

## Regulation of Hematopoietic Stem Cells by the Steel Factor/KIT Signaling Pathway

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**Abstract** Understanding the intrinsic pathways that regulate hematopoietic stem cell (HSC) proliferation and self-renewal responses to external signals offers a rational approach to developing improved strategies for HSC expansion for therapeutic applications. Such studies are also likely to reveal new targets for the treatment of human myeloid malignancies because perturbations of the biological processes that control normal HSC self-renewal divisions are believed to drive the propagation of many of these diseases. Here, we review recent findings that point to the importance of using stringent functional criteria to define HSCs as cells with longterm repopulating activity and evidence that activation of the KIT receptor and many downstream effectors serve as major regulators of changing HSC proliferative and self-renewal behavior during development.

### Background

Hematopoietic stem cells (HSC) constitute a rare, self-sustaining population that appears early in the development of the embryo and is then responsible for mature blood cell production throughout life. HSC numbers can be regulated extrinsically by three mechanisms: (a) altered exposure to factors that control their viability, (b) altered exposure to factors that control their continuing stem cell functionality, and (c) altered exposure to factors that control their proliferative status. Exposure of HSCs to different concentrations of soluble steel factor (SF, also known as stem cell factor, mast cell growth factor, or KIT ligand) is one mechanism that can regulate HSC self-renewal divisions *in vitro* (1, 2). SF is also an important (but not exclusive) physiologic regulator of HSC activity *in vivo* (3–6). Interestingly, during development, many properties of HSCs change, one of which is a marked decrease in SF sensitivity (6, 7). Here, we review current understanding of HSC heterogeneity, how HSCs respond to SF, and the role of candidate downstream effectors identified as important for sustained HSC expansion *in vitro* and *in vivo*.

### HSCs Represent a Heterogeneous Subset of Multipotent Hematopoietic Cells

The first evidence of a common origin of different blood cell types was provided by both morphologic (8) and cytogenetic (9) studies of what are now recognized as clonal myeloproliferative diseases. This concept was experimentally validated by

the discovery of a rare subset of cells in mice that generate multilineage clones in the spleen of myeloablated recipients and that are sustained throughout life (10). The finding that these “colony-forming units spleen” are present in all hematopoietic tissues and exhibit some self-renewal activity when serially transplanted led to their use as a tool for establishing many basic principles expected of an HSC population. Later, genetic tracking analyses formally showed the ability of single hematopoietic cells from normal murine and human donors to establish chimerism in the blood-forming system of transplanted recipients for periods of months to years (11–14). In some of these latter studies, the ability of the original cell to produce progeny with extensive multilineage regenerative potential was also documented.

Together, these observations established the existence of HSCs in mice and humans. At the same time, they revealed wide variability in the cell outputs and longevity of individual clones produced in similarly transplanted recipients. This heterogeneous behavior brought into focus the uncertainty of defining HSCs by the regenerative activity they display because such retrospective approaches cannot discriminate behavioral differences caused by variations in the types or sequence of extrinsic cues received by the original cells versus their preexisting intrinsic heterogeneity and/or the role of stochastic events. These issues remain incompletely resolved, although experiments with purified HSCs have recently helped to clarify the situation.

These experiments have revealed the existence of a population, quantifiable in suspensions of unknown purity as longterm competitive repopulating units (15, 16) that, when transplanted as single isolated cells into irradiated recipients, consistently display both longterm lymphomyeloid-repopulating activity and extensive self-renewal activity (7, 17–21). HSCs, thus defined, are phenotypically distinct from multilineage hematopoietic cells with shorter-lived regenerative properties including most colony-forming units spleen (22). Tracking of the clonal progeny of multiple single HSCs through two to three serial transplants has also revealed their possession of specific lineage preferences that can be propagated over many self-renewal divisions *in vivo*. However, these

