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Hematopoietic Stem Cells, Their Niche, and the Concept of Co-Culture Systems: A Critical Review

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Abstract

Hematopoietic stem cells (HSCs) have the ability to self-renew and give rise to all lineages of blood cells. HSCs reside in niches that are local tissue microenvironments that maintain and regulate them. Although much progress has been made in elucidating the location and cellular components of the HSC niche, however it still remains incompletely defined. Transplantation using HSCs has been applied for the treatment of several diseases but with limited success. Furthermore, although human HSC transplantation has been widely used to rescue the patients after cytoablative therapies, quantitative *in vivo* human assays for hematopoietic cells have been considered to be neither ethical nor practical. Since HSCs persist in small quantities in the body, understanding the mechanism that govern their fate is essential for the advancement of HSC expansion and transplantation in the future. Since bone marrow is the primary site of HSC maintenance and hematopoiesis, defining the niche components that work in concert to regulate hematopoiesis is crucial to improve regeneration following injury or following HSC transplantation and to also understand how disordered niche function could contribute to disease. In recent years, there has been a growing realization of the limitations in identifying the primitive HSCs by its phenotype alone and therefore the concept of co-culture systems (functional *in vitro* assays) has become increasingly important to demonstrate the presence of primitive hematopoietic cells by estimating their biological functions. This system has provided a basis for the development of powerful assay procedures for expanding, quantitating and distinguishing cells at discrete stages of early hematopoietic cell differentiation.

***Keywords*:** Hematopoietic stem cells, Mesenchymal stem cells, Hematopoietic stem cell niche, Co-culture

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Introduction

In the past decade, stem cell research has received immense attention not only because they play a central role in normal developmental processes as

well as in pathological processes like cancer, but also due to the possibility that extensive medical application in the field of regeneration of damaged and diseased organs may revolutionize the future of medicine [1]. Unlike embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), adult stem cells have been successfully used in research and therapy [2]. However, the greatest challenges in the field of adult stem cell therapy is to firstly reduce the risk associated with the stem cell transplantation [3] and secondly to increase the number of HSCs for large scale clinical transplantation [4]. It is therefore imperative to decipher the mechanisms that regulate the stem cell fate in order to achieve a better control over their behavior after their therapeutic application leading to a more successful and predictive outcome. The focus of this review is to give a thorough understanding about the hematopoietic stem cells (HSCs), the mesenchymal stem cells (MSCs), the HSC niches and to explore the concept and challenges of co-culture systems to facilitate *ex vivo* expansion of HSCs.

Hematopoietic Stem Cells

HSCs are defined as primitive cells that are capable of both unlimited self-renewal and differentiation into all types of hematopoietic cells belonging to both myeloid and lymphoid lineages in a regulated manner as per the physiological demand. While on one hand it is necessary to ensure the maintenance of a persistent pool of regenerating cells, on the other hand it is equally crucial to tightly regulate the HSC divisions. Unchecked growth of immature cells has been thought to represent a paradigm for malignant outgrowth, at least for AML (acute myeloid leukemia) and CML (chronic myeloid leukemia) [5].

Historically, the HSCs are the most rigorously characterized adult stem cells, and the hematopoietic system has served as a principal model structure of stem-cell biology for several decades [6]. HSCs are an ideal target for autologous and allogeneic transplantation, purging strategies, gene transfer and – with reports on the plasticity of HSCs, though still equivocal – for tissue regenerative therapies. The understanding of mechanisms underlying their extensive proliferative capacity, their multi-lineage differentiation and self-renewal, and their further characterization is being keenly pursued in the fields of hematological research [7].

Bone Marrow (BM), mobilized peripheral blood (MPBL), umbilical cord blood (UCB) and fetal liver (FL) comprise different sources of HSCs. Consistent with their ontological status, these various stem cell sources differ in regard to their collection methods, their re-constitutive and immunogenic characteristics (which are based on the proportion of early pluripotent and self-renewing stem cells to lineage-committed late progenitor cells) and on the number and characteristics of accompanying “accessory cells” [8, 10]. The use of FL as a potential source of HSCs has been under the constant scrutiny of ethical committees, as in several countries medical termination of pregnancy is either considered illegal or is forbidden from a religious or a cultural point of view. As a result, its use in a research set up as a promising source of HSCs, has met with constant skepticism, making it difficult to obtain HSCs from this tissue [9]. In the related setting, it has been observed that the use of MPBL stem cells results in a faster engraftment, thereby, conferring a survival benefit in advanced diseases, but at the same time increasing the possibility of chronic graft versus-host-disease (GVHD) owing to the high lymphocyte content. While UCB stem cells have been the simplest to collect and have allowed the greatest flexibility in human leukocyte antigen (HLA) matching, they yield the lowest stem cell dose, leading to the slowest engraftment as compared to the BM or the MBPL [10]. Study of BM-derived HSCs has further revealed that although these stem cells show the highest clonogenic potential and proliferative capacity as compared to the HSCs derived from UCB and MPBL, BM transplantation (BMT) requires HLA matching, and the incidence of graft failure and GVDH are very high in case of partially matched transplants [11]. FL HSCs on the other hand, have shown a number of pronounced phenotypic and functional differences with adult HSCs. These fetal HSCs divide rapidly and give more robust and rapid reconstitution of irradiated recipients relative to adult HSCs. They also seem to differ in the expression of specific markers such as Mac-1, CD144 and AA4.1 as well as in their general gene expression profile. There have also been clear

