REVIEW

Complexities of modeling the bone marrow microenvironment to facilitate hematopoietic research

Caroline Busch[a], Kudzai Nyamondo[b], and Helen Wheadon[a]*

[a]*Paul O'Gorman Leukaemia Research Centre, School of Cancer Sciences, University of Glasgow, Glasgow, United Kingdom;* [b]*Wellcome-Sanger Institute, Wellcome Genome Campus, Hinxton, Cambridgeshire, United Kingdom*

Hematopoiesis occurs in the bone marrow (BM), within a specialized microenvironment referred to as the stem cell niche, where the hematopoietic stem cells (HSCs) reside and are regulated for quiescence, self-renewal and differentiation through intrinsic and extrinsic mechanisms. The BM contains at least two distinctive HSC-supportive niches: an endosteal osteoblastic niche that supports quiescence and self-renewal and a more vascular/perisinusoidal niche that promotes proliferation and differentiation. Both associate with supporting mesenchymal stromal cells. Within the more hypoxic osteoblastic niche, HSCs specifically interact with the osteoblasts that line the endosteal surface, which secrete several important HSC quiescence and maintenance regulatory factors. In vivo imaging indicates that the HSCs and progenitors located further away, in the vicinity of sinusoidal endothelial cells, are more proliferative. Here, HSCs interact with endothelial cells via specific cell adhesion molecules. Endothelial cells also secrete several factors important for HSC homeostasis and proliferation. In addition, HSCs and mesenchymal stromal cells are embedded within the extracellular matrix (ECM), an important network of proteins such as collagen, elastin, laminin, proteoglycans, vitronectin, and fibronectin. The ECM provides mechanical characteristics such as stiffness and elasticity important for cell behavior regulation. ECM proteins are also able to bind, sequester, display, and distribute growth factors across the BM, thus directly affecting stem cell fate and regulation of hematopoiesis. These important physical and chemical features of the BM require careful consideration when creating three-dimensional models of the BM.

HIGHLIGHTS

- A detailed overview of how cells within the bone marrow microenvironment (BMM) support hematopoietic stem cells.
- The mechanical properties of the extracellular matrix influence stem cell maintenance within the BMM and ex vivo.
- Considerations are provided for constructing BMM models.

BONE MARROW MICROENVIROMENT

Human hematopoiesis occurs in the bone marrow (BM) of the axial skeleton, which encompasses the cranium, sternum, ribs, vertebrae, and ilium [1]. Hematopoietic activity occurs in the tissue of the red marrow, which is supported and regulated by a unique nonhematopoietic cellular network/milieu. Initially red marrow is evenly distributed, but it becomes restricted to the proximal regions of the bone with age and is replaced by the fatty yellow marrow [2]. Hematopoiesis is supported by the BM microenvironment (BMM), a vascularized space made up of nonhematopoietic cells and an extracellular matrix (ECM) that regulates hematopoietic stem cell (HSC) activity. Nonhematopoietic cells include osteolineage cells, leptin receptor $(LepR)^{+}$ mesenchymal stromal cells (MSCs), nerve cells, and vascular endothelial and sinusoidal cells. These cells are spatially organized into distinct niches, creating a unique microenvironment for hematopoietic stem and progenitor cell (HSPC) development and maintenance [1,3-5]. The interaction between HSPCs within the different niche areas is

Abbreviations: Ang-1, Angiopoietin 1; AEC, Arteriolar endothelial cell; BM, Bone marrow; BMM, Bone marrow microenvironment; BMP4, Bone morphogenetic protein 4; CXCL12, CXC-chemokine ligand 12; CXCR4, CXCL12-CXC receptor 4; ECM, Extracellular matrix; FGF, Fibroblast growth factor; FN, Fibronectin; GAG, Glycosaminoglycan; G-CSF, Granulocyte colony-stimulating factor; GF, Growth factor; HA, Hyaluronic acid; HSC, Hematopoietic stem cell; HSPC, Hematopoietic stem and progenitor cell; HEF, Hepatocyte growth factor; Il-7, Interleukon-7; LerR, Leptin receptor; MET, HGF receptor; MK, Megakaryocyte; MSC, Mesenchymal stromal cell; MSSC, Multipotent stromal stem cell; NG2, Neural glial antigen; OS, Osteochondrogenic progenitor; PDMS, Polydimethylsiloxane; Prx1, Paired-related homeobox 1; PEG, Poly (ethylene glycol); PVA, Polyvinyl alcohol; SC-RNA-Seq, Single-cell RNA-sequencing; SEC, Sinusoid endothelial cell; SCF, Stem cell factor; SNS, Sympathetic nervous system; TGFβ, Transforming growth factor beta; TPO, Thrombopoietin; Treg, Regulatory T-cell; VCAM-1, Vascular cell adhesion molecule 1; VEGF, Vascular endothelial growth factor; VN, Vitronectin

Address correspondence to Professor Helen Wheadon, Paul O'Gorman Leukaemia Research Centre, Gartnavel Hospital, University of Glasgow, 21 Shelley Road, Glasgow, G12 0ZD, United Kingdom; E-mail: Helen.Wheadon@glasgow.ac.uk

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mediated by cell-surface receptors, adhesion molecules, and the exchange of cytokines and growth factors (GFs), as illustrated in Figure 1 [4]. Below, we discuss how each component influences hematopoiesis and their requirements when recapitulating aspects of the BMM ex vivo.

