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- A detailed overview of how cells within the bone marrow microenvironment (BMM) support HSCs
- How the mechanical properties of the extracellular matrix influence stem cell maintenance within the BMM and *ex vivo*
- Considerations when constructing BMM models

Complexities of modelling the bone marrow microenvironment to facilitate haematopoietic research

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Abstract

Haematopoiesis occurs in the bone marrow (BM), within a specialised microenvironment referred to as the stem cell niche, where the haematopoietic 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*, which supports quiescence and self-renewal and a more *vascular/peri-sinusoidal niche* that promotes proliferation and differentiation. Both associate with supporting mesenchymal stromal cells (MSCs). 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 MSCs 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 behaviour 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 haematopoiesis. 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 HSCs
- How the mechanical properties of the extracellular matrix influence stem cell maintenance within the BMM and *ex vivo*
- Considerations when constructing BMM models

Bone marrow microenvironment

Human haematopoiesis occurs in the bone marrow (BM) of the axial skeleton, which encompasses the cranium, sternum, ribs, vertebrae, and ilium [1]. Haematopoietic activity occurs in the tissue of the red marrow, which is supported and regulated by a unique non-haematopoietic cellular network/milieu. Initially red marrow is evenly distributed, but it becomes restricted to the proximal regions of the bone with age and replaced by the fatty yellow marrow [2]. Haematopoiesis is supported by the BM microenvironment (BMM), a vascularised space made up of non-haematopoietic cells and an extracellular matrix (ECM), which regulates haematopoietic stem cell (HSC) activity. Non-haematopoietic cells include osteolineage cells, leptin receptor $(LepR)^+$ mesenchymal stromal cells (MSCs), nerve cells, vascular endothelial and sinusoidal cells. These cells are spatially organised into distinct niches creating a unique microenvironment for haematopoietic stem and progenitor cell (HSPC) development and maintenance [1, 3-5]. The interaction between HSPCs within the different niche areas is mediated by cell surface receptors, adhesion molecules and the exchange of cytokines and growth factors (GFs), illustrated in Figure 1 [4]. Below, we discuss how each of the components influences haematopoiesis and their requirement when recapitulating aspects of the BMM *ex vivo*.

Cellular components of the bone marrow niche

Mesenchymal stromal cells

One main non-haematopoietic cell type, which is indispensable for the regulation of the BM niche and the support of HSCs is the MSC. MSCs make 0.001-0.01% of the total BM cell numbers and are required for tissue regeneration and immunomodulation [6]. MSCs are multipotent cells with tri-lineage differentiation capacity, leading to the formation of osteoblasts, adipocytes and chondrocytes. The International Society for Cellular Therapy outlined the criteria MSCs must fulfil to be classified as such: (a) MSCs 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; (c) they must be capable to differentiate 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 peri-sinusoidal 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 leptin receptor (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 and within this group, populations showing unique expression of CXCL12 can emerge [1]. Those found to

localise 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 haematopoietic lineage-restricted progenitors but not HSCs [15]. Another population of mostly quiescent collagen expressing $LepR^+$ MSCs are found in close proximity to the peri-arteriolar and trabecular bone surface where they have a bias towards osteolineage differentiation. Expression of PTEN and more recently osteolectin were found to specify the priming of $LepR^+$ cells towards osteolineage differentiation and contribute towards HSC mobilization [11, 14].