differences between fetal and adult HSCs in the regulation of basic stem cell properties such as self-renewal and developmental potential [12]. In summary, the foregoing observations indicate that each source of hematopoietic cells has different intrinsic properties, closely correlated with ontogenetic age, which are a vital determinant for phenotypic characteristics, lineage commitments, immunogenicity as well as proliferative potentials [13].

Although the mechanistics of differentiation potential of HSCs has been extensively characterized over decades, their self-renewal potential still remains poorly understood, mostly because of the lack of culture conditions in which the HSCs can self-renew *in vitro* without differentiation [14-15]. Several genes influencing self-renewal have been identified, and yet, to date no specific gene has been described that when activated enhances the HSC self-renewal akin to natural processes and when inactivated promotes either HSC commitment and differentiation towards a specific lineage or pushes it to apoptosis [16]. Specific markers that correlate with the unique functional properties of HSCs have not yet been identified, and as a result, the detection and enumeration of "functional" HSCs requires the use of retrospective assays [7, 17]. In humans, clinical protocols involving enrichment for HSCs generally utilize cells expressing CD34 [18-20], an antigen that is expressed on ~0.2–3% of the nucleated cells in cord blood (CB), BM and mobilized peripheral blood (MBL) [21-23]. Experimentally, further isolation and characterization of Lin^- $CD34^+$ subpopulations have defined more primitive precursors with hematopoietic repopulating activity that express combinations of the CD59 surface antigen related to Sca-1, the vascular endothelial growth factor receptor-2 (VEGFR-2 or KDR), and low levels of c-Kit (CD117), Thy-1 (CD90), and the CD38 surface antigen [24-30].

The cell surface sialomucin-like adhesion molecule, CD34, has been used as a convenient marker for HSCs, since $CD34^+$ cells have been shown to possess colony forming potential in short-term assays, to maintain long-term colony-forming potential in *in vitro* cultures and to allow the establishment of multi-lineage differentiation in immuno-compromised mice [31]. Since the exact biological function of this molecule has not yet been established, it is considered as a surrogate marker. Studies in murine and human models have indicated that $CD34^-$ HSCs exist as well, which possess engraftment potential and distinct HSC characteristics [7, 32] and further studies have clearly shown that these cells give rise to $CD34^+$ cells [33]. Another cell-surface glycoprotein, CD133, the human homolog of mouse prominin-1, was identified as a selective human HSC surface molecule using a monoclonal antibody recognizing a particular glycosylated form of prominin-1 designated as AC133/CD133 [34-35]. CD133 has been shown to be expressed on primitive human progenitor cells, which do not share any previously described HSC surface antigen. CD133 may be a marker for a more primitive cell subset as the CD133 expressing cells generate $CD34^+$ cells in liquid culture and, more importantly, engraft and produce progeny in the BM of non-obese diabetic/severe combined immuno-deficiency (NOD/SCID) mice [35]. Notably, the extremely rare $CD34^-$ candidate HSCs (Wang et al. 2003) reside within the $CD133^+$ fraction [36]. In fact, reports indicate that CD133 is perhaps a pan-adult stem cell marker [37-38] and is also expressed by many types of cancer stem cells [39-40].

SLAM (CD150, CD244 and CD48), is a new family of cell-surface receptors of the immunoglobulin (Ig) superfamily, which are tandemly arrayed at a single locus on chromosome-1 [41-42]. These molecules, which act as cell–cell interacting and signaling molecules, have been known to regulate the proliferation and activation of lymphocytes [43-44] and leukocytes [41, 45]. Highly purified HSCs were shown to possess a $CD150^+CD244^-CD48^-$ phenotype while non self-renewing multi-potent hematopoietic progenitors were found to be $CD244^+CD150^-CD48^-$ while the most restricted progenitors were $CD244^+CD48^+CD150^-$ [46]. Since these receptors are differentially expressed among hematopoietic progenitors in a way that correlates with progenitor primitiveness, HSCs can be purified accordingly by using a simple combination of monoclonal antibodies directed against these three receptors [47]. This strategy has been extended to isolate the HSCs from embryonic and fetal tissues (which was initially impossible because of the developmental variations in the anatomical localization and cell-surface marker

profile of these cells) [12, 48] and also from femur and spleen tissue sections of mice [46]. Because the of the ability of SLAM scheme to greatly augment HSC purity, it has been proposed that the SLAM family of cell-surface markers might be used as part of an HSC isolation scheme instead of previously established methods [49]. However, this proposal has yet to receive an international agreement.

