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Engineering strategies to achieve efficient *in vitro* expansion of haematopoietic stem cells: development and improvement

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Haematopoietic stem cells are the basis for building and maintaining lifelong haematopoietic mechanisms and an important resource for the treatment of blood disorders. Haematopoietic niches are a microenvironment in the body where stem cells tend to accumulate, with some nurse cells protecting and regulating stem cells. On the basis of biology, materials science, and engineering, researchers have constructed stem cell niches to address the current clinical shortage of stem cells and to explore stem cell behaviour for biomedical research. Herein, three main resource categories involved in haematopoietic stem cell niche engineering are reviewed: first, the basic approach to construct bionic cell culture environments is to use cytokines, nurse cells or extracellular matrix; second, microscale technologies are applied to mimic the properties of natural stem cell niches; and finally, biomaterials are used to construct the three-dimensional extracellular matrix-like culture environment.

1. Introduction

Haematopoietic stem cells (HSCs) are rare multipotent stem cells with self-renewal ability that can constantly maintain the homeostasis of mature blood cells and quickly differentiate to replenish immune cells in the case of inflammation and/or injury.[1–3] The key to this process is the self-renewal and differentiation of HSCs.[4] It is demonstrated that HSCs grow in a niche, a specific microenvironment that regulates their growth and proliferation.[5,6] The HSC niche accommodates cellular, chemical and physical components surrounding HSCs, influencing their synergy and signal transmission stimuli received from support cells.[7]

The HSC niche is a principal node for haematopoiesis and blood formation and provides clues for understanding the blood system, oncogenesis and inevitable ageing.[4,8] From the initial HSCs to mature cells, HSC diversity starts in the HSC niche and then gradually but robustly develops, and is related to the stemness, maintenance of stemness, quiescent state, and unilineage differentiation of HSCs.[4,9–11] In addition, a prominent induction mechanism in the HSC niche plays a guiding role in the study of blood cell cancerogenesis.[12–14] The HSC niche can also reflect the relationship between ageing and haematopoietic cell regeneration and can be used to explore HSC's potential for rejuvenation (Table 1).[15–17]

In addition to their importance in biomedical research, HSCs are also crucial in clinical use.[18,19] Currently, the main curative treatment for haematologic and lymphoid cancers, and many other disorders such as autoimmune disorders, amyloidosis and aplastic anaemia, is HSC transplantation (HSCT).[18,20] However, the shortage of HSCs limits HSCT in clinical applications. Therefore, the ultimate goal of studying the HSC niche is to address the shortage of therapeutic HSC sources for use in blood engraftment. For this purpose, the general solution relies on the efficient and high-quality expansion of HSCs *in vitro*; that is, the efficient proliferation of abundant therapeutic cells while maintaining the HSC phenotype by preventing differentiation is important. This solution is embodied by an artificial niche engineered for HSCs.

In this review, we focus on the engineering methods to achieve effective HSC amplification *in vitro* involving three parts. First, the characteristics of the HSC bone marrow niche and then details of the biochemical stimulation strategies based on static culture and 3D culture approaches are introduced. Then the role of microscale technology in advancing the development of HSC artificial niches is reviewed. Finally, the materials used to construct the *in vitro* culture system and the pros and cons of the culture system construction methods are summarized (Table 2).

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Table 1 Engineering strategies and platforms in response to the characteristics of the HSC niche

Features of niche	Corresponding strategies	Specific platforms
Biochemical environment	Regulating cytokines or chemicals	Direct addition or block of growth factors[44,45]
Nursing cells environment	Simulating cell–cell interaction through coculture	Coculture system based mesenchymal stem cells (MSCs) or umbilical vein endothelial cells (UVECs)[46]
ECM environment	Simulating cell-matrix interactions	Matrix coating platforms, nanostructured substrates, decellularized scaffolds[47]
Hypoxic environment	Constructing hypoxic niche	Hypoxic incubator or maintenance of low oxidative stress[48,49]
High-quality nutrient exchange environment	Microscale technologies	Microspheres, microporous structure, microchannels, microfluidics and bioprinting[50]
3D environment	Scaffold-based culture	Hydrogel scaffolds[51]

Table 2 The pros and cons of biochemical stimulation and improvement methods

Stimulation	Advantages	Disadvantages	Improvements
Cytokine regulation	Straightforward and quick	Causing cells to lose stemness[64,96]	Sulfated scaffolds
Coculture	No additional cytokines required	Difficulties in detecting potential pathways[103]	Nursing cell encapsulation
Matrix coating platforms	Provides a native biochemical environment	Lack of spatial complexity[124]	Decellularized materials
Hypoxic niche	Closing physiological oxygen conditions	Single factor dominates[86]	Combining other technologies