CXCL12-abundant-reticular (CAR) cells, which are derived from $LepR^+$ cells, produce the majority of CXCL12 in the BM and localise close to the sinusoids [16]. Initial seeding of HSCs in the BM is 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 towards 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 little expression of CXCL12 and SCF, 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 haematopoiesis 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 whilst others like Ang-1 indirectly influence HSC behaviour 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., 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., 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 pre-fibroblasts. Trajectory analysis predicted a hierarchical organisation consisting of two interlinked differentiation trajectories with MSSC at the apex. The differences in these progenitors were further characterised via colony forming capacity and their ability to differentiate into osteoblasts, adipocytes and chondrocytes. *In silico* cell-to-cell interaction analysis predicted that haematopoietic 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. BM biopsies in-situ localisation analysis identified that SPP1-expressing OCs were located close to the endosteal region and CD271 single positive stromal cells including MSSCs were localized in the perivascular and stromal regions, respectively, suggested the possibility that different stromal cells provide specialized niches for hematopoietic cells in different locations [21]. These studies provide a 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 whilst osteocytes which have a limited differential capacity are incorporated into the bone architecture as they mature. It was the first population of BM cells associated with the regulation of HSPC cells, which 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 haematopoiesis through the provision of various supportive GFs. Localization studies [25-27] have mapped a subset of primitive HSCs to be preferential localization in the endosteal regions of the trabecular bone. 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]. Whilst Notch signalling in HSCs has been reported to be dispensable in adult haematopoiesis, specific interaction between HSCs and Jag1-producing osteoblasts has been observed to be one of the mechanisms which can promote HSC quiescence [1, 28]. There are additional HSC maintenance factors expressed by osteoblasts such as thrombopoietin (TPO) and angiopoietin 1 (ANGPT1) which 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 haematopoietic cells [33]. Interestingly, work by Zhao et al., proposes a role in the protection of unique quiescent HSC

subsets against chemotherapeutic stress from N-cadherin expressing MSCs [27]. These cells which have tri-lineage differentiation capacity are found in close proximity to the endosteal surface of the trabecular bone region and exposure to stress made them biased towards an osteoblast differentiation programme. This is further supported by work from Dominici et al., which showed preferential megakaryocyte (MK)-mediated expansion of this N-cadherin-expressing population in irradiated mice to re-establish a supportive endosteal niche for HSC reconstitution [7]. Overall, this emphasizes the supportive role played by osteolineage 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 which manage HSC and HSPC activity [1, 17, 34, 35]; deletion of these factors interrupts HSC maintenance at steady state *in vivo*. Endothelial cells can be further subdivided into arteriolar (AEC) and sinusoid endothelial cells (SEC) [1, 36], with AEC producing almost all the endothelial-derived SCF. Although the overall abundance of endothelial cells is comparable to MSCs, the expression of CXCL12 and SCF is much lower [37, 38]. In addition to SCF, AEC 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 puts nearby HSPCs in a quiescent state. Whereas 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 endothelial cells via 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. Though nerve fibres are not mandatory for the maintenance of HSCs, they are vital for the regeneration of haematopoiesis after chemotherapy [44]. Neural signals also modulate the process of haematopoiesis by controlling the circadian-mediated trafficking of HSPCs from the BM and regulating HSC quiescence [1]. HSPC release into the circulation happens in a circadian manner, this is 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 MSC 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 favour of osteoclasts, which mediate bone resorption. Consequently, CXCL12 is downregulated in the process, which increases HSC egress from the BM [37, 49]. In addition, non-myelinating Schwann cells regulate HSC quiescence through their activation of TGFβ leading to cell-cycle arrest [50].

Additional BM cells which support HSCs

Another stroma-derived component interacting with HSCs are adipocytes. With aging, the BM of adults becomes increasingly fatty. Adiponectin, a protein secreted by adipocytes, inhibits haematopoietic activity and impairs proliferation [51]. This in combination with transplantation data, which show a quicker BM recovery when treating mice with an adipocyte inhibitor suggest 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. Localisation studies using 3D-images have observed a co-localisation of a HSC subset with MKs and depletion of the latter induces HSC proliferation, indicating MKs are important in HSC quiescence [52]. Quiescence of HSCs 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 remodelling by relocalising 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 pre and post radioblation, led to enhanced MK function and HSC engraftment in mice, via reducing the duration of regeneration to re-establish a quiescent state [1, 54].

Other cell types that play a role in HSC behaviour include macrophages, which can 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 and are attracted by CXCR4/CXCL12 axis and 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 haematopoiesis 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 evidenced from allogenic stem cell transplant, where they co-localize with HSC directly after transplant, promoting 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 (MS)-based quantification methods are providing valuable information on the cells within the BMM. A comprehensive study by Hennrich et al., analysed 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, 56 identified to 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 characterise MSC subpopulations [58].