CELLULAR COMPONENTS OF THE BONE MARROW NICHE

Mesenchymal Stromal Cells

One main nonhematopoietic cell type that is indispensable for the regulation of the BM niche and the support of HSCs is the MSC. MSCs comprise 0.001−0.01% of the total BM cell numbers and are required for tissue regeneration and immunomodulation [6]. MSCs are multipotent cells with trilineage differentiation capacity, leading to the formation of osteoblasts, adipocytes and chondrocytes. The International Society for Cellular Therapy outlined the criteria that cells must fulfill to be classified as MSCs: (a) cells must be adherent to plastic when cultured; (b) they must express the cell-surface markers CD73, CD90, and CD105 and lack the expression of CD14 or CD11b, CD34, CD45, and CD79α or CD19; and (c) they must be capable of differentiating into bone, fat, and cartilage [7]. Adult BM MSC populations can be further sorted using the markers Lin^-, $CD45^-$, $CD271^+$ and $CD140a^{-/lo}$ with the expression of $CD146^+$ distinguishing perisinusoidal from endosteal $CD146^{-/lo}$ MSCs [8,9].

A great degree of heterogeneity exists within the MSC population with functionally distinct subtypes being identifiable by their expression and abundance of LepR, CXC-chemokine ligand 12 (CXCL12), Nestin, stem cell factor (SCF), neural glial antigen 2 (NG2) and paired-related homeobox 1 (Prx1) [1,10-13]. LepR-expressing cells form the largest subgroup of MSCs; within this group, populations showing unique expression of CXCL12 can emerge [1]. Those cells found to localize around sinusoids and arterioles are adipocyte-biased cells, which depending on extrinsic cues, serve as a source of factors required for HSC maintenance and retention within the BM, such as SCF, CXCL12, various interleukins, and bone morphogenetic protein 4 (BMP4) [11,14]. Conditional deletion of SCF from $LepR^+$ cells results in the depletion of hematopoietic lineage-restricted progenitors but not HSCs [15]. Another population of mostly quiescent collagen-expressing $LepR^+$ MSCs are found in close proximity to the periarteriolar and trabecular bone surface where they have a bias toward osteolineage differentiation. Expression of PTEN and more recently osteolectin were found to specify the priming of $LepR^+$ cells toward osteolineage differentiation and contribute toward HSC mobilization [11,14].

CXCL12-abundant-reticular (CAR) cells, which are derived from $LepR^+$ cells, produce the majority of CXCL12 in the BM and localize close to the sinusoids [16]. Initial seeding of HSCs in the BM occurs under the influence of the CXCL12-CXC receptor-4 (CXCR4) axis with $LepR^+$ CAR cells contributing to the accumulation of CXCL12 in the BM and creating a chemoattractant gradient for HSCs [5,12]. Conditional deletion of CXCL12 in $LepR^+$ cells has been observed to deplete the lymphoid progenitor pool and mobilize HSPCs toward extramedullary sites, thereby illustrating the important role played by the former in the provision of CXCL12 [12,17]. $Nestin^+$ MSCs, which overlap in expression with NG2, are commonly found around periarteriolar niches and show minimal CXCL12 and SCF expression, with conditional deletion having little impact in overall HSC abundance [16,18]. Instead, depletion of a particular lymphoid-biased HSC subset is observed, suggesting they release lymphoid-supportive factors [19]. A supportive role during the early stages of hematopoiesis being established in the BM has also been suggested based on their widespread distribution in the BMM [15]. They also express angiopoietin 1 (Ang-1), osteopontin, interleukin-7 (IL-7), and vascular cell adhesion molecule 1 (VCAM-1) [1]. Some of these factors play a role in HSC maintenance and quiescence, whereas others like Ang-1 indirectly influence HSC behavior through their impact on BM homeostasis [13].

Recent studies have performed high resolution single-cell RNA-Sequencing (sc-RNA-Seq) on BM stromal populations. Xie et al. [20] identified ten distinct clusters in the MSC population, and through hierarchical clustering followed by trajectory branch analysis divided these into three subpopulations; (i) Stemness cluster (ii) Functional cluster (iii) Proliferative cluster, with the $CD26^+$ stemness cluster having the ability to differentiate into the other subpopulations. The $CMKLR1^+$ functional cluster displayed immunoregulatory properties and osteogenic differentiation but lower potential for adipogenic differentiation and proliferation [20]. A separate study by Li et al. [21], identified nine potential stromal progenitor populations. Further interrogation using defined stromal markers identified six phenotypically distinct cell types within these populations; multipotent stromal stem cells (MSSC), highly adipocytic gene-expressing progenitors, balanced progenitors, pre-osteoblasts, osteochondrogenic progenitors (OS) and prefibroblasts. Trajectory analysis predicted a hierarchical organization consisting of two interlinked differentiation trajectories with MSSC at the apex. The differences in these progenitors were further characterized using colony-forming capacity and their ability to differentiate into osteoblasts, adipocytes and chondrocytes. In silico cell-to-cell interaction analysis predicted that hematopoietic cells were maintained by different stromal populations through diverse but nevertheless stromal cell-specific pathways. Interestingly, HSPCs were predicted to interact with MSSC via CXCL12-CXCR4 and with OS cells via SPP1-CD44 crosstalk. In situ localization analysis of BM biopsies identified that SPP1-expressing OCs were located close to the endosteal region and that CD271-positive stromal cells, including MSSCs, were localized in the perivascular and stromal regions, suggesting the possibility that different stromal cells provide specialized niches for hematopoietic cells in different locations [21]. These studies provide novel insight into the distinct MSC subpopulations and the intricate role they play in HSC maintenance and BMM homeostasis.

