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SELF-RENEWAL AND DIFFERENTIATION OF HEMATOPOIETIC STEM CELLS: A MOLECULAR APPROACH (A review)*

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Two characteristics define a hematopoietic stem cell: the ability to differentiate into all hematopoietic lineages, and the ability to maintain hematopoiesis over a life span by a self-renewal process. The mechanisms that regulate the fate of blood-forming cells *in vivo*, however, are poorly understood. Despite the ability to culture hematopoietic progenitor cells (committed to particular lineages), *in vitro* culture of self-renewing multipotent stem cells has not yet been achieved. What is clear that both intrinsic and extrinsic signals regulate hematopoietic stem cell fate and some of these signals have now been identified, which will be highlighted in this review.

Keywords: asymmetric cell division, commitment, *hiwi*, Notch, *PTEN*, transcription factors

Introduction

Stem cells have the unique capacity not only to give rise to more stem cells (self-renewal) but also to generate differentiated progeny. They are present at all stages of development and probably exist in all multicellular organisms. In the blastocyst stage of the embryo before implantation, the inner cell mass contains cells that will become the fetus. Some of these cells are pluripotent stem cells that give rise to all types of somatic and germ-line cells. When these pluripotent cells are grown *in vitro*, they become embryonic stem-cell lines [1, 2].

The developmental stages of pluripotent embryonic cells and multipotent tissue-specific stem cells, such as hematopoietic stem cells (HSC), are still unclear. Pluripotent stem cells generate germ-line stem cells plus tissue-specific stem cells, perhaps by way of an intermediate class of multisomatic stem cells, which would differ

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from pluripotent cells by contributing to all somatic lineages, but not the germ line. Multipotent tissue-specific stem cells can be found from the fetal stage onward. In adults, they can participate in the renewal and regeneration of tissues, and during fetal life they may be units of tissue generation. It is likely that there are specific stem cells for most, if not all, tissues [3, 4, 5]. The past few years have seen the advent of several paradigm shifts in stem cell biology, some of which will be highlighted in this review. The basic questions to be addressed include the following: What determines the self-renewal capacity of HSC? How do HSC choose their fates? To what extent do cell-autonomous programs in HSC, versus the sequential presentation of instructive signals by the microenvironment, underlie the orderly generation of different lympho-hematopoietic cells? And just how broad is the developmental potential of HSC?

The cell

Currently, there is no single phenotypic characteristic that can be used to define uniquely human or murine HSC. However, using a variety of separation techniques and functional assays, numerous laboratories have established a pattern of surface antigen expression that can be used largely to isolate HSC from the majority of bone marrow or peripheral blood cells. The human stem-cell phenotype includes expression of CD34, CD133 and CD90 (Thy-1) in the absence of a series of other antigens that mark differentiating lineages (Lin); one widely applied criterion is the absence of CD38 on cells that express CD34. In the mouse, expression of Sca-1, c-kit and Thy-1.1 in the absence of lineage markers segregates a multipotent population of primitive cells from the majority of committed progenitors and mature cells [6, 7]. Other phenotypic characteristics, such as low-level staining by the activation-sensitive probe rhodamine 123 or the active efflux of both rhodamine 123 and Hoechst 33342 dyes, have also been successfully used to define a primitive subset (side population or SP) of bone marrow cells and even to predict the existence of HSC that are CD34 negative [8, 9].

The inability to purify stem cells to a homogeneous population makes the use of functional HSC assays necessary. In the mouse, short-term repopulating cells (STRC) are measured as cells that prevent death of lethally irradiated recipients for a limited period of time (typically <12–16 weeks), whereas the numbers of long-term repopulating cells (LTRC) are estimated by their multilineage hematopoietic reconstitution of the recipients for lifetime or their successful transplantation into a secondary recipient. The most definitive measure of mouse LTRC is their ability to compete for engraftment against other HSC. In this competitive repopulation assay lethally irradiated recipients are transplanted with limiting numbers of syngeneic marked “test” cells, together with unmarked “helper” cells. By scoring the function of

recipients with lympho-myeloid repopulation by the test cells at various time points as a function of the number of transplanted cells, the number of individual "competitive repopulating units (CRU)" in a cell suspension can be determined by application of Poisson statistics. If competitive repopulation is done using low numbers of putative HSC, it is possible to measure the repopulating ability of a single stem cell. Because *in vivo* repopulation experiments cannot be done in humans, surrogate assays and xenogeneic transplant models have been developed to enumerate human HSC [10, 11, 12].

