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Engineering the hematopoietic stem cell niche: Frontiers in biomaterial science

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Abstract

Hematopoietic stem cells (HSCs) play a crucial role in the generation of the body's blood and immune cells. This process takes place primarily in the bone marrow in specialized 'niche' microenvironments, which provide signals responsible for maintaining a balance between HSC quiescence, self-renewal, and lineage specification required for life-long hematopoiesis. While our understanding of these signaling mechanisms continues to improve, our ability to engineer them in vitro for the expansion of clinically relevant HSC populations is still lacking. In this review, we focus on development of biomaterials-based culture platforms for in vitro study of interactions between HSCs and their local microenvironment. The tools and techniques used for both examining HSC-niche interactions as well as applying these findings towards controlled HSC expansion or directed differentiation in 2D and 3D platforms are discussed. These novel techniques hold the potential to push the existing boundaries of HSC cultures towards high-throughput, real-time, and single-cell level biomimetic approaches that enable a more nuanced understanding of HSC regulation and function. Their application in conjunction with innovative biomaterial platforms can pave the way for engineering artificial bone marrow niches for clinical applications as well as elucidating the pathology of blood-related cancers and disorders.

Keywords

Biomimetics; Hematopoietic stem cells; Hydrogel and scaffold fabrication; Stem cell fate; Stem cell niche engineering

1 Hematopoiesis and the hematopoietic stem cell

Hematopoiesis is the physiological process where a small number of hematopoietic stem cells (HSCs) continuously generate the body's full complement of blood and immune cells. During fetal development, primitive hematopoiesis is first evident in the yolk sac followed by definitive hematopoiesis emerging from the aorta-gonad-mesonephros (AGM) region,

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then the placenta, fetal liver, spleen, and finally in the bone marrow (BM) [1]. As a result, subsequent postnatal regulation of HSC fate decisions takes place in unique regions of the bone marrow termed niches [2–8]. First proposed by Schofield in 1978 to explain the differential engraftment potential of HSCs isolated from the bone marrow versus spleen of mice [2, 9], the concept of the niche, and indeed niche engineering, extends today to a much wider range of stem cell populations.

Hematopoietic homeostasis is maintained by a highly-regulated process responsible for generating the full spectrum of myeloid (e.g. erythrocytes, platelets, granulocytes, macrophages) and lymphoid (e.g. T-cells, B-cells, natural killer cells, dendritic cells) cells (Fig. 1). Here, deeply quiescent, long term repopulating HSCs (LT-HSCs), maintained in *quiescent* niches, and active, self-renewing short term repopulating HSCs (ST-HSCs), maintained in more prevalent *active* niches, are responsible for this balance [10]. The subsequent differentiation hierarchy produces all mature blood (e.g. erythrocytes, macrophages, platelets) and immune cells (e.g. B-cells, T-cells), typically in excess of 2.5×10^9 red blood cells (RBCs), 2.5×10^9 platelets, and 1.0×10^9 granulocytes per kg of body weight per day [9]. In addition to their primary responsibility for maintaining hematopoietic homeostasis, HSCs also mobilize to and home back from the peripheral blood, either via biomolecular signals such as granulocyte colony-stimulating factor (G-CSF) or in response to trauma. Here, HSCs exit the bone marrow and circulate through the blood system. Circulating HSCs also contribute to hematopoiesis, and are able to trigger enhanced hematopoietic cell proliferation and/or differentiation in times of stress [11, 12].

While the genetic information required to direct HSC behaviors such as quiescence, self-renewal, or differentiation is contained within its DNA, signals presented by the niche, namely from surrounding cells, the extracellular matrix (ECM), and ECM-bound or diffusible biomolecules are required to trigger these events [5, 7, 13–16]. Understanding the cascade of signals required for HSC maintenance holds significant basic science and translational value. Historically, the study of HSC niches has been restricted to in vivo efforts that selectively defunctionalize elements of the marrow or simplistic 2D cultures. Both have limited capacity for examining hierarchies and synergies. Yet, as the prototype mammalian stem cell and given its long-history of clinical use, we are armed with well-established metrics (e.g. immunophenotype, functional) to assess HSC fate [17]. A unique opportunity therefore exists to develop biomaterials to elucidate mechanisms of niche action as well as act as a rheostat to regulate HSC fate. Such an artificial bone marrow could facilitate therapeutic expansion of HSCs as well as blood and immune cells, and also the study of the etiology and treatment of hematologic diseases. In this review, we discuss in vitro biomaterial-based culture platforms used to study the interactions of HSCs with their environment. Tools and techniques used to investigate the niche interactions as well as expand HSC populations will be described. Finally, we offer a perspective on biomaterial platforms that hold the potential to push the existing boundaries of HSC culture towards high-throughput expansion and directed differentiation.

2 Clinical significance of hematopoietic stem cells

While HSCs are responsible for producing billions of hematopoietic cells a day, mutations in this process can lead to a range of pathologies such as leukemia, myelodysplasia, or bone marrow failure. While once a diagnosis of hematopoietic disease was devastating and almost ubiquitously fatal, hematopoietic stem cell transplantation (HSCT) now offers hope, but also the very real possibility of life-threatening complications. Myeloablative therapy is often the only avenue for treating hematopoietic disease. Here, the dose of chemotherapy and radiation required to treat the patient also destroys the patient's hematopoietic system. Pioneered in the 1950s by E. Donnall Thomas [18] who later received the Nobel Prize (1990), whole bone marrow or a HSC fraction taken from the patient (autograft) or a matched donor (allograft) can be infused into the patient after myeloablative therapy. These cells *home* to the marrow cavity where they then *engraft* in discrete niches to rebuild the marrow [19, 20]. Three major sources of donor HSCs for transplant come from *autologous*, *allogeneic*, or *umbilical cord blood*. In autologous transplants, the patient's own HSCs are isolated from the marrow directly or can be induced to mobilize into the peripheral blood for harvest. For some forms of hematopoietic pathologies, it is not possible to use autologous cells; here, allogeneic HSCs isolated from a matched donor or banked cord blood can be used [21]. Umbilical cord blood is another source of HSCs for transplantation that is particularly attractive for those patients without a suitable donor, but the use of more than one unit of cord blood may be necessary to secure adequate numbers of cells for a comparable outcome [22].