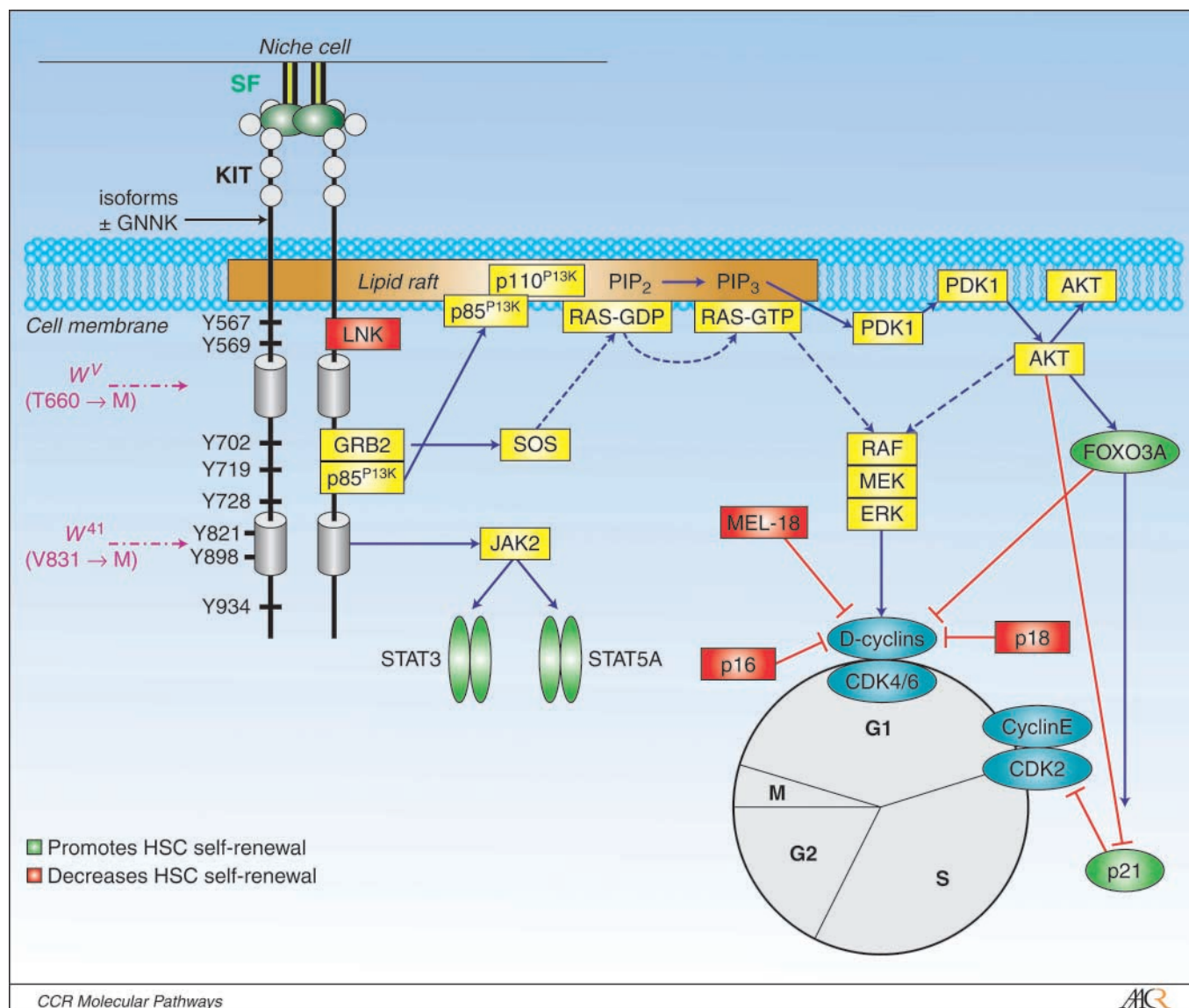
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**Fig. 1.** Schematic representation of key signaling events activated in primitive hematopoietic cells exposed to SF (here shown as two membrane-bound molecules bound to a dimerized receptor complex). Blue arrows, pathway-promoting activities; red stop lines, inhibiting activities.

differentiation programs can also change rapidly, both *in vivo* (e.g., at 3 weeks after birth in mice) and under certain culture conditions (7, 21, 23). These findings support a model in which distinct, although possibly overlapping, molecular mechanisms regulate lineage preferences and their maintenance (self-renewal), with the option that lineage preferences may be initiated before, rather than after, self-renewal potential is lost.

