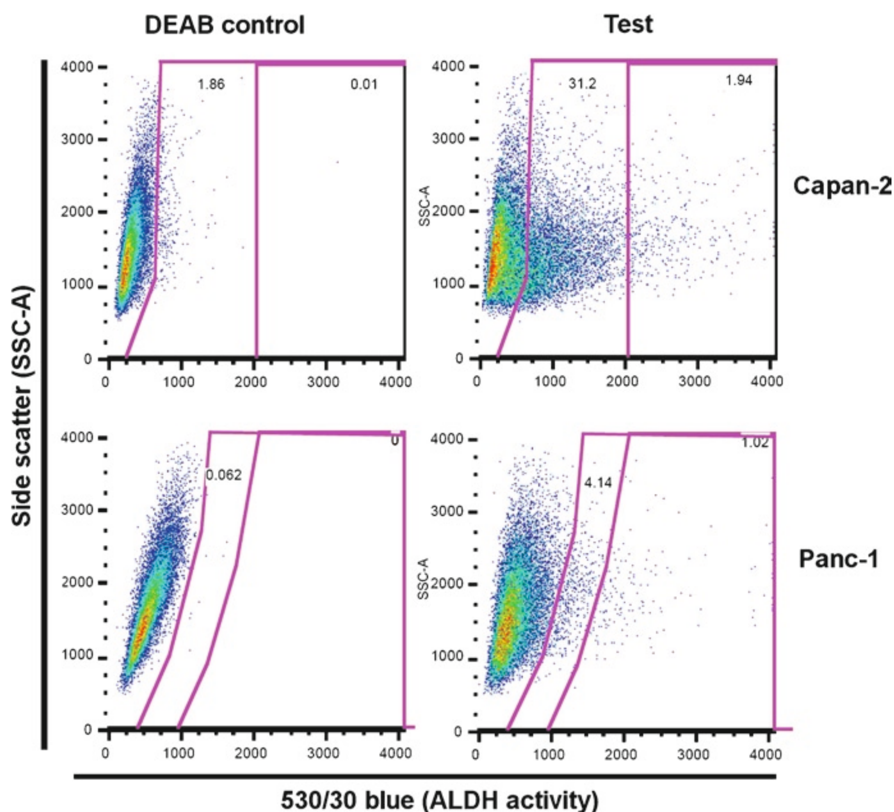

Fig. 8.3 Analysis of two pancreatic cell lines for ALDH activity using the ALDEFLUOR kit. ALDH^{-} (*area to the far left of each plot*), ALDH^{+} (*middle pink gates*) and ALDH^{++} cells (*far right pink gates*) were detected in Capan-2 and Panc-1 cell lines using ALDEFLUOR. Percentage of live single cell population contained in each gate shown. Data captured with BD LSRII with subsequent FlowJo analysis, ~100,000 events shown per plot. Note a wide side scatter in the ALDH^{+} subpopulation, a feature typical of malignant cells

(Christ et al. 2007). Other studies have suggested that the most primitive HSCs in hUCB reside in the ALDH^{bri}CD34^{+} cell fraction (Storms et al. 2005), while the ALDH^{bri}CD34^{-} cell fraction does not reliably repopulate NOD/SCID mice; in the bone marrow, this latter subset appears to be committed towards erythroid differentiation (Mirabelli et al. 2008). Lin^{-}ALDH^{bri} cells in hUCB also comprise the CD34^{+}CD38^{-} and the CD34^{+}CD133^{+} subpopulations (Hess et al. 2004), thus the ALDH^{bri}/CD133^{+} subpopulation will also produce robust haematopoietic reconstitution of immunodeficient mice (Hess et al. 2008). The numbers of ALDH^{bri} cells also correlate with CD133^{+}/CD34^{+} cell numbers in fresh human peripheral blood, even after cryopreservation, so the marker clearly has a therapeutic utility with respect to endothelial progenitor cells (EPCs) (Povsic et al. 2009), although only 65% of CD133^{+}/CD34^{+} cells actually expressed high levels of ALDH (Povsic et al. 2007). Somewhat surprisingly, in a flap ischemia model in immunocompromised mice, it was in fact the ALDH^{lo} rather than the ALDH^{bri} subpopulation from sorted

hUCB EPCs that were best able to minimize the necrosis within the most hypoxic area of the flap (Nagano et al. 2007). This ability appeared to be related to the hypoxic up-regulation of the likes of VEGF, CXCR4 and GLUT-1 by HIFs in the ALDH^{lo} rather than the ALDH^{bri} EPCs.

A growing body of evidence suggests that various haematopoietic cells selected on the basis of high ALDH activity can be used in a variety of cell therapy applications. For example, human bone marrow mononuclear cells, sorted on the basis of SSC^{lo}ALDH^{bri}, make up 1.2% of all nucleated cells, and these cells are massively enriched in haematopoietic colony-forming ability as well as in endothelial progenitors and CFU-F (Gentry et al. 2007). Results from pre-clinical trials conducted by Aldagen Inc. also suggest that the ALDH^{bri} cell population in human cord blood might provide chemical signals that promote angiogenesis (see http://www.aldagen.com). Likewise, the ALDH^{bri} subpopulation from human bone marrow was found to be most effective at promoting revascularization in an ischemic limb model in immunodeficient mice, though there was no integration of these cells into the neovessels (Capoccia et al. 2009). Likewise, improved vascularization has been seen in an immunodeficient mouse model of myocardial infarction when ALDH^{bri} cells from hUCB were injected intravenously 1 day after infarction, again with little or no integration of these cells into the neovessels (Sondergaard et al. 2010).

8.2.2 ALDH Activity in Non-Haematopoietic Organs

High ALDH activity has also been used to detect putative stem/progenitor cells in a variety of solid organs. In the murine pancreas, centroacinar/terminal duct cells have been proposed as a pancreatic precursor population that can be identified by high activity of ALDH: these cells did not show lineage-specific markers, but were enriched for progenitor markers such as Sox9, Sca-1, c-Met and nestin (Rovira et al. 2009). Furthermore, these cells were shown to be capable of multilineage differentiation in vitro, forming pancreatospheres at clonal density containing both exocrine (amylase-positive) and endocrine (C-peptide-positive) lineages, and their numbers expanded dramatically in the regenerating tubular complexes observed during caerulein-induced pancreatitis. In the human colon, immunohistochemistry has been used to locate ALDH1^{+} cells; one study found up to 15% of colonic epithelial cells were immunoreactive and although some were at the crypt base, worryingly (for a supposed stem cell marker) some were more than ten cell positions higher (Carpentino et al. 2009). On the other hand, Huang et al. (2009) observed a much tighter distribution of ALDH1^{+} cells at the crypt base, comprising 6% of all epithelial cells, and these appeared to be a sub-set of the more widely distributed CD44^{+} and CD133^{+} cell populations.

High ALDH activity was also found to be a feature of neural stem cells (Corti et al. 2006). An ALDH^{br} side scatter low (SSC^{lo}) can be isolated from murine-cultured neurospheres capable of further highly efficient neurosphere formation and trilineage potential; these cells were generally negative for differentiation antigens, but the majority expressed nestin, Sox2 and Musashi (Corti et al. 2006).

In the prostate, Burger et al. (2009) have isolated an ALDH[br] population from mouse prostate that were predominantly basally located, comprising 8% of the total population, and these were enriched for cells that efficiently generated complete prostatic tissue in an in vivo reconstitution assay, when transplanted along with urogenital sinus mesenchyme under the kidney capsule. Most cells expressed Sca-1 to a varying degree, but one third expressed very high levels of this murine stem cell marker. In normal human mammary epithelia, the ALDH[bri] population averages 8% and exclusively contains the clonogenic cells that can generate both CD10-positive (myoepithelial) and EpCAM-positive (luminal) cells, as well as double negative cells in mammosphere culture (Ginestier et al. 2007); additionally only the ALDH[bri] and not the ALDH^{lo} cells were capable of *in vivo* outgrowth in the cleared fat pads of NOD/SCID mice. These ALDH1-positive cells appeared to be located at the bifurcation points of terminal duct lobular units (TDLUs) and did not express CK18 (a luminal marker) or smooth muscle actin (SMA, a myoepithelial marker).

8.3 The Side Population (SP)

ALDHs are not the only cytoprotective molecules present in adult stem cells: a novel method for the isolation of HSCs based on the ability of HSCs to efflux the fluorescent dye Hoechst 33342 (Ho33342) was initially developed by Margaret Goodell and co-authors in the mid 1990s (Goodell et al. 1996). The "side population" (SP) is usually a small population of cells separable by flow cytometry due to their ability to exclude the stoichiometric DNA-intercalating supra-vital dye Hoechst (Ho) 33342 (Goodell et al. 1996). Ho33342, its isomer Ho33258, and the similar drug diaminophenylindole (DAPI) are useful in studies of cell cycle profiling, evaluation of DNA content, and chromatin structure (Park et al. 1985) though Ho33342 has usually been used for SP studies. A key feature of the SP cell phenotype is the presence of membrane ABC transporters (Alison 2003; Challen and Little 2006). These are ATP-binding cassette (ABC) proteins, which efflux xenobiotics across cell membranes against a concentration gradient, and pump into the extracellular space: an activity that can be inhibited by verapamil and reserpine (Higgins 2007). One function attributed to this phenomenon is the ability to efflux signalling molecules that may induce differentiation of stem cells (Bunting 2002), and thus assist in maintaining the stem cell state in haematopoietic cells. As cells that actively efflux Ho33342 form a distinct population on the side of the scatter profile on FACS analysis, they were designated the SP (Fig. 8.4). The SP phenotype of human and murine HSCs and other stem cell populations has been shown to be a direct result of high expression and activity of proteins of the ABC transporter family, primarily ABCG2 (ABC G2, also known as BCRP1 [breast cancer resistance protein]) (Zhou et al. 2001, 2002; Kim et al. 2002; Scharenberg et al. 2002). There are 49 human ABC transporters, organized into seven families, A–G (see http://www.nutrigene.4t.com/humanabc.htm), and the expression of ABC transporters such as ABCB1 (also known as the multidrug resistance protein 1 [MDR1],

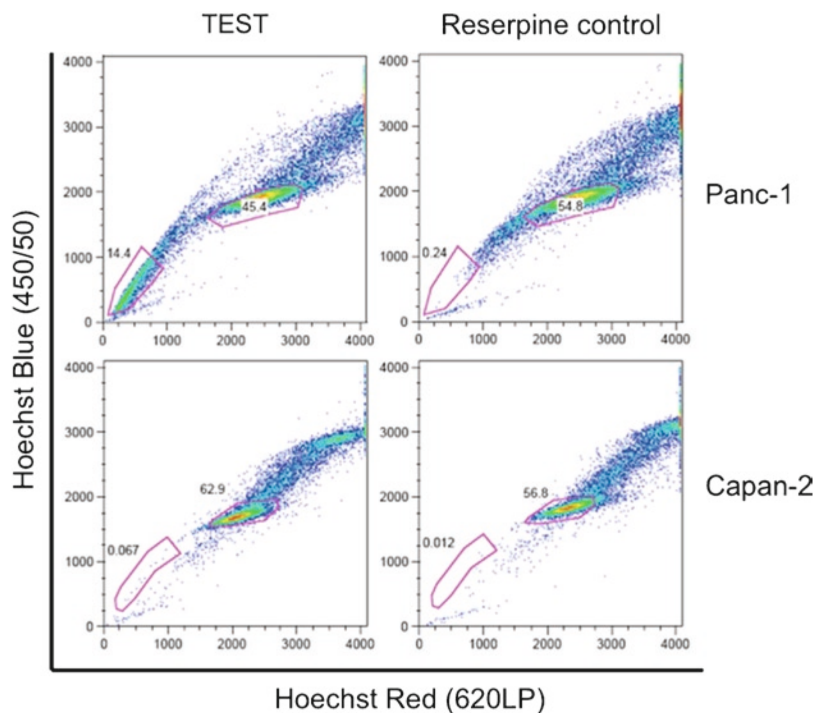

Fig. 8.4 Side population analysis of two pancreatic cancer cell lines, Panc-1 and Capan-2. A small SP (*lower pink* gates) was seen in both lines (*left*), which was inhibited by addition of reserpine (*right*). *Upper pink* gates delineate the G1 population of each line, to enable correct placement of the SP gate on control plots. Numbers indicate the percentage of the live singlet population contained by each gate. Representative data; captured using MoFlo FACS machine and CellQuest software, with subsequent analysis using FlowJo analysis

or P-glycoprotein), ABCC1 (also known as the multidrug resistance associated protein 1 [MRP1]), and ABCG2 is further associated with MDR in cancer cells (Litman et al. 2000), largely because high ABC transporter activity facilitates effective efflux of chemotherapeutic compounds out of cells, thereby lowering exposure and increasing resistance (Hirschmann-Jax et al. 2004, 2005).

The SP of many solid organs, including the breast, liver and kidney, often represents a population enriched for clonogenic activity and other stem cell traits (Shimano et al. 2003; Clarke 2005; Smalley and Clarke 2005; Challen et al. 2006), and consequently isolation of cells with the SP phenotype is now commonly employed as a method for prospective identification of stem cells (Alison 2003; Challen and Little 2006). High expression of ABC transporters is not an exclusive property of stem cells however: cells of the kidney cortical tubules and placental syncytiotrophoblasts express high levels, as do hepatocytes that transport bile acids into the biliary canaliculi, where a similar cytoprotective function is envisaged (Fetsch et al. 2006).

8.3.1 SP Cells in Haematopoietic Cells

Although the SP of murine haematopoietic cells has long been considered an adequate discriminator of HSCs with long-term repopulating ability, there is emerging evidence that different regions of the SP have different functional potentials (Weksberg et al. 2008). It was noted that there was heterogeneous expression of the signalling lymphocytic activation molecule (SLAM) family molecule CD150 within the SP^{KLS}, with more $CD150^{+}$ cells in the lower SP. Though all were long-term repopulating HSCs (LT-HSC), the $CD150^{-}$ SP^{KLS} cells were more skewed towards lymphoid cell production, whereas the $CD150^{+}$ SP^{KLS} were more biased towards myeloid cell production. Such observations have led to the speculation that rather than the existence of a single multipotential HSC (the "unitarian" hypothesis), there maybe clonal diversity within the HSC pool with distinct subtypes biased towards the myeloid or lymphoid lineages – these could be delineated as the lower-SP^{KLS} (myeloid) and upper SP^{KLS} (lymphoid) (Challen et al. 2010). As might be expected, heterogeneity within the SP can be attributed to both the rate of dye uptake between cells as well as the rate of efflux (Ibrahim et al. 2007). Although the SP is often based upon high ABCG2 activity, this does not appear to be the case for hUCB SP cells that make up about 0.4% of the mononuclear fraction (Alt et al. 2009). Here the SP cells do not express ABCG2 and the $ABCG2^{+}$ cells did not have any progenitor cell activity as adjudged by the ability to form "cobblestone" areas in culture.