Mesenchymal Stem Cells

The presence of non-hematopoietic stem cells in the BM was first suggested by the observations of the German pathologist Clonheim 130 years ago. His work raised the possibility that BM may be the source of fibroblasts that deposit collagen fibers as a part of the normal process of wound repair [50]. But it was through the work of Friedenstein and his coworkers in 1968 that the mesenchymal cells came to be known as an important entity of the marrow tissue [51]. The currently popular concept of mesenchymal stem cells [MSCs, a term first coined by Caplan (1991)] can be traced to the classical experiments demonstrating that the transplantation of BM cells to heterotopic anatomical sites results in a *de novo* generation of ectopic bone and marrow [52]. MSCs are therefore defined as multipotenial stem cells capable of forming not only bone, cartilage and other mesenchymal tissues (such as osteoblasts, chondrocytes and adipocytes), but also cardiomyocytes and neural precursors [53-54]. Moreover, MSCs are a component of the BM stroma that have been shown to express cell adhesion molecules and support hematopoiesis in culture by providing suitable cytokines, growth factors and extracellular matrix (ECM) proteins [55-57]. They also support the development of hematopoietic colonies *in vitro* [57]. Different groups have shown that MSCs can inhibit T-cell responses induced by mitogens or alloantigens [58-60] and this has led to their potential application in the reduction of graft vs. host reactions in the allo-transplantation settings [61].

The multi-potential nature of these cells, their easy isolation and culture as well as their high *ex vivo* expansive ability makes these cells an attractive therapeutic tool in diverse fields like tissue engineering and gene therapy [62-63]. Although their broad differentiation potential makes them ideal candidates for therapeutic applications, the precise signaling pathways that determine their differentiated fate are not fully understood. Also our knowledge about signals required for MSC mobilization and migration to the injured sites lags behind the extensive experience with the HSCs [64]. Certain studies point towards developmental signaling cues that may be important in regulating stem cell self-renewal and differentiation programs [65]. Etheridge et al (2004) [65] have shown that autocrine Wnt signaling operates in primitive MSC populations and this regulates mesenchymal lineage specification and processes (such as proliferation and motility, self-renewal, differentiation and generation of cell polarity). Hilton et al. (2008) [66] have shown that Notch signaling pathway maintains a pool of mesenchymal progenitors by suppressing osteoblast differentiation. On similar grounds Jian *et al* (2006) [67] have also shown that inhibition of osteogenic differentiation by transforming growth factor-β1 (TGF-β1) is potentially important for the control of self-renewal and differentiation of MSCs and that there might be a functional role of a novel form of cross-talk between the TGF-β1 and Wnt signaling pathways in regulating the activities of human MSCs. Neuss *et al* (2004) [64] have advocated the importance of hepatocyte growth factor (HGF) and its cognate receptor (c-met)-mediated signaling system in adult human MSCs and its role in cell mobilization, tissue repair and wound healing.

Unlike their well characterized neighbors, the HSCs, which have been prospectively isolated and extensively studied at the single cell level both *in vitro* and *in vivo*, MSCs have only been defined and isolated by physical and functional properties *in vitro*. Consequently, little is known about their phenotypic and functional characteristics *in vivo* [57, 68-69]. Nevertheless, MSCs have been reported to be uniformly positive for SH2, SH3, CD29, CD44, CD71, CD90, CD106, CD120a , CD124 [53]; CD73, CD105 [70] and a range of other markers like CD271, mesencymal stem cell antigen-1 (MSCA-1), CD56 [69]; SSEA-4, an early embryonic glycolipid antigen commonly used as a marker for undifferentiated pluripotent human embryonic stem cells and cleavage to blastocyst stage embryos also identifies the adult mesenchymal stem cell population [71] and STRO-1,

a marker for the isolation of highly enriched population of stromal stem cells in combination with an antibody directed to vascular cell adhesion molecule-1 (VCAM-1/CD106) [72]. In contrast, MSCs are negative for other markers of the hematopoietic lineage, including the lipopolysaccharide receptor CD14, CD34, the leukocyte common antigen CD45 and the endothelial antigen CD144 [53, 70].

MSCs are usually isolated from the BM [32, 73-74], UCB [75-77] adipose tissues (AT) [78], amniotic fluid (AF) [79] and placenta [80-81]. MSCs isolated from all these sites are shown to exhibit a phenotypic heterogeneity [82], and yet, the studies involving comparative global gene expression profiles of MSCs isolated from AT, UCB and BM have yielded no phenotypic differences when examined by flow cytometry using a panel of 22 surface antigen markers [83]. These differences may have resulted from the differences in the methodology and culture conditions used in the MSC growth.

