Stem Cells International

Heterogeneity, Self-Renewal, and Differentiation of Hematopoietic Stem Cells

Guest Editors: Roland Jurecic, Linheng Li, and R. Keith Humphries

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Editorial Board

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Editorial

Heterogeneity, Self-Renewal, and Differentiation of Hematopoietic Stem Cells

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Better understanding of how hematopoietic stem cells (HSCs) balance self-renewal and differentiation is vital for their application in treatment of various diseases and regenerative medicine. During the last decade tremendous advances have been made in identifying a complex network of cellular and molecular factors that influence self-renewal and differentiation of HSCs and characterizing the cellular and molecular components of niches which harbor HSCs. Improving our understanding of the cellular and molecular mechanisms that regulate self-renewal and differentiation of HSCs will have a profound impact on experimental and clinical HSCs research and clinical application of HSCs. Thus, *Stem Cells International* set out to publish a special issue devoted to the topic of *heterogeneity, self-renewal, and differentiation of hematopoietic stem cells*.

The ongoing research is continuously increasing our knowledge about HSC niches in the bone marrow and in particular about cell types constituting niches and a plethora of molecules and signals that make up the synapse between HSCs and niche cells. A series of three review articles in this issue discuss the role of hematopoietic niches in regulating behavior and function of hematopoietic stem cells and the role of niches in development of leukemias.

K. S. Tieu et al. present a review of quantitative approaches to understanding stem cell niche signaling in the hematopoietic system, as well as in other tissues under conditions of homeostasis and carcinogenesis. They explain the benefits of mathematical models in advancing our understanding of the mechanisms that regulate stem cell fate and how this regulation changes in cancer development. K. S. Tieu and colleagues are also highlighting the synergistic relationship between mathematical predictions and experimental validation and address the potential for mathematical models to predict and optimize therapies targeting the stem cell niche.

In their review "*Osteoblastic and vascular endothelial niches, their control on normal hematopoietic stem cells, and their consequences on the development of leukemia*" B. S. Guerrouahen and colleagues provide some common cellular and molecular principles applying to the osteoblastic and vascular hematopoietic niches and discuss altered microenvironment signaling leading to myeloid lineage disease. They also review the emerging evidence for the role of microenvironment in supporting the leukemia-initiating cells (LICs) and the influence of the microenvironment on chemotherapy resistance.

The two predominant niches in the bone marrow, the endosteal and vascular niches, are thought to regulate the self-renewal and differentiation of distinct HSC populations, and also dictate HSC behavior with respect to homeostatic requirements and exogenous stresses. In their review article "*The Haematopoietic stem cell niche: new insights into the mechanisms regulating haematopoietic stem cell behaviour*" A. J. Lilly and colleagues discuss recent research into the cellular

and molecular components of endosteal and vascular niches. Taking into account the crosstalk and overlap between cell types and signaling in these two niches, J. Lilly et al. propose that endosteal and vascular niches should be viewed as subcompartments of a single HSC niche. The review by H. C. O'Neill et al. from Australia focuses on the spleen microenvironment as a site of development of novel dendritic-like cells which are phenotypically and functionally distinct from other described antigen-presenting cells. The discovery that the lineage origin and the progenitors for this new type of tissue-specific antigen-presenting cells differ from that of other known dendritic and myeloid cell types suggests that spleen represents a distinct microenvironment for development of a novel myeloid cell type arising from HSCs or progenitors endogenous to spleen. This paper also highlights the need to explore in more detail the contribution of the spleen and its microenvironment to steady-state hematopoiesis.

β-Thalassemia is characterized by reduction or absence of β-globin production, resulting in anemia. Current therapies include blood transfusion combined with iron chelation, BM transplantation which is restricted by the matched donor limitation, and gene therapy with β-globin lentiviral vectors. The review by E. Drakopoulou et al. presents the current status of gene therapy for β-thalassemia, its success and limitations, and the novel promising strategies available involving the therapeutic role of HSCs. This paper reviews achievements in improving vector safety and efficiency to stably transduce HSCs, while minimizing insertional mutagenesis. The authors also discuss strategies that result in higher numbers of genetically modified HSCs, including manipulation of the ex vivo HSC culture conditions, use of mobilized HSC, and generation of HSCs from patient-specific induced pluripotent stem (iPS) cells. These novel strategies could set the ground for more successful β-thalassemia gene therapy clinical trials.

The papers in this special issue highlight both the advancements and challenges in understanding the role of niches in HSC biology and fate and in the use of HSCs in disease treatment.

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Review Article

Stem Cell Niche Dynamics: From Homeostasis to Carcinogenesis

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The stem cell microenvironment is involved in regulating the fate of the stem cell with respect to self-renewal, quiescence, and differentiation. Mathematical models are helpful in understanding how key pathways regulate the dynamics of stem cell maintenance and homeostasis. This tight regulation and maintenance of stem cell number is thought to break down during carcinogenesis. As a result, the stem cell niche has become a novel target of cancer therapeutics. Developing a quantitative understanding of the regulatory pathways that guide stem cell behavior will be vital to understanding how these systems change under conditions of stress, inflammation, and cancer initiation. Predictions from mathematical modeling can be used as a clinical tool to guide therapy design. We present a survey of mathematical models used to study stem cell population dynamics and stem cell niche regulation, both in the hematopoietic system and other tissues. Highlighting the quantitative aspects of stem cell biology, we describe compelling questions that can be addressed with modeling. Finally, we discuss experimental systems, most notably *Drosophila*, that can best be used to validate mathematical predictions.

1. Introduction

The hematopoietic stem cell niche is an important regulator of stem cell fate. There are complex signaling pathways, such as Notch, Wnt, and Hedgehog, that carefully regulate stem cell renewal, differentiation, and quiescence [1–3]. Mathematical models can be useful in studying the dynamics of stem cell maintenance. Quantitative models can provide information about cell population dynamics, regulatory feedback of interacting networks, and spatial considerations related to the structural relationships between stem cells and their progeny with cells of the microenvironment.

Errors in stem cell division rate or in the balance between self-renewal and differentiation may result in tissue overgrowth or depletion [4]. One novel target of cancer therapeutics is the stem cell niche [5, 6]. Stem cell niche signaling inhibitors are being designed with the idea that regulatory signals that are active in stem cell niche homeostasis may go awry during carcinogenesis [6–8]. Understanding the biology and dynamics of stem cell behavior under normal conditions and examining how the dynamics change under conditions of stress is essential to our understanding of how these mechanisms might change during carcinogenesis.

Mathematical and physical models have been used to study stem cell population dynamics and the regulation of stem cell fate through niche signaling with great success. We present a review of quantitative approaches to understanding stem cell niche signaling in the hematopoietic system, as well as in other tissues under conditions of homeostasis and carcinogenesis. We explain the benefits of mathematical models in advancing our understanding of the mechanisms of regulation of stem cell fate and how this regulation changes in cancer development. We describe models that incorporate spatial aspects of the regulation of asymmetric division and compare normal conditions to carcinogenesis. We highlight the synergistic relationship between mathematical predictions and experimental validation and illustrate *Drosophila* as a model system for quantitative studies of the stem cell niche. Finally, we address the potential for mathematical models to predict and optimize therapies targeting the stem cell niche.

2. Quantitative Aspects of the Hematopoietic Stem Cell Niche

Hematopoietic stem cells (HSCs) are a dynamically well characterized stem cell population. The hematopoietic system was the first system in which multipotency, or the ability for a single HSC to regenerate all of the different cell types within the tissue, was described. A second defining characteristic for stem cells, self-renewal, has also been demonstrated in HSCs. Self-renewal is the ability of the HSC to generate a genetically identical copy of itself during cell division. This can occur asymmetrically, giving rise to one identical copy and one partially differentiated daughter cell, or symmetrically, giving rise to two identical copies of itself. Single HSCs have been shown to be self-renewing, multipotent, and to cycle with slow kinetics. Extrapolation from feline and murine data suggests a symmetric birth rate for human HSCs of once every 42 weeks [9]. Quiescence, the state of not dividing, allows HSCs to avoid mutation accumulation and contributes to their long lifespan. In contrast to senescence, where the cell loses its ability to undergo division, a cell can reawaken from the state of quiescence to an activated state where it can again undergo self-renewal.

The stem cell microenvironment regulates stem cell self-renewal, differentiation, quiescence, and activation. While little in situ information is known about the anatomy and structural relationships of the hematopoietic stem cell and its niche, there is a growing amount of experimental information about the behavior of signaling systems that govern HSC fate.

Population dynamics models have been successfully used to model the human hematopoietic system in both health and disease [9–17]. Using stochastic and deterministic models, significant progress has been made in understanding the dynamics of cancer initiation and progression [18, 19] and the sequential order of mutation accumulation [20]. Mathematical models have also been useful in modeling leukemic stem cell and progenitor population changes in response to therapy and the development of resistance [14].

An ongoing debate in hematopoietic stem cell biology concerns how much variability exists in hematopoietic stem cell fate [21]. Stochastic models have been used to study the dynamics of clonal repopulation [22] following hematopoietic stem cell transplant. In these models, trajectories of hematopoietic stem cell counts as well as progenitor and differentiated cell counts are generated and compared with observed cell counts. Rates of self-renewal, differentiation, and elimination of cells are estimated. Stochastic trajectories are found to match experimental results. These models predict that hematopoiesis is probabilistic in nature and that clonal dominance can occur by chance. These models could be enhanced by examining regulators of stem cell fate by the microenvironment. Stochastic simulation can be used to incorporate elements of the stem cell niche, such as surrounding stromal cells and signaling pathways, and model cell-cell and cell-environment interactions. These models could identify regulators of stem cell fate and explore the dynamics of this regulation.

Chronic myelogenous leukemia (CML) represents a nice system to quantitatively study hematopoietic stem cell and progenitor dynamics. CML is the first malignancy recognized as a stem cell disorder. The translocation t(9;22) is present in leukemic stem cells, multipotent progenitors, and their progeny of the myeloid lineage. This translocation leads to transcription of the BCR-ABL fusion oncogene which is thought to regulate cell survival. Therapy inhibiting BCR-ABL is one of the first examples where chronic administration of a molecularly targeted therapy has led to a dramatic clinical response. This response is observed in all phases of the disease.

Mathematical models have been used to demonstrate that leukemic stem cells are not targeted by imatinib therapy [14], and that successful therapy must target leukemic stem cells [12]. Other models have highlighted the importance of leukemic stem cell quiescence as a mechanism leading to therapeutic resistance [13].

In a study of chronic myelogenous leukemia under targeted therapy, Michor et al. [14] describe the dynamics of leukemic stem cells and the development of resistance using a Moran process model. Based on calculated rates of death and differentiation using data of biphasic decline of BCR-ABL transcripts, they conclude that the leukemic stem cell compartment is not sensitive to therapy. An alternative explanation is provided by Komarova and Wodarz [13], using a stochastic model in which quiescence and reactivation of leukemic stem cells are considered. In this work, the biphasic decline of BCR-ABL transcripts is explained by the elimination of active leukemic stem cells, followed by the slower elimination of quiescent leukemic stem cells following their reactivation. This study offers hope that targeted therapy, used in combination with potential therapies that lead to activation of quiescent cells, could eradicate the stem cell-like compartment of a tumor. These models could be expanded by modeling the contribution of the microenvironment that regulates quiescence and activation of stem cells. Validation of these models will require experimental determination of rates of quiescence and reactivation to obtain accurate parameters for modeling.

Birth-death process models have been used to study extinction of leukemic and normal hematopoietic stem cells under therapy targeting leukemic stem cells. These models conclude that the killing efficiency of a therapy is a major determinant of the mean time to extinction of leukemic stem cells (optimal duration), while the selectivity of a therapy predicts the average number of normal hematopoietic stem cells at the time of leukemic stem cell extinction (safety) [23]. Incorporating quiescence in these models reveals that a successful therapy needs to target both active and quiescent leukemic stem cells.

We extended this model to consider combination of therapy targeting leukemic stem cells, and their niche was considered using stochastic simulation. Because stem cell self-renewal is expected to decrease with Wnt-inhibitor therapy, we modeled the addition of niche-targeted therapy as a decrease in birth rates of leukemic stem cells. We found that this combination can be effective in eliminating the leukemic stem cell compartment, even when the effects

of BCR-ABL-targeted therapy on stem cells are modest. We anticipate that extension of these models to include regulatory feedback of the stem cell microenvironment using stochastic reaction kinetic methods would be very helpful in modeling dynamics of niche-targeted therapies.

The hematopoietic stem cell niche has been studied in the healthy hematopoietic system. A model based on self-organizing principles demonstrates the importance of asymmetry in determining stem cell fate and concludes that stem cell fate is only predictable in describing populations rather than individual cellular fates [24]. Deterministic models are useful in simulating proliferation and differentiation of all populations comprising the stem cell niche [25]. These studies conclude that kinetics are highly variable because of the relatively small number of cells proliferating and differentiating in the niche. Experimental studies have examined the role of Wnt signaling in regulation of normal hematopoietic regeneration [26]. We expect the combination of mathematical modeling with experimental validation to prove useful in modeling the pathways under normal conditions and dysregulation of these pathways during stress, inflammation, and carcinogenesis.

Figure 1 describes the elements of the HSC niche and an accompanying schematic representation of a mathematical model of the niche. The model captures the key regulatory components of niche dynamics, including cell population sizes and the signaling pathways that regulate them.

3. *Drosophila* as a Classic Model System

Drosophila represents an excellent model system to study stem cells, their microenvironment, and the tight regulation of homeostasis through different signaling pathways. The male *Drosophila* germ line population is a classic system used to study properties of the stem cell niche [27, 28]. The power of this model includes the ability to quantify cell populations over time, the relatively quick repletion of lost cells with newly differentiated cells, and the ability to experimentally observe spatial effects. These quantitative aspects, as well as its simple, well-characterized lineages, make the *Drosophila* experimental system ideally suited for the development and validation of mathematical modeling. Finally, vertebrate and invertebrate digestive systems show extensive similarities in their developments, cellular makeup, and genetic control [29].

Mathematical and physical models have been used to study regulation of stem cell fate through niche signaling in the *Drosophila* blood and midgut [30], as well as in the *Drosophila* eye [31] and the *Drosophila* embryo [32], with great success. Studies of the stem cell niche in model systems such as *Drosophila* have revealed adhesive interactions, cell cycle modifications, and intercellular signals that operate to control stem cell behavior [4, 33]. These interactions have been studied quantitatively. For example, Wnt and Notch play pivotal roles in stem cell regulation in the *Drosophila* intestine [30, 34]. In addition, the APC gene has been shown to regulate *Drosophila* intestinal stem cell proliferation [35]. APC is well known to play a role in human colon carcinogenesis, and mathematical models have shown that stem cell proliferation leads to colon tumor formation in humans [36, 37].

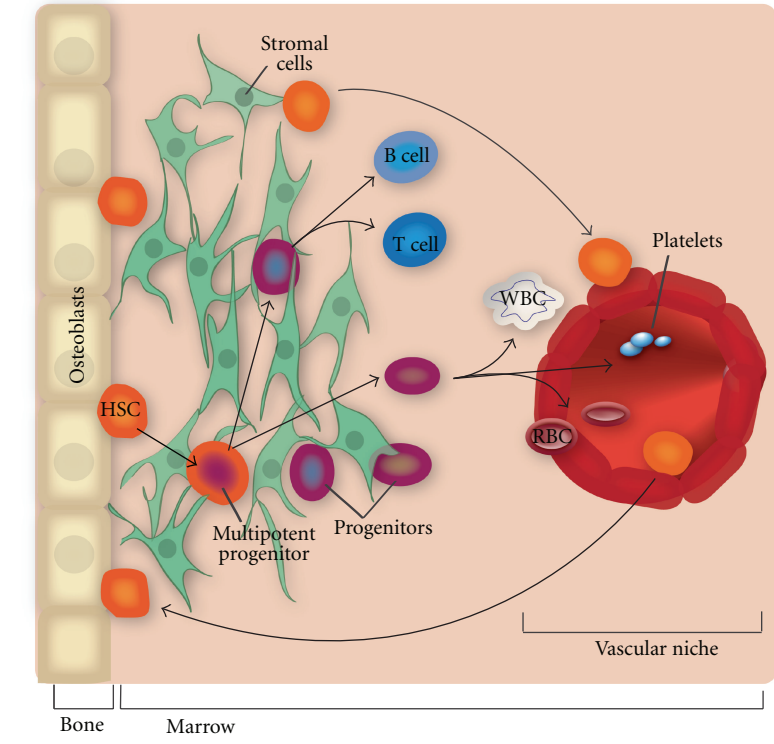
The spatially patterned self-renewal and differentiation of stem cells has been extensively studied in *Drosophila* embryonic studies of development [32, 38–40]. The spatial orientation of stem cells has been visualized in *Drosophila* brain and testes and has recently been shown to be of great importance in experimental models of neuroblastoma growth in *Drosophila* [41]. We anticipate that the combination of spatial effects simulation and direct visualization of the *Drosophila* midgut through experiment will advance our understanding of the interaction of alterations in signaling pathways and spatial effects in carcinogenesis.

4. Extension to Inflammation and Carcinogenesis across Tissues

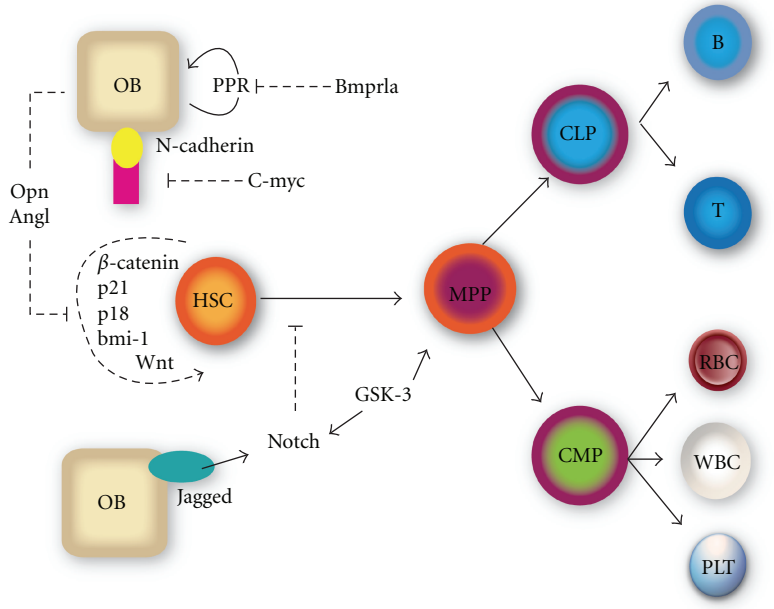
Unifying features of stem cell niche regulation are observed across tissues and across organisms [42, 43]. Figures 1, 2, and 3 compare the structural and signaling elements of the stem cell niche across the hematopoietic, intestine, and breast tissues. While little is known about the structural orientation of the human hematopoietic stem cell niche 1, much has been learned about the signaling pathways in both the bone and vasculature that regulate HSC fate. Osteoblasts (OBs) express osteopontin which negatively regulates HSC proliferation. Tie2/angiopoietin signaling regulates HSC anchorage and quiescence, and adherence to osteoblasts. HSCs and OBs are increased via the parathyroid hormone-related protein receptor (PPR) expressed in OBs. OBs express N-cadherin which forms a beta-catenin adherens complex with HSCs. C-myc negatively regulates N-cadherin in differentiating HSCs and promotes differentiation and displacement from the endosteum. OBs express Jagged-1, a Notch receptor that when bound inhibits differentiation that usually accompanies Wnt-induced HSC proliferation. GSK-3 activity enhances HSC progenitor activity and may control asymmetric cell division by modulating Notch and Wnt signaling pathways.

