

Genetic programs regulating HSC specification, maintenance and expansion

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All mature blood cells originate from a small population of self-renewing pluripotent hematopoietic stem cells (HSCs). The capacity to self-renew characterizes all stem cells, whether normal or neoplastic. Interestingly, recent studies suggest that self-renewal is essential for tumor cell maintenance, implicating that this process has therapeutic relevance. Unfortunately, the molecular bases for self-renewal of vertebrate cells remain poorly defined. This article will focus on the developmental mechanisms underlying fetal and adult HSC homeostasis. Specifically, distinctions between genetic programs regulating HSC specification (identity), self-renewal (in both fetal and adult) and differentiation/commitment will be discussed with a special emphasis on transcriptional and chromatin regulators.

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Self-renewal: a defining property of the hematopoietic stem cell?

The hematopoietic stem cell (HSC) can be operationally defined as a long-term repopulating cell with both lymphoid (T and B) and myeloid potential (reviewed in Orlic and Bodine, 1994). The first evidence for the existence of such a cell-type came from experiments by Ray Owen and co-workers, in 1945, which showed that bovine fraternal twins, sharing a single placenta and blood circulation, retained production of blood cells genetically defined to be from both throughout their life (Owen, 1945). Twenty years later, elegant experiments by Till, McCulloch, Wu, Becker, Siminovitch and co-workers demonstrated that adult bone marrow contained single cells that had the ability to form macroscopic nodules of myeloerythroid cells on the spleen, 8–12 days after intravenous injection into myelo-

ablated recipients (Becker *et al.*, 1963; Siminovitch *et al.*, 1963). These spleen-colony-forming-units (CFU-Ss) were shown to be clonal (Wu *et al.*, 1968a, b) and, in many cases, could generate similar colonies upon transplantation into secondary recipients (Siminovitch *et al.*, 1963). As they shared several characteristics attributed to HSCs (including high proliferative potential, multipotentiality and self-renewal ability), CFU-S were initially considered to be HSCs (Siminovitch *et al.*, 1963). The validity of the CFU-S assay to detect HSCs with long-term repopulating potential was questioned after the discovery that some of these cells were capable of only unilineage differentiation and/or lacked the ability to self-renew (functional heterogeneity). Although most of the cells possessed the ability to differentiate into the erythrocyte and myeloid lineages, their lymphoid potential remained controversial (Wu *et al.*, 1968a, b; Lala and Johnson, 1978; Lepault *et al.*, 1993). It is now clear that most CFU-S cells in the adult bone marrow are committed myeloid progenitors (Worton *et al.*, 1969; Jones *et al.*, 1989), which can be physically separated from more primitive cells with long-term lympho-myeloid repopulating potential (Mulder and Visser, 1987; Visser and de Vries, 1988; Jones *et al.*, 1990; Spangrude *et al.*, 1991; van der Loo *et al.*, 1994). Although the CFU-S assay played a key role in the development of concepts of primitive hematopoietic cell organization and regulation, its inability to analyse pure stem cells meant that most of their functions were implied rather than directly analysed.

The first attempts at purifying the HSC came from experiments carried out by Till and McCulloch (Worton *et al.*, 1969; van Bekkum *et al.*, 1979) in the Netherlands. From this work, it has become possible to routinely identify and isolate highly purified murine and human HSCs based mainly on characteristic cell surface proteins that are either present (Sca-1 and c-kit) or absent (using markers of lineage committed cells such as CD38, Mac-1 and CD8) (reviewed in Weissman, 2002).

Despite the progress that has been made in identifying and obtaining enriched HSC populations, analysis of the population dynamics and cell cycle kinetics of HSCs remains difficult. One of the most intriguing properties of adult HSCs is a robust maintenance of the dynamic equilibrium between self-renewal and differentiation

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(Morrison *et al.*, 1997). Under homeostatic conditions *in vivo*, most HSCs are quiescent, as demonstrated by their relative resistance to killing by the cytotoxic drug 5-fluorouracil (5-FU) when compared to committed progenitor cells (Hodgson and Bradley, 1979; Lerner and Harrison, 1990). When they enter cycle, HSCs can divide asymmetrically or symmetrically, resulting in different HSC fates (see Faubert *et al.*, in this issue). Stem cell maintenance divisions give rise to one daughter HSC with essentially identical biological properties (a process referred to as self-renewal) and one committed daughter cell. The committed daughter cell enters a transient state of rapid cellular proliferation and, upon exhaustion of its proliferative potential, withdraws from the cell cycle and progressively acquires the specialized characteristics of a predetermined blood cell-type. Although the relative influence of intrinsic versus extrinsic factors on HSC self-renewal remains to be determined, it has been easier to identify the environmental factors having a negative impact on this process than those that enhance it. Thus, most *in vivo* culture conditions defined to date lead to depletion of the HSC pool by favoring symmetric divisions (generation of two daughter differentiated cells) and concomitant expansion of committed progenitor populations (Morrison *et al.*, 1997; see also Sauvageau, Iscove and Humphries, in this issue).

Several studies using retroviral marking have demonstrated the ability of HSCs to undergo self-renewal divisions (Lemischka *et al.*, 1986; Jordan and Lemischka, 1990; Keller and Snodgrass, 1990; Fraser *et al.*, 1992). Although most of these studies failed to accurately quantify the magnitude of self-renewal events, considerable evidence suggests that this property is not unlimited (see Bell and van Zant, in this issue). First, following bone marrow transplantation, the HSC pool is not found to regenerate to levels higher than 10% of normal pretransplantation values, despite a complete regeneration of bone marrow cellularity and progenitor cell numbers (Harrison *et al.*, 1978, 1990; Harrison and Astle, 1982; Mauch and Hellman, 1989; Pawliuk *et al.*, 1996). Some investigators have suggested the involvement of negative feedback mechanisms imposed *in vivo* by more mature cells as a possible mechanism that could prematurely inhibit HSC expansion following transplantation (Iscove and Nawa, 1997). Alternatively, this loss of long-term repopulating ability may result from damage to the recipient's microenvironment inflicted by the conditioning regimen (i.e. irradiation). However, experiments performed in the anemic (WW^v) recipient mouse strain, which possesses a normal microenvironment but poorly competitive hematopoietic cells due to a mutation in the *c-kit* ligand receptor (Chabot *et al.*, 1988), rather suggest that this defect is intrinsic to the transplanted cells themselves (Harrison and Astle, 1982; Gardner *et al.*, 1988).

