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Role of secreted factors in the regulation of hematopoietic stem cells by the bone marrow microenvironment

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1. ABSTRACT

The stem cell microenvironment (*in vivo* known as niche) is a specific space in the bone marrow (BM), which nurses hematopoietic stem cells and regulates their self-renewal and differentiation using extrinsic cues, such as secreted factors. The niche plays a major role in regulating the number of blood cells and also protects stem cells against excessive proliferation. Till date, several possible secreted regulators of HSC function have been reported. Many of these were originally isolated from stromal cells and the cell lines isolated from hematopoietic tissues. These secreted factors act in concert and not only regulate HSC, but also the niche cells. It has also become clear that deregulation of the niche function is a potential cooperating factor during the development of hematological malignancies. An understanding of how the niche participates in HSC maintenance and repair through soluble factors can offer new opportunities for the development of novel therapeutic tools against hematological malignancies.

2. INTRODUCTION

Hematopoietic stem cells (HSC) reside in the bone marrow proximal to bone marrow stroma cells (BMSC). Many studies have shown that BMSC regulate the behavior of HSC. BMSC are comprised of a variety of different cell populations (fibroblasts, reticular cells, endothelial cells, adipocytes and osteoblasts) that, in concert, support maintenance of HSCs throughout the lifetime of an adult individual. *In vitro*, it has been confirmed that BMSCs produce a plethora of soluble factors, such as cytokines and growth factors (e.g., IL3, GM-CSF), as well as form physical interactions (e.g., adhesion) with hematopoietic stem and progenitor cells and extracellular matrix (ECM) molecules (1). This complex of the bone marrow microenvironment thus regulates HSC self-renewal and multilineage differentiation (2). The regulatory mechanisms involved depend on a complex interplay of cell-autonomous and cell extrinsic regulatory mechanisms the outlines of which are still to be resolved in detail (3).

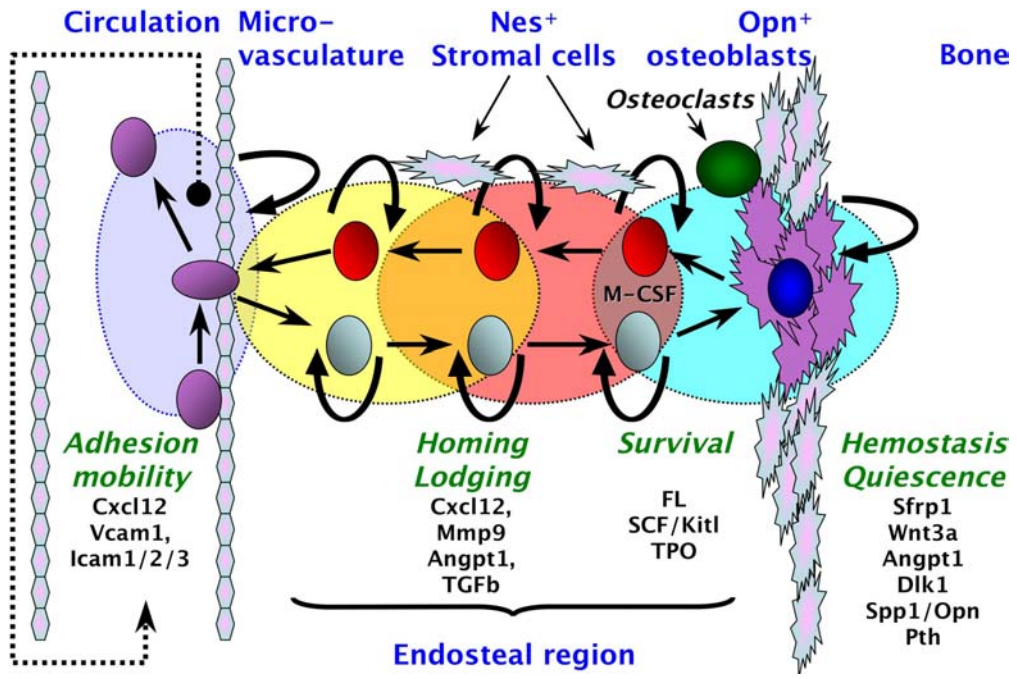

Figure 1. Soluble factors in niche and their regulation of various processes. The figure is a simple illustration of factors that act mainly in hemostasis and quiescence (extreme right) of primitive stem cells (SFRP1, Wnt3a, Angpt1, Dlk1, Spp1/Opn and Ptn). In the endosteal region there are a variety of factors playing part apparently. The in nestin positive stroma cells HSC are acted upon by soluble factors that maintain the survival (FL, SCF, TPO) and homing/lodging as well (Cxcl12, Mmp9, Angpt1, TGFb). Near to endothelium some adhesion molecules play a major role (Left)(Cxcl12, Vam1, Icam 1/2/3).

In vivo studies have extensively documented the concept of the HSC microenvironment, or niche, described as a three-dimensional cellular unit, close to the endosteal region of bone marrow (BM) (4, 5). In this niche, HSC are protected from differentiation and loss of stem cell function possibly by induction of quiescence (6). Since bone-forming osteoblasts are critical for maintaining the size of the HSC pool *in vivo*, it is thought that osteoblasts are critically active in a functional HSC niche (6, 7). However, as alluded to above, the niche represents a heterogeneous cellular unit. Within this unit, other cell types, which include endothelial cells (8), neural cells (9) as well as feedback mechanisms among mature and immature hematopoietic cells (10), also play an important role. This review largely focuses on soluble factors, which integrate the interactions occurring among HSCs and different cell types of the microenvironment.