Though HSCT represents the frontline of hematologic disease treatment, deficits in engraftment significantly reduce patient survival. The first year post-transplant is particularly critical. Infections, severe graft-versus-host-disease (GVHD) and relapse contribute to patient mortality, but critical hurdles remain due to *low homing efficiency* to the marrow cavity as well as *failure to re-engraft* [23, 24]. Defects in trafficking and engraftment between niches can negatively impact long-term homeostasis (bone marrow exhaustion) and patient survival [25–27]. Owing to the use of the patient's own cells, autologous transplants can have survival rates exceeding 80%. However, the survival rate for allogeneic transplants varies greatly based on donor match, ranging between 30% and 70% survival at five years [22]. Typically in humans, primitive HSC populations are identified by the expression of the CD34 antigen (CD34⁺ HSCs) and are commonly used for isolating HSCs for transplant. Here, the number of CD34⁺ HSCs present in transplanted marrow has been linked to successful engraftment and patient survival [22, 28, 29].

Given the potential for HSCT to treat a wider range of hematologic and oncologic diseases, it is essential to develop new approaches to overcome the mortality associated with poor engraftment and long-term homeostasis. Engineered HSC niches may also have significant potential for the study of leukemic stem cells (LSCs), a proposed mediator for leukemia found in close association with HSCs within the bone marrow microenvironment [30]. Although the origin, expansion, and effective therapeutic targeting of LSCs is still debated, improved understanding of native HSC niches and mechanisms by which they affect normal hematopoiesis may greatly assist our understanding of LSCs in the context of disease and therapy [30–33].

3 Isolation, identification, and functional metrics of HSCs

Adult HSCs are found primarily in the bone marrow [13], though HSCs can also be isolated from umbilical cord blood (UCB) or peripheral blood, most notably in the context of HSCT [34]. Like many stem cell populations, a major concern is the correct identification of a rare cell subtype ($<0.005\%$ of the bone marrow cells) [35]. While cocktails of characteristic cell surface antigens allow isolation of cells enriched for hematopoietic stem and progenitor cell (HSPC) sub-populations via fluorescence-activated cell sorting (FACS) [17, 35], it remains challenging to identify bona fide HSCs. Of primary concern in the context of engineering hematopoietic stem cell fate decisions is isolating deeply quiescent LT-HSCs and active, self-renewing ST-HSCs. For mouse, one of the most common HSC sub-fraction is that expressing $\text{Lin}^{-}\text{Sca1}^{+}\text{cKit}^{+}$ (LSK), enriched for HSPCs [13]. However, increased specificity can be obtained through the use of additional markers such as CD150, CD244, and CD48 [35]. A more comprehensive list of early hematopoietic progenitors and surface antigen expressions is provided in Table 1.

Despite advances in identifying surface antigen combinations that allow scientists to isolate LT-HSC populations of increasing purity, repopulation assays remain the gold standard for confirming putative HSCs [36]. Here, the ability to reconstitute lethally-irradiated marrow via a donor HSC population validates the presence of functional HSCs, with limiting dilution or competitive repopulation assays often used to estimate the fraction of functional HSCs within a culture [36, 37]. Additional in vitro functional assays such as the colony-forming unit (CFU), long-term culture-initiating cell (LTC-IC), and cobblestone area-forming cell (CAFC) assays are often used as a surrogate due to matters of costs and convenience [37]. However, even these assays require long incubation times with a large number of putative HSCs. These strategies are therefore limited in their capacity to examine the heterogeneity of HSC cultures or in rapidly determining functional changes in situ [38], making it challenging to dynamically assess HSC fate decisions in response to microenvironmental cues.

4 Hematopoietic stem cell niches in the bone marrow

The soft marrow found within the highly vascularized trabecular bone serves as the principal site of hematopoiesis [39]. Significant effort has been made over the past decades aimed at identifying definitive HSC niches within the marrow. While the identity and composition of discrete niches remain unclear, putative HSCs have been identified near the endosteal surface of trabecular bone structures (*endosteal niche*), in the central marrow cavity (*central medullary niche*), as well as in close proximity to vascular structures such as the sinusoidal endothelium (*perivascular niche*) or arterioles (*arteriolar niche*) [5, 13, 40–44]. Due to the proximity of these anatomical regions within the marrow it is not clear if these niches exist as overlapping or mutually exclusive microenvironments. Nonetheless, these subtle localization differences within the bone marrow support the contention that *quiescent* and *active* niches exist in close spatial register across the marrow (Fig. 2). Mimicking the niche as a coordinated entity of action requires understanding HSC fate decisions in response to multiplexed cell, biophysical, and biomolecular signals. Here we describe putative microenvironmental signals which may contribute to native HSC niches. While experiments

knocking out elements of this network (e.g. in vivo knockdown) can identify central features, they cannot provide insight regarding de novo design of an artificial marrow.

4.1 Cellular components of the HSC niche

4.1.1 Osteoblasts—Osteoblasts have been proposed to play an important role in regulating HSC maintenance. Premature osteoblasts, which highly populate the endosteum, can maintain HSCs and enhance the HSC pool [45–47]. In addition, human osteoblastic cells have been shown to support hematopoietic progenitor cell cultures ex vivo [45]. Interestingly, different subsets of osteoblasts were found to differentially affect HSC response. While constitutive activation of parathyroid hormone protein receptors (PPRs) in mature osteoblasts had no impact on HSC maintenance or function [48], precursor osteoblastic cells such as Nestin⁺ mesenchymal stem cells (MSCs) [16] or Runx2^{hi} osteoblasts [49] were able to enrich HSCs via expression of angiopoietin-1 (Ang-1), stem cell factor (SCF), chemokine (C-X-C motif) ligand 12 (CXCL12) known to support HSC quiescence [50] and self-renewal [16, 42, 51]. Moreover, primitive HSPCs show preferential homing and engraftment to sites adjacent to the endosteum, highly populated by osteoblasts and osteoblastic progenitor cells [6, 40, 41, 51, 52]. Here, advances in imaging technologies have allowed direct visualization of HSPCs in close proximity to the endosteum [40, 41].