8.3.2 SP Cells in Skeletomuscular Tissues

In murine skeletal muscle, SP cells are separate from the committed myogenic progenitors known as satellite cells, cells that express CD34/Pax7/M-cadherin (Asakura et al. 2002). Alone, these SP cells show no myogenic specification in vitro, but do so in the presence of myocytes, and furthermore when they are injected into the regenerating tibialis anterior muscle of scid/bg mice after cardiotoxin treatment, they give rise to both myocytes and satellite cells – two lines of evidence that SP cells require inductive signals from myocytes. The relationship in skeletal muscle of SP cells to the recently described group of Pax7-negative, Peg3/Pw1-positive interstitial cells, also capable of contributing to the myogenic and satellite lineages in vivo is presently unclear (Mitchell et al. 2010). An interstitial location of SP cells has been reported for mouse hindlimb skeletal muscle, making up 1–5% of all cells, and most of these expressed Abcg2 (Tanaka et al. 2009). Interestingly, 5–10% of these cells expressed Syndecan-3 and Syndecan-4 and were enriched for CD34, Pax7 and Abcg2 expression. This so-called "satellite SP" subpopulation gave rise to most satellite cells and could terminally differentiate into myocytes after transplantation into the mdx mouse, a model of dystrophin-deficient muscular dystrophy. The role of SP cells in promoting muscle regeneration may also be indirect (Motohashi et al. 2008); co-transplantation of $CD31^{-}$/$CD45^{-}$ SP cells with satellite cell-derived myoblasts into the damaged muscles of NOD/SCID

or mdx mice promoted the migration and proliferation of myoblasts, seemingly linked to the MMP-2 and gelatinase activity of the SP cells. Perhaps unsurprisingly, SP numbers in skeletal muscle are very dependent upon muscle type and species and can vary between 0.25 and 9%. Moreover stem cell markers, such as CD34 and Sca-1, and satellite markers, such as Pax7 and M-cadherin, can be expressed by both the SP and main population (MP) cells (Kallestad and McLoon 2010). As noted by these authors, higher doses of Ho33342 dye (up to 12 μg/ml) result in cell death, but the "trade-off" for the higher doses is supposedly a greater degree of cellular homogeneity within the SP.

SP cells are also found in the rodent heart. In the rat heart, they make up 1.2% of cells, most are proliferatively quiescent, expressing Abcg2 and are found in a perivascular location (Oyama et al. 2007). After intravenous injection, these cells were able to migrate to the cryoinjured heart where they contributed in a small way to cardiomyocytes (4%) and endothelia (7%), but making a larger contribution to smooth muscle cells (29%). In the mouse following cryoinjury to the heart, resident CD45-negative SP cells increase in number associated with increased Abcg2 expression (Martin et al. 2008); this enhanced expression was connected with up-regulation of HIF-2α due to oxidative stresses, and Abcg2 was thought to initiate a cytoprotective programme through induction of antioxidant stress pathways. Abcg2 has also been described to promote the survival and proliferation of mouse heart SP cells while at the same time inhibiting their lineage commitment (Pfister et al. 2008); Mdr1 was shown to be the main molecular determinant of this SP.

An SP has also been found in other so-called soft tissues. Within the superficial zone of bovine articular cartilage, an SP comprising 0.1% of the population was found, and this was absent from the deeper zones (Hattori et al. 2007); ex vivo these cells could be expanded and differentiated towards superficial and midzonal chondrocytes under the influence of BMP7. Bovine synovial tissue also has an SP (comprising 2% of the cell population), and these cells were very enriched for some traditional stem cell markers such as CD34, c-Kit, Abcg2 and Flk-1 (Teramura et al. 2008). These cells could also be expanded ex vivo, yet still show trilineage potential to form chondrocytes, osteocytes and myocytes. An SP of 2.5% has also been found among the stromal vascular fraction of mouse fat, and like MSCs they also displayed trilineage potential (Ramos et al. 2009). Some 75% of these cells co-expressed Sca-1 and CD90, and when injected intradermally beneath a fresh excised skin wound they appeared to reduce dermal scarring.

8.3.3 SP Cells in Epithelia

A number of studies have reported the existence of a small SP in the urogenital tract, but not necessarily correlated this population with the functional attributes of stem cells, namely clonogenicity and multilineage differentiation. For example, an SP in mouse kidney of 0.2% has been reported, with the niche apparently located adjacent to proximal tubules comprising both SP cells and macrophages (Challen

and Little 2006). In porcine and human kidneys, an SP of 2.1 and 1.3% respectively has been noted (Inowa et al. 2008). A slightly larger (3.8%) SP has also been reported for human kidney (Addla et al. 2008), and these cells were markedly enriched for clonogenicity in adherent culture. In the human prostate gland, an SP of 0.5–3% of the total epithelium has been found, presumably related to an ABCG2-positive subpopulation comprising 7% of the basal epithelium (Pascal et al. 2007). This SP was enriched for *ABCG2* mRNA expression, along with other markers such as *BMI-1*, *TERT* and *Nestin*. It would be interesting to see if other proposed markers for prostatic stem cells, e.g. the α2β1 integrins (CD49b/CD29) or CD133, overlapped with the SP. The human endometrium undergoes hundreds of rounds of regeneration, and since this involves formation of a new stratum functionalis from gland stumps in the basalis, it would be logical to assume that stem cells are located in this latter compartment (reviewed in Gargett et al. 2008). Bipotential SP cells have been found in this region that slowly proliferated in culture, able to form gland-like structures composed of both CD9-positive (epithelial) and CD13-positive (stromal) cells (Kato et al. 2007). Endometrial SP cells are more common in the early proliferative phase of the menstrual cycle compared to the late secretory phase (Masuda et al. 2010), and these cells can also form organized endometrial tissue when transplanted under the kidney capsule, MP cells could not do this. Intriguingly, a few $ABCG2^+/CD31^+$ cells were found among the endothelial cells of small vessels in the stratum functionalis (tissue that is shed), leading to speculation that these cells could be the founder cells of endometriosis (ectopic endometrial tissue).

SP cells have also been found in classic endocrine tissues; an SP of 1.4% has been noted in mouse thyroid, seemingly based upon Abcg2 expression, located in interfollicular spaces and enriched for stem cell markers such as nucleostemin and Oct4 (Hoshi et al. 2007). Mouse anterior pituitary gland also contains an SP, and this could be separated into a Sca-1-positive and negative fraction; surprisingly it was only the latter cells that were enriched for stem cell markers (Sox2, Sox9, Lgr5, CD133), able to form spheres (pituispheres) and demonstrate multipotency in terms of endocrine-producing cells (Chen et al. 2009). This SP appeared to be based upon Abcg2 expression, with lower expression in the Sca-1-negative cells corresponding to lower efficacy of Hoechst 33342 dye efflux.

SP cells have also been proposed as stem cell markers in the digestive system. Without doubt, recent data from the laboratory of Hans Clevers has provided the most robust evidence for the identity of both small and large intestinal stem cells in the mouse. In both tissues, rapidly cycling cells at the crypt base express the Wnt target gene *Lgr5*, and lineage tracing has demonstrated that these cells can produce long-lived cell clones containing all the appropriate cell lineages (Barker et al. 2007). Furthermore, single Lgr5-positive cells can produce organoids ex vivo that recapitulate normal crypt:villus organization (Sato et al. 2009). On the other hand, similarly clonogenic and multipotential stem cells that express Bmi-1 have been identified, also in the mouse, positioned at cell positions 4–5 above the small intestinal crypt base (Sangiorgi and Capecchi 2008), but these were slowly cycling cells, possibly acting as a reserve stem cell population. These so-called "+4" cells

may correspond to the CD45-negative SP cells in the mouse that are ablated by doxorubicin and increase in number after this chemotherapeutic treatment (Dekaney et al. 2009) and after ileocaecal resection (Dekaney et al. 2007). Mouse small intestinal SP cells are also enriched for Notch 1 transcripts, although these cells appeared to be located not only at the base of the crypt but also somewhat higher, suggesting that the SP lacks specificity for stem cells, indeed this SP also contained mature endocrine cells (Gulati et al. 2008). An SP has also been isolated from human colonic crypts (0.7% of all epithelial cells) that was enriched for many putative stem cell markers including Musashi1, CD133 and the β1 integrin (Samuel et al. 2009); interestingly these cells in vitro very efficiently adhered to primary pericryptal myofibroblasts (the niche cells for the crypt).

Epithelial cells with SP characteristics have also been identified in the adult human pancreas, where they occur with low frequency (Poliakova et al. 2004), and these have been suggested to represent a sub-fraction of a putative intra-islet precursor population (Lechner et al. 2002), although this remains to be verified. An SP of 3% among the non-parenchymal cell fraction of dog liver has been recorded, though 70% of these were CD45-positive, suggestive of a haematopoietic origin (Arends et al. 2009): both the $CD45^{-}$ and $CD45^{+}$ SPs showed up-regulation of hepatic progenitor/cholangiocyte genes encoding the likes of Abcg2, Fn14 (receptor for TWEAK, see Alison et al. 2009) and c-Kit. Dental pulp contains SP cells, and when these porcine cells were transplanted into a mouse with hind limb ischemia, both vasculogenesis and angiogenesis were improved but without the SP cells actually incorporating into the vessel walls (Iohara et al. 2008). Human dental pulp also contains SP cells, and when cultured under hypoxic conditions for 48 h, their frequency increases from 1 to 5% (Wang et al. 2010).

A small undifferentiated SP has been found in both human (0.18% of all epithelial cells) and mouse (0.45% of all epithelial cells) mammary tissue (Alvi et al. 2003); the mouse mammary SP appeared to be largely dependent upon Abcg2 activity, and when these cells were transplanted at limiting dilution to cleared mammary fat pads, they generated both ductal epithelia and lobuloalveolar structures (including myoepithelial and luminal cells). An SP with a $CD44^{lo}Cd24^{lo}$ phenotype has also been isolated from primary human mammospheres (Dey et al. 2009), but these cells failed to generate new mammospheres, perhaps because of dye toxicity.

A small SP (0.3% of all keratinocytes) has also been isolated from human epidermis (a stratified squamous epithelium) that expressed the basal marker K14 (Terunuma et al. 2007); however, the stem cell credentials of this population are in doubt since the $\alpha6^{bri}CD71^{dim}$ subpopulation (a well-recognized clonogenic population in the skin) was vastly superior to the SP in both colony formation in vitro and in long-term repopulating assays in vivo. In the pseudostratified and simple epithelial layers of the human trachea and bronchi, an SP of 0.12% has been uncovered (Hackett et al. 2008), but this population increased to over 4% in the airways of asthmatics. Furthermore, only the SP seeded at low density could form a multilayered differentiated epithelium in air-liquid interface cultures, with expression of basal markers such as ΔNp63 and CK5, and luminal cells expressing CK18 (ciliated cells) and MUC5AC (goblet cells).

Many regions of the eye appear to have SP cells. In the retina of the rat, very rare SP cells are present (0.002% of cells), and by either over-expression of the Notch intracellular domain (NICD) or γ-secretase inhibition it was clear that the SP phenotype was dependent upon Notch signalling to maintain Abcg2 expression (Bhattacharya et al. 2007). Rabbit and human conjunctiva also possess a largely non-cycling SP (<2%) that is enriched for embryonic and self-renewal gene expression, although the location of the SP was not clear (Akinci et al. 2009). Also in the rabbit, the associated limbal region of the cornea is reported to have an SP of 0.4%, enriched for CD61, Abcg2 and nucleostemin; additionally these cells showed high expression of nectin-3, an immunoglobulin-like cell-cell adhesion molecule (Kusanagi et al. 2009). A non-cycling SP has also been found in the mouse lens, located in the germinative zone around the equator of the lens (Oka et al. 2010); these cells were enriched for the expression of Abcg2, Sca-1, nestin, the β1 integrin and p75NTR.

8.4 Conclusions

This chapter has reviewed two cytoprotective attributes of stem cells, namely high ALDH activity and high ABC transporter activity, the latter giving rise to the SP phenotype. These two strategies are commonly operative in many so-called cancer stem cells contributing to the chemoresistance and MDR of these cells. In normal tissues, there is no doubt that both mechanisms can be operative, though the use of these as “markers” of adult stem cells has mainly been reported within haematopoietic cells; this bias may be a technical issue since both markers are usually assessed by FACS analysis that necessitates a monodispersed cell population. Many solid organs have cells highly expressive of these two activities, these cells are often enriched for so-called “stemness” genes, tantalizing evidence for a stem cell state, but many organs await a more robust proof of stemness, namely the demonstration of the clonogenicity and multipotentiality of these cells. Although this chapter has exclusively referred to the SP as a population able to efflux Hoechst 33342, the reader should be aware that the SP can also be detected using a similar DNA binding dye called Dyecycle violet (Telford 2010); this dye has a longer excitation maximum than Hoechst 33342, permitting detection by a violet laser source in the flow cytometer, a much cheaper option than flow cytometers equipped with ultraviolet lasers for optimum Hoechst 33342 fluorescence.

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Part III
Regulation of Life Span and Immortalization

Chapter 9
Regulation of Life Span in Adult Stem Cells

Philip Davy and Rich Allsopp

Abstract Unlike pluripotent stem cells in the early embryo, many types of somatic stem cells in the adult, such as hematopoietic, neural, and hair follicle stem cells, appear to have a limited life span. There are a number of different intrinsic and extrinsic factors that can affect the regulation of somatic stem cell life span, including the accumulation of DNA damage, oxidative stress from reactive oxygen species, the attrition of telomeres, aberrant changes in gene expression driven by age-related epigenetic shifts, and loss of function of the stem cell niche with age. There are also a number of potential events that define the end-point to stem cell life span, namely cell senescence, apoptosis, terminal differentiation, and tumorigenic transformation. The principal factors affecting the rate of aging in a somatic stem cell as well as the mechanism of life span termination are dependent on the type of stem cell. The development of new methods to extend or attenuate somatic stem cell life span holds substantial promise as effective means to treat the age-related or disease-specific decline in numbers of certain stem cells, or to eliminate cancer stem cells as an anticancer therapy, respectively.

Keywords Adult stem cells • DNA damage • Epigenetics • Replicative life span • Somatic stem cells • Telomeres • Telomerase

Abbreviations

Arf	Alternative reading frame tumor suppressor
ATM	Ataxia telangiectasia-mutated
ES	Embryonic stem
G-CSF	Granulocyte colony-stimulating factor

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HDF	Human diploid fibroblast
HSC	Hematopoietic stem cell
iPS	Induced pluripotent stem
NAC	*N*-acetyl-L-cysteine
NSC	Neural stem cell
PI3K	Phosphoinositide-3 kinase
rDNA	Extrachromosomal ribosomal DNA
ROS	Reactive oxygen species
Tert	Telomerase reverse transcriptase
TR	Telomerase

9.1 Introduction

Throughout life, somatic stem cells function to provide continuous replenishment of damaged or dead cells. This is a particularly important role for stem cells in highly proliferative tissues, where depletion of the stem cell pool could lead to a rapid decline in tissue function. Some diseases have been associated with a decline in specific stem cell pools, and in some tissues, stem cell numbers appear to decline with age. Therefore, the identification and characterization of factors which affect or regulate stem cell life span is important.

