The elements of stem cell self-renewal: a genetic perspective

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Every day, the body produces billions of new blood cells. Each of these is derived from a rare cell in the bone marrow called the hematopoietic stem cell (HSC). Because most mature blood cells have a limited lifespan, the ability of HSCs to self-renew and replenish the mature cell compartment is critical to sustaining life. While great progress has been made in isolating HSCs and defining their functional and phenotypic characteristics, the molecular mechanisms that regulate their self-renewal remain a mystery. Over the last few years, alterations in HSC frequency and self-renewal capacity in transgenic and knock-out mice have led to the identification of novel mediators of HSC homeostasis in vivo. These genetically modified mice have revealed that maintenance of survival, proliferation, quiescence, and normal telomere length all contribute to the self-renewal of HSCs. They also highlight the need to test in context of the normal microenvironment the role of signaling molecules such as Notch and Wnt, which have emerged recently as important regulators of HSC self-renewal. The emerging picture these data provide of the regulation of self-renewal in HSCs has provided a better understanding of the basic biology of stem cells and holds promise for designing strategies to improve bone marrow transplantation.

IDENTIFICATION AND ISOLATION OF HEMATOPOIETIC STEM CELLS

Hematopoietic stem cells (HSCs) are responsible for generating all the cells of the blood. Since their original identification almost 40 years ago (1), our understanding of HSCs has progressed at a remarkable rate. HSCs have been successfully isolated from the bone marrow, and stages through which they progress during commitment have been mapped in detail. Many different methods have been utilized to isolate these cells, including fluorescence activated cell sorting (FACS), based on the expression of Rhodamine 123 (2), Hoechst 33342 (3), wheat germ agglutinin (4), CD34 (5), and Flk-2 (6,7). One approach that has been used extensively has been to utilize antibodies to a variety of cell surface molecules, such as c-kit, Sca-1, and Thy1.1, and hematopoietic lineage markers like B220, CD3, and Mac-1 (lin). This approach has identified a small population of cells (c-kit⁺ Sca-1⁺ lin^{lo/-} Thy1.1^{lo}) (8,9) that migrate to the appropriate microenvironments and undergo rapid expansion and differentiation upon transplantation into lethally irradiated mice. In the short term, they provide radioprotection, generating the erythroid and myeloid cells that are necessary for survival; subsequently, they begin to self-renew and generate other hematopoietic cells and can maintain steady-state hematopoiesis for the lifetime of the animal (9,10). The HSCs identified by such methods make up 0.05% of the total bone marrow

and have been further separated into two populations: (i) long-term HSCs (c-kit⁺ Sca⁺ Lin⁻ Thy1.1^{lo} cells, present at 1:10000 in the marrow); and (ii) short-term HSCs (c-kit⁺ Sca⁺ Lin^{lo} Thy1.1^{lo} cells, present at a frequency of 1:2000 in the marrow). In general and for the purposes of this review, cells that can self-renew and are capable of reconstitution of all hematopoietic lineages are referred to as stem cells, and those that have more restricted differentiation potential and can generate only a subset of lineages are referred to as progenitors.

CONTROL OF HEMATOPOIETIC STEM CELL SELF-RENEWAL

Although HSCs readily undergo self-renewal in vivo, it has been extremely difficult to identify the signals that regulate this process and recapitulate them in vitro. Over the last few years, alterations in HSC numbers and self-renewal capacity in transgenic and knock-out mice have identified new mediators of HSC homeostasis in vivo. Here we review these studies and discuss their implications for a better understanding of the molecular control of self-renewal in vivo. If these findings can be extended to human HSCs, they may advance our ability to manipulate self-renewal ex vivo and have significant impact on improving transplantation-based therapies.