It now appears that HSC may accurately be defined as multipotent transplantable cells with variable replicative potential that are subject to developmental changes and unpredictable (stochastic?) components in their behaviour as well as poorly characterized interactions with the hematopoietic microenvironment from the host.

The microenvironment

The hematopoietic microenvironment of the bone marrow is extremely complex. The realization that stable, custom microenvironments might control HSC led *Schoefild* to call such regions "niches". It comprises many different cell types (e.g. macrophages, adipocytes, fibroblasts, reticulocytes), which lay down an extracellular matrix (ECM) of key importance to stem and progenitor cells. Stem cells express integrins, which interact with counterreceptors and also extracellular matrix molecules (e.g. fibronectin) within the stromal environment. This interaction therefore provides an adhesive interface between the hematopoietic and stromal cells. Also, morphogens and cytokines associate specifically with matrix molecules (e.g. heparan sulfate) and might be "presented" to primitive hematopoietic cells within their niches. There is also evidence of gap-junction connections between hematopoietic cells and stromal cells. These closely interrelated regulatory pathways can be simplified as cell: cell, cell: factor and cell: ECM interactions [13, 14, 15].

Defining the components of stem cell niches becomes even more complex when it is considered that in some instances more than one stem cell may reside within a niche. This appears to be the case for the bone marrow, which houses not only HSC, but also mesenchymal stem cells. These cells can replicate as undifferentiated cells, but possess the capacity to differentiate into lineages of mesenchymal tissues, including bone, cartilage, fat, tendon, muscle and marrow stroma [3, 16, 17]. This raises the intriguing possibility that different stem cell populations can respond differentially to cues within a niche, and perhaps even influence each other. Overall, this finding adds additional challenges to defining the molecular characteristics of stem cell niches.

Decision, decision, decision…

Although *in vivo* data indicate that HSC can undergo a moderate to large number of fully self-renewing divisions wherein the actual number of HSC can increase, it has been impossible to reproduce this in an *in vitro* setting, and not for lack of trying. Despite a plethora of culture systems, growth factors and stroma cell lines, the best results reported to date only lead to the maintenance of stem cell activity for limited times and/or to very modest increases in cell numbers. Many soluble growth factors have been identified that play a role in controlling proliferation and differentiation of HSC. Expansion of STRC but only very modest expansion of cells that establish hematopoiesis in secondary recipients are observed when murine or human HSC are cultured in the presence of combinations of potent hematopoietic cytokines such as c-Kit and Flt3-ligands, thrombopoietin, IL-3, IL-6 and IL-7. This suggests that known "early acting" cytokines, as the sole stimulus, do not allow cell division without the loss of repopulating ability [2, 18, 19].

During or after cell division, the two daughter cells of a HSC have to decide their fate. They can either choose the same (symmetric division) or different (asymmetric cell division) fates. The fates between which they choose are to remain an HSC (self-renewal), commit to differentiation or to undergo apoptosis. In addition, there is the decision to stay in the bone marrow or to migrate out (mobilization). The balance between these cell-fate decisions determines the size of the HSC compartment. It is probably less important in determining the production of mature blood cells because the number of HSC available for differentiation under steady-state conditions greatly exceeds the number necessary to produce the daily required number of mature cells [20, 21, 22].

Key regulatory molecules

The genes, factors and signal-transduction pathways involved in the HSC cell-fate decision are still unclear, but much work is being done to elucidate these processes. Some have argued that the decision to self-renew without differentiation is stochastic. If this is true, no factor(s) could alter stem cell fate decisions (18, 19). However, activation of certain genetic programs clearly increases the self-renewal ability of HSC.

Homeobox genes

Homeobox genes are regulatory genes encoding nuclear proteins that act as transcription factors during normal development. The homeobox is a 183-bp DNA

sequence, originally identified in *Drosophila*, coding for a 61-amino-acid domain defined as homeodomain (HD); it is usually located at the terminal or sub-terminal position of the corresponding homeoprotein, and is responsible for recognizing and binding sequence-specific DNA motifs. The specificity of this binding allows homeoproteins to activate or repress the expression of batteries of downstream effector target genes. Through sequence similarities within the HDs and flanking regions, different HD types or classes are distinguishable, each one characterizing a homeobox gene family. Novel and divergent homeobox genes are being continuously isolated. Recent indications suggest that homeobox genes constitute as much as 0.1–0.2% of the whole vertebrate genome. The role of homeobox-containing genes in embryonic development has been extensively investigated since their discovery. Furthermore, several indications suggest the involvement of these gene families in crucial biological processes of adult eukaryotic cells such as the control of cell identity, cell growth and differentiation, cell-cell and cell-ECM interactions [23, 24].