Figure 2 depicts the intestinal stem cell niche of *Drosophila*. Here, we see four key cellular populations: intestinal stem cells (ISCs), enteroblasts (EBs), enterocytes (ECs), and enteroendocrine (EE) cells. It has been previously established that ISCs can self-renew under the influence of the Wnt signaling pathway [44] and can asymmetrically divide giving rise to one partially differentiated EB cell and one ISC, under the influence of the Delta/Notch signaling pathway. EBs can then differentiate into either EC cells or EE cells. There is feedback from the EB population to the ISC population, which inhibits self-renewal and differentiation, in order to maintain stable population sizes under the normal conditions of homeostasis [45]. The EC population also interacts with the ISC population via Jak/Stat signaling feedback, which increases self-renewal and differentiation, in conditions when EC loss occurs [45].

Finally, both structural and signaling aspects of the breast stem cell niche are shown in Figure 3. The hedgehog (Hh) pathway is required for normal development of the mammary gland and regulates self-renewal of human mammary



(a)



(b)

Figure 1: Quantitative aspects of the hematopoietic stem cell (HSC) niche. The left panel provides a structural picture of the niche, while the right panel shows a schematic representation of a mathematical model for the regulation of hematopoietic stem cell fate. The model incorporates population counts and signaling pathways that may play a role in regulating stem cell population dynamics. Cellular populations comprising the bone and vascular niches include osteoblasts (OBs), endothelial cells, HSCs, multipotent progenitors (MPPs), common myeloid progenitors (CMPs), common lymphoid progenitors(CLPs), and differentiated cells. Signaling from Wnt, β-catenin, p21, p18, and bmi-1 regulate self-renewal, while Notch and GSK3 feedback from progenitors inhibit differentiation that usually accompanies self-renewal. Signaling from osteoblasts includes osteopontin (Opn) expression that inhibits HSC self-renewal, parathyroid hormone-related protein (PPR) which increases HSCs, N-cadherin which binds β-catenin, and Tie2/angiopoietin which regulates quiescence.

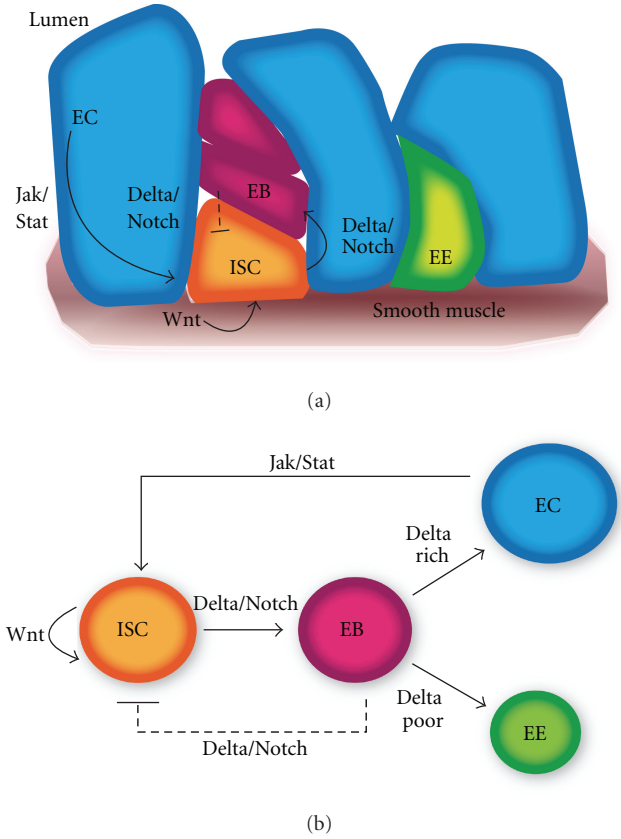


FIGURE 2: Structural and dynamic aspects of the *Drosophila* intestinal stem cell (ISC) niche. The left panel shows a structural picture of the *Drosophila* intestine, while the right panel reveals population and regulatory elements of a mathematical model for ISC regulation. Populations of the intestinal stem cell niche in the *Drosophila* include ISCs, enteroblasts (EBs), enteroendocrine cells (EE), and enterocytes (ECs). Wnt signaling from underlying smooth muscle and Notch feedback from EB regulate ISC self-renewal, while Jak/Stat feedback from damaged ECs increases ISC self-renewal.

stem cells (MSCs) [46–48]. Hh also targets endothelial cells and induces angiogenesis by promoting endothelial progenitor proliferation and migration. Wnt signaling regulates proliferation, apoptosis, and differentiation and maintains stem cells in a self-renewing state. Notch promotes self-renewal in normal mammary stem cells [46, 49]. Notch3 is expressed in epithelial progenitors, and Notch4 is expressed in bipotent progenitors. Markers of mammary stem cells include ALDH1 expression, and Sca-1. There is a significant correlation between expression of ALDH1 and HER2 overexpression [50].

The common signaling pathways that control stem cell self-renewal in these pathways, such as Notch, Wnt, and Hedgehog, are known to play a role in carcinogenesis [2, 41]. A growing body of evidence from a variety of solid tumors suggests that the first carcinogenic cell within a tumor possesses stem cell properties, including self-renewal, increased cell survival, limitless replicative potential, and the ability to produce differentiating cells [51–60]. However, it is unclear whether accumulation of mutations within a tumor cell with stem cell properties or extrinsic factors originating in the tumor microenvironment drive tumor progression [61, 62]. Understanding niche signaling pathways under normal conditions, and in response to inflammation and stress response, is vital to understanding how they may go awry in carcinogenesis.

The known link between inflammation and cancer may involve the regulation of stem cell fate by inflammatory cytokines [63]. IL-1, IL-6, and IL-8 are known to activate Stat3/NF-κB pathways in tumor and stromal cells. Positive feedback loops are formed involving further cytokine production which can drive cancer stem cell self-renewal [63]. These networks can be nicely modeled using stochastic reaction kinetics. Predictions from these models could be used to guide therapy design.

Dysregulation of normal homeostatic processes in the human hematopoietic stem cell niche may lead to enhanced self-renewal and proliferation, enforced quiescence, and resistance to chemotherapeutic agents. Leukemic stem cells have been shown to infiltrate the normal HSC niche by direct invasion or secretion of substances such as stem cell factor [6]. Leukemic stem cells may also exhibit dysregulated homing and engraftment, leading to alternative niche formation [6]. Future mathematical models of leukemic stem cell dynamics should take into account the stem cell niche.

Cytokine/Jak/Stat signaling has recently been shown to mediate regeneration and response to stress in the *Drosophila* midgut [45, 64]. Mathematical models of proliferation and differentiation of *Drosophila* intestinal stem cells have examined the dynamics of Wnt and Notch signaling [30], but have not yet examined the feedback of Jak/Stat signaling from the differentiated enterocytes to intestinal stem cells. Mathematical models of the human intestinal stem cell niche have shown that dysregulated colonic crypt dynamics cases stem cell overpopulation and initiate colon cancer [36]. Symmetric division of cancer stem cells has been shown to be a key mechanism of tumor growth to target in therapeutic approaches [37].

In mammalian systems, MyD88 and RAS signaling have been shown to lead to mouse and human cell transformation [65]. These signaling pathways are known to be involved with inflammation and also play a direct role in cell cycle control. The link between inflammation and carcinogenesis needs to be studied quantitatively.

Alterations in Wnt signaling contribute to excess proliferation of mammary progenitor cells leading to cancer [66]. Unregulated Notch signaling in the mouse mammary gland leads to tumor formation. Increased expression of Notch in ductal carcinoma is associated with shorter time to recurrence [67]. Breast density is an important risk factor for breast tumor development [68], suggesting a role of the stem cell microenvironment in carcinogenesis. Growth factors secreted by fibroblasts influence mammary stem cell behavior. Endothelial cell and adipocytes may also influence stem cell behavior. CCL5 secretion by mesenchymal stem cells influences stem cell self-renewal. Alterations in Notch signaling are thought to play a role in breast cancer development.

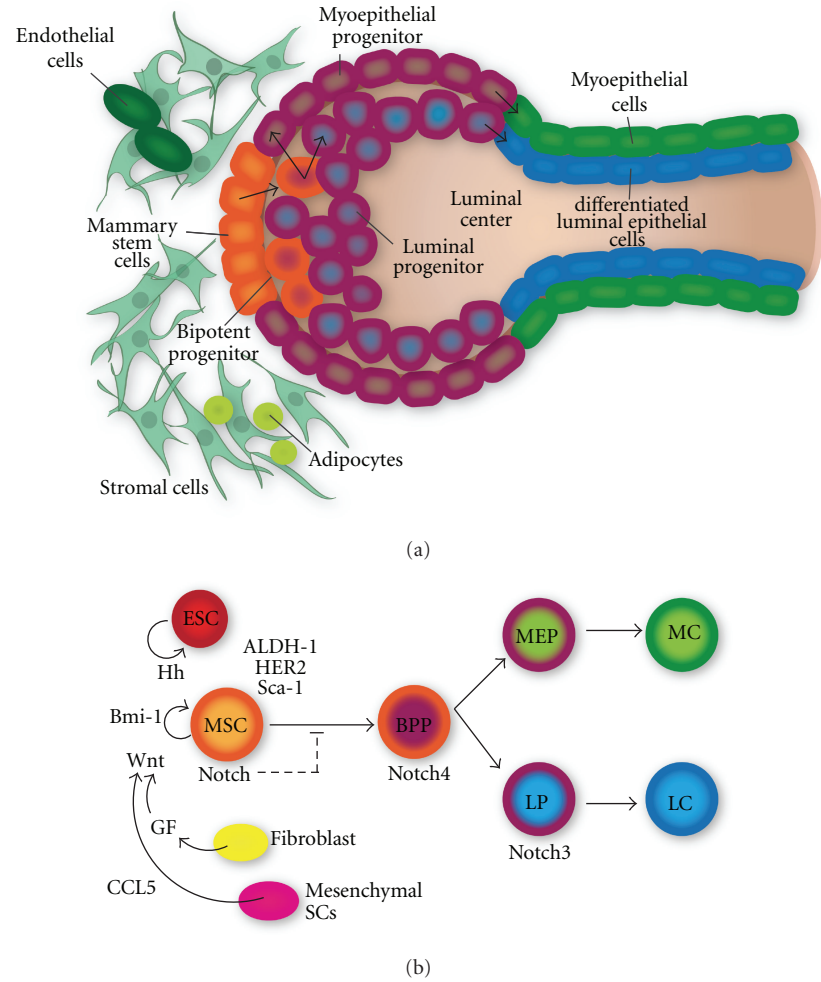


Figure 3: Model of the breast stem cell niche including structural elements (left panel) and mathematical model (right panel). Key populations of the mammary stem cell niche include mammary stem cells (MSCs), mesenchymal stem cells, endothelial stem cells (ESCs), bipotent progenitor (BPP), luminal progenitor (LP), myoepithelial progenitor (MEP), myoepithelial cells (MCs), luminal epithelial cells (LCs), and stromal cells. Wnt, Notch, and Hedgehog (Hh) signaling play a role in MSC self-renewal. Regulatory signals from growth factors (GFs) secreted by fibroblasts and CCL signaling from mesenchymal stem cells also regulate MSC fate.

Combination of theory and experiment has shed light on stromal-tumor interactions in the human breast [69]. In the breast, ductal cells secrete TGF-beta and fibroblasts secrete EGF. During carcinogenesis, TGF-beta then transforms fibroblasts into myofibroblasts, which in turn secrete higher EGF. Mathematical modeling has shown that this feedback system increases proliferation of tumor cells, and theoretical results match experimental validation well.

Mathematical models have also shed light on the interactions between the stem and nonstem compartments of solid tumors and their effects on the heterogeneous growth of solid tumors. These models show that apoptosis of nonstem cells paradoxically leads to tumor growth and progression [70, 71].

Cancer cell plasticity is an important consideration in the study cancer stem-like cells in oncology. The finding that nonstem cells can dedifferentiate to a stem-like state in mammary cell lines [72] has important implications in defining cancer stem-like cells and identifying therapies to target them. Markov models have recently proven very helpful in calculating rates of dedifferentiation of mammary epithelial cells to stem-like cells [73]. Consideration of microenvironmental signaling that regulates these transitions will greatly enhance these models and their predictions.

5. Spatial Considerations in Modeling Stem Cell Regulation

Spindle orientation is well known to play a role in stem cell fate [74]. Asymmetric division is regulated by maintaining the stem cell orientation, and this is regulated by its spatial relationship with the cells of the niche. Induction of brain tumor growth has been demonstrated by altering stem-cell asymmetric division in *Drosophila* melanogaster [41]. Loss of cell polarity and cancer are tightly correlated [4]. In stem cells, loss of polarity leads to impairment of asymmetric cell

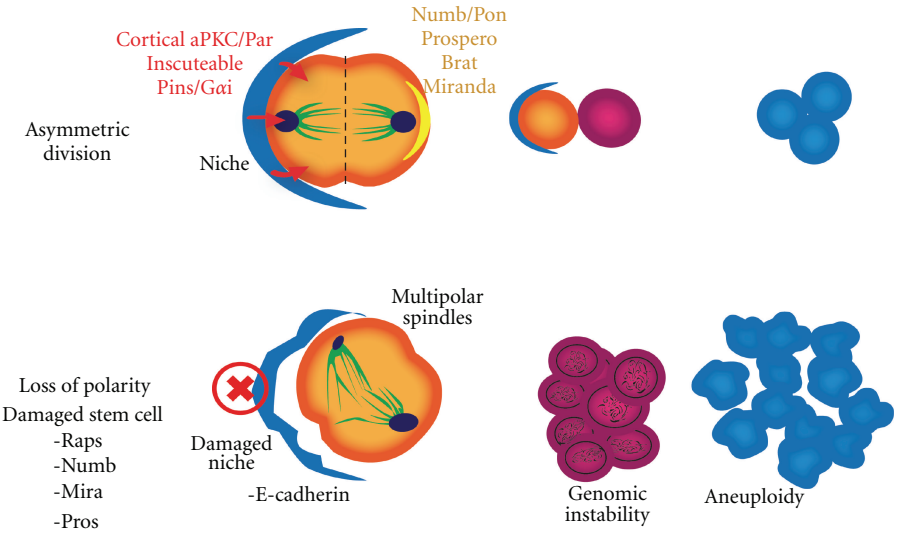


Figure 4: Stem cell polarity: homeostasis and dysregulation. Regulation of asymmetric division in the stem cell niche. The left panel represents spatial regulation of normal homeostasis, while the right panel demonstrates the loss of this asymmetry during carcinogenesis.

division, altering cell fates, rendering daughter cells unable to respond to the mechanisms that control proliferation. The tumor suppressor p53 regulates polarity of self-renewing divisions in mammary stem cells [75]. Figure 4 displays regulation of stem cell asymmetric division under normal homeostatic conditions and the loss of this regulation during carcinogenesis. Labeling of template strands in stem cells of small intestine crypts using tritiated thymidine reveals selective retention of parental DNA strands and loss of newly synthesized strands during stem cell division [76]. This mechanism provides the stem cell with protection from DNA replication errors during asymmetric division. Loss of asymmetric division may lead to loss of this protection against chromosomal instability.

Mathematical models that allow for the inclusion of spatial effects are necessary in order to study this loss of asymmetry in the stem cell and its relation to carcinogenesis. Classic models of spatial effects on development in *Drosophila* have examined reaction diffusion equations [38, 39]. While multiscale models are more recently being used to study complex biologic systems and their genetic regulation, most of the methods used assume a well-stirred system and have not allowed for consideration of spatial effects until recently. Incorporating a spatial component into stochastic simulation methods is an exciting frontier in stochastic reaction kinetics [77, 78]. A stochastic reaction-diffusion equation is used in place of the chemical master equation and is sampled in the stochastic simulation. These methods have been shown to be successful in modeling spatial effects in genetic regulatory networks [78].

6. Conclusions

Mathematical models have proven useful in characterizing stem cell and progenitor cell population dynamics, and in understanding the interacting components of the stem cell niche. Identifying quantitative characteristics of the stem cell microenvironment that are generalizable across tissues, as well as those distinct to each system, will be necessary to help define the emerging concept of the stem cell niche. Modeling the components of the stem cell niche and their interactions will advance our understanding of the tight regulation of stem cell fate. In turn, it will allow us to predict and validate responses to stress, inflammation, and carcinogenesis. In addition to quantifying population distributions and feedback networks, it will be necessary and informative to incorporate spatial aspects that govern asymmetric versus symmetric stem cell self-renewal. We expect that the combination of predictive modeling and experimental validation will prove useful in our understanding of the regulatory components of stem cell maintenance and the changes that occur in response to treatments designed to target the stem cell niche.

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Review Article

Osteoblastic and Vascular Endothelial Niches, Their Control on Normal Hematopoietic Stem Cells, and Their Consequences on the Development of Leukemia

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Stem cell self-renewal is regulated by intrinsic mechanisms and extrinsic signals mediated via specialized microenvironments called "niches." The best-characterized stem cell is the hematopoietic stem cell (HSC). Self-renewal and differentiation ability of HSC are regulated by two major elements: endosteal and vascular regulatory elements. The osteoblastic niche localized at the inner surface of the bone cavity might serve as a reservoir for long-term HSC storage in a quiescent state. Whereas the vascular niche, which consists of sinusoidal endothelial cell lining blood vessel, provides an environment for short-term HSC proliferation and differentiation. Both niches act together to maintain hematopoietic homeostasis. In this paper, we provide some principles applying to the hematopoietic niches, which will be useful in the study and understanding of other stem cell niches. We will discuss altered microenvironment signaling leading to myeloid lineage disease. And finally, we will review some data on the development of acute myeloid leukemia from a subpopulation called leukemia-initiating cells (LIC), and we will discuss on the emerging evidences supporting the influence of the microenvironment on chemotherapy resistance.

1. Introduction

Stem cells are self-renewing, and multipotent or pluripotent cells present in different compartments of the body, responsible for the growth, maintenance, and repair of the different tissues. The stem cells reside in a special microenvironment; the so-called "niche," composed of cellular structures and extracellular matrix in which those cells are tightly regulated regarding their balance between maintenance of stemness, expansion, and differentiation [1].

One of the best characterized stem cell is the hematopoietic stem cells (HSC) and in 1978, Schofield proposed the "niche" hypothesis to the HSC bone marrow (BM) microenvironment [2]. HSCs function to generate a lifelong supply of all blood cell types. The ability to reconstitute all the blood-cell lineages in lethally irradiated mice is the most common assay to assess the stemness in HSC [3]. And furthermore, the maintenance of this property upon serial transplantation shows the capability of these cells to undergo self-renewal and to give rise to a differentiated progeny at the same time. In the past few years, the microenvironment, which regulates HSCs, has been characterized. Within adult BM, self-renewal and differentiation are regulated by two major cellular components: osteoblasts and vascular endothelial cells [4]. In this review, we provide some principles applying to the hematopoietic niches, which will be useful in the study and understanding of other stem cell niches. Furthermore, the involvement of the niches in the development of leukemia and its resistance to treatment will be described.