Osteolineage Cells

Osteolineage cells originate from $LepR^+$ MSCs and are identifiable by their expression of alkaline phosphatase, osteopontin, and osteocalcin with differences in abundance being indicative of the various differentiation states [22,23]. They are found along the endosteal surface with a heterogenous pool of osteoblasts lining the surface, whereas osteocytes, which have a limited differential capacity, are incorporated into the bone architecture as they mature. Osteolineage cells were the first population of BM cells associated with the regulation of HSPC cells and were reported to be enriched in the endosteal zone [1,24]. The microenvironment formed by these and other closely associating cells is known as the endosteal niche, playing a role in the maintenance of hematopoiesis through the provision of various supportive GFs. Localization studies have mapped a subset of primitive HSCs to be preferentially localized in the endosteal regions of the trabecular

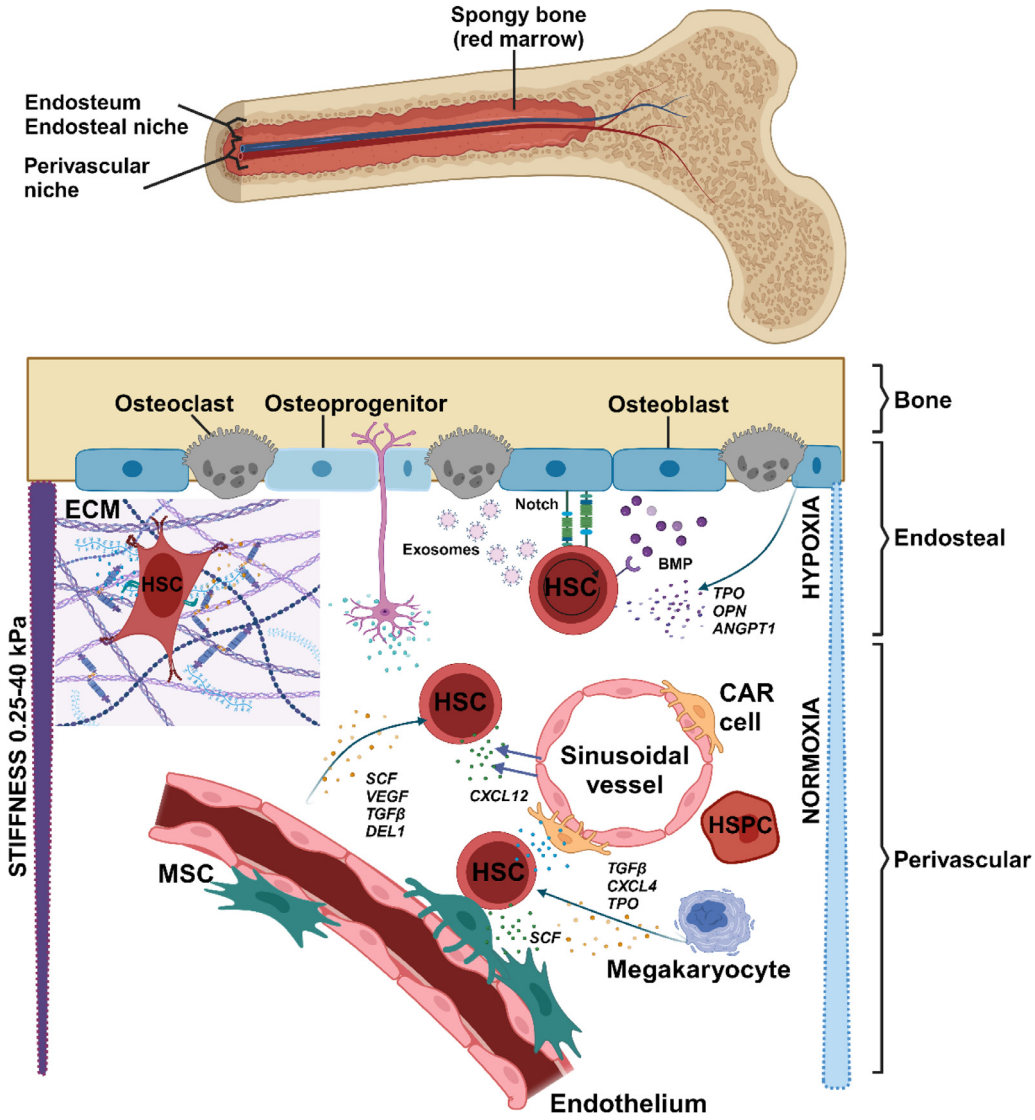

Figure 1 Schematic overview of HSC interactions in the bone marrow microenvironment.

The BM is the primary location for hematopoiesis and is located in the cancellous portion of long bones. The interface between bones and BM is the endosteum. The BM is highly vascularized with arterioles and sinusoids, which meet in the transition zone. Sinusoids have the task of enabling HSCs to leave the BM and enter the circulation. The ECM consists of collagenase and noncollagenase (laminin, VN, FN, tenascin, and elastin) proteins and proteoglycans with long glycosaminoglycan (GAG) side chains. Cell −ECM interactions are enabled through cell-specific receptors called integrins. Integrins ligate to peptide motifs of the ECM, thus triggering phosphorylation cascades that enable direct connections between integrins and the actin cytoskeleton. The BM niche consists of a variety of cell types, all of which influence the cell fate of HSCs and HSPCs. The niche can be subdivided into two zones, the more rigid hypoxic endosteal osteoblastic niche and the more oxygenated less rigid perivascular niche. In the perivascular zone, different types of stroma cell populations are localized around the arterioles and sinusoids, and all of these cells express factors such as SCF and CXCL12, thus supporting HSCs. Both sinusoids and arterioles are lined with ECs that also express HSC-supporting factors. In the endosteal zone, osteoblasts secrete HSC-supporting or -inhibiting factors, such as TPO, OPN, BMP, and ANGPT1. Additionally, the progeny of HSCs can directly stimulate HSCs. HSC localization within the niche is associated with either a more quiescence, slowly cycling state within the endosteal zone or a more active proliferating cell state within the perivascular zone. Created with BioRender.com.