THE ROLE OF SURVIVAL IN SELF-RENEWAL

For stem cells to self-renew, they must be able to proliferate extensively without dying or differentiating. Regulators of these processes—proliferation, differentiation, and death—have all been implicated in control of HSC self-renewal. The importance of apoptosis in this process is most clearly seen in studies of *H2K-BCL-2* transgenic mice (11,12). *BCL-2* transgenic mice have a higher proportion of long term HSCs in the bone marrow than wild-type mice. However, these HSCs cycle at a lower rate, suggesting that the increased cell number is a consequence of inhibiting cell death alone and not of increased proliferation. In fact, the lower number of cells in cycle also suggests that Bcl-2 is preventing their entry into the cell cycle and maintaining cells in a quiescent state as observed in other cell types (13,14). At a functional level, HSCs from the *H2K-BCL-2* mice display increased colony formation and protection from growth factor-deprived death in vitro as well as greater contribution to the total blood pool in vivo compared to wild-type mice in competitive transplantation experiments (11,12). These data show that anti-apoptotic signals and perhaps increased quiescence contribute to regulating stem cell numbers as well as the efficiency of reconstitution.

The importance of normal survival mechanisms to maintain self-renewal is underscored by the decreased self-renewal exhibited in *Bmi1*-deficient mice (15,16). *Bmi1*, a polycomb group transcription factor involved in repressing expression of homeobox genes, is overexpressed in cancer cells and can cooperate with other oncoproteins such as myc to contribute to leukemogenesis. *Bmi1*-deficient mice display hypocellular bone marrow, and while they are found to have similar numbers of multipotent progenitors as wild-type mice, they display a 10-fold reduction of postnatal KTLS HSCs (cells that are c-kit⁺Thy1.1^{lo}Lin⁻Sca-1⁺). In competitive repopulation experiments in which bone marrow HSCs from *Bmi1*^{-/-} mice were injected into irradiated mice in conjunction with bone marrow HSCs from wild-type mice, mutant HSCs were able to reconstitute the hematopoietic system only transiently, and all *Bmi1*^{-/-}-derived hematopoietic cells were depleted by 10 weeks. These data suggest an intrinsic requirement for *Bmi1* in the maintenance of HSC self-renewal. Gene expression analysis of wild-type and *Bmi1*^{-/-} bone marrow cells demonstrates that the loss of *Bmi1* led to the up-regulation of p16^{Ink4a} and p19^{Arf}, genes derived from the ink4a locus and previously shown to be targets of *Bmi1* in embryonic fibroblasts (17). The enforced expression of p19^{Arf} led to increased cell death of HSCs, which could be inhibited by the loss of p53. Additionally, the overexpression of p16 led to an inhibition of HSC proliferation. These data suggest that growth arrest due to p16 up-regulation coupled with the apoptotic consequences of p19 overexpression may lead to the impaired self-renewal observed in *Bmi1*-deficient HSCs. Since p16 and p19 have been previously suggested to mediate *Bmi1* action in oncogenesis, this study reiterates the parallels that lie between the molecular mechanisms that regulate processes of self-renewal and transformation (18). Additionally, the recent finding that *Bmi1* also regulates stem cell renewal of the peripheral and central nervous systems suggests that *Bmi1* can act as a signal for stem cell maintenance in diverse tissues (19).

PROLIFERATION AND QUIESCENCE IN SELF-RENEWAL

While apoptosis clearly plays a role in maintaining self-renewal, regulation of normal proliferation is also critical. The homeobox genes *HOXB3* and *HOXB4* appear to play a direct role in proliferation. *HoxB4* has been recently implicated as a mediator of expansion of primitive hematopoietic stem cells ex vivo (20). In contrast, *HoxB3* overexpression has been found to lead to a block in lymphoid development and leads to a myeloproliferative disorder. The common expression of both genes in CD34⁺ progenitors prompted Bjornsson et al. (21) to delete the two contiguous genes that were flanked by loxP sites and then targeted by the Cre recombinase expression. While *HoxB4*^{-/-} mice have previously been shown to have skeletal abnormalities leading to lethality, the conventional mutation has not been previously reported to have any effects on hematopoiesis (22). Interestingly, the *HoxB3/B4* mutant does not display the skeletal abnormalities observed in *HoxB4*^{-/-} mice, suggesting a fundamental difference in the nature of the deletion that remains unresolved (21). On the other hand, *HoxB3/B4*^{-/-} mice have significantly decreased cellularity in multiple hematopoietic organs, including the spleen and bone marrow (21). While HSCs from *HoxB3/B4*^{-/-} mice do not display any defects in migration or differences in frequency of HSCs under conditions in which cells are forced to undergo in vitro expansion, primitive hematopoietic progenitors from *HoxB4/B3* mutant mice display reduced proliferative ability. Additionally, twice as many mutant HSCs as wild-type HSCs remain in G0/G1 phase of the cell cycle after treatment with the cytotoxic drug cyclophosphamide. These results suggest that the coordinate loss of both *HoxB3* and *B4* leads to a delayed activation and recruitment into proliferation after hematopoietic stress (21). However, given the robust ability of ectopically expressed *HoxB4* to maintain the self-renewing state of HSCs in vitro and in vivo after transplantation (20), the lack of more profound defects in the *HoxB3/B4*^{-/-} mice is puzzling and may suggest some level of redundancy within the Hox gene family.