9.1.1 Aging and Life Span of Somatic Cells

Until the seminal work by Hayflick and Moorhead (1961) in the late 1950s and early 1960s, all somatic cells were thought to have an indefinite replicative life span. Careful analysis of the replicative capacity of normal human diploid fibroblast (HDF) strains established from dozens of different donors using stress-free and consistent culture conditions clearly demonstrated that these cells have a limited capacity to divide (Hayflick and Moorhead 1961), now referred to as the Hayflick limit. Furthermore, Hayflick and Moorhead (1961) showed that while the life span for different HDF strains varied among donors, aliquots of cryopreserved cells from the same donor always had the same replicative capacity in vitro when subsequently thawed and passaged continuously. In the decades to follow, many independent studies from laboratories around the world showed that the cellular life span for numerous different types of somatic cell strains established from different mammalian species, including humans, is limited.

The terminal stage of the life span for somatic cells in vitro is referred to as cellular senescence, which for cultured HDFs is an irreversible state of growth arrest accompanied by considerable phenotypic changes including a marked increase in cytoplasmic volume, accumulation of lipofuscin, and gross changes in global gene expression (Goldstein 1990; Cristofalo and Pignolo 1996; West et al. 1996).

A number of different theories have been proposed to account for aging and senescence at the organismal and cellular level. These theories include both stochastic theories which predict that cell aging and senescence are caused by the random accumulation of damage, and programmed theories which predict that cell life span is limited by an internal biological clock that is intrinsic to the very nature of the cell. Several notable stochastic theories have been proposed, including: (i) the free radical theory of aging, first described by Denham Harman (1956), proposing aging is caused by the accumulation of cellular damage caused by reactive oxygen species (ROS); (ii) the mitochondrial theory of aging, also proposed initially by Denham Harman (1972), which more specifically states that aging is caused by the accumulation of ROS-mediated damage to the mitochondrial genome; (iii) the error catastrophe theory of aging, described by Leslie Orgel (1963), proposing that the accumulation of damaged proteins causes cell aging; and (iv) the genetic mutation/damage theories, which propose that the gradual accumulation of DNA mutations and/or damage causes aging (Szilard 1959; Alexander 1967; Ames et al. 1993; Best 2009). Programmed theories include the gradual accumulation of extrachromosomal ribosomal DNA (rDNA) circles, observed in some strains of yeast, which could limit life span by leaching out factors required for normal cell function and otherwise interfering the essential cellular processes (Sinclair and Guarente 1997), and the gradual attrition of telomeres, genetic elements which cap and protect the ends of chromosomes as a function of cell division, as first described by Olovnikov (1971), where the continued erosion of the chromosomal end would inevitably have eventual deleterious consequences. It has now been shown that some of these theories are likely not relevant to aging of mammalian cells, at least certain types of cells. In particular, the error catastrophe theory of aging has largely been disproven as a cause of senescence in human fibroblasts where no evidence for the accumulation of damaged proteins with age has been found (Stanulis-Praeger 1987; Goldstein 1990) and the extrachromosomal rDNA circle accumulation theory of aging lacks any support in species other than yeast (Kaeberlein et al. 2004).

9.1.2 Aging and Life Span of Stem Cells

The first indication that stem cells, particularly adult somatic stem cells, also have a limited life span came from the work by Becker et al. (1963) and colleagues who reported a decline in the colony-forming ability of murine bone marrow (BM) cells during serial transplantation. Subsequently, studies by Harrison and colleagues at the Jackson Laboratory assessed the life span of murine BM stem cells in serial transplant experiments and showed that, while the replicative life span of these cells is considerable, these cells can only successfully reconstitute irradiated mice 4–6 times depending on the strain (Harrison and Astle 1982). More recent serial transplantation studies using phenotypically defined hematopoietic stem cells (HSC) have confirmed these earlier results (Allsopp et al. 2001). Though not as well

characterized as blood stem cells, other types of stem cells also appear to have a limited life span. Melanocyte stem cells and epidermal stem cells that reside in the bulge region of the hair follicle show loss of numbers or loss of proliferation, respectively, as a function of age (Nishimura et al. 2005; Flores et al. 2005), which likely contributes to the graying of hair in adult humans. Studies in mice have also provided evidence indicating that intestinal stem cells in the crypts of Lieberkuhn exhibit loss of regenerative capacity during aging (Martin et al. 1998). Age-related loss of both numbers and proliferative capacity has also been reported for murine neural stem cells (NSC) in the subventricular zone during aging (Molofsky et al. 2006) and granule progenitor cells from the dentate gyrus region of the brains of old rats also have been shown to have reduced replicative potential (Kuhn et al. 1996). Muscle stem cells (satellite cells) also show a decline in regenerative capacity in older mice (Conboy et al. 2003).

The mechanisms that have been proposed to account for the limited life span of somatic cells are also valid for adult somatic stem cells. However, the unique properties and function of stem cells allow for additional possible effectors of stem cell life span, most notably, (i) aging of the stem cell niche, which will be discussed in more detail later, and (ii) asymmetric chromosomal segregation during asymmetric division of certain types of stem cells. The immortal strand hypothesis, as first described by Cairns (1975), proposes that through asymmetric segregation of chromosomes during asymmetric stem cell division, stem cells selectively inherit the older DNA strands with the newly synthesized, error-prone DNA strands going to the committed daughter cells. This thereby provides stem cells with a mechanism to slow the accumulation of DNA damage. However, at present, most of the studies that provide support for this hypothesis have been performed in mammalian species in which the stem cell populations are still poorly defined, and a number of studies have also failed to detect evidence for asymmetric chromosomal segregation in some stem cells (Rando 2007; Morrison and Spradling 2008). Thus, further discussion of the effect of aging on this particular potential effector of stem cell life span must await future studies to provide more definitive evidence for selective chromosomal segregation during stem cell division. In this light, it is worth noting that Falconer et al. (2010) have recently reported the development of a reliable method to identify sister chromatids and its use to track their segregation during cell division. This initial study also provides exciting new evidence of nonrandom chromosome segregation in select types of adult somatic cells, potentially including colon stem cells (Falconer et al. 2010).

9.1.3 Telomeres and Cell Senescence

The loss of telomeric DNA has now been shown to ultimately limit the replicative life span of numerous types of somatic cells (Harley 2002), thus this particular effector of stem cell life span warrants additional discussion. Olovnikov (1971) initially proposed that loss of DNA from chromosomal ends would ultimately cause

cell senescence upon the eventual loss of essential genetic sequences. Twenty years after this initial hypothesis was proposed, a number of independent studies demonstrated that the terminal telomeric DNA tract gradually diminishes in size in actively dividing human cells, both in vitro and in vivo (Harley et al. 1990; Hastie et al. 1990; Lindsey et al. 1991; Allsopp et al. 1992). The exceptions to this were germ line cells, in particular male germ cells, and cancer cells, which both express the enzymatic complex telomerase (Counter et al. 1992; Allsopp et al. 1992). Telomerase is a specialized reverse transcriptase that functions to complete telomere replication, which the normal DNA replication enzymes in the cell are unable to perform (Fig. 9.1) (Chan and Blackburn 2004). Therefore, both germ line cells and cancer cells are able to maintain telomere length indefinitely, whereas the lack of telomerase in normal human somatic cells precludes telomere length maintenance

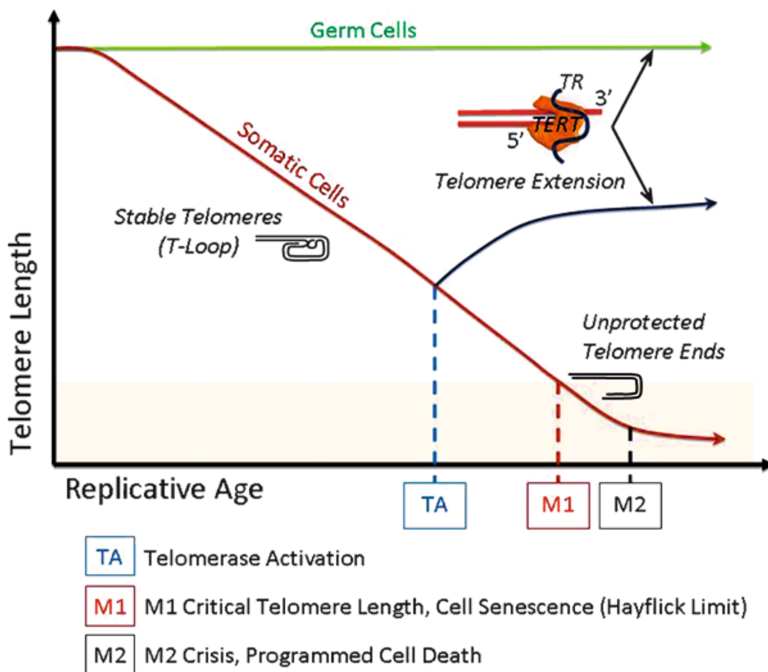

Fig. 9.1 Telomere maintenance and cell senescence. During replicative aging of most somatic cells, telomeres gradually shorten due to a lack of sufficient telomerase to complete the replication of telomeric DNA (*red line*). Once one or more telomeres become too short to form a protective cap (T-loop), the chromosomal terminus becomes unprotected and signals are sent to initiate cell senescence. Telomerase is readily abundant in the germ line where it functions to maintain long telomeres throughout life, and therefore, these cells do not encounter telomere-induced cell senescence (*green line*). Overexpression of Tert in somatic cells prior to senescence has been shown to be sufficient to restore telomerase activity in somatic cells, allowing the lengthening or maintenance of telomeres and the capacity to bypass the M1 checkpoint, or Hayflick limit (*blue line*). Most cancer cells that have acquired the ability to bypass both the M1 and M2 checkpoints have also reactivated telomerase to allow unrestricted growth. See text for further details

during replicative aging (Fig. 9.1). The telomerase complex is composed of several components, including an RNA component that provides a template for the extension of the G-rich telomeric strand (Greider and Blackburn 1989; Feng et al. 1995) and a catalytic component called telomerase reverse transcriptase (Tert) (Nakamura et al. 1997), both of which are essential for telomerase activity. The successful identification and cloning of the telomerase RNA component and Tert in the mid-1990s finally allowed direct testing of Olovnikovs' hypothesis. Numerous studies have now shown that ectopic expression of Tert in different types of cultured human somatic cells is sufficient to reactivate telomerase, allow maintenance of telomeric DNA, and postpone replicative senescence indefinitely (Harley 2002) (Fig. 9.1). Direct evidence for the importance of telomerase in the regulation of stem cell life span comes from studies that have assessed telomerase activity levels in embryonic stem (ES) cells and induced pluripotent stem (iPS) cells (Kim et al. 1994; Takahashi and Yamanaka 2006; Marion et al. 2009). In all of these studies, both ES cells and iPS have been shown to express high levels of telomerase. Moreover, genetic ablation of telomerase in murine ES cells has been shown to lead directly to gradual loss of telomeric DNA (Niida et al. 1998).

In this chapter we will describe both plausible intrinsic and extrinsic factors that affect the life span in adult somatic stem cells, the terminal events that limit stem cell life span, and potential therapeutic approaches to extend or shorten stem cell life span.

9.2 Intrinsic Mechanisms of Life Span Regulation in Stem Cells

Presently, intrinsic mechanisms of life span regulation in somatic stem cells are better established than extrinsic mechanisms and therefore will be described first. The intrinsic and extrinsic factors which are likely to play a prominent role in regulating stem cell life span, and which are discussed in greater detail here, are illustrated in Fig. 9.2.

9.2.1 Accumulation of DNA Damage

Here, we define DNA damage as any genetic alteration to nuclear DNA-epigenetic alterations and ROS-mediated damage to mitochondrial DNA will be discussed in the sections to follow. A variety of sources of DNA damage have been identified to date, including (i) spontaneous errors during DNA replication, which result in the incorporation of mismatches if left unrepaired; (ii) radiation, including ultraviolet and gamma radiation, which results in the creation of random thymine dimers in the genome as well as single- and double-strand DNA breaks; (iii) ROS

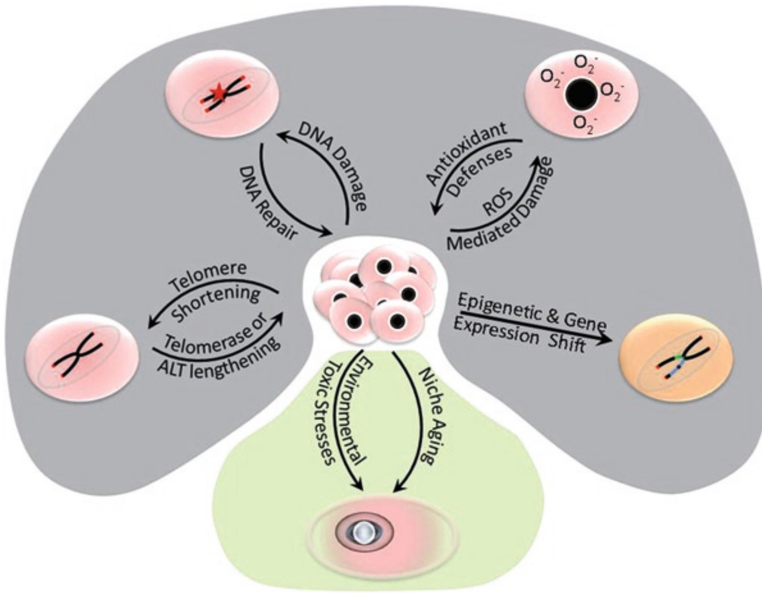

Fig. 9.2 Effectors of stem cell life span. Healthy young somatic stem cells (*center*) will encounter many repairable and irreparable changes throughout life, both intrinsic (*upper, shaded gray*) and extrinsic (*lower, shaded green*). Mechanisms to ameliorate some of these changes may also be active in somatic stem cells, including telomerase to slow the rate of telomere shortening, antioxidant defenses to slow accumulation of ROS-mediated damage, and cell cycle checkpoints plus DNA repair mechanisms to correct some of the damage incurred by the genome. See text for further details

(this particular source of damage is discussed in greater detail in the next section), which can cause single-strand DNA breaks as well as covalent modifications to DNA; and (iv) environmental hazards, such as alkylating agents, and other reactive compounds that can cause depurination and depyramidation of the nucleotides, as well as cytosine deamination. A number of repair mechanisms have evolved in eukaryotic cells to amend DNA damage, including base excision repair complexes, mismatch repair complexes, as well as repair complexes to repair nicks or breaks in the DNA. Like committed somatic cells, adult stem cells are also susceptible to these sources of DNA damage. The collective accumulation of damage to the genome from these sources of damage over time has led to the somatic mutation theory of aging where the gradual accumulation of mutations in the genome over time contributes to the rate of aging (Szilard 1959; Alexander 1967).