A major concern is that nearly all HSC assays assessing self-renewal rely on the generation of functionally mature cells, and therefore provide a retrospective rather than a current view of potential HSC attributes. In a transplantation setting, the accuracy of the HSC readout relies on the efficiency of the

transplanted cells to home and engraft to the specialized niches of the bone marrow microenvironment (Benveniste *et al.*, 2003). The heterogeneity of the HSC compartment further complicates the interpretation of such experimental designs. Age-related and strain-specific (Van Zant *et al.*, 1991; Phillips *et al.*, 1992) differences in HSC numbers and/or competitive abilities have been reported (reviewed in Geiger and Van Zant (2002). Moreover, a functional decline in the proliferative potential of HSCs derived from the fetal liver, umbilical cord (at birth) and adult bone marrow indicates ontogeny-related differences in HSC function (Pawliuk *et al.*, 1996; Rebel *et al.*, 1996a,b; Harrison *et al.*, 1997). Whether this heterogeneity represents true intrinsic quantitative and/or qualitative differences in HSC properties, or in the expression of this potential due to stochastic events, remains unclear.

Genetic programs specifying HSCs

At least three distinct genetic programs are required for the functionality of the blood system. These include: (a) the specification of HSCs, (b) their self-renewal and (c) their commitment–proliferation–differentiation (Figure 1). Several genes regulating HSC specification (Figure 1a) or differentiation (Figure 1c) have been identified (reviewed in Cantor and Orkin, 2001; Kondo *et al.*, 2003) but, as discussed below, determinants of HSC self-renewal remain poorly characterized (Figure 1b). In the next sections, we will review the role of key nuclear factors underlying the molecular programs of HSC specification, self-renewal expansion (SR-E) and maintenance (SR-M).

Genes specifying HSCs

During mouse ontogeny, the first blood cells, embryonic (or primitive) erythrocytes, arise within the blood

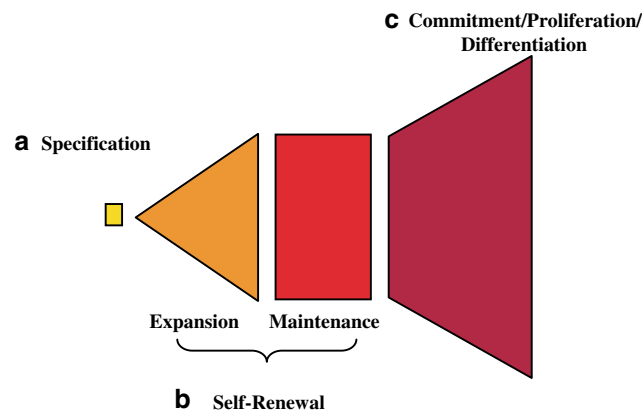


Figure 1 Genetic programs in hematopoiesis. At least three genetic programs are required for the development of the blood system. (a) The specification of HSCs (yellow) (genesis), (b) the self-renewal, including expansion (orange) and maintenance (red) of HSCs as described in Figure 4 and (c) the commitment, proliferation and differentiation of HSCs (dark red)

islands of the extraembryonic yolk sac at embryonic day 7.5 (E7.5). By E11.5, hematopoiesis shifts to the fetal liver, where adult (or definitive) red cells, as well as cells of other hematopoietic lineages, first appear. The site of origin of the HSCs has been less certain. Whereas it was previously accepted that HSCs and progenitors migrate from the yolk sac to the fetal liver during development, more recent studies relying on cell transplantation to reconstitute hematopoiesis in adult recipients assign an intraembryonic source for definitive (adult) hematopoiesis within the intraembryonic para-aortic splanchnopleura (PS) and aortic-gonadal-mesonephros (AGM) regions (Godin *et al.*, 1993; Medvinsky *et al.*, 1993; Medvinsky and Dzierzak, 1996). HSCs arising in these areas are believed to migrate to and colonize the fetal liver and spleen, where they continue to differentiate into recognizable hematopoietic precursors. After birth, definitive hematopoiesis is primarily confined to the bone marrow, and in some pathological conditions, also to extramedullary sites such as the spleen, the liver and occasionally the lung and brain. The presence of multipotential progenitors in the blood of E10 embryos suggests that migration and colonization are mediated via the circulation (Delassus and Cumano, 1996). A unique origin of HSCs is challenged by recent evidence demonstrating long-term repopulation by yolk sac (extra-embryonic) progenitors as assayed by reconstitution of fetal recipient animals (Yoder *et al.*, 1997). Hence, the origin of adult hematopoietic cells (and in particular definitive HSCs) within the specific vascular regions of the mammalian embryo body remains highly speculative. Nevertheless, the development of a stable functioning hematopoietic system reflexes complex processes involving cellular differentiation, as well as temporal and spatial control of migration, homing, self-renewal/proliferation and survival of HSCs.