2. The Osteoblastic Niche

The concurrence of hematopoiesis and osteogenesis in BM suggests a close relationship between bone-forming cells and hematopoietic cells [5].

2.1. In Vivo Evidence of the Osteoblastic Niche. In the BM, quiescent or slow-cycling HSC have been identified close to the endosteal surface in the trabecular bone; constituting a reservoir of HSC that can be mobilized and restore the hematopoiesis in response of tissue injury. Celso and collaborators demonstrated that after transplantation, an HSC pool was observed in the area closest to the endosteum. They also showed that as differentiation progressed, they homed into distinct locations [6]. Xie and collaborators showed that transplanted HSCs were homing to the endosteum in the BM, which maintains and promotes HSC [7]. We have *in vivo* evidence of an osteoblastic niche, which might serve as a reservoir for HSC storage.

2.2. Secreted Factors. The bone-forming osteoblasts are crucial players for the homeostasis of the hematopoietic tissue with its high turnover. Indeed, osteoblasts express several cell-signaling molecules such as BMP4, Jagged-1, and angiopoietin-1 (Ang1), which are important for HSC self-renewal, survival, and maintenance [8, 9] and have been proposed to control HSC fate. Ang1 in osteoblasts interacts with Tie-2, a type of receptor tyrosine kinase that is expressed in HSC, and the Tie-2/Ang1 interaction activates β1-integrin and N-cadherin. This enhanced adhesion contributes to the maintenance of the stem cell quiescence. Osteopontin, a matrix glycoprotein expressed by the osteoblasts supports the adhesion of HSC to the osteoblastic niche and negatively regulates HSC proliferation, contributing to the maintenance of a quiescent state [10]. Furthermore, osteoblasts in the bone marrow produce a significant amount of membrane-bound stem cell factor (SCF), which has the capacity to enhance the adhesion of HSC to stromal cells [11], suggesting that osteoblasts can influence the adhesive characteristics of the endosteal niche by modulating the functional state of specific integrins. SCF binds and activate c-KIT, which is highly expressed by label retaining HSC. It has been previously shown that mutations which affect the membrane bound SCF have an influence on migration and differentiation of different cells including hematopoietic cells [12]. In addition a high concentration of Ca^{2+} in the BM endosteal region mediates HSC homing and lodging in the osteoblastic niche by attracting HSCs through the calcium sensor receptor that is highly expressed in HSCs [13].

2.3. Membrane-Bound Factors. In 2003, the work leaded by two research groups supported the idea that osteoblasts control the number HSC by regulating the size of the niche. Calvi and his collaborators were able to show a direct contact between osteoblasts and HSCs in some *in vivo* studies using mutants, with increased or decreased number of osteoblasts [14]. This study showed that an increase in the number of osteoblasts by administration of parathyroid hormone (PTH) produce high levels of notch ligand Jagged-1 and increase the number of HSC. Zhang and his collaborators showed also that it was possible to increase or decrease the HSC number by modulating the osteoblast number. They also showed that HSC and osteoblasts bind each other via adhesion molecules such as N-cadherin. Although not all osteoblasts function as the HSC niche, and a subset expressing a high level of N-Cadherin function as a key component. These cells expressing a high level of N-Cadherin are located in the bone surface and named: spindle-shaped N-cadherin+CD45− osteoblastic cells (SNO) [15]. Wilson and collaborators found that the majority of HSCs/progenitors lodge in the endosteum and attach to N-Cadherin+ osteoblastic cells 15 hours after transplantation, and HSCs harboring a c-Myc mutation increases N-Cadherin expression, enhancing adhesion to osteoblasts [16]. However, the fact that hematopoiesis appears normal in N-cadherin-deficient mice [17] and the localization of a significant amount of HSC in proximity of the sinusoidal walls in the BM support the hypothesis of another specialized microenvironment affecting HSC regulation: the vascular niche [18, 19].

3. The Vascular Niche

Some evidences about the existence of a vascular niche were already present, since in case of injury HSC can detach from the endosteum and migrate to the vascular region of the BM from where they restore hematopoiesis [20]. The vascular niche promotes proliferation, differentiation of actively cycling, and short-term HSCs [21]. The most purified HSCs, fractioned as CD150+ CD48− CD41− Lin− cells, were found in majority to be associated with the sinusoidal endothelium lining blood vessels, suggesting that endothelial cells create a cellular niche for HSCs [18].

3.1. Hemangioblasts Differentiate toward HSC and Endothelial Cells. A close developmental relationship between the hematopoietic and the endothelial lineages has been shown in the study led by Keller's group [22]. HSCs seem to originate from a perivascular progenitor during embryonic development. Moreover, the presence of HSC in the yolk sac, aorta-gonad-mesonephros (AGM) region and vitelline arteries suggests that HSCs reside and undergo hematopoiesis in association with blood vessels. HSCs are found in close contact with endothelial cells at any time during development. The endothelial cells harvested from these regions have been shown to support the expansion of adult HSC LSK ($Lineage^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$) cells *in vitro* [23, 24]. In contrast, endothelial cells isolated from adult organs do not display an HSC supportive activity *in vitro* [25]. These observations support a strong embryologically interdependence between HSCs and endothelial cells of BM sinusoids and indicate that these cells hold distinct functional characteristics from endothelial cells present in other tissues.

3.2. In Vivo Evidence of the Vascular Niche. On the one hand, conditional deletion of vascular endothelial growth factor receptor 2 (VEGF2) in adult mice, inhibits regeneration of sinusoidal endothelial cells, and prevents hematopoietic reconstitution in irradiated animals [26]. And, on the other hand, thrombopoietin knock-out mice used by Rafii's group showed remarkably that CXCL12 (SDF-1, stromal cell-derived factor-1) and FGF-4 (fibroblast growth factor-4) promotes megakaryocytopoiesis, restoring platelet production.

Among other things, SDF-1 and FGF-4 induced upregulation of adhesion molecules, including VLA4/VCAM1, facilitating localization to the vascular niche [27].

3.3. Secreted Factors. The vascular niche has been shown to produce factors important for mobilization, homing, and engraftment of HSC, such as SDF-1 important for mobilization, homing, and engraftment for HSCs [27]. The role of primary human endothelial cells (PECs) in the regulation of hematopoiesis, has been highlighted in 2006 by a study led by Yeoh et al. [28]. This research group showed that the cell-autonomous capacity to support homeostasis was hidden in the presence of serum and angiogenic factors (VEGF-A and FGF2), essential to maintain PECs for coculture with hematopoietic stem progenitors (HSPs). To overcome this issue, Rafii's group generated a model to study the vascular niche, where endothelial cells could propagate, while maintaining their long-term angiogenic profile without oncogenic transformation. Indeed, they transduced human primary endothelial cells (PECs) with the adenovirus gene, early region 4 encoded open reading frame-1 (E4ORF1) [29], which leads to constitutive activation of Akt that allows for long-term culture. E4ORF1-transduced PECs expand HSCs *in vitro* through secretion of stem cell active angiocrine factors that sustain self-renewal of KLS cells [30]. Selective activation of Akt in the endothelial cells of adult mice increases the number of HSCs and accelerates hematopoietic recovery after myeloablation along with upregulation of a specific set of hematopoietic stem and progenitor cells (HSPCs) angiocrine factors, like FGF2, BMP4, Ang1, IGFBP2, and DHH [31].

3.4. Membrane-Bound Factors. During the bone marrow transplantation, the ability of HSCs to "home" and engraft in the recipient's bone marrow requires a cascade of events which includes specific molecular recognition. Adhesion molecules on the HSCs involved in the process of rolling are VLA4 (CD49d), LFA-1 (CD11a), and hyaluronan binding-cellular adhesion molecule (HCAM/CD44) whereas the complementary binding partners on the BM endothelial cells are VCAM-1, ICAM-1 (CD54), and E- and P-selectin (CD62E and CD62P) [32]. The notch signaling pathway involves the activity of notch transmembrane receptors 1, 3, and 4, which interact with membrane-bound ligands, Delta1, 2, and 4, and Serrate/Jagged-1 and 2. Ligand binding induces proteolytic cleavage of notch receptor by a gamma-secretase complex causing the subsequent translocation of the notch intracellular domain (NICD) to the nucleus, where it will activate the transcription of downstream target genes such as Hes1 (hairy enhancer of split homolog-1) and Hey1 and Hey2 (Hes-related protein) [33]. Notch-1 is expressed on LT-HSCs and maintains HSCs in an undifferentiated state [14, 34, 35]. Overexpression of the intracellular domain of notch enhances HSC self-renewal [36] whereas loss of notch signaling impairs the maintenance of HSCs both *in vitro and in vivo* [14, 37]. Butler and his collaborators blocked angiogenic pathways, which led to inhibition of notch ligand expression and a decrease of LT-HSCs expansion [30]. Altogether, these observations suggest that endothelial cells enhance proliferation of HSCs and play an important role in maintaining the pool.

4. Common Components and Differences

CXCL12/CXCR4 signaling plays important roles in HSC trafficking and HSC mobilization. Sugiyama and his collaborators [38] demonstrated that HSCs were specifically located adjacent to cells expressing a high level of CXCL12, which surrounded the sinusoidal endothelial cells. They named these cells CXCL12-abundant reticular cells (CAR). They also demonstrated that a depletion of CXCR4 led to a reduction of HSC pool. Selective short-term ablation of CAR cells within bone marrow has suggested that they play a crucial role in proliferation, maintenance in an undifferentiated state. HSCs have been shown to be associated with CAR cells in the sinusoidal region and are located between CAR and osteoblastic cells in the endosteal region, suggesting that those cells might be an important component of both the osteoblastic and the vascular niches in adult BM.

The major difference between both microenvironments is the oxygen level. Higher in the vascular niche than in the osteoblastic niche under hypoxia, HSCs would move to the vascular niche and resume then cell cycle in order to restore hematopoiesis [39]. HSCs can then come back to the osteoblastic niche where they would be maintained in the G0 state once again [40]. The role of the hypoxia has recently been demonstrated. Using a transgenic $VEGF^{\delta/\delta}$ mice model where the hypoxia response element in the VEGF promoter is enable to bind HIF; the authors demonstrated impairment of HSC function and confirmed that one important role of the hypoxic niche is to provide HSCs intrinsically with the survival factor VEGFA [41].

The osteoblastic niche localized at the inner surface of the bone cavity and with abundant osteoblasts, might serve as a reservoir for long-term HSC storage in a quiescent state. Whereas the vascular niche, which consists of sinusoidal endothelial cell lining blood vessel, provides an environment for short-term HSC proliferation and differentiation. Both niches act together to maintain hematopoietic homeostasis or restore it after damage.

5. Altered Microenvironment Leads to Myeloid Lineage Diseases

Several studies have provided insights into the role of altered microenvironment signaling leading to myelofibrosis, myeloma, and myelodysplastic syndromes. It has been shown that alterations of the microenvironment, such as decreased expression of the retinoblastoma gene in cells of the BM microenvironment, can induce a myeloproliferative disorder [42]. Latillade reviews key data suggesting that an imbalance between endosteal and vascular niches may be important in idiopathic disorder characterized by bone marrow fibrosis (primary myelofibrosis), leading to the development of clonal stem cell proliferation [43].

Wnt signaling pathway plays a critical role in the pathogenesis of myeloma. A dramatic effect of directly targeting this pathway in osteoblasts has been clearly demonstrated, showing that it could prevent *in vivo*, the development of myeloma disease within bone. Increasing Wnt signaling in the bone marrow microenvironment inhibits the development of myeloma and reduces tumor burden in bone *in vivo* [44]. Microenvironment perturbations play also a role in myelodysplastic syndromes (MDS). Raaijmakers and his collaborators have developed transgenic mice with a dicer knock out only in osteoblast progenitor to investigate if altered niche caused myelodysplasia [45]. Although dicer, which encodes a critical enzyme in the formation of microRNAs, was not deleted from the hematopoietic cells, these mice developed a form of myelodysplastic disease. To identify the mechanism by which dicer deletion in osteoblast progenitors may cause MDS, the investigators performed gene expression analysis. Differentially expressed genes and pathways included cytokines and stress response pathways, including significant downregulation of the Shwachman-Bodian-Diamond (sbds) gene, which is linked to the human Shwachman-Diamond syndrome. Although the mechanism linking dicer deletion to sbds expression is not clear, the investigators showed that knockdown of just the Sbds gene in osteoblast progenitors reproduces much of the MDS phenotype.

6. Consequences of the Niches in the Development of Leukemia and Resistance to Treatment

AML is the most common leukemia according to the SEER program of the National Cancer institute. AML is a cancer of the myeloid line of blood cells. AML is a stem cell disease with a hierarchy comparable to normal hematopoiesis. In parallel with leukemogenesis events, the niche provides an environment with signals that favor cell proliferation and growth. Although, recent line of evidences suggest that the tumor cells are heterogeneous and indicate that only a fraction of the tumor cells "the leukemia-initiating cells" (LIC) are mostly in a quiescent state and are responsible for the maintenance of the neoplasm [46].

6.1. In Vivo Evidence of Niches for Leukemia Cells. It has been demonstrated that the normal hematopoiesis disappear in leukemic patients, disturbing the BM niches [47]. Both niches play a critical role in the survival, proliferation, and differentiation of LSC. The group led by Ninomiya showed the homing proliferation and survival sites of leukemia cells in immunodeficiencies mice. Indeed, when transplanted, leukemia cells initially localized on the surface of the osteoblasts in the inner vascular and diaphyseal region. Under administration of a high dose of cytarabine leukemia cells clustered and adhered to the blood vessels suggesting that leukemia cells receive antiapoptotic signals from vascular niche as well [48]. The molecular mechanisms for maintaining quiescence of normal HSCs may also facilitate LSC survival.

6.2. Secreted Factors. Similar to regeneration of hematopoietic cells, leukemic cells may also take advantage of pro-survival signals conveyed by the osteoblastic and vascular niches to support their proliferation and invasive potential. Osteoblasts provide a source of osteopontin and SDF-1, which may induce migration of CXCR4-expressing LSCs towards the osteoblastic niche. The interaction between SDF-1 and its receptor on leukemic progenitor cells contributes to their homing to the BM microenvironment. Endothelial cells are protective of leukemia and increased levels of angiogenesis in the BM of AML patients have been reported [49]. Moreover VEGF acts as an autocrine and paracrine growth factor in some AMLs that express the VEGFR2 [50, 51]. VEGF is a hypoxia-inducible growth factor that stimulates the formation of new blood vessels. As an adaptive response to hypoxia, new vessels are generated by angiogenesis and the proliferation of endothelial cells is stimulated. By secretion of cytokines, including IL-6, IL-3, G-CSF, GM-CSF, and nitric oxide (NO) [52, 53], endothelial cells were shown to promote leukemic proliferation, suggesting that activated angiogenic endothelial cells could provoke leukemic cell growth through paracrine signaling.

6.3. Membrane Bound Factors. The attachment of AML cells to the BM microenvironment through interaction between very late antigen-4 on leukemic cells and fibronectin on stromal cells has been shown to be crucial for the persistence of minimal residual disease in AML [54]. CXCR4 levels are significantly elevated in leukemic cells from patients with AML [55] and CXCR4 expression is associated with poor outcome [56, 57]. It has been shown that administration of anti-CXCR4 antibody to a NOD/SCID mice engraft with primary AML cells resulted in a dramatic decrease in the level of human AML cells in different sites/BM, blood, and spleen. Interestingly, the levels of normal human progenitor cells were not affected [58]. Dick and colleagues showed that anti-CD44 antibody-treated NOD/SCID mice transplanted with AML exhibited a significantly lower rate of disease onset [59]. This indicates that CD44 is essential for the homing and engraftment of the LSC to the niche. To summarize the role of the microenvironment in leukemia and its normal physiologic role in supporting HSC it has been summarized in Figure 1.

6.4. Microenvironment and Resistance to Chemotherapy. From a clinical perspective, it has been demonstrated that LSCs are substantially more resistant to standard forms of chemotherapy than bulk leukemia populations. In order to optimize the treatment, it is mandatory to characterize the interaction of leukemic cells with their microenvironment in the bone marrow. The tumor microenvironment is increasingly being recognized as a critical factor in mediating drug resistance. Resistance may occur through the soluble release of growth factors and by contact-dependent mechanisms via cell-cell and cell-extracellular matrix interactions. The term, cell adhesion-mediated drug resistance (CAM-DR) has been used to describe the phenotype in which interaction of malignant cells with stroma confers resistance to chemotherapy. CAM-DR has been described in vitro for both primary

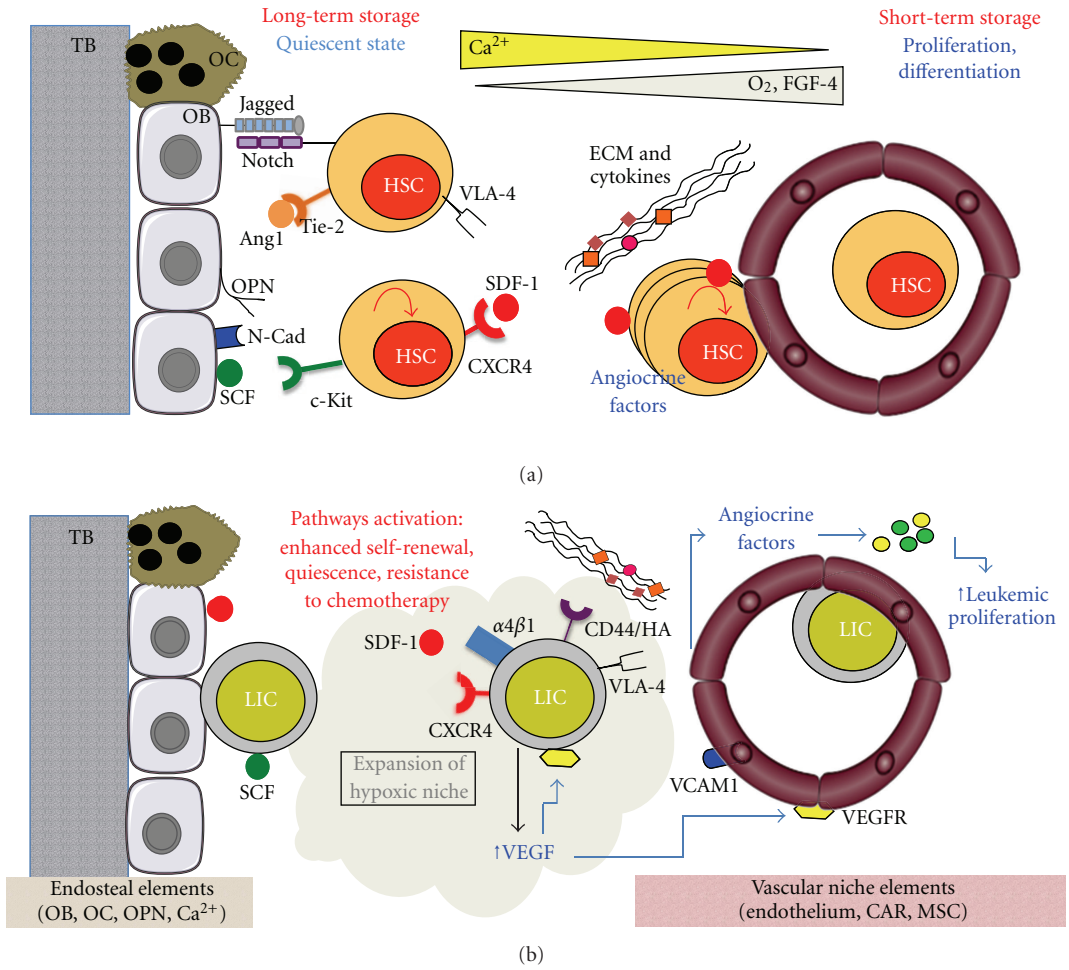

Figure 1: Schematic representation of the (a) HSC, normal stem cell and (b) LIC, leukemia-initiating cell interactions with the microenvironment. Niches provide support for self-renewal, quiescence, homing, enfrafment, and proliferation. As shown, HSCs and LSC reside in the osteoblastic or vascular niche. HSCs and LSC interact with multiple cell types (OB, OC, EC, CAR, MSC) within the BM microenvironment. Although schematically represented separately, the endosteal, and vascular niches are physically and functionally mutually involved. Candidate niche mechanisms, which regulate HSC function are also shown, including Jagged/notch, SDF1/CXCR4, SCF/c-Kit signaling. LIC quiescence, survival, and expansion are influenced by receptor kinases, adhesive receptors and signaling via matrix mediated/bound chemokines and cytokines (IL6, G-CSF, GM-CSF, KIT L, NOTCH L). LIC may secrete substance like SCF, infiltrate the niches, and take advantage of the normal hemostatic process, which enhances self-renewal and proliferation. Growth factor and other adhesion receptors signals can be targeted to overcome chemoresistance. Oxygen level, higher in the vascular niche than the osteoblastic niche under hypoxia. LIC proliferation results in expansion of hypoxic niche. HSC: hematopoietic Stem Cell, OPN: osteopontin, LIC: leukemia-initiating Cell, N-Cad: N-Cadherin, OC: osteoclast, VLA-4: very late antigen 4, OB: osteoblast, VEGFR: vascular endothelial growth factor receptor, TB: trabecular bone, SDF1: stromal growth factor 1, SCF: stem cell factor, Ang1: angiopoietin 1, HA: hyaluronic acid, ECM: extracellular matrix.

tumors and cell lines derived from several hematologic malignancies [60]. There is an increase in neovessel density in the marrow of patients with leukemia; suggesting that crosstalk between leukemic and the activated vascular niche contribute to disease progression. Dr. Rafii and others have shown that inhibition of angiogenesis effectively blocks the progression of subsets of leukemias. Clinical trials have been initiated to evaluate the use of antiangiogenic agents in combination with standard chemotherapy to treat leukemias.