This possibility is supported by the more severe defects in HSC renewal and function that occur in *Pbx-1*-deficient HSCs. *Pbx-1*, a homeodomain protein and the mammalian homolog of the extradenticle protein, serves as a cofactor for Hox proteins and enhances their DNA binding affinities (22a). *Pbx-1*^{-/-} mice die of anemia and other developmental abnormalities at embryonic day 15–16. While establishment of primitive and definitive hematopoiesis is intact in *Pbx-1*-deficient mice, HSC containing populations from *Pbx-1*^{-/-} mice were unable to reconstitute the peripheral hematopoietic system in competitive repopulation experiments (22a) and exhibit reduced colony formation in vitro and colony-forming units-spleen (CFU-S) formation in vivo. Cell cycle analysis of more differentiated progenitor populations suggests a clear reduction in the ability of *Pbx-1*-deficient cells to proliferate and enter cell cycle, suggesting by extension that the loss of *Pbx-1* may also lead to impaired proliferation and subsequent exhaustion of HSCs. These also relate well to the defects in cell cycle recruitment observed in the Hox-deficient mice since Hox and Pbx may act as a transcriptional complex to al-

low normal HSC cell cycling and renewal. However, the fact that the defects in *Pbx*^{-/-} cells are more severe suggests that Pbx is needed as a cofactor for a number of Hox proteins, and its loss results in several nonfunctional Hox proteins. This is also supported by the complete failure of hematopoiesis observed in mice deficient for Mll (23,24), a transcription factor that regulates expression of multiple *Hox* genes.

While the ability to self-renew extensively is a hallmark of HSCs, the need to do so in vivo is limited by the high proliferative capacity of progenitor populations. Thus, paradoxically, HSCs in vivo are relatively quiescent. Only a small fraction of HSCs (1%–5% for long-term HSCs, and up to 10%–13% for short-term HSCs) are in cycle under normal homeostatic conditions (3,25). In fact, these abilities appear to be intricately connected, in that the ability to remain quiescent appears to be crucial for sustaining the long-term self-renewal of HSCs. This was revealed by the analysis of mice deficient for p21^{cip1/waf1}, a cyclin-dependant kinase inhibitor. In p21-deficient mice, a greater proportion of lineage-negative cells, which includes the HSC population, were in cycle compared to the wild-type population (26). p21-deficient HSCs exhibited reduced in vitro colony formation, as well as loss of reconstitution and accelerated death in serial transplantation experiments, compared to wild-type bone marrow cells. In contrast to its role in other cell types where its deletion would be predicted to lead to increased cell cycle and thus higher numbers of cells, the loss of p21 in HSCs increased entry of HSCs into cycle and led to exhaustion and ultimate loss of the population. This suggests that excess cycling, which could potentially lead to more stem cells, cannot do so indefinitely because of a timed senescence event, such as one determined by telomere length (discussed below). Interestingly, while p21-deficient mice have increased numbers of HSCs in cycle, the loss of another cyclin-dependant kinase inhibitor, p27^{kip1}, exclusively affects progenitor proliferation and pool size but not HSCs (27). Thus, while both p21 and p27 are ubiquitously expressed, these data suggest that cell cycle kinetics and the maintenance of quiescence in stem and progenitor cell populations are regulated by distinct mechanisms. These data also identify for the first time the critical role of quiescence in maintenance of long-term self-renewal.

