Stem Cells Use Distinct Self-renewal Programs at Different Ages

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Stem cells expand in number during development and persist throughout life by undergoing self-renewing divisions. The question of how stem cells self-renew throughout life is a fundamental problem in cell biology, with broad implications for understanding development, tissue regeneration, cancer, and aging. Recent insights demonstrate that self-renewal programs depend on key transcriptional regulators that are often shared among stem cells in different tissues but that often change between stem cells at different stages of life: Embryonic, fetal, young adult, and old adult stem cells are maintained by different self-renewal programs. Self-renewal programs change over time to contend with changes in tissue growth and repair demands as well as the increasing risk of malignant transformation during aging. The downstream mechanisms by which these programs regulate the cell cycle, developmental potential, and timing of differentiation are just starting to be elucidated. One key requirement for self-renewal is repression of the p16^{Ink4a} and p19^{Arf} tumor suppressors. This is accomplished by overlapping transcriptional regulators whose expression and function change with age, so as to maintain self-renewal potential throughout life while allowing increased expression of p16^{Ink4a} and p19^{Arf} in aging stem cells. This reduces stem cell function in aging tissues but also reduces cancer incidence.

Stem cells maintain themselves throughout life by undergoing self-renewing divisions in which a mother stem cell gives rise to one or two daughter stem cells that have a developmental potential indistinguishable from that of the mother cell (Molofsky et al. 2004). In the hematopoietic system, stem cell self-renewal can be measured *in vivo* by the ability of single hematopoietic stem cells (HSCs) to give long-term multilineage reconstitution of irradiated mice and for the progeny of these cells to give long-term multilineage reconstitution of multiple secondary recipients. Individual HSCs have extensive self-renewal capacity (Harrison and Astle 1982; Morrison et al. 1997), although this capacity declines with age (Chen et al. 2000; Janzen et al. 2006). In the nervous system, self-renewal is measured by subcloning multilineage colonies that arise from single stem cells to determine the number of multipotent daughter cells that are formed by individual multipotent mother cells (Bixby et al. 2002; Kruger et al. 2002; Molofsky et al. 2003, 2005, 2006). As in the hematopoietic system, neural stem cells can self-renew extensively, but this self-renewal potential declines with age (Kruger et al. 2002; Molofsky et al. 2006). Many of the same genes are required for hematopoietic and neural stem cell self-renewal, demonstrating that although self-renewal is measured differently in the two systems, both assays reflect the function of a core program of self-renewal regulators that is often shared between tissues.

Stem cells change their properties and their regulation over time, consistent with the changing demands of tissue growth. Stem cell numbers must expand rapidly during development to support tissue formation and growth. Both HSCs and neural stem cells are thought to undergo rapid, symmetric self-renewing divisions to enable this expansion during fetal development (Chenn and McConnell 1995; Morrison et al. 1995; Noctor et al. 2004). However,

as tissue growth slows postnatally, stem cell division also slows, and a higher proportion of the divisions are likely to be asymmetric (for review, see Morrison and Kimble 2006). Throughout adulthood, stem cells in many tissues appear to be quiescent most of the time but regularly enter cycle to maintain tissue homeostasis (Morrison and Kimble 2006). Self-renewal programs must therefore change between the fetal, neonatal, and young adult stages to accommodate developmental changes in the frequency and symmetry of stem cell division (Table 1).

Additional changes occur in stem cells between young adulthood and old adulthood as stem cells struggle to maintain homeostasis within aging tissues and to avoid neoplastic transformation. Genetic mutations (Rossi et al. 2007) and damage from reactive oxygen species (Ito et al. 2006) accumulate with age in stem cells, impairing stem cell function and increasing the risk of neoplastic transformation. There are also changes during aging in the rate at which stem cells divide, the fates they are likely to acquire, their frequency, and their self-renewal potential (Kuhn et al. 1996; Morrison et al. 1996; de Haan et al. 1997; de Haan and Van Zant 1999; Enwere et al. 2004; Rossi et al. 2005; Janzen et al. 2006; Molofsky et al. 2006). The overall effect of these changes is to reduce stem cell function and regenerative capacity in aging tissues. These changes likely reflect a combination of environmental changes (Conboy et al. 2003, 2005; Brack et al. 2007; Carlson et al. 2008), cell-intrinsic changes in stem cell regulation (Janzen et al. 2006; Krishnamurthy et al. 2006; Molofsky et al. 2006), and the accumulation of genetic and epigenetic damage within stem cells (Rossi et al. 2007). Stem cell self-renewal programs must therefore change during aging to cope with these environmental changes, to adjust to the changing demands of tissue homeostasis, and to avoid transformation (Table 1).

We review evidence that self-renewal programs differ among embryonic, fetal, young adult, and old adult stem cells. Our goal is not to comprehensively review self-renewal mechanisms but rather to focus on a subset of key age-specific mechanisms. To accomplish this, we focus primarily on embryonic stem (ES) cells, germ-line stem cells, HSCs, and neural stem cells.

EMBRYONIC STEM CELL SELF-RENEWAL

The zygote is totipotent and can form all lineages of the embryo including the extraembryonic trophoblast and primitive endoderm. It is not clear whether totipotent stem cells self-renew in vivo as cells begin to specialize within the embryo by the 32-cell stage (3 days in mice) (Johnson and McConnell 2004). By implantation (4 days in mice), the blastocyst includes the extraembryonic trophoblast as well as the embryonic inner cell mass (Yamanaka et al. 2006). The inner cell mass contains epiblast cells that when cultured give rise to pluripotent ES cell lines. ES cells self-renew indefinitely in culture and are pluripotent, giving rise to cells of all three germ layers, although they are not totipotent because they are unable to form extraembryonic tissues. Epiblast cells exist only in a limited window of development and although these cells must maintain pluripotency, it is not clear whether they actually execute self-renewing divisions in vivo. It is thus possible that the in vitro ES cell self-renewal program incorporates mechanisms that regulate pluripotency in vivo, whereas other aspects of the self-renewal program are acquired in culture.