bone [25-27]. This specific association was suggested to support their self-renewal capacity with resident osteoblasts in that area showing enrichment of Notch ligands Jag1, Jag2, and Dll4 [25]. Although Notch signaling in HSCs has been reported to be dispensable in adult hematopoiesis, a specific interaction between HSCs and Jag1-producing osteoblasts has been observed to be one of the mechanisms that can promote HSC quiescence [1,28]. There are additional HSC maintenance factors expressed by osteoblasts such as thrombopoietin (TPO) and angiopoietin 1 (ANGPT1) that regulate HSC quiescence [29-32]. This is further supported by in vitro studies which showed osteoblasts were capable of supporting the immature phenotype of primitive hematopoietic cells [33]. Interestingly, work by Zhao et al. [27] proposes a role in the protection of unique quiescent HSC subsets against chemotherapeutic stress from N-cadherin expressing MSCs. These cells that have trilineage differentiation capacity are found in close proximity to the endosteal surface of the trabecular bone region and exposure to stress make them biased toward an osteoblast differentiation program. This is further supported by work from Dominici et al. [7], showing preferential megakaryocyte (MK)-mediated expansion of this N-cadherin-expressing population in irradiated mice to re-establish a supportive endosteal niche for HSC reconstitution. Overall, this emphasizes the supportive role played by osteolineage cells in HSC maintenance.

Endothelial Cells

The BMM has an abundance of endothelial cells (ECs) that line the inside of blood vessels and produce factor such as Notch, CXCL12, SCF, and pleiotrophin that manage HSC and HSPC activity [1,17,34,35]; deletion of these factors interrupts HSC maintenance at steady-state in vivo. ECs can be further subdivided into arteriolar ECs (AECs) and sinusoid ECs (SECs) [1,36], with AECs producing almost all the endothelial-derived SCF. Although the overall abundance of ECs is comparable to MSCs, the expression of CXCL12 and SCF is much lower [37,38]. In addition to SCF, AECs also express the glycoprotein developmental endothelial locus (DEL1) that supports HSC proliferation and myeloid lineage progression [39]. Differing permeability of arterioles and sinusoids to blood plasma affect the localization of HSCs in the BM through reactive oxygen species (ROS); less-permeable AECs result in low ROS levels, which induces a quiescent state in nearby HSPCs. In contrast, cells in the vicinity of the more "leaky" and higher ROS-presenting SECs induces activation, migration, and differentiation [1,40]. Another avenue that drives HSC cycling is their direct interaction with ECs through E-selectin [41,42]. MKs have also been noted to exist near sinusoids and promote HSC quiescence through expression of transforming growth factor beta (TGFβ), CXCL4, and TPO [43].

Nerve Cells

Sympathetic and sensory nerves innervate both the bone and the BM. Although nerve fibers are not mandatory for the maintenance of HSCs, they are vital for the regeneration of hematopoiesis after chemotherapy [44]. Neural signals also modulate the process of hematopoiesis by controlling the circadian-mediated trafficking of HSPCs from the BM and regulating HSC quiescence [1]. HSPC release into the circulation occurs in a circadian manner in response to adrenergic signals from the sympathetic nervous system (SNS) that regulates the inhibition of CXCL12 expression by stromal cell populations [45]. Ablation of the adrenergic neurotransmitters can inhibit the release of HSPCs out of the BM [46]. Granulocyte colony-stimulating factor (G-CSF) secretion further manages the levels of CXCL12 through proteolytic cleavage, enabling HSPCs to egress [47,48]. Sympathetic nerves also interact with MSCs and osteolineage cells expressing β2 or β3 adrenergic receptors [18,37]. This interaction is mediated by catecholamines, such as norepinephrine sourced from the sympathetic nerves, which target the adrenergic receptors and suppresses MSC and immature osteoblast activity in favor of osteoclasts that mediate bone resorption. Consequently, CXCL12 is downregulated in the process, which increases HSC egress from the BM [37,49]. In addition, nonmyelinating Schwann cells regulate HSC quiescence through their activation of TGFβ, leading to cell-cycle arrest [50].

Additional BM Cells That Support HSCs

Adipocytes are another stroma-derived component interacting with HSCs. With aging, the BM of adults becomes increasingly fatty. Adiponectin, a protein secreted by adipocytes, inhibits hematopoietic activity and impairs proliferation [51]. This feature in combination with transplantation data that show a quicker BM recovery when treating mice with an adipocyte inhibitor suggests that adipocytes have a negative regulatory role of HSCs in the BM.

In addition to the stroma-derived niche components, the progeny from HSCs can play a role in regulation of HSCs. Localization studies using three-dimensional (3D) images have observed a colocalization of a HSC subset with MKs, and depletion of the latter induces HSC proliferation, indicating that MKs are important in HSC quiescence [52]. HSC quiescence is potentially regulated by MKs through the secretion of factors such as CXCL4, TGFβ, and TPO [1,52,53]. After a lethal dose of radiation, MKs support niche remodeling by relocalizing to the endosteal surface of the BMM; this is mediated by high TPO levels secreted by the osteoblasts and CD41 integrin-mediated adhesion. MKs then promote osteolineage expansion through the secretion of PDGFβ. Administration of TPO before and after radioablation led to enhanced MK function and HSC engraftment in mice by reducing the duration of regeneration to re-establish a quiescent state [1,54].

