

Regulation of Hematopoietic Stem Cell Self-Renewal

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ABSTRACT

Every day, billions of new blood cells are produced in the body, each one derived from a hematopoietic stem cell (HSC). Because most mature blood stem cells have a limited life span, the ability of HSCs to perpetuate themselves through self-renewal and generate new blood cells for the lifetime of an organism is critical to sustaining life. A key problem in hematopoietic stem cell biology is how HSC self-renewal is regulated. Recent evidence suggests that signaling pathways classically involved in embryonic development — such as the Wnt signaling pathway — play an important role in regulating stem cell self-renewal. The Wnt signaling pathway has been shown to regulate stem cell fate choice in a variety of organs, including the skin, the nervous system, and the hematopoietic system. In the hematopoietic system, stimulation of hematopoietic progenitors and stem cells with soluble Wnt proteins or downstream activators of the Wnt signaling pathway leads to their expansion. Future studies focusing on the mechanism of action of the Wnt signaling pathway and its interaction with other pathways are needed to gain further insight into the regulation of stem cell self-renewal, not only in the hematopoietic system but also in a variety of other tissues.

I. Isolation and Characterization of Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) originally were identified functionally by Till and McCulloch (1961). A variety of methods have been used to isolate HSCs from the bone marrow, the primary site of hematopoiesis in the adult (Visser *et al.*, 1984; Spangrude and Johnson, 1990; Goodell *et al.*, 1996; Osawa *et al.*, 1996; Adolfsson *et al.*, 2001; Christensen and Weissman, 2001). One approach has been to utilize antibodies to a variety of HSC-associated cell surface molecules (e.g., c-kit, Sca-1, Thy1.1) and hematopoietic lineage markers such as B220, CD3, and Mac-1. Using this approach, all the HSC activity in the bone marrow has been found to reside within a small population of cells, characterized by their expression of low or undetectable levels of hematopoietic lineage markers ($lin^{lo/-}$), high levels of c-kit and Sca-1, and low levels of Thy1.1 ($lin^{lo/-}c-kit^+Sca-1^+Thy1.1^{lo}$) (Spangrude *et al.*, 1988; Uchida and Weissman, 1992). Upon injection into lethally irradiated mice, these cells migrate to the appropriate microenvironments and undergo rapid expansion and differentiation. 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 (Figure 1) and can maintain steady-state hematopoiesis for the lifetime of the animal (Uchida and Weissman, 1992; Morrison and Weissman, 1994). The HSCs identified by such methods make up 0.05% of the total bone marrow. They have been separated further into two populations: one with long-term repopulating ability ($\text{Lin}^- \text{ckit}^+ \text{Sca}^+ \text{Thy1.1}^{\text{lo}}$ cells, present at a frequency of 1:10,000 in the marrow) and the other with short-term repopulating ability ($\text{Lin}^{\text{lo}} \text{ckit}^+ \text{Sca}^+ \text{Thy1.1}^{\text{lo}}$ cells, present at a frequency of 1:2000 in the marrow). While both these populations can confer radioprotection, only the long-term-HSCs can provide reconstitution beyond 10 weeks (Morrison and Weissman, 1994).