Another study isolated MSCs and osteoblasts from the BM of healthy donors and cultured them in serum free media for 48 hrs to collect the supernatant (conditioned media) for analysis. They identified a total of 1,379 proteins for the MSCs and the osteoblasts, with more than 90% similarity between the two cell types. The majority of released proteins fell 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); 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 haematopoietic homeostasis.

Serum free and chemically defined ex vivo expansion of HSCs

Several studies have been conducted to optimise 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 to be essential for HSPC expansion, with higher

concentrations of TPO (100ng/mL) and lower concentrations of SCF (10ng/mL) being optimal. Expansion was further enhanced by culturing on fibronectin (FN) and replacing serum albumin with the synthetic caprolactam-based polymer, polyvinyl alcohol (PVA). For long-term expansion, full media changes were necessary to sustain long-term HSC activity, as secreted cytokines and chemokines were identified to be inducing differentiation, especially IL-6 and CCL2-4 [60]. Although effective for mouse HSPCs expansion, results for human HSPCs was more limited, with a 3- to 4-fold expansion observed. By examining the signalling pathways activated by TPO and SCF, differences between mouse and human HSPCs were observed. Most notably decreased levels of PI3K and AKT activation 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 to clinical translation in the future [62].

The Extracellular matrix (ECM)

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 growth factors (GFs) across the BM, thus directly affecting stem cell fate and regulation of haematopoiesis [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 behaviour regulation. Consequently, ECM composition and structure plays a vital part 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, 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, while other non-collagenous proteins such as FN, laminin, tenascin and elastin only make 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 vascularised zone where more laminin is present [65, 66]. Further supporting 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 (CS/DS), keratan sulfate and hyaluronate [68].

Changes in mechanical properties within the BMM directly affects residing cells; for example stem cell behaviour is dependent on tissue stiffness, which is in turn dependent on the ECM composition and organisation [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 characterised elastic and viscoelastic properties of cortical and trabecular bones measured in 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 1-100 Pa [69-71]. The structural complexity of bone impedes mechanical measurements, therefore approaches such as sample freezing, dehydration, jet washing, polishing, homogenising, 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 (35^0C) between perivascular and endosteal niche ranging from 0.25-24.7 kPa [74]. More recent work analysing 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 analysed using Atomic Force Microscopy [72]. The overall elastic moduli measurements were much lower compared to previous studies by other groups. Chen *et al*. reported the 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 viscoelastic properties of all four regions, supporting the theory that the BM is rather viscoelastic than purely elastic. Similar to their findings analysing 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 a lot higher than elasticity values due to previous measurements not taking into account viscous effects. Additionally 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 can get 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 can 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 "solid phase" ligand using heparan sulfate as a co-factor, 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, comprised of an alpha and beta subunit combination [80]. HSCs and HSPCs express a variety of integrins. They are a family of transmembrane receptors involved in ECM-HSC interaction, adhesion/anchorage and homing of HSCs. Synergistic signalling with integrins and GF receptors has been observed, meaning that cells bind via integrins to the ECM, which in return presents GFs in close proximity, which can be simultaneously bound via matching GF receptors on the cell [79]. The integrins VLA-4 and VLA-5 are specific for FN, $\alpha 6\beta 1$ specific to laminin and $\alpha 2\beta 1$ to collagen [81]. Integrin-FN/collagen interaction can result in a blockage of cell cycle progression in 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 - 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 a slower cell expansion compared to liquid culture, enhanced colony-forming unit cell

(CFU-C) potential, indicating sustained differentiation potential [83]. In comparison to two dimensional (2D) cultures, these three dimensional (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