The hematopoietic system continues to evolve as a model; however, concerning the microenvironment, numerous critical issues remain to be addressed. A critical role for notch in hematopoiesis was initially suggested after detection of the human NOTCH-1 gene in CD34+ or CD34+ lin− human hematopoietic precursors [61]. Then several reports [36, 62, 63] have shown that NOTCH-1 plays a key regulatory role in hematopoiesis and suggest that notch ligands will be useful reagents for improving *ex vivo* culture of stem/progenitor cells. In 2010, a research group leaded by Bernstein reports the development of an optimized, clinically feasible methodology for generating cord blood stem/progenitor cells for clinical evaluation. *Ex vivo* culture of CD34+ cord blood stem/progenitor cells in the presence of notch ligand resulted in a greater than 100-fold increase in CD34+ cells that

repopulate immunodeficient mice. This work was the first demonstration of rapid hematopoietic engraftment derived from *ex vivo* expanded hematopoietic progenitors. A recent study has demonstrated for the first time that the activation of notch pathway may indicate a poor prognosis in AML [64]. Especially, NOTCH-1, Jagged-1 and Delta1 expression may be relevant prognostic markers in intermediate risk AML. Nowadays, the most clinically arguably advanced approach for influencing leukemia microenvironment is targeting the angiogenesis. For example, therapies combining a monoclonal antibody neutralizing VEGF, bevacizumab with other chemotherapies as (cytarabine and mitoxantrone) improve the overall response rate of relapse and refractory AML [65]. Recently, new antiangiogenic agents are in use; among them, tyrosine kinase inhibitors (such as sorafenib for targeting VEGFR) and anticytokine drugs (such as thalidomide). On the one hand, those therapies can affect vascular endothelial niche but on the other hand, they may induce chemoresistance through the increase of hypoxia. In conclusion, targeting leukemic cells and cells in their surrounding microenvironment might be useful to observe a reduction of the leukemic burden. Going forward, it is challenging to revisit many of the concepts derived from work in the blood field in other organ systems. This will probably increase our knowledge on the network controlling self-renewal and differentiation, the hallmarks of stem cells.

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Review Article

Spleen as a Site for Hematopoiesis of a Distinct Antigen Presenting Cell Type

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While spleen and other secondary tissue sites contribute to hematopoiesis, the nature of cells produced and the environment under which this happens are not fully defined. Evidence is reviewed here for hematopoiesis occurring in the spleen microenvironment leading to the production of tissue-specific antigen presenting cells. The novel dendritic-like cell identified in spleen is phenotypically and functionally distinct from other described antigen presenting cells. In order to identify these cells as distinct, it has been necessary to show that their lineage origin and progenitors differ from that of other known dendritic and myeloid cell types. The spleen therefore represents a distinct microenvironment for hematopoiesis of a novel myeloid cell arising from self-renewing hematopoietic stem cells (HSC) or progenitors endogenous to spleen.

1. Hematopoiesis in the Mouse Model

Hematopoietic stem cells arise in the embryo from an hemangioblast present in yolk sac and aorta-gonad-mesonephros. They then migrate into fetal liver and spleen, entering bone marrow in the late embryonic period [1]. Numbers of HSC increase in bone marrow and spleen in the neonatal period and are then maintained in those organs for life [2, 3]. HSC migration into bone marrow at birth then determines bone as the dominant site for hematopoiesis in adults. HSC have also been identified in extramedullary sites in the steady state and small numbers also mobilize through blood and lymph into sites like liver, spleen, brain, lung and intestine [4, 5]. Infection, inflammation, or drug treatment can lead to expansion in the number of hematopoietic stem/progenitor cells (HSPC) circulating in blood and entering tissues like spleen and liver [6, 7]. Despite the small numbers of HSPC present in extramedullary tissues in the steady state, this fact should not discount their importance or potential contribution to the development of the immune system and immune responsiveness. Findings from this lab indicate that spleen in the steady state contains HSC [2] and that these undergo myelopoiesis within spleen giving rise to a novel type of dendritic-like antigen presenting cell [8].

2. The Hematopoietic Stem Cell

Bone marrow is a very rich source of HSC and for decades bone marrow transplants have provided HSC for regeneration of the hematopoietic system in humans. HSC have distinguishing properties of self-renewal, asymmetric cell division, and ability to clonally regenerate all hematopoietic cell types. Methodology for sorting HSC from bone marrow using specific antibodies and flow cytometry [9] has become a standard technique in many labs. HSC are heterogeneous, comprising cells with both long-term and short-term reconstitution capacity [10, 11]. Long-term reconstituting HSC are commonly isolated from murine bone marrow as lineage $(Lin)^-Sca\text{-}1^+c\text{-}kit^+Flt3^-$ cells [11] and can be further purified as the $CD150^+CD48^-$ subset of $Lin^-Sca1^+ckit^+$ bone marrow [12, 13]. Epigenetic imprinting of HSC also contributes to differences in hematopoietic potential [14]. Given the level of heterogeneity amongst HSC, it is important to consider them in relation to their tissue niche

of origin, their distinct hematopoietic potential, and to use this knowledge to inform HSC transplantation in the clinic. Most studies have concentrated on the more prevalent HSC in bone marrow with less attention given to HSC in extramedullary sites like spleen. While HSC from tissue sites like bone marrow, cord blood and spleen can reconstitute all cell lineages, there has been no comprehensive study of their relative capacity to produce a range of hematopoietic cell subsets. The hematopoietic support capacity of different tissue niches is also a factor not yet investigated in terms of hematopoietic output. Recent evidence that spleen supports the development of distinct dendritic-like antigen presenting cells suggests that spleen may contribute in a distinct, tissue-specific manner to hematopoiesis.

3. The Spleen in Hematopoiesis

HSC represent a rare subset in adult mouse spleen. They are however readily detectable by their long-term reconstitution ability upon adoptive transfer into irradiated host mice [2, 3] (Figure 1). The weaker contribution of splenic HSC compared with bone-marrow-derived HSC to hematopoiesis in the normal animal has been reported [15]. It has long been assumed that spleen fills the role of an emergency or backup site for cell development at times of stress or disease. Indeed, the importance of bone marrow over spleen in hematopoiesis is evident since neonatally splenectomised mice can maintain normal bone marrow hematopoiesis [16]. However, a distinct role for spleen in hematopoiesis has not yet been fully investigated in terms of development of individual cell subsets. During embryogenesis, spleen harbours myeloid cells seeding from fetal liver and does not appear to become colonized with HSC until around the time of birth [3]. Evidence that splenectomy in newborn mice results in a dramatic increase in colony forming cells in liver suggests that HSC entering spleen at this time may seed directly into spleen from fetal liver rather than from bone marrow [3]. Spleen could therefore become colonized by HSC during development resulting in an endogenous HSC population. Such a model would not however preclude later or additional entry of bone-marrow-derived HSC at times of stress or inflammation, or following stem cell transplantation.

Spleen is also a central organ for development of dendritic cells (DC) that initiate immune responses by taking up and presenting antigen to lymphocytes. Murine spleen contains multiple subsets of DC including the CD8α^- and CD8α^+ conventional(c)DC, plasmacytoid(p)DC [17], and regulatory DC which are not well characterised [18]. Monocyte-derived DC are also found in spleen but only under conditions of inflammation [19]. Very few markers distinguish these subsets and they are best distinguished by their distinct function in immunity. Both cDC subsets are powerful T-cell activators, although the CD8α^- cDC are located in the marginal zone of spleen where antigen enters from blood, while the CD8α^+ cDC are located in the T-cell areas where they are poised for T-cell stimulation. The CD8α^- cDC preferentially activate a T-helper(h)2 response with T-cell production of interleukin(IL)-4 and IL-10, while CD8α^+ cDC preferentially induce a Th1 response marked by interferon (IFN)-γ production [20, 21]. While both subsets of cDC can activate CD4$^+$ and CD8$^+$ T cells, the CD8α^+ cDC subset has been shown to be superior at antigen cross-presentation [22]. Plasmacytoid DC are distinct in that they reside mainly in blood in a precursor form, often referred to as p-preDC. They are distinct by their capacity to produce high levels of IFN-α in response to viruses [23]. Regulatory DCs represent a poorly defined class of DCs which are inhibitory rather than stimulatory for T cells, producing factors like IL-10 and nitric oxide [18, 24]. In contrast to cDC and pDC, mo-DCs are not detectable in spleen in the resting state. They develop from blood monocytes in response to inflammatory factors. Tumour necrosis factor α/inducible nitric oxide synthase producing (Tip) DC derive from monocytes and enter tissues where they function as strong T-cell activators [25].

An essential element of DC biology is the definition of progenitors and precursors since this underpins the formation of lineages of cells with distinct function. Initially cDC and pDC progenitors were defined as a Flt3$^+$ subset amongst common myeloid and lymphoid progenitors (CMP/CLP) in bone marrow [26]. Recent evidence now points to a monocyte/dendritic progenitor (MDP) in bone marrow which gives rise to all monocyte/macrophage and dendritic type cells [27, 28], as well as a more committed common dendritic progenitor (CDP) for cDC and pDC in bone marrow [29, 30]. While CDP and MDP are not present in spleen or blood [31], spleen does harbour more differentiated cDC precursors which have a high turnover and are replaced by blood-borne precursors migrating from bone marrow [32]. Studies with parabiotic mice that share a circulatory system, suggest that some splenic DC might arise from endogenous progenitors [33], although others have questioned that result [34]. The presence of an endogenous hematopoietic progenitor in spleen that differentiates to give a novel and distinct dendritic-like cell is now indicated by the findings described below.

4. A Novel Antigen Presenting Cell Subset in Spleen

Distinct myeloid dendritic-like cells have been discovered as a subset in murine spleen [35] on the basis of similarity with dendritic-like cells identified previously which develop in splenic longterm cultures (LTC) [36–38]. LTC-derived DC and the *in vivo* subset of "L-DC", have a characteristic immature phenotype as CD11c^{lo}CD11b^{hi}MHC-II$^-$CD8α^- cells, distinguishing them from cDC, pDC, and monocytes [35] (Figure 2). They are large, endocytic cells specialized in cross-presentation of antigen to CD8$^+$ T cells, a function usually attributed to CD8α^+ cDC [39]. LTC-DC are also distinct by their very weak ability to activate CD4$^+$ T cells and their MHC-II$^-$ phenotype, properties which distinguish them from all of the common subsets of CD8α^+ cDC, CD8α^- cDC, and pDC [35, 37, 40]. Since LTC-DC can be derived from *GM-CSF*$^{-/-}$ mice [41], they do not resemble monocyte-derived DC that develop in response to inflammatory factors like GM-CSF and TNF-α [19].

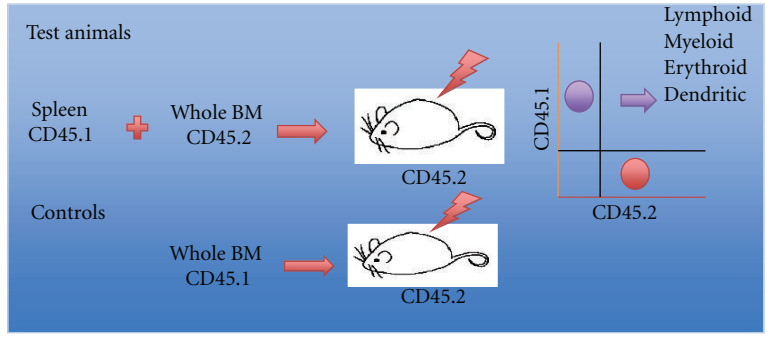

Figure 1: Hematopoietic reconstitution assay for detecting the presence of HSC. The hematopoietic system of lethally irradiated host mice can be fully reconstituted with lymphoid, myeloid, erythroid, and dendritic cells following adoptive transfer of bone marrow cells as a source of HSC. In order to assess the presence of HSC or other hematopoietic progenitors in a test population of cells, CD45-allotype distinct cells are adoptively transferred with assessment of the hematopoietic reconstitution capacity of this specific population by flow cytometry. In most cases, test animals are also given an inoculum of host type bone marrow to ensure survival. HSC with long-term reconstitution potential produce progeny out to 25 weeks, while HSC with short-term potential are assessed after 8 weeks.

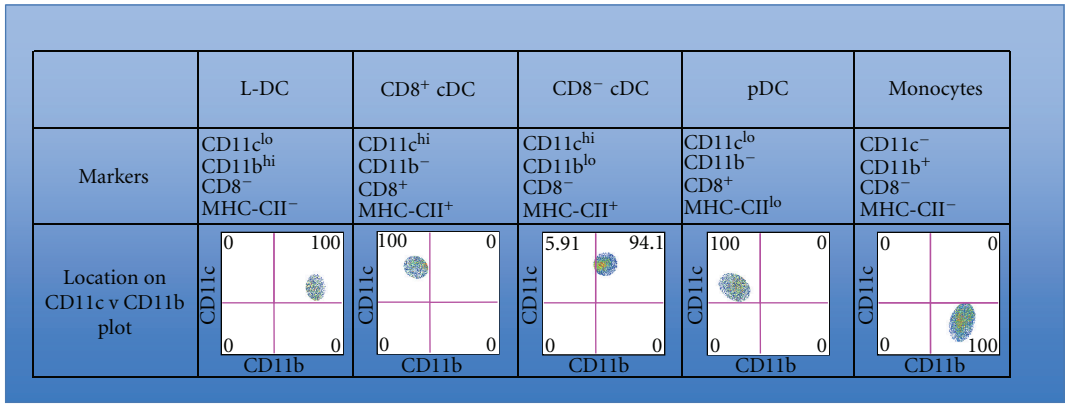

Figure 2: Classification of DC and myeloid subsets in spleen. Subsets in spleen can be identified flow cytometrically in terms of their phenotype following staining with specific antibody to cell surface markers. The different subsets can be identified based on variant expression of CD11c, CD11b, CD8α and MHC-II. The phenotype of L-DC is shown by comparison with pDC, cDC and a broad monocyte population. The different subsets can also be located in distinct positions on a bivariate CD11c versus CD11b scatter plot.

An isolation procedure was optimised to reveal the *in vivo* equivalent cell type to LTC-DC [35]. This procedure distinguished CD8α^+ cDC, CD8α^- cDC, pDC and monocytes on the basis of CD11b, CD11c, MHC-II and CD8α expression, from a population of cells of large size (FSC^{hi}) resembling LTC-DC by their CD11c^{lo}CD11b^{hi}MHC-II$^-$CD8α^- phenotype and their high endocytic capacity for soluble antigen (Figure 3). These cells have been tentatively named "L-DC," since they resemble a DC more than a myeloid cell. L-DC are phenotypically and functionally distinct from monocytes/macrophages, cDC and pDC. They are distinct as CD11c$^+$ cells that are highly cross-presenting, a property which distinguishes them from common subsets of macrophages and monocytes [40]. Recent evidence for uniform expression of F4/80 and CD11c, but not Ly6G, also distinguishes L-DC from neutrophils [42]. The immune potential of this *in vivo* subset is under continued investigation in terms of both T-cell and B-cell immunity. Already we know that L-DC share similar antigen cross-presenting function for CD8$^+$ T-cell activation with LTC-DC [35]. Like LTC-DC, they are also unable to activate CD4$^+$ T cells, consistent with the absence of MHC-II expression [37, 40]. Despite their distinct functional potential, the lineage origin of L-DC relative to other known splenic myeloid and DC subsets, is still unknown.

The distinctiveness of L-DC from monocytes, cDC subsets and granulocytes has been confirmed by transcriptome analysis of subsets sorted as shown in Figure 3. Principal Components Analysis of the data indicated a broad separation of overall gene expression between L-DC and other subsets, with CD8α^+ and CD8α^- cDC the most closely related subsets (Figure 4). L-DC specifically express high levels of *Treml4*, a gene recently identified as expressed by CD8α^+ but not CD8α^- cDC and by splenic red pulp and metallophilic macrophages [43]. This gene encodes a receptor that binds late apoptotic and necrotic cells, consistent with cross-presenting capacity related to the uptake of dead and dying cells from blood. The relationship between L-DC and other DC and macrophage cell types is currently under investigation.