The consequences of DNA damage depend on the efficiency of repair and severity of damage (i.e., the number of damaging events at a given time). There are three possible scenarios by which a cell, including stem cells, could respond to DNA damage. The occurrence of even a single DNA damaging event is sufficient to trigger checkpoint control mechanisms, including DNA damage signaling pathways and DNA repair pathways. These pathways will lead to a pause in the progression of

the cell cycle (if the cell is actively dividing), allowing time for the DNA repair complexes to repair the damage. Once repaired, the cell is released from cell cycle arrest and is free to continue with business as usual. Hence, low levels of DNA damage may be tolerated by stem cells and are not predicted to effect stem cell life span. On the other hand, if the number of DNA damaging events is sufficiently high, the DNA repair machinery will not be able to efficiently repair all of the damage, leading to the prolonged activation of checkpoint control pathways or the failure to detect some of the damaged sites. In this scenario, prolonged stimulation of checkpoint control mechanisms is likely to lead to hyperactivation of the p53/Arf stress response pathway and lead to either apoptosis or senescence, and a reduction in stem cell life span. The other possible consequence of elevated levels of DNA damaging events is that one or more sites of damage fail to be detected by the DNA damage surveillance machinery. In this scenario, the site of damage could potentially lead to the incorporation of a permanent mutation in the genome, or if the damage is a single- or double-strand break, gross chromosomal rearrangements and elevated genomic instability. These events will predispose the stem cell to neoplasia and could potentially cause an extension of the cell life span, if the mutations eventually cause inactivation of the checkpoint control pathways, concomitant with dysregulation of stem cell function (also see Fig. 9.3).

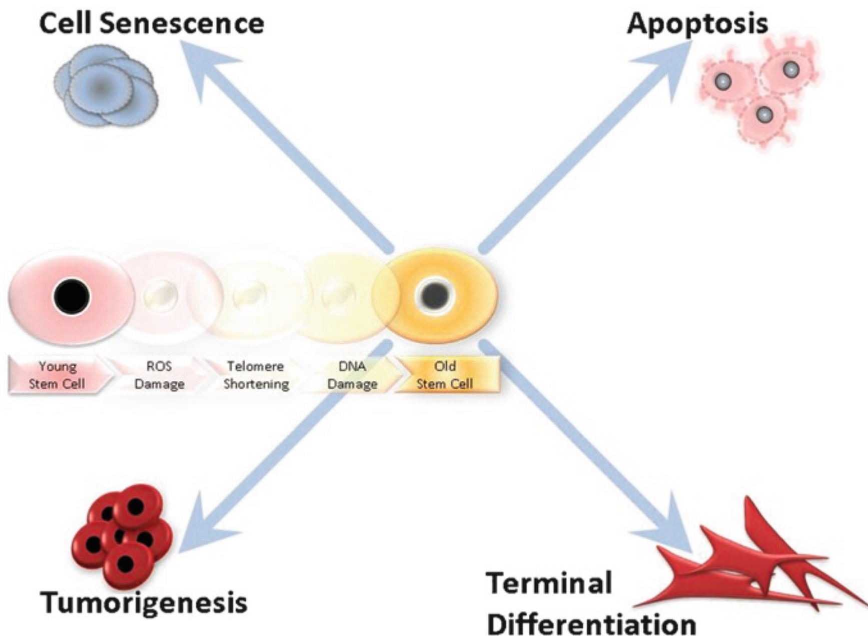

Fig. 9.3 Terminal end-points in the somatic stem cell life span. The progressive aging of somatic stem cells may lead to one of four possible end-points: (1) cell senescence; (2) apoptosis; (3) terminal differentiation; or (4) tumorigenic transformation. The types of damage or deleterious changes that accrue in stem cell during aging are also illustrated (in no particular order). See text for more details

A number of recent studies provide evidence that genes involved in the cellular response to DNA damage including Ku80 (Rossi et al. 2007), XPD (Rossi et al. 2007), ATR (Ruzankina et al. 2007), and BRCA2 (Navarro et al. 2006) have a significant role in regulating stem cell life span. Deficiencies in any of these genes have been shown to lead to premature exhaustion of HSC repopulating activity following transplant (Rossi et al. 2007; Navarro et al. 2006) or a decline in HSC number with increasing age (Ruzankina et al. 2007). On the other hand, deficiency in genes required for checkpoint control, such as the tumor suppressor p53 or the cyclin-dependent kinase inhibitor $p16^{In4a}$, has been associated with an extension of stem cell life span (Molofsky et al. 2006; Janzen et al. 2006; Dumble et al. 2007). One issue that remains to be addressed is the relevance of DNA damage to the life span of adult somatic stem cells under normal physiological conditions. This is a somewhat difficult question to address because of scarcity of adult stem cells and the low frequency of sites of DNA damage observed in many cells under normal conditions (~1–5 sites per million base pairs). However, most adult somatic stem cell compartments remain quiescent for extended periods of time, and therefore, may be more susceptible to the accumulation of DNA damage and the effect on life span since many forms of DNA damage are only efficiently detected and repaired in mitotically active cells.

9.2.2 Reactive Oxygen Species

One of the first theories of aging (Harman 1956, 1972) hypothesized that highly reactive-free radicals, generated primarily via oxygen metabolism in the mitochondria, contribute significantly to the aging process by damaging not just the mitochondria, but potentially any molecule in the cell. Moreover, numerous studies, primarily performed in Drosophila, have now provided a considerable amount of data suggesting that the increased longevity imparted by long-term caloric restriction is directly associated with the reduced accumulation of oxidative lesions (Sohal and Weindruch 1996), and that caloric restriction protects cells from oxidative damage (Kim et al. 2002). Notably, experiments that make lab animals either more susceptible or resistant to oxidative stress have been shown to be associated with a reduction or extension of life span, respectively (Orr and Sohal 1994; Migliaccio et al. 1999). ROS are primarily generated in the mitochondria during respiration; however, the extent to which oxidative damage to the mitochondrial genome affects cellular or organismal life span is still somewhat controversial (Kujoth et al. 2005; Vermulst et al. 2007).

Recently, the results from a number of studies suggest that ROS levels have a strong effect on stem cell life span, especially HSCs. The first such study by Ito et al. (2004) showed that conditional deficiency in HSCs of the ataxia telangiectasia-mutated (ATM) gene, a member of the phosphoinositide-3 kinase (PI3K) family that functions as a master regulator of cell cycle checkpoint pathways, causes a dramatic reduction, relative to HSCs from wild-type littermates, in proliferative

capacity and the ability of HSCs to reconstitute lethally irradiate mice following transplantation. Importantly, this effect of ATM-deficiency on HSC function and proliferative life span was associated with elevated levels of ROS in the cells and was largely reversible upon treatment of the mice with the antioxidant *N*-acetyl-L-cysteine (NAC) (Ito et al. 2004). The compromised function of HSCs in the ATM-deficient mice was also shown to be associated with elevated levels of p16^{Ink4a}, indicative of growth arrest (Ito et al. 2004). Subsequent independent studies have also shown that disruption of genes encoding Foxo transcription factors, which are important downstream targets of PI3K-Akt cell cycle checkpoint pathways, cause elevated apoptosis and reduced numbers of HSCs in mice, as well as increased intracellular levels of ROS in these cells (Tothova et al. 2007; Miyamoto et al. 2007). Furthermore, the reduced numbers of HSCs and low viability of these Foxo-deficient stem cells could be restored upon treatment of the mice with NAC, similar to the findings observed in the ATM-deficient mice (Tothova et al. 2007). One Foxo transcription factor in particular, Foxo3a, seems to play a significant role in protecting HSCs from ROS-mediated damage (Miyamoto et al. 2007). Another recent study has shown that mice maintained on a caloric restriction regimen, known to induce a systemic reduction in ROS-mediated damage, show signs of improved HSC function with age, although this effect was dependent upon the mouse strain (i.e., genotype-specific) (Ertl et al. 2008).

None of these latter studies directly addresses the question as to whether ROS-mediated damage could affect the life span of stem cells under normal physiological conditions. However, evidence for a potential effect of ROS on stem cell life span under normal physiological conditions has recently emerged. In one study by Rossi and colleagues, they reported a marked increase in HSCs from aged mice and in the number of genomic DNA foci positive for the phosphorylated form of the histone H2A variant H2AX (γ-H2AX), an established marker of ROS-mediated DNA damage (Rossi et al. 2007). Another recent study has shown that HSCs likely reside in hypoxic niches (Parmar et al. 2007), implying that stem cells, at least HSCs, may be particularly sensitive to ROS-mediated damage. Specifically, murine HSCs were observed to stain positively for the hypoxic marker pimonidazole and showed increased sensitivity, relative to other BM cells, to the hypoxia cytotoxic agent tirapazamine (Parmar et al. 2007). Thus, stem cells may require long-term protection from the rate of accumulation of ROS-mediated damage to ensure their proliferative potential is maintained throughout life.

Together, these studies point towards ROS having a potentially significant effect on stem cell life span, although it remains to be established whether ROS have similar effects in stem cells other than HSCs, and also whether human stem cells show signs of accumulated ROS-mediated damage during aging.

9.2.3 Epigenetic Alterations

Age-related changes in the epigenetic status of cell can have a profound effect on global gene expression. A diverse number of studies have reported significant

changes in gene expression as a function of age including changes in expression of genes involved in stress responses and DNA repair (Lu et al. 2004; Lee et al. 2000). Longitudinal studies of genetically identical monozygotic twins have also demonstrated substantial age-related differences in gene expression, which must be largely, if not entirely, attributed to epigenetic changes in the genome with age (Fraga et al. 2005). However, until recently, no studies have been performed to directly assess changes in gene expression in aging stem cells and the potential effect of epigenetic alterations on stem cell life span.

The first study to directly assess changes in gene expression associated with aging in somatic stem cells was performed in murine HSCs by Rossi et al. (2005). In this study, no substantial age-related changes in expression of genes involved in stress response pathways or DNA repair were observed. However, changes in gene expression consistent with a shift to increased myeloid potential and decreased lymphoid potential of HSCs from aged animals were reported. Furthermore, an increase in expression of genes involved in leukemic transformation was noted in HSCs from aged animals. These observations were proposed to potentially contribute to the increased incidence of myeloid leukemia observed in the elderly (Rossi et al. 2005). A similar study that was reported shortly thereafter by Chambers et al. (2007) found a slightly different pattern of age-related changes in gene expression in HSCs. Notably, unlike the study by Rossi et al. (2005), decreased expression of genes involved in DNA repair and stress response pathways was observed in HSCs from older mice. The authors attribute the differences in results between these two studies to differences in the methods used to purify the HSCs and perform the array analysis. Chambers et al. (2007) also reported significant age-related changes in the expression of genes involved in regulating the epigenetic state of the genome, such as down-regulation of the SWI/SNF-related chromatin remodeling genes in HSCs from aged animals. Together, these recent studies provide exciting initial evidence for age-related epigenetic alterations in gene expression in stem cells; however, many more studies need to be performed to rigorously assess the effect of epigenetic alterations in gene expression on stem cell life span.

9.2.4 Telomeres and Telomerase

As described above, the absence of sufficient levels of telomerase in eukaryotic cells prevents, with few exceptions, the maintenance of telomeres upon cell proliferation. The ultimate consequence of continuous telomere erosion is either cell senescence or cell death. Thus, unless telomere shortening is prevented via activation of telomerase or by other relatively rare telomerase-independent mechanisms, lack of telomerase in somatic cells will ultimately limit cell life span. This phenomenon, referred to as telomere-induced senescence, has been observed in numerous types of somatic cells spanning a number of different eukaryotic species from yeast to humans. The very ability of telomerase activation, typically via up-regulation of Tert which is limiting for telomerase activity in most human somatic cells, to

immortalize many types of somatic cells in vitro (Harley 2002) suggests that this enzyme may also play a key role in regulating somatic stem cell life span as well.

The early observations of gradual telomere shortening during aging of human somatic cells in vitro and in vivo led to initial speculation that telomere shortening may limit the life span of all normal somatic cells including somatic stem cells, particularly in highly proliferative tissues such as blood and skin. One of the first direct indications that telomere erosion occurs in somatic stem cells comes from a study by Vaziri et al. (1994) who assessed telomere length during in vitro and in vivo aging of human hematopoietic progenitors. In both long-term cultures of CD34+CD38−Lin− hematopoietic progenitors and in the analysis of this BM population directly from both young and old donors, telomere length was observed to decrease as a function of replicative age (Vaziri et al. 1994). More convincing evidence for the role of telomerase in regulating somatic stem cell life span came from a study of the telomerase (TR)-deficient mouse strain developed by the Greider and de Pinho labs. In this study, late generation TR knock-out (−/−) mice, in which many cells harbor one or more dysfunctional telomeres, were shown to have a number of systemic defects, particularly in highly proliferative tissues such as the male germ line, intestinal epithelial cells, skin cells, and hematopoietic cells (Lee et al. 1998), which all require the intermittent proliferation of tissue-specific stem cells to restore cells lost to death or excessive damage throughout life.

However, these studies had not yet directly assessed the role of telomerase in regulation of stem cell life span. Perhaps not surprisingly, the first studies to accomplish this were performed in murine embryonic stem cells (mES) in vitro (Niida et al. 1998) and HSCs in vivo (Samper et al. 2002; Allsopp et al. 2003), two of the most well-characterized types of stem cells to date. In mES cell lines, Niida et al. (1998) assessed the effect of genetic ablation of the TR gene on telomere length maintenance during long-term passaging of these cells. Unlike wild-type mES cells, the TR−/− mES cells were only capable of a finite number of population doublings (~400), and analysis of telomere length at regular population doubling intervals during culture showed a clear gradual loss of telomere length. Moreover, at late passages, the TR−/− mES cells were shown to have significant signs of telomere dysfunction including the absence of detectable telomeric signal on the end of at least one chromosome in most cells, as well as aneuploidy (Niida et al. 1998). Subsequent studies to assess the role of telomerase in HSCs were performed in telomerase-deficient mice, both TR−/− (Samper et al. 2002; Allsopp et al. 2003) and Tert−/− (Allsopp et al. 2003) strains. In these studies, long-term in vitro and in vivo proliferative assays were performed to assess the effect of telomerase deficiency on telomere length and HSC life span. All of the data indicated a markedly reduced capacity of the telomerase-deficient HSCs to proliferate, for example, both TR−/− and Tert−/− HSC were capable of just two rounds of serial transplantation as compared to four rounds for HSCs from wild-type littermates (Allsopp et al. 2003). Furthermore, continuous proliferation of telomerase-deficient HSCs was accompanied by both telomere shortening and eventual increased incidence of telomere dysfunction (Samper et al. 2002; Allsopp et al. 2003). Defects in telomerase in humans have also been indicated as likely having an effect on HSC life span.

In particular, mutations in the TR component and/or Tert which compromise telomerase activity have been associated with BM failure and markedly reduced telomere lengths in hematopoietic cells from patients with dyskeratosis congenita (Mitchell et al. 1999; Du et al. 2009) as well as reduced telomere lengths in hematopoietic cells from patients with aplastic anemia (Calado et al. 2009). Together, these studies show that maintenance of telomere reserve is crucial for the long-term maintenance of replicative life span for at least some types of stem cells.