Not only HSCs, but also 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 favours osteogenesis and a softer substrate favours adipogenesis. The degree of cell spreading is important for differentiation, as demonstrated when single cells were cultured on micro-patterned islands (1024, 2025 and 10,000 μm^2) consisting of fibronectin printed onto polydimethylsiloxane (PDMS) substrates. Using a mixed media which favoured 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 favouring osteogenesis and pentagonal symmetry with long concave curves favouring 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 behaviour 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 filodopia-rich morphology and undergo neuro-induction whereas stiff gels (~25-40 kPa) resulted in polygonal MSCs similar in morphology to osteoblasts which expressed osteogenic differentiation markers [80]. Soft substrates have been shown to cause a lack of stress fibres and focal adhesions points in MSCs, with more rigid substrates being highly adhesive and favouring 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 is able to strengthen the clonal proliferation of MSCs [94]. MSCs as a stromal population produce ECM proteins such as collagen type I, FN and osteopontin, ultimately manipulating the ECM in its composition. 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].

Modelling the BMM

Expansion and maintenance of HSCs properties *ex vivo* has been challenging, due to their tendency to differentiate and lose their self-renewal capacity over time once removed from the BMM. This is due to the lack of biophysical and biochemical cues from the native BMM. Recent advances in the field have identified important signalling 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 modelling the BMM. *In vitro* modelling of the BMM is emerging to be an important concept in biomedical research for studying normal and malignant haematopoiesis. 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-to-cell interactions, structural differences, the composition of ECMs and availability of extrinsic molecular cues from GFs and cytokines. In reconstructing the BMM *ex vivo*, firstly 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 endothelial cells 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 behaviour. 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 way to allow 3D culture of cells which would normally be adherent in 2D culture. MSCs cultured in spheroids display many differences compared to 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 VEGF, HGF and CXCL12 amongst 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 modelling

[100]. By using hydrogels, cells are cultured within a network of swollen polymeric fibres, with either natural or synthetic backbones. Formation strategies include spontaneous gelation or photo-initiated formation. Examples for biological scaffolds are 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 fibronectins. Synthetic scaffolds based on polymers can negate unwanted interactions between ECM and cells. They 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, poly (ethylene glycol) (PEG) is often used due to its non-toxicity and non-immunogenicity [101, 103]. Additional ECM specific proteins can be incorporated into PEG gels such as FN, which can serve for GF presentation [102]. Another bioengineering approach is to use synthetic materials (porous tantalum, polyurethane, poly D L-lactide-co-glycolide, polyethersulfone and non-woven 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 prior to introducing cells to the system. These synthetic scaffolds have shown some promise in supporting HSCs *in vitro* [85, 105-107]. In addition, the modelling of the endosteal or perivascular niche can be recapitulated by adding additional osteoblast or endothelial cells, however careful consideration of growth requirements and seeding densities are required when constructing multi-cellular models. Immune regulation by T-cells, natural killer (NK) cells and macrophages could also be investigated in 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 signalling.

Conclusion

Compared to traditional 2D culture systems, 3D models offer by far a more powerful toolbox, which 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 haematopoiesis, disease development and subsequent therapeutic targeting of haematological malignancies in the future.