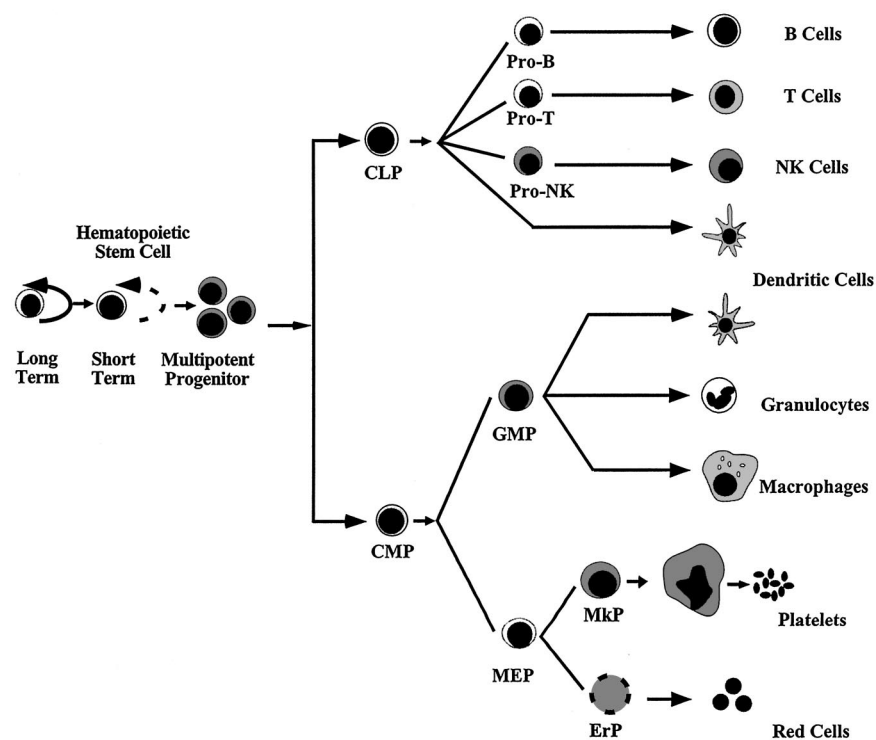


FIG. 1. Hematopoietic stem cell development. Hematopoietic stem cells can be classified into long-term, short-term, and multipotent progenitors, based on the extent of their self-renewal abilities. These cells go through a number of proliferative and commitment steps to give rise to all the myeloid and lymphoid lineages of the blood. Abbreviations: common lymphocyte progenitor (CLP); common myeloid progenitor (CMP); granulocyte monocyte progenitor (GMP); megakaryocyte erythrocyte progenitor (MEP). [Adapted from Reya T, Morrison SJ, Clarke MF, Weissman IL 2001 Stem cells, cancer, and cancer stem cells. *Nature* 414:105–111.]

II. Hematopoietic Stem Cell Self-Renewal

While the phenotypic and functional properties of HSCs have been extensively characterized (for reviews, see Morrison *et al.*, 1995; Weissman, 2000), a fundamental question that remains is how self-renewal is regulated. In most cases, combinations of growth factors that can induce extensive proliferation are unable to prevent differentiation of HSCs in long-term cultures. Although progress has been made in identifying conditions that maintain HSC activity in culture for a brief period of time (Miller and Eaves, 1997), it has proven exceedingly difficult to identify combinations of growth factors that cause significant expansion in culture in the number of progenitors with transplantable HSC activity.

Recent *in vitro* and *in vivo* studies investigating intracellular factors that mediate self-renewal have significantly advanced our understanding of HSC development. Ectopic expression of the transcription factor HoxB4 has been shown to be able to increase the numbers of transplantable hematopoietic stem cells both *in vitro* and *in vivo* (Sauvageau *et al.*, 1995; Antonchuk *et al.*, 2002). Alterations in HSC growth in transgenic and knockout mice also have identified potential mediators of HSC homeostasis *in vivo*. Mice that overexpress the antiapoptotic gene bcl-2 (Domen *et al.*, 2000) have increased numbers of HSCs, suggesting that such antiapoptotic signals may contribute to regulating stem cell numbers. Mice lacking the G1 checkpoint regulator p21 display a higher rate of HSC proliferation and differentiation and a lower self-renewal capacity, suggesting that p21 is required for maintaining HSC quiescence and that, in its absence, HSCs rapidly proliferate and differentiate to more-committed lineages (Cheng *et al.*, 2000). While these are clearly important mediators of HSC development, the upstream signal that may control their activity remains unclear.

Signaling pathways classically studied in context of embryonic development — such as the Notch, Sonic hedgehog, and Wnt signaling pathways — have emerged as candidates for regulating self-renewal. The expression of constitutively active Notch1 in hematopoietic progenitors has been shown to lead to the establishment of at least some immortalized, cytokine-dependent cell lines that retain the potential to generate both lymphoid and myeloid cells *in vitro* and in long-term mouse reconstitution assays (Varnum-Finney *et al.*, 2000). More recently, it was demonstrated that HSCs infected with Notch expand *in vivo* (Stier *et al.*, 2002). While in many cases Notch1 expression in HSCs can lead to the rapid formation of T-cell leukemias *in vivo*, using RAG1^{-/-} mice to prevent lymphocyte development allowed the effects on HSC expansion to be revealed in this study (Stier *et al.*, 2002). Sonic hedgehog, another pathway studied classically as a regulator of embryonic development, also has emerged as a potential mediator of HSC development. Human cells highly enriched for hematopoietic progenitors (CD34⁺Lin⁻CD38⁻) exhibited increased self-renewal in response to

Sonic hedgehog signaling *in vitro*, albeit in combination with a cocktail of six other growth factors (Bhardwaj *et al.*, 2001). The fact that both Notch and Sonic hedgehog activation has been observed to inhibit the differentiation of progenitors in many different systems by acting to maintain progenitors in an undifferentiated state (Artavanis-Tsakonas *et al.*, 1995; Harris *et al.*, 1997; Rowitch *et al.*, 1999; Wechsler-Reya and Scott, 1999) certainly supports the idea that these signals may promote self-renewal in a variety of tissues.