Oct4, Nanog, and Sox2 are transcription factors that compose a core regulatory circuit required for ES cell pluripotency and self-renewal (Jaenisch and Young 2008). These three proteins bind and autoregulate their own promoters, regulate the expression of other pluripotency genes, and repress early differentiation genes (Nichols et al. 1998; Boyer et al. 2005; Loh et al. 2006; Wang et al. 2006; Mitsui et al. 2007). Nanog stabilizes the pluripotent state, reduces the propensity of ES cells to

spontaneously differentiate, and is required for ES cells to contribute to the germ line (Chambers et al. 2007). Nanog, Oct4, and Sox2 induction also reprograms adult cells to pluripotency (Takahashi and Yamanaka 2006; Takahashi et al. 2007; Yu et al. 2007b; Park et al. 2008).

These core transcriptional regulators do not control self-renewal on their own. Caspase-3 is an intracellular protease that is activated during apoptosis and in ES cells upon differentiation. Caspase-3 is necessary and sufficient for ES cell differentiation, promoting this in part through the proteolytic cleavage of Nanog (Fujita et al. 2008). The discovery that caspase-3 regulates ES cell differentiation led to the discovery of Ronin. Ronin is a zinc finger DNA-binding protein that is also necessary and sufficient for ES cell self-renewal. Like Nanog, Ronin is cleaved by caspase-3 during differentiation (Dejosez et al. 2008). Ronin acts with other proteins to epigenetically repress gene expression independently of Oct4, Sox2, and Nanog. Thus, multiple pathways independently regulate ES cell self-renewal and pluripotency, and caspase-3 activity attenuates at least two of these to initiate differentiation.

Oct4, Nanog, Sox2, and Ronin are also required for the formation of the inner cell mass in vivo. Oct4 expression is restricted to the inner cell mass during early embryonic development and is rapidly shut off as differentiation occurs (Lengner et al. 2007). *Oct4*-deficient embryos fail to develop beyond the blastocyst stage and lack a pluripotent inner cell mass, which instead gives rise to extraembryonic trophoblast cells (Nichols et al. 1998). *Nanog* expression is largely restricted to the inner cell mass and early germ-line cells (Chambers et al. 2003; Mitsui et al. 2003). *Nanog*-deficient embryos fail to develop beyond the blastocyst stage and lack pluripotent epiblasts, although *Nanog*-deficient ES cells can contribute widely to somatic tissues outside of the germ line (Chambers et al. 2003; Mitsui et al. 2003). *Sox2*-deficient embryos also lack pluripotent epiblast cells (Avilion et al. 2003). Ronin expression is primarily detected within the inner cell mass. *Ronin* deficiency leads to peri-implantation lethality and prevents pluripotent cells from growing out of the

Table 1. Examples of genes that regulate stem cell self-renewal at distinct developmental stages

Gene	Gene product	Stem cells	References
<i>Nanog</i>	homeodomain transcription factor	ES cells	Mitsui et al. (2003); Chambers et al. (2007)
<i>Pouf1/Oct4</i>	Pou domain transcription factor	ES cells	Nichols et al. (1998); Lengner et al. (2007)
<i>Thap11/Ronin</i>	Thap domain epigenetic regulator	ES cells	Dejosez et al. (2008)
<i>Caspase-3</i>	protease	ES cells, HSCs	Fujita et al. (2008); Janzen et al. (2008)
<i>Zfx</i>	zinc finger transcription factor	ES cells, HSCs	Galan-Caridad et al. (2007)
<i>Sox17</i>	transcription factor	fetal HSCs	Kim et al. (2007)
<i>Hmga2</i>	chromatin regulator	fetal and young adult neural stem cells	Nishino et al. (2008)
<i>Bmi-1</i>	Polycomb epigenetic regulator	many adult stem cells	Lessard and Sauvageau (2003); Molofsky et al. (2003); Park et al. (2003)
<i>Klotho</i>	Wnt Inhibitor	old HSCs, satellite muscle cells	Liu et al. (2007)
<i>Angiopoietin-1</i>	cytokine	adult HSCs	Puri and Bernstein (2003); Arai et al. (2004)
<i>thrombopoietin</i>	cytokine	adult HSCs	Qian et al. (2007); Yoshihara et al. (2007)
<i>Gfi-1</i>	transcription factor	adult HSCs	Hock et al. (2004a)
<i>Tel/Etv6</i>	transcription factor	adult HSCs	Hock et al. (2004b)
<i>let-7b</i>	microRNA	old neural stem cells	Nishino et al. (2008)
<i>p16^{Ink4a}</i>	cyclin-dependent kinase inhibitor	many old stem cells	Janzen et al. (2006); Krishnamurthy et al. (2006); Molofsky et al. (2006)

inner cell mass in culture (Dejosez et al. 2008). These studies demonstrate that aspects of the ES cell self-renewal program that are required in culture are also required for the formation of the inner cell mass and for epiblast pluripotency in vivo.

Other genes that are required for the self-renewal of ES cells in culture are not necessary for the generation of the inner cell mass in vivo. Standard protocols for the derivation and propagation of ES cells use leukemia inhibitory factor (LIF), which promotes self-renewal by activating STAT3 (signal transducer and activator of transcription 3) via the gp130 receptor (Niwa et al. 1998; Ying et al. 2003). Although these genes are required for the self-renewal of ES cells in vitro, LIF and gp130 are dispensable for embryonic development in vivo (Li et al. 1995; Ware et al. 1995; Yoshida et al. 1996; Dani et al. 1998). The requirement for LIF signaling in culture may reflect the need to block differentiation-inducing cues in culture that do not exist in vivo; addition of chemical inhibitors of fibroblast growth factor-4 (FGF-4)-induced ERK (extracellular signal-regulated kinase) phosphorylation can maintain the pluripotency of ES cells in the absence of LIF and serum (Ying et al. 2008). A series of transcriptional regulators including Tbx3, Tc11, and Esrrb (Ivanova et al. 2006) as well as Zfx (Galan-Caridad et al. 2007) are also required for ES cell self-renewal in culture but not early embryonic development in vivo. These studies illustrate the context dependence of self-renewal programs and the fact that mechanisms required for self-renewal in culture do not necessarily regulate pluripotency or self-renewal under physiological conditions in vivo.