Hematopoietic Stem Cell Niche

The concept of HSC niche was first proposed by Schofield in 1978 after an analysis of findings on the spleen colony-forming cell (CFU-S) [88]. He proposed that stem cells are fixed tissue cells that are prevented from differentiation and continue to proliferate as stem cells within a functionally and spatially characterized "niche". [84-85]. The HSCs persist throughout the life of an organism dynamically regulating their numbers after injury by undergoing rapid self-renewing divisions and this is mediated by both cell-intrinsic [86] as well as cell-extrinsic [87] mechanisms. With respect to the cell-extrinsic mechanisms, HSCs are thought to reside within the "niches", which are specialized micro-environments within the hematopoietic tissues created by supporting cells that express membrane-bound and secreted factors that promote HSC maintenance (survival and self-renewal), and regulate HSC migration, quiescence and differentiation [88].

A large body of evidence suggests that HSCs and hematopoietic progenitor cells (HPCs) are not randomly distributed in the BM but rather are localized close to the endosteum of the bone [89-91] and around blood vessels [46, 92-93]. A prevalent idea in the field suggests that two niche compartments, the osteoblastic and vascular niches may house distinct types of stem cells [94]. Whereas the endosteal zone is thought to favor quiescence, the centrally located vascular niche perhaps serves as a location that allows differentiation and ultimate mobilization to the peripheral circulation [84]. Studies have demonstrated that 'osteoblasts' comprise a crucial component of the HSC niche in the endosteal compartment [91, 95-97] while cells of other lineages including 'endothelial cells' most likely participate in niche functions in the vascular compartment [46, 98-100]. Moreover, it is likely that the coordinated activities of the niche (be it endosteal or vascular) and the stem cells collectively are responsible for integrating the delicate balance between hematopoiesis and tissue turnover [101].

Anatomy of the Bone Marrow

HSCs reside primarily within the bone marrow during adulthood. The BM is a complex organ containing many different hematopoietic and non-hematopoietic cell types. Hematopoiesis occurs within the medullary cavity, surrounded by a shell of vascularized and innervated cancellous bone. Minute projections of bone (trabeculae) are found throughout the trabecular zone of bone, such that many cells in this region are close to the bone surface. The interface of bone and BM is known as the endosteum, and this is covered by bone-lining cells that can differentiate into bone forming osteoblasts. Although these bone-lining cells are often described as osteoblasts in the literature on the HSC niches, the bone-lining cells are heterogeneous in their degree of differentiation, and only a minority of these cells is actually the bone-synthesizing osteoblasts [102-103]. The endosteum is also marked by the presence of bone-resorbing osteoclasts. Osteoblasts and osteoclasts are present in a dynamic equilibrium under steady-state conditions, but either partner can increase or decrease in frequency depending on whether bone is being formed or being remodeled [104-105]. The endosteal surface is heavily vascularized, raising the possibility that vascular cells also have an important role in regulating hematopoietic progenitors near the

endosteum. It is likely that the interaction of bone cells, hematopoietic cells and vascular cells at the endosteum regulates both hematopoiesis and bone formation [102].

Vascular cells also probably contribute to the creation of HSC niches in extramedullary tissues. A close interaction between HSCs and endothelial cells is not unexpected because both lineages arise from a common embryonic precursor, the hemangioblast [106]. HSCs are present throughout the adult life in extramedullary tissues, such as the liver and spleen [107], and extramedullary hematopoiesis can flourish in these tissues for long periods of time, despite the absence of bone or endosteum [108-109]. This implies that there are cells other than osteoblasts and osteoclasts that can create environments capable of sustaining adult HSCs. Most HSCs mobilized to the adult spleen localize adjacent to sinusoids [46], suggesting that the HSCs in the extramedullary tissues may reside within perivascular niches. Sinusoids are specialized blood vessels (venuoles) that are present in hematopoietic tissues and through which the venous circulation occurs. The walls of the sinusoids are composed of fenestrated endothelial cells through which hematopoietic cells can enter and exit the circulation [102, 110]. Kopp *et al* (2005) [84] have additionally explained that BM sinusoidal endothelial cells (BMECs) are functionally and phenotypically distinct from micro-vasculature endothelial cells of other organs, as endothelial cells that are isolated from various adult non-hematopoietic organs have little or no ability to maintain the HSCs *in vitro* [111]. A study conducted by Kiel *et al* (2005) [46] found that the HSCs that are attached to the sinusoidal endothelium are $CD150^{+}$; which raises the possibility that probably a vascular BM HSC niche might also exist during homeostasis.

A vascular BM HSC niche has previously been predicted to form during HSC mobilization after myeloablation. It was observed that the quiescent HSCs detached from the endosteal niche and migrated towards the centre of the BM to the vascular zone from where they re-established the hematopoiesis [84, 112-113].