Other cell types that play a role in HSC behavior include macrophages, which regulate HSC retention by regulating osteolineage cells and MSCs. G-CSF has been demonstrated to transiently ablate osteoblast-supportive endosteal macrophages, leading to the suppression of osteoblasts and bone formation. This in turn inhibits the expression of HSC-supportive cytokines at the endosteum, leading to HSPC egress into the peripheral blood. Thus, macrophages play a critical role in maintaining the endosteal HSC niche and potentially function as antagonists to the SNS, enhancing retention of HSCs in the BM [1,55].

Regulatory T-cells (T_{reg}) are present in high numbers in the BMM, are attracted by the CXCR4/CXCL12 axis, and are retained by their high expression of CD44, which binds to hyaluronan in the BMM. T_{reg} secrete IL-10 and adenosine, which play an important role in regulating hematopoiesis and stromal cell development. In particular, T_{reg} suppress HSC proliferation and help maintain quiescence [56]. T_{reg} may also play a role in HSC retention as evidenced in allogenic stem cell transplant studies, where they colocalize with HSCs directly after transplant and promote survival by secreting IL-10, an immunoregulatory cytokine. Depletion of the T_{reg} population resulted in a loss of allo-HSCs, due to the loss of immune privilege mediated

through adenosine during transplantation, allowing allo-HSC engraftment [1,57].

The Secretome and HSC Maintenance Ex Vivo

Proteomics studies using advanced mass spectrometry-based quantification methods are providing valuable information on the cells within the BMM. A comprehensive study by Hennrich et al. [58] analyzed the proteome of 59 BM samples from individuals of different ages. Of the ~12,000 proteins identified, only a fraction (8.3%; 578 proteins) of the proteome was expressed in a strictly cell-specific manner. MSCs had the most distinct proteome with 452 proteins uniquely expressed, and 56 of these proteins play a role in the organization of the ECM and HSPC homing. This study identified 17 novel proteins involved in HSPC early differentiation processes (myeloid, lymphoid) and pluripotency regulation and several new cell-surface proteins with the potential to characterize MSC subpopulations [58].

Another study isolated MSCs and osteoblasts from the BM of healthy donors and cultured them in serum-free media for 48 hours to collect the supernatant (conditioned media) for analysis. They identified a total of 1,379 proteins in the MSCs and the osteoblasts, with more than 90% similarity between the two cell types. The majority of released proteins were classified into the following categories: ECM, especially fibrillar and nonfibrillar collagens; enzymes including several proteases, complement factors, and protease inhibitors; proteins involved in stabilization and posttranscriptional modification of other proteins; intracellular functions (intracellular transport and/or exocytosis, protein synthesis, nuclear protein interactions, and cellular metabolism); and cytokines, soluble cytokine receptors, and soluble adhesion molecules [59]. Proteomic studies provide valuable insight into the structurally and functionally diverse milieu of proteins released in the BMM important for sustaining HSCs and hematopoietic homeostasis.

Serum-free and Chemically Defined Ex Vivo Expansion of HSCs

Several studies have been conducted to optimize long-term ex vivo culture of HSCs, with functionality measured by performing competitive transplantation into lethally irradiated recipient mice. Fundamental research identified TPO and SCF as essential for HSPC expansion, with higher concentrations of TPO (100 ng/mL) and lower concentrations of SCF (10 ng/mL) being optimal. Expansion was further enhanced by culturing on fibronectin (FN) and replacing serum albumin with the synthetic caprolactam-based polymer polyvinyl alcohol. For long-term expansion, full media changes were necessary to sustain long-term HSC activity, as secreted cytokines and chemokines, especially IL-6 and CCL2-4, induce differentiation [60]. Although effective for mouse HSPCs expansion, results for human HSPCs was more limited, with a threefold to fourfold expansion observed. By examining the signaling pathways activated by TPO and SCF, differences between mouse and human HSPCs were observed. Most notably, decreased levels of PI3K and AKT activation were noted in human HSCs. By replacing SCF with the chemical agonists 740Y-P (a PI3K activator) and TPO with the THPO-receptor agonists (THPO-RAs) butyzamide and preventing $CD41^+$ MK differentiation using the pyrimidoindole derivative UM171, long-term HSPC expansion capable of serial engraftment in xenotransplantation assays was achieved. Using this chemically defined cocktail, HSPC proliferation was sustained over a 30-day culture by around 14-fold. Validation that the HSC population had expanded was confirmed by performing split-clone transplantation assays and sc-RNA-Seq analysis [61]. This ability to culture HSPCs using the MK inhibitors StemRegenin 1 and UM171 has led to advances in gene editing, especially studies mapping the clonogenic output and multilineage repopulating capacity of HSPC, paving the way for clinical translation in the future [62].

THE EXTRACELLULAR MATRIX

The ECM is primarily composed of water, proteins, and polysaccharides and provides more than just structural support for tissues and organs. ECM proteins are able to bind, sequester, display, and distribute GFs across the BM, thus directly affecting stem cell fate and regulation of hematopoiesis [4]. ECM-cell receptor adhesion via integrins has been the subject of significant study. Additionally, mechanical characteristics such as stiffness and elasticity have delivered insights into cell behavior regulation. Consequently, the ECM composition and structure play vital roles in cell polarity, differentiation, proliferation, and survival [63].

Characteristics and Composition of the ECM

The two main classes of macromolecules in the ECM are fibrous proteins (collagen and elastin) and glycoproteins (laminin, proteoglycans, vitronectin (VN), and FN); these macromolecules are predominantly produced by stroma cells [4]. The most abundant components of the ECM are collagens type I-XI, whereas other noncollagenous proteins, such as FN, laminin, tenascin, and elastin, only comprise approximately 10−15% of total proteins in the ECM [64]. The distribution of ECM protein changes between the endosteal and the perivascular niche; the endosteal niche consists primarily of collagen type I and FN in contrast to the more vascularized zone where more laminin is present [65,66]. Further supporting the integrity of the BM ECM integrity are the glycoprotein proteoglycans with large glycosaminoglycan (GAG) side chains. GAGs are polysaccharides chains of repeated disaccharides that are anchored to the core protein [67]. In total, there are four families of GAGs: heparin/heparan sulfate, chondroitin/dermatan sulfate, keratan sulfate, and hyaluronate [68].