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Authorship

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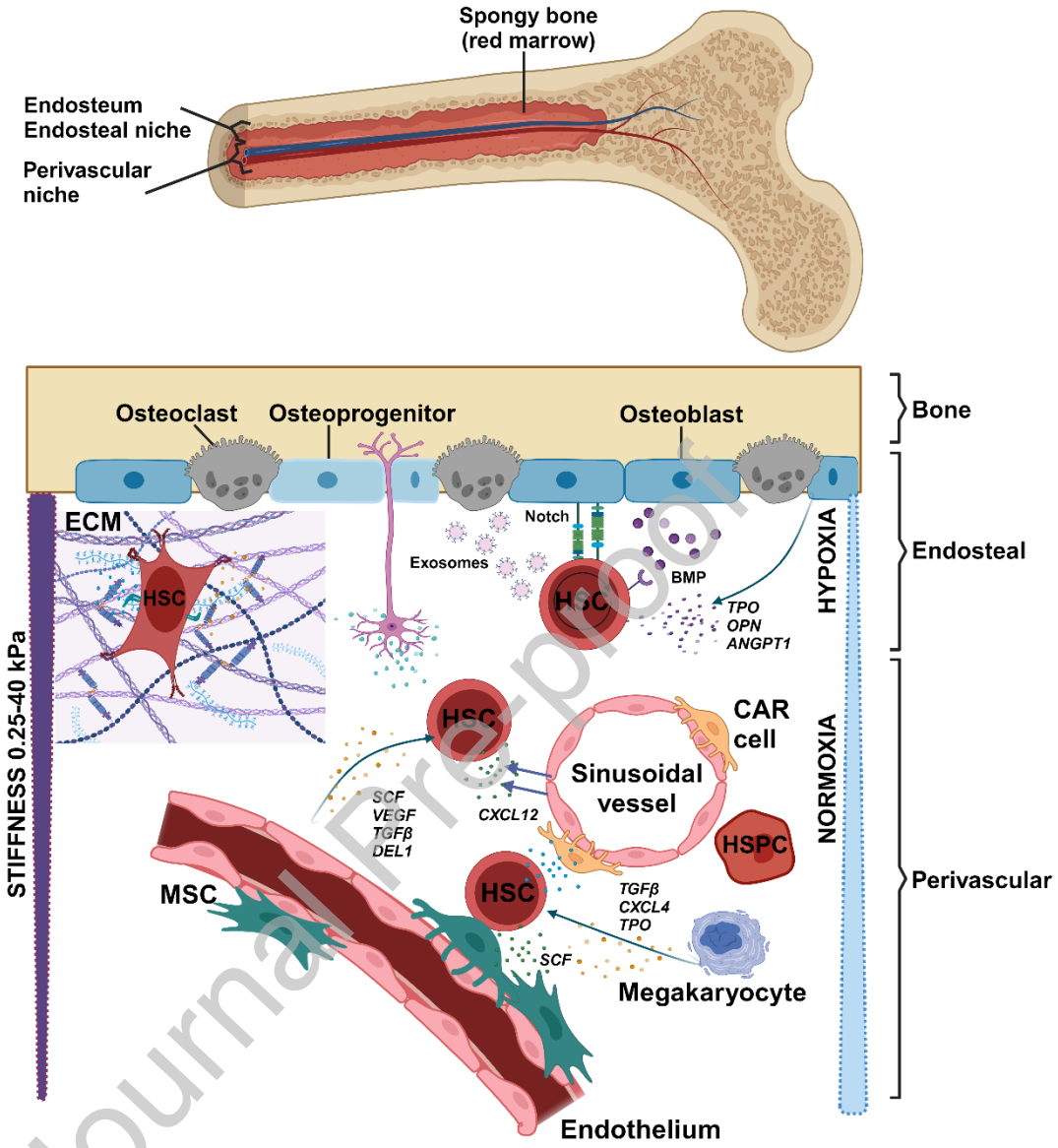
Abbreviations:

Ang-1	Angiopoietin 1
AEC	Arteriolar endothelial cells
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	Haematopoietic stem cells
HSPC	Haematopoietic 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)