Some key elements of the pluripotent self-renewal program are restricted to ES cells and epiblast cells, whereas others are not. Although Nanog and Oct4 regulate the maintenance of pluripotency in ES cells and epiblast cells, they are not required for the function of fetal or adult somatic stem cells (Chambers et al. 2007; Lengner et al. 2007). Sox2, on the other hand, is expressed by fetal and adult neural stem cells and is required for their self-renewal (Graham et al. 2003; Suh et al. 2007). Caspase-3 deficiency increases adult HSC numbers (Janzen et al. 2008) just as it promotes the maintenance of ES cells (Fujita et al. 2008). The Zfx transcription factor provides a particularly interesting example because it is required for the self-renewal of adult (but not fetal) HSCs in addition to ES cells (Galan-Caridad et al. 2007). It is not yet clear whether Ronin regulates somatic stem cell function. These studies demonstrate that although elements of the pluripotent stem cell self-renewal program can be used by somatic stem cells at later stages of development, other features seem to uniquely define the pluripotent state.

FETAL AND NEONATAL STEM CELL SELF-RENEWAL

Fetal development represents a distinct stage marked by tissue specialization and tremendous growth. Consistent with this, the pluripotent cells of the embryo are replaced by a series of tissue-specific multipotent stem cells with developmental potentials that match the demands of their growing tissues. As a result, fetal self-

renewal programs acquire tissue-specific components that regulate multipotency and differentiation in response to tissue-specific cues. But although the developmental potential of fetal somatic stem cells is narrower than their embryonic progenitors, fetal stem cells remain highly mitotically active. HSCs from the fetal liver (Morrison et al. 1995) and neural crest stem cells from the developing peripheral nervous system (Morrison et al. 1999; Bixby et al. 2002) divide rapidly in vivo and appear to undergo frequent self-renewing divisions. The absolute numbers of stem cells in many tissues expand considerably during fetal development, and imaging of progenitor divisions suggests that this expansion is driven initially by symmetric self-renewing divisions, followed by asymmetric divisions that generate differentiated progeny (Chenn and McConnell 1995; Lechler and Fuchs 2005; Morrison and Kimble 2006). Fetal self-renewal programs must therefore change relative to embryonic self-renewal to account for tissue specialization as well as developmental changes in the symmetry of division.

Fetal Germ-line Stem Cells

The existence of fetal-specific self-renewal programs is illustrated by spermatogonial stem cells (Oatley and Brinster 2008). Primordial germ cells are specified from epiblast cells early in embryogenesis, then migrate to the genital ridges by E11.5. Primordial germ cells cycle rapidly and expand their numbers from 100 cells at E8.5 to 25,000 cells at E13.5. Once in the genital ridges, primordial germ cells form gonocytes that commit to a sex-specific fate. In male testis, fetal gonocytes briefly stop dividing, but they resume self-renewal by postnatal day 3 when they become spermatogonial stem cells.

This lineage illustrates the changes in fetal self-renewal programs that occur over time. *Nanos3* and *Nanos2* encode zinc finger proteins, thought to act as RNA-binding translational repressors, that are required for the maintenance/expansion of primordial germ cells and gonocytes, respectively (Tsuda et al. 2003). *Nanos3* is expressed in primordial germ cells as early as E7.5, whereas *Nanos2* is only detected in gonocytes after colonization of the genital ridges. In the absence of *Nanos3*, the number of primordial germ cells is normal at E7.5 but depleted by E15.5. In the absence of *Nanos2*, primordial germ cell numbers are normal at E14.5 but no spermatogonial stem cells are present in the testis by 4 weeks after birth. *Nanos3* is required to prevent cell death during primordial germ cell migration (Suzuki et al. 2008). *Nanos2* is required for specification of male-specific germ cell fate (Suzuki and Saga 2008) in addition to gonocyte survival (Tsuda et al. 2003). These results show that *Nanos3* and *Nanos2* are dispensable for the specification of primordial germ cells but are sequentially required for the maintenance of germ-line stem cells during development. Although *Nanos3* and *Nanos2* are required by germ-line stem cells during development, mice lacking these genes have no other overt phenotype, suggesting that *Nanos3* and *Nanos2* are not required by other somatic stem cells (Tsuda et al. 2003). The genes that *Nanos3* and *Nanos2* repress in order to ensure stem cell maintenance are not yet known.

Fetal and Neonatal Hematopoietic Stem Cells

Fetal and neonatal HSCs have a unique self-renewal program that depends on the Sox17 transcriptional regulator (Kim et al. 2007). *Sox17* is a Sox family high-mobility-group transcription factor that is expressed in fetal and neonatal HSCs but is absent from adult HSCs. Conditional deletion of *Sox17* in fetal mice using *Tie2-Cre* or in neonatal mice using *Mx1-Cre* led to the rapid depletion of HSCs and hematopoietic failure (Fig. 1). However, when *Sox17* was conditionally deleted from HSCs using *Mx1-Cre* 6 weeks after birth, no defect was observed in HSC frequency, function, or hematopoiesis. These findings demonstrate that *Sox17* is required for the maintenance of fetal and neonatal HSCs but not adult HSCs. This demonstrates that there is a distinct fetal/neonatal HSC self-renewal program and raises the question of what program takes over for the maintenance

of adult HSCs. Additional experiments are required to elucidate the mechanisms by which Sox17 promotes HSC maintenance.

Sox17 is also a marker of fetal identity in HSCs. Sox17 is expressed by all fetal and neonatal HSCs but by less than 1% of other cells in the fetal liver or neonatal bone marrow (Fig. 1A). At 3–4 weeks after birth, HSCs go through a concerted developmental change in which they transition from a rapidly dividing fetal phenotype to a slowly dividing adult phenotype (Bowie et al. 2006, 2007). This is also when Sox17 expression is extinguished by HSCs (Kim et al. 2007). Moreover, examination of HSCs in the middle of this transition shows that the Sox17⁺ subset retains fetal characteristics, whereas the Sox17⁻ subset in the same bones of the same mice have acquired adult characteristics (see Fig. 1D). A key transcriptional regulator of HSC identity and function thus changes between the fetal and young adult stages.