In an effort to identify novel signaling pathways that regulate stem cell self-renewal, we analyzed the transcription factor profile of mouse bone marrow HSCs. HSCs express high levels of GATA-2 and SCL, PU.1 (Akashi *et al.*, 2000), and lymphoid enhancer factor-1 (LEF-1) (S.H. Cheshier, K. Li, I.L. Weissman, unpublished results). The expression of LEF-1, the transcriptional mediator of Wnt signaling, in HSCs and the expression of Wnts in the bone marrow (Reya *et al.*, 2000), together with our previous findings that Wnt signaling regulates early B-cell growth (Reya *et al.*, 2000), suggested that Wnts may control hematopoietic stem cell self-renewal.

III. Wnt Signaling

Wnt proteins represent a growing family of secreted signaling molecules that are expressed in diverse tissues and have been shown to influence multiple processes in vertebrate and invertebrate development (reviewed in Cadigan and Nusse, 1997). The founding member of the family, Int-1 (now called Wnt1), was identified as a protooncogene (Nusse and Varmus, 1982). Wnt proteins have been shown to regulate segment polarity in *Drosophila* (Siegfried and Perrimon, 1994) and axis specification in *Xenopus* (Moon *et al.*, 1997). In the mouse, Wnt proteins are widely expressed. Mutations in *Wnt* genes result in defects in limb, somite, and axis formation and abnormal development of brain, kidney, and reproductive tract (Parr and McMahon, 1994; Monkley *et al.*, 1996; Yoshikawa *et al.*, 1997; Miller and Sassoon, 1998; Liu *et al.*, 1999). In addition to its importance in normal development, dysregulation of the Wnt pathway can have potent oncogenic effects in tissues such as colon, breast, prostate, and skin (Tsukamoto *et al.*, 1988; Korinek *et al.*, 1997; Morin *et al.*, 1997; Polakis, 2000).

Wnts act by binding to two types of receptor molecules at the surface of a cell (Figure 2). One is the Frizzled family of seven-pass transmembrane proteins, which contain a cysteine-rich extracellular domain that binds to Wnt proteins (Wodarz and Nusse, 1998). The second is a subset of the low-density lipoprotein receptor-related protein (or the LRP) family, specifically, LRP-5 and LRP-6 (called arrow in *Drosophila*) (Pinson *et al.*, 2000; Tamai *et al.*, 2000; Wehrli *et al.*, 2000), a single-pass transmembrane protein. Experiments have provided evidence that both LRP-5/6 and Frizzled are needed to functionally activate the

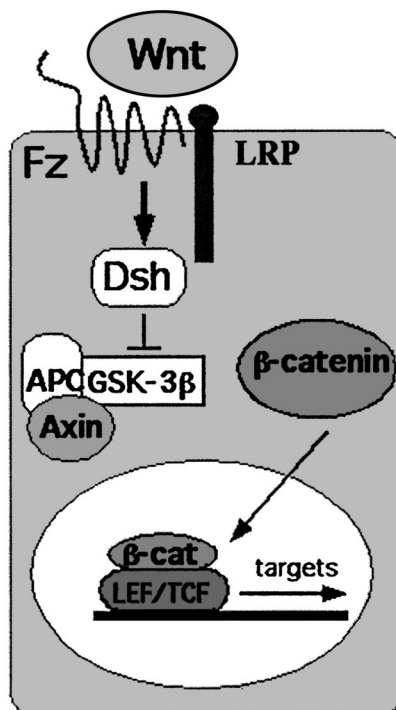


FIG. 2. Wnt signaling pathway. In the absence of Wnt signaling, the key regulator of the pathway, beta-catenin, is complexed with glycogen synthase kinase-3 beta (GSK-3 β), Axin, and adenomatosis polyposis coli (APC) as part of the destruction complex. This leads to the phosphorylation, ubiquitination, and degradation of β -catenin. The binding of Wnt to its coreceptors, Frizzled and LRP, leads to the dissociation of the destruction complex and stabilization of β -catenin, followed by its translocation to the nucleus and activation of target genes in collaboration with the LEF/TCF family of transcription factors.

downstream components of the canonical pathway (reviewed in Pandur and Kuhl, 2001).

