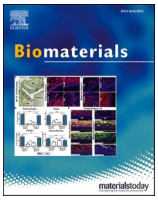
Biomaterials 286 (2022) 121568

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials



Current insights into the bone marrow niche: From biology *in vivo* to bioengineering *ex vivo*

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ARTICLE INFO

Keywords:
Hematopoietic stem cells
Bone marrow niche
Niche engineering
Biomaterials

ABSTRACT

Hematopoietic stem cells (HSCs) are fundamental to the generation of the body's blood and immune cells. They reside primarily within the bone marrow (BM) niche microenvironment, which provides signals responsible for the regulation of HSC activities. While our understanding of these signalling mechanisms continues to improve, our ability to recapitulate them *in vitro* to harness the clinical potential of the HSC populations is still lacking. Recent studies have applied novel engineering techniques combined with traditional *in vitro* work to establish *ex vivo* BM niche models. These models exhibit promising potential for research and clinical applications. In this review, BM niche factors that regulate the HSCs *in vivo* are discussed and their applications in the engineering of BM biomaterial-based platforms are considered. Many questions remain regarding the heterogeneity of niche components and the interactions of HSCs with their microenvironment. A greater understanding of the niche would help to elucidate these remaining questions, leading to the development of novel therapeutic tools.

1. Introduction

Hematopoietic stem cells (HSCs) are a rare cell population that is characterized by their ability to self-renew and differentiate into cells of the blood system throughout the lifetime of the organism [1–3]. They primarily reside in a specialized microenvironment within the bone marrow (BM), termed the 'BM niche' [4]. The BM niche orchestrates HSC maintenance, proliferation, self-renewal and differentiation [5]. Over the past few decades, extensive work has been carried out to study BM niche physiology and elucidate how niche components regulate HSC behaviours (as reviewed in Refs. [6,7]). Notably, once removed from the *in vivo* niche and placed in *in-vitro* culture, HSCs rapidly lose their self-renewal capacity and multipotency [8,9]. Given the complexity of the BM niche microenvironment, the application of appropriate *ex vivo* models which could mimic the *in vivo* niche organization is limited, causing limited efficiency in maintaining long-term repopulating HSCs *in vitro*. This restricts research on HSCs, haematopoiesis, stemness and haematological diseases.

Recently, biomaterial and bioengineering approaches have provided novel strategies to reconstruct the *in vivo*-like niche for HSCs [10–12]. In general, these strategies aim to create two-dimensional (2D) or three-dimensional (3D) biomaterial-based cell culture systems, that recapitulate or mimic *ex vivo* biochemical and biophysical components from the BM niche. In the last few years, there has been a substantial increase in the development of more advanced platforms, such as microfluidic organ on chips (OOC) devices [13–16]. It is worth noting that several of these models have demonstrated their ability to support HSC proliferation and maintenance with satisfying outcomes to some extent [14,17]. Furthermore, these platforms have been utilized to recapitulate healthy or malignant haematopoiesis states in humans, enabling researchers to study the effects of clinically relevant drug or radiation exposures on BM, without requiring initial tests in animals [18,19]. Therefore, these models are a promising step toward non-animal-based technologies (NATs).

Here, we review the progress of the past several years in understanding the BM niche, emphasizing the cellular composition and biophysical cues within the BM niche that underlie HSC-niche communication during maintenance and differentiation. Moreover,

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https://doi.org/10.1016/j.biomaterials.2022.121568
Received 3 November 2021; Received in revised form 26 April 2022; Accepted 3 May 2022
Available online 7 May 2022

advanced techniques from recent BM-model engineering approaches which utilize several of these elements *in vitro* (2D and 3D biomaterial-based techniques) are compared. Their promising applications in research and in the clinic are highlighted as well. Finally, challenges and future directions of BM niche biology and BM niche bioengineering will be discussed.

2. Bone marrow niche biology

The components of the BM niche are highly heterogeneous. There has been a significant focus over the past several decades aiming to identify definitive HSC niche components within the BM microenvironment, including soluble factors, cellular contributions, oxygen tension, ECM (Extracellular Matrix) and physical factors (Fig. 1). The contribution of these factors is summarized below.

Schematic representation of the adult BM niche, showing various cell types and niche factors that directly or indirectly regulate HSC activities. Vascular endothelial cells and associated stromal cells, such as peri-arteriolar Nestin^{+} cells and NG2^{+} cells, perisinusoidal CAR cells and LepR^{+} cells, are key regulators of HSC maintenance. Osteoblasts line the endosteal surface and are also associated with HSC maintenance. Schwann cells may contribute to HSC quiescence. Hematopoietic cells, such as macrophages and megakaryocytes, are examples of HSC-derived progeny that can feedback and contribute to HSC maintenance or mobilization. These cells regulate HSC activities mostly via paracrine signals. The distribution of ECM across the BM is not random but highly organized. FN and collagen are closely associated with the bone matrix, while laminin is enriched along the vascular basement membrane. The central BM is soft (between 0.1 and 1 kPa), whereas the endosteum region is rigid (up to >35 kPa). The BM is hypoxic, despite being heavily vascularized. The deep sinusoidal regions have been found to have the lowest oxygen tension, and the endosteal regions have slightly higher oxygen tension. Hypoxia has been shown to affect HSCs in the BM niche.

BM, Bone marrow; HSC, hematopoietic stem cell; CAR cell, CXC-chemokine ligand 12 (CXCL12)-abundant reticular (CAR) cells; LepR + cell, leptin receptor (LEPR)-positive cells; NG2+ cells, neural–glial antigen 2 (NG2)-positive cells; SCF, Stem Cell Factor; CXCL12, C-X-C Motif Chemokine Ligand 12; TPO, Thrombopoietin; OPN, Osteopontin; Angpt-1, Angiopoietin 1; Vcam1, Vascular cell adhesion protein 1; G-CSF, Granulocyte colony-stimulating factor; TGF-β1, Transforming growth factor beta 1; DARC, Duffy antigen receptor for chemokines; FGF, Fibroblast Growth Factor; ECM, Extracellular Matrix; FN, Fibronectin; LM, Laminin; Col, Collagen.

2.1. Constituents of the HSC niche

2.1.1. Growth factors

Growth factors (GFs) are a broad class of small, secreted proteins. They act on specific target cell receptors and trigger intracellular signalling cascades that ultimately influence cellular behaviours [20]. In the BM niche, many niche-derived GFs have been described to orchestrate HSC behaviours such as maintenance, retention, proliferation, and mobilization [7]. The main niche GFs, their cellular sources and functions within the niche are summarized in Table 1.

In general, these GFs mediate effects through binding to their specific receptors on HSCs. For example, CXC-chemokine ligand 12 (CXCL12) can bind to CXC-chemokine receptor 4 (CXCR4)-expressing HSCs [21,22], and stem cell factor (SCF) mediates its effects through binding and activating the KIT receptor on HSCs [23]. Studies that disrupt GF-receptor binding, via antibody blocking or deleting expression using genetic approaches, demonstrate the BM niche to be significantly altered and normal haematopoiesis dramatically damaged [24–28]. Different GFs have been shown to modulate different HSC behaviours. For instance, SCF mainly promotes HSC maintenance [29], while Notch ligands induce HSC proliferation [30–32]. CXCL12 and Vascular cell adhesion protein 1 (Vcam1) regulate HSC retention and mobilization [28,33]. These suggest that the balance of HSC activities is tightly controlled by GF contributions. Further to this, niche GF homeostasis

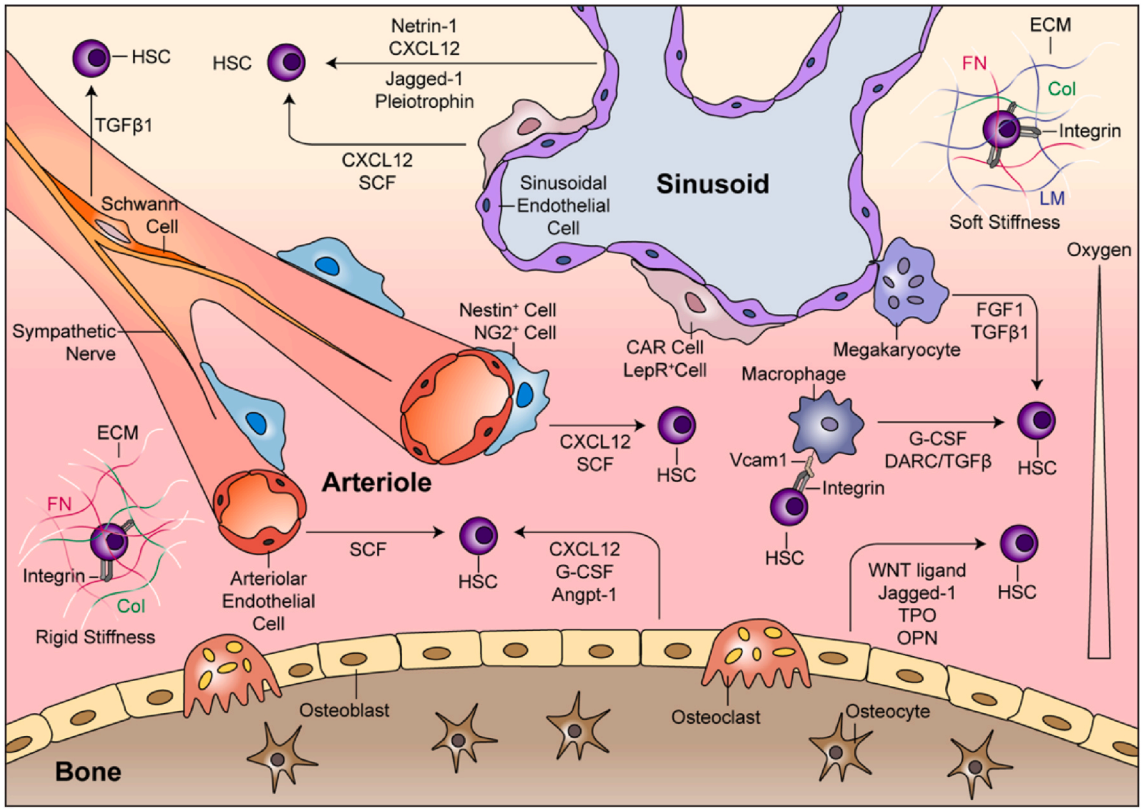

Fig. 1. A schematic of the HSC niche in adult BM.

Table 1
Locally secreted factors associated with HSC regulation in the BM niche.