4.1.2 Vascular endothelial cells—In vivo studies also suggest vascular endothelial cells are critical for maintenance of HSC functional phenotype [53–55]. Imaging analyses reveal that HSPCs can be found co-localized with the marrow vasculature [35, 56] even when they are in endosteal regions [40, 41, 44]. This is not surprising considering the dense vascularization within the bone marrow. The functional importance of vascular endothelial cells may be particularly important in the context of HSC mobilization as well as homing and (re) engraftment. Anatomically, the artery entering cortical bone splits into ascending and descending branches that run axially along the central marrow cavity, then radially towards the endosteal region in a network of smaller Sca-1⁺ arterioles and capillaries that anastomose with Sca-1^{lo} irregularly-shaped venous sinusoids (diameter ~50–100 µm) [12, 15, 40, 44, 57]. These venous sinusoids drain via the central longitudinal vein, creating a circular flow of blood supply within the bone marrow [57]. Bone marrow-derived endothelial cells have shown limited capacity to facilitate HSC expansion in vitro [53, 54] while non-marrow derived endothelial cells showed varying degrees of support [58, 59]. Limited in vivo data suggests the perivascular niche may be particularly important in HSC activation, with enhanced HSC recruitment to the perivascular region observed following bone marrow stress [59, 60].

4.1.3 Additional sources of cellular signals—Several other cell types have also been implicated with different aspects of HSC quiescence, self-renewal, and lineage specification. These include CXCL12-abundant reticular (CAR) cells, macrophages, adipocytes, and neuronal cells [10, 15, 61]. However identifying regulatory features of the niche remains a challenge, due both to their rarity (<0.005% of marrow) [35] as well as their activity: HSCs traffic to/from the peripheral blood and between niches [62, 63]. Clearly, decoding how the cellular niche affects HSC fate requires examining both explicit HSC-niche cell interactions as well as the history of these interactions, which will be difficult to meet in vivo.

4.2 Biomolecular signals within the niche

A wide range of biomolecular cues have been correlated with HSC localization within the marrow [5–7]. Cytokines including SCF, thrombopoietin (TPO), fms-related tyrosine kinase 3-ligand (Flt3L), interleukin-3 (IL-3), IL-6, IL-11, and G-CSF [9, 64–67] are known mediators of quiescence [50], self-renewal [55], and engraftment [68] *in vivo*. More recently, *in vitro* cultures using combinations of these factors [9, 69, 70] have led to selective expansion of cells with enhanced repopulation capacity after transplantation into irradiated recipients, though this ability was lost in progressively longer (>9 days) cultures [65]. Interestingly, matrix-immobilized forms of these cytokines (e.g. macrophage colony-stimulating factor or M-CSF [71], SCF [72, 73]) may provide better support for long-term *in vitro* expansion of HSCs than soluble forms. A wider range of additional factors, notably Ang-1, OPN, IL-7, and VCAM [10] can also affect HSC fate decisions.

In addition to selective promotion of HSC quiescence vs. self-renewal, biomolecular signals such as hypoxia, G-CSF, CXCL12, CXCL2, SCF, and a wide range of interleukins (IL-1/6/7/8/12) have been implicated in HSC homing, migration, and retention within the bone marrow niche [10, 51, 74]. A body of work suggests that local regions of hypoxia may be particularly important in quiescent niches that promote lower ATP levels in HSCs although the presence of a hypoxic niche within the bone marrow is challenged by more recent studies [75–80]. Similarly, CXCL12 (also known as SDF-1) is a potent chemokine expressed by several types of HSC niche cells, notably CAR cells which are found largely in perivascular regions [81, 82]. The CXCL12/CXCR4 signaling cascade plays an essential role in HSC homing, mobilization, and retention, with disruptions linked to HSC mobilization defects [10, 83], a reduction in HSPCs and a marked loss of long-term repopulation potential [84]. A number of studies have identified many growth factors (e.g. VEGF, FGF, PDGF, TGF- β , EGF, IGF) as playing potential roles in HSC migration, mobilization, quiescence, and self-renewal, [9, 85–87]. These efforts have identified both a range of biomolecular cues and phenotypes they affect *in vivo*, and have even motivated studies attempting to model the kinetics of biomolecular signals on HSC fate using advanced bioreactor culture tools [88]. While these findings inspire current efforts in engineering stem cell culture platforms that direct HSC fate, results have yet to be translated to systems which direct HSC fate via bio-molecular cues alone.

4.3 Niche extracellular matrix environment

The ECM is a complex organization of insoluble proteins that defines the structural and mechanical environment within tissues. Like many other stem cell populations [89, 90], there is increasing evidence that the local matrix environment provides extrinsic cues which can mediate HSC differentiation, lineage specification, proliferation, and apoptosis (cell death) [91–93]. Several ECM proteins including fibronectin [94], collagen [91–93] and laminin [95] as well as ECM remodeling proteins such as matrix metalloproteinases (MMPs) [92, 96] have been implicated with modulating HSC functions and/or hematopoiesis. HSCs and their differentiated progeny express a range of integrins including $\alpha 4\beta 1$, $\alpha 5\beta 1$, $\alpha L\beta 2$, $\alpha M\beta 2$, and Reference [9]. Integrin-mediated HSC-ECM interactions and downstream signaling pathways have been implicated in HSC quiescence, differentiation, and mobilization (for a more detailed review on the role of ECM and other adhesion

molecules on HSC fate, refer to [97]). More recently, it was also found that the expression of several adhesion molecules in HSCs may be differentially regulated during development vs. homeostasis, suggesting that the disparate adhesive signatures of HSC subsets may be related to differences in observed functional capacities [97]. These adhesion molecules include transforming-growth-factor- β inducible gene h3 (BIGH3), CD164, CD166, biglycan, and embigin. Additionally, other constituents of the ECM including OPN, dystroglycan, and heparin sulfate proteoglycans have been linked to ECM-based HSC regulation [9, 98–101].