Figure 1. Sox17 is required for the self-renewal of fetal HSCs but not adult HSCs. (A) Green fluorescent protein (GFP) and c-Kit expression from unfractionated fetal liver or bone marrow cells analyzed by flow cytometry from *Sox17*^{GFP/+} mice. Boxes indicate GFP⁺ (red) and GFP⁻ (green) cells. GFP⁺ cells represented fewer than 1% of cells in the fetal liver and were no longer detectable 8 weeks after birth in the bone marrow. (B) E11.5 embryos that lack Sox17 (*Sox17*^{GFP/GFP}) have a near-complete lack of hematopoiesis in the yolk sac or embryo. (C) *Sox17* was conditionally deleted (in *Mx1-Cre*⁺ *Sox17*^{GFP/GFP} mice) to test its role in hematopoiesis in neonatal (left) or young adult (right) mice. Although all neonatal mice died within 1 week of *Sox17* deletion, *Sox17* deficiency did not affect the viability of young adult mice. After *Sox17* deletion, HSCs were dramatically depleted in the bone marrow and spleen of neonatal mice, whereas HSCs were unaffected in young adult mice. (D) HSCs uniformly express *Sox17* 2 weeks after birth but uniformly lack expression by 8 weeks after birth (inferred based on GFP expression from the *Sox17*^{GFP} knockin allele). Four weeks after birth, when HSCs are making the transition from a fetal to an adult phenotype, half of the HSCs express *Sox17*. The *Sox17* expressing HSCs are rapidly dividing and express fetal HSC markers, whereas the subset of HSCs that lack *Sox17* expression are slowly dividing and express adult HSC markers. These results suggest that Sox17 is a marker of fetal/neonatal identity in HSCs. (Reprinted from Kim et al. 2007 [© Cell Press].)

Fetal Neural Stem Cells

Multipotent and self-renewing central nervous system (CNS) stem cells first arise in the context of the embryonic neuroepithelium. As neurogenesis begins, stem cells acquire a radial glial morphology with cell bodies in the ventricular zone and long processes that extend to the pial surface (Anthony et al. 2004). These radial glia often appear to divide asymmetrically, forming a radial glial daughter as well as a daughter cell fated to undergo neuronal differentiation (Noctor et al. 2004). Many of the daughter cells fated to form neurons amplify their progeny through subsequent symmetric divisions (Miyata et al. 2002; Haubensak et al. 2004; Noctor et al. 2004). Radial glial cells persist in the ventricle zone until the postnatal appearance of adult neural stem cells in a sub-ventricular zone (Doetsch et al. 1999; Imura et al. 2003; Garcia et al. 2004). Lineage mapping has shown that adult neural cells are derived from fetal radial glial cells (Merkle et al. 2004). Neural stem cells thus undergo changes in their morphology and localization throughout fetal development.

Neural stem cells are fated to form an array of progeny at different stages of fetal development, starting with different types of neurons and ending with gliogenesis (Temple 2001). These changes in fate are likely regulated by both environmental and stem-cell-intrinsic changes that occur over time. In support of this, neural progenitors that are heterochronically transplanted into the CNS of older recipients form neurons appropriate to the age of their new environment, but older progenitors cannot form earlier subtypes of neurons upon transplantation into a younger developing forebrain (Frantz and McConnell 1996; Desai and McConnell 2000). This neuronal fate restriction is controlled by several components including *FoxG1*. *FoxG1* is expressed by neural progenitors after the production of layer-1 neurons and represses layer-1 neuronal fate. Deletion of *FoxG1* causes layer-1 neurons to be continually produced during later developmental stages, where they are normally no longer produced (Hanashima et al. 2004; Shen et al. 2006).

The narrowing of neuronal subtype potential that occurs during cortical development suggests that multipotent forebrain cells do not generate identical progeny when they self-renew. Indeed, neural stem cells appear to undergo developmental restrictions throughout life but retain multipotency and proliferative potential. The observation that neural stem cells depend on many of the same self-renewal mechanisms that are used by HSCs (and that even HSCs undergo developmental changes in lymphocyte subtype potential over time) suggests that they depend on a core “self-renewal” program despite the fact that developmental potential changes over time. Existing data suggest that this core self-renewal program can be modified by factors that restrict neuronal subtype potential (or lymphocyte subtype potential in the case of HSCs). Self-renewal mechanisms are therefore not necessarily designed to generate identical progeny but rather to generate undifferentiated progeny that remain multipotent and self-renewing.

ADULT STEM CELL SELF-RENEWAL

Adult stem cells exhibit several differences relative to fetal stem cells that affect the regulation of self-renewal. In contrast to the rapid division of fetal stem cells, adult stem cells are often relatively quiescent. Adult mouse HSCs are quiescent most of the time, although all HSCs appear to enter the cycle on a regular basis and contribute to hematopoiesis (Cheshier et al. 1999; Kiel et al. 2007). As a result, adult self-renewal programs commonly feature mechanisms that promote quiescence. Adult stem cells often have less self-renewal potential than fetal stem cells from the same tissue. HSCs and neural crest stem cells from fetal mice have more self-renewal potential than those from adult mice (Chen et al. 2000; Kruger et al. 2002). Differences in self-renewal programs must therefore account for the difference in self-renewal potential. Finally, adult stem cells often have a narrower developmental potential than fetal stem cells in the same tissues. Adult HSCs remain multipotent and capable of generating lymphocytes but they lose the ability to make certain subtypes of lymphocytes that are only made by fetal HSCs (Hayakawa et al. 1985; Ikuta et al. 1990; Kantor et al. 1992). Neural crest stem cells from the adult gut remain multipotent and able to make neurons, but they lose the ability to make certain subtypes of neurons that are only made in the fetal gut (Kruger et al. 2002). As a result, multipotency of adult stem cells differs from that of fetal stem cells. For all of these reasons, self-renewal programs must be regulated differently in adult stem cells as compared to fetal stem cells.

Adult Germ-line Stem Cells

Cell signaling and transcriptional regulation distinguish the self-renewal of adult spermatogonial stem cells from their fetal counterparts. Glial-cell-line-derived neurotrophic factor (GDNF) is critical for the self-renewal of spermatogonial stem cells but not for the expansion of their fetal progenitors. Sertoli cells in the testis secrete GDNF (Tadokoro et al. 2002), whereas the spermatogonial stem cells express the GDNF coreceptors *Ret* and *Gfra1* (Naughton et al. 2006). In the absence of *Gdnf*, *Ret*, or *Gfra1*, primordial germ cells colonize the testis normally and are present in normal numbers at birth, but spermatogonial stem cells fail to self-renew postnatally and become depleted (Naughton et al. 2006). Adult spermatogonial stem cells also require the zinc finger transcription factor *Plzf* (Buaas et al. 2004; Costoya et al. 2004) and the TFIID RNA polymerase complex component *Taf4b* (Falender et al. 2005). Mice lacking these genes exhibit normal spermatogonial development and initially produce mature sperm, but spermatogonial stem cells fail to self-renew and spermatogenesis is depleted (Costoya et al. 2004; Falender et al. 2005). Extrinsic and intrinsic factors that control spermatogonial stem cell self-renewal thus differ between adult and fetal stages.