9.3 Extrinsic Mechanisms of Life Span Regulation in Stem Cells

There are various forms of environmental hazards, including radiation, toxic compounds, and acute or chronic infections that are certain to affect the life span of both mature cells and somatic stem cells alike to some degree. Another extrinsic effect that is unique to stem cells and is beginning to emerge as a major influential factor on the rate of stem cell aging is aging-related changes in the stem cell niche.

Early indications that the aging of the stem cell niche could also affect stem cell aging and life span came from comparison of young and old BM stromal cells, an important cellular component of the HSC niche (Stephan et al. 1998). In this study, stromal cells from old mice showed a significant reduction in their ability to support B-lymphopoiesis ex vivo, which may be partially explained by reduced excretion of IL-7 from these cells with age. Subsequent studies involving reciprocal transplantation of HSCs between young and old mice showed that young HSCs transplanted into old recipients caused a skewing towards the myeloid lineage (Liang et al. 2005), as has been shown to be part of the normal aging process in mice (Morrison et al. 1996) and that transplantation of HSCs from old mice into young mice is able to partially restore the efficiency of old HSCs to produce new B cell subpopulations (Labrie et al. 2004). Furthermore, transplantation of HSC from young mice into old telomerase-deficient (TR−/−) mice revealed a marked loss of both homing efficiency and B-lymphopoiesis, whereas transplantation of HSCs from TR−/− mice, which show an accelerated skewing to myeloid development as compared to HSCs from wild-type mice, into young wild-type recipients caused restoration of the lymphoid potential of these cells (Ju et al. 2007). Stromal cell production of the cytokine granulocyte colony-stimulating factor (G-CSF) was also observed to be elevated in TR−/− mice as compared to wild-type mice, and administration of G-CSF to wild-type mice caused a similar HSC phenotype as observed in old TR−/− mice, including reduced efficiency and impaired B-lymphopoiesis (Ju et al. 2007). This latter study suggests that telomere shortening in vivo may have a significant effect on the HSC stem cell niche in the elderly.

Analysis of the effect of parabiosis between young and old mice, where the vasculature of young and old mice is surgically connected to allow exposure of cells in the old mice to serum from younger mice and vice versa, also indicates that aging of the niche may also effect the life span of other somatic stem cells, and in

particular, muscle satellite stem cells (Conboy et al. 2005). The reduced ability of aged muscle satellite cells to respond to injury has been attributed to an age-related loss of these cells, which is potentially attributed to a decline in Delta-Notch signaling (Shefer et al. 2006; Conboy et al. 2003). The parabiotic experiments by Conboy et al. (2005) showed that exposure of the satellite cells in the old mice to a younger systemic environment was able to regenerate the number and proliferative potential of these cells via a mechanism that involves restoration of levels of the Notch ligand Delta in the old satellite cells.

While studies to assess the age-related changes of the stem cell niche are just getting underway, initial results already suggest that these changes likely have a significant influence on stem cell life span.

9.4 Mechanisms of Termination of Stem Cell Life Span

As mentioned in the previous section, there are several possible end-points to stem cell life span. Specifically, and as discussed in greater detail in this section, a stem cell could feasibly succumb to cell senescence, apoptosis, tumorigenesis, or terminal differentiation.

To date, there is little evidence in support of cell senescence, wherein a stem cell enters a state of terminal growth arrest yet remains viable, playing a significant role in the termination of stem cell life span. Using β-galactosidase as a biomarker for senescent cells, an increase in the frequency of senescent cells has been detected in the dermis of humans during aging (Dimri et al. 1995). However, it is uncertain what fraction, if any, of these senescent skin cells are actual stem cells. Another well-established marker of senescent cells is $p16^{Ink4a}$ (Alcorta et al. 1996; Wong and Riabowol 1996; Itahana et al. 2003). This senescence biomarker has been shown to accumulate as a function of age in stem cells including NSCs (Molofsky et al. 2006) and HSCs (Janzen et al. 2006). Another senescence biomarker, γ-H2AX, has also been shown to increase in HSCs from old mice (Rossi et al. 2007). However, the majority of HSCs from old mice retain the ability to proliferate as assessed in culture experiments ex vivo and in transplantation experiments (Morrison et al. 1996). HSCs deficient in the proto-oncogene Bmi1, a member of the *Polycomb* group of transcriptional repressors that functions to suppress the *Ink4a* locus in cells including HSCs (Park et al. 2003), have also been shown to initiate cell division just as readily as wild-type HSCs (Iwama et al. 2004). It is important to note that while these latter studies suggest that stem cells do not undergo cell senescence, at least during aging in vivo, it is also possible that the signaling of cell senescence in stem cells may induce a change in cell surface phenotype that could prevent their detection, or senescent stem cells may specifically be cleared in vivo by immune surveillance machinery. Furthermore, studies in mice suggesting that the HSC pool may either increase or decline in number depending on the mouse strain further confound our understanding of the occurrence of cell senescence in somatic stem cells, at least in the case of murine HSCs.

A number of studies have provided both indirect and direct evidence that the terminal stage of stem cell life span may involve activation of programmed cell death, or apoptosis. First, a number of studies in mice have shown that some somatic stem cell populations may decline with age including NSCs (Maslov et al. 2004; Molofsky et al. 2006) and HSCs (Chen et al. 2000; Geiger et al. 2001), though, at least for HSCs, these observations appear to be strain-specific (Morrison et al. 1996; Chen et al. 2000). Second, HSCs from mice strains with premature aging phenotypes, harboring specific defects in either DNA repair genes or genes encoding components of telomerase, showed an age-dependent increase in apoptosis. Third, mice rendered susceptible to ROS-mediated damage via genetic ablation of FOXO transcription factors were shown to have an elevated incidence of apoptosis in HSCs (Tothova et al. 2007). An important question still remaining is whether apoptosis occurs at a higher frequency in stem cells from old organisms under normal physiological conditions.

The life span of a normal somatic stem cell could also terminate upon age-dependent transformation into a cancer stem cell. The best evidence for this possible stem cell fate also comes from studies of HSCs. Preliminary studies indicate that DNA damage accumulates in stem cells during aging, at least in murine HSCs (Rossi et al. 2007), and it is reasonable to speculate that some of these mutations could lead to tumor suppressor inactivation or activation of protooncogenes. One of the first comparative studies of the behavior of HSCs from young and old mice showed that HSCs from old mice are more numerous and also more mitotically active (Morrison et al. 1996), leading to the initial proposal that HSCs from old mice may be predisposed to develop leukemia. This hypothesis is in agreement with the general observation of increased incidence of leukemia, particularly myeloid leukemia, with age in mice and humans. In further support of this hypothesis, it has long been known that there is a bias towards myelopoiesis during human aging (Williams et al. 1983), and recent studies have also shown that murine HSCs from old animals have greatly diminished lymphoid potential and are much more biased towards a myeloid potential (Rossi et al. 2005; Cho et al. 2008).

To date, evidence of age-related loss of stem cells due to a skewing towards terminal differentiation as opposed to self-renewal has been very limited. Results from a recent study by Inomata et al. (2009), however, indicate that this potential mechanism of termination of stem cell life span may be important during aging of melanocyte stem cells. This study showed that inducing elevated levels of DNA damage in the coats of mice by exposure to genotoxic stress (ionizing radiation) caused these stem cells to differentiate, without any appreciable increase in the incidence of apoptosis or cell senescence. This implies that the decline in melanocyte stem cell numbers observed during aging of mice (Nishimura et al. 2005) may be primarily accounted for by an increased occurrence of terminal differentiation of these cells.

Thus, the defining end-point in a somatic stem cells' life span is likely to be dependent on the type of stem cell. Additional studies will be required to assess whether the mechanisms of life span termination observed in stem cells from lab animals are also relevant to human stem cells.

9.5 Therapeutic Approaches to the Regulation of Stem Cell Life Span

The development of therapies designed to alter stem cell life span may be beneficial for the treatment of age-related diseases and symptoms of aging. In particular, therapies designed to extend stem cell life span may be useful to treat diseases associated with a decline in specific stem cell populations, as well as to counter the decline in certain stem cell populations with age. Therapies designed to shorten stem cell life span may provide a novel approach to treat cancer by targeting the cancer stem cells.

9.5.1 Methods to Extend Stem Cell Life Span

In light of the various events that can affect the rate of aging and life span in stem cells as discussed here, there are a number of potential therapies that could be proposed. If activation of $p16^{Ink4a}$ affects the life span of human HSCs as observed in mice (Molofsky et al. 2006), it may be possible to extend life span of these stem cells by selectively inhibiting $p16^{Ink4a}$, for example, by developing therapies to transiently upregulate Bmi1. However, long-term inhibition of $p16^{Ink4a}$ or activation of Bmi1 predispose cells towards tumorigenesis, so this approach would require very careful design and a considerable amount of caution to ensure there is no significant risk for cancer development. An alternative and potentially more efficacious approach may be to slow the accumulation of damage in stem cells during aging. A recent study in mice by Matheu et al. (2007) has shown that enhancing the levels of p53 and Arf (alternative reading frame tumor suppressor) by increasing gene dosage while maintaining endogenous methods of regulation can extend organismal life span. Furthermore, this extension in life span is accompanied by a reduction in the rate of accumulation of damage in cells during aging (Matheu et al. 2007). Results from other studies have indicated that suppressing the levels of ROS-mediated damage in cells may also potentially promote life span extension in stem cells. For example, transgenic experiments designed to increase antioxidant defenses in mice, e.g., by elevating the levels of either catalase or thioredoxin, result in a significant extension of life span in these animals (Mitsui et al. 2002; Schriner et al. 2005). Thus, methods designed to moderately enhance the p53/Arf stress response pathway or boost defenses against ROS-mediated damage could potentially extend stem cell life span. A possible additional beneficial effect of these approaches is increased resistance to cancer by reducing the amount of damage to the genome during aging (Matheu et al. 2007).

None of the therapies described above are expected to prevent the attrition of telomeres during replicative aging of stem cells. Therefore, therapies designed to maintain telomere length via activation of telomerase are also desired for optimal extension of stem cell life span. Evidence that activation of telomerase may extend

stem cell life span comes from a recent study showing that up-regulation of Tert in cancer-resistant mice delays aging-related changes and extends the median life span (Tomás-Loba et al. 2008). A recent study by Fauce et al. (2008) reports the use of a small molecule activator of telomerase to slow telomere loss and enhance the proliferative potential of CD8+ T lymphocytes from HIV-infected individuals. Thus, pharmacologic approaches to activate telomerase in stem cells and in the stem cell niche may soon be available, and are likely to be critical for long-term life span extension in stem cells. While there is no significant evidence that suggests telomerase activation has prooncogenic effects in human cells, there have been reports of potentially protumorigenic effects of chronic telomerase activation in mice (González-Suárez et al. 2001; Artandi et al. 2002). Therefore, methods to transiently activate telomerase in stem cells will be most desirable.

9.5.2 Methods to Shorten Stem Cell Life Span

There are a number of viable methods to shorten the life span of stem cells, specifically cancer stem cells, including stimulating bursts of ROS-mediated damage in these cells, targeting the antioxidant defenses, and inhibiting telomerase activity. Regarding the latter approach, a potent and specific telomerase inhibitor, GRN163L identified by Geron corporation, has been used successfully to inhibit telomerase in cancer cells in vitro and in animal models and is now being tested in ongoing clinical trials (Harley 2008). It will be important for future studies to assess whether small molecule compounds such as GRN163L are effective at specifically limiting the life span of cancer stem cells. Therapies designed to reduce cancer stem cell life span are likely to complement the growing list of other new anticancer strategies.

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Chapter 10
The Cancer Stem Cell Paradigm

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Abstract Following the discovery that leukemic cells exhibit properties of hematopoietic stem cells, the prospective isolation of stem-like cells with high tumorigenicity has been reported for a variety of tumors. These "cancer stem cells" (CSCs) are so named because they exhibit the capacity for sustained self-renewal and possess the ability to regenerate transplanted tumor masses resembling the primary tumor in immunodeficient mice. However, the existence of CSCs remains contentious in the field of cancer biology, in part because of the application of inconsistent and inaccurate definitions and disputes over terminology. Herein, we review the discovery of CSCs, examine in detail their physical and functional characteristics, the mechanisms that lead to their formation, and how their contribution to solid tumor formation impacts cancer therapies.

Keywords Adult stem cells • Cancer stem cells • Epigenetics • Glioblastoma • Xenografts

Abbreviations

AML	Acute myeloid leukemia
APML	Acute promyelocytic leukemia
CML	Chronic myeloid leukemia
CSC	Cancer stem cell
ECM	Extracellular matrix
FACS	Fluorescence-activated cell sorting
HIF	Hypoxia-inducible factor
HSC	Hematopoietic stem cell

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LSC	Leukemic stem cell
RGC	Radial glial cells
SSEA-1	Stage-specific embryonic antigen 1
SVZ	Subventricular zone
TNFAIP3	Tumor necrosis factor inducible protein 3
VEGF	Vascular endothelial growth factor

10.1 Introduction

Cancerous lesions have been thought to originate in a clonal fashion, to develop from a single mutated cell with the ability to persist and generate phenotypically similar progeny due to the survival advantage afforded by a particular genetic mutation, with subsequent clonal evolution from within this population due to the accumulation of favorable mutations (Barr and Fialkow 1973; Nowell 1976). Due to the obvious expansion of tumor cells and the clear contribution of these cells to tumor progression, cancer researchers historically may have taken a reductionist view of tumors, focusing largely on neoplastic cellular growth and often assuming a defined distinction between malignant and healthy tissue (Hanahan and Weinberg 2000). Though much valuable research has emerged from the study of expanding tumor cells, the successful incorporation of antivascular (de Groot and Yung 2008; Saltz et al. 2008) or immunomodulatory (Mitsiades et al. 2009) drugs into anticancer therapeutic regimens suggests that this perspective warrants further examination. In fact, tumors contain a diverse range of stromal, immune, and vascular cells, while the neoplastic cell compartment also displays a wide assortment of phenotypic and even genotypic variability (Fulci et al. 2002). For example, it has long been appreciated that neoplastic cells within the same brain tumor can display a wide range of morphologies (Bailey and Cushing 1926). It may be appropriate to consider a tumor as an aberrant organ, containing a wide range of supportive cells that contribute to the tumor macroenvironment, while a substantial heterogeneity exists within the neoplastic parenchyma as well (Carmeliet and Jain 2000; Reya et al. 2001).

Two major theories have been proposed to explain the propagation of, and selective evolution for, neoplastic cells within tumors (Reya et al. 2001). The stochastic theory of tumor evolution posits that almost every cell in a tumor maintains tumorigenic potential, and it is the Darwinian selection for genotypically distinct clones of tumor cells generated through stochastic mutation events that determines the ultimate cellular phenotype of the original or secondary tumor lesions. The implication of this model is that the accumulation of mutations is nondeterministic, so it is impossible to predict which cells will possess the necessary mutations to become tumor promoting (or even tumor initiating).