The transcriptional machinery governing early HSC function is undoubtedly very complex. Genes involved in specifying HSCs during early embryogenesis include: *SCL/tal-1* (Shivdasani *et al.*, 1995) (*stem cell leukemia hematopoietic transcription factor*) and *Rbtn2* (Warren *et al.*, 1994) (also known as *Lmo-2* or *tig-2*), which are essential for primitive and definitive hematopoiesis and *AML-1* (Okuda *et al.*, 1996; Lacaud *et al.*, 2002) (also known as *RUNX1/CBFA2 and PEBP2B*), that is specifically required for definitive hematopoiesis. Other factors appear to be more lineage-specific in action such as *GATA-3*, *Ikaros*, *PU.1*, *GATA-1*, *CBP*, *Atf4*, *c-myb*, *T-bet* and *E2A*, as their absence affects specific hematopoietic lineages (see Table 1).

The b-HLH *SCL/tal-1* transcription factor is essential for the establishment of primitive hematopoiesis. Mice lacking *SCL/tal-1* are embryonic lethal and show an absence of yolk sac-derived hematopoiesis (Robb *et al.*, 1995, 1996; Shivdasani *et al.*, 1995; Porcher *et al.*, 1996). Using a conditional gene targeting approach, Orkin and co-workers recently established that *SCL1/tal-1* is critical for the genesis of HSCs, but that its continued expression is dispensable for HSC function (Mikkola *et al.*, 2003). This suggests that distinct classes of HSC regulatory factors may exist: those required for their

genesis (i.e. identity), and those specifically required for later functions, such as long-term repopulating activity (self-renewal) and multipotency (see below).

The LIM domain-containing *Rbtn2/Lmo-2* protein (which is known to form a heterocomplex together with *SCL/tal-1*) (Valge-Archer *et al.*, 1994; Wadman *et al.*, 1994) also acts very early in ontogeny, as mice deficient for this gene lack all lineages of both primitive and definitive hematopoiesis, although yolk sac-derived macrophages have been observed (Warren *et al.*, 1994) (see Table 1).

Mice nullizygous for *AML-1* are embryonic lethal and lack fetal liver-derived (definitive) hematopoiesis. However, primitive hematopoiesis is not affected since large nucleated erythrocytes are present in the embryo. *AML-1*^{-/-} ES cells fail to contribute to adult hematopoietic tissues in chimeras (Okuda *et al.*, 1996; Wang *et al.*, 1996a, b, c). Inducible gene-targeting experiments demonstrated that *AML-1* is dispensable for adult HSC function (Ichikawa *et al.*, 2004). *AML-1* appears to be required for the proper temporal and spatial localization of stem cells in the embryo (Cai *et al.*, 2000). Deletion of *Cbfb*, which enhances the binding activity of *AML-1* *in vivo*, is also embryonic lethal. Surprisingly, its effect on fetal liver hematopoiesis is less drastic, suggesting that *AML-1* can operate in part without *Cbfb* (Sasaki *et al.*, 1996; Wang *et al.*, 1996a, b, c). Examination of the contribution of these genetic axes to later HSC properties (such as HSC self-renewal, proliferation and multipotency) is eagerly awaited.

Intrinsic regulators of fetal HSC self-renewal

After their specification early in ontogeny (see above and Yoder in this issue), HSCs undergo two rounds of mobilization: first, to the embryonic fetal liver where they expand and second, to the bone marrow where they are maintained throughout adult life. Expansion in the fetal liver implies symmetrical HSC divisions referred herein as 'SR-E' for self-renewal expansion. HSC maintenance in the bone marrow suggests asymmetrical divisions named hereafter 'SR-M' for self-renewal maintenance (see next section and Figure 2). Although not proven yet, evidence suggests that these two developmental processes may be intrinsically regulated by distinct sets of genetic determinants.

Embryonic/fetal liver HSC SR-E is necessary to increase the pool size of the mobilized stem cell population. HSCs deficient in the genetic program necessary for SR-E will not expand, but depending on the intensity of the defect, they may generate enough mature blood elements to ensure the viability of an animal. This hypothesis is supported by serial transplantation experiments, which indicated that hematopoietic mouse chimeras reconstituted with as few as 280 HSCs (evaluated by the competitive-repopulating unit (CRU) assay and representing around 3% of the normal pool size) are viable for a prolonged period of time (Pawliuk *et al.*, 1996; U Thorsteinsdottir and GS,

