

## Local signals in stem cell-based bone marrow regeneration

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The cellular basis of bone marrow (BM) tissue development and regeneration is mediated through hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). Local interplays between hematopoietic cells and BM stromal cells (BMSCs) determine the reconstitution of hematopoiesis after myelosuppression. Here we review the BM local signals in control of BM regeneration after insults. Hematopoietic growth factors (HGFs) and cytokines produced by BMSCs are primary factors in regulation of BM hematopoiesis. Morphogens which are critical to early embryo development in multiple species have been added to the family of HSCs regulators, including families of Wnt proteins, Notch ligands, BMPs, and Hedgehogs. Global gene expression analysis of HSCs and BMSCs has begun to reveal signature groups of genes for both cell types. More importantly, analysis of global gene expression coupled with biochemical and biological studies of local signals during BM regeneration have strongly suggested that HGFs and cytokines may not be the primary local regulators for BM recovery, rather chemokines (SDF-1, FGF-4) and angiogenic growth factors (VEGF-A, Ang-1) play instructive roles in BM reconstitution after myelosuppression. A new direction of management of BM toxicity is emerging from the identification of BM regenerative regulators.

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### Introduction

Bone marrow (BM) regeneration after chemotherapy involves participation of hierarchical arrays of hematopoietic cells as well as bone marrow stromal cells, including fat cells, fibroblasts, endothelial cells, osteoblasts, osteoclasts and their precursors developed possibly from mesenchymal stem cells. Hematopoiesis is regulated by a complex network of cytokines, growth factors, and chemokines. Recent studies demonstrate that the development-related Notch and Wnt signaling pathways also play key roles in hematopoiesis. Recent progress in autocrine/paracrine mechanisms regulating BM hematopoiesis and stromapoesis (making of the stromal cells) and their interplays in the context of BM regeneration are reviewed.

### Cellular composition of BM

Bone marrow is a soft, spongy material containing blood, fat, connective tissue, blood vessels and small segments of bone. The connective tissue forms a delicate meshwork within the marrow cavity of bones, and it is permeated by numerous thin-walled blood vessels. Within the spaces of this tissue, the immature and adult stages of blood cells exist. The small segments of bone are termed trabecular bone; a meshwork of delicate bony plates and strands within which the hematopoietic tissue and its stromal cells are suspended. The trabecular surfaces are covered by a layer of endosteal cells, osteoblasts, and osteoclasts. The spaces with varying size and shape that lie between adjacent trabeculae comprise the basic structural units of hematopoiesis. A sinusoidal network arborizes throughout the intertrabecular spaces. The trabeculae, arterioles and venules form the structural framework of BM, around which hematopoiesis develops in the intersinusoidal spaces [1].

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Hematopoietic cells in BM can be recognized histologically as granulopoietic cells, monopoietic cells, erythropoietic cells, megakaryopoietic cells, and other cells including lymphocytes, plasma cells and mast cells. Bone marrow stromal cells consist of a heterogeneous mixture of adipocytes, fibroblasts, endothelial cells, osteoblasts, osteoclasts, and macrophage-like cells, together with a complex extracellular matrix including fibronectin, haemonection, and proteoglycans [1].

### Stem cell-based BM development

Two types of tissue-specific stem cells reside in BM, including hematopoietic stem cells (HSC) and mesenchymal stem cell (MSC). The two cell types and their progenies interact closely to form a cellular system of inter-, intra-, and/or mutually-dependent relationships. Much of the research activity in BM has focused on the characterization of these specific cellular system and their relationships.

### HSCs reside in BM

The hematopoietic system is an embryologic derivative of mesoderm. In vertebrates, hematopoiesis takes place at successive anatomic sites. Embryonic hematopoiesis occurs in the blood islands of the yolk sac. Adult hematopoiesis is transient in the fetal liver but later sustained throughout life in the BM. Indeed, experiments more than 30 years ago described the sequential blood-making organs [2]. Blood cells are continuously produced throughout the lifetime from a rare population of multipotential HSCs. Almost 75% of HSCs are quiescent in normal BM [3]. HSCs have two characteristics: they give rise to additional HSCs through self-renewal and also undergo differentiation to progenitor cells that become variously committed to different hematopoietic lineages [4]. Cells of the lymphoid lineages (B and T lymphocytes, natural killer cells), and cells of the erythroid/myeloid lineages (erythrocytes, megakaryocytes, platelets, granulocytes, monocytes, macrophages, osteoclasts, and dendritic cells) are all descendants of a multipotential HSC [4-6].