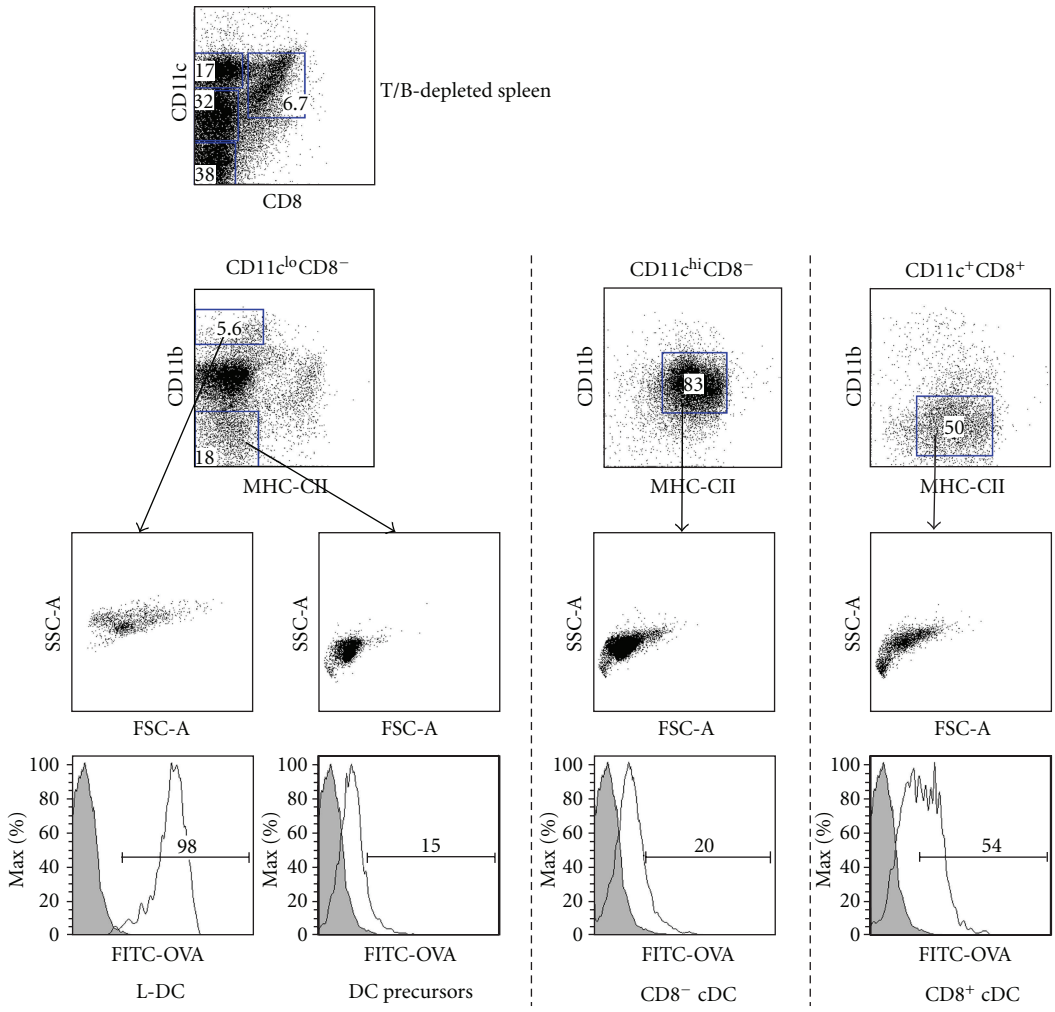

Figure 3: Isolation of L-DC (LIVE-DC) in spleen amongst other DC subsets. Spleens were depleted of T and B cells using antibody-coated magnetic beads and then stained with antibodies specific for CD11c, CD11b, CD8α and MHC-II ahead of flow cytometric analysis. DC subsets were identified based on multiple parameters of forward scatter (FSC), side scatter (SSC), marker expression and endocytosis after intravenous administration of FITC-OVA (0.6 mg/mouse) 24 hours previously.

5. Hematopoiesis Leading to L-DC Production *In Vitro*

Coculture assays are now used instead of LTC to produce L-DC *in vitro*. These involve overlay of Lin$^-$ BM or T/B-cell-depleted spleen cell suspensions over competent splenic stroma [40]. The splenic stromal line STX3 and its 5G3 clone originated from a long-term splenic culture that had lost hematopoietic progenitors over time and passage [40]. The 5G3 clone of STX3 has been characterized as an immature endothelial-like cell [44, 45], unique in its phenotype and function as a supporter of *in vitro* hematopoiesis of L-DC. There also appears to be no equivalent stromal cell line reported in the literature. Cells produced in cocultures over 35 days both phenotypically and functionally resemble LTC-DC as well as the *in vivo* L-DC subset [8, 40]. Cocultures can therefore be used to identify L-DC progenitors amongst sorted cell subsets by their capacity to seed stroma for continued L-DC production *in vitro*. Indeed, the continuous nature of LTC which produce dendritic-like cells over several years [36], as well as the ability of STX3 or 5G3 stroma to support *in vitro* hematopoiesis of L-DC for up to a year [46], suggests that the L-DC progenitor may represent a self-renewing HSPC.

The development of L-DC in LTC depends on the maintenance of a population of small progenitors [38, 47]. In fact, the requirement for differentiation of progenitors was demonstrated by sorting the small progenitor population in LTC and showing that these cells produced L-DC upon transfer to stromal cocultures [38]. L-DC were however shown to undergo several rounds of proliferation in bromodeoxyuridine labeling experiments [47]. The production of dendritic-like cells by differentiation from HSPC maintained within LTC as opposed to proliferation of L-DC was also confirmed by gene profiling of subtracted libraries prepared between the purified small and large cells maintained in LTC [48]. These studies showed that small cells resemble HSPC, while large cells reflect myeloid dendritic cells. More

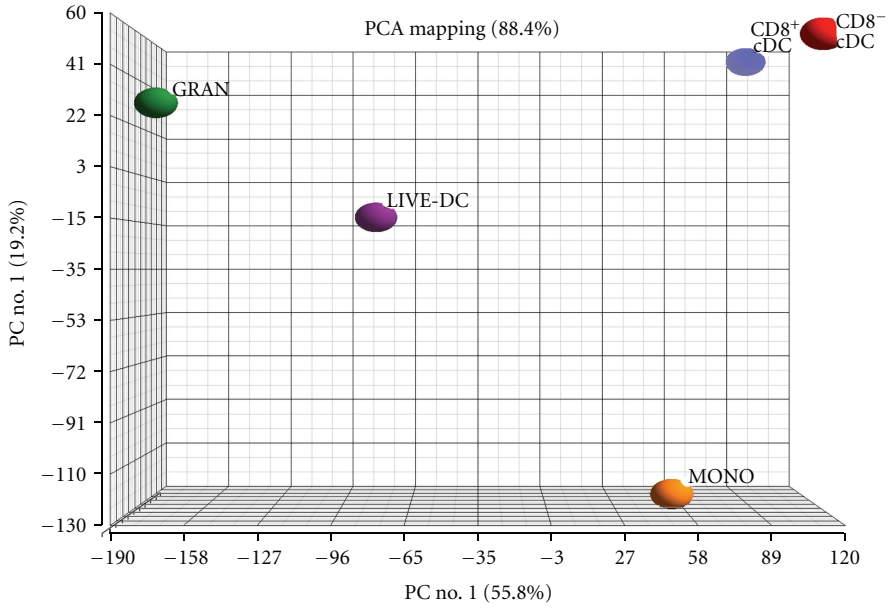

Figure 4: Relatedness of transcriptome between L-DC (LIVE-DC) and other spleen myeloid subsets. Myeloid and DC subsets were isolated from spleen by antibody staining and cell sorting using flow cytometry as demonstrated in Figure 3. RNA was prepared from DC subsets described in Figure 2, and from granulocytes (GRAN) as Gr-1$^+$CD11b$^+$CD11c$^-$ cells and monocytes (MONO) as CD11b$^+$CD11c$^-$MHC-II$^-$ cells. RNA was used to probe Affymetrix Gene 1.0ST microarrays. Data show Principal Components Analysis (1&2) reflecting degree of relatedness in overall gene expression between subsets.

recent studies have involved phenotypic characterization of progenitors. Over many experiments, progenitor activity was found to be enriched within the subset of Lin$^-$ckit$^+$CD34$^{-/+}$ "small" cells in LTC, a phenotype reflective of described subsets of HSC differing in terms of long-term or short-term reconstituting capacity [11]. Highly purified, sorted HSC from bone marrow have now been shown to seed STX3 cocultures to produce L-DC, but only when contact is maintained with stroma [46]. Cells produced were found to phenotypically resemble L-DC. Overall, these results align the L-DC progenitor with the HSC subset in bone marrow. This finding is particularly important in terms of the relationship between the L-DC progenitor and the progenitors of cDC and pDC which have been identified as MDP, CDP and myeloid progenitors (MP), which are present in bone marrow but not spleen [31]. One hypothesis is that splenic niches are distinct from bone marrow niches and can support the direct differentiation of HSC to give L-DC in the apparent absence of committed myeloid or dendritic progenitors like the MDP, CDP and MP. The relationship between these progenitors and a putative L-DC progenitor in spleen is not known and warrants further investigation.

6. Identification of L-DC Progenitors in Spleen

To delineate the L-DC progenitor in mouse spleen, Lin$^-$ splenocytes were assessed for expression of ckit and other markers of early hematopoietic progenitors followed by sorting of subsets of interest. Sorted subsets were each assessed for their hematopoietic potential in cocultures of competent stroma. They were also tested *in vivo* for capacity to reconstitute lethally irradiated CD45-allotype distinct host mice also given syngeneic bone marrow to aid recovery [8] (Figure 1). The Lin$^-$ckit^{lo} spleen subset produced exclusively L-DC in cocultures, and the Lin$^-$ckit^{hi} subset produced predominantly L-DC with a few cDC-like cells that were not maintained over time. However, only Lin$^-$ckit^{hi} splenocytes, and not the Lin$^-$ckit^{lo} or Lin$^-$ckit$^-$ subsets, showed multi-lineage reconstitution *in vivo* consistent with the presence of HSC [8]. The Lin$^-$ckit^{hi} population of spleen therefore contains long-term reconstituting HSC as well as L-DC progenitors, while the Lin$^-$ckit^{lo} fraction contains only L-DC progenitors. One explanation is that there is a common progenitor within each of these two populations. Another is that there are distinct progenitors within the two subsets and that the stroma supports differentiation of ckit^{hi} progenitors to give ckit^{lo} progenitors. The relationship between the L-DC progenitor and the HSC subset in spleen is unclear at present. These findings are also consistent with evidence that bone-marrow-derived HSC can seed stroma for L-DC production and that the L-DC progenitor maintained in LTC is represented by a Lin$^-$ckit$^+$CD34$^{+/-}$ subset. Splenic stroma therefore appears to impose specific differentiative capacity on HSC, irrespective of their origin from spleen or bone marrow. In terms of the characterization of L-DC progenitors endogenous to spleen, a first goal will be to fractionate subsets of splenic Lin$^-$ckit$^+$ cells further and to identify the minimum subset that produces L-DC in cocultures.

7. Does Hematopoiesis in Spleen Involve an Endogenous Progenitor?

Many studies now indicate a specific role for splenic niches in the development of the novel antigen presenting cell subset, namely L-DC. While the *in vitro* evidence is direct and compelling, it is a harder task to delineate L-DC production *in vivo* as dependent on hematopoiesis occurring in spleen and arising from an HSPC endogenous to spleen. In experiments involving adoptive transfer of stem cells into irradiated mice (Figure 1), the issue of where in the animal hematopoiesis occurs is not clear. Transferred stem cells can migrate to and localize in multiple niches in spleen and bone marrow. In these sites, they can undergo hematopoiesis and then migrate into other lymphoid areas. Another more direct method to delineate hematopoiesis specific to splenic HSC involves ectopic grafting of allotype-distinct spleen or spleen fragments under the kidney capsule of splenectomized mice and monitoring the output of donor graft-derived mature cells on the basis of allotype markers. We recently published successful engraftment of neonatal spleen fragments under the kidney capsule of splenectomized mice [49]. Most grafts retained donor-type HSC and showed development of some donor-type myeloid cells at 2 weeks. However, recruitment of host HSC, lymphocytes and myeloid cells into grafts was also evident [49]. The spleen microenvironment therefore appears to support myelopoiesis and possibly L-DC development. The development of individual subsets of myeloid cells is yet to be analysed more completely using this model.

Indirect evidence that spleen supports hematopoiesis and the specific differentiation of L-DC also comes from chimera studies. These studies compared the relative ability of splenic versus bone marrow HSC to generate the different myeloid and DC subsets including L-DC [2]. In several studies, analysis of the relative numbers of donor : host progeny cells representing each of the common DC and myeloid subsets produced in chimeras identified a distinct developmental bias towards donor L-DC over other myeloid and DC subsets [2]. The only explanation for this finding is that splenic niches preferentially support the development of L-DC from HSC. Another consideration is whether the microenvironment in which HSC develop conditions their hematopoietic capacity. In terms of stem cell transplantation, it will be important to determine whether HSC arising in BM, spleen, or fetal liver can adopt distinct differentiative capacity after residence in splenic niches.

8. Conclusion

A history of published work now supports the hypothesis that the splenic L-DC subset described here represents a functionally distinct tissue-specific antigen presenting cell type that develops within spleen from endogenous HSPC. Its development is entirely dependent on endothelial niches within spleen although bone-marrow-derived HSC can also differentiate within this niche to produce L-DC. The hypothesis that spleen contains an endogenous HSC laid down during embryogenesis would be consistent with evidence for tissue-specific hematopoiesis of L-DC.

The identification of a novel antigen presenting cell subset with unique immune function is of significant immunological interest. It is therefore important to define the hematopoietic pathways in spleen that lead to development of these dendritic-like cells, especially since these cells do not appear to have a lineage origin in common with other described cDC and myeloid subsets. Indeed, there are now a number of examples in the literature of tissue-specific antigen presenting cells developing from endogenous progenitors maintained in distinct tissue sites. For example, Langerhans cells in skin arise from epidermal progenitors that colonize embryonic skin [50], and microglia represent antigen presenting cells in brain which develop from self-renewing progenitors induced to proliferate after inflammation [51]. These examples support a model for compartmentalization of the immune response by the production of tissue-specific antigen presenting cells, and specialization of immunity in tissues colonized by particular pathogens. A full understanding of hematopoiesis occurring in extramedullary sites will be essential knowledge for therapeutic practice in immunotherapy and indeed in HSC transplantation.

Abbreviations

HSC: Hematopoietic stem cells
HSPC: Hematopoietic stem/progenitor cells
DC: Dendritic cells
cDC: Conventional DC
pDC: Plasmacytoid DC
CMP: Common myeloid progenitors
CLP: Common lymphoid progenitors
CDP: Common dendritic progenitors
MDP: Myeloid/dendritic progenitors
MP: Myeloid progenitors.

Conflict of Interests

The authors declare no financial or commercial conflict of interests.

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Review Article

The Ongoing Challenge of Hematopoietic Stem Cell-Based Gene Therapy for β-Thalassemia

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β-thalassemia is characterized by reduced or absence of β-globin production, resulting in anemia. Current therapies include blood transfusion combined with iron chelation. BM transplantation, although curative, is restricted by the matched donor limitation. Gene therapy, on the other hand, is promising, and its success lies primarily on designing efficient globin vectors that can effectively and stably transduce HSCs. The major breakthrough in β-thalassemia gene therapy occurred a decade ago with the development of globin LVs. Since then, researchers focused on designing efficient and safe vectors, which can successfully deliver the therapeutic transgene, demonstrating no insertional mutagenesis. Furthermore, as human HSCs have intrinsic barriers to HIV-1 infection, attention is drawn towards their *ex vivo* manipulation, aiming to achieve higher yield of genetically modified HSCs. This paper presents the current status of gene therapy for β-thalassemia, its success and limitations, and the novel promising strategies available involving the therapeutic role of HSCs.

1. Introduction

The β-thalassemias represent inherited, monogenic anemias, arising from autosomal recessive mutations, affecting the synthesis of the β-chain of hemoglobin [1]. They are characterized by reduction or absence of β-chain synthesis, resulting in excess of α-chain molecules, which precipitate in red blood precursors, leading to impaired erythrocyte maturation, mechanical damage, and ultimately to apoptosis [2]. Thalassemias are caused by more than 200 mutations affecting the human β-globin gene and are most prevalent in the Mediterranean region, the Middle East, India, and South East Asia, representing a serious health problem. Lately, due to population migration, β-thalassemia presents a clinical problem also in UK, US, and Australia. Globally, it is estimated that there are 80 million carriers [3].

β-thalassemic phenotype is very heterogeneous and directly linked to the genotype. In heterozygotic state, the outcome is consistent with clinically normal individuals, who are largely unaware of their genetic condition. Inheritance of two copies of β-thalassemia genes causes thalassemia major and usually results in life-threatening anemia and transfusion-dependence treatment for survival. Intermediate clinical forms of the disease exist as thalassemia intermedia, which is characterised by moderately severe anemia with occasional need for blood transfusion.

Current therapies for β-thalassemia include blood transfusions together with life-long iron chelation and hydroxyurea treatment for fetal hemoglobin (HbF) induction. Although these strategies have improved patients' mortality and have significantly delayed the onset of iron-related organ failure, treatment noncompliance is common, leading to cardiac, hepatic, or endocrine failure [4]. Allogeneic hematopoietic stem cell (HSC) transplantation of human leukocyte antigen- (HLA-) matched sibling donors can be curative, reaching cure rates up to 90% in patients younger than 17 years of age [5]. However, it is associated with a number of drawbacks, such as the limited matched related donors and the need for long-term immunosuppression to prevent, treat or delay graft-versus-host disease (GVHD), often associated

with allogeneic HSC transplantation. Therefore, an alternative molecular strategy based on gene therapy is undoubtedly a radical approach that overcomes all the above limitations.

2. Designing Effective Vectors for β-Thalassemia Gene Therapy

Most research efforts towards gene therapy for β-thalassemia have focused on employing retroviral vectors as a means of gene delivery, since these are capable to integrate into the target cell genome, resulting in stable and long-term expression. However, the integration has to be targeted and should occur in a specific manner in order to avoid poor gene expression or even silencing. Therefore, for gene therapy for β-thalassemia to become an effective and realistic therapeutic approach, the following very important criteria need to be met:

(I) the therapeutic vector should exhibit stability, high titer and erythroid-lineage specificity, via the utilization of respective regulatory elements,

(II) the transgene must be expressed in therapeutic and sustained levels,

(III) the therapy itself should be safe and efficient in terms of viral transduction.

Early attempts back in the 1980s and 1990s utilized gammaretroviral vectors to achieve stable and high-level transgene expression, however, with no success. More specifically, Williams et al. [6] managed to introduce a marker gene into murine HSCs, using a vector, derived from murine leukemia virus (MLV) after replacing *gag, pol,* and *env* gene with the transgene of interest, while later on, investigators utilized these vectors to drive expression of β-globin genes into murine HSCs [7–9]. The outcome, however, was unsatisfactory, as poor gene-transfer efficiency was obtained, with levels of β-globin reaching 0%–2% of the endogenous RNA levels. In an attempt to increase β-globin expression, Novak et al. [10] incorporated into MLV the newly identified powerful DNA-enhancer elements from the β-globin locus control region (LCR), which is found to be essential for high-level expression of globin genes [11, 12]. Unfortunately, poor vector production and genetic instability of the viral vector genome were observed under these experimental conditions, primarily due to the large size of the fragments incorporated into the vector. Extensive mutagenesis studies of the transduced β-globin gene by Leboulch et al. [13] identified a 372 bp intronic segment and multiple reverse polyadenylation and splicing events, which were responsible for low viral titers and instability of proviral transmission, upon infection. Around the same time, Sadelain et al. [14] managed to generate a high-titer retroviral vector that expressed high levels of β-globin in an erythroid-specific manner, by combining the human β-globin gene and the LCR core hypersensitive sites (HS) 2, 3, and 4; however, the group failed to reduce positional variability of expression. Taken together, the above findings indicated that vector instability might be caused by splicing of the retroviral RNA genome, a consequence of cryptic splice sites within the genomic sequences [13, 14]. A way to circumvent the above was to turn towards lentiviral vectors (LVs), as the latter, in addition to the common Gag, Pol, and Env proteins, also encode Tat and Rev. A major function of Rev is to mediate nucleoplasmic export of unspliced viral RNA, allowing thus the production of full-length viral RNA genomes [15]. Therefore, Rev expression in a LV packaging line could prevent splicing of a β-globin vector, containing large genomic fragments, leading thus to vector stability.