Like many tissues, there is significant structural, mechanical, and biochemical diversity within the marrow [9, 94]. For example, the composition of the marrow varies significantly across discrete anatomical regions, with higher levels of fibronectin reported near the endosteum while higher expression of laminin is observed in the perivascular space [12]. Notably, fibronectin has been shown to promote long-term maintenance and expansion of HSCs in vitro [60, 102]. Further, the bone marrow presents a range of mechanical properties, with encapsulated cells experiencing elastic moduli near the bone surface (osteoid and collagenous bone) on order of 40–50 kPa while those in the central medullary region (adipocytes; fatty marrow) on order of 3 kPa [103]. HSCs within the marrow and those mobilized within the peripheral blood also experience additional biophysical forces including hydrostatic pressure and fluid shear stress [89, 104, 105].

5 Engineering the hematopoietic stem cell niche

Mimicking the niche as a coordinated entity of action requires understanding HSC fate decisions in response to multiplexed cell, biophysical, and biomolecular signals. Experiments knocking out elements of this network (e.g. in vivo knockdown) can identify central features but cannot provide insight regarding de novo design of an artificial marrow. Replicating how niche signals impact hematopoiesis therefore requires new tools to dissect synergies and hierarchies. To date, a variety of experimental platforms have been described to examine the impact of extracellular cues on HSC fate decisions. Design criteria for such systems include: (i) ease of fabrication; (ii) ability to examine combinations of cues; (iii) scalable to study small numbers of rare populations or to scale-up for clinical use, and (iv) increasingly the capacity for high-throughput analyses. Three-dimensional biomaterial platforms are increasingly used in an effort to capture physiological conditions of native niches [106], and will be discussed later in this chapter. However, a wide range of two-dimensional culture approaches have been used to present well-defined cues along with ease of high-throughput, in situ analyses.

5.1 Two-dimensional biomaterial platforms

While matrix mechanics has become a primary design principle for many stem cell populations (e.g. MSCs [89], embryonic stem cells (ESCs) [90], neural stem cells [107]), the impact of matrix mechanics on HSC fate remains poorly explored [5, 9]. Nevertheless, the known mechanical diversity across the marrow [94, 105] and the localization of specialized HSC niches within and across these regions [40] suggest that biophysical cues may provide important extrinsic signals to regulate HSC fate. With the majority of efforts to expand HSCs in culture largely unsuccessful without the use of feeder cells, the addition of niche-

inspired structural or mechanical cues has increasingly gained interest as a potential approach to engineer HSC fate decisions [108] (Fig. 3).

Indeed, recent evidence suggests that varying subsets of HSCs can respond to changes in substrate elasticity [109–111], topography [112–114], and lateral spacing of ligands [115, 116] (Fig. 3A). For instance, Holst et al. showed that human and mouse whole bone marrow cells cultured on tropoelastin-coated substrates result in significant enrichment in HSC fractions compared to bare tissue culture plates due to changes in substrate elasticity [109]. Similarly, culturing HSCs on electrospun polyethersulfone nanofibers [114] or microcavities with a diameter of 15 μm or smaller [112, 113] led to increased expansion due to changes in substrate topography. And our group showed that the shape, spreading, and cytoskeletal organization of murine HSCs increase when they are cultured on stiffer collagen-coated substrates or on substrates coated with higher collagen density, with similar changes observed in 3D collagen gels with varying stiffness [110]. Increasingly, high-throughput analytical techniques (e.g. microarray) have been used to parse the effect of combinations of niche factors on HSC fate [117–119]. For example, Lutolf et al. used arrays of hydrogel-based microwells to trace the division kinetics of both HSCs and their differentiated progeny, revealing that HSC division kinetics respond to local changes in protein cues such as TPO, Wnt3a, IL-11, and N-Cadherin [117] (Fig. 3B).

5.2 Co-culture with niche cells

The co-culture of HSCs with niche cell populations, typically denoted as ‘feeder cells’, is a common strategy used to support HSC growth and differentiation in vitro. Stromal cells are commonly included in early efforts aimed at ex vivo expansion of HSCs [69, 120]. Here, culture with bone marrow-derived stromal cells [71, 121], MSCs [122], osteoblasts [45], and vascular endothelial cells [53, 54, 58] have been shown to effect long-term HSC maintenance and expansion. Feeder cell layers are commonly used to promote the differentiation of HSCs into myeloid (e.g. RBCs [123], megakaryocytic cells [124]) and lymphoid (e.g. NK cells [125], B cells [126], T cells [127]) lineages. The effect of feeder and stromal cell co-cultures can be ascribed to both direct (e.g. cell-cell contact) and indirect (e.g. paracrine) signals. Taqvi et al. induced HSC differentiation to T cell precursors via co-culture with stromal cells and the presentation of surface-bound Notch ligand or delta-like ligand 4 (DLL4), using microbeads decorated with DLL4 to activate Notch signaling [127]. Miharada et al. reported that erythroblast nucleation could be achieved by culturing human CD34⁺ cells with only cytokines for four successive passages [128]. Recent efforts by Zandstra et al. using 2D liquid cultures demonstrated rapid diffusive transport of signaling molecules between cells, indicating that cell-cell signaling can dynamically perturb both intercellular signaling pathways and the resultant hematopoietic cell fate decisions [129] (Fig. 3E). Despite these findings, the use of feeder cell co-culture remains the primary approach used in investigations attempting to induce directed differentiation of HSCs into lymphoid or myeloid lineages.