Table 1 Nuclear factors affecting intrinsic HSC properties

Mutated gene	Hematopoietic phenotype	ES cell chimera	Hematopoietic chimera	Genetic program(s) affected in HSC		
				Specification	SR-E	SR-M
<i>Aml1</i>	Embryonic lethality, no effect on primitive hematopoiesis, absence of definitive hematopoiesis (Okuda <i>et al.</i> , 1996; Wang <i>et al.</i> , 1996a, b, c)	Differentiation into primitive erythrocytes <i>in vitro</i> , no contribution to definitive hematopoiesis <i>in vivo</i> (Okuda <i>et al.</i> , 1996)	Reconstitution (Cai <i>et al.</i> , 2000; Ichikawa <i>et al.</i> , 2004)	+	?	–
<i>Atf4</i>	Early birth lethality, presence of primitive hematopoiesis, impaired fetal liver hematopoiesis (Masuoka and Townes, 2002)	NA	NA	–	?	?
<i>Bmi-1</i>	Young adulthood lethality, hypoplasia of hematopoietic organs, impaired lymphopoiesis (van der Lugt <i>et al.</i> , 1994)	NA	No reconstitution (Lessard and Sauvageau, 2003; Park <i>et al.</i> , 2003)	–	–/?	+
<i>Cbfb</i>	Embryonic lethality, primitive hematopoiesis normal, impaired definitive hematopoiesis (Sasaki <i>et al.</i> , 1996; Wang <i>et al.</i> , 1996a, b, c)	No contribution to adult hematopoietic tissues (Wang <i>et al.</i> , 1996a, b, c)	NA	–	?	?
<i>Cbp</i>	Embryonic lethality, impaired primitive hematopoiesis, impaired early definitive hematopoiesis (Oike <i>et al.</i> , 1999; Kung <i>et al.</i> , 2000; Tanaka <i>et al.</i> , 2000)	Impaired contribution to definitive hematopoiesis (Rebel <i>et al.</i> , 2002)	Reduced (Rebel <i>et al.</i> , 2002)	–	+/?	–
<i>C/Ebpa</i>	Early birth death, lack mature granulocytes (Zhang <i>et al.</i> , 1997)	NA	Reconstitution (Zhang <i>et al.</i> , 1997; Lessard and Sauvageau, 2003)	–	–/?	–
<i>C-Myb</i>	Embryonic lethality, primitive hematopoiesis normal, impaired fetal liver hematopoiesis, presence of megakaryocytes (Mucenski <i>et al.</i> , 1991; Sumner <i>et al.</i> , 2000; Emambokus <i>et al.</i> , 2003)	Contribution to early fetal liver hematopoiesis, no contribution to mature hematopoiesis, generation of double-negative thymocytes in Rag-2 ^{-/-} chimeras (Allen <i>et al.</i> , 1999; Clarke <i>et al.</i> , 2000; Sumner <i>et al.</i> , 2000)	NA	–	–/?	–/?
<i>E2A</i>	High number postnatal death, impaired B-cell development (Bain <i>et al.</i> , 1994; Zhuang <i>et al.</i> , 1994)	NA	NA	–	?	–
<i>Gata-2</i>	Embryonic lethality, reduced number of primitive erythrocytes, reduced number of yolk sac (YS) colonies (Tsai <i>et al.</i> , 1994)	Contribution to primitive erythropoiesis, no contribution to fetal liver, adult hematopoietic organs and mature RBCs, presence of lymphocytes in the spleen (Tsai <i>et al.</i> , 1994; Tsai and Orkin, 1997)	NA	–	–/?	–/?
<i>Ikaros</i>	Live up to 4 months, impaired lymphopoiesis, erythropoiesis and myelopoiesis nonaffected (Wang <i>et al.</i> , 1996a, b, c; Wu <i>et al.</i> , 1997; Lopez <i>et al.</i> , 2002; Georgopoulos <i>et al.</i> , 1994)	NA	Reduced (Wu <i>et al.</i> , 1997; Nichogiannopoulou <i>et al.</i> , 1999)	–	–	–
<i>Lmo2</i>	Embryonic lethality, absence of primitive erythropoiesis, presence of YS derived macrophages (Warren <i>et al.</i> , 1994)	ES cells are able to form macrophages <i>in vitro</i> , no reconstitution to adult hematopoietic tissues (Warren <i>et al.</i> , 1994; Yamada <i>et al.</i> , 1998)	NA	–	?	?
<i>Meis1</i>	Embryonic lethality, absence of megakaryocytes, reduced fetal liver CFCs (Hisa <i>et al.</i> , 2004)	NA	No reconstitution (Hisa <i>et al.</i> , 2004)	–	+/?	–/?

Table 1 (continued)

Mutated gene	Hematopoietic phenotype	ES cell chimera	Hematopoietic chimera	Genetic program(s) affected in HSC		
				Specification	SR-E	SR-M
<i>Pu.1</i>	Embryonic lethality to early birth death, multilineage defects (Scott <i>et al.</i> , 1994; McKercher <i>et al.</i> , 1996)	Contribution to fetal erythropoiesis, no contribution to adult hematopoietic tissues (Scott <i>et al.</i> , 1997) NA	No reconstitution (Fisher <i>et al.</i> , 1999)	-	?	-
<i>Rae-28</i>	Embryonic lethality, impaired lymphopoiesis (Takahara <i>et al.</i> , 1997; Tokimasa <i>et al.</i> , 2001)	NA	Reduced (Tokimasa <i>et al.</i> , 2001; Ohta <i>et al.</i> , 2002)	-	+/?	-
<i>Scl</i>	Embryonic lethality, no development of primitive and definitive hematopoiesis (Robb <i>et al.</i> , 1995; Shivdasani <i>et al.</i> , 1995)	No contribution to hematopoiesis (Porcher <i>et al.</i> , 1996; Robb <i>et al.</i> , 1996) NA	Reconstitution (Mikkola <i>et al.</i> , 2003)	+	-	-
<i>T-bet</i>	Lack of Th1 commitment (Finotto <i>et al.</i> , 2002)	NA	NA	-	?	-
<i>Tel</i>	Embryonic lethality, similar number of yolk sac (YS) primitive and definitive colonies (Wang <i>et al.</i> , 1997)	Contribution to fetal liver hematopoiesis, no contribution to bone marrow myelopoiesis and erythropoiesis, impaired contribution to lymphogenesis, no contribution to adult hematopoietic tissues (Wang <i>et al.</i> , 1997, 1998)	NA	-	-	?

BM: bone marrow; CFC: colony-forming cell; ES cell: embryonic stem cell; FL: fetal liver; RBC: red blood cell; SR-E: self-renewal expansion; SR-M: self-renewal maintenance; YS: yolk sac; NA: not applicable. Specific program is affected (+), nonaffected (-), may be affected (+/?), or may not be affected (-/?)