A major breakthrough in the gene therapy field for hemoglobinopathies took place when May et al. [16] and Pawliuk et al. [17] constructed an HIV-based vector with the β-globin gene along with its LCR and managed to achieve high titers, which in turn allowed high expression of the therapeutic gene and thus disease amelioration. Following this approach, several groups working also on hemoglobinopathies employed β-globin LVs in their studies, obtaining significant results, leading to correction of β-thalassemia [18] in murine models, as will be discussed below. Although the above vectors were able to ameliorate the phenotype of β-thalassemia, the observation that compound thalassemic patients with the syndrome of hereditary persistence of fetal hemoglobin (HPFH) typically have less anemia, milder clinical symptoms, and are often transfusion-independent, drew the attention towards constructing γ-globin vectors [19].

Persons and colleagues designed such vectors, containing also the extended β-globin LCR, and managed to show significant correction of the thalassemic phenotype [20]. However, globin expression by these vectors was inconsistent because of chromosomal position effects albeit the curative effect they were capable to demonstrate, and eventually led to the use of chromatin insulators [21].

Insulators represent DNA elements capable to shield the therapeutic gene from the negative and/or positive effects of the surrounding DNA, leading thus to higher and more consistent expression and reducing also vector genotoxicity by preventing the viral regulatory elements to interfere with the expression of flanking genes. A recent study has shown that improved and more consistent globin gene expression can be obtained when a 1.2 kb DNA element from the chicken β-globin locus (cHS4) is incorporated into the globin LV design [21]. Unfortunately, this vector design can lead to a significant reduction in vector production and titer and seriously compromise practical use; the mechanism underlying this decrease has been recently elucidated for the first time [21]. However, when Hanawa et al. [22], included only the 0.25 kb core element of cHS4, it was shown that the specific element could rescue vector titer by alleviating a postentry block to reverse transcription associated with the 1.2 kb element. Also, in an orientation-dependent manner, the 0.25 kb core element significantly increased transgene expression from an internal promoter due to improved transcriptional termination. This element also demonstrated barrier activity, reducing variability of expression due to position effects. Similarly, Lisowski and Sadelain [23] showed that the incorporation of HS1 element enhances the therapeutic efficacy of the globin gene transfer in murine β-thalassemia, compared to HS2-HS3-HS4 alone and, therefore, can lead to even higher globin expression with lower vector copy numbers.

Also, for safety reasons and in order to avoid insertional mutagenesis complications as in the SCID clinical trial [24], all research groups have employed a self inactivating (SIN) configuration in their vectors, by deleting large fragments of the U3 region within the vectors' long terminal repeat (LTR).

3. Hematopoietic Stem Cells (HSCs) and Lentiviral Globin Vectors

A schematic representation of the HSC-based gene therapy for β-thalassemia is shown in Figure 1. HSCs represent a minor population of the adult bone marrow, accounting for 1 in 2,500 to 1 in 10,000 cells in the adult mouse [25, 26]. These cells remain relatively quiescent most of their lives, with murine HSCs entering cell cycle every 1-2 months [27], while in primates and humans, the turnover rate is even slower reaching 1-2 years [28]. Due to the above features, gene therapy for hemoglobinopathies focused on employing such vectors for gene delivery, as they can efficiently infect nondividing cells, and thus manage to deliver the transgene of interest [29]. However, it should be noted that although LVs may be able to infect HSCs in G_o phase, it has been clearly shown that HSCs exiting G_o and entering G_1b phase are more readily transduced [30], possibly due to the fact that reverse transcription occurs at this stage [31].

3.1. Correction of Murine β-Thalassemia. As mentioned above, the major breakthrough in the correction of β-thalassemia came from the group of Sadelain in New York, where they managed to correct thalassemia intermedia in a murine model [16] and later in rescuing lethality in a thalassemia major model [32], using TNS9 β-globin vector. In parallel, Pawliuk et al. [17] demonstrated that lentiviral-mediated stem cell transfer of an antisickling variant of the human β-globin chain resulted in hematologic correction and diminished end-organ damage in murine sickle cell disease (SCD). Similarly, Imren et al. [33] using a β-globin vector showed that β-globin expression reached approximately 32% of the total hemoglobin, while in the case of the GLOBE vector, the Ferrari group showed β-globin expression ranging from 14 to 37% [18].

Correction of β-thalassemia was also achieved using γ-globin vectors, as mentioned previously, with the Persons group being the first to construct and test such vector. In their first attempt, using a noninsulated vector, they managed to correct thalassemia intermedia in the murine model. However, the phenotypic correction varied due to chromosomal positioning effects and vector copy number [20]. In an attempt to address these issues, Arumugam et al. [21] incorporated the cHS4 insulator in the D432β-4γ vector and succeeded in increasing the transgene's expression, in the expense of viral titers though. Similarly, Hanawa et al. [34] demonstrated that animals receiving transplants of β-thalassemic stem cells transduced with a new LV, containing 3.2 kb of LCR sequences, expressed high levels of fetal hemoglobin, ranging from 17% to 33%, with an average vector copy number of 1.3. The above strategy led to a mean increase in hemoglobin concentration (26 g/L) and enhanced amelioration of other hematologic parameters [34].

Lastly, Zhao et al. from the Persons group went further and incorporated a drug-resistance gene, methylguanine methyltransferase (MGMT), in the γ-globin vector [35], demonstrating amelioration of murine β-thalassemia and enrichment of the corrected HSC compartment, employing *in vitro* and *in vivo* selection, following drug treatment. However, despite the successful drug-induced enrichment of the HSC pool in the above study, findings from other investigators tend to suggest that MGMT selection approach may not always result in the desired outcome and quite often is accompanied by drug-related toxicity. More specifically, Larochelle et al. [36] failed to induce selection of long-term repopulating HSCs in rhesus macaques and showed that the alkylating agent bis-chloroethyl nitrosourea (BCNU) can result in significant nonhematopoietic toxicity, such as pulmonary congestion/edema and necrohemorrhagic colitis. In addition, recent work by Giordano et al. [37] using a murine serial transplant model demonstrated that MGMT selection approach can also lead to insertional mutagenesis and clonal dominance. Taken together, the above data indicate that although MGMT selection approach can be an effective strategy for enrichment of the corrected HSC compartment, its drug-related toxicity and insertional mutagenesis potential represent considerable risk factors for use in human clinical trials.

3.2. In Vitro Studies with Human HSCs. Both β-globin and γ-globin vectors have been used in correcting human erythropoiesis in erythroid cultures and immunodeficient mice. In a detailed study, Puthenveetil et al. [38] tested a lentiviral vector carrying the human β-globin expression cassette, flanked by a chromatin insulator in transfusion-dependent human thalassemia major, and demonstrated that normal amounts of human β-globin were expressed in erythroid cells produced *in vitro*; erythropoiesis was restored and apoptosis significantly reduced. These gene-corrected human β-thalassemia progenitor cells were then transplanted into immunodeficient mice, where they were capable of establishing normal erythropoiesis. In addition, Roselli et al. [39], using the GLOBE vector, reported successful correction of thalassemia major in human cells by achieving high transduction frequency, restoration of hemoglobin A synthesis, rescue from apoptosis, and correction of ineffective erythropoiesis.

Similarly, Persons and colleagues, using different γ-globin vectors and an *in vitro* model of human erythropoiesis, showed recently that both lentiviral-mediated γ-globin gene addition and genetic reactivation of endogenous γ-globin genes have the potential to provide therapeutic HbF levels to patients with β-globin deficiency [40, 41]. Finally, the group of Anagnou, to address the issues of low titer, variable expression, and gene silencing, affecting the gene therapy vectors for hemoglobinopathies, has successfully used the HPFH-2 enhancer in a series of oncoretroviral vectors [42]. Based on these data, the same group [43] generated a novel insulated SIN LV designated as GGHI,

containing the Aγ-globin gene with the −117 HPFH point mutation and the HPFH-2 enhancer. This vector managed to produce efficient amounts of HbF resulting in phenotypic correction in erythroid cultures of CD34$^+$ HSCs isolated from peripheral blood (PB) or bone marrow (BM) [43]. In this specific vector design, the incorporation of the full-length 1.2 kb cHS4 in the U3 region and the SIN configuration had no apparent effect on viral titer, since it reached 2×10^8 TU/mL. Efforts have also been made in the ability of γ-globin vectors pseudotyped with different envelope glycoproteins to transduce human HSCs [44] that resulted in the notion that the vesicular stomatitis virus glycoprotein (VSVG) is more effective in transducing engrafting cells than other gammaretroviral glycoproteins, supporting thus its use in clinical-grade vectors [44].

3.3. The First Clinical Trial. The first human clinical trial using a β-globin LV commenced in France on June 7, 2007, where Leboulch and colleagues selected two β-thalassemia patients who underwent transplantation of LV-transduced HSCs [45, 46]. The first one, a 28-year-old patient experienced a period of prolonged aplasia likely due to the technical handling of the cells, without relation to the gene therapy vector, and despite the absence of any adverse effects, required the administration of untransduced cells kept as a backup, in order to avoid putative infections. As a result, the lentiviral-modified cells did not reach a significant level in PB, neither did the therapeutic hemoglobin, leading to no conclusions regarding the specific patient.

The second patient in the study was a 18-year-old male suffering from HbE/β° thalassemia, a form of the disorder in which hemoglobin production is severely compromised. He was transfusion-dependent since the age of three, requiring 160 mL of packed erythrocytes/kg/year. He received 4×10^6 CD34$^+$ cells/kg [46]. The levels of genetically modified cells increased from 2% in the first few months to 11% at 33 months posttransplant. The above rise was also observed in the levels of normal β-globin protein, with 10%-20% of HSCs being genetically modified, leading thus to improved production and quality of red blood cells. Remarkably, a year after the treatment, the patient no longer required blood transfusions. He was last transfused on June 6, 2008, and four years after transplantation, despite being slightly anemic and undergoing repeated phlebotomies for the decrease of iron overload, the patient does not require blood transfusions, which means that this single case can be viewed as a clinical success.

However, despite the successful outcome of the second patient, Cavazzana-Calvo et al. reported that one hematopoietic clone, harboring a vector insertion into the HMGA2 gene showed clonal dominance [46]. At 20 months post transplantation, the specific clone accounted for 50% of the genetically modified HSCs; its contribution, however, to the circulating red blood cells' pool remains at around 3%. The potential clinical relevance of the alteration of *HMGA2* expression by the vector integration is highlighted by the fact that this gene may function as a potential oncogene in various types of cancer. However, it remains unclear whether the vector insertion into this gene has actually resulted in the relatively high contribution of this clone to hematopoiesis, since it is conceivable that the above observation may simply reflect the consequences of engraftment from a small number of transduced HSCs.

In summary, the above patient represents the proof of principle that gene therapy can be a successful therapeutic approach. This successful clinical trial demonstrated that large amounts of a therapeutic protein can be produced *in vivo* in a lineage-specific manner and validated somatic gene transfer using a lentiviral SIN vector for transducing long-term repopulating HSCs. It also demonstrated that somatic gene transfer *ex vivo* can provide transfusion independence for patients with severe forms of thalassemia.

4. Enhancing Lentiviral Gene Transfer Efficiency in Human and Nonhuman Primate HSCs

As discussed above, studies from many laboratories have shown that murine HSCs can be genetically modified using both β-globin and γ-globin LVs and engraft, resulting in amelioration of β-thalassemia. However, this is not the case for both human and nonhuman primate HSCs. These types are more resistant to lentiviral gene transfer and so far, no more than 10%–15% of genetically modified peripheral blood cells have been achieved. Recent work on rhesus macaques [47] using GFP LV demonstrated an average of 7% LV-bearing peripheral blood cells, a finding that comes into agreement with previous studies on pigtail macaques [48, 49]. Similarly, the 18-year-old patient in the first clinical trial discussed above showed an average of 15% genetically modified HSCs [46], supporting even further the notion that higher percentages of genetically modified HSCs in the case of human and nonhuman primates is not an easy task. The most effective strategies shown to increase HSC yield are described below and are also shown in Figure 1.

4.1. HSCs and Cellular Factors. The resistance of primitive human and nonhuman primate HSCs to infection by HIV-based vectors has drawn a lot of attention in the past decade. Recent work by the Naldini group [50] demonstrated that lentiviral gene transfer is limited by the proteasome through the regulation of one or more of the cellular postentry steps of the vector particle. Experiments involving the proteasome inhibitor MG132 during LV transduction demonstrated a 4-fold increase in gene transfer into human CD34$^+$ cells [50]. In this study, the transient and reversible inhibition of proteasome function did not lead to any obvious HSC defects. However, as proteasome is the major cellular proteolytic machinery, inhibition of its function may lead to cellular toxicity, through rapid accumulation of proteins in cells, resulting in impairment of their survival. Thus, the use of proteasome inhibitors as a means of increasing lentiviral HSC transduction needs further investigation, before it is considered safe to use in clinical trials. Lastly, in a different study by Zhang et al. [51], cell-cycle protein p21, which is found at high levels in HSCs, was identified as a unique molecular barrier to HIV infection, through its

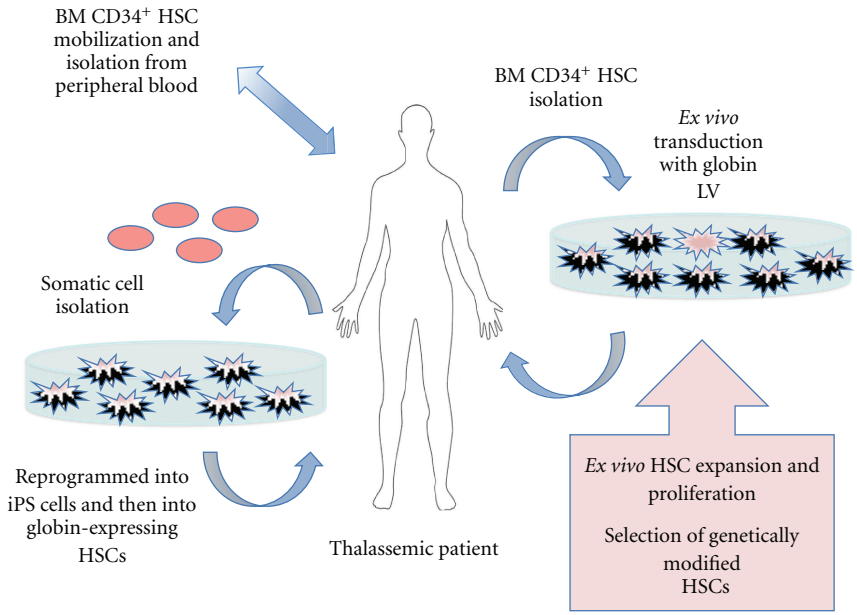

Figure 1: Schematic representation of HSC-based gene therapy for β-thalassemia. BM CD34$^+$ cells are removed from the patient (right panel), transduced *in vitro* with the therapeutic globin LV, carrying the preferred envelope glycoproteins, and returned to the patient intravenously. At this stage, and primarily in the case of human HSCs, *ex vivo* expansion and proliferation can be induced; alternatively, selection of genetically modified HSCs may take place. Both strategies can lead to increased yield of corrected HSCs, which will contribute to the HSC compartment, when returned to the patient. Alternative strategies for obtaining higher yield of CD34$^+$ cells are continuously emerging (left panel). These include mobilization of BM CD34$^+$ HSCs with mainly G-CSF and then isolation of these cells from peripheral blood. Moreover, iPS cell strategy suggests that somatic cells from the patient can be reprogrammed to iPS cells and after genetic manipulation, involving genetic correction for the mutations by homologous recombination, these cells can give rise to globin-producing HSCs, following directed hematopoietic differentiation.

interactions with the viral integrase, leading to inhibition of chromosomal integration [51].

4.2. HSC Cycling and Expansion Ex Vivo . Apart from cellular factors being important for lentiviral gene transfer into human HSCs, another crucial characteristic that impairs the efficiency of the gene transfer is the low levels of *ex vivo* HSC cycling. As mentioned earlier, human HSCs remain quiescent longer than murine ones, with the former entering G_1 every 30–40 weeks [28], compared to just 4–8 weeks [27] in the murine case. These findings, together with the fact that HSCs are more readily transduced when they enter cell cycle, point towards the need for *in vitro* proliferation and expansion of HSCs destined for lentiviral gene transfer. Major efforts have focused on research into novel culture conditions, which can lead to HSC expansion and proliferation. Promising candidates for these features are angiopoietin-like proteins, which are shown to induce *ex vivo* expansion of human cells that repopulate immunodeficient mice [52, 53]. Also, transcription factors such as HOXB4 [54] and NUP98-HOX fusion protein [55] have also been shown to induce HSC expansion. Furthermore, a purine derivative called stem regenin1 (SR1) was also shown to promote *ex vivo* expansion of CD34$^+$ cells, by acting as an aryl hydrocarbon receptor (AHR) antagonist, leading to a 50-fold increase in CD34$^+$ cells and a 17-fold increase in cells that retained their ability to engraft immunodeficient mice [56]. SR1 was shown to directly bind AHR and inhibit AHR signaling, which is implicated in hematopoiesis regulation. Recent work by Wang et al. [57] showed that inhibition of mitogen-activated protein kinase (MAPK) p38 could also result in *ex vivo* expansion of HSCs. This increase in HSC expansion is likely attributable to the p38 inhibitor-mediated inhibition of HSC apoptosis and senescence and to the upregulation of HOXB4 and CXCR4. Although the above study used murine lineage negative cells, the fact that resulted in HOXB4 upregulation, already shown to be essential for human HSC expansion, suggests that it is very likely to be effective in human HSCs case as well.

4.3. Selection of Genetically Modified HSCs. As mentioned above, increasing the levels of genetically modified human HSCs following lentiviral gene transfer is not an easy task. Various approaches have been employed in order to achieve this goal. Zhao et al. [35] incorporated the drug-resistance gene MGMT, which confers resistance to several potent hematopoietic toxins such as BCNU, in their γ-globin LV and managed to show significant amelioration of the disease in the murine model [35]. The globin-expressing HSCs were selected *in vivo* by cytotoxic drug administration and reached high levels, sufficient to ameliorate the disease. Moreover, this system allowed also an *in vitro* selection of the transduced cells, prior to transplantation, which led to enrichment of the γ-globin-expressing cells compartment.

However, despite the successful application of the above approach in murine models, there are major caveats for clinical applications, primarily due to the alkylating agents-related genotoxicity and the insertional mutagenesis potential, as discussed previously in this paper. Lastly, it is feasible in a system where cell numbers are not a limiting step, which usually is not the case for human HSCs.