Role of Endosteal and Vascular Niches in HSC Maintenance

Osteoblasts secrete factors that regulate HSC maintenance - positive regulators such as angiopoietin [97] and thrombopoietin [113-114] and negative regulators such as osteopontin [115-117] and (C-X-C motif) ligand 12 (CXCL12) [118]. Functional studies indicate that angiopoietin and thrombopoietin promote HSC quiescence [97, 114, 119] whereas CXCL12 regulates HSC migration and localization in the BM [118, 120]. Angiopoietin is also secreted by megakaryocytes [121]. The major sources of thrombopoietin are the liver and kidney, from which thrombopoietin is thought to enter the BM through the circulation, although BM stromal cells may also secrete thrombopoietin during hematopoietic stress [122-123]. These observations raise the question of whether the osteoblasts are really the only physiologically important source of these factors for the HSC maintenance or other cells also promote the HSC maintenance by secreting these factors. Also, some state that as none of these factors have yet been conditionally deleted from the osteoblasts, they may not be the only major source of the secretory factors in the BM [102]. Another school of thought argues that perivascular cells could also be critical sources of angiopoietin and CXCL12 for HSCs that reside at or near the endosteal surface and much of the thrombopoietin in the endosteal region may enter the BM through circulation. Given this possibility, there may not be any dichotomy between endosteal and perivascular niches [124]. Thus, until and unless these factors are temporarily deleted from the specific cell types it will be difficult to find out which cells are the actual physiological sources of these soluble factors!

i) Molecular Cross-Talk in the Endosteal Niche

A fundamental question in the maintenance of the HSCs is whether HSCs require cell-cell contact with the supporting cells (whether endosteal or endothelial). One widely discussed model has shown that the HSCs do adhere to specialized spindle shaped osteoblasts using N-cadherin-mediated homotypic adhesion and that the HSCs are acutely dependent upon this interaction for their maintenance [94, 96,

110, 125]. Because β-catenin is the intracellular portion of N-cadherin and a crucial component of the Wnt signaling pathway, the role of Wnt signaling pathway in HSCs has also been studied in great detail [126-129]. However, the importance of canonical Wnt signaling during hematopoiesis has been questioned because β-catenin has been found to be dispensable for HSC function [130].

Previous research widely hypothesized that Notch ligands were a critical component of the HSC niches and promoted HSC maintenance by activating expression of Notch receptors on the HSCs. Gain-of-function experiments suggested that Notch signaling increases self-renewal and decreases differentiation of the hematopoietic progenitors. In these experiments, elevated levels of Notch signaling were achieved through exposure of progenitors to the Notch ligands *in vitro* [131-134]. Later when the osteoblasts were found to express the Notch ligand, it was proposed that they probably played a pivotal role to promote HSC maintenance by activating Notch expression by the HSCs [135]. Osteoblasts produce hematopoietic growth factors and are activated by parathyroid hormone (PTH) [136]. In 2003, Calvi *et al* [91] were successful in showing that on exposure to PTH, the osteoblasts upregulated Jagged 1, which concomitantly led to an increase in the HSC number. However, in the same year, Zhang *et al* (2003) [96] showed that a conditional deletion of both Jagged1 and Notch1 from BM cells did not affect the HSC maintenance *in vivo*. A study conducted by Maillard *et al* (2008) [137] further confirmed that, although the gain of function experiments demonstrated a potential role of Notch signaling to expand primitive hematopoietic progenitors, the canonical Notch signaling is dispensable for the maintenance of adult HSCs. Hence it remains possible that the HSCs require Notch signaling only under certain specific circumstances and Notch activation may not be a crucial general requirement of the niche [110]. Together, these data indicate that signaling events occurring between HSCs and osteoblasts are more complex than has been previously assumed and may involve factors other than the Notch signals.

If N-cadherin and Notch ligands are not critically required for the HSC maintenance, it is possible that the endosteal cells promote HSC maintenance by secreting diffusible factors that act indirectly or at a distance [102]. The role of osteoclasts [138] and the calcium ions that are generated by bone resorption [117] have also been implicated in the HSC maintenance and localization. Osteoclasts secrete proteases such as matrix metalloproteinase 9 (MMP9) and cathepsin K, as well as growth factors such as CXCL12 [138] that regulate HSC maintenance and localization within the BM [120, 124, 139-140].

Through live cell imaging, Gillette *et al* (2009) [141] were able to successfully characterize both the site of contact between osteoblasts and hematopoietic stem progenitor cells (HSPCs) and also the events (related to TGF-β signaling) taking place at this site resulting in the downstream modulation of responses important for niche maintenance. They specifically showed that the portions of the HPC uropod membrane at the contact site are actively endocytosed by the osteoblasts and delivered to Smad anchor for receptor activation (SARA)-positive, signaling endosomes. In response to this intercellular transfer, osteoblasts show a decreased Smad signaling and a greater production of the BM chemokine, stromal derived factor-1 (SDF1).