Changes in mechanical properties within the BMM directly affect residing cells; for example, stem cell behavior is dependent on tissue stiffness, which is in turn dependent on the ECM composition and organization [63]. Methods such as rheology are able to determine the stiffness of material; however, as they often require dismembering the sample, studying the stiffness of an intact cavity brings many hurdles. There have been numerous studies that have characterized elastic and viscoelastic properties of cortical and trabecular bones measured as the Young/elastic modulus (ratio of stress to strain) and reported in Pascal (Pa). Measurements of the BM alone consider it to be a purely viscous tissue with reported values ranging from 1 to 100 Pa [69-71]. The structural complexity of bone impedes mechanical measurements; therefore, approaches such as sample freezing, dehydration, jet washing, polishing, homogenizing, sectioning, and fracturing have been explored [72]. Some studies showed that cells encapsulated close to the bone surface are under an elastic modulus of 40−50 kPa, whereas the central region presents about 3 kPa [73]. Other work investigating porcine BM used a minimal deconstructed sample approach and also found a heterogeneity within the BM. The group reported a Young modulus at physiological temperature (35 °C) between perivascular and endosteal niche ranging from 0.25

to 24.7 kPa [74]. More recent work analyzing murine bone samples used another minimal sample processing approach on four distinct regions of interest (cortical bone, growth plate, metaphysis, and BM in the diaphysis). These regions were analyzed using atomic force microscopy [72]. The overall elastic moduli measurements were much lower compared with previous studies by other groups. Chen et al. reported a mean Young modulus for the BM in the diaphysis of 0.14 kPa, indicating that the BM is very soft [72]. In addition, they determined the viscoelastic properties of all four regions, supporting the theory that the BM is rather viscoelastic than purely elastic. Similar to their findings analyzing the elasticity, all regions also displayed a high heterogeneity in regards of viscoelasticity. Analyses found a mean viscoelasticity of the BM of 0.52 kPa, which is considerably higher than elasticity values because previous measurements did not take into account viscous effects. In addition to elastic and viscoelastic properties, biophysical forces such as hydrostatic pressure and fluid shear stress are also additional factors within the BM directly affecting HSCs [73,75-77].

ECM Function

The ECM can function as a reservoir for GFs that are distributed and presented by proteins and proteoglycans of the ECM to HSCs [78]. This enables direct cell adherence to the ECM. Metalloproteinases secreted by cells remodel the components of the ECM and thus induce the release of GFs. A long-lasting view was that proteoglycans act as a sink/net for GFs that, once released, are present in soluble form [78,79]. However, some GFs actually bind to their matching receptors at the cell membrane as a "solid phase" ligand using heparan sulfate as a cofactor, indicating that the GFs are bound and presented by GAG chains of the ECM [78,79]. Examples of GF presentation are fibroblast growth factors (FGF) and vascular endothelial growth factor (VEGF) that bind to the proteoglycan heparin and heparan sulfate and are therefore presented to their matching receptors. Furthermore, GFs can be bound directly by the ECM proteins themselves. FN and VN can both bind directly to hepatocyte growth factor (HGF), forming complexes of the HGF receptor (Met) with integrins, resulting in enhanced cell migration [78].

ECM–HSC Interaction

As well as the influence of other cell types, the ECM is proposed to exhibit extrinsic cues that can influence HSC differentiation, lineage commitment, proliferation and apoptosis [73]. Cell–ECM contact is enabled through integrins expressed on the cell-surface that are comprised of an alpha and beta subunit [80]. HSCs and HSPCs express a variety of integrins. They are a family of transmembrane receptors involved in ECM–HSC interactions as well as adhesion/anchorage and homing of HSCs. Synergistic signaling with integrins and GF receptors has been observed; cells bind via integrins to the ECM, which subsequently presents GFs in close proximity that can be simultaneously bound via matching GF receptors on the cell [79]. The integrins VLA-4 and VLA-5 are specific for FN, α6β1 is specific for laminin, and α2β1 is specific for collagen [81]. Integrin–FN/collagen interactions can result in a blockage of cell-cycle progression at the S phase in HSCs [82,83]. Further, FN has been shown to promote long-term maintenance and expansion of HSCs in vitro [84,85].

After integrin/ECM binding, integrin clustering can lead to the formation of supramolecular complexes that form focal adhesion points. These adhesion points connect the ECM with the actin cytoskeleton of the cell [78]. Thus, integrins connect the extracellular environment with the intracellular cytoskeleton, which has a direct impact on cell migration, proliferation, quiescence, survival, and differentiation [63]. One example for regulating HSC homing is hyaluronic acid (HA), which binds the surface marker CD44 in HSCs. Cytoskeletal linker proteins further link all signals coming from the CD44 receptor to the cell's actin cytoskeleton, thus trigging transduction pathways that can activate adhesion molecules [86]. Culturing HSCs in ECM components such as collagen I results in slower cell expansion compared with liquid culture and enhanced colony-forming unit cell potential, indicating sustained differentiation potential [83]. Compared with two-dimensional (2D) cultures, these 3D cultures display an upregulation of genes involved in GF and cytokine transcription well known to maintain and regulate HSCs and their cell-cycle activity [83].