2. Haematopoietic stem cells and their natural niche

HSCs first appear in the aorta-gonad-mesonephros (AGM) region, and later, with the transfer of haematopoiesis functional sites, HSCs emerge in the foetal liver and mainly participate in circulation in the body.[21,22] Finally, with the development of the body, the bone marrow becomes the basis of the haematopoiesis system. In addition to this path, the placenta is believed to influence haematopoiesis during the period from AGM initiation to the foetal liver emergence.[4] Compared to HSCs in the AGM region, HSCs in the placenta tend to differentiate into progenitors. HSCs in the bone marrow are largely quiescent and are mainly involved in the circulatory system, except those in the foetal liver.[4] Under normal conditions, the behaviours of stem cells are critically regulated by both intrinsic factors and extrinsic cues from the surrounding microenvironment *in vivo*; these areas of external regulatory signalling are termed stem cell niches. HSCs are mainly located in the bone marrow during adulthood. In bone marrow, human HSCs are located either next to osteoblasts on the endophytic wall of the bone or adjacent to the endothelial cells (ECs) of sinusoidal blood vessels in marrow fluid.[23] A summary of the bone marrow niche is shown in Fig. 1.

It is reported that HSCs were attached to spindle-shaped N-cadherin^{+} CD45^{-} osteoblasts (SNO) in the bone niche for a long time and that the number of SNO cells determined the number of HSCs.[24] Researchers believe that SNO cells may support HSCs through the specific adhesion of N-cadherin and β-catenin and play critical regulatory roles affecting HSC function through notch activation by generating Jagged1.[7,24] N-cadherin is also the main target of angiopoietin-1 (Ang-1)/Tie-2 signalling,

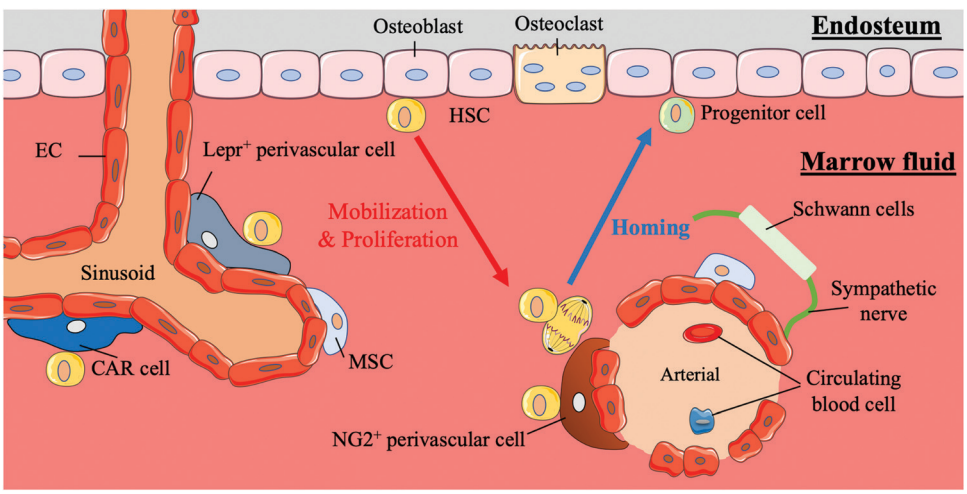

Fig. 1 A sketch of the haematopoietic stem cell bone marrow niche.

which can facilitate the preservation of HSC quiescence.[25] SNO cells and the bone cavity create a microenvironment suitable for long-term HSC proliferation while maintaining the HSC phenotype. The bone niche also plays an anchoring role in the homing process of HSCs. Chemokine ligand 12 (CXCL12, also known as stromal cell-derived factor (SDF)-1) secreted by osteoblasts or other nurse cells recruits HSCs to the bone marrow and directs them to their appropriate niche by interacting with the CXCR4 receptor expressed by HSCs.[26,27]

HSCs also gather around blood vessels inside the bone marrow.[28] This perivascular location near the endosteum is called the vascular niche.[29] In the vascular niche, stromal cells and endothelial cells surround HSCs and their progeny forming a protective environment and maintaining the HSC population by secreting CXCL12, stem cell factor (SCF, also known as KITL) and probably other cytokines.[23,30,31] Among these support cells, a particular type of perivascular stromal cell can secrete a large amount of CXCL12 and contact HSCs directly, CXCL2-abundant reticular (CAR) cells, which surround endothelial cells on sinusoids and arterioles or are located near the bone niche of HSCs.[32] The localization of HSCs in the quiescent and activation state differs. Quiescent HSCs are associated with small arterial regions where neural-glial antigen 2-positive ($NG2^+$) periarteriolar cells are located. In contrast, activated HSCs are clustered near the perisinusoidal region expressing a leptin receptor (Lepr).[33] Other cells, including macrophages, megakaryocytes, sympathetic nerves, and non-myelinating Schwann cells, also play roles in regulating the vascular niche.[34,35]