Defects in self-renewal also occur in mice lacking Rae28, which is a polycomb group gene (28), and Creb binding protein (CBP) (29), which is a histone acetyltransferase. Rae28 is a homolog of the *Drosophila* polyhomeotic gene and a member of the polycomb complex 1. Rae28-deficient fetal liver cells repopulate the hematopoietic system poorly compared to wild-type fetal liver cells in transplantation assays and display reduced ability to radioprotect and form colonies in vitro and in vivo. Intriguingly, these hematopoietic cells do not appear to have altered apoptosis or proliferation (at least in unfractionated fetal liver, although whether rare progenitor and HSC fractions may have defects remains to be tested). Similarly CBP^{+/-} mice have multiple phenotypic abnormalities, including hematopoietic failure (30), and CBP^{-/-} HSCs are much less efficient than wild-type HSCs in reconstituting irradiated secondary recipients. Additionally, the surface molecule Sca-1 (used extensively to isolate HSCs) appears to contribute functionally to enhance its self-renewal capacity; *Sca-1*^{-/-} mice

display a disadvantage during competitive repopulation experiments and deficiencies during serial transplantation (31). Whether the defects in CBP-deficient and Sca1-deficient mice are due to alterations in cell death, proliferation, migration, or differentiation remains untested, and the genes that lie downstream of these molecules and may be responsible for the HSC defects seen in Rae28, CBP, and Sca-1 mutant mice will be important areas of future investigation.

TELOMERES AND SENESCENCE AS MODULATORS OF SELF-RENEWAL

Another important component of self-renewal is the maintenance of telomere length. In many cancers, the ability to proliferate limitlessly is dependent in part on the activation of telomerase, which enables the cell to maintain telomere length and subvert a normal state of senescence. While normal somatic cells do not express telomerase, high telomerase activity has also been associated with a high degree of self-renewal in HSCs (32), and telomere length decreases with repeated transplantation of HSCs (33). Thus, if one mechanism of stem cell exhaustion is the reduction of telomere length, one prediction would be that increased or decreased telomere length may lead to greater or lesser self-renewal activity, respectively. This idea was tested recently by examining the extent of serial transplantation that can be carried out by HSCs from telomerase reverse transcriptase (TERT)-deficient and transgenic mice. While wild-type HSCs can maintain reconstitution ability for four rounds of serial transplantation, TERT^{-/-} HSCs exhaust prematurely after two rounds of serial transplantation (34), suggesting that telomere length is indeed critical for maintaining the self-renewal ability of HSCs.

Whether extending the length of telomeres allows HSCs to extend their natural lifespan was tested by the generation of a transgenic mouse in which TERT was overexpressed using an H2K promoter (35). Overexpression of telomerase was found to increase telomere lengths in the transgenic mouse, and transgenic mice clearly maintained longer telomeres with serial transplantation. However, neither wild-type nor transgenic mouse bone marrow were able to contribute to more than four rounds of serial transplantation. This suggests that extending telomere length alone is not sufficient to extend self-renewal capacity of HSCs and that other factors must contribute to the clock that limits the HSC lifespan.

HEMATOPOIETIC SPECIFICATION AND MAINTENANCE: NOTCH, SHH, AND WNT PATHWAYS

While the above discussion has focused on the genes that influence HSC self-renewal through analysis of genetically altered mice, another group of genes and signaling pathways have been identified as regulators of HSC self-renewal through ectopic overexpression and subsequent in vitro and in vivo analysis. These include elements of the Notch, Sonic Hedgehog (Shh), and Wnt signaling pathways, as well as HoxB4, which has been discussed above. The expression of constitutively active Notch1 in populations containing

hematopoietic progenitors allows the establishment of immortalized cell lines that retain the potential to generate both lymphoid and myeloid cells in vitro and in long-term mouse reconstitution assays (36). Similarly, overexpression of activated Notch1 in Rag-deficient Sca1⁺Lin⁻ cells allows the increased generation of HSCs in vitro and in vivo; while the cells remain multipotential, they display preferential differentiation towards the lymphoid lineage underscoring Notch1's role in enhancing lymphoid development (37). Finally, human bone marrow cells enriched for HSCs exhibit increased engraftment in vivo after being cultured in the presence of the Notch1 ligand Jagged1 in context of other growth factors (38). The fact that Notch activation has been observed to inhibit the differentiation of progenitors in many different systems (reviewed in Reference 39) supports the idea that Notch activation may promote stem cell self-renewal.