6 Adding a third dimension to artificial hematopoietic stem cell niches

As the marrow is a fully three-dimensional tissue, recent focus has shifted to developing 3D biomaterials in order to achieve added control over the spatial and temporal presentation of

niche-inspired signals (Fig. 4). Such efforts are complicated by an as-of-yet still unclear picture of HSC-matrix engagement within the native marrow. Diffusive biotransport limitations introduce further complexity with regards to identifying doses of soluble biomolecules and using biomaterial design to leverage known autocrine-paracrine feedback loops originally identified in diffusion unlimited liquid cultures [88, 129].

6.1 Scaffold-based three-dimensional biomaterials

Low-density, open-cell foam structures such as those presented by tissue engineering scaffolds have long been adopted as analogs of the trabecular bone [130–132]. A range of fabrication approaches can be used (e.g. lyophilization [133], selective laser sintering [134]; colloidal crystal templating [135]; salt leaching [136]; electrospinning [137]) to manipulate pore architecture (size, shape, porosity) and mechanical properties. Such instructive signals have been used to induce a wide range of desired cell behaviors such as attachment [138], migration [137, 139], and (mesenchymal) stem cell lineage specification [140].

Recently, such constructs have been adapted as culture platforms for HSCs, in many ways an attempt to replicate the endosteal niche [141–143]. Taqvi et al. demonstrated that the hematopoietic differentiation potential of mouse ESCs on 3D PLLA scaffolds was dependent on polymer concentration and pore size [136]. ESCs tended to differentiate into HSCs in scaffolds with smaller pore sizes and higher polymer concentrations (corresponding to higher stiffness). Ferreira et al. have used a broad range of materials (PCL, PLGA, fibrin, collagen) as scaffold cultures for UCB-derived CD34⁺ HSCs [144], showing selective expansion of HSCs in fibrin scaffolds in the presence of UCB-MSCs as support cells. Interestingly, the 3D PLGA scaffolds led to poor HSC survival, likely a response to poor cell adhesion. Selective changes to scaffold porosity, fiber size, or fiber stiffness did not affect HSC migration and/or adhesion, though random fiber orientations appeared to limit HSC adhesion (HSC-HSC and HSC-substrate). Co-culture with MSCs led to increased numbers of CD34⁺ HSCs, a theme consistent with many other studies [142, 145, 146].

Scaffold topology has also been shown to affect HSC behavior. Nichols et al. described scaffolds fabricated with an inverted colloidal crystal (ICC) geometry to mimic aspects of the native niche (Fig. 4A) [135]. Co-cultures of stromal cells and CD34⁺ HSCs in these scaffolds supported cell expansion and promoted increased B cell differentiation compared to 2D controls. Similarly, Tan et al. [147] used bio-derived bone to co-culture CD34⁺ HSCs with bone marrow MSCs in an attempt to replicate the native osteoblastic niche. Results indicated that the 3D scaffold led to a five- to seven-fold increase in the frequency of LTC-IC numbers (an indicator of primitive HSCs) over two weeks of culture, significantly higher than in 2D control cultures. Together, these results suggest the network architecture of porous scaffolds can affect HSC fate decisions, but that co-culture with niche cells is often a requirement for selective expansion.

6.1.1 Culture environment—Apart from variations in biophysical properties, several groups have also investigated the effects of oxygen concentration on HSC culture. While the oxygen concentration within the niche is a topic of ongoing debate [148], it is generally agreed that HSPCs retain hypoxic features and that hypoxic conditions may better maintain

HSCs in vitro [97]. Several studies performed on 2D substrates have demonstrated better maintenance and expansion of the repopulating HSC fraction under hypoxic conditions [149–151]. The effect of oxygen on hematopoietic lineage specification was also explored in 3D constructs [152, 153]. Miyoshi et al. found that BM mononuclear cells cultured in collagen-coated, porous reticulated polyvinyl formal (PVF) resin scaffolds under different amounts of oxygen supply (10% vs. 20%) showed similar cell expansion over a short term (<2 weeks) but lower oxygen supply (10%) led to higher cell expansion over longer culture periods (three weeks) [153]. In another study, the presence of oxygen gradient within 3D constructs, as opposed to a constant hypoxic environment, was found to be important for maintaining a functional niche-like environment [152]. Abolishing oxygen gradient via a re-oxygenation agent perfluorotributylamine or by exposing the cultures to a uniform hypoxic condition (1% oxygen) induced a significant drop in the number of HSCs (CD34⁺CD38⁻) within the cultures.

Perfusion systems are often integrated with 3D porous scaffolds for tissue engineering applications [130, 154, 155]. Not surprisingly, their effects have been explored in the context of HSC niche engineering. Recent efforts to expand HSCs with bone marrow stromal cells on hydroxyapatite ceramic scaffolds showed perfusion enabled uniform seeding and long term culture up to several weeks [156–159], resulting in a higher number of CFU-GEMM colonies vs. 2D controls. More recent efforts have involved the use of a multi-compartment hollow-fiber membrane based perfusion bioreactor for the long-term culture of BM mononuclear cells [160]. Although the bioreactor induced a stable expansion of hematopoietic progenitors, the effect seemed to be totally driven by the perfusion and not the scaffold architecture.

6.2 Hydrogel-based three-dimensional biomaterials

Hydrogels offer a separate 3D biomaterial platform for HSC niche engineering. Here, cells are commonly encapsulated within a dense network of swollen polymeric fibers. Primary design criteria are choice of both the polymeric backbone (e.g. synthetic vs. natural) as well as the method for forming the hydrogel (e.g. spontaneous gelation, photo-initiated). Both natural (e.g. collagen [161]) and synthetic (e.g. polyethylene glycol – PEG [162, 163]) polymers have been used, though particular care is required in relation to HSC cytotoxicity (as opposed to many other stem cell populations) in response to photo-polymerization [163]. Like most HSC cultures, successful platforms often utilize feeder or stromal cell co-cultures. Leisten et al. described collagen gels to test the relative effects of bone marrow (BM) vs. umbilical cord (UC) MSCs on HSC activity [142], finding BM-MSCs to be more efficient in maintaining a primitive HSC population. Interestingly, cells found at the gel periphery and in the surrounding media were further differentiated versus those deep within the gel, suggesting the matrix environment plays a critical role in HSC phenotype maintenance. Like with scaffold platforms, early efforts have concentrated on engineering hydrogel environments to mimic the endosteal niche environment near the trabecular bone surface. For example, Raic et al. created a macroporous PEG hydrogel functionalized with the adhesion peptide RGD using salt leaching to examine co-cultures of MSC and HSCs (Fig. 4B) [141]. As with previous efforts, the greatest expansion of primitive HSCs was observed with co-culture in presence of BM-MSCs vs. UC-MSCs.