In the absence of a Wnt signal, beta-catenin is associated with a large, multiprotein complex (the “destruction complex”) that includes the scaffold protein Axin and the serine/threonine kinase, glycogen synthase kinase-3 beta (GSK-3 β). In this complex, β -catenin is phosphorylated at its NH₂ terminus by GSK-3 β and thereby targeted for ubiquitination and degradation by proteasomes (Cadigan and Nusse, 1997). Axin is a key component of this complex, since it acts as a scaffold to significantly enhance the ability of GSK-3 β to phosphorylate β -catenin. Binding of Wnt proteins to their receptors inhibits phosphorylation of β -catenin by GSK-3 β . This prevents β -catenin’s degradation and results in

stabilization and accumulation of β -catenin in the cytosol (Willert and Nusse, 1998). β -catenin then translocates to the nucleus, where it binds to members of the LEF/T-cell factor (TCF) family of transcription factors. LEF/TCF proteins normally are associated with the transcriptional repressor, Groucho (Cavallo *et al.*, 1998; Roose *et al.*, 1998), which suppresses their activity and represses gene transcription. Binding of β -catenin relieves this repression and allows LEF/TCF factors to induce expression of the appropriate target genes (reviewed in Eastman and Grosschedl, 1999).

IV. Wnt Signaling in Stem Cell and Progenitor Cell Development

The possibility that the Wnt signaling pathway might contribute to the regulation of stem cell self-renewal in the hematopoietic system is supported by studies of nonhematopoietic progenitors and stem cells. For example, cultured human keratinocytes with higher proliferative potential have been shown to have increased levels of β -catenin, compared to those with lower proliferative capacity. Moreover, retroviral transduction of a constitutively activated form of β -catenin (mutated such that it cannot be degraded) results in increased self-renewal of epidermal progenitors/stem cells (Zhu and Watt, 1999). Transgenic mice overexpressing activated β -catenin in epidermal stem cells develop hair follicle tumors (Gat *et al.*, 1998). Moreover, the Wnt signaling pathway may be required in the maintenance or self-renewal of stem cells of the gut, based on the finding that TCF-4-deficient mice progressively exhaust the undifferentiated progenitors in the crypts of the gut epithelium during fetal development (Korinek *et al.*, 1998). Finally, transgenic mice that overexpress activated β -catenin on a nestin promoter display increased cell-cycle entry of neural precursors and, consequently, a larger brain with characteristics of higher mammals (Chenn and Walsh, 2002).

Wnt signaling also has been implicated in the maintenance and renewal of committed precursors of a variety of lineages. Wnt10B has been shown to maintain preadipocytes in an undifferentiated state, while inhibition of Wnt signaling allows their differentiation into adipocytes (Ross *et al.*, 2000). Moreover, we have shown that Wnt3A can induce proliferation of B-cell precursors in a LEF-1-dependent manner and that these precursors are depleted in LEF-1-deficient mice (Reya *et al.*, 2000). Cumulatively, these findings suggest that the Wnt signaling pathway may regulate self-renewal of stem and progenitor cells in a variety of tissues (summarized in Table I). However, Wnt signaling also has been implicated in lineage commitment of stem cells, most notably, in the skin (Huelsenken *et al.*, 2001). Thus, its influence on self-renewal is likely to be context specific and does not preclude an ability to regulate lineage commitment as well.

TABLE I
Summary of Selected Studies Demonstrating a Role for Wnt Signaling in Stem and Progenitor Cell Development from Diverse Mammalian Tissues