3. SECRETED SOLUBLE FACTORS PRODUCED BY THE NICHE

Osteoblasts produce soluble hematopoietic supportive–secreted and cell-associated factors (e.g., chemokines, interleukins, and interferon's) that overlap in their functions and allow HSCs to derive regulatory information from the niche. Functionally similar to the osteoblastic niche, vascular endothelial cells maintain HSCs *in vitro* (11, 12) and are required for hematopoiesis *in vivo* (8, 13). Lodgment of HSCs at the endosteal region appears to favor quiescence or dormancy (14), whereas the more central vascular niche serves as a location that allows differentiation and subsequent mobilization to the peripheral circulation (15). The precise primary cells, which are responsible for modulating HSC function within the adult niche, have not been isolated. It has been described that the presence of spindle-shaped N-cadherin+CD45- osteoblastic (SNO) cells may correlate with HSC function (7). Another population of cells has been described to produce a major soluble factor, which keeps HSC in their niches and attracts circulating HSC to the niche: the chemokine SDF-1α (Cxcl12). This chemokine is mainly produced by Cxcl12-abundant reticular (CAR) cells, which can be found at the endosteal surface, but also near vascular endothelial cells in the bone marrow (16).

A third population of cells express high levels of nestin and show potential to differentiate in different mesenchymal lineages (mesenchymal stem cells), suggesting that the activity of HSC and mesenchymal stem cells are regulated in a coordinated fashion (17). The SNO, CAR, and nestin$^+$ cells may express additional regulatory components that influence stem cell function, which include cell-cell receptors, soluble and cell surface–associated cytokines, and growth factors. All the so far determined and undetermined factors are influenced directly or indirectly by mechanical, systemic (e.g., PTH), and local (e.g., chemokines, BMPs, Angpt1) signals that regulate niche function (18, 19). In the paragraphs below, we will describe some of these factors and their possible functions of HSC self-renewal and differentiation (Figure 1).

3.1. SECRETED REGULATORS MAINTAINING HSC ACTIVITY

3.1.1. Factors involved in stem cell maintenance/homeostasis

It is still unclear what exactly constitutes "niche function" and what factors are required to uphold functional capacity for niche cells. During the passed years, many laboratories, including our own (20-22), have generated new cell lines which may serve as models for the different components of the niche and which identifies possible regulators produced by HSC-supportive stromal cells. Most of these cell lines have been generated from the mouse from different embryonic tissues (23) as well as adult hematopoietic niches (24). In addition, cell lines from human sources have also been explored (25-27). Extensive gene expression analyses are generating a picture of which potential soluble regulators are required for stem cell maintenance, including modulators of Wingless (Wnt) and Insulin-like growth factor (Igf) signaling, secreted proteases, novel chemokines and small cytokines (21, 22, 28). For example, these studies showed that the chemokine Cxcl15 (WECHE) affects cell cycle of repopulating cells (29). Also, it has become clear that stromal membrane receptors like Dlk1(30), a possible ligand of the Notch pathway, and Kirrel3 (31), can be cleaved and shedded to act as soluble factors, to increase formation of cobblestone area (32) and stem cell support function of stromal cells.

Our own studies showed over expression of several modulators of the Wnt signaling pathway. A loss of one of these, secreted frizzled-related protein1 (Sfrp1), in stromal cells increase production of hematopoietic progenitors in co-culture experiments. In mice deficient in Sfrp1, deregulated hemostasis and an increase in the number of quiescent stem cells (LSK: Lin- Il7r- Sca-1+ Kit+) cells was noted, and this was associated with a decrease in the expression level of β-catenin in these cells *in vivo*. Serial transplantations of wild type HSCs into Sfrp knockout mice showed a decrease of stem cells (Cd34- LSK: Cd34- Lin- IL-7R- Sca-1+ Kit+) and multipotent progenitor cells (MPP: Lin- IL-7R- Sca-1- Kit+), and a functional loss of HSC self-renewal when Sfrp1 is deficient in the microenvironment. These data demonstrated the role of Sfrp1 as a HSC homeostasis-maintaining protein through extrinsic regulation of β-catenin (33) (see also below).

3.1.2. Factors involved in HSC self-renewal and quiescence

3.1.2.1. The niche and HSC self-renewal

In recent years much knowledge has accumulated using mouse knockout and transgenic models. It has been demonstrated that specification of hematopoiesis as well as hematopoietic self-renewal are intimately linked to developmental pathways (Notch, Wnt, Bmp), cell cycle and epigenetic control. The precise relevance of each of the components of these developmental pathways is, however, still to be elucidated. Though the role of the pathways in self-renewal and HSC maintenance are undisputed, unexpected redundancies among pathways seem to exist. For instance, HSC can engraft and self-renew without canonical Notch (34), or Wnt (35, 36) signaling pathways. Furthermore, disruption of the TGF/BMP signaling pathway, by deletion of the central signaling intermediate Smad4, impairs HSC engraftment (37) but it is unclear at which level HSC behavior is affected. Thus, other signaling intermediates may be involved in cross-linking different pathways to promote self-renewal of adult HSC.