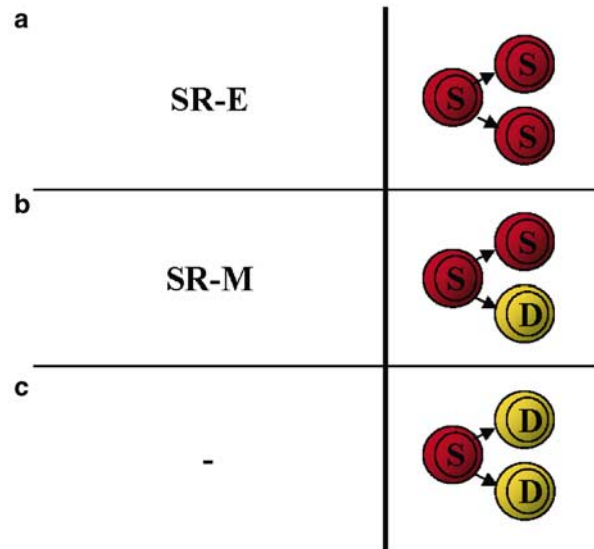


Figure 2 Symmetrical versus asymmetrical divisions of HSCs. (a) SR-E for self-renewal expansion results from a symmetrical HSC division. (b) SR-M for self-renewal maintenance occurs when HSC undergoes asymmetrical division. This process most likely characterizes adult-type HSCs, which occupy their niche in the bone marrow environment. (c) In this situation, symmetrical division occurs, leading to the production of two differentiated cells (or apoptotic cells) and HSC depletion. This mostly occurs when HSCs are grown *in vitro* under currently available conditions. D: differentiated cell (yellow); S: stem cell (red); SR-E: self-renewal expansion; SR-M: self-renewal maintenance

unpublished observation; see also Sauvageau, Humphries and Iscoe, in this issue). In a competitive environment, such as that observed in chimeric mice derived from mutant ES cell implementation, HSCs with impaired SR-E properties may be difficult to detect (Figure 3). Therefore, HSC specification versus SR-E deficiencies could be confused. Figure 3b illustrates an experimental design (involving HSC trans-complementation and *in vivo* reconstitution strategies), which may allow to distinguish between HSC specification and SR-E developmental defects and/or evaluate the 'full' SR-E potential of genetically modified (mutant) HSCs.

Among the genetic factors involved in regulating fetal liver HSC function, the *Meis1* locus, which encodes a homeobox gene of the TALE family, may be specifically involved in regulating SR-E. *Meis1* is highly expressed in fetal liver Sca-1⁺ Lin⁻ cells that are enriched for HSC activity (Pineault *et al.*, 2002). *Meis1* null mice are embryonic lethal and display a reduction in fetal liver cell counts and lineage-specific differentiation defects. Furthermore, *Meis1*^{-/-} fetal liver cells are unable to radioprotect lethally irradiated hosts and show an impaired potential to compete in reconstitution assays (Hisa *et al.*, 2004). Further studies including homing and complementation experiments (as those described in Figure 3b) will provide additional information on the role *Meis1* may play in regulating HSC self-renewal.

Overexpression of *Hoxb4* leads to an important *in vivo* and *ex vivo* expansion of HSCs when compared

Experimental condition	Specification defect	SR-E defect	SR-M defect
Loss of Function	Embryonic lethal phenotype. No stem cells are generated.	Mice may be viable. HSCs generated in the embryo sustain the production of differentiated cells.	Mice die as young adults of progressive anemia.
Chimeras	ES cells-derived hematopoiesis is undetectable.	ES cells derived hematopoiesis may be detectable at low levels in embryos and adults. Reduce competitiveness in comparison with wild type ES cells.	ES derived HSCs contribute to fetal but poorly to adult hematopoiesis.
Stem Cell Transplantation (adoptive transfer without complementation)	N.A.	No reconstitution unless very large cell doses are administered. Expansion is required for transplantation.	No long-term reconstitution. HSCs are not maintained and therefore no differentiated cells are produced in the transplanted mice.
Adoptive Transfer with Complementation ⁽¹⁾	N.A.	Reconstitution. Complementation and transplantation at limit dilution identify a severe decline in FL HSC numbers (b).	Reconstitution. Complementation and transplantation at limit dilution show normal HSC number in FL (c).

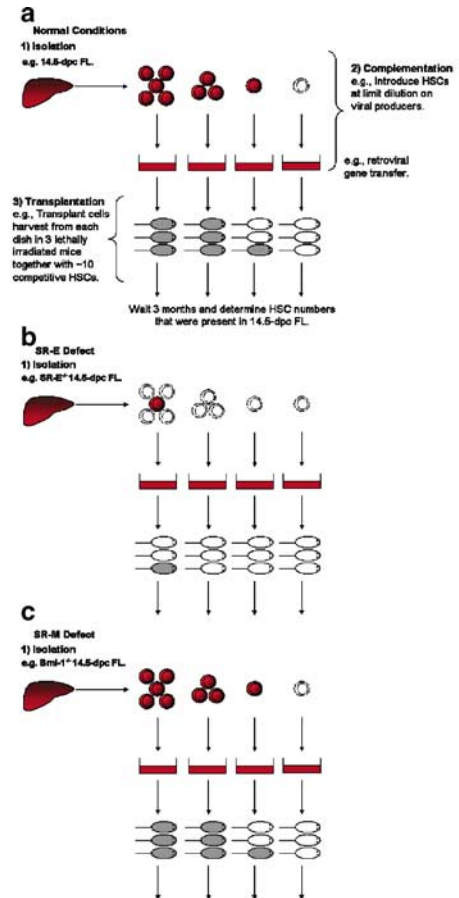


Figure 3 Bases for HSC specification, SR-E and SR-M. (1) Complementation can be performed by reintroducing the gene in 'trans' using, for example, retroviral gene transfer to 14.5-dpc fetal liver cells. Quantitative analysis is performed as indicated in the cartoon. ES cell: embryonic stem cell, FL: fetal liver, SR-E: self-renewal expansion and SR-M: self-renewal maintenance

to nontransduced cells (see Sauvageau, Humphries and Iscove, in this issue). As observed with control HSCs, much of the expansion occurring with *Hoxb4*-transduced cells is observed during the first few weeks following transplantation (Antonchuk *et al.*, 2001). This suggests that the SR-E program, which is operational during embryogenesis, may be reactivated following HSC transplantation and that certain *Hox* genes may play a role in this process (Figure 4). Ongoing studies using *Hox* gene mutant mice should help clarify this issue. Interestingly, HSCs engineered to overexpress an activated form of *STAT3* (*STAT3c*) behave similarly to those transduced with *Hoxb4*, potentially suggesting the activation of a common genetic program (Oh *et al.*, 2003). Moreover, Hox proteins interact with Pbx which itself interacts with Meis1 forming a trimeric nuclear complex involved in target gene regulation (Swift *et al.*, 1998; Jacobs *et al.*, 1999; Shen *et al.*, 1999; Liu *et al.*, 2001). The demonstration of a genetic interaction between these transcription factors in the control of fetal liver HSC self-renewal is much awaited.