| Stimulus | Cell type | Effect | Assay | Reference |
|---------------------------------------|-----------------------|-----------------------------------|-----------------|--|
| Activated β -catenin | Keratinocytes | Inhibition of differentiation | <i>In vitro</i> | Zhu <i>et al.</i> (1999) <i>Development</i> 126:2285 |
| Wnt10B | Preadipocytes | Inhibition of differentiation | <i>In vitro</i> | Ross <i>et al.</i> (2000) <i>Science</i> 289:950 |
| Wnt3A | B-cell progenitors | Induction of proliferation | <i>In vitro</i> | Reya <i>et al.</i> (2000) <i>Immunity</i> 13:15 |
| Lef-1 knockout | B-cell progenitors | Depletion of progenitors | <i>In vivo</i> | Reya <i>et al.</i> (2000) <i>Immunity</i> 13:15 |
| Tcf-4 knockout | Intestinal stem cells | Depletion of stem cells | <i>In vivo</i> | Korinek <i>et al.</i> (1998) <i>Nat Genet</i> 4:379 |
| Activated β -catenin transgenic | Follicular stem cells | Hair follicle and tumor formation | <i>In vivo</i> | Gat <i>et al.</i> (1998) <i>Cell</i> 95:605 |
| β -catenin knockout | Follicular stem cells | Cell fate alteration | <i>In vivo</i> | Huelsken <i>et al.</i> (2001) <i>Cell</i> 105:533 |

V. Wnt Signaling in Hematopoietic Stem Cell and Progenitor Cell Development

While the Wnt pathway has been shown to play a critical role in the development of a variety of organs and lineages, relatively little is known about its function in the hematopoietic system. Recent evidence strongly suggests that Wnt signaling has an important regulatory role in hematopoietic progenitors/stem cells during both fetal and adult development.

During fetal hematopoiesis, Wnt proteins – specifically, Wnt5A and Wnt10B — are expressed in the yolk sac and the fetal liver, both sites of hematopoiesis in the embryo (Austin *et al.*, 1997). Moreover, hematopoietic progenitor populations were found to express Wnt10B, suggesting that Wnts may be utilized in an autocrine manner. Conditioned media containing Wnt1, Wnt5A, or Wnt10B stimulated an 11-fold expansion of fetal liver progenitors in synergy with the stem cell growth factor, SLF (Austin *et al.*, 1997). To determine whether the cells that proliferated as a consequence of Wnt stimulation *in vitro* maintained immature characteristics, cells were tested in semisolid colony-forming assays and found to have increased colony-forming ability, especially of blast cell-containing colonies. This result suggested that treatment with Wnt proteins

retained immature functional characteristics, at least as determined by *in vitro* assays. Consistent with the effects of soluble Wnt proteins in mouse hematopoiesis are their effects in human hematopoiesis (Van Den Berg *et al.*, 1998). The exposure of CD34⁺Lin⁻ human hematopoietic progenitors to Wnt5A, which was found to be expressed in these precursors, promoted the expansion of undifferentiated progenitors in the presence of stromal cells. The presence of undifferentiated progenitors was determined by *in vitro* colony-formation assays, which revealed a 10- to 20-fold higher number of colony-forming unit (CFU)-Mix colonies, which reflect the presence of an immature population. These *in vitro* experiments clearly point to an increase in immature progenitors in response to Wnt protein stimulation; however, further experiments with *in vivo* transplants will enable a better distinction between the expansion of hematopoietic progenitors and stem cells.

In contrast to these studies, some groups have reported that soluble Wnt proteins can inhibit self-renewal of hematopoietic progenitors. In an attempt to determine the role of Wnts in the context of the stromal cell microenvironment, one group (Yamane *et al.*, 2001) utilized Wnt3A-conditioned media in Dexter cultures and noted a decrease in the numbers of B lineage and myeloid lineage cells. These data are difficult to interpret due to the complex nature of exposing both stromal elements and hematopoietic cells to Wnt3A and the inability to distinguish direct and indirect effects. In a separate system using the quail mesodermal cell line QCE6 and avian whole-bone marrow, Wnt11 was shown to preferentially specify erythroid lineage fate in avian whole-bone marrow cultures (Brandon *et al.*, 2000). While both these studies seem to indicate that Wnt proteins can influence lineage decisions, purified populations of stem cells will need to be tested in the context of purified Wnt proteins to make this conclusion definitively.

These studies (summarized in Table II) are difficult to compare directly due to the wide variation in the species used, the hematopoietic populations studied, the Wnt stimulus utilized, and the assays performed. The use of a highly purified, defined population of primary cells is critical to understanding the true nature of Wnt effects on HSCs, since an indirect effect through non-HSCs in a mixed population could confound interpretation of results. Moreover, due to the complexity of the Wnt pathway, it will be important to analyze the role of different components of the pathway to determine whether canonical Wnt signaling regulates HSC development. Finally, the use of a system in which the effects on HSCs can be assayed through both *in vitro* and *in vivo* approaches is critical to establishing the true effects of modulating Wnt signaling in HSCs. Many *in vitro* assays cannot distinguish between the activity of a fully uncommitted stem cell and an early committed progenitor cell (e.g., a common myeloid progenitor).