Since self-renewal requires cell division it is likely that the cell cycle machinery is somehow involved in self-renewal. Indeed, deficiency of cell cycle regulators like Cdkn1a ($p21^{Waf1}$), Cdkn1b ($p27^{Kip1}$), Cdkn2a ($p16^{Ink4a}$), Cdkn2c ($p18^{Ink4c}$), as well as cofactors like p53 and Rb strongly affect HSC self-renewal, where Cdkn2 acts as negative regulator and the other factors mentioned as positive regulators of self-renewal (38) (reviewed by Orford and Scadden). Besides, these drivers of cell cycle and factors involved in chromatin stabilization during cell division also have a great impact on HSC self-renewal. Factors like Ezh2, Mll, and the polycomb-group complex 1 (PcG1) genes Bmi1, Ring1b, and Rae28 are all required for stem cell maintenance as their deletion causes HSC exhaustion (39) (reviewed by Zon). As it turns out, PcG1 regulates proteasome-mediated degradation of the Cdkns and upregulate geminin, a new player in HSC maintenance and self-renewal (40) suggesting a regulatory feedback mechanism between PcG1 and Cdkn signaling.

3.2.1.2. The niche and HSC quiescence

Although there is progression in understanding the relative significance of the above intrinsic signaling pathways involved in HSC regulation, it is still unresolved how extrinsic signals affect these. Understanding extrinsic signaling is important in predicting the effects of extrinsic manipulation on HSC. One of such manipulation is the attempt of many investigators to *ex-vivo* expand HSC, or to restore hematopoiesis after hematopoietic damage. Regarding the expansion of HSC, there are currently no known growth factors or consistent conditions that allow a sustained numerical increase of HSC in culture. The major cause of this is the precise mechanisms by which extrinsic signals are coupled to intrinsic signaling are not known in detail.

The factors critically involved in niche-dependent HSC quiescence are slowly being uncovered. HSCs expressing the receptor Mpl or the tyrosine kinase Tie2 are quiescent, resistant to apoptosis and comprised within the, so-called, side population of HSC (41, 73). In addition, Mpl+ and Tie2+ HSC were found to be in direct contact with BM osteoblasts. Primitive behavior of Tie2+ HSC has been implicated both *in vitro* and maintained *in vivo* in the formation of cobblestone and induction of long-term repopulating activity of HSCs. Furthermore, secretion of the Tie2 ligand Angpt1 and the Mpl ligand TPO in the BM niche enhances the ability of HSCs to become quiescent and induces adhesion to bone, resulting in protection of the HSC compartment from myelosuppressive stress (41, 72, 73).

3.1.3. Ligands of receptor tyrosine kinases and their role in niche

A variety of soluble growth factors which have been shown to enhance the proliferation of HSC have been dubbed early-acting factors (42). These factors include multi-CSF (interleukin 3, Il3), GM-CSF (Gmcsf), FMS-like tyrosine Kinase-3 ligand (FL, Flt3l), stem cell factor (Kit ligand (Kitl) or SCF), erythropoietin (Epo), interleukin-6 (IL6) and thrombopoietin (Thpo/TPO) (43, 44). The effects produced by these growth factors are dependant on their concentration as well as combination of each one of these components (45).

3.1.3.1. KIT ligand (SCF)

The SCF receptor Kit is expressed by long-term repopulating stem cells (46). SCF is produced by both fibroblastic cells and endothelial cells and is expressed at development sites such as fetal liver and bone marrow (46, 47). The null alleles of stem cell factor and its receptor ckit, (Sl and W), cause embryonic lethality associated with severe anemia, but viable alleles which lack membrane-bound SCF, or in which point mutations impair c-Kit tyrosine kinase activity, results in the reduced numbers of CFU-s in the bone marrow of mice harboring Sl or W mutant (46). SCF has been shown to increase adhesion and thus may play a large role in ensuring that HSCs remain in the niche (47). A small percentage of HSCs regularly leave the bone marrow to enter circulation and then return to their niche in the bone marrow (48). The concentration gradients of SCF, along with the chemokine SDF-1, allow HSCs to find their way back to the niche (49). Moreover, different concentrations of SCF affect HSC differently, such that high SCF concentrations are required to promote self-renewal (50). In addition, the involvement of SCF in survival, mobility, and possibly self-renewal of HSCs in culture and in the HSC niche likely reflects its complex relationship with respect to different cell fates of HSCs.

Triggering of Kit by extrinsic SCF leads to phosphorylation of the intracellular domain of Kit, which contains at least eight phosphorylation sites (51). SCF stimulation starts the Ras/Mapk pathway, as well as the Pi3k/Akt pathway. As a single agent, SCF causes prolonged activation of the Ras/Mapk pathway, leading to loss of stem cells (52). Since it is known that SCF synergizes with other cytokines to maintain HSC, it is possible that these, in fact, cut prolonged Mapk activation short. Through binding to Jak2, Kit also activates Stat3 and Stat5, both associated with promotion of self-renewal and cell cycle progression.

3.1.3.2. FLT3 ligand (FL, FLT3LG)

The ligand of the Fms-like tyrosin kinase receptor 3 (Flt3 ligand, FL) is expressed in bone marrow fibroblasts and in stromal cells of adherent layers of long-term BMC (bone marrow culture). However, mice lacking Flk2/Flt3 develop normally. This statement is in line with the absence of the Flt3 receptor on the long-term repopulating stem cells (53), suggesting Flt3 is not directly involved in HSC quiescence or maintenance under steady state conditions. On the other hand, bone marrow from mutant mice exhibit 5-fold reduction in competitive repopulating potential relative to the wild-type marrow, that shows the importance of Flt3l for hematopoietic regeneration (54). In contrast to murine HSC, human HSC capable of long-term engraftment of immunodeficient mice do express FLT3 (55). Though murine HSC may not be directly susceptible to FL secretion, human HSC may respond to FL in the niche.