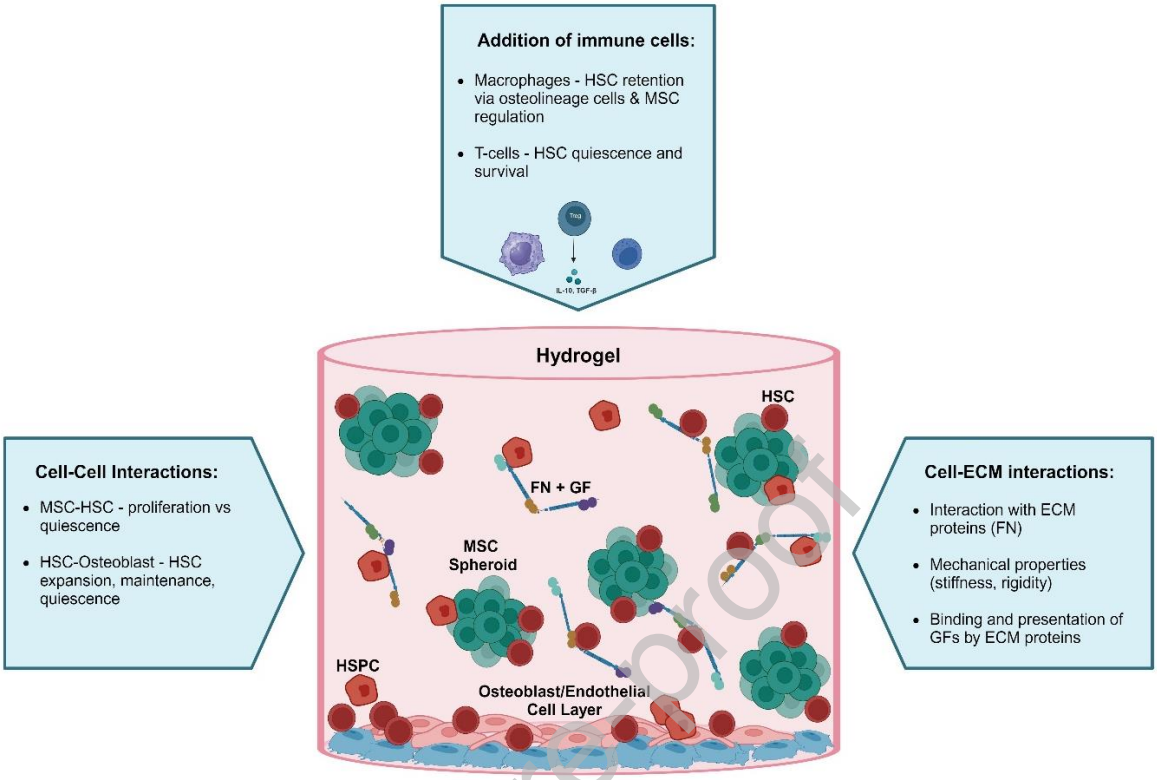
PVA	Polyvinyl alcohol
SC-RNA-Seq	Single-cell RNA-Sequencing
SEC	Sinusoid endothelial cells
SCF	Stem cell factor
SNS	Sympathetic nervous system
TGFb	Transforming growth factor beta
TPO	Thrombopoietin
Treg	Regulatory T-cell
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VN	Vitronectin

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



The BM is the primary location for haematopoiesis and is 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 circulation. The ECM consist of collagenase and non-collagenase (laminin, VN, FN, tenascin, and elastin) proteins and proteoglycans with long glycosaminoglycan (GAG) side chains. Cell-ECM interaction is enabled through cell-specific receptors, called integrins. Integrins ligate to peptide motifs of the ECM, thus triggering phosphorylation cascades that enables direct connection between integrins and the actin cytoskeleton. The BM niche consists of a variety of cell types, which can all influence the cell fate of HSCs and HSPCs. The niche can be sub-divided into two zones, the more rigid hypoxic endosteal osteoblastic niche and the more oxygenated less rigid peri-vascular niche. In the peri-vascular zone different types of stroma cell populations are localized around the arterioles and sinusoids, all expressing factors such as SCF and CXCL12 and thus supporting HSC. Both sinusoids and arterioles are lined with endothelial cells, also expressing HSC supporting factors. In the endosteal zone osteoblasts secrete HSC-supporting or inhibiting factors such as TPO, OPN, BMP and ANGPT1. Additionally, the HSCs own progeny can directly stimulate HSCs. Localization of HSCs 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 peri-vascular zone. Created with BioRender.com.

Figure 2: Schematic diagram representing 3D in vitro approaches to BMM modelling



3D models need to recapitulate the important physical and chemical features of the BMM in order to sustain HSC properties ex vivo. 3D models often focus on the formation of MSC spheroids which are embedded into hydrogels (natural or synthetic), to provide the physical features of the BMM (stiffness, rigidity, 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 prior to gelation, or 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.

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