Several lines of evidence indicate that appropriate homeobox-gene expression is required for normal hematopoiesis. For example, it is well known that overexpression of HOXA10 in murine hematopoietic cells perturbs both myeloid and lymphoid differentiation and leads to acute myeloid leukemia [25, 26]. Recently, *Antonchuk* et al. [27] have shown the potency of *HOXB4* to enable high-level *ex vivo* HSC expansion. Cultures of normal murine bone marrow cells experienced large HSC losses over 10-14 days. In sharp contrast, cultures of *HOXB4*-transduced cells achieved rapid, extensive, and highly polyclonal HSC expansion, resulting in over 1000-fold higher levels relative to controls and a 40-fold net HSC increase. Importantly, these HSC retained full lympho-myeloid repopulating potential and enhanced *in vivo* regenerative potential, demonstrating the feasibility of achieving significant *ex vivo* expansion of HSC without functional impairment. Therefore, these data suggest that it should be possible to develop a HOXB4 protein that can act as an HSC-specific mitogen.

Another homeobox transcription factor expressed in hematopoietic stem/progenitor cells but absent in lineage-committed bone marrow cells is the *Pitx2*. Thus, it may also play a role in hematopoietic stem cell biology. During organogenesis, *Pitx2* is expressed in the left lateral plate mesoderm and, subsequently, in the left heart and gut of the mouse, chick and *Xenopus* embryos. Moreover, *PITX2* mutations cause Rieger syndrome in human. Thus, it seems to serve as a critical downstream transcription target that mediates left-right asymmetry in vertebrates [28].

Hiwi (piwi)

Stem cells can sometimes be identified quite precisely by their morphology or location. In the *Drosophila* ovary, for example, stem and nonstem daughters have a well-defined orientation with respect to the surrounding cells. The adult ovary consists of approximately fifteen ovarioles, each with a specialized structure, the germarium, situated at the most anterior tip. Each germarium contains 2 to 3 germline stem cells (GSC) that are in direct contact with specialized somatic cells, the basal terminal filament cells. GSC undergo asymmetric divisions to produce a daughter stem cell and a differentiated daughter cell, a cytoblast. A number of genes including *dpp, piwi, pumilio*, and fs(1)Yb have been identified and shown to be essential for GSC maintenance. Among these genes, *piwi* has been of special interest. It has recently been demonstrated to be an essential stem cell gene in *Drosophila* and to be expressed in tissues belonging to many species including human. The *Drosophila piwi* gene is required for asymmetric division of GSC but is not required for differentiation of committed daughter cells. Expression of *piwi* in adjacent somatic cells, terminal filament cells, regulates GSC division [29]. *Sharma* et al. [30] have demonstrated first, that the human equivalent of *piwi*, termed *hiwi*, is expressed in a variety of human tissues including primitive hematopoietic cells. The gene was cloned from human bone marrow $CD34^+$ cells and the HIWI protein was shown to have 52% homology with the *Drosophila* PIWI protein at the amino acid level. They have also shown that *hiwi* expression within the hematopoietic compartment is unique to the most primitive ($CD34^+$) progenitors and is diminished or absent in more differentiated cells. Furthermore, when $CD34^+$ cells were placed in conditions that favoured differentiation *in vitro, hiwi* gene expression declined synchronously with cellular differentiation. Therefore, *hiwi* may be an important developmental regulator, which, in part, underlies the unique biologic properties associated with HSC.

The conservation of the *piwi*-mediated mechanism appears to extend to the plant kingdom as well. The overall homology between PIWI and ZWILLE (ZLL) and argonaute (AGO) is worth noting. Intriguingly, ZLL is essential for maintaining stem cells of the shoot meristem in an undifferentiated state during the transition from embryo-specific development to repetitive organ formation through the self-perpetuating shoot meristem division. AGO also plays an important role in maintaining normal apical shoot meristem function [31]. Thus, the homology between *piwi*, *hiwi*, *ZLL* and *ago* suggests the existence of a novel family of "stem-cell-specific genes" essential for stem cell renewal in diverse organisms (moreover, they belong to a family of molecules implicated in post-transcriptional gene silencing) [32].