The second paradigm is informed by the hierarchical organization central to normal stem cell function. This hierarchical theory of tumor organization suggests that cellular tumor initiation and propagation capacity are stratified, with the most

tumorigenic cell type at the top, giving rise to less tumorigenic progeny. In this model, the state of a cancer cell and its position in the hierarchy may be controlled by microenvironmental context, transcriptional activation of specific genes, or alterations of epigenetic regulatory mechanisms. This suggests that the tumorigenic population can be predicted and prospectively enriched (discussed later in this chapter).

Though often presented as competing and mutually exclusive theories, the actual organization and evolution of heterogeneity within tumors likely derive from elements of both. One could reasonably envision tumors as evolving according to Darwinian selection for cells exhibiting advantageous mutations, while it is well known that a wide range of nongenetically determined phenotypes can exist within tumors. Until recently, however, the diversity of nongenetically determined tumorigenicity had not been evaluated.

10.2 History of the Cancer Stem Cell Hypothesis

Over 150 years ago, Rudolf Virchow observed the cellular diversity present in neoplastic growths and theorized that a tumor could arise from mutated embryonic stem cells that lie dormant in adult tissue. This pioneer of modern pathology posited that, following an unknown signal, ESCs "reactivate" and form a growing lesion comprised of ESCs (Virchow 1963). Although we now know this not to be true for the majority of tumors, this theory is an early form of the cancer stem cell (CSC) paradigm. Similarly, tumors have long been recognized to contain differentiation patterns reminiscent of stem cell-derived hierarchies (Bailey and Cushing 1926).

Despite these early theories and observations regarding stem-like cells in cancer, the contribution of stem-like cells to tumor initiation and maintenance remained hypothetical until the prospective isolation of leukemic cells resembling hematopoietic stem cells (HSCs) by John Dick and colleagues (Lapidot et al. 1994; Bonnet and Dick 1997). Increased tumorigenicity and augmented stem cell-like characteristics were identified in acute myeloid leukemia (AML) cells that were positive for the HSC marker CD34 and negative for the lymphocyte differentiation marker CD38 (Bonnet and Dick 1997). Prospectively isolated CD34+/CD38− AML cells were several orders of magnitude more tumorigenic than cells not displaying this permutation of cell surface markers and appeared to demonstrate stem-like characteristics such as prolonged self-renewal and a capacity for producing progeny that displayed a range of differentiation markers.

Though a series of papers utilized competitive assays of leukemic stem cell (LSC) repopulation to further characterize the properties and cell surface markers of LSCs (Brecher et al. 1993; Sirard et al. 1996; Sutherland et al. 1996), the presence of highly tumorigenic CSC subpopulations within solid tumors remained speculative until 2003 when a series of groups reported the prospective isolation and characterization of such cells deriving from various brain tumors, including glioblastoma and medulloblastoma (Singh et al. 2003, 2004; Hemmati et al. 2003). Concurrently,

identification of stem-like subpopulations with increased tumorigenicity in transplantation assays was reported for malignant breast tumors (Al-Hajj et al. 2003). Since these initial studies, the prospective isolation of stem-like cells with high tumorigenicity has been reported for a variety of tumors including colon, head and neck, hepatocellular carcinoma, ovarian carcinoma, and melanoma (Fang et al. 2005, 2010; O'Brien et al. 2007; Ricci-Vitiani et al. 2007; Wang et al. 2007; Haraguchi et al. 2008; Tirino et al. 2008, 2009; Wright et al. 2008; Bertolini et al. 2009; Curley et al. 2009; Puglisi et al. 2009; Held et al. 2010; Hu et al. 2010; Tomuleasa et al. 2010; Zhu et al. 2010). While the cell surface molecules effective for prospective isolation of these populations often varied between tissues, the properties of the isolated stem-like tumorigenic subpopulations remained reasonably consistent. The isolated CSCs have the capacity for sustained self-renewal and possess the ability to regenerate transplanted tumor masses resembling the primary tumor in immunodeficient mice.

10.3 Stem-Like Behaviors and Signaling Provide Selective Advantages for Cancer Cells and Tumor Growth

Cancer cells have a tremendous propensity to access gene expression patterns and cell behaviors that confer growth or survival advantages for either individual cells or the tumor as a whole. As such, it is not surprising that a selection advantage exists for cancer cells able to access the proproliferative, promigratory, and proinvasive characteristics associated with stem cell gene expression patterns and cellular behaviors. For example, in differentiated epithelium, normal E-cadherin-mediated intercellular junctions can restrict the growth of cells, direct cellular polarity, and maintain an appropriately restricted differentiation state (Gottardi et al. 2001). Dysplastic cells able to release from these restrictive adherens junctions acquire more primitive, less differentiated morphologies. These cells are thought to have a selective advantage for cellular invasion and metastasis, and may possess stem cell-related Wnt pathway activation occurring due to cytoplasmic accumulation of beta-catenin released from its association with E-cadherin in adherens junctions (Ceteci et al. 2007). Indeed, metastasis is promoted by downregulation of E-cadherin through miR-9 regulation by MYC (Ma et al. 2010), a protein implicated in CSC biology (Wang et al. 2008).

In addition to such selective advantages afforded to individual cancer cells by hyperactivation of stem cell-related signaling pathways and networks, systems biology approaches to tumors suggest that cellular diversity and functional redundancy provided by stem cell-like hierarchies within tumors can substantially enhance the resilience of the tumor as a whole (Sottoriva et al. 2010). The DNA of every organism contains the requisite genetic material for initiating complex embryonic developmental programs allowing for multipotency and self-renewal. Over the course of development, a series of inhibitory epigenetic adjustments leads to the silencing or damping of these developmental programs in all but a very select number of normal stem cells in the body. Similarly, within many tumors, pathologists

have noted the presence of cellular hierarchies that resemble stem cell-derived differentiation cascades or morphologically resemble developmentally primitive stages in organ development (Sell and Pierce 1994). The neoplastic relaxation of epigenetic rules through either mutations or nongenetic changes permits cell populations in tumors to reaccess embryonic or developmental patterns, and it has been hypothesized that these stem cell-informed metapatterns can contribute to the persistence of the tumor overall (Widschwendter et al. 2007; Shukla et al. 2008; Teschendorff et al. 2010).

10.4 Cancer Stem Cell Properties

Despite their resemblance to normal stem cells, CSCs are cancerous and thus the "rules" of normal cellular physiology do not strictly apply. Therefore, although CSCs resemble normal stem cells in several ways, they are not defined by the exact criteria used to characterize normal stem cells. CSCs are defined by functional characteristics; in order to be a CSC, a cancer cell must be able to: (1) self-renew and (2) generate a phenocopy of the parental tumor in transplantation assays (Reya et al. 2001); Clarke et al. 2006.

One criterion required of both normal and CSCs is an ability to "self-renew," or generate at least one mitotic daughter cell capable of maintaining the stem cell phenotype exhibited by the parental cell. For normal stem cells, this means that at least one daughter cell must have the capacity for self-renewal as well as the capacity for multilineage differentiation. For CSCs, self-renewal implies that at least one daughter cell retains the capacity for self-renewal as well as the ability to generate tumors that resemble the parental tumor in transplantation assays.

Normal stem cells are defined by a capacity for multilineage differentiation – that is, they must be multipotent. In contrast, CSCs have no such absolute requirement for differentiation into discretely different cellular lineages. Many tumor cells aberrantly differentiate (i.e., they may simultaneously express differentiation markers for multiple lineages), while other tumors tend to display cells that exhibit one primary differentiation state, and still other tumors may display a panoply of distinctly different tumor cells with characteristics of not only the parental tissue but also differentiation markers resembling other tissues. Instead, CSCs are defined as cells with the ability to generate tumors resembling the parental tumor in transplantation assays. In the most pure sense, this would be manifested by the growth of a second tumor in a human patient that is distinct from the primary mass (e.g., growth of a second gliomatous lesion in the contralateral brain hemisphere of a patient with a frontal lobe glioma) or through regeneration of a tumor after surgical or chemoradiotherapy-based ablation. In an academic sense, the ability for CSCs to generate tumors resembling the primary mass is measured by the propensity of cells to initiate xenografted tumors in immunocompromised mouse models that resemble the heterogeneity and characteristics of the parental human tumor.

There are many other characteristics that may frequently cosegregate with the required properties of CSCs, while not being diagnostic of CSCs. Just as the

original identification of LSCs took advantage of HSC surface marker expression, CSCs from many types of tumors express cell surface markers typically seen on the surface of normal embryonic or adult stem cells. For example, much work has been invested in determining the cell surface marker expression patterns most effective for isolation of AML stem cells (Lapidot et al. 1994; Sutherland et al. 1996; Florian et al. 2006). The current "state of the art" expression pattern displayed by AML stem cells mimics cell surface markers patterns exhibited by normal HSCs; while human HSCs are contained in the lineage-CD34+CD38−CD90− population, LSCs from AML are segregated by CD34+CD38− cell surface marker expression profiles. Similarly (as discussed in further detail below), CSCs from solid tumors appear to be effectively segregated into tumorigenic and nontumorigenic populations based on cell surface expression of stem cell-related markers.

Interestingly, stem-like tumorigenic populations within tumors appear to employ transcriptionally regulated signaling networks typically active within embryonic, induced pluripotent, or adult stem cells, such as the signaling networks related to the stem transcription factors Oct4, Sox2, c-Myc, Bmi1, or Nanog. Similarly, CSCs often activate one or more critical stem-related signaling pathways including Notch, Wnt/Beta-catenin, or Hedgehog (Reya et al. 2001). Several groups have assessed the gene expression profiles exhibited by CSCs and normal adult or embryonic stem cells to show that overall signatures are often similar, though may segregate into several different global patterns (e.g., glioblastoma CSCs may resemble either neuronal or embryonic stem cells). The ability to access stem cell-related signaling pathways provides selective advantages for CSCs, including the ability for sustained proliferation and enhanced migratory capacity. However, though they may access stem cell signaling programs, CSCs are not defined by this characteristic.

10.5 Isolation and Identification of Cancer Stem Cells

To call a cell a CSC, it must have the functional capacity for self-renewal and the ability to generate tumors that recapitulate the primary mass in transplantation assays. Therefore, to designate a single cell a CSC, it is necessary to utilize it in functional assays, which presents a fundamental limitation to assessing the characteristics demonstrated by that particular cell in an unaltered form. For this reason, CSCs are studied in the context of cell populations known to be enriched for tumorigenic and self-renewal capacity; single cells are isolated from a primary tumor specimen by mechanical digestion and enzymatic digestion and then they are labeled and selected by a variety of methods to obtain populations of CSC-enriched cells. These cell populations can then be verified as "CSC-enriched" and the relative frequency of CSCs assessed by tumorigenesis and self-renewal assays as described below, and the remainder of these cells evaluated experimentally in vitro or in vivo.

Various cell surface markers or labeling techniques can be utilized to segregate CSC-enriched populations from CSC-depleted populations, depending on the type and grade of tumor, the tissue from which a tumor derives, the species of

the tumor-bearing animal, and the cytogenetic and gene expression profiles within that tumor. Cell surface markers can be labeled with fluorophore-conjugated antibodies and then can be sorted using fluorescence-activated cell sorting (FACS) to obtain populations demonstrating particular cell surface marker expression patterns that are known to enrich for CSCs or nonstem cancer cells. Alternatively, cells positive for a single cell surface marker can be isolated using magnetic bead-conjugated antibodies and magnetic selection columns. For example, CD133 (Prominin1) has been used to enrich for CSCs in brain (Hemmati et al. 2003; Singh et al. 2003, 2004), colon (O'Brien et al. 2007; Ricci-Vitiani et al. 2007; Haraguchi et al. 2008; Puglisi et al. 2009; Fang et al. 2010), ovarian (Curley et al. 2009), breast (Wright et al. 2008), lung (Bertolini et al. 2009; Tirino et al. 2009), bone (Tirino et al. 2008), and liver cancers (Zhu et al. 2010). Stage-specific embryonic antigen 1 (SSEA-1; also known as CD15) has also been used to enrich for CSCs in brain tumors (Read et al. 2009; Son et al. 2009; Ward et al. 2009). CD44 has also been found to be an important selection marker for colon (Du et al. 2008; Haraguchi et al. 2008; Chu et al. 2009), breast (Al-Hajj et al. 2003; Wright et al. 2008), and pancreatic (Li et al. 2007) CSCs. Other non-surface protein marker-based systems are also available to isolate CSCs. Partially due to overexpression of ATP-binding cassette type drug transporters (ABC transporters), CSCs from some solid tumors display characteristic dye efflux patterns that permit them to be identified based on the relative rate of vital dye (e.g., Hoescht) efflux based on FACS in combination with cell size and scatter characteristics (Zhou et al. 2001; Hirschmann-Jax et al. 2004; Komuro et al. 2007; Wang et al. 2007; Wang et al. 2009c; Yao et al. 2010). Still other tumors display characteristic enzymatic activities (e.g., aldehyde dehydrogenase activity) that can be utilized with fluorescent substrates to identify and select populations by FACS (Ginestier et al. 2007; Huang et al. 2009). While useful for enriching for CSCs, expression of a CSC marker alone is not sufficient to characterize cells as CSCs in the absence of functional assays. In addition, it is important to note that none of these markers is likely sufficient to obtain a homogeneous population of CSCs.

10.6 In Vitro and In Vivo CSC Maintenance

Optimized in vitro cell culture conditions are critical to the maintenance of CSCs ex vivo. Although standard cell culture conditions involve serum supplementation, recent results have suggested that culturing cells in serum-free, nonadherent conditions in the presence of various growth factors such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) can enrich for CSCs in certain tumor types in the absence of marker selection and is necessary for the maintenance of CSC-enriched populations. Tumor cells isolated and then cultured in stem cell permissive medium (serum-free, growth factor enriched medium) maintain tumorigenic capacity and gene expression profiles that resemble the original tumor. In contrast, culturing cells in serum-containing medium usually results in increased

tumor cell differentiation, a reduction in tumor formation and self-renewal capacity, and divergence from the gene expression profile displayed by the parental tumor (Lee et al. 2006).

Oxygen availability is one lab tissue culture parameter that is often overlooked in cell biology, cancer biology, and stem cell biology despite its role as a critical environmental factor in regulating cell behavior. Typical conditions for a cell incubator are 37°C, 5% carbon dioxide for maintaining a pH of 7.4 (when combined with sodium bicarbonate-containing cell culture media), and abundant atmospheric oxygen tensions of 21% (150 mmHg). Conversely, oxygen tension is tightly restricted within normal tissue to around 2–9% (approximately 40 mmHg) in the tissue space. Even in cancerous tissue, with any associated hyper-angiogenic vascular growth, the tumor bulk experiences approximately 7% oxygen. This can drop to hypoxic (≤2% O_2) or near anoxic (≤0.02% O_2) levels in regions of necrosis (Bertout et al. 2008). Since the early twentieth century, when Otto Warburg observed that cancer cells may preferentially generate energy through anaerobic glycolysis, the behavior of cancer cells in sub-atmospheric oxygen conditions has been of immense interest (Semenza et al. 2001). Further, Lacassagne and colleagues demonstrated that normal cells under hypoxia had radioresistant properties (Lacassagne 1954), and further evidence suggested that radiation activates hypoxia-inducible factors (HIFs) to regulated radioresistance (Moeller et al. 2004). These findings emphasize the importance of understanding the role of oxygen in cancer cell behaviors such as therapeutic response and indicate that the scientific community must not overlook such microenvironmental factors when studying CSCs.