Since KIT and FLT3 are highly related receptor tyrosine kinase receptors, it is not surprising that FL triggers very similar signaling pathways as SCF, including Ras/Mapk, Pi3k/Akt and Stat signaling (56, 57). Despite these similarities with SCF-stimulated signals, FL as a single factor fails to stimulate HSC to proliferate (58). Interestingly, although ubiquitination is a general mechanism by which the level of receptor-type tyrosine kinases (like Kit and Csfr1) responses is regulated (59), activation of FL seems particularly dependant on the E3 ubiquitin-protein ligase Cbl. Inactivation of Cbl leads to FL-dependent myeloid transformation (60) suggesting that in those cases, FL production by the niche promotes leukemia development (see also below). Thus, although the responses to SCF and FL differ, though there are currently no definitive insights in the differences in activation of downstream signaling intermediates of Kit and Flt3 in HSC.

3.1.3.3 Macrophage colony stimulating factor (M-CSF)

A third member of the Kit/Flt3 family is Cfsr1, which is triggered by its ligand Csf1 (M-CSF). M-CSF/Csf1 exists as different splice variants and cleavage products and is expressed by fibroblastic cells, including osteoblastic cells as well as endothelial cells as well as several stromal cell lines (61). Mice deficient in Csf1 or its receptor Csf1r show increased numbers hematopoietic progenitors but an almost complete loss of monocytes/macrophages, suggesting that this ligand pair is dispensable for hematopoiesis, but required for myeloid differentiation (61). Indeed, development of osteoclasts requires the presence of M-CSF. Considering that osteoclasts are important cells in bone remodeling (see below), expression of M-CSF may indirectly regulate the niche. Like Kit and Flt3, the Csfr1 has a multiple phosphorylation site, which upon activation with M-CSF directs the already mentioned Jak2/Stat, Ras/Mapk and Pi3k/Akt signaling pathways.

M-CSF is also one of the few factors that are known to be involved directly in hematopoietic specification, together with G-CSF. Elegant recent experiments using sorted granulocyte/monocyte bipotent cells together with single-cell tracking, showed that M-CSF instructs the decision towards a monocytic cell fate (62). Since the niche produces M-CSF, this also means that not only does the niche regulates HSC quiescence, it is also actively involved in regulating hematopoietic cell fate.

3.1.4. Angiopoietins and angiopoietin-like secreted factors

Angiopoietin-1 (Angpt1), a previously reported ligand of Tie2 (63) is predominantly expressed by osteoblastic cells in the endosteum (64). Tie2/Angpt1

signaling activates β1-integrin and N-cadherin in LSK-Tie2+ cells and promotes HSC interactions with extracellular matrix and cellular components of the niche (41). Where SCF and FL mostly trigger proliferation and a regenerative response, the Tie2 ligands are mostly involved in maintaining quiescence (41). Quiescence or slow cell cycling of HSCs induced by Tie2/Angpt1 signaling contributes to the maintenance of long-term repopulating ability of HSCs and for the protection of the HSC compartment from various cellular stresses. Triggering of Tie2 by Angpt1 or 2 has overlapping signals with Kit and Flt3: Ras/Mapk and Pi3k/Akt activation. However, triggering of Tie2 also leads to NF-□B-dependent inflammatory responses (65). How NF-□B ties into HSC self-renewal is unclear at the moment.

In murine fetal liver, a CD3+ Dlk1+ population of cells exists which stimulates HSC expansion (66, 67). These cells secreted high levels of two Angpt family members Angptl2 and 3. The Angptls are mainly involved in lipid metabolism as inhibitors of lipoprotein lipase. In combination with Igf2, these Angptls expand HSC (66). In adults, angptl3 is expressed in the niche, mainly by sinusoidal endothelial cells. It was recently shown that activity of the Angptl factors is not restricted to fetal hematopoiesis. In adult Angptl3-deficient mice, HSC show decreased number and quiescence. Moreover, Angptl3-deficient stromal cells show diminished capacity of support HSC in culture (68). These results strongly suggest the involvement of the Angptl secreted factors in HSC maintenance. So far only Ikaros, a transcription factor known to regulate HSC self-renewal (69), was implicated as Angptl3 downstream intermediate (68). Considering these observations, it would be of interest to find out which receptors (and, thus, signaling pathways) are involved in this response and how signals from the niche modulates these pathways.

3.1.5. Thrombopoietin (TPO)

The early acting factor thrombopoietin (Thpo, TPO) was cloned as a factor mainly affecting thrombopoiesis (70). Many of the molecular pathways that mediate TPO action have been explored. Like all other members of the hematopoietic cytokine receptor family, on binding of this hormone to cells, members of the JAK family of kinases are activated. This in turn phosphorylates the TPO receptor, generating docking sites for second messengers that affect multiple signaling pathways (71). TPO produced by osteoblasts in the BM niche has also been reported as an important mediator to maintain quiescence of BM HSCs. A high expression of the TPO receptor MPL in the side population (SP) of HSC revealed the modulation of thrombopoietin on HSC cell cycle progression on the osteoblast surface. This study demonstrated that a single cytokine could theoretically regulate HSC in the presence of postnatal niche cells (72, 73).