PTEN and SHIP

PTEN (for phosphatase and tensin homolog deleted on chromosome ten) was identified as a candidate tumor suppressor on chromosome 10q22-24. Loss of heterozygosity at this locus is observed in several spontaneous human malignancies including myeloid and lymphoid neoplasms, making it, like *p53*, one of the most commonly mutated genes in human cancer. The PTEN protein is a lipid phosphatase that dephosphorylates the D3 position of phosphatidylinositol 3,4,5-triphosphate (PIP_3), a product of phosphatidylinositol 3-kinase (PI3K). Thus, PTEN lowers the amount of the PI3K product, PIP_3, within cells and antagonizes PI3K-mediated cellular signaling pathways. There also may be additional PTEN substrates because PTEN can also dephosphorylate both phosphotyrosine- and phosphoserine/threonine-containing substrates *in vitro*. However, negative regulation of PIP_3 is the critical determinant for control of tumor growth. PI3K and its product PIP_3 regulate many cellular processes including proliferation, transcriptional regulation, glucose metabolism, cell migration, and protein synthesis, and they also protect against apoptosis [33, 34]. Recently, *Groszer* et al. [35] have added another important cellular process to this list: the negative regulation of neural stem cell proliferation. They found that mice lacking PTEN exhibit enlarged, histoarchitecturally abnormal brains, which resulted from increased cell proliferation, decreased cell death and enlarged cell size. Neurosphere cultures revealed a greater proliferation capacity for $Pten^{-/-}$ central nervous system stem cells, which can be attributed, at least in part, to a shortened cell cycle. However, cell fate commitments of the stem cells were largely undisturbed.

Genetic inactivation of lipid phosphatese called SHIP1 (src homology 2-containing inositol phosphatase) – which dephosphorylates the D5 position of PIP_3 and thus (in the same way as PTEN) negatively regulates PI3K signaling – results in enhanced proliferation and differentiation of HSC in response to growth factors, and a reduction of apoptosis of myeloid cells. In contrast to PTEN, however, there is no evidence that inactivation of SHIP1 or other D5 lipid phosphateses cause malignancies. Rather, inactivation of SHIP1 results in progressive myeloid hyperplasia, and inactivation of its cousin, SHIP2, leads to early neonatal death due to increased insulin receptor signaling and hypoglycemia [36]. Therefore, transient inactivation of PTEN (and/or SHIP1/SHIP2) could provide a booster shot for a rare stem cell population needed to self-renewal. The caveat is that such an approach would have to circumvent the procancer consequences of lipid phosphatase inactivation in other cell types [34].

Notch

The Notch family comprises a group of highly conserved proteins that functions both as cell surface receptors and direct regulators of gene transcription. Notch signalling is the pre-eminent pathway that directs binary cell fate decisions in the embryo. Notch signaling limits the number of cells adopting a particular fate and leaves some progenitors uncommitted but competent to adopt alternative fates. Further, the lateral inhibitory activity characteristic of Notch could contribute to the well-known, but "mysterious", ability of paired daughter cells to adopt different fates both *in vivo* and *in vitro*. Thus, stochastic or random, stem-cell commitment could be explained by the type of signaling observed with Notch [37, 38].

Notch was first described in *Drosophila* as a single transmembrane protein that functions as a receptor for two different ligands, Delta and Serrate. Vertebrates express multiple Notch receptors (Notch1-4) and ligands (Delta-like 1, 3, 4 and Jagged (Serrate)-1, 2). Interaction between cells expressing Notch and cells expressing Notch ligands result in proteolytic cleavage of Notch within or close to its transmembrane domain. This releases the intracellular domain of Notch, which translocates to the nucleus and interacts with the CBF1 transcription factor, converting it from a repressor to an activator of gene transcription. Genes that are transcriptionally regulated by Notch include genes that can either inhibit or promote cell differentiation. For example, HES gene products are transcriptional repressors that are a conserved downstream target of Notch signaling and function, in part, by repressing the transcription of tissue-specific bHLH transcription factors that influence lineage commitment and other events. (The transcriptional regulation of Notch by Notch signaling is part of a positive feedback loop.) [39, 40]