ECM–MSC Interaction

In addition to HSCs, MSCs are fine-tuned by the mechanical properties of the BM ECM. Stiffness, surface mobility, and topography are key differentiation factors for MSCs [87-90]. A rigid substrate favors osteogenesis, whereas a softer substrate favors adipogenesis. The degree of cell spreading is important for differentiation, as demonstrated when single cells were cultured on micropatterned islands (1,024, 2,025, and 10,000 μm^2) consisting of FN printed onto polydimethylsiloxane substrates. Using a mixed media that favored both osteogenesis and adipogenesis, there was a distinct shift from predominantly adipogenesis with the small islands to osteogenesis with the large islands [79]. The shape of the space has also been shown to influence MSC differentiation with rectangles with high aspect ratios favoring osteogenesis and pentagonal symmetry with long concave curves favoring adipogenesis [82]. In another study, MSC differentiation was evaluated through culture on various topographies. Polymethylmethacrylate substratum was embossed with nanopits in different configurations to evaluate osteoblastic differentiation of MSCs in the absence of osteogenic stimuli. Highly ordered nanotopographies produced low to negligible cellular adhesion and osteoblastic differentiation. Cells on random nanotopographies exhibited a more osteoblastic morphology, whereas a disordered nanodisplaced topography significantly increasing osteospecific differentiation [81]. MSC cell behavior can also be altered using cell compliant polyacrylamide gels incorporating type 1 collagen to change the stiffness. Soft gels (~0.1–1 kPa) caused MSCs to adhere, spread, and exhibit a branched filopodia-rich morphology and undergo neuro-induction, whereas stiff gels (~25–40 kPa) resulted in polygonal MSCs similar in morphology to osteoblasts that expressed osteogenic differentiation markers [80]. Soft substrates have been shown to cause a lack of stress fibers and focal adhesions points in MSCs, with more rigid substrates being highly adhesive and favoring osteogenesis [91-93]. Understanding and adjusting these factors can facilitate a multipotent state of MSCs, supporting the HSCs during homeostasis and stress situations. Culturing MSCs in a collagen-containing matrix strengthens the clonal proliferation of MSCs [94]. As a stromal population, MSCs produce ECM proteins, such as collagen type I, FN, and osteopontin, ultimately manipulating the composition of the ECM. MSCs encapsulated into hydrogels secrete and assemble ECM proteins that influence not only other cells residing in the ECM but also themselves by altering the rigidity of the surrounding microenvironment [95].

MODELING THE BMM

Expansion and maintenance of HSCs properties ex vivo have been challenging given the tendency of these cells to differentiate and lose their self-renewal capacity over time once removed from the BMM. This feature is due to the lack of biophysical and biochemical cues from the native BMM. Recent advances in the field have identified important signaling pathways required to sustain phenotypically functional HSPCs ex vivo. This has paved the way to develop chemically defined media, polymers and ECM components necessary to enhance HSPCs expansion ex vivo, fundamental for future translational medicine approaches such as gene editing and for modeling the BMM. In vitro modeling of the BMM is emerging as an important concept in biomedical research for studying normal and malignant hematopoiesis. Recent developments in biomaterial and bioengineering approaches are enabling researchers to reconstruct elements of the BMM in vitro. However, simulating the complexity of the BMM in vitro is extremely challenging when taking into account; architecture, cell composition, cell–cell interactions, structural differences, and the composition of the ECM as well as the availability of extrinsic molecular cues from GFs and cytokines. To reconstruct the BMM ex vivo, first, we need to take into consideration different cellular compartments as well as the scaffold/ECM of the niche. Specifically, one has to include cell–cell interactions known to support HSCs. Therefore, the inclusion of stroma cells, such as MSCs and ECs, is indispensable. The function of the ECM in stability and release of GFs also needs to be considered, in particular incorporation of natural ECM components, such as different types of collagen, laminin, FN, or VN, or synthetic matrixes. The stiffness of these components must closely match the BM niche, as material stiffness and elasticity directly affects cellular behavior. In addition, soluble factors, such as GFs, cytokines, and chemokines, need to be able to freely diffuse through any matrix to be fully accessible to all cells. To incorporate the majority of these characteristics, we must move away from traditional 2D tissue cultures and focus on more physiological 3D approaches (Figure 2). Many 3D approaches focus on the formation of spheroids. Spheroids are cellular aggregates and the most common method to allow 3D culture of cells that would normally be adherent in 2D culture. MSCs cultured in spheroids display many differences compared with cultures in monolayers, including altered cell morphology and size, changes in expression of cell-surface antigens, altered gene expression profiles, and enhanced ability to differentiate to osteogenic and adipogenic lineages [96-99]. In particular, MSC spheroids exhibit enhanced quiescence, stemness, and expression of