TPO-mediated signaling synergistically induces HSC proliferation with other cytokines. TPO acts as survival factor for HSC (74) and as a single factor, supports long-term maintenance HSCs in stroma-dependent Dexter-type culture of bone marrow cells (75). The number of megakaryocyte, granulocyte-macrophage erythroid, and multilineage progenitors are significantly reduced in the bone marrow, spleen, and peripheral blood of either TPO (76) or Mpl deficient mice (77). This phenotype could be recovered with the administration of recombinant murine TPO to TPO-deficient mice and control littermate mice. It also significantly increased the absolute number of myeloid, erythroid, and mixed progenitors in bone marrow and spleen. Furthermore, the megakaryocytopoietic activities of other cytokines in cells from animals without a functional TPO or c-mpl gene have been shown both *in vitro* and *in vivo*. Thrombopoietin has been shown to tightly regulate HSC cell-cycle progression at the endosteal surface, and either inhibition or stimulation of Mpl/THPO signaling in LT-HSCs showed reciprocal expression of cell cycle regulators (76, 78, 79).

TPO stimulates Ras/Mapk, Pi3k/Akt and Stat signaling in HSC, and is in that respect similar to SCF and FL. However, where Mapk activation leads to sustained Erk phosphorylation, TPO-induced phosphorylation leads to a transient activation (80), one reason to explain that TPO sustains self-renewal and SCF, as a single factor. The TPO receptor MPL is also intimately linked to the adaptor Lnk (81). This adaptor seems to be required for the ability of TPO to sustain HSC through Jak2 (82). In myeloid dysplasia, Lnk missense and deletion have been found which increase Jak2/Stat5 signaling (83), suggesting that myeloid disease, the niche has diminished capacity to regulate Jak/Stat signaling through secretion of TPO.

3.1.6. Wnt stimulators and Wnt inhibitors

Wnt proteins are secreted by osteoblast cells in the hematopoietic niche and bind to frizzled receptors (Fzd4 and Fzd6) on the surface of HSCs. Signaling through the canonical Wnt pathway is mostly regulated through signals affecting β-catenin degradation (84-86). Mice deficient in the canonical Wnt factor Wnt3a die at E12.5 of embryonic gestation. Although Wnt factors are known to be extrinsic regulators in Drosophila, functionality of Wnt3a-/- HSC was not restored in a wild-type environment, suggesting an intrinsic, rather than an extrinsic defect in Wnt3a-deficiency (87). Deletion of Wnt5a, a major mediator of non-canonical Wnt signaling is also embryonically lethal. Animals with haploinsufficiency develop myeloid leukemia and B cell lymphomas (88). Although the specific role of the niche was not investigated, it seems likely that secreted Wnt5a is required for maintenance of normal (89). Interestingly, Wnt5a stimulation of HSC resulted in an increase of □-catenin, suggesting that Wnt5a stimulates canonical, rather than non-canonical pathways in HSC.

Beta-catenin is the major regulator of the evolutionary conserved canonical Wnt signaling pathway. Defects in degradation lead to prolonged stabilization of β-catenin levels in HSCs. Prolonged stabilization results to a loss of HSC dormancy, a concomitant loss of HSC function, and a differentiation block (90). However, loss of both β- and γ-catenin does not seem to have any affect on HSC behavior (35). Thus, for proper HSC function, catenin

levels need to be low. This view is also supported by reports showing that deficiency in the upstream catenin kinase Gsk3 or its ubiquitinase Ubc13 causes similar HSC defects as catenin over expression (91, 92).

The Wnt signaling pathway is not the only pathway that involves HSC. Indeed, modulation of β-catenin also affects niche function. A non-functional β-catenin induces reduced ability to support early HSCs accompanied by a diminished number of osteoblast and reduction in associated growth factor *in vivo* (93). Conversely, over expression of β-catenin improves the ability of stromal cells to support HSC maintenance in culture (94). Both these reports demonstrate that β-catenin plays an important role in the niche for the generation of osteoblasts, as well as for the preservation of early hematopoietic cells in the HSC niche (93).

The significance of Wnt signaling for HSC numbers and function is highlighted by observations of HSC regulation by different components of this pathway. Inhibition of Wnt/ β-catenin in bone marrow microenvironment created by the osteoblast-specific over expression of Dkk1 resulted in the increase of the number of proliferating HSCs and the reduction in the ability to reconstitute the system of irradiated recipient mice, showing that microenvironment-related Wnt/β-catenin activity is crucial for the maintenance of HSC quiescence (95). Our own work with Sfrp1 knockout mice revealed diminished HSC self-renewal in the absence of niche Sfrp1. Surprisingly, Sfrp1 deficiency also lowered β-catenin levels in HSC isolated from their niche environment, suggesting that *in vivo*, Sfrp1 may, in fact, act as a Wnt stimulator. This hypothesis warrants further investigation, since it has been shown that Sfrp1 may directly bind to Fzd receptors, some of which are expressed on HSC, like Fzd4 (89). Thus, the fine-tuning of Wnt/β-catenin activity might be crucial for the balance between maintenance of HSC quiescence and differentiation (96).