Adult Hematopoietic Stem Cells

Many self-renewal mechanisms are conserved between fetal and adult HSCs. A number of transcriptional regula-

tors including Rae28 (Ohta et al. 2002; Kim et al. 2004b), Meis1 (Hisa et al. 2004; Kirito et al. 2004; Azcoitia et al. 2005), c-Myb (Mucenski et al. 1991; Sandberg et al. 2005), Cbp (Rebel et al. 2002), Gata-2 (Tsai et al. 1994; Rodrigues et al. 2005), PU.1 (Kim et al. 2004a; Iwasaki et al. 2005), and Mll (McMahon et al. 2007) are required for the self-renewal of both fetal and adult HSCs. This demonstrates that self-renewal programs do not undergo wholesale changes between fetal development and adulthood but rather are modified to account for developmental differences in stem cell function.

Nonetheless, there are key differences in self-renewal regulators among fetal and adult HSCs. One prominent example is *Bmi-1*, which is consistently required for the self-renewal of all postnatal stem cell populations examined so far, including HSCs and neural stem cells (Lessard and Sauvageau 2003; Molofsky et al. 2003; Park et al. 2003). *Bmi-1* is a proto-oncogene and a component of the Polycomb repressive complex 1 that regulates chromatin structure by recruiting epigenetic regulators to specific loci (Valk-Lingbeek et al. 2004). *Bmi-1* is not required for the formation or maintenance of stem cells during fetal development because *Bmi-1*-deficient mice are born with normal numbers of stem cells. Rather, *Bmi-1*-deficient stem cells exhibit a postnatal self-renewal defect that leads to the depletion of stem cells by early adulthood. By postnatal day 30, HSCs are depleted from the bone marrow of *Bmi-1*-deficient mice, and bone marrow cells from these mice are unable to reconstitute irradiated recipient mice (Park et al. 2003). *Bmi-1*-deficient mice generally die by postnatal day 30, exhibiting growth retardation, hematopoietic failure, and neurological problems (ataxia, seizures) (van der Lugt et al. 1994).

One major mechanism by which *Bmi-1* promotes the self-renewal of HSCs and other postnatal stem cell populations is through repression of the *p16^{Ink4a}* and *p19^{Arf}* tumor suppressors (Fig. 2) (Jacobs et al. 1999; Bruggeman et al. 2005; Molofsky et al. 2005; Akala et al. 2008). *p16^{Ink4a}* is a cyclin-dependent kinase inhibitor that promotes Rb (retinoblastoma) activation, slowing cell cycle progression or inducing cellular senescence (Lowe and Sherr 2003). *p19^{Arf}* promotes p53 activity, also slowing cell cycle progression or inducing cellular senescence. *p16^{Ink4a}* and *p19^{Arf}* expression generally cannot be detected in fetal or young adult tissue, but these gene products are readily detected in postnatal stem cells in the absence of *Bmi-1* (Molofsky et al. 2003; Park et al. 2003). Deletion of *p16^{Ink4a}* and/or *p19^{Arf}* partially rescues the self-renewal defects observed in various stem cell populations, including HSCs and neural stem cells (Jacobs et al. 1999; Bruggeman et al. 2005; Molofsky et al. 2005; Oguro et al. 2006; Akala et al. 2008). These results demonstrate that repression of *p16^{Ink4a}* and *p19^{Arf}* is a fundamental requirement for the maintenance of adult stem cells.

Additional transcriptional regulators also distinguish adult from fetal HSC self-renewal. Gfi1 (Hock et al. 2004a), Tel/Etv6 (Hock et al. 2004b), and FoxO (Miyamoto et al. 2007; Tothova et al. 2007) gene products are all required for the maintenance of adult but not fetal HSCs. Gfi1 promotes the quiescence of adult HSCs, whereas Tel/Etv6 promotes their survival. FoxO tran-

Figure 2. *p16^{Ink4a}* expression increases with age and negatively regulates stem cell frequency and function. (A) *p16^{Ink4a}* expression increases with age in uncultured subventricular zone cells. (B) The decline in stem cell frequency during aging is at least partially rescued by *p16^{Ink4a}* deficiency. (C,D) The decline in olfactory bulb neurogenesis during aging is partially rescued by *p16^{Ink4a}* deficiency (based on the frequency of bromodeoxyuridine [BrdU]-containing NeuN⁺ neurons in the olfactory bulb). (Reprinted from Molofsky et al. 2006 [© Nature Publishing Group].)

scription factors promote the expression of enzymes that reduce levels of reactive oxygen species, such that in the absence of FoxO function, reactive oxygen species accumulate and HSCs are depleted. These results emphasize the importance of diverse transcription factors to regulate the maintenance of stem cell integrity in a developmentally regulated manner.

Fetal and adult HSCs also differ in their dependence on growth factors and signaling molecules. Thrombopoietin and its receptor Mpl promote the quiescence and maintenance of adult HSCs, but neither gene is required for the maintenance of fetal HSCs (Qian et al. 2007; Yoshihara et al. 2007). Angiopoietin-1 and its receptor Tie2 are similarly reported to promote the quiescence and maintenance of adult HSCs, but they are not required for the maintenance of fetal HSCs (Puri and Bernstein 2003; Arai et al. 2004). The ligands thrombopoietin and angiopoietin-1 are expressed in the bone marrow, and their corresponding receptors are expressed on HSCs. These results demonstrate that both cell-extrinsic and -intrinsic components of signaling networks regulate differences in stem cell maintenance between fetal and adult HSCs.