Growth Factor	HSC Receptor	Niche cellular Source	Impact on HSCs	Reference
SCF	c-Kit	ECs	Induce HSC maintenance and promote HSC recovery after myeloablation	[37–39]
		CAR cells	Direct HSC engraftment	[40]
		Nestin$^+$ cells	Induce HSC maintenance	[29,37,41]
		LepR$^+$ cells	Promote HSC maintenance and enhance HSC regeneration after irradiated	[29,37,38,42]
		NG2$^+$ cells	Promote HSC maintenance	[29]
CXCL12	CXCR4	CAR cells	Promote HSC maintenance	[43]
		Nestin$^+$ cells	Induce HSC maintenance and retention	[28,29]
		LepR$^+$ cells	Promote HSC retention	[28]
		ECs	Maintain HSC quiescence, self-renew and retention	[28,29,43,44]
		NG2$^+$ cells	Maintain HSCs quiescence and retention	[29]
TPO	MPL	OBs	Maintain HSC quiescence	[45,46]
OPN	CD44	OBs	Restrict HSC pool	[47,48]
Angpt-1	Tie-2	OBs	Maintain HSC quiescence and self-renewal, and enhance survival under stressed	[49]
		LepR$^+$ cells	Promote HSC recovery after irradiation	[50]
Vcam1	VLA-4/ Integrin α4β1	ECs	Direct HSC homing	[51]
		Macrophage	Promote HSC retention	[33]
G-CSF	G-CSF receptor	OBs	Maintain HSC quiescence	[52]
		Macrophages	Maintain HSC retention	[53]
TGF-β1	TGF-β receptors	Schwann cells	Maintain HSC quiescence and self-renewal	[54]
		Megakaryocytes	Maintain HSC quiescence and enhance HSC expansion under stress	[36]
Notch ligand Jagged-1	Notch receptor	OBs	Support HSC self-renewal	[55]
		ECs	Support HSC self-renewal and proliferation	[30–32]
WNT ligands	Fizzled receptors	OBs	Maintain HSC quiescence and enhance HSC recovery under stress	[56]
Pleiotrophin	RPTP-β/ζ	ECs	Enhances self-renewal and BM retention, and accelerate hematopoietic recovery following myelosuppression	[57–59]
Netrin-1	Neogenin-1	ECs	Maintain HSC quiescence and self-renewal	[60–62]
DARC/TGFβ	CD82	Macrophage	Promote HSC quiescence	[63]
FGF1	FGF receptor	Megakaryocytes	Promote HSC proliferation enhance HSC recovery under stressed	[64,65]
FGF2		Unknown	Promote HSC recovery after stress	[66]

BM, Bone marrow; HSC, hematopoietic stem cell; OBs, Osteoblasts; CAR cells, CXC- chemokine ligand 12 (CXCL12)-abundant reticular (CAR) cells; ECs, Endothelial cells; LepR + cells, leptin receptor (LepR)-positive cells; NG2+ cells, neural–glial antigen 2 (NG2)-positive cells; SCF, Stem Cell Factor; CXCL12, C-X-C Motif Chemokine Ligand 12; TPO, Thrombopoietin; MPL, myeloproliferative leukaemia protein; OPN, Osteopontin; Angpt-1, Angiopoietin 1; Vcam1, Vascular cell adhesion protein 1; VLA-4, Very Late Antigen-4; G-CSF, Granulocyte colony-stimulating factor; TGF-β1, Transforming growth factor beta 1; RPTP-β/ζ, receptor protein tyrosine phosphatase (RPTP) beta/zeta; DARC, Duffy antigen receptor for chemokines; FGF, Fibroblast Growth Factor.

and biological functions can also be altered by stress, such as ageing, chemotherapy and irradiation (as reviewed in Ref. [34]). For example, some GFs are dispensable during normal haematopoiesis homeostasis but are required to promote hematopoietic regeneration after injury. These include fibroblast growth factor 1 (FGF1) and transforming growth factor (TGF) β1, which are shown to promote HSC expansion and re-establish HSC quiescence following chemotherapy treatment [35,36]. Thus, a detailed GF profile of the BM niche, how these GFs change in response to diverse stresses, and how they could be harnessed to *e.g.* promote hematopoietic recovery post-irradiation remains a focus for investigation.

2.1.2. Cellular components

Difficulties toward reconstituting the BM niche *in vitro* relate to its substantial cellular diversity. Classic genetic approaches have already contributed greatly to our understanding of BM niche cellular architecture, such as the deletion of molecular markers and Cre-mediated lineage tracing, followed by imaging or functional readouts. However, limited marker-based approaches probably result in the labelling of heterogeneous populations [67,68]. In the last few years, single-cell RNA-sequencing (scRNA seq), has shown promising potential to overcome such shortcomings, providing us with more detailed and systematic information in BM cellular architecture [69,70]. In general, stromal cellular components of the BM niche are rare (approximately 2% of total BM cells) but highly heterogeneous, mainly containing osteoblasts (OBs), mesenchymal stromal cells (MSCs), endothelial cells (ECs), fibroblasts, pericytes, nerve cells and smooth muscle cells [69–71]. Several MSC subpopulations have been identified as well, such as CXCL12-abundant reticular cells (CAR cells) [24], leptin receptor (LepR)+cells [37], nestin$^+$ cells [72], and neural–glial antigen 2 (NG2)+ cells [73].

2.1.2.1. Hematopoietic cells. The BM niche harbours various hematopoietic lineage cells. This includes HSCs, multipotent progenitors (MPPs), common lymphoid progenitors (CLPs), common myeloid progenitors (CMPs), as well as their differentiated progeny [7]. The HSC differentiation landscape follows a strict hierarchy, in that the most primitive HSCs and MPPs sit on the top; lineage-committed progenitors, such as CLPs and CMPs sit at the middle, and the terminally differentiated mature cells sit at the bottom [68,74]. Such hierarchical development involves progressive loss of self-renewal capacity, proliferation

ability, and lineage potentials, ultimately giving rise to various cell populations, including megakaryocytes, erythrocytes, monocytes, lymphoid cells, neutrophils, basophils, mast cells, and eosinophils, which are critical to maintain haematopoiesis homeostasis and refresh the blood and immune system throughout our life [68,75–78].

It should be noted that primitive HSCs can be classified into two subsets, long-term HSCs (LT-HSCs) and short-term HSCs (ST-HSCs) [79]. The main difference between these two cell populations is their capacity to re-establish haematopoiesis of the recipient after transplantation. Post transplantation and engraftment, ST-HSCs contribute to blood system reconstitution for approximately 3 months only [80], whereas LT-HSCs are able to engraft and re-establish hematopoietic homeostasis long-term and can undergo further serial transplantation [80]. LT-HSCs are, therefore, the most clinically valuable HSC subset. Yet, LT-HSCs are rare in the BM niche, and currently, there exist no reliable strategies to isolate and expand the subset. Phenotypically ST- and LT-HSCs are similar and cannot be distinguished using the well-defined surface markers typically used to separate hematopoietic lineages by flow cytometry. Recent years have seen investigation into identifying gene signatures and markers, such as CD49f (also known as integrin α6) [81], that are expressed by/on LT-HSCs, but not ST-HSCs, leading to potentially more refined identification, and thus investigation, of these two subsets [82].

In the BM niche, HSC activities are further regulated via their progeny. For example, conditional deletion of megakaryocytes significantly leads to HSC activation and proliferation, indicating that megakaryocytes may be closely associated with maintaining HSC quiescence. This regulation may act through the secretion of chemokines by the differentiated cells such as CXC-chemokine ligand 4 (CXCL4) [83], TGFβ1 [36,84] and FGF1 [64,65] (Table 1). Interestingly, megakaryocytes can also selectively regulate myeloid-biased von Willebrand factor (vWf)$^+$ HSCs, but not lymphoid-biased vWf$^-$ HSCs [85]. Conditional deletion of megakaryocytes can promote vWf$^+$ HSCs expansion and ablate their self-renewal capacity and reconstitution potential, whereas deletion had no effect on vWf$^-$ HSCs [85]. These results suggest that distinct niches for HSC subpopulations and their progeny may exist, which have distinct developmental potential. Collectively, HSCs are regulated in a feedback loop by their progeny.

2.1.2.2. OBs. OBs were the first cell population shown to influence HSC frequency both *in vitro* and *in vivo*. *In vivo*, a population of HSCs were shown to associate exclusively with cells with an osteoblastic phenotype that line the long bone. Then in a knockout mouse model lacking bone morphogenetic protein (BMP) receptor I, an increase in this HSC population was observed [86]. Further to this, Calvi et al. demonstrated that augmenting parathyroid hormone (PTH) signalling to increase osteoprogenitor or pre-OBs activation, could enrich LSK (Lin$^-$ Sca-1$^+$ c-Kit$^+$) cells (HSC-like cells) *in vivo* [55]. Further studies indicate that OB ablation could lead to reduced quiescence, long-term engraftment, and self-renewal capacity of HSCs [87]. These effects are mediated by the secretion of various molecules, such as Osteopontin (OPN) [47], Thrombopoietin (TPO) [46], granulocyte-colony stimulating factor (G-CSF) [52], Angiopoietin 1 (Angpt-1) [49] and Wnt ligands [56], notably except for CXCL12 or SCF [28,37,43]. Thus, collective evidence suggests a role of paracrine signalling by OBs in regulating HSC activities.

However, the evidence implicating OBs as direct regulators of HSC functions is still lacking. This regulation was thought to be a result of interactions between HSCs and OBs mediated by the adhesion molecule N-cadherin [86,88,89]. Conditional deletion of N-cadherin$^+$ OBs impairs HSC maintenance during steady state and delay haematopoiesis recovery under stress stimulation [86,88,89]. Subsequent studies dispute these findings, showing that conditional N-cadherin deficiency from either OBs or HSCs, has no effect on haematopoiesis [90,91]. Moreover, *in vivo* imaging studies using validated HSC markers or labelled HSCs illustrate that few are in direct contact with OBs [92–94], suggesting that endogenous HSCs may not be spatially associated with OBs. Thus, taken together, OBs in the BM niche play a regulatory role in HSC activities, yet these regulations are probably not achieved via direct signalling but rather via distant signalling.

2.1.2.3. CAR cells. Within the CXCL12-GFP knock-in mice, a small population of reticular cells are identified with high GFP signals, indicating a high level of CXCL12 expression, termed as CAR cells [95]. CAR cells are found surrounding vascular endothelial cells and tightly in contact with HSCs [95]. They play an essential role in maintaining the HSC pool. Conditional depletion of CAR cells in transgenic mice models leads to a severe reduction of HSC number [95]. Intriguingly, compared to those isolated from the wild type mice, HSCs from CAR cell-depleted mice remain in a quiescent state *in vivo*, with relatively lower expression of cell-cycle-promoting genes, suggesting that CAR cells may encourage HSCs proliferation [95,96].

CAR cells are not homogenous. They express both adipocytic and osteogenic genes and consequently can differentiate into adipocytes or osteoblastic cells [95]. Based on sequencing data, there are two subsets of CAR cells within the BM identified: adipo-lineage and osteo-lineage cells, despite both sharing similar overall transcriptomic profiles [70,97]. Adipo-CAR cells are preferentially located adjacent to sinusoidal endothelial cells (SECs), while osteo-CAR cells are localized to non-vascular regions or cover arteriolar endothelial cells (AECs) [70]. The difference in the spatial distribution of these two CAR subsets further indicates that they may contribute to different functional perivascular niches [70].

2.1.2.4. LepR$^+$ cells. SCF-GFP knock-in mice provide a tool to explore the cellular source of SCF throughout the BM *in vivo* [37]. Immunofluorescence images of BM sections from the SCF-GFP knock-in mice show that SCF-GFP-expressing cells mainly surround sinusoids, overlapping with highly restricted LepR expression [37]. Thus, perivascular LepR$^+$ stromal cells appear to be the dominant source of SCF within the BM and play an essential role in HSC maintenance. When SCF is deleted from LepR$^+$ cells, the HSC number is significantly reduced [37,38] and their subsequent reconstitution capacity is impaired [29,37,42]. Thus, SCF from LepR$^+$ cells is required to maintain HSCs' self-renewal. Besides SCF, as LepR$^+$ cells partially overlap with CAR cells, these cells also contribute to CXCL12 production [28,69]. However, unlike SCF, CXCL12 secreted by LepR$^+$ cells is not required to maintain HSCs, but to retain HSCs within the BM compartment. Conditional deletion of *CXCL12* from LepR $^+$ cells has no effect on the BM cellularity and HSC frequency, except for an increased number of HSCs in the blood and spleen [28,29].

LepR$^+$ stromal cells are shown to be highly enriched for MSCs, uniformly expressing several BM MSC markers *e.g.* platelet-derived growth factor receptor α (PDGFRα) and CD51 (also known as integrin αV) [98,99]. Fate mapping shows that they can give rise to bone and adipocytes, serving as the main source of OBs and adipocytes in adult BM [98]. Recently, via scRNA seq, LepR$^+$ cells could be further distinguished into four subsets [69]. All four subsets express *SCF* and *Anpgt1* comparably, while multiple other genes, such as *CXCL12*, *LepR* and *Gremlin*, are significantly different [69]. The detailed function of these individual subpopulations on HSCs activities remains unclear.

2.1.2.5. Nestin$^+$ cells. Nestin, an intermediate filament protein, is a characteristic marker of MSCs that secrete important HSC maintenance factors [100]. In Nestin-GFP knock-in mice, nestin$^+$ cells are distributed along the vasculature throughout the BM [72,73]. The vast majority of HSCs are shown to be preferentially adjacent to the nestin$^+$ cells, and the conditional deletion of these cells can lead to a reduced HSC pool in the BM [72,73]. Thus, nestin$^+$ cells are critical for the control of HSC quiescence and haematopoiesis. Compared to the nestin$^-$ cells, nestin$^+$

cells express higher levels of HSC maintenance-related genes, such as *CXCL12*, *SCF*, *IL-7*, *Vcam1*, *Opn*, and *Angpt1* [72,73]. However, conditional deletion of *SCF* or *CXCL12* from nestin $^{+}$ cells has no effect on HSC activities in the BM, highlighting functional redundancy within the niche and the importance of heterogeneity [28,37]. nestin^{+} cells are therefore required for HSC activities, but the mechanism is still unclear.