In vivo, many types of normal adult stem cells reside in specialized tissue locations, or niches, that may provide microenvironmental cues to control the fate of stem cells and play a critical role in balancing self-renewal, proliferation, differentiation, and survival of stem cells. In these stem cell niches, multiple components concertedly affect stem cell behavior, including cell-to-cell interaction, diffusible factors, and extracellular matrix (ECM) composition (Watt and Hogan 2000; Voog and Jones 2010). The ECM in particular has importance in maintaining the localization of stem cells at the stem cell permissive niche, while it also can modify the mechanical tension and cytoskeletal structure of the stem cells. These changes can affect stem cell proliferation and fate decision (Mammoto and Ingber 2009). Indeed, careful selection of ECM used in the defined culture system allows selection of stem cells, and attachment to the ECM helps maintenance of stem cells (Sun et al. 2008; Hall et al. 2008; Campos et al. 2004). Though the self-renewal of CSCs might be less dependent on niche and more autonomous than normal stem cells due to intrinsic genetic alteration (Li and Neaves 2006), there are data to indicate that microenvironmental cues, and the interaction of CSCs with the ECM in particular, can contribute to maintenance of CSCs (Iwasaki and Suda 2009). For example, disruption of the ECM-receptor CD44 suppresses the ability of LSCs to engraft and propagate disease in the bone marrow (Jin et al. 2006; Krause et al. 2006). In brain tumors, glioma stem cell marker-positive cells are enriched in perivascular area (Calabrese et al. 2007), which is also known to be a laminin-rich niche important for the maintenance of normal neural stem cells (Shen et al. 2008). Our recent work

suggests that glioma CSCs expressing the laminin receptor integrin alpha 6 are preferentially localized in perivascular regions in human glioma specimens, and the expression of integrin alpha 6 and possibly its ability to interact with the ECM is critical to the survival and phenotype of glioma CSCs (Lathia et al. 2010). These data indicate that the ECM is a critical component of the CSC niche, and may be critical to the maintenance of the phenotype of these cells. As such, several groups have made efforts to adapt the use of ECM to in vitro CSC tissue culture protocols in order to maintain this critical niche-CSC interaction. Growth of cancer cells in 3-dimensional cultures in ECM-rich matrigel may permit the growth of heterogeneous hierarchical cultures from CSCs (Alvero et al. 2009; Fong et al. 2009; Pandit et al. 2009; Yeung et al. 2010). Further, inclusion of the ECM molecule laminin in the serum-free culture media of glioma CSCs appears to maintain the cell growth, self-renewal, and tumorigenic characteristics of glioma CSCs while permitting their expansion in an adherent culture (Pollard et al. 2009; Fael Al-Mayhani et al. 2009). Though the exact cocktail of ECM proteins that is optimal for CSC culture is still being evaluated, adaptation of this component of the in vivo niche to in vitro culture will likely have an important role in the development of more representative in vitro culture conditions for examining the biology of CSCs.

The expression profiles, behaviors, and cellular composition of human tumor cell populations can be altered by in vitro cell culture, and there are clear microenvironmental cues that are not present in in vitro cell culture conditions. Therefore, human tumor cells can be propagated in a less-artificial (though still far from perfect) manner by xenografting human cells into immunocompromised mice and amplifying tumor cells in an in vivo environment. In this way, tumor cells can be propagated to maintain substantial cellular diversity more accurately than in vitro culture, which subjects cells to the selective pressures of plastic tissue culture dishes and abnormal environmental stresses encountered in standard lab tissue culture environments, as described above. Orthotopic xenografting, or implantation of human tumor cells from a particular organ in the corresponding mouse organ, is often considered desirable for the maintenance of tumors in an organ-appropriate environment. However, the technical requirements of orthotopic xenograft establishment can be difficult or even prohibitive, depending on the anatomic location and tumor type. As such, nonorthotopic tumor cell implantation (e.g., subcutaneous or under the kidney capsule) can be utilized to capitalize on the maintenance of heterogeneity that in vivo tumor propagation affords if anatomically appropriate implantation is not possible (Morton and Houghton 2007; Fujii et al. 2008).

10.7 Debate Surrounding Cancer Stem Cells

Evidence from a wide range of both human and murine tumor specimens suggests that some tumor cells have an augmented capacity for self-renewal as well as an amplified ability to initiate tumors that phenocopy the parental tumor. However, the

existence of CSCs remains contentious in the field of cancer biology, in part because of the application of inconsistent and inaccurate definitions and disputes over terminology as discussed below.

10.7.1 Terminology

Though we utilize the term "CSC" to refer to the subset of highly tumorigenic, stem-like cancer cells within many tumors, this terminology often generates confusion or even controversy. As previously discussed, the term "CSC" refers to the functional characteristics of secondary tumor initiation capacity and prolonged self-renewal exhibited by a subpopulation of cells within the neoplastic compartment of many tumors. However, the use of the term "stem cell" raises issues among researchers from many scientific disciplines as well as the lay population, since it is clear that CSCs do not strictly adhere to the rules applied to normal stem cells. This has resulted in much confusion over the proper name for this population of cells. As such, CSCs have also been commonly referred to as tumor-initiating cells, tumor propagating cells, and stem-like cancer cells; similarly, each additional name bears inconsistencies, limitations, and insufficiencies (Bansal and Banerjee 2009; Komuro et al. 2007). Generation of widespread agreement on this subject has been limited by our inability to elucidate and replicate definitive characteristics among multiple different patient specimens, tumor classifications, and grade levels (Gupta et al. 2009). However, we must remember that these cells are cancer cells and, by definition, are transformed in ways that do not require them to follow the normal pathways or characteristics of the cells from which they came. Therefore, we do not expect a CSC to exhibit all of the expected characteristics or functional properties of normal stem cells. Instead, we can use their aberrant resemblance to stem cells to guide the selection of more appropriate research methods, evaluation of cellular behaviors, and identification of previously hidden therapeutic targets. Similarly, it is critical to the development of nontoxic CSC-directed therapies that biological and signaling differences between CSCs and normal stem cells guide the selection of therapeutic elements that will not generate undue toxicity within normal stem cell populations (Dingli and Michor 2006; Chumsri and Burger 2008).

10.7.2 Rarity

Original descriptions of the prospective isolation of CSC-enriched populations in human leukemias, brain tumors, and other tissues identified relatively small populations (i.e., 0.5–5% of the total tumor cell population) that possessed the defining characteristics of CSCs. However, further studies have described tumors that exhibit a much higher proportion of cells that satisfy the definitive requirements of

CSCs, and some genetically driven mouse tumors display very high percentages of cells possessing CSC characteristics (Quintana et al. 2008; Zhang et al. 2008; Tamase et al. 2009; Ward et al. 2009). Although the original descriptions of CSCs were notable for the prospective isolation of a “rare” population of CSCs within a tumor, the prevalence of CSCs is irrelevant to the designation of cells as CSCs.

10.7.3 Cellular Origin of Tumors

Though they are called CSCs, current evidence suggests that tissue stem cells, transit-amplifying cells, committed progenitors, or even postmitotic differentiated cells may serve as targets for oncogenic mutations and yield tumors that possess CSCs, depending on the model, organ, and oncogenic stimulus employed. Though it is clear that each of these cell types is *sufficient* to generate tumors that possess cells fulfilling the CSC-defining characteristics, the identity or even the existence of what cell type is *necessary* for generation of tumors with CSCs remains unclear. Retrospective identification of the cell-of-origin in already established tumors is challenging or even unattainable. However, though the animal cancer models currently available provide valuable insights into mechanisms of cancer initiation, they do not always permit definitive conclusions regarding what is necessary for the initiation of tumors possessing the hierarchical organization often seen in human tumors.

Proponents of a stem cell as the cell-of-origin for CSCs reason that in most tissues where malignancies commonly arise (such as hematopoietic system or the gut epithelium), stem cells are the only population that persists long enough to accumulate multiple oncogenic mutations needed for transformation, whereas restricted progenitors and differentiated cells appear to be relatively short-lived. Moreover, tissue stem cells already have an activated self-renewal program, which is critical to eventual CSC populations. The immunophenotype of LSCs in AML patients was characterized as CD34+/CD38−, suggesting that normal primitive cells (Lin−/CD34+/CD38−/CD90+), rather than committed progenitor cells, may be the target for leukemic transformation (Lapidot et al. 1994; Bonnet and Dick 1997). In line with these findings, Perez-Caro and colleagues have recently shown that restricted expression of the p120BCR-ABL oncogene in Sca1^{+} HSCs is sufficient to establish the phenotype and biology of chronic myeloid leukemia (CML) in a mouse model, though comparing these findings to those of other groups suggests that perhaps this oncogenic stimulus in Sca1^{+} HSCs leads to a preleukemic state and additional alterations are necessary for full induction of CML (Perez-Caro et al. 2009). Two recent studies on tumorigenesis in the intestines further support a stem cell origin for CSCs. The intestinal stem cells residing in the base of the crypt spawn highly proliferative transit amplifying cells that migrate upward as they differentiate and finally reach the top of the villus where fully differentiated cells are shed into the intestinal lumen. The mouse model of colorectal cancer developed in Hans Clevers’ lab employed selective tumor

suppressor inactivation of Wnt pathway self-renewal regulator *Apc* in slowly cycling *Lgr5*-expressing intestinal stem cells (Barker et al. 2009). Deletion of *Apc* in long-lived crypt stem cells rapidly leads to the formation of multiple microadenomas in the gut that progress to macroadenomas within weeks. In contrast, inactivation of *Apc* in short-lived transit-amplifying cells rarely results in full-blown cancer. These findings were further corroborated by the study led by Richard Gilbertson that took advantage of restricted expression of Prominin-1 gene in crypt stem cells of the small intestine (Zhu et al. 2009). Activation of Wnt signaling in Prominin-1$^+$ stem cells by the expression of mutant β-catenin resulted in disruption of crypt architecture characterized by focal high-grade intraepithelial neoplasia and formation of crypt adenomas. Though heterogeneous tumors can initiate from oncogenic stimulation of normal stem cells, these studies did not specifically look at the behavior of CSC fractions within these tumors.

Despite this evidence that adult stem cells can serve as the target for genetic events leading to malignant transformation and establishment of heterogeneous lesions, considerable evidence also supports the potential for committed progenitors or even mature cells to serve as cells-of-origin for CSCs. As the adult stem cells within a given tissue are usually scarce and have relatively slow cell cycling rates, the stem cell population may not be suitable for successful genetic evolution to complete cancer formation. On the other hand, it is easy to envision highly proliferative progenitor cells rapidly accumulating the multiple mutations necessary not only for reacquisition of self-renewal machinery but also for malignant transformation (Gao 2008; Gupta et al. 2009). Multiple mouse models of leukemia employ promoter elements of myeloid lineage-specific genes to target oncogenic transgene expression specifically to committed myeloid cells, which indeed support this notion (Passegué et al. 2003). For example, mice expressing the fusion gene PML/RARα from the human *MRP8* promoter develop preleukemic state that eventually progresses to acute promyelocytic leukemia (APML) (Brown et al. 1997); interestingly, just as in human promyelocytic leukemia, administration of all-trans retinoic acid to these cells can result in irreversible differentiation of the tumor-maintaining cancerous promyeloblasts. Similarly, transgenic mice expressing AML1-ETO fusion protein develop AML if additional alterations are induced by mutagenic treatment (Yuan et al. 2001), suggesting that more transforming events are necessary to fully convert progenitor cells into LSCs (Lobo et al. 2007). Similarities in gene expression profiles and cell surface immunophenotypes between ependymal tumors and mitotically active ependymal precursor radial glial cells (RGC) suggest a RGC origin to these tumors (Taylor et al. 2005). Studies of a mouse glioma model with a brain-targeted *TP53* deletion mutation also suggest that, although the mutant p53 protein is expressed at detectable levels in neural stem cells in the subventricular zone (SVZ), it is the Nestin+/GFAP−/Olig2+ transit-amplifying progenitor-like cells that expand in the corpus callosum peri-SVZ to initiate glioma formation (Wang et al. 2009a, b, c). Jamieson, Weissman, and colleagues provided strong evidence for the ability to generate CSC. In their study, the expansion of the granulocyte-macrophage progenitors was associated with progression to the blast crisis (Jamieson et al. 2004). Moreover, these were

the cells that exemplified amplification of the BCR-ABL fusion oncogene, whereas in HSCs the level of the BCR-ABL transcript remained largely unchanged, suggesting that a committed granulocyte-macrophage progenitor may acquire self-renewal capacity and thus “reacquire” stem-like properties due to the effect of later mutations (Passegué et al. 2003; Lobo et al. 2007).

10.7.4 What Makes a CSC? Genomic Alteration vs. Epigenomic Alteration vs. Plasticity

Though one of the defining characteristics of CSCs is the ability to initiate tumors that resemble the parental lesion, it is critical to recognize that this definition does not require CSCs at any point during the natural history of a tumor to be equivalent to, or even phenotypically similar to, the original tumor initiating cell. Oncogenic stimuli or tumor suppressor deletions may be sufficient or even necessary to induce a tumor when applied to a cell of a particular differentiation state (as discussed above). However, this does not necessarily mean that CSCs observed within that tumor at a later point originate and evolve from cancerous progeny displaying the same differentiation state as the cell initiating the tumor.

Keeping this in mind, it is not clear how the CSC population in a tumor is formed. One possibility is that this tumorigenic population arises following alterations or mutations in the cell’s genetic structure. In this “genomic alteration” model, a cell requires mutations in key regulatory genes such as loss of p53 or activation of Oct4 that allows it to escape growth restriction and apoptotic signals in order to acquire self-renewal and tumorigenic potential (Levings et al. 2009). It is possible that, after sustaining such genetic modifications and acquisition of the associated phenotypic characteristics, a cell is then considered a CSC and can self-renew and propagate the parental tumor. However, this model suggests that the selection of the CSC population is nondeterministic, as acquiring mutations happens stochastically.