### Self-renewal and multipotency of HSCs

Upon division of a stem cell, at least one daughter cell retains the state of this stem cell. This capacity is called self-renewal. Symmetric divisions expand the number of HSCs, whereas asymmetric divisions retain HSC potential in one daughter cell, generating further differentiated progeny in the other daughter cell. Divisions that generate two differentiated progeny daughter cells delete the HSC potential. At different times of ontogeny and in different

environments, the probability of an HSC dividing either symmetrically, asymmetrically, or fully differentiating is believed to vary [7]. It has been a matter of much debate on whether the local signals in the BM environment are able to influence the type of division, and thereby the number of BM HSCs [4, 6, 8, 9].

### Local signals regulating BM HSCs numbers

Three BM local signaling molecules have recently been reported to regulate the number of BM HSCs. Wnt proteins produced by BM stromal cells activate their cognate Frizzled receptors of HSCs. Recombinant Wnt3a protein was demonstrated in a HSC ex-vivo system to induce self-renewal of HSCs [10]. The second local signaling pathway is the bone morphogenic proteins (BMPs). Conditional knockout of the BMP1a receptor in the mouse led to an increase of trabecular bone, osteoblasts, and the number of HSCs [11]. It is believed that the osteoblasts constituted the HSCs niche, and the niche size controlled the number of HSCs. The third local signaling molecule is the Notch pathway. Notch ligand Jagged1, expressed by osteoblasts, activated the Notch pathway in HSCs and increased the stem cell numbers [12]. Interestingly, the number of osteoblasts in the BM stromal elements could be easily manipulated by exposure of the BM to parathyroid hormone *in vivo* and in tissue culture of BM cells. Activation of parathyroid receptor increased the number of osteoblasts, hence the number of HSC niches. Notch pathway activation is responsible for the observed increase of HSCs. Cultivation of stromal cells with parathyroid hormone provides a clinical strategy to expand HSCs by manipulating the number of osteoblasts *ex-vivo*. Therefore, it is expected that clinical expansion of HSCs may be achieved by utilizing the newly identified local signals controlling the number of HSCs.

### Lineage commitment of MSCs in BM

The BM cells of nonhemopoietic tissues are referred to as mesenchymal cells or bone marrow stromal cells (BMSCs). It is a heterogeneous population of BM cells. Among them, MSCs are a rare population of cells capable of differentiating into different cell types defined as mesenchymal cells [13]. Other cell types of BMSCs include macrophages, endothelial cells, fibroblasts, adipocytes, osteoblasts, and osteoclasts. BMSCs provide growth factors, cell to cell interactions, and matrix proteins essential for the maintenance, growth, and differentiation of HSCs [14-16]. Functional defects in the BMSCs would therefore be expected to impact negatively on hematopoiesis. MSCs are a rare population (approximately 0.001%-0.01%) of adult human BM [17]. MSCs contain homogeneous fibro-

blast-like cells, which do not express typical hematopoietic lineage markers (CD14, CD34, and CD45), but are consistently positive for CD13, CD28, CD33, CD44, CD105, CD166, and HLA class 1. There appears to be significant heterogeneity in normal individuals in the ability of MSC to expand in culture and to maintain multipotentiality [18-20]. The number of putative MSCs decreases markedly in aging [21, 22]. Therefore, MSCs undergo two parallel and probably interdependent processes during prolonged cultivation. One is replicative aging; another is a decline in differentiation potential. MSCs can be induced to differentiate into bone, adipose tissue, cartilage, muscle, and endothelium, if they are cultured under specific permissive conditions [23, 24]. However, it is not clear to what extent MSCs contribute to a number of other endogenous mesenchymal cells, such as fibroblasts, endothelial cells, osteoblasts, and adipocytes, in BM tissue. Can a single MSC repopulate the BM nonhematopoietic tissue? Further studies of the relationship between MSCs and their progenies will shed light on the lineage development of BM nonhematopoietic cells.

### **Stem cell-based BM regeneration**

Radiotherapy and chemotherapy are common therapeutic modalities for cancer. Unfortunately, these therapies are not tumor-specific. Normal tissues, particularly BM, are extremely vulnerable to cytotoxicity caused by these therapies [25]. An acute and transient myelosuppression is a common side effect of radiotherapy and chemotherapy, which primarily damage the rapidly proliferating hematopoietic progenitors and their more mature progeny. Persistent myelosuppression or BM failure occurring in some patients after radiotherapy and chemotherapy is an indication of injury to HSCs and/or MSCs.