Interestingly, the predictions made by these in vitro studies are contradicted by the phenotype observed in mice that are deficient for Notch1 (40), as well as inducible Notch1 knock-outs (41). In these mice there are no detectable defects in definitive HSC self-renewal or maintenance. However, analysis of embryonic Notch1-deficient mice suggests a defect in the establishment of HSC fate (40). It is of course possible that Notch1 is redundant with other Notch family members or that there is a deficiency in self-renewal that may become appar-

ent only through serial transplants; these possibilities have not yet been tested directly. That it may play a role that has not yet been detected is supported by very recent studies that reveal the ability of Notch signaling to regulate HSC numbers in context of the microenvironment or stem cell "niche" (42). However, another interpretation that cannot be ruled out is that while Notch1 may have the ability to drive HSCs into self-renewing divisions, it normally does not do so in vivo; furthermore, it may be important only for specification of hematopoietic fate, but not HSC maintenance. The possibility that the molecular basis of self-renewal and specification are distinct is supported by the observation that mice mutant for the stem cell leukemia (SCL) gene during embryonic life when HSC fate is being specified display a complete absence of all hematopoietic lineage cells (43), but mice in which SCL expression is deleted after HSC specification (44) are able to maintain hematopoiesis, and HSC self-renewal progresses normally.

Similar to Notch signaling, Wnt and Shh are signaling molecules that control many developmental processes and are dysregulated in many human cancers. They have also recently been implicated in the regulation of HSC self-renewal. Human HSCs (CD34⁺Lin⁻CD38⁻) exhibit increased self-renewal in response to Shh signaling in vitro (45). Shh promotes proliferation and retards differentiation of normal cerebellar pre-

cursors and other central nervous system (CNS) precursors (46,47), and mutations in the Shh signaling pathway lead to medulloblastoma (48) and basal cell carcinoma (49), suggesting this pathway may promote self-renewal in a variety of normal and cancerous tissues as well. Recent evidence also suggests that Wnt signaling has an important regulatory role in hematopoietic progenitors/stem cells during both fetal and adult development (reviewed in Reference 50). Experiments conducted in both mouse and human progenitors suggested an increase in immature in vitro colony formation in response to conditioned media containing Wnt proteins (51,52). Furthermore, stimulating Wnt signaling by overexpression of activated β -catenin (a downstream mediator of Wnt signaling) in long-term cultures of purified HSCs expands the pool of HSCs as determined by phenotype in vitro and functional transplantation in vivo (53). Moreover, ectopic expression of Axin, an inhibitor that acts by degrading β -catenin, leads to the inhibition of HSC proliferation and reduced reconstitution in vivo, suggesting that this pathway is also important for maintenance of HSC proliferation and survival in vitro and in vivo. Purified HSCs proliferate in response to soluble Wnt protein in synergy with stem cell factor (SCF) while maintaining the

Figure 1. Overview of selected genes shown to influence elements of hematopoietic stem and progenitor cell function and self-renewal through the use of genetic mutants. Asterisks indicate those genes that have not been directly demonstrated to regulate the function indicated in hematopoietic stem cells (HSCs) but are predicted to do so based on function in related cell types.

Table 1. Summary of Selected Mutant Mice that Exhibit Alterations in HSC Function