We have undertaken a set of experiments to identify methodically the role of Wnts in HSCs. We retrovirally transduced downstream components of the Wnt

TABLE II
Summary of Selected Studies Demonstrating a Role for Wnt Signaling in Hematopoietic Stem and Progenitor Cell Development

| Stimulus | Cell type | Effect | Reference |
|----------------------------|---------------------------------|-----------------------------|---|
| Wnt5A + SLF | Fetal liver enriched precursors | Increased progenitors | Austin <i>et al.</i> (1997) Blood 89:3624 |
| Wnt5A + SLF + feeder cells | Bone marrow enriched precursors | Increased progenitors | Van Den Berg <i>et al.</i> (1998) Blood 92:3189 |
| Wnt3A + stromal cells | Bone marrow unenriched | Inhibition of hematopoiesis | Yamane <i>et al.</i> (2001) J Immunol 167:765 |
| Wnt5A or Wnt11A | Bone marrow nonadherent | Increased differentiation | Brandon <i>et al.</i> (2000) Blood 96:4132 |

pathway into highly purified mouse bone marrow HSCs and analyzed their effects by *in vitro* and *in vivo* assays. Through this approach, we found that overexpression of activated β -catenin in long-term cultures of HSCs expands the pool of HSCs, as determined by both phenotype *in vitro* and ability to reconstitute the hematopoietic system *in vivo* (T. Reya, J. Domen, D. Scherer, A.W. Duncan, K. Willert, R. Nusse, H. Weissman, unpublished results). Activated β -catenin induced cells to enter the cell cycle and grow in long-term cultures for 1 to 4 weeks, while control HSCs did not survive beyond 48 hours (Table III). These expanded HSCs also retained the functional characteristics of HSCs, following transplant into allelically distinct irradiated mice. Moreover, ectopic expression of Axin, an inhibitor that acts by degrading β -catenin, led to inhibition of HSC proliferation, increased cell death of HSCs *in vitro*, and reduced reconstitution *in vivo*. These studies demonstrated the ability of downstream components of the Wnt pathway to regulate bone marrow HSC function. Moreover, using Wnt3A protein, we found that HSCs expand dramatically to soluble Wnt protein (up to 100-fold), while maintaining the phenotypic characteristics of HSCs (K. Willert, J. Brown, E. Danenberg, R. Nusse, unpublished results). Cumulatively, our studies, together with those of others described here, indicate that Wnt signaling is important in the maintenance and self-renewal of hematopoietic stem and progenitor cells.

VI. Perspectives

The studies that have examined the role of Wnt signaling in the hematopoietic stem and progenitor cell growth and cell-fate decisions indicate that the Wnt

TABLE III
Summary of Studies Carried Out by Activating and Inhibiting Wnt Signaling in Purified HSC, by Using Retroviral Transduction of Downstream Mediators

| | Phenotype of cells <i>in vitro</i> | Transplantation ability <i>in vivo</i> |
|--------------|---------------------------------------|---|
| Control | +++ | +++ |
| Beta-catenin | ++++++ | ++++++ |
| Axin | + | + |

[The relative extent of cell proliferation, while maintaining the surface phenotype of HSCs (Lin⁻ c-kit⁺ sca-1⁺ Thy1^{lo}), and their ability to reconstitute the hematopoietic system of lethally irradiated recipient mice are indicated by the frequency of + signs.]

signaling pathway plays an important role in the hematopoietic system. These studies have raised many key questions about the mechanisms of HSC regulation. For example, in order to understand the mechanisms by which Wnt and β -catenin promote self-renewal, molecular analysis of the targets that are regulated in HSCs is needed. Moreover, given that multiple signaling pathways (e.g., Notch, Sonic hedgehog) and transcription factors (e.g., HoxB4) are emerging as regulators of HSC self-renewal, it will be important to determine if and how these signals are integrated to regulate HSC development. It is exciting to speculate that the Wnt, Notch, and Sonic hedgehog pathways may regulate similar targets genes in HSCs or may act in a hierarchical manner to regulate self-renewal. Answering these questions will enable us to understand the signals that regulate HSCs specifically and stem-cell growth in general. This will, in the long term, lead to the ability to create methods to maintain the undifferentiated state of hematopoietic stem cells in culture and have numerous practical ramifications for transplantation and regenerative therapies.

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