The role of the membrane-bound stem cell factor (SCF) cannot be ignored in this regard as SCF is a potent stimulator of adhesion of HPCs to stromal cells [142-143]. When SCF is expressed by the osteoblasts, it causes a sustained activation of KIT [expressed at high levels by long-term repopulating (LTR) HSCs as well as other stem cells] [144] which further leads to the expression of very late antigens 4 & 5 (VLA4 & VLA5 aka integrins α4β1 and α5β1) indicating that the membrane-bound SCF can affect the adhesive properties of the endosteal niche by modifying the functional state of specific integrins [145]. Collectively, these data indicate that membrane-bound SCF is an essential component of the endosteal HSC niche that maintains the long-term HSC activity in the adult BM.

ii) Regulation of HSCs by Vascular Cells

The vasculature has a crucial role in the formation and the expansion of HSCs during embryonic development, and probably in the regulation of adult HSCs. Therefore, a close developmental relationship between the

hematopoietic and the endothelial lineages is not surprising [106, 146] as HSCs appear to arise from a perivascular progenitor during embryonic development [147-152].

Similar to osteoblasts, endothelial cells also promote the maintenance of HSCs in culture [112, 153] and normal endothelial-cell function is a requisite for hematopoiesis *in vivo* [154]. BMECs constitutively express cytokines such as CXCL12 and adhesion molecules such as endothelial-cell (E)-selectin and vascular cell adhesion molecule 1 (VCAM1) that are important for HSC mobilization, homing and engraftment [113, 155-156]. BMECs are also known to support survival, proliferation and differentiation of myeloid and megakaryocyte progenitors [84, 113,157-158], whereas primary BM stromal cells on the other hand release factors that inhibit megakaryocyte maturation [159]. These data indicate that megakaryocyte lineage development (and possibly the development of other myeloid-cell types) might be predominantly initiated at the vascular niche itself [84]. Another possibility is that the pool of HSCs located in the vascular and self-renewing endosteal niches are freely exchanged to maintain homeostasis in a constantly changing hematopoietic environment [110].

The following study by Sugiyama *et al* [124] will further validate the meaning of the above statement. In 2006, Sugiyama *et al* [124] found that the perivascular reticular cells in the BM expressed very high levels of CXCL12 (CAR cells). They further confirmed that a high proportion of HSCs localized perivascularly, and that the subset of HSCs that localized to the endosteum or elsewhere were consistently in contact with the CXCL12 expressing reticular cells. The presence of CXCL12-secreting reticular cells in both the perivascular and endosteal environments, therefore, provides a potentially unifying mechanism for the maintenance of HSCs in both the sites, and reminds us that the most morphologically recognizable cell types in a particular environment (such as osteoblasts and endothelial cells) need not be the cells that are functionally important for the creation of a niche. Nonetheless, there are no genetic data confirming that these reticular cells are a functionally more important source of CXCL12 for the maintenance of HSCs than the osteoblasts or the osteoclasts, so the argument that they create a niche for the HSCs is not yet conclusive [102].

In 2004, a study by Visnjic *et al* [160] showed that deletion of osteoblasts resulted in extramedullary hematopoiesis, thereby implying that the vascular BM HSC niche alone might not be sufficient to maintain the long-term hematopoiesis. This indicates that in the BM, the vascular niche might be a secondary niche, requiring an influx of HSCs from the primary endosteal niches. Collectively, the above discussion lucidly illustrates that both niches – the vascular and the endosteal – strongly cooperate to control HSC quiescence and its self renewing activity (and therefore HSC number), as well as the production of early progenitors to maintain homeostasis or re-establish it after injury.

iii) Other Contributions to the Niche

Using SLAM family markers, Kiel *et al* (2005) [46] found that 38% of HSCs in the extramedullary tissues and 26% of HSCs in the BM were not detectable to be in contact with either the sinusoids or the endosteum. Therefore, it is possible that there are yet certain undiscovered distinct niches that we do not know of! There is also a probability that these undetected HSCs are simply migrating and hence illusive. It is important to mention the role of adipocytes here as negative regulators of the hematopoietic stem cells and that antagonizing marrow adipogenesis may enhance hematopoietic recovery in a clinical BM transplantation set up [161].

Another interesting concept is the presence of hypoxic micro-environments in the BM and the tendency of HSCs to preferentially occupy these hypoxic regions [162]. Although much of the endosteum is highly vascularized, certain studies do show that there are regions of the endosteum which appear to be hypoxic [162-164]. Hypoxia appears to regulate hematopoiesis in the BM by maintaining important HSC functions, such as cell cycle control, survival, metabolism, and protection against oxidative stress [165]. However, additional studies, especially those related to molecular mechanisms of hypoxia-mediated regulation, will be required to fully characterize the hypoxic regions within the BM and the manner in which they regulate the HSCs in order

to eventually improve the therapeutic expansion of HSCs.