Thus, much remains to be done to determine whether HSC self-renewal expansion (SR-E) is affected by the loss of function of several transcription factors involved in the specification of early hematopoietic cells (see

Table 1). It is hoped that complementary experimental strategies, such as those reported by Mikkola *et al.* (2003) or described in Figure 3b, will help clarify this issue.

Intrinsic regulators of adult HSC self-renewal

Until recently, very little was known about the genetic mechanisms that bring about the intrinsic programs of SR-M in early bone marrow hematopoietic cells. Accumulating evidence, from a number of recent studies, is now pointing to nuclear factors such as the *Polycomb-Group* (*PcG*) genes *Bmi-1* and *Mph-1/Rae-28*, *GATA-2* and *TEL* for potentially regulating this process. It is likely that these molecular programs will be distinct from those involved in regulating fetal liver HSC SR-E.

Recent studies indicated that the *Polycomb Group* (*PcG*) gene *Bmi-1* is dispensable for HSC specification and SR-E (both fetal and adult), but absolutely required for their *in vivo* maintenance (SR-M?). During embryonic/fetal liver (FL) (Park *et al.*, 2003) and adult (Lessard *et al.*, 1998; Lessard and Sauvageau, 2003) hematopoiesis, expression of the *Bmi-1* gene is highly enriched in

stem and multipotent progenitor cells (such as primitive human (CD34⁺ CD45⁻ CD71⁻) and mouse (Sca-1⁺ Lin⁻) bone marrow cells). Nullizyosity for the *Bmi-1* gene in mice leads to severe aplastic anemia due to a progressive impairment of bone marrow HSC function (van der Lugt *et al.*, 1994; Lessard *et al.*, 1999). Consequently, *Bmi-1* null mutants surviving beyond the first week of birth suffer from pneumonia and infections of the intestinal tract and die within 20 weeks of birth. Bone marrow-derived committed progenitors (i.e. myeloid colony-forming cells or CFCs) lacking *Bmi-1* are severely reduced in numbers and in their proliferative potential (van der Lugt *et al.*, 1994). Furthermore, the number and proliferative capacity of primitive myeloid (LTC-IC) and lymphoid (WW-IC) bone marrow progenitors in these mice are severely reduced (to 4 and 1% of wild-type levels, respectively) (Lessard *et al.*, 1999). Retroviral expression of *Bmi-1* in *Bmi-1*^{-/-} fetal liver cells completely rescued the absolute numbers of high and low proliferative potential myeloid colony-forming cells (HPP- and LPP-CFCs) to wild-type levels, indicating that *Bmi-1* is dispensable for the generation of FL-derived myeloid progenitors, but absolutely essential for their full proliferative activity (Lessard and Sauvageau, 2003). Similarly, transplantation studies performed at limiting dilution (LD) in sublethally irradiated recipients confirmed the presence of similar numbers of cells with long-term repopulating potential (LRC) in E14.5 *Bmi-1*^{-/-} fetal livers relative to controls. Importantly, the detection of the *Bmi-1*^{-/-} FL-derived HSCs, at 16 weeks post-transplantation, was strictly dependent on the retroviral expression of *Bmi-1*, indicating that *Bmi-1* is dispensable for the genesis of fetal liver-derived HSCs, but absolutely required for their maintenance. The long-term (16 wks) and pluripotent potential of these cells confirmed that the rescue was occurring at the HSC level (Lessard and Sauvageau, 2003). Clarke and co-workers also demonstrated an inability of bone marrow (BM) and E14.5 FL-derived *Bmi-1*^{-/-} cells to contribute to long-term hematopoiesis in reconstitution (FL and BM) as well as competitive (FL) experiments, suggesting a cell autonomous impairment of their SR-M potential (Park *et al.*, 2003). Of note, the proliferative defect in progenitors derived from *Bmi-1*^{-/-} E14.5 fetal livers (FLs) was much less pronounced than that observed in the bone marrow, suggesting a progressive impairment of the proliferative potential of hematopoietic cells lacking this gene (van der Lugt *et al.*, 1994; Lessard *et al.*, 1999). The apparent progressive instability of the HSC phenotype in the absence of *Bmi-1* may reflect complex epigenetic regulatory circuits established in a context-dependent manner during hematopoiesis. As these results are derived from the analysis of transplantation chimeras, fetal liver and bone marrow, *Bmi-1*^{-/-} HSC SR-M defects are likely cell-autonomous and hematopoietic cell specific. These findings, however, do not exclude additional functions of *Bmi-1* specific to cells of the microenvironment, as these would not be detected in such experimental settings. Together, these studies suggest that *Bmi-1* might be dispensable for HSC

specification and SR-E but required for HSC maintenance (Figure 2, SR-M?). Initial experiments indicated that *Bmi-1* lacks the capacity to induce HSC expansion when overexpressed (JL and GS, unpublished observation), reinforcing the notion that *Bmi-1* (SR-M) and *Hoxb4* (SR-E) may regulate distinct self-renewal programs (Figures 2 and 3).