7 Integrating advanced analytical approaches into artificial niches

The small scale that makes many artificial HSC niches advantageous for studying a relatively rare cell population introduces a new issue: the heterogeneity of HSC response to a given cue. While not surprising that HSCs may exhibit a range of responses to a niche signal, this heterogeneity will likely be magnified in multi-cue environments and therefore must be defined [164]. Functional metrics of HSC fate such as limiting dilution repopulation or CFU assays can estimate the fraction of short term vs. long term HSCs in a population [36], but cannot quantify heterogeneity of response. This problem is exacerbated by the lack of in situ analysis tool to dynamically assess functional changes in HSCs. New methods are needed to gather accurate, quantitative, time-resolved information for single cells or small groups of HSCs within a continuum of niche environments. Such efforts are being explored by several groups, with early results already adding important nuanced information that may otherwise be masked in traditional population-level ensemble studies.

Dr. Timm Schroeder has been pioneering efforts in tracing HSC responses via advanced imaging techniques that allow the user to continuously track single HSCs in response to real-time perturbation. These efforts have provided critical insight into how HSCs behave at the single cell level [38, 165–168] (Fig. 3C). For example, these efforts allowed them to document not only lineage specification choices of myeloid progenitors to exogenous cytokines (e.g. G-CSF, M-SCF) but also the rate of those fate decisions [169]. These tools also allowed examination of the origin of mammalian blood; through continuous tracing of endothelial cell division processes, they revealed hemogenic endothelial cells arising from cells already expressing endothelial markers in the endothelium [170]. Together, these efforts enable identification of new HSC subtypes and provide the basis for evaluating HSC heterogeneity [166, 171].

Separate efforts exploiting array-based methods that enable high-throughput analysis of single cells are increasingly allowing investigators to parallelize studies of HSC-niche interactions. Lecault et al. demonstrated a microfluidic-based platform containing thousands of nanoliter-scale chambers with an automated media exchange system and capabilities for tracing HSC responses within discrete chambers via live-cell imaging [118]. Using this platform, they demonstrated the inherent heterogeneities associated with HSC clone growth patterns. This effort also demonstrated that while the local SCF concentration did not influence division kinetics as HSCs exited quiescence, SCF doses needed to be increased to keep the dividing HSCs viable (Fig. 3D). Lutolf et al. described a hydrogel-based microarray platform that enabled both rapid analysis of HSC proliferation kinetics [117] and a microfluidic trap for capturing HSCs for post-culture single-cell analysis [172].

8 Building complexity into artificial hematopoietic stem cell niches

Mimicking the niche as a coordinated entity of action requires understanding HSC fate decisions in response to multiplexed cell, biophysical, and biomolecular signals. Efforts for building artificial HSC niches must increasingly engineer the balance between a small number of quiescent niches and more prevalent active niches that promote mobilization, homing/engraftment, self-renewal, and lineage commitment. Replicating how niche signals

impact hematopoiesis requires new tools to dissect synergies and hierarchies, offering motivation for advanced biomaterial development. The existence of anatomical gradients across the marrow suggests a need for approaches to replicate distinct niche environments and the biophysical gradations linking these niches.

8.1 Composite niche cultures

Early efforts targeting increasing the complexity of engineered HSC niches concentrated on co-presenting multiple niche-inspired signals and developing constructs that when implanted in vivo facilitated the formation of an ectopic, HSC-containing marrow cavity. Bladergroen et al. demonstrated subcutaneous culture of collagen scaffolds loaded with stromal cell-derived factor 1 alpha (SDF-1 α) in the back of mice [173] led to higher recruitment of hematopoietic progenitor cells, but that very few LT-HSCs were recruited or maintained. Moving towards more niche-like analogs, Torisawa et al. described a bone marrow-on-a-chip platform to replicate the in vivo HSC niche environment (Fig. 4C) [174]. Integrating demineralized bone powder, BMP2 and BMP4 into a collagen scaffold, this composite device was subcutaneously transplanted into mice, resulting in the creation of a new bone-encased marrow compartment containing hematopoietic cells. Interestingly, this composite could be explanted and retained in culture in vitro, offering new possibilities in the realm of study of hematopoiesis and drug responses of HSCs in vitro.

8.2 High throughput methods

Co-culture experiments typically use large populations of cells and often entail population-level analysis of cell responses, requirements that are not always feasible when examining HSC-niche cell interactions using the well-characterized murine hematopoietic system [17]. While some 2D and liquid culture approaches have demonstrated small-volume, array-based platforms for high-throughput HSC analysis [117, 118], significant effort is still needed to translate such approaches to fully three-dimensional artificial HSC niches. Recent promising efforts by Cook et al. [145] described a high-throughput method for co-culturing of HSCs with niche cells (MSCs) in biomaterial-free 3D aggregates. These ‘micromarrow’ could be created using defined ratios of HSCs:MSCs and cultured over seven days. Improved maintenance of HSC phenotype and increased expression of key niche factors (SCF, Ang-1, Jagged-1, and SDF-1) were observed in the aggregates relative to both HSC-only 3D cultures and 2D controls.