3.1.7. Notch ligands

Notch signaling plays an important part in both HSC regulation and in osteoblastic cells. Apart from the multiple *in vitro* studies (97-100), evidence for a role of Notch signaling between HSCs and the bone marrow microenvironment *in vivo* remains ambiguous. Several lines of evidence strongly suggest a role for Notch in the interactions between HSC and the niche. First, the Notch ligand Jagged1 is expressed in BM stromal cells and murine osteoblastic cells and increased Jagged 1 (Jag1) in human stroma is sufficient to expand HSC (97). Additionally Jag1 is expressed by human-derived CD146+ cells multipotent stromal cells (101), which can form a hematopoietic supportive niche *in vivo* (64). In addition, MSC uniquely express Notch3, suggesting possible feedback mechanisms between MSC and cells with which they interact. Although the functionality of Jag1 in these cells was not examined, the observation that HSC and MSC form a niche *in vivo* (17) further supports the idea that Notch signaling in the niche is important in interaction with HSC.

In contrast to this data, other investigators reported findings which suggest that neither Jagged1 nor Notch signaling are required for maintaining HSC populations. For instance, in mouse studies in which Jag1 was conditionally deleted no differences in basal levels of HSC was noted (102). Further loss of Notch1 on HSC also did not influence engraftment and HSC self-renewal (102). One explanation for this finding could be that Jag1-associated Notch signaling is mediated by other signaling elements in this pathway. However, expression of a dominant-negative isoform of mastermind (DNMAML), ruled out the possibility of compensation by alternative Notch receptors, indicating the dispensability of notch signaling for the maintenance of HSC at steady state (34).

However, Notch signaling was shown to be required for megakaryocytic differentiation (103). HSC-supportive cell lines are characterized by expression of the putative Notch ligand Dlk1 (22, 30). Dlk1 (Pref-1) is an imprinted gene which is also expressed in HSC and its action appears to be to limit proliferation and differentiation (164). Interestingly, studies in $Dlk^{-/-}$ mice suggest that proper B-cell development requires the presence of Dlk1. Whether Dlk1 is a true ligand for the Notch receptors is unknown at present. Inactivation of Mind bomb-1 (Mib1), which is essential for Notch ligand endocytosis and subsequent Notch activation in the microenvironment, results in myeloproliferative disease. These results imply that defective Notch signaling in non-hematopoietic microenvironmental cells can be the cause of the myeloproliferative disease. These experiments also indicate that, although Notch signaling in HSC may be dispensable for normal hematopoiesis, Notch signaling may be required for HSC support in the microenvironment (104).

3.2. Secreted Factors involved in stem cell adhesion and lodging

HSC lodge very close to the endosteal surface (105-108), indicating that, on the one hand, HSC home very efficiently to the niche, and, on the other hand, the endosteal niche is the preferred environment of HSC. Homing and adhesion depend on a complex of subsequent mechanisms involving rolling, firm adhesion and transendothelial migration. Stromal derived factor-1 (Sdf-1 or Cxcl12) is a chemokine produced by osteoblasts, fibroblasts, and endothelial cells in response to injury. Cxcl12 and its receptor: Cxcr4 are critically involved in chemotaxis of HSC to the bone marrow during development, and their absence cause defective myelopoiesis and B-lymphopoiesis (109). Moreover, Cxcl12 is a critical factor in guiding the matrix metalloproteinase 9 (Mmp9) dependent transendothelial migration of hematopoietic cells into the bone marrow microenvironment (110).

Once within the microenvironment, Cxcl12 regulates lodgment of HSC within the endosteal region, where, in collaboration with TGF, it controls the HSC quiescence/cycling switch through Cxcr4 (111, 112). It has been suggested, that interference with Cxcl12 or Cxcr4 may obstruct HSC function. Indeed, in HSC mobilization,

for instance by the growth factor G-CSF, HSC proliferation is preceded by degradation of Cxcl12 by exo-proteases such as cathepsin K (113) and X (114), suggesting that the loss of Cxcl12 is, at least in part, responsible for the mobilization of stem cells. Also, modulators of Cxcl12 expression, such as Txnip (115), or Cxcr4, such as Pim1 (116), have been shown to regulate critically HSC adhesiveness and homing. Indeed, Txnip-deficiency reduces responsiveness to Cxcl12, culminating in HSC exhaustion. Interestingly, Txnip-deficient HSC shows hyperactive Wnt signaling, suggesting a link between HSC adhesion and Wnt signaling.

Several different factors regulate adhesion of HSC to niche cells. Indeed, it has been demonstrated that direct cell-to-cell adhesions are important mechanisms for the survival of HSC in microenvironment (117). Other secreted factors may also influence adhesion through so-called inside-out signaling mechanisms that regulate the affinity of adhesive receptors. The most important adhesive receptor is the α4β1 integrin (VLA-4)(118), which binds mainly to its ligand Vcam1 and also to fibronectin (Fn). Adhesive activity of the α5β1 integrin VLA-5 to Fn is inducible (119). Examples of this kind of regulation are the modulation of the affinity of the integrins, VLA-4 and VLA-5 to their respective ligands Vcam1 and Fn by Il3 (120), Gmcsf and Scf (118, 121), and Tpo (122). In addition, different adhesive receptors may cross-talk to regulate affinity of VLA-4 and -5 (119). The interesting aspect of these studies is that the induction of adhesion does not last, but subsides [66]. The significance of this observation is unclear, but opens the possibility that cells move within the niche and are only tightly bound to certain cells in the niche for a limited period of time. These studies show that secreted factors may improve HSC survival in the niche at least in part by modulating their adhesive behavior, and that loss of adhesion may constitute a differentiation-promoting environment.