Adult Neural Stem Cells

Neural stem cells are maintained throughout adult life in two regions of the CNS and in at least two regions of the peripheral nervous system (PNS). Within the CNS, stem cells are maintained in the lateral wall of the lateral ventricle (Doetsch et al. 1999; Sanai et al. 2004) where they undergo neurogenesis throughout life, forming interneurons that migrate into the olfactory bulb (Sawamoto et al. 2006; Curtis et al. 2007). These new interneurons synapse on the projection neurons that connect the olfactory bulb to the cortex and likely regulate the ability to learn to discriminate chemically similar odor-

ants (Magavi et al. 2005; Alonso et al. 2006). Stem cells also persist through adult life in the subgranular layer of the dentate gyrus (Palmer et al. 1997) where they form interneurons (Eriksson et al. 1998; van Praag et al. 2002). The new neurons that incorporate into the adult dentate gyrus appear to regulate certain forms of spatial learning and memory (Shors et al. 2001; Zhang et al. 2008). Within the PNS, multipotent and self-renewing neural crest stem cells persist throughout adult life in the wall of the gut (Kruger et al. 2002), although the physiological function of these stem cells remains unclear. A distinct neural-crest-derived stem cell population persists throughout life in the carotid body, where it gives rise to new glomus cells in response to oxygen stress (Pardal et al. 2007).

Just as in the hematopoietic system, some components of the neural stem cell self-renewal program are required by stem cells at all stages of development. For example, the Sox2 transcriptional regulator that is required for ES cell self-renewal is also required for the maintenance of undifferentiated neural stem cells at all stages from embryonic development through adulthood (Pevny and Placzek 2005). A loss of Sox2 function causes premature differentiation and depletion of neural stem cells at both fetal and adult stages, with a consequent impairment of neurogenesis (Bylund et al. 2003; Graham et al. 2003; Ferri et al. 2004). Sox2 is not alone in this regard because Numb and Numbl-like also appear to be required throughout fetal and adult life for the self-renewal of forebrain neural stem cells (Petersen et al. 2002, 2004; Shen et al. 2002; Kuo et al. 2006).

Some gene products are required throughout life by stem cells, but their precise function may change in concert with developmental changes in stem cells. For example, the orphan nuclear receptor Tlx is required to regulate cell cycle progression and fate determination within fetal and adult neural progenitors (Miyawaki et al. 2004; Roy et al. 2004; Shi et al. 2004); however, the precise ways in which these progenitors depend on Tlx varies depending on the location and time during development. In the retina, Tlx is required to regulate the proliferation of progenitors during fetal development and then to regulate glial differentiation postnatally (Miyawaki et al. 2004). In the developing cortex, Tlx is required to promote proliferation and to delay neurogenesis by ventricular zone progenitors, such that in the absence of Tlx, undifferentiated cells become depleted by premature neurogenesis and outer layers of cortical neurons are severely reduced (Roy et al. 2004). Within the adult subventricular zone, Tlx is required by stem cells to maintain self-renewal potential and to avoid premature glial differentiation (Shi et al. 2004). Thus, the way in which stem cells use Tlx to retain an undifferentiated state changes with location and time during development.

Other key transcriptional regulators are required by adult but not fetal stem cells. In the absence of Bmi-1, mice are born with normal numbers of neural stem cells, but p16^{Ink4a} and p19^{Arf} become derepressed postnatally, leading to the depletion of stem cells and neurogenesis from the CNS and PNS (Molofsky et al. 2003, 2005; Bruggeman et al. 2005). This demonstrates that neural stem cells share with HSCs a common dependence on transcriptional regu-

latory mechanisms that distinguish adult stem cell maintenance from fetal stem cell maintenance.

AGING STEM CELL SELF-RENEWAL

Stem cells must persist throughout life in numerous tissues to maintain tissue regenerative capacity. Yet, these cells must cope with ongoing change during adulthood. Genetic and epigenetic damage accumulates throughout life from reactive oxygen species, radiation, and chemical exposure. This damage impairs cellular function. For example, mitochondria accumulate damage with age, leading to a decline in mitochondrial function (Guarente 2008). Cells must compensate for this damage and declining function. In concert, the risk of neoplastic transformation increases with age as mutations accumulate. Cells must counteract this by augmenting tumor suppressor mechanisms during aging. Finally, there are systemic and local environmental changes within tissues during aging to which stem cells must adapt. For all of these reasons, stem cell self-renewal mechanisms change during aging.

Aging Hematopoietic Stem Cells

The properties of HSCs change in several ways as these cells age. The frequency of HSCs changes with age, although the nature of the change depends on the mouse strain: In some strains, such as C57BL, HSCs expand in number during aging (Morrison et al. 1996; Sudo et al. 2000; Yilmaz et al. 2006), whereas in other strains, HSCs become depleted during aging (de Haan and Van Zant 1999; Geiger and Van Zant 2002). HSC cell cycle status also changes during aging in a strain-dependent manner, with C57BL HSCs exhibiting increased cycling (Morrison et al. 1996; de Haan et al. 1997; Yilmaz et al. 2006). Aging HSCs also acquire an engraftment defect in which individual old HSCs are only one-third as likely as young HSCs to engraft in young irradiated recipient mice after transplantation (Morrison et al. 1996; Liang et al. 2005; Yilmaz et al. 2006). Nonetheless, individual reconstituting HSCs give similar overall levels of reconstitution, irrespective of whether they are from young or old donors. Finally, fate determination is affected because HSC differentiation appears skewed toward the production of myeloid cells at the expense of lymphoid cells in old mice (Morrison et al. 1996; Liang et al. 2005; Rossi et al. 2005). HSCs thus undergo many cell-intrinsic alterations in their functional properties during aging.

Despite ongoing Bmi-1 expression, p16^{Ink4a} is induced in aging stem cells, reducing the cells' self-renewal potential (Janzen et al. 2006). p16^{Ink4a} is expressed by HSCs from old, but not young, mice. p16^{Ink4a} deficiency does not affect HSCs from young mice, but it increases the frequency, survival, self-renewal, and repopulating ability of HSCs from old mice. p16^{Ink4a} expression thus distinguishes old HSCs from young HSCs, demonstrating that the self-renewal of stem cells during aging is modified by increased tumor suppressor function. p19^{Arf} expression also increases with age in a variety of tissues (Krishnamurthy et al. 2004), but whether p19^{Arf} regulates stem cell aging has not yet been tested.