Gene	Description	Type of Mutant	Effects on HSC Function In Vivo	Effects on HSC Function In Vitro	References (No.)
<i>Bmi-1</i>	Polycomb group transcription factor	Knock-out	Reduced HSC frequency in bone marrow. Inability to contribute to PB in repopulation assay. Up-regulation of p16 ^{Ink4a} and p19 ^{Arf} .	Reduction in progenitor frequency by CFC assay. Overexpression of p16 ^{Ink4a} decreased HSC proliferation and overexpression of p19 ^{Arf} increased p53-dependent cell death.	(15,16)
<i>p21</i>	Cyclin-dependent kinase inhibitor	Knock-out	Increased cycling of HSC containing fraction. Premature exhaustion during serial transplantation assays.	Increased stem cell numbers by CAFC assay. Normal numbers of committed progenitors by CFC assay.	(26)
<i>p27</i>	Cyclin-dependent kinase inhibitor	Knock-out	Committed progenitors but not stem cells have increased fraction of cells in cycle. Preferential outgrowth of progenitor and mature cells during competitive repopulation assay.	Expansion of progenitor population indicated by CFC assay.	(27)
<i>CBP</i>	Transcriptional co-activator	Knock-out	CBP ^{+/-} HSCs exhaust prematurely during serial transplantation. No PB contribution from CBP ^{-/-} cells in blastocyst chimeras.	No defect in hematopoietic differentiation of CBP ^{-/-} ES cells.	(29)
<i>HoxB3/B4</i>	Transcription factors	Knock-out	Delayed HSC regenerative capacity in competitive repopulation assay. Reduced number of cells in cycle after cytoxin treatment.	Small reduction in proliferative capacity of HSC containing fraction.	(21)
<i>Pbx-1</i>	Transcription cofactor for Hox and Meis family proteins	Knock-out	Reduced ability of FL HSCs to repopulate peripheral blood in competitive reconstitution assays.	Reduced proliferative ability of committed progenitors.	(7)
<i>Rae28</i>	Polycomb group transcription factor	Knock-out	Reduced CFU-S. Reduced CRU when FL cells used in transplantation assays. Reduced CRU found in bone marrow after serial transplantation.	Reduced number of CFC in methylcellulose assay.	(28)
<i>Sca-1</i>	Cell-surface antigen	Knock-out	Reduced frequency of CFU-S. Reduced repopulation efficiency in competitive and serial transplantation experiments.	Reduced numbers of CFU-GEMM.	(31)
<i>TERT</i>	Catalytic component of telomerase	Knock-out	Enhanced telomere shortening in HSCs during serial transplantation. Deficient HSCs exhaust prematurely after two rounds of serial transplantation.	NA	(34)
<i>TERT</i>	Catalytic component of telomerase	Transgenic	No difference in HSC frequency or extent of serial transplantation.	NA	(35)
<i>BCL2</i>	Inhibitor of cell death	Transgenic	Higher frequency of LT-HSCs in bone marrow. Greater contribution from HSCs to total blood pool in competitive reconstitution assays.	Increased plating efficiency and protection against growth factor-deprivation apoptosis.	(11)

CAFC, cobblestone area-forming cell; CBP, Creb binding protein; CFC, colony-forming cell; CFU-GEMM, colony-forming unit-granulocyte, erythrocyte, monocyte, macrophage; CFU-S, colony-forming unit-spleen; CRU, competitive repopulation units; ES, embryonic stem; FL, fetal liver; HSC, hematopoietic stem cell; LT-HSC, long-term HSC; PB, peripheral blood; NA, not applicable.

phenotypic and functional characteristics of HSCs, suggesting that multiple elements of this pathway can act as stem cell growth factors (54). Interestingly, studies also suggest that certain Wnt family members (specifically Wnt5A) may also have a positive impact on human hematopoietic progenitor and stem cell growth when injected in xenograft models in vivo (55). The studies described above indicate that Wnt signaling is important in the maintenance and self-renewal of hematopoietic stem and progenitor cells. Importantly, besides its role in the hematopoietic system, Wnt signaling also regulates self-renewal and maintenance of stem cells and progenitors in tissues as diverse as the nervous system, the gut, and the human epidermis (reviewed in Reference 50). Since Notch, Shh, and Wnt appear to be strong inducers of self-renewal in multiple contexts, it will be extremely important for future studies to focus on defining their role in HSCs in context of the native microenvironment through the use of transgenic and knock-out mouse models.

PERSPECTIVES

At a cellular level, a stem cell must survive, proliferate, and remain undifferentiated in order to self-renew successfully. But what does self-renewal mean at a molecular level? The molecular program must include genes that regulate the cell cycle, those that prevent cell death, as well as those that inhibit differentiation. The data discussed in this review point to several elements that appear to control separate components of this self-renewal process, such as survival, proliferation, or quiescence. These genes appear to act in HSCs in much the same manner as they do in other tissues and cells. However, the ability of HSCs to renew long term is unique and a property that is not shared by most other cells. Thus, even if the machinery in stem cells and other somatic cells is the same, it is tempting to speculate that there are master regulators uniquely expressed in stem cells that can coordinate these programs in their entirety to maintain self-renewal ability throughout life. If such master regulators could be identified and harnessed, it would revolutionize our understanding of stem cell biology and our ability to utilize stem cells for therapy.

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