Tight adhesion of HSC to the niche is almost certainly mediated through signaling through the Rho GTPase Cdc42 that not only regulates adhesion (123), but also HSC proliferation (124). In addition, Cdc42 is required for proper B cell development (125). The niche itself is not a passive adhesive substrate in the process of HSC lodging. Indeed, niche cells defective in annexin 2 (Anxa2) expression fail to support HSC adhesion, and HSC show defective survival in Anxa2-deficient animals (126). In addition, as noted above, Wnt signaling is of critical importance as an intrinsic determinant in the balance of HSC proliferation and differentiation. There is now also evidence that canonical Wnt signals modulate HSC supportive activity of non-hematopoietic niche cells. Indeed, over expression of β-catenin in stromal cells, significantly improved their ability to support HSC in culture (93, 94). Since Wnt signaling is required for skeletogenesis (reviewed by Macsai *et al* (127), and Williams and Insogna (128) this pathway may not only directly regulate HSC, but also, the size of the niche.

3.3 Secreted factors that retain stem cell pool and niche size

As described above, niche factors determine the balance between HSC quiescence and cell cycle entry, with subsequent differentiation. Thus, beside the factors that control quiescence, other factors constrain the size of the stem cell pool. Since osteoblasts are important in this process, it was hypothesized that continuous activation of osteoblasts would affect HSC behavior. Indeed, when osteoblasts are activated by parathyroid hormone, many factors are secreted, including Sfrp4, Bmp4, Jag1, TGF□ and Opn (129, 130). Studies of Opn-deficient mice demonstrated that in the absence of Opn, the number of HSC increases in a stromal cell-dependent manner. This, in turn, is associated with increased stromal Jag1 and Angpt1 expression and a reduction in primitive hematopoietic cell apoptosis. Activating osteoblasts with parathyroid hormone in the absence of Opn induces a strong increase in stem cells, indicating that physiological Opn limits the affects of niche activation on HSC cycling (130).

Although the size of the niche is relatively poorly researched, one could speculate that secreted factors that affect remodeling of trabecular bone, will also affect niche size. Remodeling of bone is a complex process in which bone is formed by osteoblasts and osteocytes, and also resorped by osteoclasts. Obvious factors responsible in this respect are Wnt3a and its inhibitors Dkk1 and Sfrp1. As described in detail above, all these factors have known affects on HSC self-renewal. Wnt factors not only directly affect HSC but also affect bone remodeling. Both Dkk1 and Wnt3a increase osteoblast proliferation, and where Dkk1 stimulates osteoclast differentiation (131), Sfrp1 has been shown to inhibit Rankl-dependent osteoclast differentiation (132, 133). Thus, like in regulation of HSC, Dkk1 and Sfrp1 appear to exhibit opposite affects on bone remodeling. Beta catenin itself promotes differentiation of MSC into early osteoblasts (134). Other mediators that are thought to regulate hematopoietic stem cells at least in part through altered bone remodeling are Serpina1 (135), Timp3 (136) and Ebf2 (137).

Bone remodeling is also altered during stem cell mobilization. Clearly mobilization protocols using the growth factor G-CSF affect hematopoietic cells. It has been shown that HSC mobilize in part because of the niche remodeling. During mobilization, Opn-expressing osteoblasts are depleted and once the mobilizing agent is removed, rapid expansion of these cells occur (138). This process primarily depends on tropic endosteal macrophages (139). Thus G-CSF causes HSC proliferation and mobilization by temporary remodeling of the niche.

4. THE CANCER STEM CELL NICHE

The behavior of cancer stem cells (CSC) resembles that of normal stem cells. Although it has long been thought that cancer stem cells were actively cycling cells, it is now clear that quiescent leukemia stem cells exist (140, 141). Niche cells regulate CSC in much the same way as normal HSC. Nevertheless, the precise role for the niche in cancer development is still a mystery. Some

studies show that knocking out the extrinsically-regulated phosphatase Pten, results in mobilization and eventual depletion of normal HSCs with the generation of CSC (142) (143). This observation is associated with an increased HSC mobilization and development of leukemia. In such cases, it is well possible that the CSC niche functions normally in the presence of tumor cells. Tumor cells may already have been selected for secondary mutations that promote their survival. For instance, downregulation of the ubiquitinase Cbl increases expression of survival- and proliferation-promoting receptor tyrosine kinases such as Kit and Flt3. Activating mutations in kinases such as Pim1, which regulates Cxcr4 expression (116) may promote proliferation, and thus, the emergence of additional mutations. Several lines of evidence strongly suggest that intrinsical stromal dysfunction is a cooperating factor in the development of leukemia. Deficiency of the NF-□B signaling intermediate Ikba (144), the Notch pathway mediator Mib1 (104) and the cell cycle regulators Rb (145) and Rarg (146) cause a non-cell autonomous myeloproliferation involving deregulation of HSC homing, and alterations in Notch, and Tnf signaling in HSC.

More recent work has elegantly shown that the osteoprogenitor-specific absence of Dicer1 and Sbds causes myelodysplasia with increased hematopoietic cell turnover, which in some animals developed into myeloid sarcomas (147). In all these models, it seems likely that intrinsic changes in the stromal cells lead to altered extrinsic signaling. However, the nature of the changes in these signals have not been explored. Jag1 expression is altered in the $Pten^{-/-}$, $Ikba^{-/-}$ as well as the $Dicer1^{-/-}$ mice, suggesting Notch signaling is commonly involved in the hematopoietic deregulation. However, the secreted factors differentially expressed in Dicer-deficient osteoprogenitors do not include known Notch ligands. Thus, stromal dysfunction may collaborate with CSC in the development of leukemia. But, the alterations in secreted factors between the CSC niche and the normal HSC still needs to be elucidated in detail.