Our lab has recently integrated microfluidic-forming and orthogonal hydrogel chemistries to generate, then sustain in culture, libraries of optically-translucent 3D biomaterials containing overlapping patterns of cell, biophysical, and biomolecular cues inspired by the marrow (Fig. 4D) [161, 175]. The intent behind this approach is to capture distinct niche environments and the biophysical gradations linking these niches. The transition from the endosteal to the vascular niche includes a transition in matrix mechanics [104, 105], ECM proteins [110] and physiological factors (Ca²⁺) [1] as well as niche cells [40, 176, 177]. Therefore, a common theme across the marrow is the presence of spatial gradations in niche-associated structural, biomolecular, and cellular components. Ranging in volume from <20 μ L to >150 μ L, these hydrogels are small enough (thickness: 500 μ m) to enable in situ analyses via confocal microscopy, yet large enough to support quantitative analyses of cells

isolated from distinct regions across the construct, termed ‘zipcodes,’ via FACS, transcriptomic, ELISA, and cell signaling (e.g., Western) metrics [161, 178]. We also demonstrated a series of imaging modalities to characterize HSC fate decisions at multiple scales within the BM: whole construct fluorescence to examine cell populations and in situ analysis of single cells via multi-photon imaging [161]. Adaptable across multiple hydrogel chemistries such as methacrylamide-modified gelatin (GelMA) and collagen, we further demonstrated approaches to functionalize the hydrogel backbone with activity inducing growth factors (e.g., SCF) to enhance long term survival of HSCs within the fully 3D construct. Excitingly, this platform allows control over spatial (distance-dependent) and temporal (biomolecule supplementation, sequestration) aspects of the niche environment in ways not obtainable by existing experimental platforms. Moving forward, efforts using a defined library of microfluidic platforms of increasing complexity may allow us to dissect how combinations of microenvironmental signals impact quiescence vs. self-renewal and lineage commitment. Functional bone marrow mimics are critical for expanding therapeutic HSC populations, generating blood and immune cells, and investigating the etiology, growth, and treatment for a range of hematopoietic pathologies.

9 Future opportunities and concluding remarks

Stem cell engineering research is in the midst of a paradigm shift. Historically, the study of HSC niches has been restricted to in vivo efforts that selectively defunctionalize elements of the marrow or simplistic 2D cultures. Both have limited capacity for examining hierarchies and synergies. Yet, as the prototype mammalian stem cell and given its long-history of clinical use, we are armed with well-established metrics (e.g. immuno-phenotype, functional) to assess murine HSC fate [17]. A unique opportunity therefore exists to develop biomaterials to elucidate mechanisms of niche action as well as act as a rheostat to regulate HSC fate. While we have described a range of tissue engineering inspired approaches to generate artificial bone marrow niches of increasingly complexity for ex vivo HSC culture, the development of analytical methods able to quantify HSC fate with high fidelity at the single cell level within these constructs represents a new frontier. Such methods need to gather accurate, quantitative information from single HSCs within a continuum of niche environments.

Recently, our group has described the use of two label-free techniques for tracing stem cell activity. Label-free Photonic Crystal Enhanced Microscopy (PCEM) is becoming increasingly popular for measuring cell intrinsic properties (e.g. number of cells on a biosensor; sub-cellular distributions of focal adhesions; discriminating cell types) [179–182]. It allows the user to gather time-resolved metrics at $<1\ \mu\text{m}$ resolution of the adhesive signature of cells and underlying substrates, allowing the user to trace interactions between a target stem cell and its matrix and the surrounding cellular environment [183, 184]. Additionally we have demonstrated that time-of-flight secondary ion mass spectrometry/multivariate analysis (TOF-SIMS/MVA) can be used to identify the differentiation state of primary murine hematopoietic cells, successfully segmenting hematopoietic stem and progenitor cells (HSPCs), common lymphoid progenitors (CLPs), and mature B cells [185]. These results suggest that label-free methods are amenable towards label-free tracing and classification of HSC fate at the single cell level, and may eventually allow time-lapse

tracing of single HSCs in response to combinations of niche signals, circumventing the need for fixing or unstable fluorescent dyes.

Moving forward, engineered HSC niches need to be developed to meet unique requirements of the HSC system and to leverage the full spectrum of current HSC fate analysis techniques (Fig. 5). Prospects for success are then inextricably linked to an ability to reproducibly create coincident patterns of cell, matrix, and biomolecules cues. Efforts over the last decade have shown tissue engineering approaches can be used to regulate critical HSC fate decisions. But much work is still required. However, engineered HSC niches offer the ability to tackle a number of critical problems. **Precision medicine platform:** artificial HSC niches stocked with patient-derived biopsy cells to explore hypotheses regarding the impact of marrow niches on the dormancy, survival, and malignancy of hematopoietic pathologies (e.g., leukemia). **HSC ageing and HSCT:** The upper age limit for HSCT was recently raised from 55 to 70–75 years of age, but at the expense of reduced-intensity or non-myeloablative therapies that increase the potential for relapse. Engineering HSC niches may provide valuable insight regarding preconditioning protocols for donor HSCs (or niches cells) to facilitate engraftment.

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Brendan A.C. Harley received a B.S. in Engineering Sciences from Harvard University, a Sc.D. in Mechanical Engineering from MIT, and was a postdoctoral fellow in the Joint Program for Transfusion Medicine at Children's Hospital Boston. In 2008 he joined the faculty at the University of Illinois at Urbana-Champaign with appointments in the Chemical and Biomolecular Engineering Department and the Carl R. Woese Institute for Genomic Biology. His lab is developing approaches to engineer biomaterials at the structural and biomolecular level in order to replicate the dynamic, spatially-patterned properties of native tissues. His lab has received funding from the NSF, NIH, American Cancer Society, and the AO Foundation.