### **BM cellular damages after radiotherapy and chemotherapy**

Exposure to high doses of radiation leads to severe toxicity to BM. Several histopathologic studies have shown that BM becomes hypocellular with vascular congestion and hemorrhage during the first hours and days after irradiation [26]. Doses exceeding 2 Gy markedly suppress mouse BM function, leading to pancytopenia and immunosuppression. Meng *et al.* have found that exposure of BM mononuclear cells to radiation inhibits the frequency of HSCs in association with the induction of apoptosis in HSCs and their progeny [27]. Pre-incubation of the cells with z-VAD, a broad-spectrum caspase inhibitor, significantly decreased the radiation-induced apoptosis [27]. These findings are in agreement with previous studies showing that over-ex-

pression of an anti-apoptotic protein or down-regulation of a pro-apoptotic protein in hematopoietic cells attenuated the hematopoietic effects of radiation [28, 29]. The studies suggest that radiation-induced BM suppression is caused primarily by apoptosis of BM HSCs and progenitors.

Cytotoxic agents used in cancer treatment have adverse effects on hematopoiesis. A large number of non-cell-cycle dependent drugs (e.g., alkylating agents) may lead to progressive depletion of HSCs, resulting in impaired BM reserve capacity and long-term cytopenia. Cell-cycle dependent drugs (e.g., cytarabine) tend to cause transient cytopenia due to their preferential killing of highly proliferating hematopoietic progenitors. They have little effect on the largely quiescent HSCs.

### **BM regeneration after radiotherapy and chemotherapy**

BM suppression in the settings of cancer radiotherapy and chemotherapy has several possible etiologies [30], including direct injury to HSCs or their progenies, structural or functional damage to BMSCs and the related vasculature, perturbation of BM function by the disease (e.g., leukemia), and an inherent defect in BM HSCs and/or MSCs associated with disease (e.g., leukemia) [31]. BM regeneration depends on two factors: a sufficient number of HSCs and stromal cells. Stromal cells are known to be damaged by radiotherapy and chemotherapy. However little is known on the extent of this damage and on the regeneration ability of the stromal cells. It usually takes a much longer time to regenerate stromal cells, if possible, than to regenerate hematopoietic cells [32]. The difficulties inherent in experimental and clinical studies of stromal cell alterations make it difficult to assess their contribution to BM regeneration. Currently, we have no means to protect stromal cells from damage or enhance their recovery. It is therefore understandable that a great deal of effort has been focused on the factors which enhance the regeneration of BM hematopoietic cells.

### **Local signals in BM development and regeneration**

Regulation of a cellular kinetic system may be controlled by three types of factors: stimulatory factors, which trigger cells into proliferation; differentiation factors, which provoke specific maturation; and inhibitors. In a stable system, regulation requires negative feedback. This signal directly inhibits proliferation of the parent cells or suppresses a stimulatory process. BM is a dispersed tissue and its control is predominantly exerted by soluble factors. A network of finely tuned regulatory factors is required to control normal hematopoiesis and regeneration after an insult.

**Table 1** Partial list of soluble factors that regulate normal BM hematopoiesis.

Factors that regulate HSCs	Positive regulators	IL-3, IL-6, IL-11, SCF, GM-CSF, FL, TPO, Wnt-3, Delta-1, Delta-4, Jagged-1, Shh, BMP4, SDF-1
	Negative regulators	MIP-1, TGF- $\beta$
Myelopoietic factors	Positive regulators	GM-CSF, G-CSF, M-CSF, EPO, TPO, SCF/KL, FL, TNF, LIF, IL-12, IL-11, IL-6, IL-5, IL-4, IL-3, IL-1, SDF-1, FGF-4
	Negative regulators	TNF, IL-4, TGF- $\beta$ , IFN, MIP-1, IL-10, IL-13
Lymphopoietic factors	Positive regulators	IL-2, IL-4, IL-7
	Negative regulators	TGF- $\beta$ , IL-4

### Soluble factors that regulate BM hematopoiesis

The development of hematopoietic cells is regulated by hematopoietic growth factors, cytokines, and chemokines secreted by BM stromal cells and hematopoietic cells [33-38]. They are summarized in Table 1.