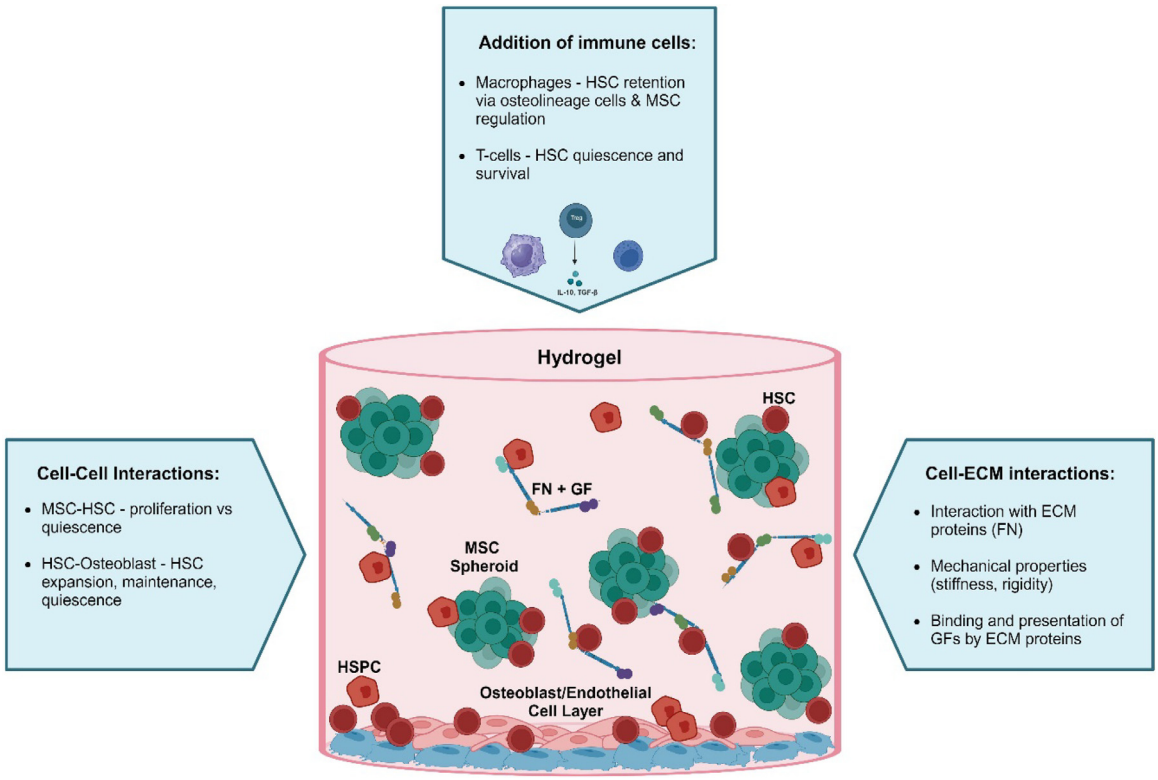

Figure 2 Schematic diagram representing 3D in vitro approaches to BMM modeling. Three-dimensional models need to recapitulate the important physical and chemical features of the BMM to sustain HSC properties ex vivo. Three-dimensional models often focus on the formation of MSC spheroids that are embedded into hydrogels (natural or synthetic) to provide the physical features of the BMM (stiffness, rigidity, and elasticity). Inclusion of additional ECM proteins (collagen, laminin, FN, VN, or synthetic matrixes) can further enhance stability and release of GFs. Additional cell populations can either be seeded in the bottom of the well before gelation, seeded in the liquid hydrogel solution before gelation, or introduced by seeding on top of the matrix after hydrogel gelation. Important considerations when constructing a 3D model are cell-binding sites, cell migration, and the ability of GFs and nutrients to freely diffuse through the system. Created with BioRender.com.

VEGF, HGF, and CXCL12 among other factors known to sustain HSCs. Including ECM components into spheroid models increases the applicability of cell types and the regulation of spheroid formation and also enables better disease modeling [100]. Using hydrogels, cells are cultured within a network of swollen polymeric fibers, with either natural or synthetic backbones. Formation strategies include spontaneous gelation or photo-initiated formation. Examples for biological scaffolds include Matrigel™, collagen, alginate, and fibrin [101]. Cells can either be seeded on top of a matrix or resuspended in the liquid hydrogel solution before gelation. Biological scaffolds are more than just physical support; they can also deliver GFs, hormones, and other compounds regulating the residing cells [102,103]. Application-specific scaffolds, therefore, need to be carefully chosen based on their composition. If no scaffold is provided for their growth, the spheroids are forced to produce their own ECM, containing collagen, hyaluronan, and FNs. Synthetic scaffolds based on polymers can negate unwanted interactions between ECM and cells. These scaffolds enable a more controllable matrix that can be tuned in regard to stiffness and degradability. Biologically inert polymer hydrogels circumvent problems arising from biological hydrogels. For example, polyethylene glycol (PEG) is often used due to its nontoxicity and nonimmunogenicity [101,103]. Additional ECM specific proteins can be incorporated into PEG gels, such as FN, which is useful for GF presentation [102]. Another bioengineering approach is to use synthetic materials (porous tantalum, polyurethane, poly D L-lactide-co-glycolide, polyethersulfone and nonwoven polyethylene terephthalate fabric) to recreate the honeycomb-like architecture of the BM using soft or rigid scaffolds [103]. Synthetic materials provide a large surface area for cell adhesion and increased porosity, allowing cell migration and nutrient exchange [104]. ECM proteins need to be incorporated onto the synthetic scaffolds to overcome the lack of cell-binding sites before introducing cells to the system. These synthetic scaffolds have shown some promise in supporting HSCs in vitro [85,105-107]. In addition, the modeling of the endosteal or perivascular niche can be recapitulated by adding additional osteoblast or ECs; however, careful consideration of growth requirements and seeding densities are required when constructing multicellular models. Immune regulation by T-cells, natural killer cells, and macrophages could also be investigated using a more definitive model. The introduction of additional HSC-supporting cells would provide a higher levels of cellular and molecular complexity to replicate microenvironmental-induced signaling.

CONCLUSION

Compared with traditional 2D culture systems, 3D models offer a more powerful system that can reflect in vivo cell morphology, cell polarity, gene expression, and tissue architecture, thus serving as a bridge between in vitro and in vivo models. By recapitulating the BMM properties using 3D in vitro systems, it will better inform our understanding of the role played by the BM in steady-state hematopoiesis, disease development, and subsequent therapeutic targeting of hematological malignancies in the future.

Conflicts of Interest Disclosure

The authors declare no competing interests associated with the manuscript.

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Authorship Contribution

CB and KN wrote parts of the manuscript. HW prepared the figures and helped write the manuscript.

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