The HSC compartment of fetal and young adult mice can thus now be functionally defined as a restricted compartment of distinct multipotent cells that universally display extensive self-renewal activity when transplanted into irradiated recipients. However, these cells seem to be preprogrammed to display particular patterns of differentiation. Unfortunately, the combination of phenotypic markers used to obtain these cells at very high purities cannot yet be assumed to measure, or even detect, cells with the same properties in uncharacterized cell suspensions. This is because many of the markers in question

show variable expression on HSCs according to the activation status of the HSCs and may also be variably expressed on non-HSCs (24–26). Future identification of molecular markers that stably associate with self-renewing HSCs independent of their cycling status or differentiation program should help to elucidate the mechanism that allows for long-term maintenance of HSC activity.

### Role of SF in Regulating HSCs

SF is a transmembrane growth factor encoded by the *Sl* gene. SF binds to and activates a type III transmembrane receptor tyrosine kinase called KIT (also referred to as CD117; see Fig. 1). KIT contains a split intracellular kinase domain and is encoded by a transcriptional unit found at the *W* locus. Both SF and its receptor can be expressed as different isoforms with different activities and can be cleaved proteolytically to yield soluble forms with similar binding affinity (27, 28).

Even before the products encoded by the *W* and *Sl* loci were known to represent a receptor-ligand pair, studies of the defects caused by mutations at both loci had pointed to their involvement in HSC regulation. For example, both fetal and adult hematopoietic tissues from mice carrying mutations within the kinase domain of *Kit* (e.g., see Fig. 1) show reduced colony-forming units spleen/HSC activity (29). Mice with a *W41/W41* genotype are of particular interest because they are viable and fertile (in contrast to those with more severe *W*-mutations; ref. 30) but still have significantly reduced HSC numbers (10- to 20-fold). As a result, sublethally irradiated adult *W41/W41* mice can be used as hosts to detect transplanted (wild-type) HSCs with the same sensitivity as lethally irradiated wild-type hosts given a minimal radioprotective transplant (31, 32). In contrast, *Sl*-mutant mice, which have deletions in the SF genomic sequence (33), have a defect in the microenvironmental niche that supports the regenerative activity of colony-forming units spleen/HSCs (34).

HSCs from all stages of development express the same levels of the KIT receptor on the cell surface regardless of their cycling status or position in the cell cycle (3, 6, 35, 36). *In vitro*, the ability of different concentrations of soluble SF to modulate HSC self-renewal divisions directly has been shown using highly purified starting populations and subsequent *in vivo* readouts of retained or lost HSC activity (1, 2, 18). Moreover, these effects on HSC self-renewal can be elicited even in the absence of changes in HSC viability or cell cycle progression. These experiments have further shown that the self-renewal responses of fetal and adult murine HSCs to soluble SF in serum-free suspension cultures are both steeply SF concentration-dependent above and below an optimum level, but their specific sensitivities to SF are markedly different. Fetal HSCs are 6-fold more sensitive to SF than their adult counterparts with maximum maintenance of fetal HSC activity in medium containing 50 ng/mL of SF (only) compared with the 300 ng/mL of SF (+20 ng/mL IL-11) required to achieve a similar result with adult HSCs (6). The different SF sensitivity displayed by fetal and adult HSCs is likely due to differences in how KIT-activated signals are transmitted to downstream intracellular targets in these cells because both express similar levels of KIT and, under their respective optimal conditions of SF stimulation, they show no differences in apoptosis and divide with the same cell cycle times (7). *In vivo*, fetal and adult HSCs show marked differences in their cycling activity (35, 37) and also in their self-renewal and differentiation patterns posttransplant (7, 21). All of these properties change abruptly between 3 and 4 weeks after birth in a fashion that seems to be intrinsically determined and preprogrammed. Changes in mechanisms that mediate SF signals may thus offer an attractive explanation for why they display different biological properties *in vivo*. Interestingly, indirect evidence exists for a similar switch mechanism operating in humans (38) and nonhuman primates (39), as inferred from the finding of an abrupt change in the rate of decline of circulating granulocyte telomeres within the first year after birth.