Abbreviations

Ang-1	angiopoietin-1
BM	bone marrow
CAR cells	CXCL12 ⁺ abundant reticular cells
CFU	colony-forming unit
CXCL12	chemokine C-X-C motif ligand 12
DLL4	delta-like ligand 4

ECM	extracellular matrix
ESC	embryonic stem cell
FACS	fluorescence-activated cell sorting
G-CSF	granulocyte colony-stimulating factor
HSC	hematopoietic stem cell
HSCT	hematopoietic stem cell transplantation
HSPC	hematopoietic stem and progenitor cell
LSC	leukemic stem cell
LSK	Lin ⁻ Sca1 ⁺ cKit ⁺ cells
LTC-IC	long-term culture-initiating cell
LT-HSC	long term repopulating hematopoietic stem cell
MSC	mesenchymal stem cell
OPN	osteopontin
PEG	polyethylene glycol
RBC	red blood cell
SCF	stem cell factor
SDF-1	stromal cell-derived factor-1
ST-HSC	short term repopulating hematopoietic stem cell
TPO	thrombopoietin
UCB	umbilical cord blood
UC	Umbilical cord

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Figure 1. Schematic of the HSC differentiation hierarchy. MPP, multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte-erythrocyte progenitor; GMP, granulocyte-macrophage progenitor. Schematic inspired by [8, 31].

Figure 2.

Schematic of the bone marrow niche. HSCs are known to localize within discrete microenvironments across the marrow which present a wide range of biophysical and biomolecular cues. The endosteal niche, associated closer to the trabecular bone is home to more quiescent HSCs whereas the perivascular niche houses more actively cycling HSCs.

Figure 3.

(A) Human CD133⁺HSCs show increased cell-matrix interactions when cultured in collagen-coated (COLI) versus heparin-coated (HE) micro-cavities. Scale bar: 5 μ m. (B) Tracing division kinetics in hydrogel-coated microwells. (C) Traditional end-point assays are unable to trace the dynamics associated with HSC fate decisions. (D) HSCs cultured in liquid suspension within a microfluidic array platform to trace division kinetics. HSC division kinetics in response to temporal control of low and high doses of stem cell factor (SF) in the media. (E) A challenge in maintaining HSCs in vitro is rapid production of inhibitory feedback signals (SF) by differentiated progeny in the culture. Diagram demonstrates the effect of paracrine signals from differentiated progeny on HSC self-renewal or proliferation. HPC, hematopoietic progenitor cell. Figures reproduced (with permission) from: (A) [113], (B) [117], (C) [165], (D) [118], (E) [88].

Figure 4.

(A) Culture of CD34⁺HSCs (red) within silicate (left) and poly(acrylamide) hydrogels (right) fabricated via inverted colloidal crystal (ICC) templating. Scale bar: 100 μ m. (B) SEM image (left) of CD34⁺hematopoietic stem and progenitor cell (HSPC, red) cultured with bone marrow MSCs (purple) in macroporous PEGDA hydrogels. HSPCs showed improved retention in 3D constructs as a result of MSC co-culture. Scale bar: 20 μ m. (C) Subcutaneous implantation of an engineered bone marrow-on-a-chip containing demineralized bone powder, collagen I, BMP2, and BMP4. Engineered marrow after eight weeks of implantation as an ectopic marrow (bottom, left) vs. in vitro culture (bottom, right). Scale bar: 2 mm. (D) Microfluidic templating approach to generate engineered bone marrow containing overlapping gradients of cell, matrix, and biomolecular signals for in vitro expansion of murine HSCs. Figures reproduced (with permission) from: (A) [135], (B) [141], (C) [174], (D) [161].

Figure 5.

Ongoing challenges and opportunities for an engineered hematopoietic stem cell niche. Successful platforms must facilitate selective incorporation of a range of instructive signals (e.g., biochemical, niche cell, biophysical cues). The platforms must be readily adaptable and scalable. Increasingly, they must facilitate the use of enhanced analytical techniques to trace HSC responses to defined combinations of niche-inspired signals at multi-scales. Despite these challenges, artificial hematopoietic stem cell niches are poised to provide significant new insight regarding mechanisms by which native marrow niches affect HSC fate. They may also be used as translational tools to expand clinical cell populations or improve the diagnosis and treatment of many hematopoietic diseases. Elements of the 'adaptable platforms' figure are reproduced (with permission) from [161] (top), [189] (bottom).

Table 1

Combinations of cell surface markers to identify discrete human vs. mouse hematopoietic stem and progenitor populations.

	Human	Mouse
HSPC	Lin ⁻ CD34 ⁺ CD38 ⁻	Lin ⁻ Sca-1 ⁺ c-Kit ⁺ (LSK; 0.05% of BM)
HSC	Lin ⁻ CD34 ⁺ CD38 ⁻ CD90 ⁺ CD45RA ⁻	Lin ⁻ Sca-1 ⁺ c-Kit ⁺ Flk2 ⁻ CD34 ⁻ Slamf1 ⁺
MPP	Lin ⁻ CD34 ⁺ CD38 ⁻ CD90 ⁻ CD45RA ⁻	Lin ⁻ Sca-1 ⁺ c-Kit ⁺ Flk2 ⁺ CD34 ⁺
CLP	Lin ⁻ CD34 ⁺ CD38 ⁺ CD10 ⁺	Lin ⁻ Flk2 ⁺ IL7R α ⁺ CD34 ⁺
CMP	Lin ⁻ CD34 ⁺ CD38 ⁺ IL3R α ^{low} CD45RA ⁻	Lin ⁻ Sca-1 ⁻ c-Kit ⁺ Fc γ II/IIIR ^{lo} CD34 ⁺
MEP	Lin ⁻ CD34 ⁺ CD38 ⁺ IL3R α ⁻ CD45RA ⁻	Lin ⁻ Sca-1 ⁻ c-Kit ⁺ Fc γ II/IIIR ⁻ CD34 ⁻
GMP	Lin ⁻ CD34 ⁺ CD38 ⁺ IL3R α ⁺ CD45RA ⁻	Lin ⁻ Sca-1 ⁻ c-Kit ⁺ Fc γ II/IIIR ^{hi} CD34 ⁺

MPP, multipotent progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; MEP, megakaryocyte-erythrocyte progenitor; GMP, granulocyte-macrophage progenitor. Information adapted from [17, 186–188].