Interest in a possible role for Notch in hematopoiesis originated with the finding that Notch1 is expressed in human $CD34^+$ stem/progenitor cells [41], and that a t(7;9) chromosomal translocation resulting in constitutively active Notch1 occurs in a subset of T-lineage ALL [42, 43]. The finding that soluble recombinant Notch ligand Jagged1 is capable of maintaining the survival and expanding human HSC population capable of pluripotent reconstituting capacity strengthened a role for Notch in hematopoietic lineage fate decisions. In general, exposure of mouse and human HSC to Notch ligands induces their self-renewal and expansion without markedly altering their differentiative potential. All four Notch transcripts are expressed in hematopoietic cells. Notch1 is preferentially expressed on thymocytes and also detected on marrow precursors. In contrast, Notch2 is found on both thymocytes and B-cells as well as precursor cells. Notch3 is expressed on various types of hematopoietic cells. Expression of Notch4 is dominant in endothelial cells but it is also expressed on macrophages. Normally, HSC

express higher levels of Notch2 than Notch1, raising the possibility that individual Notch family members might have distinct roles in HSC self-renewal and differentiation. However, a careful study of the expression patterns of the various members of this family in HSC, and of the behaviour of purified HSC that have been stimulated with various ligands, should clarify the exact role of this receptor family in cell-fate decisions of HSC and more committed progenitors [22, 44, 45, 46].

Morphogens

By definition, morphogens are molecules that specify different cell fates in a concentration-dependent manner. Three classes of secreted proteins, namely hedgehogs, Wnts and bone morphogenetic proteins fit this criterion in that they activate transcription of distinct target genes in a dose-dependent manner, thereby enabling cells to adopt different fates [47].

The vertebrate hedgehog family is represented by at least three members: Desert hedgehog, Indian hedgehog (Ihh) and Sonic hedgehog (Shh). (The original *hedgehog* gene, identified in *Drosophila*, is named after its mutant phenotype: the embryo is covered with pointy denticles, resembling a hedgehog.) As shown by *Bhardway* et al. (48), Shh regulates hematopoiesis in a dose-dependent manner. In cell cultures, the addition of Shh increases the number of blood cells capable of repopulating nonobese diabetic-severe combined immunodeficient (NOD/SCID) mice. Conversely, cytokine-dependent differentiation was inhibited by the addition of Shh-blocking antibodies. The effects of Shh are exerted by establishing BMP homeostasis in the bone marrow; Shh inhibits both BMP-4 and a BMP inhibitor called Noggin. Noggin is able to block the mitogenic effects of Shh, whereas antibodies to Shh cannot block the proliferative effect of BMP-4. This suggests that much of the influence of Shh on hematopoiesis is mediated through BMP. Nevertheless, Shh induces the expansion of repopulating cells while BMP cannot, which implies that Shh has additional effects that are independent of BMP. Thus, for HSC in the bone marrow, the regulation of BMP and Noggin by Shh "fine-tunes" the local effective concentration of BMP [48].

Another member of the Hedgehog family, Indian, is produced by visceral endoderm, can induce formation of blood and endothelial cells in explant cultures and can reprogram prospective neuroectoderm (anterior epiblast) along hematopoietic and endothelial (posterior) lineages. Downstream targets of the hedgehog signaling pathway are upregulated in anterior epiblast cultured in the presence of Ihh protein, as is *Bmp4*, which may mediate the effects of Ihh. Blocking Ihh function in primitive endoderm inhibits activation of hematopoiesis and vasculogenesis in the adjacent

epiblast, suggesting that Ihh is an endogenous signal that plays a key role in the development of the earliest hemato-vascular system [49].

Another particularly interesting pathway that has also been shown to regulate self-renewal in different organs is the Wnt signalling pathway. Wnt proteins are intercellular signalling molecules that regulate development in several organisms and contribute to cancer initiation when dysregulated. The expression of Wnt proteins in the bone marrow suggests that they may influence HSC as well. Using highly purified mouse bone marrow HSC, it has been shown that overexpression of activated β-catenin (a downstream activator of the Wnt signalling pathway) in long-term cultures of HSC expands the pool of transplantable stem cells determined by both phenotype ($Sca1^{+}c\text{-}kit^{+}Thy1.1^{low}Lin^{-}$) and function (ability to reconstitute the hematopoietic system *in vivo*). Moreover, ectopic expression of Axin, an inhibitor of Wnt signalling, leads to inhibition of HSC proliferation, increased death of stem cells *in vitro*, and reduced reconstitution *in vivo*. Conversely, soluble Wnt proteins from conditioned supernatants have also been shown to influence the proliferation of hematopoietic progenitors from mouse fetal liver and human bone marrow. However, the molecular mechanisms by which Wnt signalling influence stem cells remain to be elucidated [50]. It will also be important to determine whether the Notch, Shh and Wnt pathways interact to regulate hematopoietic stem and progenitor cell self-renewal.