Nestin^{+} cells are enriched for MSCs [72]. Based on nestin expression levels, nestin^{+} cells can be divided into two subsets, one strongly expressing nestin (nestin^{bright} cells), and the other expressing relatively low nestin (nestin^{low} cells) [73]. nestin^{bright} cells are distributed exclusively along arterioles and are mainly NG2 positive, which will be discussed in detail later [44,73]. nestin^{low} stromal cells are largely associated with sinusoids and do partially overlap with LepR $^{+}$ cells [101]. Thus, the distinct spatial distribution and cellular components of nestin^{bright} and nestin^{low} cells suggest their different contribution to the HSC niche, and also highlights the possible existence of the periarteriolar niche and the perisinusoidal niche, separately.

2.1.2.6. NG2^{+} cells. NG2^{+} cells are restricted to the periarteriolar nestin^{bright} population, rather than perisinusoidal nestin^{low} cells [73]. They are located exclusively near the arteriole [73]. Essential for HSC maintenance in the BM, conditional deletion of NG2^{+} cells reduces the HSC pool in the BM as well as the spleen [73]. Subsequent HSC functional analysis indicated that HSCs isolated from NG2^{+} cell-depleted mice lose their reconstitution capacity [73]. Thus, NG2^{+} cells are required for HSC maintenance and self-renewal.

Notably, HSCs near periarteriolar NG2^{+} cells exhibit relatively lower proliferative status compared to the ones near the perisinusoidal NG2^{-} stromal cells [73]. The deletion of NG2 cells switches HSCs into an active status and alter their localization away from arterioles [73,85]. Thus, NG2^{+} cells within the periarteriolar niche can promote HSC quiescence and construct a quiescent niche for HSCs. Such hematopoietic regulation of NG2^{+} cells may be achieved by the production of CXCL12, rather than by SCF [29]. Thus, NG2^{+} cells are vital in regulating HSC activities and contribute to the periarteriolar niche.

2.1.2.7. ECs. Monolayers of ECs line the lumen of blood vessels in the BM, consisting of arterioles, sinusoids, and a large network of capillaries that connect the arterial and venous systems [44,102]. BM ECs contribute to the HSC perivascular niche by secreting HSC maintenance factors, such as SCF [37]. Conditional deletion of *SCF* from ECs, results in reduced HSC frequency, followed by impairment of reconstitution capacity [37,38]. ECs can also regulate and support HSCs by Notch signalling. Specific deletion or blocking of Notch ligands (*e.g.* Jagged 1 or Jagged 2) from ECs can perturb Notch signalling within HSCs, leading to hematopoietic exhaustion and deficiency [31,101,103]. The supportive function of ECs requires cell proximity, given the fact that such regulatory effects are abolished once HSC-EC interactions are disrupted [30]. Taken together, research suggests that ECs are required in the BM to support the quiescence and self-renewal of HSCs in the perivascular niche.

2.1.2.8. Other cells and systems that contribute to HSC niche regulation. Additional cells have been found to contribute to the niche, for instance, macrophages [33,63] and neural cells [54,104,105]. As a result, several other systems participate in BM niche regulation, such as the immune system and sympathetic nervous system. Some actions may occur directly and are relatively straightforward. For example, Vcam1^{+} macrophages interact with HSCs via integrin α4 and consequently direct HSC retention [33]. Adrenergic signals act directly on HSCs expressing the β-2 adrenergic receptor (ADRβ2), promoting migration and engraftment [106]. Nociceptor neurons secrete calcitonin gene-related peptides, which binding directly to the calcitonin receptor-like receptor on HSCs, promoting granulocyte colony-stimulating factor (G-CSF) induced HSC mobilization [107]. In contrast, some regulations are indirect and may be achieved by the interaction of several synergistic cellular components. For example, nestin^{+} MSCs are linked to the sympathetic nervous system (SNS); nestin^{+} cells that express β-3 adrenergic receptor (ADRβ3) can transduce adrenergic signals, mediating circadian oscillations of CXCL12 secretion and HSC egress from the BM [72,108,109].

Taken together, there is currently evidence identifying effectively all cellular components within the BM niche as having an important role in HSC regulation. Many of these studies are based primarily on mouse models, which combined with increasingly advanced imaging techniques and computational analyses have facilitated the *in vivo* study of HSCs. Although these are powerful tools, often they are used to delete single factors in the niche cells, which could lead to compensatory behaviours from other niche components, or long-range signals from outside the BM. This should therefore be a consideration in the interpretation of such studies. These studies provide an extraordinary insight into endogenous HSC activity and niche cell behaviours, yet much remains to be elucidated before these complex networks are fully understood.

2.1.3. ECM and biophysical properties

The ECM is a complex collection of extracellular molecules such as insoluble proteins that define the structural and mechanical properties within tissues. In the BM, ECM proteins are secreted by local stromal cells and functionally mediate HSC anchorage, triggering intrinsic signals and directing HSC behaviours [110]. BM ECM is mainly composed of collagen type I-XI and other non-collagenous ECM proteins such as laminin (LM), fibronectin (FN), vitronectin and elastin [110,111]. Given the cellular and structural heterogeneity across the BM, ECM components' distributions and spatial locations are not homogenous either, yet they are also not randomly distributed (Fig. 1). Collagen I and vitronectin are particularly enriched within the endosteal region, whereas LM (especially LM containing α4, α5 chains) and collagen IV are enriched along the vascular basement membrane [112–115]. FN is abundantly found throughout the BM [112].

In addition to ECM components, local biomechanical properties differ significantly across the BM, such as matrix stiffness (Fig. 1). Using rheology, Jansen et al. have shown that the BM is predominantly elastic, with an effective Young's modulus ranging from 0.25 to 24.7 kPa at the physiological temperature [116]. Recently, Chen et al. characterized the mechanical properties of the murine BM using a more sensitive method, Atomic Force Microscopy [117]. The BM stiffness obtained by this method ranges from 1 Pa to 10^4 Pa [117], which is in line with the previous study [116]. These reports are, to our knowledge, the only two measuring the stiffness of intact BM, therefore, providing critical insight into the native mechanical environment.

Both the ECM composition and its biophysical properties are important in regulating HSC activities. Generally, cells interact with ECM via non-enzymatic integrin receptors which couple to ECM proteins [118]. This coupling allows for the transmission of force and biochemical signals from the ECM to the cell and vice versa via mechanotransduction [119]. Such inside-out and outside-in signalling ultimately regulates numerous cellular processes such as morphology changes and cell differentiation [120–122]. Due to HSCs being non-adherent cells, HSC-ECM interactions have been largely overlooked in the literature. In a recent study, the Harley group engineered BM-inspired ECM ligand-coated polyacrylamide (PAM) substrates with tuneable stiffness and demonstrated how different combinations of substrate stiffness and ECM ligand presentation can influence HSC lineage commitment [123]. They reported substrates mimicking endosteal niches (stiff ~40 kPa, high FN content) increased primitive myeloid proliferation, whereas substrates mimicking vascular niches (soft ~3 kPa, high LM content) promoted the erythroid lineage [123]. This work demonstrates that mimicking microenvironmental mechanics *ex vivo* to represent *in vivo* composition, allows investigation into its regulatory impact on HSC expansion, proliferation and functional capacity [133].

2.1.4. The role of hypoxia in the BM niche

Despite being heavily vascularized, the BM niche is hypoxic [124] (Fig. 1). Quiescent LT-HSCs localise to the endosteal region of the BM and display hypoxic phenotypes. This, coupled with the fact that active HSCs reside in the highly vascularized central cavity [125], led to the initial assumption that the endosteal region of the BM was the most hypoxic. However, this assumption was challenged by a 2014 study that directly measured the local oxygen tension of the different BM regions using two-photon phosphorescence lifetime microscopy [124]. Spencer et al. confirmed the hypoxic nature of the BM cavity, which was shown to have oxygen pressure (pO_2) ranging from 1.5 to 4.2% [124]. Unexpectedly, the endosteal zone was less hypoxic (pO_2 between 1.8 and 2.9%) than the deep sinusoidal regions (>40 μm from bone) which had a slightly lower oxygen gradient (pO_2 between 1.3 and 2.4%) [124].

The maintenance of long-term quiescence and self-renewal of stem cells within the BM niche is thought to be linked to oxygen tension. It is postulated that quiescent HSCs restrict mitochondrial respiration by oxidative phosphorylation (OXPHOS) and rely mainly on anaerobic glycolysis for their maintenance [126–128]. Both mitochondrial number and activity are reduced in quiescent HSCs, resulting in reduced OXPHOS [129–131]. When mitochondria accumulate again, HSCs convert from a quiescent to a proliferative state and lose their self-renewal potential [129,131]. In addition, mitochondrial metabolism is known to promote HSC commitment by producing reactive oxygen species (ROS) [132–135]. ROS plays a role in activating numerous cellular signalling pathways, such as phosphoinositide 3-kinase (PI3K)/Protein kinase B (AKT) [133,136,137] and p38/mitogen-activated protein kinase (MAPK) [138], which are essential pathways for HSC maintenance.

HSCs detect low oxygen tension by stabilizing hypoxia-inducible factor-1α (HIF1α) protein, a master transcriptional regulator of the cellular and systemic hypoxia response [139]. Accumulation of HIF-1α causes HSCs to adopt a quiescent phenotype and retain a long-term repopulation capacity, while a low level of HIF-1α causes HSCs to enter the cell cycle and adopt OXPHOS mitochondrial activity [126,140]. The HIF-1α stability in HSCs is not entirely dependent on the oxygen tension of cells' microenvironment [164] but is also regulated by independent, cell-specific mechanisms. HIF1α has been shown to be stably expressed by HSCs isolated from BM regions with differing *p*O2, but also by those in circulation. This suggests that cell-specific factors influence the hypoxic phenotype of HSCs [141]. Other factors have also been shown to drive HIF1α stability, demonstrating metabolic control is a fundamental process for HSC regulation. Both SCF [142] and TPO [143] reportedly drive HIF1α stabilisation in HSCs cultured under normoxic conditions. Therefore, HIF1α expression is key to regulating the metabolic phenotype of HSCs. However, this is not solely determined by the localization of HSCs in the BM niche, highlighting the importance of the metabolic machinery for their functions.

2.2. Distinct niches

Based on the preferential localization of HSCs as well as physical landmarks within BM, BM niches can be classified into three distinct niches: endosteal niche, perisinusoidal niche and periarteriolar niche (Table 2).

2.2.1. Endosteal niche

The interface between the bone and BM is called the "endosteum", covered by layers of osteoprogenitors, as well as bone-forming OBs and bone-resorbing osteoclasts [144]. The existence of an endosteal niche was first suggested by early studies preceding Schofield's niche concept decades ago [145], and later supported by evidence that activation of osteogenesis could enrich HSCs in trabecular-rich bone areas [55,86]. Subsequent immunofluorescence imaging showed that endogenous HSCs were located near or even in direct contact with some subsets of osteoblast-like cells, positive for N-cadherin, Jagged-1, and OPN [55,88,89]. By contrast, osteoclasts are dispensable for HSC mobilization and may function as negative regulators in the endosteal niche [146]. Thus, HSCs reside in the endosteal region where enriched with OBs and skeletal cells.

The existence of the endosteal niche is further supported by transplant models, which are used to track HSCs homing and re-locating to the micro-anatomy of the BM [48]. By means of transplant models combined with advanced imaging technology, transplanted exogenous HSCs have been shown to be preferentially home to the endosteal bone surfaces in recipients [88,147]. A similar HSC distribution was also observed when human HSCs were transplanted into mice [148]. The HSC homing to endosteal niches may be mediated by functional local factors, for example, Angiopoietin 1 (Ang-1) secreted from OBs binds HSC receptor Tie2 for HSC retention to the niche [49].