### Soluble factors that regulate BM stromal cells

Much less is known about soluble factors that regulate the function and growth of stromal cells comparing with hematopoietic regulators. Radiotherapy and chemotherapy often induce myelosuppression as well as regression of BM sinusoidal vessels. Kopp *et al.* reported that during chemotherapy-induced BM regeneration angiogenic factors (VEGF-A and Angiopoietin 1) support the emergence of Tie-2-positive BM neovasculature [39]. Exogenous VEGF-A and Ang-1 stimulate Tie2 expression in the regenerating BM vasculature and accelerate hematopoietic recovery. The results suggest that activation of Tie2 expression on vascular endothelium supports the assembly of sinusoidal endothelial cells during BM regeneration. BMSCs cultured *in vitro* were found to express several chemokine receptors (CCR1, CCR7, CCR9), and chemokine ligands (CCL2, CCL4, CCL5, CCL20, CXCL12, CXCL8, CX3CL1) [40]. The expressed receptors mediate biochemical and biological responses of BMSCs to the stimulation of chemokines. Extended passage of BMSCs led to the loss of receptor expression accompanied by slowing of cell growth and increased spontaneous apoptosis. The results indicate that the expression profiles of chemokines could serve as molecular markers for the functional status of cultured BMSCs.

### Global gene expression profiles of HSCs

To elucidate the HSC unique properties of self-renewal and differentiation, HSCs from different sources were highly enriched and large scale gene expression analysis was performed. The reported HSCs gene expression profiles

include mouse fetal liver HSCs [41], human and mouse fetal liver HSCs and mouse BM HSCs [42], and mouse BM HSCs [43, 44]. The global gene expression profiles of HSCs have identified growth factor receptors, cytokine receptors, hematopoietic growth factor receptors, G-protein coupled receptors (GPCR), morphogen receptors, hormone receptors, and putative genes encoding transmembrane proteins (Table 2). Analysis of the available published dataset revealed that most of the known functional receptors of HSCs were detected in the global gene expression array. The biochemical and biological functions of most of the newly identified genes encoding HSCs receptors need to be further characterized individually.

Interestingly, not only arrays of receptors are expressed by HSCs, but also a list of genes encoding for secreted proteins are detected in HSCs. These include growth factors, cytokines, hematopoietic growth factors, and morphogens (Table 3). Comparing the HSC expressed receptor and ligand genes listed in the two tables reveals possible autocrine/paracrine signaling ligand/receptor pairs: Agpt/Tie2, FGF/FGFR, PDGF/PDGFR, IL4/IL4R, Wnt/Fzd, Dll1/Notch1, Jag2/Notch1. As discussed above at

**Table 2** Partial list of known genes encoding receptors expressed by HSCs

Growth factor receptors	Eng, ErbB4, Fgfr1, Igf1r, Ngfrap1, Nrg4, Pdgfra, Pdgfrb, Tie1, Tie2, Tnfrsf4, Tnfrsf8
Cytokine receptors	Flt3, Il11ra2, Il12rb1, IL1R1, IL4Ra, IL6Ra, IL11Ra2, IFNR
Hematopoietic growth factor receptors	Kit, Mpl
GPCR genes	F2r11, F2r13, Gpr105, Gpr37, Gpr50, Gpr56, Gpr97, Gprc5b, Mmd, Opn3
Morphogen receptor genes	Fzd4, Fzd6, Fzd7, Fzd8, Notch1
Hormone receptors	Insulin R, Growth hormone R



**Table 3** Partial list of genes encoding secreted proteins expressed by HSCs.

Growth factors	Agpt, Btc, Ereg, Fgf15, Fgf18, Fgf3, Fgf7, Fgf8, Pdgfc, Vegfa, Vegfb
Cytokines	Gdf8, Ifna5, Il1f6, Il4, Scgb3a1, Scya19,
Hematopoietic growth factors	Scyb14 Tgfb3
Morphogens	Agr2, Bmp2, Bmp5, Bmp8a, Dll1, Jag2, Wnt1, Wnt6, Wnt10a, Wnt11

least three of the pairs (Agpt/Tie2, Wnt/Fzd, Jag2/Notch1) are functional in control of HSC properties. However, no direct evidence supports these apparent autocrine/paracrine pathways within the population of HSCs.