Aging Neural Stem Cells

Neural stem cell frequency, self-renewal capacity, and mitotic activity all decline with age, along with the rate of neurogenesis (Enwere et al. 2004; Maslov et al. 2004; Molofsky et al. 2006). These declines in neural stem cell frequency and function are partially caused by increasing $p16^{\text{Ink4a}}$ expression. $p16^{\text{Ink4a}}$ generally cannot be detected in fetal and young adult tissues but is induced in a variety of aging tissues (Fig. 2A) (Zindy et al. 1997; Krishnamurthy et al. 2004; Molofsky et al. 2006). $p16^{\text{Ink4a}}$ deficiency partially rescues the decline in stem cell frequency (Fig. 2B), self-renewal potential, and neurogenesis in the aging CNS without affecting stem cell function or neurogenesis in the young adult CNS (Fig. 2C,D) (Molofsky et al. 2006). The decline in stem cell function at least partially reflects an active process in which tumor suppressors are induced during aging. Because $p16^{\text{Ink4a}}$ deficiency also leads to early onset and increased incidence of malignancies, stem cells may induce $p16^{\text{Ink4a}}$ expression during aging to avoid neoplastic transformation. This raises the possibility that stem cells balance regenerative (self-renewal) capacity with the risk of transformation during aging and that the induction of tumor suppressors during aging is a compromise designed to delay the onset of cancer.

The results described above raised two questions. First, why do old adult stem cells express higher levels of $p16^{\text{Ink4a}}$ and $p19^{\text{Arf}}$ than fetal and young adult stem cells? Second, given that Bmi-1 does not regulate $p16^{\text{Ink4a}}$ and $p19^{\text{Arf}}$ expression by fetal stem cells in vivo (Molofsky et al. 2003), are there other mechanisms that repress $p16^{\text{Ink4a}}$ and $p19^{\text{Arf}}$ in fetal stem cells? We have recently discovered that the high-mobility-group transcriptional regulator Hmga2 is highly expressed in fetal neural stem cells, but expression declines in young adult stem cells and is extinguished in old adult stem cells (Fig. 3A) (Nishino et al. 2008). This decrease in expression is partly caused by the increasing expression of *let-7b* microRNA, which is known to target Hmga2 (Fig. 3F) (Lee and Dutta 2007; Mayr et al. 2007; Yu et al. 2007a). *Hmga2*-deficient mice show reduced stem cell frequency and self-renewal potential throughout the CNS and PNS of fetal and young adult mice, but not old adult mice (Fig. 3D) (Nishino et al. 2008). Furthermore, $p16^{\text{Ink4a}}$ and $p19^{\text{Arf}}$ expression is increased in *Hmga2*-deficient fetal and young adult stem cells, and deletion of $p16^{\text{Ink4a}}$ and/or $p19^{\text{Arf}}$ partially restores their self-renewal capacity (Fig. 3B,C). Hmga2 thus promotes stem cell self-renewal during early life by decreasing $p16^{\text{Ink4a}}/p19^{\text{Arf}}$ expression. These results identify a new pathway that regulates stem cell aging in which *let-7b* expression increases with age, reducing Hmga2 expression and increasing $p16^{\text{Ink4a}}/p19^{\text{Arf}}$ expression, leading to reduced stem cell function (Fig. 3G).

The requirement for Hmga2 in fetal and young adult stem cells, along with the requirement for Bmi-1 in young and old adult stem cells, demonstrates that there are overlapping transcriptional mechanisms to prevent the expression of $p16^{\text{Ink4a}}$ and $p19^{\text{Arf}}$ in stem cells. Such pathways are temporally regulated by changes in *let-7b* and Hmga2 expression to allow $p16^{\text{Ink4a}}$ and $p19^{\text{Arf}}$ expression in aging stem cells (Fig. 4). In this way, self-renewal mech-

anisms can maintain stem cells throughout life while augmenting tumor suppression in aging stem cells.

$p16^{\text{Ink4a}}$ and $p19^{\text{Arf}}$ may be broadly important to diseases of aging beyond their function in stem cells. Several genome-wide association studies of large human populations revealed that polymorphisms at the $p16^{\text{Ink4a}}/p19^{\text{Arf}}$ locus (chromosome 9p21) are associated with frailty (Melzer et al. 2007), atherosclerotic heart disease (Helgadottir et al. 2007, 2008; McPherson et al. 2007; Wellcome Trust Case Control Consortium 2007), and type-2 diabetes (Saxena et al. 2007; Wellcome Trust Case Control Consortium 2007; Zeggini et al. 2007). Although these studies do not pinpoint the polymorphisms that affect the risks of age-related diseases, there are only four genes in the vicinity of the mapped polymorphisms: $p16^{\text{Ink4a}}$, $p19^{\text{Arf}}$, $p15^{\text{Ink4b}}$, and *ANRIL* (a noncoding RNA) (Sharpless and DePinho 2007). $p16^{\text{Ink4a}}$ expression increases with age in pancreatic β cells, and $p16^{\text{Ink4a}}$ deficiency increases β -cell regenerative capacity (Krishnamurthy et al. 2006), providing a mechanism by which polymorphisms that affect $p16^{\text{Ink4a}}$ expression or activity might affect risk for type-2 diabetes. It remains unclear whether these polymorphisms influence the risk of frailty and heart disease through their effects on tissue regenerative capacity or by mechanisms that are completely independent of stem/progenitor cells.

$p16^{\text{Ink4a}}$ may not be the only tumor suppressor that influences stem cell self-renewal, tissue regenerative capacity, age-related disease, and cancer. p53 is a well-known tumor suppressor that is frequently inactivated in human cancers. A polymorphism in *p53* increases longevity and survival after cancer diagnosis in humans (Orsted et al. 2007). Overexpression of short isoforms of p53 in mice leads to early-onset aging phenotypes and decreased longevity (Tyner et al. 2002; Maier et al. 2004). These results raise the possibility that polymorphisms that increase p53 activity decrease cancer incidence but promote aging, whereas polymorphisms that decrease p53 activity increase cancer incidence but slow aging. However, bacterial artificial chromosome transgenic mice that bear a third copy of the *p53* locus or the $p15^{\text{Ink4b}}/p16^{\text{Ink4a}}/p19^{\text{Arf}}$ locus show a decreased cancer incidence but normal longevity and normal onset of aging phenotypes (Garcia-Cao et al. 2002; Matheu et al. 2004). Complicating matters further, mice that bear a third copy of the *p53* locus and a third copy of the $p15^{\text{Ink4b}}/p16^{\text{Ink4a}}/p19^{\text{Arf}}$ locus show increased longevity and delayed aging in a manner that cannot be explained by their reduced incidence of cancer (Matheu et al. 2007). These results suggest that the effects of *p53* and $p15^{\text{Ink4b}}/p16^{\text{Ink4a}}/p19^{\text{Arf}}$ expression on aging are context and dosage dependent and likely reflect a complex mix of mechanisms.