### Candidate Intrinsic Targets of SF Action

Much is known from model cell systems about the pathways that SF can activate, and many of these have been confirmed in primitive HSC-like hematopoietic cell lines (36, 40). Figure 1

summarizes the downstream signaling pathways likely to influence HSC self-renewal responses. SF binding induces receptor homodimerization and autophosphorylation of tyrosine residues in the cytoplasmic domain, which then serve as docking sites for various SH2 domain-containing signaling intermediates (41). Activation of KIT also leads to the recruitment and activation of adjacent kinases including JAK2 (42), TEC (43), and MATK (44); the tyrosine phosphatases SHP-1 and SHP-2 (45); phospholipase C; and the p85 subunit of phosphatidylinositol 3-kinase (41). The activated KIT receptor complex is then recruited transiently to cell surface lipid rafts where the p110 catalytic subunit of phosphatidylinositol 3-kinase is located (46). This allows a functional phosphatidylinositol 3-kinase holoenzyme to assemble leading to the subsequent activation of cytosolic PDK1 and AKT/PKB. This is accompanied by a reduction in PTEN levels (a negative regulator of the phosphatidylinositol 3-kinase pathway) in the rafts, thus, reinforcing the activation of the phosphatidylinositol 3-kinase pathway and multiple downstream events including inactivation of the forkhead transcription factor O3A (47) and activation of the mitogen-activated protein kinase pathway (48). SF-mediated activation of JAK2 leads, in turn, to the activation of STATs 1, 3, and 5, which then form dimers and translocate to the nucleus to alter the transcription of specific target genes (49–51). As summarized below, some of these signaling elements and their ultimate transcriptional targets seem to participate in the regulation of HSC amplification, although, in many cases, it has not yet been possible to discriminate between effects on HSC viability or mitogenesis compared with independent effects on their self-renewal control.

**Signal transducers and activators of transcription 3 and 5A.** Activation of both signal transducers and activators of transcription (STAT)3 and STAT5A positively regulate fetal and adult HSC expansion *in vivo*, as shown by studies with a dominant-negative version of STAT3 (52) and cells from *Stat5a*<sup>-/-</sup> mice (53) or human CD34<sup>+</sup> cells with RNAi-suppressed STAT5 (54, 55). Conversely, transduction of primitive hematopoietic cells with constitutively active forms of STAT3 (56) or STAT5A (56, 57) enhanced HSC self-renewal divisions under certain conditions and, in the case of STAT5A, led to a myeloproliferative syndrome *in vivo*. However, levels of STAT3 seem to be nonlimiting in HSCs because overexpression of the native form does not alter HSC amplification *in vivo* (52), and levels of STAT3 mRNA are found to be significantly higher (~2-fold) in proliferating adult HSCs than in their fetal counterparts (6).

**LNK.** LNK is one of many adapter molecules that bind to KIT after SF activation. LNK acts as a negative regulator of HSC self-renewal divisions both *in vivo*, as shown by an increased production of HSCs in *Lnk*<sup>-/-</sup> mice (58, 59), and *in vitro*, as shown by an increased frequency in symmetrical self-renewal divisions executed by *Lnk*<sup>-/-</sup> (compared with wild-type) HSCs stimulated with thrombopoietin and SF (60).