4.4. Packaging Envelope Glycoproteins. Another promising strategy for increasing human HSC lentiviral transduction is by designing vectors that carry ligands on their envelope, which match the receptors on target cell. In a recent pioneering work by Verhoeyen et al. [58], it was shown that HSC gene transfer is significantly increased when lentiviral particles are engineered to display early acting cytokines on their surface. The rationale behind the above strategy is that these modified LVs would selectively and minimally stimulate HSCs within the $CD34^+$ cell population, leading to increased transduction and thus gene transfer. Finally, the use of the alternative envelope protein RD114 [59, 60] represents another good candidate for enhancing viral transduction and might be used as an alternative to the more cytotoxic VSVG protein.

5. Alternative Strategies for Obtaining More HSCs with Less Effort

Apart from manipulating the *ex vivo* culture conditions and the HSC state, researchers have demonstrated additional means of increasing the HSC yield, such as HSC mobilization and the generation of induced pluripotent stem (iPS) cells (Figure 1) as described below.

5.1. HSC Mobilization. The term HSC mobilization refers to the forced migration of HSCs from the BM to the bloodstream. Mobilized PB can then be used as an alternative source for $CD34^+$ cells for lentiviral transduction, gene transfer, and eventual transplantation for the treatment of β-thalassemia, yielding a 3 to 4-fold enrichment [61]. Lately, this strategy has drawn the attention of many research groups, with granulocyte colony-stimulating factor (G-CSF) being the major inducer of peripheral blood stem cell (PBSC) mobilization, alone or together with chemotherapy [61, 62], and has specifically gained ground in the field of gene therapy for β-thalassemia. Particularly, Li et al. [63] assessed the administration of G-CSF in mobilizing stem and progenitor cells in thalassemic major pediatric patients and compared the kinetics of $CD34^+$ cells and lymphocyte subsets with those of healthy PBSC donors. Results showed that $CD34^+$ cells in 20 thalassemic patients and 11 healthy donors were effectively mobilized by G-CSF in concentrations of 10–16 μg/day/kg of weight. No significant difference was observed in the levels of daily stem cell counts between the two groups of subjects, demonstrating that under close monitoring of $CD34^+$ cell levels in PB, the mobilization by G-CSF and collection of PBSCs in β-thalassemia patients are feasible [63]. Recently, Yannaki et al. [64] have mobilized murine HSCs using G-CSF and showed that thalassemic mice mobilized less efficiently than their control counterparts due to increased splenic trapping of HSCs and progenitor cells. The reduced mobilization efficiency was restored when splenectomy was performed in HBB^{th-3} mice, suggesting that for human gene therapy, HSC mobilization may require more than one cycle or alternative protocols, so as to yield sufficient HSCs for genetic manipulation and transplantation.

Regarding the French ongoing clinical trial, following authorization from the regulatory agency may already use either BM-derived or peripheral blood G-CSF mobilized $CD34^+$ cells [45].

5.2. Induced Pluripotent Stem (iPS) Cells. iPS cells are generated by reprogramming a differentiated somatic cell into a pluripotent embryonic stem cell (ESC) [65]. These iPS cells, which are identical to human ESCs, have the potential to give rise to every cell type in the human body. The genes and surface proteins expressed in these cells are almost identical to those expressed in ESCs and, therefore, can be eventually used to correct mutant cells or tissues by homologous recombination. Thus, somatic cells from a patient may be isolated and reprogrammed to iPS cells, employing genes such as Oct3/4, Sox2 with either Klf4 and c-Myc or Nanog and Lin28, and after genetic manipulation and differentiation to the desired cell lineage, they could be administered back to the patient, maintaining, at least theoretically, the same properties and characteristics [65]. There are different techniques for pluripotency induction, and these are extensively reviewed by Patel and Yang [65] and have been presented in detail in a recent special issue of this journal [66]. Briefly, they include somatic cell transfer, cell fusion, reprogramming through cell extracts, and direct reprogramming using mainly viral vectors, proteins, RNAs, microRNAs (miRNAs), and small molecules.

iPS cell technology seems quite promising in the context of human gene therapy for β-thalassemia, as it provides an alternative and patient-friendly strategy for obtaining higher number of HSCs for genetic manipulation and transplantation. The first reported gene correction in the context of hemoglobinopathies, using iPS cells, was performed in an SCD mouse model. Hanna et al. [67] harvested cells from the skin of the SCD mouse and reprogrammed them to ESCs, by retrovirally delivering Oct4, Sox2, Lif4, and c-Myc genes. After removing *c-Myc* to decrease or eliminate putative tumorigenesis in treated mice, ESCs were cultured to produce BM stem cell precusors, and following replacement of the defected gene with a normal one, via homologous recombination, they were transplanted back to SCD mice. The outcome was disease amelioration in these mice, with blood and kidney function returning to normal levels. Following the murine study, Ye et al. [68] were the first to show that this issue is also feasible in humans, as they managed to reprogram skin fibroblasts from a thalassemic patient with β° thalassemia into iPS cells, and demonstrated that the latter, following gene targeting, could differentiate into hemoglobin-producing HSCs.

Such gene targeting, however, needs to be highly controlled, as randomly integrated transgenes may result in

oncogenicity, and therefore, a general need for a strategy to introduce transgenes into "safe" regions in iPS cells is imperative. A good approach to overcome this obstacle came recently from the Sadelain group [69], where they managed to induce β-globin transgene expression in iPS cell clones, where the LV had integrated in "safe harbors" throughout the genome. iPS cells in this study originated from skin fibroblast or BM mesenchymal cells from patients suffering from β-thalassemia major. In order to identify safe harbors for transgene integration in the human genome, they employed bioinformatics and functional analysis. Retrieval of safe harbor sites (i.e., safe integration sites) met the following five criteria: (i) distance of at least 50 kb from the 5′ end of any gene, (ii) distance of at least 300 kb from any cancer-related gene, (iii) distance of at least 300 kb from any miRNA coding gene, (iv) location outside a transcription unit, and (v) location outside ultraconserved regions (UCRs) of the human genome. Measurement of β-globin expression in these progenitors reached high levels, suggesting that the above strategy, once improved, can be very efficient for β-thalassemia.

6. Summary

The efficient gene delivery of therapeutic transgenes using LVs has become a milestone in the field of gene therapy for hemoglobinopathies. Improved LV design enabled successful introduction of transgenes into both murine and human HSCs, leading to amelioration of β-thalassemia in murine models, and restored erythropoiesis *in vitro*. Extensive studies in the field led also to the success of the first clinical trial in France, in June 2007, where a 18-year-old HbE/β° thalassemic patient was treated with a β-globin vector and a year after BM transplantation managed to become transfusion-independent. As of today, he remains transfusion-independent, for over three years, in spite of repeated phlebotomies aimed to decrease iron overload. Despite the efficacy of LVs to introduce therapeutic transgenes into HSCs, there is also the risk for insertional mutagenesis, and therefore, an extensive work is currently focused on generating safe vectors for gene therapy. The above is usually achieved by incorporating elements, which make the vector tissue-specific and also shield the transgene from neighbouring effects, upon integration. Regardless the need for constantly improving vector design, a lot of attention has been drawn also towards strategies that result in higher numbers of genetically modified HSCs, which will in turn contribute to the HSC pool in the patient. The latter, together with extensive research towards alternative HSC sources, such as iPS cells, will undoubtedly set the ground for more successful clinical trials.

List of Abbreviations

HbF: Fetal hemoglobin
HSC: Hematopoietic stem cells
HLA: Human leukocyte antigen
GVHD: Graft versus host disease
MLV: Murine leukemia virus
LCR: Locus control region
HS: Hypersensitive site
LV: Lentiviral vector
HPFH: Hereditary persistence of fetal hemoglobin
cHS4: Chicken hypersensitive site 4
SCID: Severe combined immunodeficiency
SIN: Self-inactivating
LTR: Long terminal repeat
SCD: Sickle cell disease
MGMT: Methyl guanine methyl transferase
BCNU: Bis-chloroethyl nitrosourea
PB: Peripheral blood
BM: Bone marrow
VSVG: Vesicular stomatitis virus glycoprotein
SR1: Stem-regenin 1
AHR: Aryl hydrocarbon receptor
MAPK: Mitogen-activated protein kinase
iPS: Induced pluripotent stem
G-CSF: Granulocyte colony-stimulating factor
PBSC: Peripheral blood stem cell
ESC: Embryonic stem cell
miRNA: microRNA
UCRs: Ultraconserved regions.

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Review Article

The Haematopoietic Stem Cell Niche: New Insights into the Mechanisms Regulating Haematopoietic Stem Cell Behaviour

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The concept of the haematopoietic stem cell (HSC) niche was formulated by Schofield in the 1970s, as a region within the bone marrow containing functional cell types that can maintain HSC potency throughout life. Since then, ongoing research has identified numerous cell types and a plethora of signals that not only maintain HSCs, but also dictate their behaviour with respect to homeostatic requirements and exogenous stresses. It has been proposed that there are endosteal and vascular niches within the bone marrow, which are thought to regulate different HSC populations. However, recent data depicts a more complicated picture, with functional crosstalk between cells in these two regions. In this review, recent research into the endosteal/vascular cell types and signals regulating HSC behaviour are considered, together with the possibility of a single subcompartmentalised niche.

1. Introduction

Haematopoietic stem cells (HSCs) are a heterogeneous group of multipotent stem cells that have the ability to self-renew and differentiate into all the functional blood cell types of the body. During vertebrate development, the first source of haematopoiesis is extraembryonic haemangioblasts. These cells predominantly generate erythrocytes and endothelial cells to supply the emerging yolk sac vasculature and have different properties to HSCs of the adult which arise later in development [1]. For this review, HSCs will be considered as cells able to confer long-term myeloid and lymphoid multilineage haematopoiesis. Transplantation studies in the mouse have demonstrated that the first HSCs that satisfy this definition emerge during the development of the embryonic blood vessels from a subset of cells called the haemogenic endothelium [2–4]. The HSCs then colonise the fetal liver, where there is HSC expansion and the appearance of characteristic cell surface markers of adult HSCs [5, 6]. Late in fetal development, HSCs home and engraft in the bone marrow, where they reside throughout adult life [7]. The bone marrow is the main site of adult haematopoiesis, although during times of stress haematopoiesis may also occur in the spleen and liver.

A multitude of studies have identified numerous intrinsic pathways that regulate HSC self-renewal and differentiation programmes [8]. However, in the 1970s, it was noted that whilst HSCs in the bone marrow drive haematopoiesis throughout the life of organisms, when they are removed from the bone marrow, they lose the ability to self renew indicating the equal dependence of HSCs on extrinsic signals [9]. This led Schofield to propose the "niche" hypothesis, which states that HSCs require the support of other cell types in the bone marrow to maintain HSC potency [9]. It is now clear that other than simply maintaining HSCs, the niche plays important roles in regulating the behaviour of HSCs with respect to homeostasis and responses to exogenous stresses. For example, under normal conditions most HSCs in the bone marrow are dormant or slowly cycling, which prevents stem cell exhaustion and maintains haematopoiesis [10, 11]. However, under periods of haematopoietic stress such as blood loss, HSCs and progenitors are activated to proliferate and differentiate to replace the lost cells [12].

Schofield's recognition of the HSC niche has fostered ongoing research trying to identify and understand the cellular and molecular components that make up the niche. At present, there is broad discussion of the possible presence of two bone marrow niches able to maintain and regulate

HSCs, which are the endosteal and vascular niches. However, whether these environments truly represent two distinct HSC niches still remains under debate.

2. The Endosteal Niche

The endosteum is the interface between bone and bone marrow. This region is lined with a heterogeneous group of osteoblastic cells at various stages of differentiation, only a fraction of which are fully mature osteoblasts able to synthesise bone. Osteoclasts, which are bone-absorbing cells, also line the endosteum and dynamically balance bone formation with the osteoblasts. Several lines of investigation have pointed to the importance of osteoblastic cells in maintaining and supporting HSCs in the niche. The coculture of HSCs and osteoblast cell lines *in vitro* results in an expansion of HSC numbers indicating enhanced *ex vivo* self-renewal [13]. Similarly, increasing the number of osteoblastic cells *in vivo* correlates with an increase in the number of HSCs [14, 15]. In addition, the cotransplantation of osteoblasts with HSCs in mice significantly enhances engraftment [16].

Osteoblastic cells synthesise a number of cytokines that appear to contribute to the maintenance and regulation of HSCs by the endosteal niche. These include thrombopoietin (THPO) and angiopoietin (Ang-1), which bind to cell surface receptors MPL and Tie2, respectively, which are expressed on HSCs. These cytokines are thought to be important as THPO and Ang-1 knockout mice have decreased numbers or defects in bone marrow HSCs [17–19]. In agreement with these data, stimulation of the MPL receptor with THPO enhances the quiescence of long term (LT)-HSCs, whilst inhibition of the receptor decreases their quiescence [20]. The THPO/MPL pathway is thought to promote quiescence by activating cyclin-dependent kinase inhibitors (CDKIs) such as $p57^{kip2}$ [21]. The interaction of Ang-1 with its receptor Tie2 has also been shown to enhance quiescence and maintain long term repopulating ability of HSCs, whilst protecting against apoptosis by activating the PI3K pathway [22].

Besides secreting important cytokines, osteoblastic cells also express a range of membrane-bound ligands and adhesion receptors which contribute to the maintenance of HSCs within the endosteal niche. Osteoblastic cells express Jagged, a ligand for Notch receptors expressed on HSCs. Activation of Notch receptors on HSCs has been shown to inhibit differentiation and enhance their self renewal capacity *in vitro* [23, 24]. Conversely, *in vivo* Notch deletion studies failed to show any significant effects of Notch signalling on HSC differentiation and self renewal, making the role of Notch in the niche controversial [25, 26]. However, it has been recently demonstrated that Notch2 knockout mice have a reduced ability to recover from bone marrow injury induced by 5-fluorouracil (5-FU) treatment as well as a decrease in repopulating HSCs in the bone marrow [27]. This suggests that Notch signalling is important *in vivo* for controlling HSC self renewal and differentiation during haematopoietic stress conditions and is masked by other factors or is redundant during homeostasis.

N-Cadherin is a calcium-dependent homophilic adhesion molecule [28], which is expressed on both immature and mature osteoblastic cell populations. Like the Jagged/Notch pathway, the role of N-Cadherin in the endosteal niche remains controversial [29, 30]. In some mouse studies neither N-Cadherin mRNA nor protein could be detected in HSCs [31], whereas other studies report its expression in both ST-HSCs and LT-HSCs [32, 33]. The N-Cadherin knock-out mouse has no apparent defects in HSC activity or haematopoiesis, indicating that HSCs do not depend on N-Cadherin for maintenance *in vivo* [34]. However, HSCs that express a dominant negative mutant N-Cadherin have a reduced ability to anchor to the endosteum and consequently a diminished capacity to repopulate bone marrow following transplantation [32]. Therefore, the role of N-Cadherin in the endosteal niche remains uncertain, but it seems to have a role in enhancing endosteal-HSC interactions following bone marrow transplantation.

Chemokines and their receptors control HSC behaviour by regulating migration, homing, and release of HSCs within the bone marrow. The best-understood chemokine is stromal-derived factor-1 (SDF-1) also called chemokine C-X-C motif ligand 12 (CXCL12). The SDF-1 receptor is the C-X-C chemokine receptor type 4 (CXCR4) and is expressed on HSCs and progenitors. The importance of SDF-1/CXCR4 signalling has been demonstrated by both $SDF\text{-}1^{-/-}$ and $CXCR4^{-/-}$ mice, which have severe defects in myelopoiesis, including a decreased number of myeloid progenitors within the bone marrow [35, 36]. SDF-1 is secreted by a variety of cells within the bone marrow including osteoblasts, endothelial cells, and scattered stromal cells [37], but it has recently been reported that osteoblasts are high secretors of SDF-1 [38]. In agreement with these data the authors found that the HSC-mobilising cytokine G-CSF exerts its long term effects partly by decreasing osteoblast activity resulting in reduced endosteal SDF-1 levels [38].

Although osteoblastic cells are the most studied in terms of HSC maintenance within the endosteal niche, many other types of cells are present. Increasing evidence suggests that bone-degrading osteoclasts play an important role in regulating HSCs within the bone marrow. The degradation of bone by osteoclasts releases factors embedded in the bone matrix. These include transforming growth factor beta 1 (TGF beta 1), bone morphogenic proteins (BMPs), and calcium ions [39–41]. TGF beta 1, BMP-2, and BMP-7 have been demonstrated to enhance the quiescence and maintenance of HSCs *in vitro* [39, 40], whilst evidence suggests that calcium ions enhance the retention of HSCs to the endosteal surface [42]. In support of these data, mice treated with bisphosphonates, which inhibit osteoclast reabsorbing activity, have diminished numbers of LT-HSCs within the bone marrow coupled with reduced HSC quiescence and enhanced differentiation [43].

Accumulating evidence now indicates that tissue-specific macrophages play important roles in supporting the development of a variety of tissues [44]. Macrophages termed osteomacs are found at the endosteum in close proximity to osteoblasts and osteoclasts [45]. Osteomacs regulate osteoblast function and are required for optimal mineralisation

in vitro and *in vivo* [45]. Depletion of osteomacs *in vivo* also results in decreased numbers of osteoblasts and a reduction in osteoblast-secreted cytokines such as Ang-1, KIT ligand, and CXCL12 [46]. These changes are accompanied by the mobilisation of HSCs from the bone marrow, indicating a central role for osteomacs in maintaining the structure and function of the endosteal niche [46]. An important unanswered question is whether osteomacs interact directly with HSCs within the endosteal niche.

3. Sympathetic Innervation of the HSC Niche

A complex organisation of neuronal fibres are found within the bone marrow, and sympathetic nervous system (SNS) activity has been reported to control bone formation [47, 48]. However, more recently, a potential role of SNS activity in the endosteal niche has been identified. Frenette and colleagues demonstrated that sulphogalactoceramide (sulfatide), a sulpholipid synthesised by ceramide galactosytransferase (CGT) in neuronal Schwann cells, could mobilise HSCs from the bone marrow [49]. To investigate the effects of sulfatide depletion on the endosteal niche and HSC mobilisation, a CGT knockout mouse was created. As expected, $CGT^{-/-}$ mice showed hematopoietic defects and decreased HSC mobilisation in response to G-CSF administration, but unexpectedly this was not directly due to impaired sulfatide release [50, 51]. Rather, the decreased ability of the $CGT^{-/-}$ mice to mobilise HSCs in response to G-CSF was found to be due to impairment of the neuronal signals that regulate osteoblast function [51]. The authors went on to demonstrate that sympathetic nervous system activity suppresses osteoblast function resulting in enhanced HSC mobilisation from the bone marrow [51].

4. The Vascular Niche

Several lines of evidence suggest that vascular environments are involved in the maintenance of HSCs as well as endosteal environments. During fetal development, fetal functioning HSCs first arise from the haemogenic endothelium of the vasculature, indicating that the two tissues are developmentally closely related [2–4]. Fetal HSCs then reside in the liver and the spleen, where early haematopoiesis takes place in the absence of osteoblasts or the endosteal niche [5, 6]. Even in adults, HSCs are present in the liver and the spleen throughout life and are capable of extramedullary haematopoiesis [52, 53]. These observations indicate that cells of vascular environments can both support HSCs and regulate their self renewal and differentiation.