The *Polycomb-Group* (*PcG*) *Mph1/Rae-28* gene product (a known nuclear partner of *Bmi-1*) also seems to play a key role in sustaining the activity of fetal (and adult?) HSCs (SR-M?). This was demonstrated by a progressive impairment in the numbers and proliferative potential of E14.5 FL-derived CFCs in *Mph1/Rae-28* mutant embryos when compared to wild-type littermates. The absolute number and proliferative potential of primitive myeloid long-term-culture-initiating cells (LTC-ICs) and colony-forming units in spleen (CFU-S₁₂) are also severely decreased (up to 20-fold) in *Mph1/Rae-28*^{-/-} fetal livers when compared to controls. Moreover, serial FL cell transplantation experiments performed at LD in sublethally irradiated congenic mice demonstrated a 15- to 20-fold decrease in CRU content/proliferative activity in *Mph1/Rae-28*^{-/-} embryos relative to controls. These studies indicate a crucial role for *Mph1/Rae-28* in maintaining the activity of HSCs during fetal hematopoiesis (Ohta *et al.*, 2002) and might explain the severe splenic/thymic hypoplasia and perinatal lethality observed in *Mph1/Rae-28*^{-/-} neonates (Takahara *et al.*, 1997; Ohta *et al.*, 2002). The zinc-finger transcription factor *GATA-2*, a member of GATA family that binds a common DNA sequence motif [T/A (GATA) A/G], also plays a critical role in maintaining the pool of multipotential progenitors and HSCs, both during embryogenesis and in the adult (Tsai *et al.*, 1994; Tsai and Orkin, 1997). *GATA-2* gene disruption makes reduction in all hematopoietic precursors, whereas enforced expression of *GATA-2* blocks normal hematopoiesis (Persons *et al.*, 1999). Similarly, the *TEL* (*translocation-Ets-leukemia* or *ETV6*) locus, which encodes an Ets family transcription factor, seems to be required specifically for hematopoiesis of all lineages within the bone marrow (SR-M?) (Wang *et al.*, 1998 and S Orkin, personal communication). Further studies will be necessary to establish whether this defect reflects an inability of *TEL*^{-/-} HSC/progenitors to migrate to the bone marrow or, more likely, to respond appropriately and/or survive within the bone marrow microenvironment.

Seminal work in *Drosophila* revealed that unequal inheritance of specific determinants (such as Numb) is the key to intrinsically determined asymmetric neural progenitor divisions and consequently, neural cell fate specification (see Faubert, Lessard and Sauvageau, in this issue). Whether a similar mechanism underlies HSC SR-M is still unknown. Future work should address whether *Bmi-1*, *Mph1/Rae-28*, *GATA-2* and *TEL* and other yet uncharacterized genetic determinants of HSC SR-M have a role in regulating the asymmetric partitioning of these cell fate determinants during asymmetric HSC divisions.

***Bmi-1* is required for the maintenance of leukemic HSCs**

There is strong support for the idea that cancer is a stem-cell disease (Dick, 2003). The similarity in the hierarchical organization of malignant and normal tissues is best characterized in the hematopoietic system. Human acute myeloid leukemia (AML) originates from a rare population of primitive cells (CD34⁺ CD38⁻) highly enriched in HSCs (Bonnet and Dick, 1997). Most leukemic cells (blasts) are limited in their proliferative capacity and must be constantly replenished by rare, self-renewing 'leukemic stem cells' (L-HSCs). So, like the normal hematopoietic system, leukemia seems to be organized as a hierarchy that originates from a stem-cell pool, which most likely retains remnants of the normal developmental program.

It has also been proposed that the initial, cancer-causing ('transforming') mutations occur in the self-renewing stem cell pool, rather than in already committed precursors. In this view, fewer mutations would be required to generate fully malignant cells if they were to originate from already self-renewing stem cells, as opposed to committed progenitors with low proliferative potential. Thus, two important findings have recently emerged from studies of stem cell biology and carcinogenesis: (1) in the process of neoplastic transformation, the genetic events responsible for disease progression must occur in a stem cell, unless one of the mutations would permit self-renewal in a downstream committed progenitor; (2) within the cancer or leukemia, only a subset of the cells that make up the tumor mass are tumorigenic – the 'cancer stem cells'

(reviewed in Reya *et al.*, 2001). These ideas predict similarities in the molecular programs of normal and cancer/AML stem cell self-renewal.

We recently demonstrated that *Bmi-1* is an essential determinant of leukemic stem and progenitor cell maintenance (Lessard and Sauvageau, 2003). Although *Bmi-1* is dispensable for the initial establishment of *Hoxa9-Meis1*-induced AML, replicative exhaustion of *Bmi-1*^{-/-} L-HSCs is reached upon transplantation into secondary recipients, leading to rapid proliferative arrest, differentiation, apoptosis of the leukemic blasts and resumption to normal host hematopoiesis. However, through high *in vitro* selective pressure, which induced epigenetic alterations of critical regulators of proliferation (including both *p19*^{ARF} and *p16*^{INK4a}), rare immortalized *Bmi-1*^{-/-} highly proliferative clones (HPCs) could be derived from *Bmi-1*^{-/-} AMLs (at a frequency of 1 versus 24% without selection in control AMLs). When assessed *in vivo*, some of these *Bmi-1*^{-/-} HPCs, initially nonleukemogenic, eventually acquired the capacity to induce AML, pointing to clonal evolution rather than selection, as the underlying mechanism responsible for leukemic progression in certain *Bmi-1*^{-/-} HPCs. Retroviral introduction of *Bmi-1* in these 'immortal' but nonleukemogenic *Bmi-1*^{-/-} HPCs could induce AML within similar latency periods than HPCs derived from control AMLs. The highly polyclonal nature and short latencies of these leukemias indicated that *Bmi-1* is sufficient to fully restore the leukemogenic potential of these clones. This demonstrates that *Bmi-1*'s function in L-HSCs involves more than cellular proliferation, which was apparently similar