Telomere/telomerase

A final example of molecular control of stem cell fate concerns an intrinsic mechanism for maintaining genomic stability and responding to DNA damage. Telomeres, the ends of chromosomes, consist of repeats of six-base DNA sequence (TTAGGG) that preserve chromosome integrity and prevent end-to-end fusions. A reverse transcriptase called telomerase is responsible for adding the TTAGGG sequence to the chromosome ends. Telomerase consists of a catalytic subunit (TERT) and an RNA template (TR). Telomerase is expressed in highly proliferative cells throughout the embryonic development and is then dramatically downregulated as cells differentiate, and is not detectable in many somatic cells in the adult. Therefore, progressive shortening of telomeres could act as a mitotic clock, counting off divisions before senescence. Stem cells may not be subject to senescence because of constitutive telomerase activity [51].

Telomeres in HSC in adult bone marrow and peripheral blood are shorter than in comparable cells from cord blood and fetal liver, suggesting attrition in the course of normal replication that occurs as a function of chronological age. Clinical observations of patients undergoing bone marrow transplantation revealed that the telomeres in the

peripheral blood mononuclear cells of the recipient were shorter than in the mononuclear cells in the donor. Hence, the transplanted stem cells seemed to have undergone extensive replication in the process of reconstituting the recipient. Analysis of telomerase activity in HSC has demonstrated a low constitutive level, with the lowest values observed in fetal liver and the highest levels in bone marrow HSC. The basal constitutive levels of telomerase are apparently insufficient to prevent the shortening of telomeres during normal replication. Although there is no indication that HSC actually reach replicative senescence under normal physiological conditions, their expansion potential may still not be infinite [52, 53]. Conversely, elderly first-generation telomerase null mice have normal hematopoiesis and no detectable abnormalities in the testis, intestine or epidermis. However, by the six generation, long-term renewal of HSC is compromised, male mice are infertile, and there is hair loss and delayed repithelialization of skin wounds [54]. Thus, although a telomerase-based clock may not control progenitor populations during the normal life span of the mouse, such a role may be important in longer-living mammals such as humans.

Master controls or control committee

Hematopoiesis is usually depicted in a hierarchical fashion, with HSC giving rise first to progenitors and then to precursors with varying commitments to multiple or single pathways. Although this representation oversimplifies hematopoietic development, it provides a useful framework in which to consider the properties of the intermediate cell populations. The critical developmental decisions are thought to be executed by transcription factors. Models, naively fashioned after the simplest interpretation of early experiments of myogenesis induced by myoD, posited unique roles of individual lineage-restricted regulators (master regulators) in commitment. Accordingly, one might anticipate the discovery of single (master) transcription factors whose expression in multipotential progenitors would lead to unilineage commitment and subsequent differentiation [55, 56]. However, there are numerous examples where seemingly committed hematopoietic cells can be induced to convert into cells of another lineage. Perhaps the greatest plasticity of this kind yet demonstrated is the *in vitro* and *in vivo* differentiation of *Pax5*-deficient pre-B I cells into various hematopoietic lineages. Transcription factor *Pax5*-deficient ($Pax5^{-/-}$) pre-B I cells can give rise *in vitro* to myeloid cells, such as macrophages, granulocytes and osteoclasts; to DCs; and to NK cells in the proper environments of cytokines and cell contacts. *In vivo* they can differentiate to all these cell types and also to erythrocytes and to thymocytes and mature T cells. Moreover, $Pax5^{-/-}$ pre-B I cells appear to have all the properties of HSC, i.e., the capacity of self-renewal, long-term reconstitution, and

multipotency. In contrast to HSC, however, which have a limited capacity to proliferate under similar *in vitro* culture conditions, *Pax5*$^{-/-}$ precursor B-cell clones can be grown on stromal cells in the presence of IL-7 for extended periods, retaining their reconstitution capacity and hematopoietic multipotency. In normal B lymphocyte development, the function of *Pax5* thus appears to be just as much to suppress promiscuous transcription of genes specific for other hematopoietic lineages as to promote the expression of B-cell specific genes [57, 58]. According to this and several other examples [59], lineage selection is not so much a matter of commitment, but rather a somewhat unstable and progressive outcome of highly plastic and combinatorial regulatory interactions that are influenced by subtle effects operating at many different levels. Prior to unilineage commitment and differentiation, hematopoietic progenitor cells express markers of both erythroid, myeloid and lymphoid lineages, including lineage-affiliated cytokine receptors, rather than only one set of lineage markers [60, 61].