2.2.2. Perivascular niche

The complex vascular network within the BM, together with various perivascular stromal cells found to support haematopoiesis, lead to the hypothesis that HSCs may also reside near the vascular region. Kiel et al. found that the SLAM family of receptors could be used to label purified HSCs and that such HSCs were located in proximity to the sinusoid endothelium, rather than to the bone surface [3]. With the powerful imaging cytometry platform, Nombela-Arrieta et al. performed a comprehensive quantitative analysis of HSC distribution within BM, and demonstrated that most HSCs were preferentially adjacent to sinusoids, closer than <10 μm, confirming the concept of a perivascular niche [141]. Since this study, multiple distinct sets of markers, such as α-catulin, Homeobox protein Hox-B5 (Hoxb5) and myelodysplasia syndrome 1 (MDS1), that each identifies HSCs to a high level of purity, demonstrated that HSCs are perivascular in adult BM, mainly residing proximal to sinusoidal blood vessels [92,149,150].

Besides sinusoids, HSCs have also been shown to be adjacent to BM arterioles. Using 3D BM confocal imaging, Kunisaki et al. have found that 37% of HSCs are located within a 20 μm distance from arterioles,

Table 2
Comparison of the endosteal niche, periarteriolar niche, perisinusoidal niche in BM.

		Endosteal niche	Perivascular niche	
			Periarteriolar niche	Perisinusoidal niche
Cellular Composition		OBs	$NG2^+$ cells $Nestin^+$ cells	CAR cells $LepR^+$ cells
		Osteoclasts	Adrenergic nerve Arteriolar ECs	$Nestin^+$ cells Sinusoidal ECs
Non-cellular composition	ECM	Fibronectin, Collagen I, Vitronectin	Laminin, Collagen IV, Fibronectin	
	Stiffness	<40 kPa	<3 kPa	
	Oxygen	pO2 (~1.8–2.9%)	pO2 (~1.3–2.4%)	
Niche functions		Modulates HSC quiescence. Homing for exogenous HSCs.	Circadian oscillations of HSC egression. Haematopoiesis regeneration after irradiation.	GF-abundant niche HSC maintenance and activation. HSC egression.

and 15% are even directly adjacent to arterioles [73]. In another parallel study, Acar et al. assessed the localization of α-catulin^{+}KIT^{+} HSCs and found that approximately 10% of HSCs were closely associated with arteriole blood vessels, whereas approximately 80% of HSCs were adjacent to sinusoids. Interestingly, in this study, only a small percentage of HSCs were located in the endosteal zone [92]. These results refine our understanding of HSCs localization, suggesting that most HSCs reside near the sinusoids, with a small frequency of HSCs residing near the arterioles in the BM.

2.2.3. Endosteal niche vs perivascular niche

There remains contention as to the true location of the HSC niche, leading to a model that presents the existence of multiple niches with different functions. It is widely believed that the endosteal niche acts as a quiescent niche, while HSCs in the perivascular niche are believed to be more 'active' [73,88,151]. Based on colony assays and rough isolation of BM fractions according to their proximity to endosteum, early studies mostly support the concept that the endosteal niche is enriched for more primitive progenitors while more differentiated cells are located near the central sinus [152–154]. Immune-histological analysis of BM specimens also supports such a differentiation gradient [155–157]. Furthermore, based on the difference in quiescent states and specific markers for LT-HSCs (Lin^{low} c-Kit^{+} Sca1^{+} CD34^{-} Flk2^{-}), LT-HSCs are known to reside closest to endosteum and OBs, while more mature subsets (Lin^{low} c-Kit^{+} Sca1^{+} CD34^{+} Flk2^{+}) reside progressively further away and subsequently egress into the peripheral bloodstream via the vasculature [48,73,147,158]. Thus, these findings suggest that the endosteal niche and perivascular niche act differently in terms of HSC hierarchical residency and establish specific niches for various progenitors in the BM.

The existence of this differentiation gradient and specific progenitor niches becomes controversial when several mature hematopoietic populations are recognized as HSC niche components, such as megakaryocytes [36,64,65] and macrophages [33,63]. These findings suggest that HSCs and their mature progenies exist together in the same BM zones or regions, rather than separate niches along a specific gradient within the microenvironment. The communication between primitive HSCs and their subsequent mature progenies is critical for their maintenance and differentiation [33,36,63–65]. Furthermore, mature OBs have been implicated in the maintenance and differentiation of certain lymphoid progenitors via CXCL12 [28,43,52,159,160], suggesting that early lymphoid restricted progenitors are located within the endosteal niche as well. Thus, whether specific niches exist for various progenitors in the BM and if so, how the spatial locations distribute, remains contentious. Moreover, it should be noted that the relatively quiescent state of the niche is dynamic rather than static. The endosteal niche maintains HSCs under normal hematopoietic conditions but promotes their expansion in response to stress [150]. Thus, the endosteal niche can be thought of as being in equilibrium whereby HSCs are normally maintained in a quiescent state to maintain the stem cell pool, however in response to stress, they can become activated to trigger HSC proliferation and differentiation to repopulate the blood and meeting physiological demands.

2.2.4. Perisinusoidal niche vs periarteriolar niche

The differences between the BM arterioles and sinusoids with respect to the resident cells, oxygen tension, and vessel wall permeability, highlights the possibility that arterioles and sinusoids in the BM could also establish functionally distinct perivascular niches; the periarteriolar niche and the perisinusoidal niche.

In the BM, LepR^{+} and CAR cells have been shown to reside mainly around sinusoids, serving as the main source of key HSC niche factors, and thus contributing to HSC maintenance and activation [37,69,70,95]. In contrast, NG2^{+} and nestin^{+} cells are located exclusively along arterioles [72,73]. Though producing less functional HSC key factors than CAR and LepR^{+} cells [69,70], NG2^{+} and nestin^{+} cells are intriguingly shown to be near quiescent HSCs, suggesting that periarteriolar may construct a quiescent niche for HSCs [72,73]. Moreover, given that NG2^{+} and nestin^{+} cells are closely associated with sympathetic nerve fibres [72,104,108], these findings suggest that periarteriolar stromal cells may participate in HSC mobilization directed by the nervous system. Besides the perivascular stromal cells, ECs within the perisinusoidal and periarteriolar niche also exhibit distinct phenotypes and functions [39]. Cytokines produced by AECs function differently compared to those from SECs [69]. Selective deletion of SCF from AECs, rather than from SECs, results in the reduction of HSC frequency and reconstitution capacity [39]. Moreover, AECs are shown to be more resilient to irradiation than SECs [161], and couple with sinusoid regeneration [162], suggesting that HSCs may depend more on periarteriolar niches during the regeneration of haematopoiesis after irradiation [6].

The physical and mechanical microenvironments within the arterioles and sinusoids are not similar to each other either. AECs line the interior of arterioles and create a mechanical barrier between the circulation and the marrow [102]. In contrast, sinusoids are characterized by a fenestrated thin-walled endothelial cell structure, which potentially increases exposure of perisinusoidal HSCs to components of blood plasma [7,163]. As such, less permeable arterial vessels can maintain HSCs in a low ROS state, whereas the more permeable sinusoids promote HSC activation and are the exclusive site for immature and mature leukocyte trafficking to and from the BM [44]. Impaired endothelial integrity can cause HSCs to adopt a proliferative state (higher ROS level and glucose uptake) and consequently mobilize and apoptosis [44]. Thus, vessel permeability is essential in maintaining homeostasis in the perisinusoidal niche and periarteriolar niche.

3. Bioengineering the bone marrow niche *ex vivo*

HSCs cannot be expanded *in vitro* with a satisfying outcome, due to their quick differentiation and loss of self-renewal capacity once removed from the BM niche microenvironment [164]. This is thought to be a result of the lack of biophysical and biochemical cues from the native niche presented in standard synthetic culture. Recently, biomaterial and bioengineering strategies have offered promising approaches to reconstruct the in vivo-like niche for HSCs in the lab/*ex vivo* for clinical use. In general, these strategies aim to create a 2D or 3D environment using material components *e.g.*, scaffolds or hydrogels, alongside other essential niche elements such as cytokines, matrix stiffness, ECM components and specific cell types (Fig. 2).

Evolution of conventional HSC cultures into complex 3D biomimetic BM niches. In conventional culture systems, HSCs are cultured in the medium supplemented with various cytokines and growth factors (I), by functionalizing the substrate with ECM proteins (II), and by feeder layers (III). In 3D cultures, HSCs alone or in co-culture with supporting cells, are cultured within spheroids (IV), or embedded in a polymer matrix or in the cavities of pores of polymer scaffolds (V), decellularized ECM (VI) or natural and synthetic hydrogels (VII). Some more sophisticated systems have been developed. Such as perfusion bioreactors to mimic blood flow, shear stress and nutrient delivery/waste removal (VIII), and organ-on-a-chip models combine various ECM, stiffnesses, cells, soluble factors and vascular systems into one multi-parameter model (IX).

3.1. 2D suspension cultures; key components for stem cell maintenance and support

3.1.1. Soluble factors for stem cell maintenance

Soluble factors have already been shown to contribute to HSC maintenance within the BM niche *in vivo* [165]. Thus, the simplest BM niche engineering model could be achieved by supplying BM cytokines into conventional culture systems, to recapitulate *in vivo* biochemical signals. Cytokines most commonly used for HSC expansion are SCF,

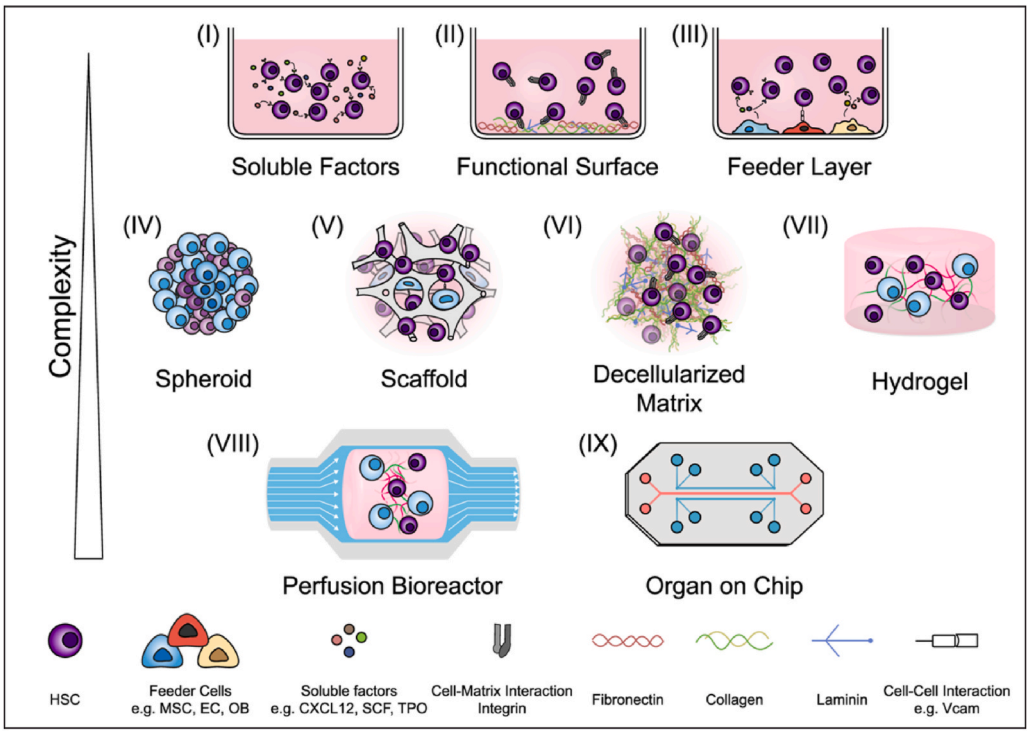

Fig. 2. Schematic drawing of the development of BM niche models.

TPO, FMS-like tyrosine kinase 3 ligand (Flt3L), angiopoietin-like proteins and IL-6 [8,9,166–171]. Additional factors were recently identified, such as nerve growth factor (NGF) and interleukin-11, which can achieve better expansion of HSCs while maintaining self-renewal capacity [168].