The bone marrow is heavily vascularised; the medullary artery feeds in to arterioles, capillaries, and then the sinusoids. Sinusoids are specialised blood vessels, which have thin walls with a fenestrated arrangement of endothelial cells to allow the passage of haematopoietic cells [54]. The sinusoids form an extensive network throughout the bone marrow, and therefore the endothelial cells which make up the sinusoids are important when considering HSC bone marrow niche. This idea has been supported by mouse endothelial cell and HSC co-culture experiments, which demonstrate that some endothelial cell populations can support the expansion of HCSs *in vitro* [55, 56]. As well as this, some endothelial cell populations can maintain severe combined immune-deficient (SCID) mouse repopulating capacity of HSCs during co-culture, [57, 58].

Important recent work indicates that endothelial cells are important for haematopoiesis *in vivo*. Yao and colleagues sought to investigate whether the reduced numbers of HSCs and defects in haematopoiesis of *gp130* knockout mice [59] were directly due to effects on HSCs or due to effects on niche endothelial cells [60]. Mice expressing Cre recombinase under control of the Tie2 promoter, which is active in HSCs and endothelial cells, were crossed with mice containing a *gp130* gene with *loxP* sites. The resulting mice had a conditional deletion of *gp130* in both HSCs and endothelial cells. These mice appeared normal at birth but had hypocellular bone marrow, developed expanded sinusoidal spaces, and died prematurely at around 1 year. Transplantation of bone marrow from *gp130* deficient mice into normal irradiated mice restored normal haematopoiesis. However, transplantation of bone marrow from normal mice into *gp130*-deficient irradiated mice did not restore normal haematopoiesis. These complimentary transplantation experiments indicate that endothelial cells are essential components of the HSC niche and that endothelial gp130 signalling is crucial for maintaining haematopoiesis [60].

Other evidence indicates that many HSCs are located near the sinusoids in the bone marrow. It has been suggested that the rapid mobilisation of HSCs within minutes of administering G-CSF argues that a large proportion of HSCs must be very close to blood vessels [61, 62]. More recently, Kiel and colleagues identified a group of markers called signalling lymphocyte activation molecule (SLAM) family markers which include CD150, CD244, and CD48, which are differentially expressed between HSC and progenitor subpopulations in mice [63]. The most immature HSCs could be precisely identified as $CD150^{+ve}$, $CD244^{-ve}$, and $CD48^{-ve}$, allowing reliable imaging of HSCs within the bone marrow for the first time [31, 63]. Strikingly, they found that around 60% of HSCs defined in this way were in contact with the endothelium of sinusoids, whereas, only around 20% were found at the endosteal surface. Overall only 10% of the total bone marrow nucleated cells were found at the sinusoid endothelium, indicating a 6-fold selective enrichment of HSCs in this area. Whilst these results point to a distinct vascular niche for HSCs within the bone marrow, virtually all HSCs (92–95%) were also found within 5 cell diameters from the sinusoid endothelium. Thus, it remains possible that HSCs at the endosteum are also effected by vascular cells [31].

Reticular cells are a group of cells that are located around the sinusoid endothelium and are important in the homing and localisation of HSCs within the bone marrow. These cells have recently been shown to be high secretors of SDF-1 (CXCL12), and as a result have been named CXCL12 abundant reticular (CAR) cells [64]. By using immunohistochemical analysis of the bone marrow of CXCL12-GFP knock in mice, Sugiyama and colleagues have shown that nearly all

(97%) of HSCs within the bone marrow were localized to CAR cells. Interestingly, 100% of the HSCs located at the endosteum were also located to CAR cells. These data suggest that CAR cells are crucial for the homing of HSCs in both the vascular and endosteal niches and together with the data of Kiel and colleagues, indicate that functionally there may be just one class of niche in the bone marrow.

Further data from Omatsu et al. (2010) suggests that CAR cells are important in supporting the proliferation of HSCs/progenitors as selective ablation of CAR cells resulted in a reduction in the number of cycling progenitors, and enhanced the quiescence of HSCs in the bone marrow [65]. As well as this, CAR cells were found to be high secretors of the proproliferative cytokine stem cell factor (SCF), and that CAR cell ablation resulted in a decreased in SCF levels [65]. The authors went on to show that CAR cells express adipogenic and osteogenic genes and can differentiate into adipocytes and osteoblasts *in vitro.* These data suggest that CAR cells are a form of adipo-osteogenic progenitor derived from mesenchymal stem cells [65]. Mesenchymal stem cell (MSC) was a term proposed by Caplan in 1991 [66] to describe the multipotent bone marrow stromal cell populations, which Owen and Friedenstein demonstrated in the 1960s and 1970s to be capable of undergoing osteoblastic differentiation to form bone [67]. Whether MSCs truly represent a stem cell capable of self renewal and multipotency on a single cell basis is not proven *in vivo* [68]. However, their participation in supporting the vascular HSC niche is better established, specifically $CD146^{+ve}$ cells that are found in the subendothelial layers of sinusoids [69]. Bone marrow-derived $CD146^{+ve}$ cells are capable of differentiating *in vitro* along multiple lineages to form osteoblasts, chondrocytes, and adipocytes, which is considered characteristic of MSCs [70], but only $CD146^{+ve}$ cells (and not other MSC-like populations) have been demonstrated conclusively and clonally to recapitulate the haematopoietic microenvironment and synthesise bone when transplanted heterotopically [69]. HSCs are also better supported *in vitro* by MSCs derived from bone marrow than MSCs derived from other tissues, for example from adipose tissue [71]. $CD146^{+ve}$ bone marrow cells express a variety of factors that support HSCs, including Jagged-1, N cadherin, stem cell factor (SCF), and SDF-1 [69].

Megakaryocytes have been implicated to be involved in regulating haematopoiesis, since mice with decreased mature megakaryocytes and platelets have other haematopoietic defects such as anaemia and myelofibrosis [72, 73]. However, the contribution of megakaryocytes in regulating HSCs by direct or indirect mechanisms is poorly understood. Megakaryocytes are located close to sinusoidal endothelial cells in the perivascular environment [74, 75], and data also suggests that they communicate with the endosteal niche. Co-culture of megakaryocytes and osteoblasts *in vitro* enhances osteoblast proliferation up to 6-fold, a mechanism requiring direct cell contact [76–78]. These data are supported by *in vivo* studies, using NF-E2 knockout mice, which have an accumulation of megakaryocytes and an increase in bone mass and osteoblasts [76, 79]. Kacena and colleagues [80] transplanted spleen cells from NF-$E2^{-/-}$ mice into irradiated mice and after 4 weeks found the same megakaryocytic and osteoblastic phenotype as the donor NF-$E2^{-/-}$ mice. These data suggest that the increase in bone mass and osteoblasts seen in the NF-$E2^{-/-}$ mice is an indirect feature, caused by an increase in megakaryocytes [80].

Recent data suggests that megakaryocytes and platelets directly regulate the behaviour of HSCs. Like HSCs, megakaryocytes and their precursors express Mpl, the receptor for TPO, and as the name thrombopoietin suggests, signalling via this pathway is necessary for megakaryocytic differentiation and platelet formation [81, 82]. Serum TPO levels inversely correlate with megakaryocyte mass suggesting that TPO levels are regulated by the amount of cells which can take up and remove TPO from the circulation via Mpl [83, 84]. This provokes an interesting question whether megakaryocytes regulate the levels of available TPO and consequently regulate HSC quiescence. De Graaf and colleagues [85] investigated this possibility by creating *myb* and *p300* mutations in mice that lead to an increase in megakaryocytes and platelets. As expected, these mutants had decreased serum TPO levels, and a striking change in TPO responsive genes in bone marrow HSCs. Furthermore, the authors found that this resulted in increased HSC cycling consistent with the decreased TPO levels. The authors conclude that the TPO regulates megakaryocytic differentiation, which in turn regulates circulating TPO levels and quiescence of HSCs [85]. This feedback mechanism could be important to maintain homeostasis during injury. For example, increased bleeding would cause a decrease in platelets resulting in an increase in circulating TPO. The increased TPO could then not only drive differentiation of megakaryocytic precursors to replace the lost platelets, but also enhance the quiescence of LT-HSCs to prevent stem cell exhaustion and protect future haematopoiesis. These data provide exciting new evidence that HSCs are regulated by their mature progeny, a process which has been poorly studied but has to be important to guide haematopoiesis with respect to the levels of mature cell populations.

5. Evidence for an Adult Haemangioblast

The relationship and proximity of HSCs to sinusoidal endothelial cells provoke the question whether adults have haemangioblasts and haemogenic endothelial cells which can renew HSC within adult bone marrow. In humans, CD34 is a marker expressed on both HSCs and endothelial progenitor cell populations. HSCs and endothelial progenitors are further enriched within the $CD34^{+ve}$ and vascular endothelial growth factor receptor 2 (KDR^{+ve}) population, whilst HSCs only are found within the $CD34^{+ve}/KDR^{-ve}$ fraction [86]. Pelosi and colleagues used limiting dilution assays to show that 5% of cells within the $CD34^{+ve}/KDR^{+ve}$ fraction could give rise to both haematopoietic and endothelial lineages, indicative of haemangioblast activity [86]. Coupled with this, it has been suggested that some HSCs expressing CD34 and leukocyte marker CD45 have haemangioblast activity *in vivo,* as transplantation of these cells into mice results in donor-derived vascularisation [87]. However, the transplantation of a small contaminating population of endothelial progenitors

cannot be fully ruled out in this study. These studies are supported by investigations in chronic myeloid leukaemia (CML), a clonal malignancy arising in the HSC compartment, characterised by expression of the BCR:ABL fusion gene. Endothelial cells from CML patients have been found that express the BCR:ABL fusion gene, suggesting that endothelial cells and HSCs have a common ancestor [88]. However, the location of the adult haemangioblasts within the bone marrow niche is unknown, as well as its contribution to renewing the HSC pool. Also, unlike in the embryo, an adult haemogenic endothelium with the potential to give rise to HSCs has not yet been discovered.

6. The Role of Small Bioactive Signalling Molecules in the HSC Niche

The majority of research into signalling mechanisms within the bone marrow microenvironment has focussed on protein molecules such as cytokines, chemokines, adhesion molecules, and their prospective receptors. However, small non-protein bioactive molecules such as eicosanoids are emerging as essential signalling mediators regulating HSC behaviour within the niche. Eicosanoids are a group of 20 carbon fatty acids that are derived from arachidonc acid and include; prostacyclins, prostaglandins, thromboxanes, endocannabinoids, and leukotrienes. Eicosanoids are synthesised by a wide range of cells and have diverse effects [89].

Prostaglandins are the best understood eicosanoids, and of which prostaglandin E2 (PGE2) is the most studied in terms of modulating HSC behaviour. PGE2 synthesis is a two-step process; COX-1 and COX-2 enzymes convert arachidonic acid into prostaglandin H2 (PGH2), which is further converted to PGE2 by prostaglandin synthases. PGE2 is secreted by efflux transporters called multi-drug resistance proteins (MDRP), where it can act as an autocrine or paracrine signal by binding to G-protein-coupled E prostanoid (EP) receptors. Four different EP receptors for PGE2 have been identified (EP1 to EP4) [90], and all four of these have been shown to be expressed on both murine and human HSCs [91]. Evidence suggests that PGE2 is secreted by a variety of cell types which are found in the bone marrow including; osteoblasts [92–94], monocytes/macrophages [95, 96], and sinusoidal endothelial cells [97, 98]. Therefore, PGE2 is likely to be found in both endosteal and vascular niches.

In seminal studies from North et al. [99], a chemical genetic screen was used to identify important factors regulating HSC formation during zebrafish embryogenesis. It was found that chemicals that increased PGE2 synthesis, such as linoleic acid, also increased HSC numbers, whilst COX inhibitors, such as celecoxib, decreased HSC numbers. In agreement with the screen, exogenous use of a stable derivative of PGE2, 16,16-dimethyl-PGE2 (dmPGE2), increased HSC formation during embryogenesis and enhanced the haematopoietic recovery of irradiated adult zebrafish. In murine models, treatment of HSCs with dmPGE2 *in vitro* also enhanced HSC repopulating capacity following transplantation into irradiated mice [99]. Follow-up experiments demonstrated that PGE2 signalling enhanced HSC wnt signalling associated with increased HSC proliferation [100]. Others found that PGE2 increased the expression of survivin in HSCs, an antiapoptotic protein which enhances HSC survival as well as progression through cell cycle [91]. In addition to this, PGE2 has been found to increase CXCR4 expression on HSCs, thereby enhancing migration to SDF-1 and homing to the bone marrow [91, 101].

Although these studies provide some of the first enticing evidence of the role of PGE2 in the HSC niche, surprisingly little is known about the involvement of other eicosanoids. Data suggests that prostaglandin D2 (PGD2), another derivative of PGH2, is the major prostaglandin in the bone marrow [102], but its effects on HSCs remain largely uninvestigated. Leukotriene synthesis has also been shown in the bone marrow [103], and there is evidence that leukotrienes can stimulate myeloid progenitor proliferation *in vitro* [104]. Therefore, further research is needed to shed light on the role of the array of small bioactive signalling molecules within the HSC niche.

7. The Role of Hypoxia in the HSC Niche

As well as the cells and other factors that make up bone marrow microenvironments, other physiological characteristics of the niche need to be considered, of which hypoxia is emerging as vital in regulating HSC behaviour. The oxygen levels of the bone marrow vary considerably, as areas close to sinusoids are highly perfused, whereas areas further away around the endosteum are poorly perfused and hypoxic. *In vitro* studies demonstrated that haematopoiesis is enhanced by low oxygen levels of around 1%, giving suggestive evidence that this may be the case *in vivo* [105, 106]. In agreement with these data, mathematical modelling approaches pointed to hypoxic environments for the location of HSCs in the bone marrow [107].

Until recently, the study of hypoxia in the bone marrow was hampered by its complex organisation and inaccessibility. Parmar and colleagues 2007 [108], used Hoechst dye (a DNA-intercalating dye that is readily taken up by live cells) to measure the perfusion of cell populations within the bone marrow. Mice were intravenously infused with a pulse of Hoechst, and then uptake into cells in the bone marrow was measured using flow cytometry. They found a striking broad continuum in the amount of Hoechst taken up with variability in the range of 1000-fold. Furthermore, cells that had taken up the least amount of Hoechst exhibited HSC-like properties in long-term culture initiating assays and highest engraftment frequency in irradiated mice [108].

Winkler et al. [109] built on the above results by analysing not only Hoechst perfusion but also the cell surface markers expressed on HSCs and on more mature progenitors. As expected, they found that the most immature HSCs were Hoechst negative and only these cells could reconstitute haematopoietic system in serial transfer experiments [109]. As only dormant HSCs are able to serially engraft [110], it suggests that HSCs away from sinusoid perfusion contain the most potent and dormant HSCs. The authors also found a phenotypically identical (by surface marker expression)

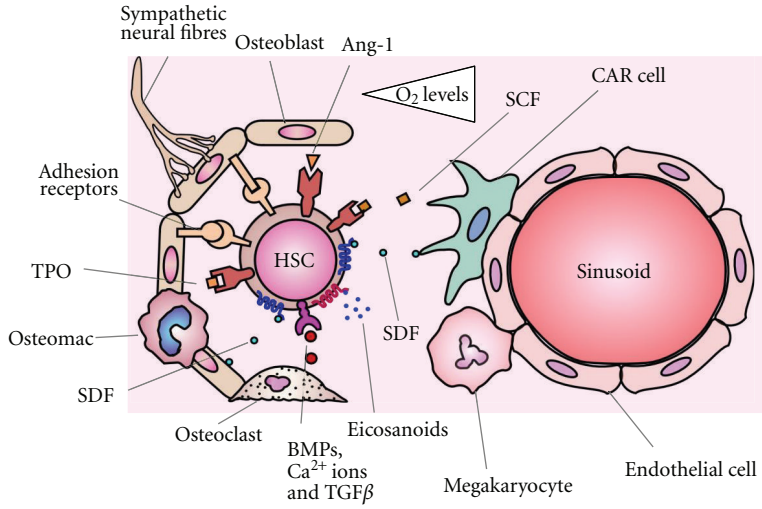

Figure 1: Endosteal and vascular niches are subcompartments of a single niche. HSCs located at the endosteum are more quiescent and have a greater self-renewal capacity due to a variety of cytokines, adhesion molecules, and hypoxia. HSCs located close to the sinusoid endothelium have reduced self-renewal capacity and are cycling more rapidly, due to higher oxygen levels and SCF. However, nearly all HSCs reside adjacent to CAR cells and in close proximity to the sinusoid endothelium. The cells of the vascular niche communicate with cells of the endosteal niche, and the subtle balance of factors from these subcompartments governs the behaviour of the HSCs.

population of Hoechst medium cells, which had ability to reconstitute haematopoiesis only in primary recipients [109].

The association of the more potent HSCs with a hypoxic environment may be linked to protection from reactive oxygen species (ROS). When oxygen is metabolised, ROS are released as a byproduct, causing DNA damage and protein miss folding, which have been associated with ageing [111]. Elevating ROS in the haematopoietic system, by creation of ataxia-telangiectasia-mutated-(ATM-) knockout mice, results in progressive bone marrow failure [112]. In agreement with these data, HSCs with low ROS levels have been shown to maintain potency throughout serial transplantation experiments, when compared to HSCs with high ROS levels [113]. Other than causing cellular damage, data suggests that ROS activates the P38 MAPK pathway, which decreases adhesion molecule expression and enhances cell cycle progression [112, 114, 115]. Collectively, these data indicate that hypoxia in the endosteal niche protects HSCs against ROS-induced damage, whilst maintaining niche-HSC interactions and quiescence. This is necessary to maintain HSC self renewal capacity and protect against stem cell exhaustion, whilst the higher oxygen levels in the vascular niche may facilitate progenitor proliferation.

8. Concluding Remarks

More than 30 years of research has uncovered an astonishingly complex array of signals within the bone marrow niche, the subtle balance of which dictates the behaviour of HSCs. Distinct endosteal and vascular environments have been identified, where generally speaking the endosteal niche supports quiescent HSCs, whereas the vascular niche facilitates the proliferation of more mature progenitors. However, even HSCs at the endosteum reside within 5 cell diameters of sinusoids [31], and virtually all endosteal HSCs are adjacent to CAR cells associated with the sinusoid endothelium [64]. Coupled with this, the endosteal niche cannot maintain adequate haematopoiesis in mice where the vascular niche has been compromised [60], supporting the accumulating data describing crosstalk and overlap between cells in these two compartments. Consequently, these data reveal that the endosteal/vascular two niches hypothesis is far too basic and possibly should be thought of as subcompartments of a single niche (Figure 1). The advent of novel technologies that now permit the study of the niche in three dimensions and in real time will no doubt accelerate the solution of this problem [116, 117]. Even so, there is still much to learn about the HSC niche with new avenues including, the role of maturing cells in guiding haematopoiesis with respect to biological need, the effect of small bioactive molecules on HSCs, and functions of the adult haemangioblasts in replenishing the HSC pool.

The knowledge gained from studying the HSC niche has important implications for health and disease. The use of cord blood HSCs to treat disorders in adults has been hampered by their relatively small numbers and shortcomings in expansion techniques of current *ex vivo* culture systems. Further understanding of the factors involved in regulating self renewal and proliferation of HSCs in the niche, would allow these to be mimicked *in vitro,* thereby enhancing expansion of HCS prior to transplantation. On the other side of the coin, the quiescence of leukaemic stem cells (LSCs) renders them largely unresponsive to current chemotherapeutic agents, leading to disease relapse. Understanding the myriad factors regulating HSC quiescence and how these signals are perturbed in leukaemia are therefore important to enable the future development of therapies to eradicate LSCs.

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