Several models may be considered to account for these findings. Perhaps the presence of multilineage markers at low levels merely reflects nonspecific activation at the transcriptional level. Arguing against this is the absence of markers of totally irrelevant programs, such as myogenic regulators (myoD). Alternatively, multilineage gene expression may be considered as a genetic harbinger of programs to follow and one of the molecular mechanisms that ensure multiple developmental options. This seems likely to be the case, as multilineage gene expression – perhaps manifest only at a low level, in random bursts or in a cell-cycle-dependent fashion – provides a cellular context in which the positive and negative activities of the respective key regulators are inventoried to generate output for differentiation. Once a tentative decision is reached, mutual reinforcement of a stable pathway for subsequent differentiation may be provided by auto-upregulation of one lineage factor and cross-antagonism of others [62, 63, 64].

Concluding remarks

It has proven surprisingly difficult to manipulate HSC without losing them as a result of differentiation or apoptosis. As yet, the combination that will allow HSC to be manipulated in a therapeutic setting remains unknown [2, 7]. Recently, it has become feasible to interrogate entire genomes for global gene expression patterns as a function of defined biologic properties. Oligonucleotide or cDNA microarrays provide rapid means to identify such patterns. Microarrays representing most, if not all, genes in the human and mouse are now commercially available. Although not yet easily affordable by many laboratories, the potential informational gained from even a limited use of

these microarrays are enormous. Not only is it feasible to rapidly and in parallel analyze the entire genome at work in different stem cell populations, but also in response to induced biologic changes in the properties of these cells [65, 66, 67]. Indeed, one can take advantage of the difficulties that encumber *ex vivo* stem cell expansion efforts to identify important stem cell regulatory molecules. However, HSC have stubbornly resisted most manipulations thus far, and it might be a while longer before the culturing of HSC is as a routine as that of embryonic stem cells today.

Table I

Assays commonly used to detect hematopoietic activity*

Colony-forming-unit cell (CFU-C) assay(s)	Committed hematopoietic progenitor cells, in the presence of appropriate growth factors are able to form visible colonies after 7–14 days in semisolid medium. CFU-C assays can be used in both murine and human systems. However, CFU-C is not reliably predictive of engraftment potential.
Colony-forming-units-spleen (CFU-S) assay	Spleen colonies formed 8–12 days after injection of test cells into a lethally irradiated host. This is the original quantitative stem cell assay in the mouse. Day 12 colonies represent more primitive cells than day 8 colonies. The limit of this assay is that early progenitor populations can form similar colonies. The colonies are visible as white nodules in the fixed spleen.
Long-term culture-initiating cell (LTC-IC) assay	In the LTC-IC assay, cell populations that contain HSC are plated onto a performed layer of stromal cells. The cultures are followed for the intermediate to long-term maintenance of hematopoietic progenitor cells (colony-forming unit cells) defined by a secondary culture in semisolid medium.
Cobble-stone-area-forming cell (CAFC) assay	In this assay, the test population is plated onto a preformed stromal cell monolayer. Stem and progenitor cells grow in a specific fashion, called cobble-stone-areas. Cultures are scored for the presence of these structures at different time points, usually 7–35 days after plating. The more primitive the input cell is, the longer it will maintain growth as cobble-stone-areas. Appearance of cobble-stones within 7 days indicates progenitor cells, while appearance after 35 days indicates a more primitive stem cell. The use of limiting-dilution statistics allows frequency estimates of different hematopoietic stem and progenitor cells.

*The "gold standard" is the long-term reconstitution of hematopoiesis in mice that had undergone a lethal preconditioning regimen, usually irradiation, to ablate or diminish endogenous hematopoiesis. Reconstitution with a cell fraction that contains functional HSC can be direct or indirect (see text).

Table II

Summary of selected transcriptional factors and their role in hematopoiesis

Factor	Type	Expression*	Hematopoietic requirement**
SCL/tal-1	BHLH	Prog, E, Meg	All (embryonic and definitive) hematopoiesis
Runx1/AML1	Runt	Prog	Definitive hematopoiesis
GATA-1	Zinc finger	Prog, E, Meg	E and Meg differentiation
GATA-2	Zinc finger	Prog, Meg	Proliferation/survival of hematopoietic stem/progenitor cells
GATA-3	Zinc finger	Prog, T cells	T cell development
PU.1	Ets	Prog, myeloid, B cells	Myeloid, T and B cell development
FOG-1	Multi-type-zinc finger	Prog, E, Meg	E and Meg differentiation
Ikaros	Zinc finger	Prog, T, B and NK cells	Lymphoid cell differentiation
Pax5	Paired box	B cells	B cell development

*Abbreviations: Prog, progenitors; E, erythroid; Meg, megakaryocyte;
***In vivo* requirements in development have generally been established by gene targeting in mice.