In addition to cytokines, several small molecules have been identified to either suppress differentiation or promote self-renewal in dividing HSCs. Through chemical genetic screens or screening of extensive small molecule libraries several small molecules have already been identified, such as Nicotinamide [172], prostaglandin E2 (PGE2) [173], StemRegenin 1 (SR1) [174], PPAR-γ antagonist GW9662 [175], polyvinyl alcohol (PVA) [176] and matricellular protein Nephroblastoma Overexpressed (NOV/CCN3) [176,177]. All of these have demonstrated the potential to induce robust proliferation *in vitro*. PGE2, nicotinamide and SR1 have passed phase I clinical trials, confirming the safety of *ex vivo* expanded cells [172,178,179]. However, later trials demonstrated that SR1-driven expansion is predominantly in the progenitor compartment, and 7 days post-HSC transplantation a transient burst of myeloid cells was observed. Further to this, it was noted that in the trial, the fraction of LT-HSC was derived from an untreated blood unit that was co-administered and not the SR1 expanded cells. This indicates the SR1-treated cells may contribute more to the progenitor pool than to the long-term HSC population [179,180]. Even though these molecules are believed to target critical pathways involved in self-renewal regulation, detailed mechanisms of action are still unknown. Thus, future efforts should aim to decipher the mechanisms within HSC self-renewal regulation and look for other novel elements, which are thought to promisingly support self-renewal, halt differentiation, promote homing, and suppress apoptosis of HSCs [181,182].

3.1.2. Functionalized surfaces to mimic ECM components

In the context of functionalized surfaces, one major advancement in functionalizing surfaces has been to physically adhere full-length ECM proteins or their short functional peptides to 2D substrates, thereby recapitulating niche ECM composition. Dao et al. first found that when HSCs interacted with FN *ex vivo*, they could traverse back to a quiescent state [183]. Celebi et al., also indicated that ECM protein could direct HSC fates, more specifically that megakaryocyte progenitor expansion was supported by LM-coated surfaces, while FN supported differentiation into erythroid progenitors *ex vivo* [184]. Strikingly, a mixed four-component (FN, LM, Collagen I and IV) ECM coating increased proliferation and myeloid differentiation when compared to a three-component coating without collagen I, suggesting that ECM-mix complexes could be more advantageous for niche engineering [184]. These studies offer insight into ECM type-related HSC activity control *in vitro* and stress the importance of reconstituting ECM components for BM niche engineering.

One study investigated HSC adhesion in terms of not only the type of ECM ligand but also the lateral, nanoscale distance between them. Using hydrogel substrates nanopatterned with ECM-peptides, it was demonstrated that for adhesion a distance lower than 45 nm was critical for the attachment of HSCs in substrates coated with RGD (Arginine-Glycine-Aspartate tripeptide, binding sequences of FN), while a larger distance was possible for longer full-length FN molecules [185], demonstrating that HSCs are sensitive to the nanoscale presentation of ECM-derived ligands. Efficient cell-peptide adhesion leads to the stabilisation of lipid rafts. Lipid-rafts are necessary to form various signalling complexes in HSCs and thus lead to the activation of molecular processes [186]. Taken together, the surface functionalization via adhesion peptide adsorption is suitable for the *ex vivo* expansion of HSCs; however, the type of ligand and its spatial presentation should be further evaluated and considered.

3.1.3. Stromal feeder layers for stem cell support

To recapitulate biomimetic niches, the cellular compartment must be considered. Co-culture of HSCs with stromal cells, typically denoted as “feeder cells”, is a common strategy for supporting HSC growth and differentiation *in vitro* [187–191]. The most widely used feeder cells in HSC cultures are MSCs [192] and ECs [30], as they express high levels of HSC-supporting factors compared to other stromal cell types [7,67]. Recent clinical trials show that umbilical cord blood (UCB) mononuclear cells expanded by mesenchymal cells co-culture, could significantly

improve neutrophil engraftment and platelet recovery when compared to unmanipulated cord blood only [193]. However, this treatment did not contribute to long-term engraftment and thus lead to no significant improvement in the survival of patients [193–195]. This is due to mesenchymal cell co-culture of HSCs leading to loss of primary LT-HSCs, which exhibit the self-renewal capacity and support long-term engraftment after transplantation [193,195]. Thus, conventional co-culture of HSCs with feeder cells does not achieve maintenance of clinically valuable HSCs in culture. More sophisticated devices should be designed to mimic other components of the native BM niche as closely as possible.

3.2. 3D systems – the importance of mimicking in vivo-like features

The HSC niche is a dynamic 3D microenvironment. Thus, various 3D tissue models have been developed to mimic *in vivo*-like features and are discussed in detail in Table 3.

3.2.1. Spheroids

Accumulating evidence suggests that spheroid-culture of BM stromal cells can facilitate cell-cell and cell-matrix interactions, generate their own ECM, and recapitulate *in vivo* gradients of oxygen and nutrients, thus mimicking the natural BM niche [228]. Several techniques have been developed for generating spheroids, including hanging drops [198,229], low-adherence vessels [41,196,197] and magnetic levitation [200,201]. For example, Lewis et al. have developed a spheroid culture system in which MSCs were labelled with magnetic nanoparticles and then multicellular spheroids were generated by magnetic levitation. Such an approach could maintain MSCs' expression of niche phenotypic markers, such as nestin and Stro-1 [200,201]. Furthermore, based on such MSC spheroids, endosteal niche and perivascular niche models were bioengineered by co-culturing with OBs or ECs. It was found that HSCs maintain quiescent MSCs in the endosteal model whilst promoting active MSCs in the vascular model [202]. Similarly, some other spheroids derived from enriched stromal cells, such as PDGFRα$^+$ CD51$^+$ MSCs and CD146$^+$ nestin$^+$ MSCs, were generated by low-adherence vessels and shown to be able to support HSC expansion, as well as maintain engraftment potential *ex vivo* [41,197]. However, several other studies show that MSC spheroids were not superior to the conventional methods in supporting HSC expansion, differentiation and engraftment potential [198,199]. These conflicting findings may be due to the oversaturated co-culture HSCs with MSCs, given that the addition of MSCs in lower numbers improved hematopoietic expansion until a maximum was reached, at which point the addition of more MSCs compromised expansion outcomes [197,199]. Taken together, spheroids may hold the potential to support HSC *in vitro*; however, stromal subpopulation, optimal MSCs/HSCs ratio and related mechanisms should be further evaluated.

3.2.2. Scaffolds

One 3D *in vitro* BM bioengineering approach is to recreate the honeycomb-like architecture of the BM using soft or rigid scaffolds. A number of synthetic materials have already been investigated, including porous tantalum [203], polyurethane (PU) [208], poly (D, L-lactide--*co*-glycolide) (PLGA) [206], polyethersulfone (PES) and non-woven polyethylene terephthalate (PET) fabric [205]. These synthetic materials are advantageous due to their large surface area for cell adhesion and increased porosity, allowing cell migration as well as nutrient exchange [206]. However, synthetic polymers lack cell-binding sites to localise signals and are therefore less biocompatible and are less suitable for cell culture. To achieve better cell adhesion as well as BM biomimicry, ECM proteins have been physically or chemically tethered onto synthetic scaffolds [205,207,208].

Overall, these scaffolds have shown promise in supporting HSCs *in vitro*, while there are still many differences to natural BM in terms of scaffold pore size and scaffold matrix components [214]. Furthermore, additional material properties, such as fibre orientation, material

Table 3
Current 3D biomimetic platforms for bone marrow niche engineering.

System	Materials & Models	Remarks	Reference
Spheroids	AggreWell 24-well-plate Co-culture with MSCs. Without cytokine addition.	3D spheroids support HSC expansion in co-culture *in vitro*.	[196]
	Ultralow-adherence plate/ dish Co-culture with Nestin$^+$ MSCs. Supply with cytokines in the medium.	Mesenspheres can expand HSCs that are capable of multilineage reconstitution and serial engraftment *in vivo*.	[41,197]
	Perfecta3D 96-well hanging drop plate. Co-culture with MSCs. Supply with cytokines in the medium.	3D spheroid systems are not superior to traditional 2D culture.	[198]
	Nonadherent microwell. Co-culture with MSCs. Supply with cytokines in the medium.	3D spheroid systems are not superior to traditional 2D culture.	[199]
	Magnetic levitation driven MSC spheroids. Encapsulated with MSC spheroids, OBs, ECs within Collagen I hydrogels.	Endosteal model supported HSC quiescence, while vascular model activated HSC differentiation.	[200–202]
Scaffolds	Tantalum-coated Carbone matrix coated by FN	Supports expansion of HPSCs with multi-lineage engraftment capability.	[203]
	PET functionalized with FN. Co-culture with MSCs. Supply cytokines within medium.	Support CD34$^+$ proliferation. Integrin engagement enhances the maintenance of CD34$^+$ progenitors.	[204,205]
	PCL coated with FN. Supply cytokines within medium.	3D PCL scaffold coated with FN is suited for expansion of HSC.	[206,207]
	Fibrins. Co-culture with MSCs.	3D fibrin scaffolds with stromal support have high potential for expansion of CD34$^+$ cells.	[206]
	PLGA Co-culture with MSCs	Hydrophobicity and porosity of the synthetic scaffolds affects cellular adhesion and expansion.	[208]
	Carbonate apatite scaffolds Co-culture with OBs	Scaffolds with micropore enhance BM-like tissue development.	[209]
	Carboxymethylcellulose scaffolds Co-culture with MSCs	Co-culture format preserves HSC function *in vitro*. Injectable *in vivo* without disrupting the cell-cell interactions established *in vitro*.	[210]
Decellularized matrix	Matrix derived from cell layer *in vitro*. Co-culture MSCs and HSCs within such matrix.	HSC expansion and MSC-HSC interactions improve within this matrix. Oxygen tension matters the HSC	[211,212]

(*continued on next page*)

Table 3 (*continued*)

System	Materials & Models	Remarks	Reference
		expansion capacity *in vitro*.	
	ECM matrix derived from cultured MSC layer, which anchored on immobilized FN surface.	HSC expansion improved, while retaining engraftment potential and differentiation capacity after transplantation.	[213]
	Decellularized bovine BM	Native 3D-architectures are preserved. Improve MSC expression of HSC supportive factors, as well as HSC adhesion and expansion.	[214]
Hydrogels	Collagen gels Encapsulated with MSCs and HSCs.	MSC source affects their support on HSCs. BM-MSC enables HSC expansion better than UCB-MSCs. Autocrine feedback enhanced HSC expansion while paracrine signals from stromal cells increased myeloid differentiation.	[215–217]
	Matrigel/Alginate gels Encapsulated OBs, MSC, endothelial cells and HSCs within the gels. Without any cytokine addition.	Heterogenous cells together achieve better HSC support, even without cytokine addition.	[218]
	Puramatrix Gel Encapsulated with MSCs and HSCs.	Maintain HSC supportive marker expression and form a functional BM niche.	[219]
	PEG gels functionalized with RGD or GFs (Interferon γ (IFN-γ), SCF) Encapsulated with MSCs and HSCs within the gels.	Positive effect of 3D culture of MSCs on the preservation of HSC stemness compared to 2D. HSCs encapsulated within 3D gels appeared to be in an undifferentiated state.	[220–222]
	GelMA gel functionalized with GFs (SCF) Encapsulate MSCs and HSCs within the gels.	Hydrogel retained HSC viability with relatively low dose of SCF. MSC density affects local matrix remodelling, further influencing HSC quiescence.	[223,224]
	Zwitterionic hydrogels Encapsulated with HSCs inside.	Zwitterionic hydrogels have the potential to facilitate HSC expansion, while maintaining self-renewal.	[225]
	GAG/PEG gels functionalized with GFs (TPO, SCF, Flt3L).	HSCs respond to increased spatial confinement with lowered proliferation and cell cycling, and higher quiescence. Maintenance can be achieved by combining biophysical and biochemical *ex vivo* culture parameters in 3D.	[226]
	HA/PEG gels	Support HSCs *in vitro* and further show to be superior in functional BM organoids formation *in vivo*.	[227]

hydrophobicity and stiffness, have also been shown to influence cellular migration and adhesion within [123,206,209,230,231]. Thus, ideal 3D scaffolds should consider engineering interconnected pore structures, controlled pore sizes, an open surface area, and surfaces that support cell adhesion and long-term survival.