Insights from Other Systems

A series of studies conducted on aging muscle have shown that the function of aging stem cells is also affected by environmental change. Muscle satellite cells poorly regenerate injured muscles in aging animals, partly due to reduced Notch signaling in aging satellite cells (Conboy

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Figure 3. *Hmga2* and *let-7b* regulate *p16*^{Ink4a}/*p19*^{Arf} expression and stem cell self-renewal in an age-related manner. (A) *Hmga2* is expressed by undifferentiated cells in the ventricular/subventricular zone and expression declines with age. Expression can no longer be detected in the subventricular zone of old adult mice. Asterisk indicates the ventricle. (B) *Hmga2* deficiency reduced the frequency (percentage of cells that formed stem cell colonies in culture) and self-renewal potential (number of secondary stem cell colonies generated per primary colony upon subcloning) of stem cells cultured from the fetal and young adult brain. *p16*^{Ink4a}/*p19*^{Arf} deficiency partially rescued these defects. Statistics reflect mean ± standard. (C) *Hmga2* deficiency reduced the rate of proliferation within the subventricular zone of young adult mice, but *p16*^{Ink4a}/*p19*^{Arf} deficiency partially rescued this defect. Numbers indicate the percentage of subventricular zone cells (mean ± s.d.) that incorporate a pulse of BrdU in vivo. (D) *Hmga2* deficiency reduced the self-renewal potential of stem cells cultured from the fetal and young adult brain but did not affect the self-renewal potential of stem cells from the old adult brain. (E) Overexpression of *let-7b*, but not *GFP* control, reduced the self-renewal of neural stem cells. (F) Deletion of the *Hmga2* 3'UTR (eliminating *let-7*-binding sites) was required to increase *Hmga2* protein levels in old neural stem cells (data not shown). This *let-7*-insensitive form of *Hmga2*, but not wild-type *Hmga2*, significantly increased the self-renewal of neural stem cells from old mice. (G) Schematic of the mechanism by which changes in *let-7b* and *Hmga2* expression during aging regulate stem cell function. (Reprinted from Nishino et al. 2008. [© Cell Press].)

et al. 2003). However, when the circulatory systems of old and young mice are parabiotically fused, Notch signaling and muscle regeneration in the old mice are restored to young levels (Conboy et al. 2005). This demonstrates that age-related changes in stem cells are governed by both local and systemic environmental changes. Further mechanistic insights into these changes were provided by the observation that Wnt signaling is

elevated in aging mice and that this contributes to the reduced regenerative capacity (Brack et al. 2007). This finding was supported by the discovery that *Klotho*-deficient mice, which exhibit elevated Wnt signaling and premature aging, lack a secreted Wnt antagonist (Liu et al. 2007). Other environmental changes also occur during aging, such as increasing TGF-β signaling. The increase in TGF-β signaling leads to increased expression of

Figure 4. Age-related changes in p16^{Ink4a} regulation. (A) Schematic of genes and processes that affect p16^{Ink4a} expression during aging. (B) A summary of p16^{Ink4a} regulatory mechanisms within stem cells.

cyclin-dependent kinase inhibitors, including p16^{Ink4a} (Carlson et al. 2008). This provides a mechanism by which environmental changes that occur during aging can regulate cell-intrinsic factors identified in prior studies.

PERSPECTIVE

For a mammal to survive, stem cells must maintain their ability to self-renew throughout life. Tissue growth and regeneration demands change dramatically over the course of an animal's life, and the genetic programs regulating stem cell self-renewal change accordingly. Stem cells from embryonic, fetal, young adult, and old adult stages use somewhat different mechanisms to regulate their self-renewal (see Table 1).

Many key mechanisms that regulate stem cell self-renewal do not generically regulate all progenitor proliferation. Deletion of *Bmi-1*, *Hmga2*, or *p16^{Ink4a}* leads to self-renewal phenotypes in multipotent stem cells without affecting the proliferation of many restricted progenitors (Molofsky et al. 2003, 2006; Nishino et al. 2008). Sox17 is absolutely required for the maintenance of fetal HSCs but is not expressed by most restricted hematopoietic progenitors in the fetal liver (Kim et al. 2007). These results demonstrate that the self-renewal of stem cells is mechanistically distinct from the proliferation of many restricted progenitors. Nonetheless, this is not a black and white difference, because some restricted progenitors, such as lym-

phocytes and cerebellar granule precursor cells, also depend on Bmi-1 (van der Lugt et al. 1994; Leung et al. 2004). This suggests that some restricted progenitors use self-renewal mechanisms that are similar to those of stem cells.

A fundamental requirement for the maintenance of stem cells throughout life is the repression of p16^{Ink4a} and p19^{Arf} tumor suppressors. This is often accomplished by stage-specific transcriptional regulatory mechanisms, such as *Hmga2* and *Bmi-1*. The regulation of p16^{Ink4a} and p19^{Arf} in a stage-specific manner confers the capacity for dynamic regulation. As the risk of cancer increases during aging, these genes are induced, reducing stem cell frequency and function in multiple tissues. Stem cell self-renewal is regulated by networks of proto-oncogenes and tumor suppressors that balance the need to maintain stem cells throughout life with the need to avoid neoplastic transformation. An important, although difficult, future question is whether the mechanisms that regulate stem cell aging also control life span and whether stem cell aging influences longevity.

The age-related changes in stem cell self-renewal programs are also likely to mean that different mutations are required for carcinogenesis at different stages of life. Cancers consistently arise from mutations that inappropriately activate self-renewal pathways (Reya et al. 2001; Pardal et al. 2003). Because self-renewal pathways change with age, we hypothesize that different mutations

are required for neoplastic transformation at different ages, irrespective of whether cancers arise from stem cells or restricted progenitors/differentiated cells. This hypothesis remains to be tested directly, but it provides one explanation for the observation that adult cancers often have different mutation spectrums as compared to the childhood versions of the same cancers (Downing and Shannon 2002). A more sophisticated understanding of self-renewal mechanisms and how they change with age could provide new anticancer strategies.

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