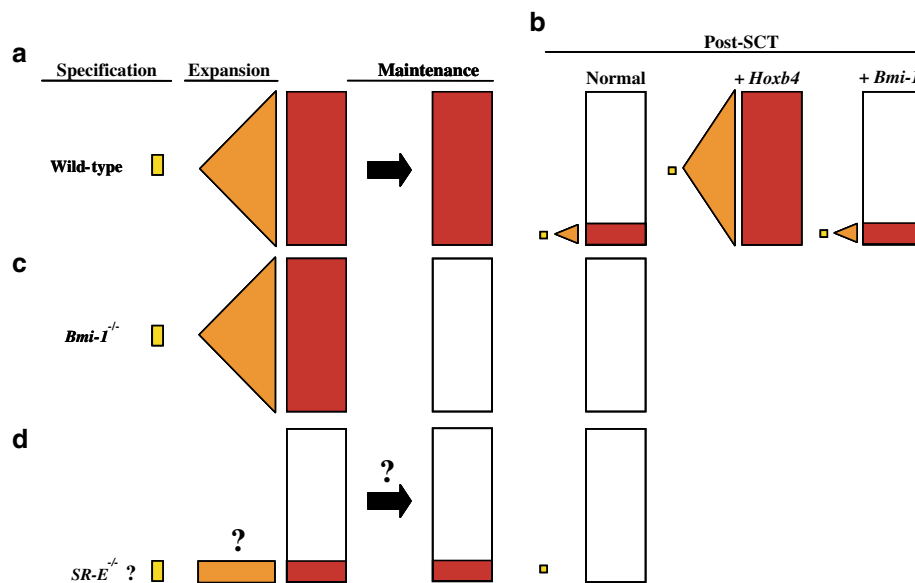


Figure 4 Self-renewal programs regulated by *Bmi-1* and *Hoxb4*. (a) Wild-type HSCs are generated (specification: yellow), expand in the embryo (expansion: orange) and are maintained during adult life (maintenance: red). (b) Wild-type stem cells can be transplanted into myeloablated recipient, where they reconstitute a fraction of the stem cell pool (post-SCT normal). HSCs overexpressing *Hoxb4* can reconstitute 100% of the stem cell pool after bone marrow transplantation into myeloablated recipient (post-SCT + *Hoxb4*) and overexpression of *Bmi-1* has no effect on the stem cell pool (post-SCT + *Bmi-1*). (c) HSCs lacking *Bmi-1* are generated, expanded but are not maintained through adult life. Since HSCs are not maintained, no stem cells transplantation is possible. (d) Stem cells deficient in the genetic program SR-E are generated but may fail to expand. As a result, HSCs cannot be transplanted into myeloablated recipient. SCT: stem cell transplantation

between the several selected HPCs derived from control and *Bmi-1*^{-/-} AMLs (Lessard and Sauvageau, 2003).

Importantly, epigenetic and genetic abrogation of *CDKN2A* (*p16*^{INK4a}) and *CDKN2B* (*p19*^{ARF}) are common lesions associated with poor prognosis in several human leukemias and mouse leukemia models (Hebert *et al.*, 1994; Maloney *et al.*, 1999; Schmitt *et al.*, 1999; Carter *et al.*, 2001; Garcia *et al.*, 2002). The observation that the leukemogenic potential of *Bmi-1*^{-/-} clones correlated with the loss of expression of *p16*^{INK4a} and *p19*^{ARF} underscores the importance of investigating whether these CKIs represent downstream regulators of *Bmi-1* function in normal and neoplastic HSCs. However, the inability of several *Bmi-1*^{-/-} clones, which lacked expression of both *p16*^{INK4a} and *p19*^{ARF} (and other CKIs), to induce AML when transplanted indicates that *Bmi-1*'s function in leukemic stem cells involves additional targets (Lessard and Sauvageau, 2003). Together, these studies indicate that *Bmi-1* is dispensable for the generation of normal and leukemic fetal liver-derived HSCs, but is absolutely necessary for their SR-M.

***Bmi-1*: a common regulator of stem cell function?**

The genetic mechanisms regulating self-renewal of the HSCs may be more generally applicable to other regenerating tissue systems. Recent findings implicated the *Notch* (Austin and Kimble, 1987; Henrique *et al.*, 1997; Varnum-Finney *et al.*, 2000), *Wnt* and *Shh* (Bhardwaj *et al.*, 2001; Wechsler-Reya and Scott, 2001; Zhang and Kalderon, 2001) signaling pathways in promoting stem cell self-renewal in a variety of different

epithelia in addition to HSCs. Interestingly, mutations of these pathways have been associated with a number of human neoplasia, including colon carcinoma and epidermal tumors (Chan *et al.*, 1999; Polakis, 2000) (*Wnt*), medulloblastoma and basal cell carcinoma (Gailani and Bale, 1999; Wechsler-Reya and Scott, 2001) (*Shh*), and T-cell leukemias (Ellisen *et al.*, 1991) (*Notch*). Supporting a potential role for *Bmi-1* in regulating the SR-M of stem cells others than the HSCs, severe skeletal and neuronal developmental defects were reported in the *Bmi-1* knockout mouse (van der Lugt *et al.*, 1994). Moreover, the overexpression of *Bmi-1* was observed in several cases of human non-small-cell lung cancer (Vonlanthen *et al.*, 2001), breast cancer cell lines (Dimri *et al.*, 2002) as well as immortalized mammary epithelial cells (MECs) (Dimri *et al.*, 2002). High-level DNA amplifications and gains of the region encompassing the human *Bmi-1* gene locus (10p13) have also been reported in several cases of head and neck carcinomas (Knuutila *et al.*, 1998). Future experiments will reveal whether the function of *Bmi-1* in regulating HSC SR-M extends to stem cells in other self-renewing tissues.

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