3.2.3. Decellularized matrices

Currently, an increasing number of BM ECM components are being highlighted as contributors to HSC regulation, although it is not yet clear what level of complexity is required to be recapitulated in *ex vivo* models [184,214,232,233]. Thus, ECM matrices derived from cultured cells or tissues have been developed with the possible advantage of accurately mimicking the *in vivo* microenvironment of the BM niche. Prewitz et al. described a methodology that permitted reliable anchorage of MSC-secreted ECM to culture carriers, which could be used to generate BM-mimicking ECM scaffolds derived from MSCs [213]. Results indicated that such decellularized ECM scaffolds showed improved potential to expand HSCs *ex vivo*, providing increased cell numbers for transplantation without a loss of long-term engraftment capacity after transplantation [213]. However, decellularization of BM tissue is difficult owing to its softness and liquid-like properties [213]. Recently, one study described one kind of ECM scaffold obtained from decellularized bovine BM [214]. Compared with artificial scaffold systems, these bio-scaffolds could preserve additional complexity of the BM niche including vascular structures, cellular niches and native chemical composition. These scaffolds provide support not only for HSC expansion, but also for MSCs, inducing the production of CXCL12 and SCF, and providing an inductive environment for multicellular co-cultures to mimic the natural BM niche [214]. Nevertheless, these decellularized matrices are often brittle to handle, making recovery of live cells difficult, and are not amenable to clinically relevant scale-up. Yet, these systems highlight the importance of retaining native-like architecture and composition of the ECM in BM-niche engineering.

3.2.4. Hydrogels

3.2.4.1. Natural gels. Compared to scaffold-based systems, hydrogel encapsulation can overcome the limitation of cell-environment infiltration. Encapsulating cells within hydrogels offers a realistic 3D microenvironment that represents the physical and mechanical complexities of the stem cell niche [10,234]. Several natural hydrogels have already been employed in bioengineered BM niche models, including collagen [216,217], fibrin [206], hyaluronic acid (HA) [227], and alginate [218]. These natural materials demonstrate good biocompatibility, are highly abundant and can interact well with cells in culture. In addition, some of these materials, such as collagen and HA, are present *in vivo* and thus are highly biomimetic [11,235]. Several hydrogel-based BM niche models also implement co-culture with supportive niche cells. This enables the study of cellular communication between niche cells and HSCs. For example, Gilchirist et al. determined the effects of varying the ratio between HSCs and MSCs (HSC: MSC 1:0, 1:1, 1:10) on HSC

lineage specification patterns and HSC proliferative cycle status [224]. In line with other reports, the presence of MSCs (HSC: MSC 1:1) shifted HSC population dynamics, with a significant increase in HSC maintenance over time compared to the absence of MSCs (HSC: MSC 1:0). However, when HSCs were co-cultured with a tenfold higher density of MSCs (HSC: MSC 1:10), hematopoietic differentiation toward the myeloid lineage was enhanced, with significantly reduced maintenance of early hematopoietic progenitors [224]. This may be due to oversaturation of the culture with MSCs leading to increased soluble factors and significant matrix remodelling, that influences HSC equilibrium.

Hydrogels also offer a platform to study the optimal cell combination in the BM niche engineering. Due to the cellular complexity of the BM niche *in vivo*, combinations of heterotypic stromal cells are said to be more advantageous in expanding and maintaining HSCs *in vitro*, compared to a single population of stromal cells. Recently, Braham et al. co-cultured HSCs with heterotypic primary human cells within the Matrigels [218]. The results indicated that the Matrigels encapsulated with differentiated adipogenic, osteogenic, and endothelial cell mixtures could optimally support HSCs maintenance as well as haematopoiesis [218]. Notably, such maintenance was achieved without further cytokine culture medium supplementation. Thus, hydrogels encapsulated with an optimized heterogeneous mix of supporting primary cells, hold the potential to decrease the supplementation of cytokines and also stress the importance of cellular complexity in HSC niche engineering.

3.2.4.2. Synthetic gels. Notably, natural hydrogels have limitations in user control over mechanical properties. They also have several limitations in their use due to batch-to-batch variability and due to being of animal origin. Therefore, synthetic materials have been investigated, with the advantage of uniform physical properties and molecular compositions, which offer reproducible and controllable mechanical properties, as well as allowing fine-tuning of degradation rates [11,236,237]. Commonly used synthetic materials include PAM, poly (ethylene oxide) (PEO), poly (ethylene glycol) (PEG), poly (*l*-lactic acid) (PLLA). Recently, another novel synthetic material, zwitterionic poly (carboxybetaine), has demonstrated excellent potential to expand HSCs with long-term repopulating ability *ex vivo* [225]. Moreover, synthetical hydrogels enable an orthogonal design allowing modification or presentation of biologically active moieties [238], such as GF immobilization. By adding PEG-functionalized SCF into gelatin methacryloyl (GelMA) hydrogels, B. Mahadik et al. have shown that, compared to the continuous soluble SCF administration, GelMA hydrogels containing covalent SCF showed significantly higher selectivity for maintaining a more primitive fraction of HSCs throughout 7 days culture. It is critical to note that this is achieved at a ~45-fold lower SCF dose than with soluble delivery [223].

Further to this, ECM proteins can also be functionalized onto synthetic polymers. For example, PEG chains containing plasmin and Matrix metalloproteinase (MMP) substrates on one end and cell adhesion peptides (*e.g.* RGD) on the other, can be photopolymerized into hydrogels through stepwise crosslinking, improving HSCs expansion and maintenance [220–222,239]. However, using just adhesive or short bioactive peptides might not fully replicate ECM properties. Prior to peptides, full-length ECM proteins present many other binding sites which are physiologically relevant [240]. This avenue has advantages given that the ECM is a supramolecular structure that is difficult to replicate by mixing purified ECM components. Recently, a synthetic 3D hydrogel was developed that incorporated full-length FN [240,241] and full-length LM [242]. In these studies, they functionalized the FN protein with PEG-maleimide or LM protein with PEG-acrylate in order to covalently crosslink the protein to the hydrogel network [240–242]. Due to the capacity of ECM proteins such as FN and LM containing potent GF binding domains, these hydrogels may mimic the native ECMs' ability to capture GFs and cytokines secreted from support cells. Biomimicking strategies such as this will offer a valuable platform for BM niche engineering in the future.

With the advantage of controllable mechanical properties, synthetic hydrogels are ideal for studying the regulation of HSC behaviour by biophysical signals within the niche such as stiffness and viscoelasticity. Gvaramia et al. encapsulated HSCs within PEG hydrogels and explored the impact of stiffness on HSC activities [226]. They showed that increased stiffness of the 3D environment resulted in increased frequency of quiescent cells [226]. This suggests that HSC proliferation and maintenance can be controlled in 3D hydrogel culture systems by optimizing the stiffness of the matrix. However, given the heterogenous gradient of stiffness exhibited within the BM [116,117], we have to consider the inherent substrate stiffness and the underlying gradient of stiffness.

Besides stiffness, controlling the viscoelastic properties of hydrogels has shown great potential in harnessing the mechanosensitive response of stem cells [243–246]. For control of key MSC behaviours, incorporation of viscous, linear polymers and steric spacing of crosslinking points are examples of strategies to modulate hydrogel viscoelasticity that can influence cell adhesion and differentiation [246–248]. However, research into the impact of viscous interaction in HSC behaviours and HSC niche engineering is still limited. Therefore, further investigations are required. In general, synthetic hydrogels may offer ideal material for niche modelling, with a realistic, reproducible, and controllable microenvironment for BM niche engineering.

3.2.4.3. Hybrid gels. Recognizing the enhanced biological activity of natural hydrogels and the increased tunability of synthetic hydrogels, hybrid hydrogels could provide synergistic benefits for bioengineering [249]. One example of such hybrid hydrogels is PEG/-Glycosaminoglycan (GAG) - based hybrid hydrogels. These hybrid hydrogels have been successfully used for several applications [250,251], for instance, for MSC differentiation [252] and for cancer and myoblasts culture [253]. Regarding BM, the study conducted by Gvaramia et al. developed a GAG-rich PEG hybrid hydrogel, which could facilitate TPO, SCF and Flt3L, presentation to HSCs [226]. Flt3L, SCF, and TPO are known to bind to the GAG heparin domain [254,255]. Thus, these cytokines could be retained within the hybrid hydrogel and subsequently support HSC culture even with low cytokine concentrations. Recently, the use of such hydrogels could maintain, expand, and differentiate human HSCs *in vitro* and were further shown to be superior in functional BM organoid formation *in vivo* [227]. Thus, by combining the features of synthetic and natural polymers, such hybrid hydrogels can offer an optimal strategy for BM niche engineering, though the research is currently limited.

3.3. Combinatorial niche models

3.3.1. Perfusion bioreactors

In vivo, the biological delivery of cytokines is a continuous process, as opposed to traditional static models [256,257]. Thus, the feeding rate and medium exchange in the conventional cell culture protocols may also perturb the maintenance and proliferation of HSCs [257,258]. Several studies have demonstrated that frequent medium changes were advantageous for cultured hematopoietic cells [259–261], perhaps because of the active removal of cell-derived negative regulators [226,257,262]. In this regard, some advanced dynamic platforms have been developed, which integrate bioreactors to allow a direct perfusion flow of medium while culturing HSCs (Table 4). Perfused systems can offer efficient nutrient supply/waste removal while mimicking interstitial flow and associated shear stress [256,257].

Perfusion bioreactors typically consist of two compartments: the chamber, in which the scaffold/hydrogel is in place, and the tubing, which allows increased nutrients diffusion and waste removal [263]. A recent study from Bourgine et al. developed an *in vitro* perfused 3D BM niche system supporting the development and maintenance of human

Table 4
Current combinatorial platforms for BM niche engineering.

System	Materials & Models	Remarks	Reference
Perfusion bioreactors	3D PEG hydrogel functionalized with RGD and flow. Co-culture MSCs and HSCs within the hydrogels.	Mimics the HSC niche under steady-state and activated conditions. Perfusion enhanced HSC expansion and erythroid differentiation.	[264]
	Hydroxyapatite scaffolds with osteoblastic MSC within one perfusion system.	MSCs deposit matrix, display osteogenic differentiation, and maintenance of niche markers. HSC expansion compared to control. HSCs localise and adhere to MSCs on scaffolds.	[256]
	Collagen gel with MSCs and fibroblasts, combining with lithography technology.	Multicellular interactions within the perivascular niche, subsequently direct HSC trafficking.	[272]
	Collagen-coated polyurethane scaffold embedded with ceramic hollow fibres.	Maintain immature hematopoietic populations and represent a physiologically relevant system of erythropoiesis.	[273]
OOC models	Hybrid *in vivo-ex vivo* model. Collagen gel containing osteoblastic factors. Engineering new bone *in vivo*, removing it whole and perfusing it with culture medium in a microfluidic device.	Reconstitute complex *in vivo* niche architecture and physiology. LT-HSCs maintained more efficiently than static stromal feeder layers.	[14,267]
	MSCs and ECs cultured within 3D decellularized bone matrix, followed by perfused with breast cancer cells in the microfluidic chip.	Mimics the perivascular niche. Established a capillary-like vascular network in the niche, further study the metastatic colonization of breast cancer.	[266]
	Culture hydroxyapatite coated zirconium oxide scaffold, comprising of MSCs and HSCs, within the microfluidic multiorgan chip culture for 28 days.	Suitable platform for long-term culture of primitive HSCs. Molecular and structural similarity to the *in vivo* BM niche.	[17]
	Culture ECM-based hydrogels encapsulated with MSC, arterial EC, sinusoid EC and OBs within an microfluid recirculating perfusion system.	HSC infused–healthy $CD34^+$ cells, lymphoma cells, and leukaemia cells exhibited a marked preference for homing to particular niche constructs.	[265]
	A channel comprised of fibrin gel containing $CD34^+$ cells and MSCs, and a parallel channel lined by human vascular endothelium and perfused with culture medium.	Establish perivascular niche to study the chemotherapy related hemotoxic and haematopoiesis related disease.	[16]
	Osteoblastic MSCs on the bottom surface of the device, and subsequent a fibrin-collagen hydrogel network containing EC and MSC on the top created an interconnected 3D microvascular network.	Presence of the endosteal niche decreased the proliferation and increased maintenance of $CD34^+$ HSCs. A high-throughput multi-niche platform.	[268]