Another model for CSC generation within tumors suggests that the generation of a tumorigenic subpopulation is selected for by an outside force on the epigenome and only those cells that alter their epigenetic structure are able to propagate the tumor as CSCs. In this model, several different genetically distinct subpopulations have the potential to become tumorigenic, but only those that are stimulated by outside stimuli (such as the microenvironment) and respond by altering their epigenetic regulatory mechanisms may acquire the characteristics to be considered CSCs. For example, it is possible that the characteristics of the tumor environment (e.g., hypoxia, reactive chemical species, ECM, etc.) may mediate changes in the epigenome to allow for increased transcription factor access to critical prosurvival factors such as Chk2 or VEGF (Bao et al. 2006a, b; Ben-Porath et al. 2008). The key difference between these two models of CSC generation is the ability of cells in the second model to be influenced by extrinsic factors. Where genomically mutated cells acquire the stem-like phenotype in a random stochastic manner, the heterogeneic populations in the epigenetic model are under a constant state of flux

as their microenvironment changes throughout the life of the tumor. This allows the tumor to adapt to changes brought on by chemoradiotherapy or surgical resection. In such cases, the plasticity of the CSCs, and the tumor as a whole, to adapt to an ever-changing microenvironment is critical for survival.

The previously described genomic and epigenomic models of CSC generation are not necessarily mutually exclusive. For example, cells may require certain genetic mutations that allow them to adapt their epigenomic structure to survive and proliferate. In other cases, the hierarchical structure of tumor subpopulations may be initially arranged by stratification of cells with specific genomic mutations that is further modified by microenvironmental influence to establish a new hierarchy of tumor initiation and self-renewal capacity. Certainly, recent experimental evidence supports the theory that the microenvironment may play a vital role in maintaining certain tumor subpopulations or cellular phenotypes (Heddleston et al. 2009; Helczynska et al. 2003; Li et al. 2009).

10.8 Glioblastoma: Deadly Tumor and Model System for Solid Tumor Cancer Stem Cells

Malignant gliomas, the most common primary brain malignancies, provide an informative solid tumor model system for characterization of CSC biology, phenotypic behavior, and underlying molecular mechanisms. Additionally, the dismal prognosis associated with malignant gliomas (median survival of 15 months with optimal therapy for the Grade IV glioblastoma multiforme subtype; Stupp et al. 2005) calls for the identification of novel antiglioma therapeutic strategies. Historically, the identification of antiglioma therapies had focused on molecular characteristics shared throughout the tumor bulk, but clinical evaluation of therapeutic approaches identified in this way have been largely disappointing, suggesting that more careful evaluation of tumor subpopulations such as brain tumor CSCs may be warranted.

The initial discovery and prospective isolation of CSCs from human brain tumors was performed by sorting based on expression of the cell surface marker CD133 (Prominin 1), which had previously been identified as a marker displayed by normal HSCs (Singh et al. 2003). The existence of brain tumor stem cells was subsequently demonstrated in pediatric brain tumors (Hemmati et al. 2003); this study also reported overlapping gene expression profiles between normal neural stem cells and brain tumor stem cells. Results from our laboratory and others have repeatedly demonstrated that $CD133^{+}$ glioma cells can exhibit the required characteristics of CSCs (self-renewal and the ability to generate secondary tumors that phenocopy the parental lesion). They also exhibit other common characteristics of CSCs, including the expression of stem cell markers (CD133, nestin, Sox2, Oct4, etc) and the capacity for multilineage differentiation potential (Hemmati et al. 2003; Singh et al. 2003, 2004; Bao et al. 2006a).

CD133 was first described as a marker of HSCs and subsequently reported as a marker of normal human neural stem cells (Corbeil et al. 1998; Uchida et al. 2000; Tamaki et al. 2002). However, CD133 is by no means the exclusive marker of glioma CSCs; we and others have identified additional cell surface markers, such as integrin a6 and SSEA-1, which have facilitated enrichment of CSC-enriched fractions from the bulk tumor population (Son et al. 2009; Lathia et al. 2010). Regardless of the specific marker employed for sorting, these markers have allowed successful enrichment of tumor cell subpopulations exhibiting typical characteristics of CSCs, enabling us to address the molecular mechanisms underlying the glioma stem cell phenotype. Over time, as intertumoral differences are more thoroughly understood and grade/cytogenetic contributions to CSCs more comprehensively evaluated, it is likely that more established protocols for optimal cell surface marker-based CSC population enrichment will be developed. Further, it is not unlikely that some permutation of the aforementioned markers or unidentified ones may most effectively enrich for brain tumor CSCs.

By studying the $CD133^{+}$ cell population in gliomas, our laboratory and others have already identified numerous critical molecular agents contributing to the CSC phenotype, many of which might represent viable targets for therapeutic intervention. A neural cell adhesion molecule, L1CAM, plays a central role in nervous system development by regulating survival, growth, and migration, among other developmental processes, and is previously reported to be overexpressed in gliomas (Izumoto et al. 1996). We have demonstrated that L1CAM is overexpressed in glioma CSCs relative to the nonstem tumor cell population, and knockdown of L1CAM expression inhibits glioma stem cell growth and survival, both in vitro and in vivo (Bao et al. 2008). Additionally, our results suggest that L1CAM mediates its effects via regulation of the stem cell transcription factor Olig2.

Another molecular target of interest is Akt, a key mediator in signaling pathways governing cell survival and invasion. Akt regulates the ABCG2 transporter, which generates the side population used to identify stem cells, and loss of PTEN increases the side population phenotype (Bleau et al. 2009). Relative to the nonstem cell tumor fraction, $CD133^{+}$ glioma cells preferentially respond to Akt inhibition as demonstrated by decreased growth and neurosphere formation associated with increased apoptosis (Eyler et al. 2008; Gallia et al. 2009). Targeting Akt also increased the survival of mice bearing glioma xenografts indicating that small molecule inhibitors of Akt may have therapeutic benefit against CSCs (Eyler et al. 2008; Gallia et al. 2009).

The role of the c-Myc oncoprotein in normal stem cell biology and malignancy is well recognized. We have found that glioma stem cells express greater levels of c-Myc relative to nonstem tumor cells and that c-Myc is required for the survival, growth, and proliferation of $CD133^{+}$ glioma cells (Wang et al. 2008). shRNA-mediated knockdown of c-Myc hampers the ability of glioma CSCs to form tumors in vivo (Wang et al. 2008). In keeping with the regulatory role of c-Myc in the cell cycle, our findings suggest that c-Myc exerts its effects on glioma CSC proliferation by regulating the expression of cyclin D1 and $p21^{WAF1/CIP1}$ (Wang et al. 2008).

Similar to these findings in glioma, evidence from transgenic mice indicated that deletion of p21$^{WAF1/CIP1}$ increased c-myc-induced mammary tumorigenesis associated with acquisition of a CSC phenotype (Liu et al. 2009).

Our recently published findings also highlight the role of A20 in maintaining glioma stem cells. A20, or Tumor Necrosis Factor inducible protein 3 (TNFAIP3), is a regulator of both cell survival and NF-kB signaling, previously described in numerous cancers as either tumor suppressive or protumorigenic, suggesting its effects are context-specific. In the context of glioma CSCs, we demonstrated that A20 expression is elevated relative to nonstem tumor cells and is required for glioma stem cell growth, neurosphere formation, and survival. A20 conferred resistance to TNFa-induced apoptosis in CD133$^+$ glioma cells, and shRNA-mediated knockdown of A20 expression in glioma CSCs mitigated tumorigenicity of glioma stem cells (Hjelmeland et al. 2010). Furthermore, A20 expression levels can serve as a prognostic marker in glioma patients; elevated A20 mRNA levels correlated with a significant reduction in patient survival (Hjelmeland et al. 2010).

In addition to these CSC targets, further anti-CSC therapies may be developed to antagonize the potent ability of CSCs to promote angiogenesis, the formation of new blood vessels. Compared to the nonstem glioma cell fraction, CD133$^+$ glioma cells form intracranial tumors with significantly greater vascularity and necrosis. Data from our laboratory and others suggest that this phenotype is dependent upon hypersecretion of vascular endothelial growth factor (VEGF) (Bao et al. 2006b; Folkins et al. 2009). As VEGF neutralizing antibodies inhibit the ability of glioma stem cells to induce human endothelial cell migration and tube formation in vitro while also inhibiting subcutaneous tumor growth and hemorrhage in vivo (Bao et al. 2006b), these data suggest the utility of antiangiogenic therapies as anti-CSC-based approaches. Indeed, the VEGF-neutralizing antibody Avastin (bevacizumab) has recently been approved for the treatment of recurrent glioblastoma, suggesting that future clinical studies will be able to determine its utility against CSCs.

As the main regulators of VEGF signaling in a variety of tumors, the HIFs are largely responsible for the general cellular response to low oxygen. They exist as heterodimers with the β subunit constitutively present in the nucleus and α subunit sequestered in the cytoplasm and subject to oxygen-dependant degradation (Iyer et al. 1998). In addition to their roles in VEGF signaling and angiogenesis, the HIFs have also been implicated in cell proliferation, survival, metabolism, and ECM function. The importance of HIF function also extends to normal stem cell biology where it has been observed that hypoxia creates a microenvironmental niche in order to support the maintenance of an adult stem cell population (Silván et al. 2009; Forristal et al. 2010). In glioma CSCs, critical roles for HIFs have been defined in CSC maintenance (Li et al. 2009; Seidel et al. 2010). Interestingly, HIF2α levels are relatively high in glioma CSCs where it is important for CSC survival and tumorigenic potential (Li et al. 2009; Seidel et al. 2010). Hypoxia target genes including erythropoietin and interleukin 6 have also been determined to play an important role in glioma CSCs (Wang et al. 2009a; Cao et al. 2010). Hypoxia also increases the expression of CSC genes, including CD133, Oct4, and Nanog (Platet et al. 2007; Heddleston et al. 2009; Soeda et al. 2009). These data

strongly suggest that oxygen tension, HIFs, and HIF target genes play much larger roles in glioma and CSC biology than previously appreciated.

Mounting evidence indicates that glioma CSCs not only contribute to tumor maintenance but also play a major role in therapeutic resistance. Glioma CSCs exhibit increased radioresistance as demonstrated by relative enrichment of the $CD133^+$ tumor cell subpopulation following ionizing radiation treatment, suggesting that glioma CSCs significantly contribute to tumor recurrence following radiotherapy (Bao et al. 2006a). Direct comparisons between glioma CSC vs. nonstem cell tumor cell subpopulations demonstrate that glioma CSCs have an enhanced ability to activate the DNA damage checkpoint response following radiation treatment (Bao et al. 2006a; Ropolo et al. 2009). More recent evidence suggests that targeting Notch signaling, a pathway known to regulate stem cell maintenance and differentiation during development, can impair glioma CSC radioresistance (Wang et al. 2010). These data suggest that glioma stem cells and the molecular pathways, which regulate stem cell maintenance, may be responsible for tumor recurrence after radiotherapy.

Taken together, investigation of the mechanisms governing brain tumor biology provides an instructive model system for the CSC paradigm. This model not only identifies novel therapeutic targets but also concomitantly enriches our understanding of glioma and CSC biology.

10.9 Conclusions and Implications for Therapeutic Approaches

While the CSC paradigm remains controversial, the theory itself leads to a greater appreciation for the contribution of intratumoral heterogeneity to tumor progression and recurrence. Certainly, it is not surprising that there are cells within a tumor that are predisposed to survive therapy or invade normal tissue. Prospective identification of these cells with subsequent determination of their differential molecular and biological properties would permit a greater understanding of tumor recurrence and metastasis than ever before. Researchers working with CSCs believe that these cells are responsible for therapeutic resistance and invasion. Thus, elucidating molecular differences in CSCs will define novel targets that have been overlooked in evaluations of the bulk tumor alone.

Novel treatments that reduce the ability of CSCs to survive or that promote differentiation will be needed to prevent CSC self-renewal in vivo. Such an anti-CSC therapy used in combination with conventional therapies, which kill most tumor cells, may improve cancer treatments to cures, or at least sustained remissions (Fig. 10.1). The importance of using dual therapies rather than those against CSCs alone is suggested by evidence that the CSC phenotype in solid cancers may be more plastic than the hierarchical model proposed for hematopoietic cancers. If hypoxia or other microenvironmental conditions that promote an undifferentiated state persist within the tumor, targeting of CSCs alone is unlikely to be sufficient to

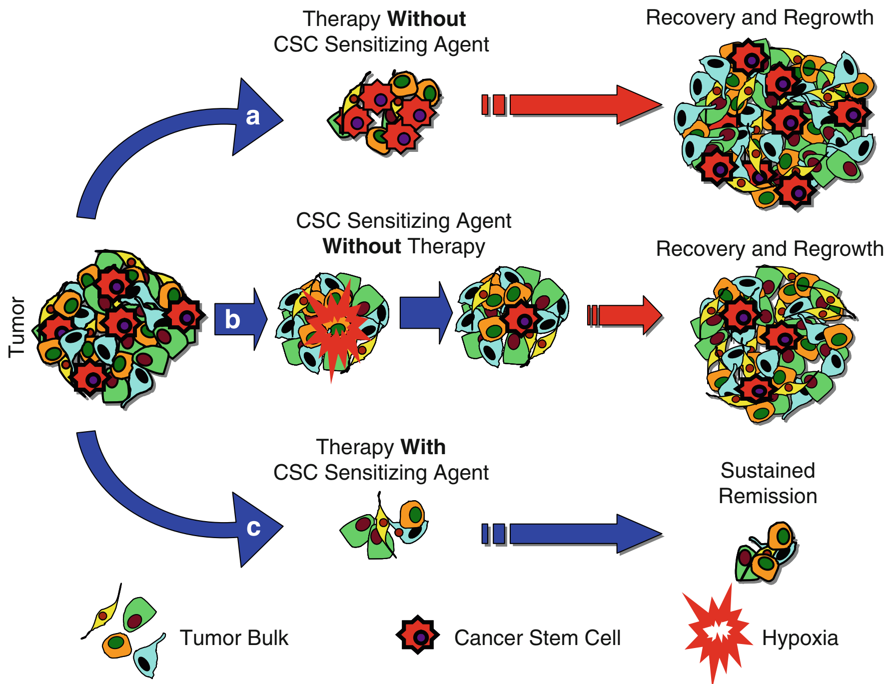

Fig. 10.1 A model for cancer stem cell (CSC) directed therapeutic approaches. (**a**) If CSCs compose the therapeutically resistant portion of the tumor, current therapies that kill the bulk of tumor cells remain ineffective due to the ability of CSCs to survive radio- and chemotherapy and repopulate the tumor. The ability to target CSCs is therefore expected to improve patient outcome. (**b**) Therapies specific to CSCs would be expected to delay tumor growth by targeting the cellular population responsible for tumor maintenance. However, nonstem tumor cells remaining after anti-CSC therapies may respond to microenvironmental conditions such as hypoxia to transition to a more stem cell like state. This plasticity of the tumor cells would likely permit tumor regrowth over time. (**c**) Therapies directed against both nonstem cells and CSCs, administered concurrently or contiguously, are therefore expected to be the most beneficial for improving patient outcome

permanently control tumor growth. It is therefore important that initial studies characterizing CSC targets are followed by preclinical studies evaluating therapies against CSCs alone and in combination with conventional treatments. These novel dual therapies offer renewed hope for tumor control, particularly for advanced disease states where current treatments remain ineffective.

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