### Global gene expression profiles of stromal cells supporting hematopoiesis

Mouse fetal liver derived stromal cell line AFT024 and 2012 can support both fetal and adult HSCs. Global gene expression of AFT024 and 2012 cell lines was compared to that of non-HSC supporting stromal cell lines [45, 46]. It is hypothesized that the cell lines that are capable of supporting HSCs differentially express genes encoding cell surface proteins and secreted proteins that mediate direct communication between HSCs and stromal cells. However, few known hematopoietic growth factor and cytokine gene products were differentially expressed in the HSC-supporting cell lines, except that the expression of *TGF-β*, *FGF*, *IL-1*, and *TNF* in the stromal cell lines was detected. The authors were able to suggest that 31 gene products constitute a core of proteins required for fetal liver stromal cells to support HSCs including rho, myosin 1c, keratin complex 1, filamin alpha, lysyl oxidase, biglycan, pleiotrophin, and delta-like 1 [45]. Following analysis of specific gene expression profiles of the HSC-supportive stromal cells, the authors concluded that “the HSC-supporting stromal cells are immature, sessile, and highly reactive after binding to integrin ligands and cytokines.” It is worth noting that the HSC-supporting cell lines did express a battery of known hematopoietic growth factors and cytokines, which were also detected in non-supportive cell lines [46]. It is therefore probable that the signature gene expression profile of HSC-supporting function may contain genes expressed in both HSC-supporting and non-supporting cell lines. This is evidenced by an observation that HSC-supportive MSCs expressed an array of cytokines and hematopoietic growth factors, including IL-6, IL-8, IL-12, IL-14, IL-15, LIF, G-CSF, GM-CSF, M-CSF, FL, and SCF [38].

### Genetic programs regulate BM regeneration after insult

In clinical settings as well as animal studies, BM regeneration is often initiated after BM suppression following radiation and cytotoxic drug treatment. Regeneration involves recovery of both hematopoietic cells and stromal elements including the BM vasculature. Given the roles of stromal cells in regulating hematopoiesis, through secreting cytokines and hematopoietic growth factors, it is expected that we should be able to detect elevated gene expression of the hematopoietic regulators. However, experiments in a murine model of chemotherapy-induced BM regeneration [47] and analysis of human BM tissue after chemotherapy [48] have failed to detect induction of G-CSF, M-CSF, GM-CSF, IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, IL-12, TNF- $\alpha$ , and TGF- $\beta$ . Furthermore, the expression of IL-6 was decreased after chemotherapy. We have performed a global gene expression survey of mouse BM during its regeneration after a single dose of 5-fluouracil treatment. M-CSF, SCF, IL-7, and TGF- $\beta$ 3 were the only four known hematopoietic regulators with more than 2-fold induction in their gene expression (unpublished data). This result is largely in agreement with published observations. The undetectable induction of most hematopoietic regulators could be explained by low sensitivity of the gene detection methods, or they are not the local signals regulating BM regeneration. If the latter is true, then there must be other unidentified molecules that regulate BM regeneration after insults. Indeed recent publications from Rafii’s laboratory demonstrated that chemokines (SDF-1 and FGF-4) and angiogenic growth factors (VEGF-A, Ang-1) are critical regulators initiating BM recovery. Myelosuppression-induced BM production of SDF-1 and VEGF-A upregulated the expression of MMP-9, a BM matrix metalloproteinase. MMP-9 was shown to release soluble Kit-ligand, which initiated the activation of quiescent HSCs to repopulate the damaged BM [49]. SDF1 and FGF-4 also enhanced localization of megakaryocyte progenitors to the vascular niche, promoting megakaryocyte maturation and thrombopoiesis during BM regeneration [50]. VEGF-A and Ang-1 stimulated Tie2 expression in BM endothelial cells, which is required for proper assembly of sinusoidal endothelial cells in neoangiogenesis during BM regeneration [39]. Therefore, through accelerating neoangiogenesis, VEGF-A and Ang-1 promote reconstruction of damaged BM structures, hence the recovery of hematopoiesis after myelosuppression. Taken together, the apparent important hematopoietic growth factors and cytokines seem not to be rate limiting molecules for BM regeneration. Rather non-hematopoietic regulators overexpressed by BMSCs in the stress situation play crucial roles in tissue regeneration.

## Prospective

Identification of genes and their products that regulate the process of tissue regeneration will contribute to the discovery of a new generation of regenerative medicine. The available myelosuppression treatments are mostly ameliorated by administration of blood and blood products, hematopoietic growth factors, or HSC transplantation. The curative treatments should provide the missing parts of the tissue regeneration machinery. Therefore, future efforts may focus on: (1) Systematic identification of all molecules participating in BM regeneration and their mechanisms of action; (2) Identification of pathological BM conditions with defined regenerative molecule defects; (3) Development of drugs based on new molecule mechanisms of BM regeneration; (4) Elucidation of relationships between hematopoietic growth factors and newly identified BM regenerative factors in mechanisms of action and in treatment of BM toxicities.

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