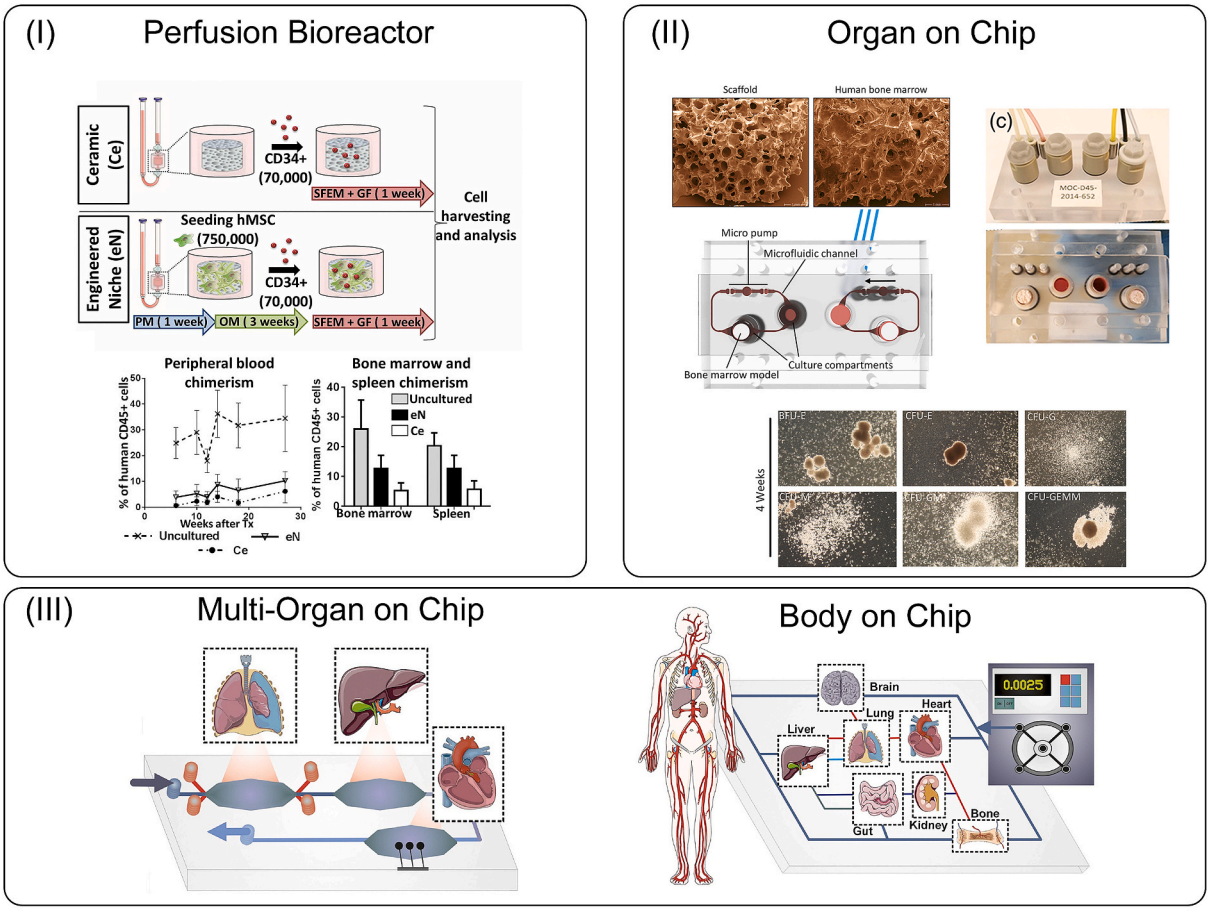

Fig. 3. Examples of BM niche models using combinational strategies.

BM analogue [256]. Their approach was to co-culture HSCs with MSCs *in vitro* within BM mimicking porous hydroxyapatite scaffolds by perfusion of the medium through a bioreactor [256]. Such engineered systems partially recapitulated structural and functional features of the human BM in defined and tuneable settings [256] (Fig. 3I). Furthermore, such a device allowed the expansion and maintenance of HSCs with *in-vivo* engraftment and multi-lineage reconstitution potential. In another parallel study, HSCs and MSCs were co-cultured in an RGD peptide functionalized PEG-hydrogel under a perfusion system [264]. This system could mimic the niche in active or steady-state conditions, which either supported HSC differentiation or HSC maintenance.

3.3.2. OOC models

In the last few years, OOC platforms have been developed and employed, in which organoid structures are combined within a microfluidic perfusion network [15]. Such microfluidic platforms permit precise manipulation of the microenvironment to deliver soluble factors to cells [15,265]. In terms of the BM, there have been several examples of attempts to produce an OOC system that captures the BM niche on a chip system [14,16,17,266] (Table 4). Torisawa et al. engineered a BM-on-a-chip device by implanting a scaffold into a mouse and allowing it to be populated by murine cells [14,267]. This construct was then removed and placed in a microfluidic device, which could continuously supply nutrients and medium. Such devices have been shown to support the maintenance of primitive hematopoietic cells and faithfully mimic the natural physiological response of living BM to clinically relevant doses of γ-radiation, whereas conventional 2D stromal-supported cultures do not [14,267]. Similarly, Sieber et al. developed another novel 3D co-culture model without hybrid *in vivo* implantation [17]. This device comprised of human MSCs and HSCs within the microfluidic OOC model enabled successful HSC culture for a period of 28 days [17]. The cultured HSCs were still functional and capable of differentiating into hematopoietic progenitors, indicating stable maintenance of functional HSCs within such a 3D model (Fig. 3II). Nonetheless, the biology of these systems is significantly simplified by only inducing one single niche cell type (*e.g.,* MSCs) to test the ability to support primitive HSCs. The addition of some other cell types should be considered.

Recently, one multi-niche microfluidic BM on-a-chip device was introduced, by sorting out four major BM niche cells (SECs, AECs, MSCs and OBs), and subsequently engineering them into four major distinct niches (perisinusoidal, periarterial, mesenchymal and osteoblastic niche) within HA and gelatin-based hydrogels. These four niche constructs were housed within one single microfluidic device system [265]. With this multi-niche platform, they showed that healthy $CD34^{+}$ cells, lymphoma cells, and leukaemia cells, exhibited a marked preference for homing to particular niche zones respectively [265]. Thus, BM on-a-chip devices provide an ideal platform to study BM physiologies and pathologies [16,265,268]. It is important to note that such OOC devices are not as structurally and functionally sophisticated as tissues *in vivo*. Furthermore, all chips should be experimentally validated in terms of their ability to recapitulate the key physiological properties of the particular organ, or specific pathophysiology of the disease state, and to do it consistently and robustly. However, we are beginning to see the development of more sophisticated, reproducible, OOC systems and even Multi-Organ on-Chip or Body on-Chip [16,268–270] (Fig. 3III). These enable more biologically relevant modelling of BM pathophysiology than traditional 2D cell culture, or of those than cannot be effectively recreated in animal models. Future development will aim to see these NATs used as an alternative to animal testing.

Advanced technologies to bioengineer BM niche models. (I) BM niche model in a perfusion bioreactor system. Such system was shown to support HSC repopulation capacity and engraftment in the long term. Reproduced with permission from Ref. [256]. (II) BM niche model in Organ on Chip. Such platform was shown to remain HSCs differentiation capacity after 4 weeks culture. Reproduced within permission from Ref. [17] Copyright 2017 John Wiley and Sons. (III) Ongoing and future work in integrating BM models with different OOC models together, to be more in line with the Multi-Organ on Chip and Body on Chip philosophy. Reproduced with permission from Ref. [271] Copyright 2020 Elsevier, reproduced under the terms of the Creative Commons CC-BY license.

3.4. Significance and applications of BM niche models

3.4.1. HSC expansion ex vivo

Based on HSC hematopoietic functions, whole BM or HSC fractions, including most recently gene-edited HSCs [274,275], can be taken from patients (autografts) or matched donors (allografts), and subsequently transplanted to recipients to reconstitute ablated or injured hematopoietic systems. This process is known as HSC transplantation therapy (HSCT) and is currently a widely used therapy for the treatment of many blood-related diseases, such as malignancies (leukaemia, multiple myeloma *etc.*) and inherited blood cell diseases (immune deficiencies, hemoglobinopathies *etc.*) [276–279]. However, there is currently unmet demand for matched donors. Only 53% of searches resulted in matched donations in the UK in 2015 [280]. Further, problems associated with low donor cell yields can often lead to the requirement of multiple donors, or graft failure [281]. The lack of reliable methods for HSC expansion and the limited supply of available HSCs presents a major obstacle for the wider application of HSCT. Thus, efficient expansion and maintenance of HSCs remain a major goal in the field [282].

Engineering *ex vivo* models to mimic the *in vivo* niche microenvironment holds promise in drawing back on these current limitations to adequately expand HSCs [169]. As discussed above, an artificial BM niche able to sufficiently recapitulate the necessary properties of the *in vivo* BM niche should in theory be able to accommodate HSC expansion *ex vivo* [164] (Fig. 4I). Thus, these systems can ultimately be developed to expand autologous patient HSCs *ex vivo,* followed by transplantation back to the donor without the need to undergo myeloablative therapy or other immunosuppressant treatments. This will enable higher yields and negate the need for a donor, by-passing the need to find a human leukocyte antigen (HLA) match and avoiding risks of graft-versus-host disease.

3.4.2. BM platform to model niche biology and pathophysiology

Current studies of HSC biology and haematopoiesis are mostly reliant on mouse models. Though mouse models offer an invaluable substitute, disparities do exist in the context of molecular, cellular and physiological mechanisms [19]. Thus, to overcome the aforementioned limitations, BM niche models provide an optimal platform for HSC biology study (Fig. 4II). Moreover, despite our enhanced understanding of HSC biology over the past several decades, many processes of HSC regulation still remain inconclusive, for instance, HSC self-renewal processes remain to be fully defined at the molecular level [80,283]. Artificial BM niches capable of maintaining populations of HSCs *ex vivo* will allow niche cells to be more easily studied, with modern bioinformatics technologies shedding light on the molecular regulation within the HSCs' activities.

Aside from using *in vitro* HSC niche models to study healthy haematopoiesis, BM models can also be used to investigate disease pathology *in vitro*, allowing for a greater understanding of how diseased cells function. Current evidence suggests that niche alterations may act as oncogenic drivers or facilitators of malignancy [284,285]. In addition, malignant cells can transform the microenvironment into a leukemic niche to support their proliferation and support leukemogenesis [285,286]. The crosstalk between malignant and/or pre-malignant cells and their niches acts as a key contributor to disease initiation, progression and resistance to therapy (as reviewed in Ref. [285]). However, most mechanisms remain to be elucidated. Artificial *in vitro* BM niche models offer a platform to study cellular interactions in detail and to explore therapeutic targets, which further deepen our understanding of the BM niche's role in BM-related diseases.