**Cell cycle regulators [Cyclins D1, 2, and 3 and Cdkn1a/p21Cip1 (p21), Cdkn2a/p16Ink4a (p16), and Cdkn2c/p18Ink4c (p18)].** Expression of the D-type cyclins is induced by mitogenic cytokines and their expression allows the formation of complexes with partner cyclin-dependent kinases leading to entry into S-phase (61). In mice, loss of all three D-type cyclins severely impairs the expansion of HSCs in the fetal liver and

posttransplant, although it is likely that this is due to an inability of the cells to transmit a mitogenic signal rather than a direct effect on the HSC self-renewal mechanism itself (62). p21, p16, and p18 are three of several cyclin-dependent kinase inhibitors with well-established roles in regulating progression of cells through specific phases of the cell cycle (61). All three have also been implicated as regulators of HSC self-renewal either directly or indirectly. Cells from adult p21<sup>-/-</sup> mice have markedly reduced numbers of colony-forming units spleen and HSC activity (63). This seems to be caused by their failure to respond to signals that normally induce HSC quiescence (64), which occurs when mice reach the age of 4 weeks (35). In contrast, p16 (65) and p18 (66) deficiencies each endow HSCs with improved longterm repopulating and self-renewal activity, and the latter can partially offset the negative consequence of p21 deficiency in HSCs (67). Interestingly, p18 mRNA is present at significantly lower (~10-fold) levels in fetal compared with proliferating adult HSCs even after the cells have been stimulated to proliferate by SF *in vitro* (6), a finding consistent with a role of p18 in mediating the observed differences between fetal and adult self-renewal activities *in vivo*.

**BMI1, PHC1 (RAE28), EZH2, and PCGF2 (MEL18).** BMI1, PHC1 (RAE28), EZH2, and PCGF2 (MEL18) are members of the polycomb family of transcriptional regulators, all four of which have been implicated in regulating HSC self-renewal activity either positively [BMI1 (68, 69), RAE28 (70), and EZH2 (71)] or negatively (MEL18; ref. 72). MEL18 is of particular interest here because it can inhibit the activity of cyclin D2 by direct physical interaction in the nucleus (73). Interestingly, both MEL18 and EZH2 transcripts are expressed at much higher levels (~10-fold) in quiescent adult compared with proliferating fetal HSCs, whereas BMI1 transcript levels are similar in both (7).

**Forkhead transcription factor A.** Forkhead transcription factor A is a member of the forkhead box family of transcription factors that have major roles in longevity and stress resistance (74). Deletion of the *Foxo3a* gene allows HSCs to be generated but inhibits both their ability to enter a quiescent state and their

survival. Interestingly, this is associated with an inhibition of p21 expression and an increase in the expression of cyclin D2 (74, 75).

### Therapeutic Implications

HSC-containing transplants from donors of all ages are the basis of thousands of annually performed life-saving therapies where they are used to re-establish normal blood formation in patients given a myeloablative treatment to eradicate a defective or malignant hematopoietic condition. Major improvements in these therapeutic strategies and others requiring the transient replacement of specific types of mature blood cells could be envisaged if current barriers to the production of large numbers of HSCs *in vitro* were overcome. In addition, accumulating evidence indicates that most human myeloid malignancies involve perturbations of pathways that regulate normal HSCs (76). All of these issues highlight the need for a more precise understanding of the intrinsic molecular anatomy of normal HSCs and how this can be altered by interactions of HSCs with fluctuating cues from their environment that signal changing needs for blood cell production.

The recent, more stringent, biological characterization of HSCs and the development of methods for their isolation in close to pure form from the fetal liver and bone marrow of young mice has made it possible to obtain more precise descriptions of the molecular differences between these two critical cell populations. The information thus acquired has opened the door to more incisive analysis of the mechanisms that regulate HSC self-renewal activity *in vivo* where SF signaling has been shown to be an important cue for change that is differently interpreted by fetal and adult HSCs. The complexity of this process, its remarkable alteration soon after birth, and the need to investigate the full relevance of these findings for normal and leukemic human HSCs remain exciting challenges for the future.

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