Co-Culture of HSCs with MSCs

i) Two-dimensional (2D) Culture System

Despite intensive research there are still no reliable methods for the expansion of primitive and self-renewing HSPCs in cultures. Although several studies have been reported in the last decade regarding *ex vivo* expansion of HSPCs, most of them have met with little success [166-168]. Based upon these previous approaches, the *ex vivo* expansion strategies can be divided into 2 categories [169]. The first category is the treatment of HSCs with various combinations of cytokines because it has been well documented that MSCs constitutively secrete various hematopoietic cytokines such as SCF, thrombopoietin (TPO), Flt-3 ligand (Flt-3L), leukemia inhibitory factor (LIF), interleukin (IL)3, IL6, IL7, IL8, IL11, IL12, IL14 and IL15, which might be supporting the self renewing cell divisions of HSCs [14, 170-172]. This school of thought emphasizes the importance of noncontact culture systems in the successful maintenance of hematopoietic cells. Oostendorp *et al* (2005) [173] for the first time investigated whether a direct HSC-stroma contact was required for the stem cell maintenance and their results showed the presence of some secreted molecules essential in the maintenance of the adult BM HSCs. Another interesting study using BM-derived hematopoietic cells and mesenchymal cells has shown that an intercellular cytokine network between the two types of cells mutually leads to their expansion [174]. The use of conditioned medium (CM) derived from MSCs therefore presents as a powerful alternate tool for therapeutic applications, like in the field of neurological disorders [175-176] and tissue engineering [177].

Although the treatment with a combination of cytokines increases the HSPC population by several folds it is difficult to maintain the HSC activity in long-term cultures even if the total number of hematopoietic cells could be expanded [169]. The second category therefore involves use of the stromal cells as a feeder layer on which the HSCs are cultured, as a number of studies have shown that contact between HSCs and stromal cells is important for maintaining the function of HSCs [178-182]. Wagner and group have done extensive work to identify the essential cellular and molecular mechanisms involved in the interaction between HPCs and stroma feeder layer, since cell-cell contact of HSPCs with the stromal cells is crucial in maintaining "stemness" of the former [78, 83, 182-185].

In 2007, Wagner et al. [184] illustrated that the primitive fractions of HPCs associated with a higher self-renewal ability, strongly expressed the adhesion molecules such as fibronectin-1, cadherin-11, VCAM1, connexin 43 (CX43), and integrins, implying their role in the specific interaction of human HPCs with MSCs than their more differentiated counterparts. In 2008 they additionally showed that the adhesion of HPCs to MSCs involves CD44, which binds to its main ligand, hyaluronic acid [185]. Further extensive research [182, 186] has corroborated their findings and has addressed the role of MSCs in the proliferation and the maintenance of HSCs *in vitro*, thereby resulting in a better understanding of the intrinsic and the extrinsic factors regulating self-renewing divisions of HSCs. Subsequent integrin profiling studies have shown that β1-integrins, especially VLA4 and VLA5, play a vital role in the early interaction of HPCs with the BM niche, thereby regulating their initial cell divisions [187]. Other studies have simultaneously demonstrated that β1-integrins are involved in the regulation of progenitor cell proliferation, survival, clonogenic growth, and maintenance during *ex vivo* culture and transduction [188-192]. Most importantly, certain studies have shown that the HSPCs cultured on MSCs show an enhanced *ex vivo* expansion potential and a higher degree of long-term engraftment as compared to the control cultures [169, 193]. This is of prime concern as establishment of a defined cell culture system that facilitates *ex vivo* expansion of the isolated HSPCs is a crucial issue in hematology and stem cell transplantation [193-195].

In 2010, Jing *et al* [196] conducted an interesting study to investigate in greater detail the spatial relationship between HSCs and MSCs during *ex vivo* expansion. In this study, MSCs served as a physical boundary of distinct compartments, and the properties

and the features of the HSCs present in different localizations were evaluated at various times. They found that the phase-bright cells located above the mesenchymal stromal cell surface was the dominant location where the HSCs proliferated, whereas the compartment beneath the mesenchymal stromal cell layer seemed to mimic the stem cell niche for more phase-dim/phase-dark immature cobblestone area-forming cells (CAFCs). The CAFCs are tightly knit group of phase dark, angular cells in the stroma [197] and experiments have revealed that only these cells growing under the stroma are capable of producing colony forming unit-granulocyte-monocyte (CFU-GM), indicating that only the cells in the CAFC areas represent the true primitive hematopoietic cells [198-199]. The visual CAFC readout eliminates the requirement of trypsinization as the wells are simply scored positive or negative for the presence of cobblestone areas at more than one time point e.g. after 5 and 8 weeks [197, 200]. CAFC frequencies have been reported in the study of stem cell storage [201], expansion [202-203], or mobilization [204]. In the clinical setting these assays have been used to assess stem cell defects e.g., in aplastic anemia [205] and Diamond Blackfan anemia [206] and to investigate bone marrow reserve after stem cell transplantation [207-208] or in patients with autoimmune cytopenias [209]. Although the data by Jing *et al* (2010) [196] provides novel and valuable insights into the construction and the function of a three-dimensional HSC-MSC co-culture micro-environment *in vitro*, however, it is still not clear whether the environment beneath the MSC layer actively keeps HSCs in an immature state or creates a niche atmosphere which specifically attracts the HSCs. Until further investigations are carried out, it will be a matter of speculation as to which mechanism(s) are involved in the migration of HSCs beneath the MSC layer thereby leading to the generation of CAFCs.