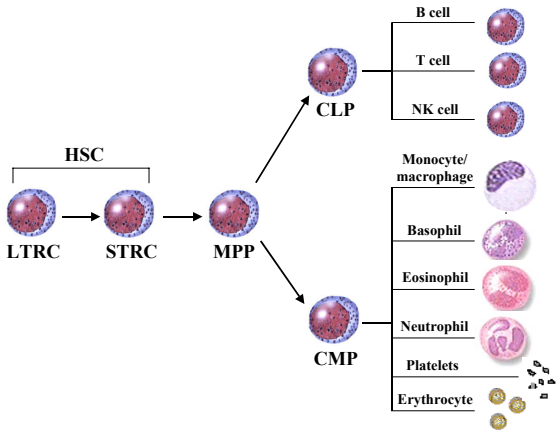

Fig. 1. An overview of the mouse hematopoietic system. HSC can be divided into cells that can reconstitute an animal for life (long-term repopulating cells, LTRC) or for limited periods of time (short-term repopulating cells, STRC). These differentiate into multipotent progenitors (MPP) that have the capacity to differentiate into a subset of hematopoietic lineages. A common lymphoid progenitor (CLP) has been clearly defined in mouse and human; the same myeloid progenitor (CMP) is less well defined. These multipotent progenitors will differentiate into progressively more restricted, committed progenitors (not shown) and become the mature hematopoietic cells of various lineages, some of which are shown

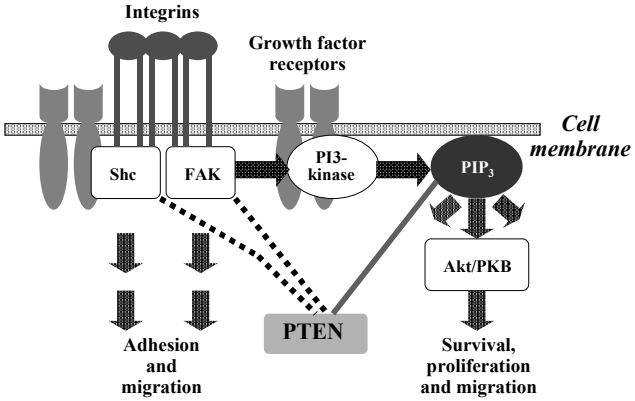

Fig. 2. Regulation of signaling by PTEN. Extracellular interactions trigger signaling from integrins and growth factor receptors. The major function of PTEN appears to be the downregulation of the PI3-kinase product PIP3, which regulates (Akt/PKB) and complex downstream pathways affecting cell growth, survival and migration. In addition, PTEN has weak protein tyrosine phosphatase activity, which may target focal adhesion kinase (FAK) and Shc, and thereby modulate other complex pathways

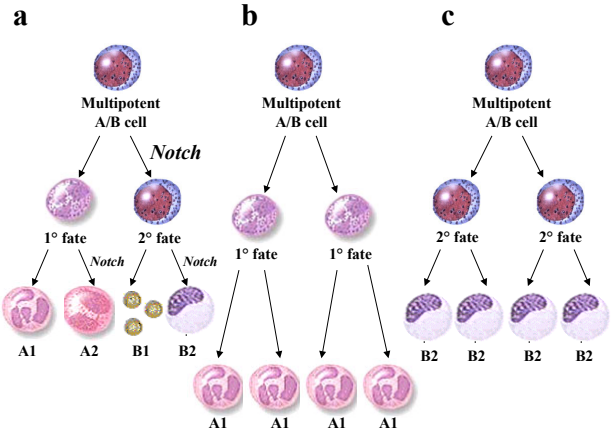

Fig. 3. Notch functions through successive cell divisions to influence the numbers and types of cells generated from a multipotent progenitor. Normal Notch expression (a) allows the A/B progenitor to give rise to cells of four distinct lineages; at each cell division, the daughter cell expressing fewer Notches adopts the primary fate, whereas the cell expressing more adopts the alternative secondary fate. The A/B progenitor gives rise to A and B cells; progeny of A cells expressing less Notch subsequently adopt the primary fate A1, whereas those expressing more Notch adopt the secondary fate A2; the same occurs for type B cells. The result is the balanced production of cells of all four lineages. When Notch activity is dysregulated, the result is the overproduction of one cell type at the expense of another. With loss of Notch function (b), all cells adopt the primary fates resulting in production of only A1 cells. With increased Notch activity (c), daughter cells adopt the secondary fates, generating only B2 cells

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