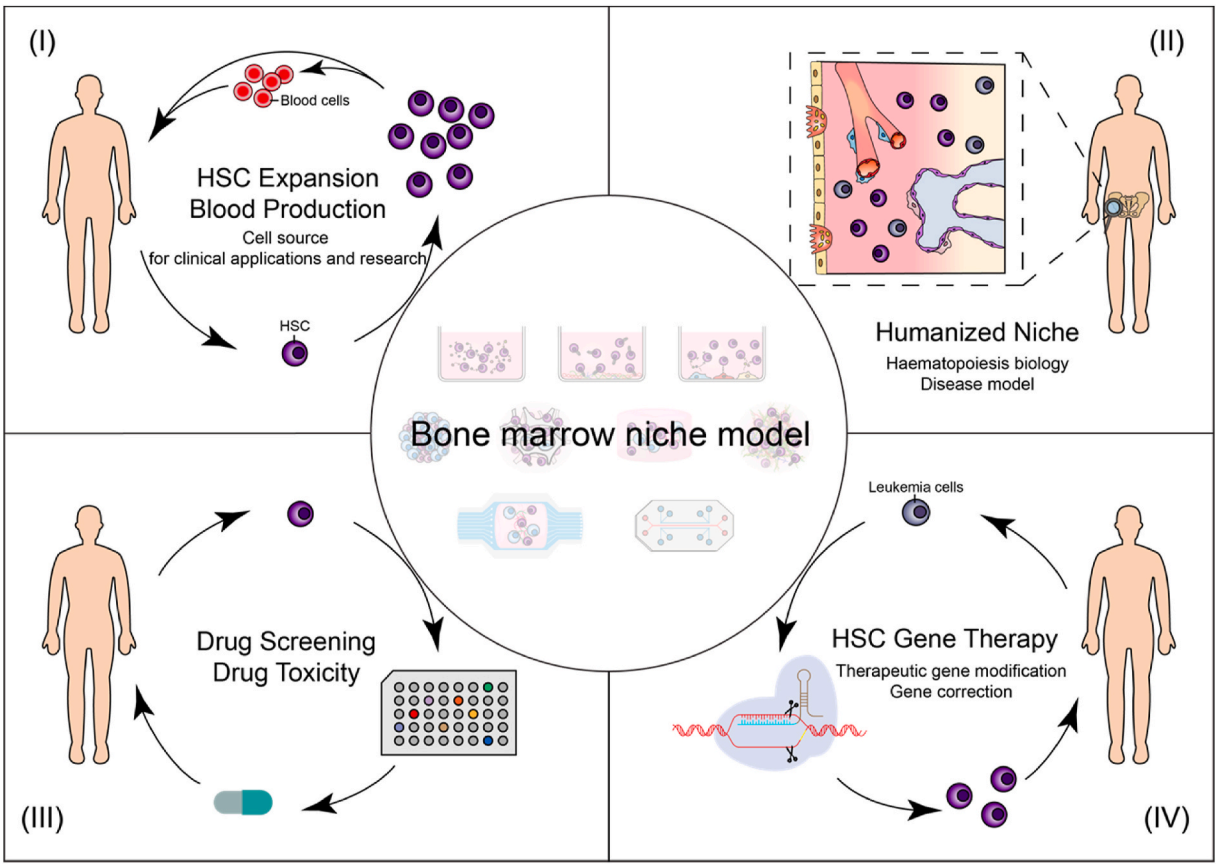

Fig. 4. Potential applications of an *in vitro* BM niche model.

3.4.3. *Manufacture of mature blood cells ex vivo*

The manufacture of mature blood cells *ex vivo*, such as erythrocytes and platelets, is particularly of interest for transfusion medicine. Currently, blood collection is largely dependent on volunteer donations, yet there remains an unmet demand for blood in the clinic [287]. Volunteer donations also risk the potential transmission of viruses and prions during the collection, culture or transfusion, leading to post-transfusion infection and thus to potential further complications [288,289]. Therefore, there is a growing need to create alternative methods for blood cell production to address these challenges and improve transfusion outcomes.

As discussed previously, the unique architecture and composition of the BM niche can affect resident progenitor cells' differentiation and function. BM niche models have been employed to guide HSC differentiation and produce clinical-grade blood cells *in vitro*. Several BM niche models have proposed their potential use for erythropoiesis (erythrocyte production) [273,290] and megakaryopoiesis (platelet production) [291,292] (as reviewed in Ref. [293]). However, each blood transfusion unit approximately contains 3×10^{11} platelets, and currently reported systems demonstrate efficiencies of platelets collected from their systems as being well below this number [292]. Yet, these models provide invaluable insight for studying the fundamental mechanisms of erythropoiesis and megakaryopoiesis *in vitro*, which can offer potential targets for making blood cell units on demand for transfusions in the future.

These studies demonstrate the potential for the development of more efficient BM models that may enable blood production on a clinically valuable and commercially viable scale. However, this is a complex and multidisciplinary task, requiring collaboration between engineers, chemists, biologists, and clinicians.

3.4.4. *In vitro drug screening platforms*

In recent decades, advances in the molecular understanding of disease processes have underpinned new potential therapeutic drugs. However, there is still a lack of *in vitro* models to accurately predict drug efficiency and potential drug toxicity prior to *in vivo* studies and clinical trials [294,295]. For example, haemato-toxicity (haem-tox) is a very common and unfortunate side effect of anti-cancer drugs with more than 50% of cancer drugs promoting haem-tox [296]. However, common simplified cell models do not recapitulate functional aspects of *in vivo* cell microenvironments, and animal models exhibit differences in physiology and drug responses compared to humans, leading to poor translation and high drug attrition rates in Pharma [19,297]. This is driving Pharma to look to NATs [294], to build using human cells and likely requiring the tissue complexity that stem cells and tissue engineering strategies can produce [298].

In vitro niche models provide an optimal pre-clinical platform to predict drug efficiency and toxicity (Fig. 4III). Recently, advances in biomedical engineering techniques lead to the development of some more sophisticated bioengineered models for drug screening, including microfluidics [299,300], xenograft models [301,302], OOC models [16], multi-organ-on-chips [269,270] and organoid systems [303]. These models have shown great advantages and better predictive powers over conventional *in vitro* tools, without the need for animal studies. One critical feature of these studies is that these models recapitulate the complex tissue structures and enable the crosstalk between cells and niche microenvironments, which are pivotal to mediating the maintenance and progression of cancer cells as well as drug resistance *in vivo* [285,304]. These niche models can be further customized by incorporating primary patient-derived cells, which provide a personalized model to predict the individualized response to *e.g.* chemotherapy regimens [299]. These *in vitro* systems, therefore, offer potential alternatives to animal studies which are still highly favoured in the world of

academia yet present numerous limitations and high costs [269,270]. Perhaps it is time for researchers and regulators to discuss the need for animal studies vs potential alternatives such as these artificial models which may offer better promise in recapitulating human responses [19].

3.4.5. Gene therapy

Recent advances in gene-editing technology have resulted in the development of the clustered regularly interspaced short palindromic repeat (CRISPR)-associated 9 (Cas9) nuclease system [305,306]. This system can be used to perform gene therapy, a process in which malfunctioning genes in cells affected by genetic disorders are corrected or counteracted *in vitro*, before the gene-corrected cells are autologously transplanted back into the patient they were originally harvested from, conveniently avoiding transplant rejection in the process [307]. Several recent clinical trials have also shown CRISPR to be a safe and effective tool in the treatment of various severe inherited diseases of the blood and solid cancers [274,275,307–309].

Applying this technology to the BM, specifically, HSCs has been shown to have vast potential for curing BM-associated disorders [307]. However, this approach is limited by delayed recovery periods, which are reportedly associated with the *ex vivo* culture of the gene-corrected HSCs [310,311]. The solution is the development of improved *ex vivo* culture conditions, such as bioengineered BM models, which can improve gene therapy success rates, while simultaneously expanding the healthy HSC population for implantation (Fig. 4IV). Further, despite the overall safety and efficacy of the CRISPR-Cas 9 system, risks of genotoxicity and off-target effects do exist, limiting its clinical applications [307,312]. A fabricated *ex vivo* BM niche model will provide a platform on which to study the accuracy, precision, and safety of gene-editing on HSCs, thereby improving gene therapy success on HSCs.

BM niche models can be applied to reconstitute *ex vivo* the stem cell regulatory environment. We propose that the resulting models will have important relevance in the following field. (I) Efficient *ex vivo* HSC expansion and blood cell production will offer better, increased and potentially less costly cell sources for research and clinical use. (II) Systems that model HSC and niche cellular processes permit to study at the molecular level, such as haematopoiesis and self-renewal mechanisms. More humanized modelling of diseases such as leukaemia, allow the study of disease progression and therapeutic targets. (III) More humanized methods for screening drug efficacy and pharmaceutical leads, negating the need for early animal models and reducing the number of high risks fails to enter costly clinical trial stages. (IV) Gene editing platforms would increase efficacy and promote the expansion of the therapeutic cells.

4. Future challenges

Over the past several decades, advances in imaging and genetic tools have rapidly increased our understanding of HSC niches and the specific cellular and molecular components that regulate HSC activities. However, much remains unclear and further investigations are required:

1. The number of currently identified stromal BM niche cells is at least twenty times greater than the number of HSCs, suggesting that mesenchymal derived populations require further defining or that the establishment of a bona fide niche requires the participation of other cellular constituents [158]. It is therefore important to elucidate the physical and functional interplay between each specific stromal cell type and HSCs, to help better understand HSC regulations.
2. Mechanotransduction plays a pivotal role in stem cell biology [313,314]. However how mechanical stimulation regulates the BM niche is still unknown. Recently, Shen et al. have uncovered that mechano-stimulation regulated SCF secretion from niche stromal cells, thereby controlling HSC maintenance and BM immune function [315]. This work provides an exciting possibility that mechano-sensing in niche-forming cells might contribute to the maintenance of HSCs and their progenitor cells. More efforts should be taken to decipher the detail of mechano-sensing stimulation within the niche.
3. Bioactive metabolites have the potential to become important research tools that can be used to control the differentiation and activities of HSCs [316] in combination with traditionally used GFs. It would therefore be interesting to investigate whether these metabolites are involved in regulating the BM niche and HSC activities *in vivo*.

As for BM niche modelling *in vitro*, we are only just beginning to appreciate the critical role of material-driven, engineered BM constructs. Though recent advances in material science, bioengineering, and biotechnology have provided details on how to adequately mimic aspects of the multifaceted HSC niche microenvironments, many challenges remain:

1. Re-creating the complexity of the multi-niche BM environment in one single culture system [317]. As discussed above, several parameters need to be taken into consideration to achieve this: (i) biochemical conditions, (ii) cellular composition, (iii) binding sites for cell attachment, (iv) nano topography and –patterning, (v) stiffness gradients, (vi) 3D architecture, and (vii) suitable supply of nutrients and oxygen [169]. However, the incorporation of all these complex parameters into one single model followed by reliable experimental readouts is a major challenge for BM niche model development.
2. Engineering microenvironments to promote HSC proliferation whilst maintaining multipotency *ex vivo*. Current *in vitro* models usually utilize co-cultures of HSCs with various BM cell types [265,268]. However, these niche cells still aren't able to promote the self-renewal of clinically valuable LT-HSCs and lead to a rapid decline of stemness.
3. Engineering ready-to-use niches for research and clinic. Currently, manufacturing and experimental implementations are relatively costly or complex, making such platforms inaccessible and not conducive to widespread use. Thus, we need to figure out solutions to make components low-cost, reusable and/or easy to dispose.
4. Most critically, as the number of models increases, they can exhibit significant variation and inconsistency between different manufacturing batches, different laboratories and even different fabricators in the same group [318]. Functionality becomes more complex and generated data carries artefactual and non-translatable risks. Standardised guidelines need to be worked out to evaluate the safety and efficiency of various models.

Thus, for applications of BM niche models, there is still a long way to go from concept to lab bench to bedside.

5. Conclusion

Taken together, the past decade has seen extraordinary progress in understanding the BM niche and the specific cellular components and molecular processes that regulate HSC activities [7]. However, much remains to be elucidated before the enormous potential of these factors is fully harnessed. Based on a better understanding of niche-specific factors regulating HSC activities *in vivo*, several novel studies have suggested that reconstructing the BM niche *ex vivo* might be feasible by means of advanced bioengineering technology. These advances would enable the development of improved, chemically, and physically defined, culture systems. The design of *ex vivo* HSC niche models and their application will ultimately improve the research and clinical use of these valuable cell types. As more is uncovered about the advantages and limitations of engineered human models, we encourage the continued collaboration between biologists, engineers, and clinicians to

develop more BM niche models and undoubtedly various modalities, strategies, and methodologies will emerge for humanized models in the near future.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This review is funded by China Scholarship Council (#201908060063), Engineering and Physical Sciences Research Council (EP/P001114/1) and Biotechnology and Biological Sciences Research Council (BB/N018419/1).

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