Dynamic *in vivo* imaging show that leukemic cell growth disrupts normal hematopoietic stem cell bone marrow niches and creates abnormal microenvironments that confiscate transplanted human $CD34^+$ (HPC-enriched) cells. $CD34^+$ cells in leukemic mice declined in number over time and failed to mobilize into the peripheral circulation in response to cytokine stimulation. The normal phenotype could be rescued by neutralizing stem cell factor (SCF) secreted by leukemic cells. Thus, $CD34^+$ cell numbers could be normalized, and their mobilization restored in leukemic mice (148). In experiments with infused childhood ALL cells, it has been noted that the leukemia cells secrete SCF that, in turn may attract the normal HSC. As a result, the CSC forms a new niche, which also harbors normal HSC. However, this CSC-induced niche does not maintain normal hematopoiesis, probably by interfering with a return to quiescence, causing a depletion of normal cells. So far, this is an isolated, but intriguing observation that awaits confirmation in other CSC model systems (149).

5. THERAPEUTIC RELEVANCE AND PERSPECTIVES

The former paragraph clearly demonstrates that stromal dysfunction contributes to leukemia development. Furthermore, once the leukemia has developed, the CSC may influence normal niche function. These observations open the possibility of targeting the niche as a novel therapy of leukemia disease. In order to ensure successful translation of this concept, the underlying signaling mechanisms for homing, lodging and transmigration of HSC out of the microenviroenment needs to be addressed more specifically. In particular, the differences between normal HSC and CSC behavior need to be addressed. Some recent model studies have begun mapping the homing, proliferation and survival sites of the human leukemia cells in comparison to normal cord blood CD34+ cells.

A crucial role of both the osteoblastic niche and vascular niche in providing anti-apoptotic signals has been described (150). As mentioned above, secreted factors influence quiescence, cell cycle entry, regeneration, as well as cell survival. The latter is most probably tied into the mechanisms regulating dormancy and likely to involve adhesive mechanisms. The activation achieved with cell-to-cell adhesion forces has been shown to be important for the persistence for both acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) CSCs (151-153). The late survival-signaling cascade is switched on with the ligation of integrins and as a result of the direct contact of leukemic CSC with BMSC downstream anti-apoptotic signaling is activating. The blockade of the adhesion regulator and integrin-linked pseudokinase ILK (154) with a specific inhibitor QLT0267 specifically inhibits the downstream signaling in leukemia cells (155). Interestingly treatment of normal hematopoietic cells appears refractive to inhibition of ILK, suggesting ILK is not involved normal hemostasis (156). Another way of disrupting adhesive interactions between leukemia CSC and the niche is by interfering with Cxcl12 signaling. Interfering with lodgment could force cells out of quiescence into cell cycle entry, which would make them susceptible to cytostatic and apoptotic therapy. Cxcr4 levels are significantly elevated in subtypes of leukemia (AML, B-CLL, B- or T- cell ALL) (157). A possible mediator of Cxcr4 up regulation is the up regulation or activated mutations of Pim1 (158) by activated FLT3 mutations. Since FL is produced by the niche, anti-FL or Pim1 inhibitor treatment are possible modalities by which leukemia may be treated in the future.

Treatment with AMD-3100, a specific antagonist of CXCR4, induces rapid and robust HSC mobilization in both humans and mice (159, 160). Recently, administration of the Cxcr4 antagonists AMD3100 or the rcp168 peptide in combination with chemotherapy has been shown to trigger the movement of APL CSCs in blood and improve overall survival (161, 162). Similar observations have been made in the case of primary CLL and AML cells. Role of E-selectin and P-selectin in the HSC homing to the BM has been well characterized in mice using function –blocking antibodies or targeted deletions of these genes. When E-selectin and β1-integrin were inactivated, the deficient

recipients revealed a profound alteration in HPC homing (> 90% reduction). Competitive assays to test homing of long-term repopulating stem cells also revealed a drastic reduction (> 99%) of the homed stem cell activity. Further homing studies with PSGL-1–deficient HPCs pre-treated with anti-4 integrin antibody revealed that PSGL-1 contributes to about 60% of E-selectin ligand–mediated homing activity (9).

So far, no therapies exist which specifically target the leukemia niche or molecules deregulated in niche cells that could contribute to leukemogenesis. Considering the myeloproliferation present in mice with deletion of Ikba, Mib1, or Pten, one could envision targeting these molecules to "quiet down" HSC. Also, since cALL cells may secrete SCF which creates a secondary leukemia, niche targeting specific early acting factors like SCF or its receptor: Kit may also be an option. Indeed, anti-SCF restores the normal localization of HSC (148) and enhances chemotoxicity in AML cell lines (163). Other niche factors known to reinforce HSC dormancy, such as the Wnt inhibitors Sfrp1 and Dkk1, are frequently down regulated in human cancer. Since over expression of Dkk1 decreases HSC self-renewal, Dkk1 agonists could be of help in interfering with self-renewal of leukemia CSC. The delineation of the networks of soluble niche factors involved in regulation of normal self-renewal, cell cycle entry, regeneration and adhesion are incomplete. The same can be said for what is known about the cellular components involved and the feedback mechanisms existing between these cells. Clearly, since disruption of normal niche function interferes with normal HSC regulation and promotes leukemogenesis, the niche is is more and more becoming an attractive target for the therapy of leukemia.

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