ii) Three-dimensional (3D) Culture System

The bone marrow provides spatially and temporarily variable signals that are known to impact the behavior of HSCs [221]. In contrast to other stem cells, the clinical application of HSCs is well established. Even though their functionality throughout life *in vivo* is regulated in a brilliant manner; controlling HSC behavior *in vitro* still remains a major task that has not been resolved with standard culture systems [219]. *In vivo*, HSCs are located in a three-dimensional (3D) environment and therefore culturing the HSCs on flat tissue-culture dishes or in suspension is considered as a highly artificial situation for these cells [222]. Studies have shown that unlike two-dimensional (2D) cell culture systems, the effects of local accumulation or depletion of diffusible factors can be reproduced in 3D culture systems [219]. The diffusion of other soluble factors like nutrients or oxygen in the natural extra-cellular matrix (ECM) (that leads to gradients) that play an important role in many cellular processes such as migration or homing [223] have been shown to be mimicked more realistically by diffusion-limited 3D cell culture systems than standard 2D cell cultures [219].

Hence in recent years, the development of artificial microenvironments mimicking important stem cell niche interactions *in vitro* has received increased attention to gain new insights into the orchestrating and the regulatory roles of the niche components [210]. Studies attempting to recapitulate the marrow physiology by constructing a three-dimensional (3D) culture system using MSCs have shown that these cultures are able to harbor a large pool of primitive HSCs with superior phenotypic and functional attributes [211-212]. Recently, complex and diverse stromal stem cell populations that contribute to HSC maintenance have been identified. Data suggests that the perivascular chemokine CXC ligand (CXCL) 12-expressing mesenchymal progenitors and endothelial cells are key cellular components of the stem cell niche in the bone marrow [213]. A biomimetic microdevice that replicates the hematopoietic microenvironment *in vitro* offers a new approach for analysis of drug responses and toxicities in bone marrow as well as for study of hematopoiesis and hematologic diseases *in vitro* [214]. Similarly it has been shown that 3D micro-pillar device that provides opportunities for mechanical interlocking may prove to be more suitable culture substrates for HSC expansion *ex vivo* [224]. Furthermore, the decoupled effects of substrate elasticity, construct dimensionality, and ligand concentration on the

biophysical properties of primary hematopoietic stem and progenitor cells (HSPCs) suggests that these parameters may be critical design criteria for the development of artificial HSC niches [225]. With better understanding of the HSC niche and development of superior 3D-culture systems, there is a high possibility that we will be able to expand HSCs *in vitro* and safely and effectively use them for transplantation in certain clinical contexts.

Future Perspective

The last decade of research has strengthened our knowledge on HSCs and the hematopoietic microenvironment (niche) by answering some basic questions regarding the molecular and cellular nature of the HSCs and the niche in which they reside. Although there are parts of the niche which remain ambiguous due to overlapping cell populations, however with the pace of the current work, it is likely that additional components will be discovered and soon we will be able to compare distinct HSC niches that have different functions. For example, transcriptome studies by Gazit et al, 2013 [226], have successfully demonstrated for the first time that several factors in fact act collectively for maintaining HSC multipotency in the bone marrow microenvironment. These transcriptome programs could provide unique insights into the molecular mechanisms that regulate stem cell core properties in distinct HSC niche domains. Such information would be helpful in identifying growth factors that are synthesized by the specific niche domain *in vivo* and to develop novel methods for achieving HSC expansion, thereby improving their clinical utility.

With our growing understanding of the bone marrow niche another set of questions worth pondering upon is the manner in which the niche participates in diseases of stem cell failure, such as aplastic anemia or neoplasia [215]. Is the niche hostile towards progenitors in the diseased state or do the deregulated progenitors alter the hematopoietic microenvironment are some of the questions that remain unanswered! 2D and 3D culture systems provide unique approaches for the investigation of the regulation and maintenance of early hematopoietic progenitors under conditions that reproduce many aspects of the marrow microenvironment [216]. Studies have shown that such co-culture systems may perhaps help to create specialized *in vitro* niches, thereby modulating stem cell functions, by either activating or inhibiting specific signaling pathways [211]. The co-cultured cells harbor primitive HSCs that possess a superior *in vivo* engraftment potential [193, 211, 217-218], although it has been reported that the 2D culture systems lack the autocrine and paracrine signaling that are important regulating mechanisms during hematopoiesis [219]. Nevertheless, such *in vitro* co-culture systems that amplify the HSCs for extended periods without having lose their self-renewal capacity are promising approaches for clinical application in the future [218].

Once standardized, e*x vivo* expansion of human HSCs will surely have a positive impact on the field of regenerative medicine [220]. The bone marrow niche has grown up; and it is only a matter of time till we develop new advanced approaches in studying its intricately complex architecture in regulating HSCs, shall we be successful in unraveling its complete role in normal and diseased hematological conditions.

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