The intricate physical and chemical environment inside the bone marrow niche is crucial to the regulation of HSCs. The extracellular matrix (ECM) is a complex system involved in the development of the inner three-dimensional (3D) structure of bone marrow and composed of collagen, proteoglycans, glycoproteins and glycosaminoglycans.[36] The ECM not only provides structural scaffolding but also regulates stem cell behaviour. Different ECM molecules change the adhesion, proliferation, survival, migration and differentiation of stem cells.[37–41] Simultaneously, changes in the stiffness of the bone marrow can affect the behaviour of HSCs. Changes in the external physical environment can be detected by stem cells through complex mechanical sensing systems, and modulate the subsequent transcription process.[42] The influence of changes in oxygen content on HSCs cannot be ignored either. The hypoxic tension in the bone marrow niche is considered to be an important reason for maintaining HSC stemness.[43] This local heterogeneity with a steep gradient influences the behaviours and fates of HSCs.

The natural HSC niche has complex regulatory behaviours with many biochemical and physical components participating in this process. The simulation and in-depth exploration of this platform are of great significance to biomedicine and also to clinical medicine. Thus, in combination with the properties of the HSC niche, there are responsive engineering strategies to simulate in order to create a culture system more suitable for HSC *in vitro* amplification and maintenance of the phenotype, which will be elaborated in detail in the following sections.

3. Biochemical stimulation

3.1 Regulating cytokines or chemicals to mimic the bone marrow niche

The development and growth of HSCs are believed to be regulated by a wide range of biomolecules. Cytokines, including SCF, FMS-related tyrosine kinase 3 ligand (Flt3L), granulocyte colony-stimulating factor (G-CSF), interleukin (IL)-3, IL-6, IL-11 and thrombopoietin (TPO), are known regulators that contribute to quiescence maintenance, self-renewal regulation and migration of HSCs *in vivo*.[45,52–54] The combination of these cytokines can achieve diverse selective amplification of HSCs in an *in vitro* environment, among which the combination of Flt3L, SCF and TPO has been commonly used. For instance, the number of $CD34^+$ cells can be increased by 28 ± 9-fold *in vitro* in a 10 days treatment of cultured $CD34^+/CD38^-$ cells with the combination of the above three cytokines.[55] However, no matter how these cytokines are combined, the effect on long-term culturing of HSCs *in vitro* is not significant.[56] Interestingly, compared to soluble cytokines, the matrix-immobilized form of cytokines shows a better supportive effect on the long-term culture of HSCs *in vitro*.[57] Mahadik *et al.* used acrylate-functionalized polyethylene glycol (PEG) tethers to covalently immobilize SCF and proved that the effect of covalent SCF on cell phenotype maintenance is stronger than that of continuous SCF.[58] Immobilized cytokines such as cell-surface SCF and cell-surface CSF-1 were proven to play supporting roles in the long-term culture of HSCs *in vitro* by transduction technology according to Friel *et al.*[59]

Other cytokines have different regulatory effects on HSCs and haematopoietic progenitor cells (HPCs). Tumour necrosis factor-alpha (TNF-α) not only induces the apoptosis of myeloid progenitor cells but also promotes HSC survival and bone marrow differentiation by activating p65 nuclear factor κB (NF-κB)-dependent gene programmes.[60,61] This specificity can prevent necroptosis and induce immune regulation. However, whether TNF-α itself or other ligands induced by TNF-α directly act on HSCs to exert the aforementioned protective function remains to be determined.[60]

Transforming growth factor beta (TGF-β) is one of the most effective inhibitors restricting the growth of HSCs *in vitro* and is also considered to be the main cytokine maintaining the quiescent state of HSCs.[62] TGF-β not only independently controls the cell cycle of HSCs through the upregulation of $p57^{Kip2}$ but also inhibits the proliferation of HCSs by changing the expression of cytokine receptors and upregulating inhibitors of cyclin-dependent kinases.[44] Akhkand *et al.* blocked the expression of TGF-β receptor II on $CD34^+$ cells to increase the expansion rate of $CD34^+$ cells.[63] In a culture environment with only growth factors, the expansion factor of transfected $CD34^+$ cells measured on the eighth day was 28.5 fold, while the expansion factor of $CD34^+$ cells was only 10.8 fold.[63]

GSK3 inhibits Wnt/β-catenin signal transduction through the degradation of β-catenin triggered by phosphorylation and inhibits the expansion of HSCs *in vitro*. However, interestingly, GSK3 inhibitors can induce β-catenin activity to promote HSC proliferation and in turn stimulate bone marrow cells to

produce inflammatory cytokines, which can reduce HSC proliferation by inducing p38 activation.[64] Li *et al.* directly blocked the GSK3 signalling pathways. In impure HSC populations, the number of $CD34^+$ cells in the blocking environment was significantly increased compared with that in the unblocked environment, with a 9- to 11-fold difference after 12 days.[64]

In addition to cytokines, synthetic chemical molecules can be applied to replace specific components of the culture medium. Polyvinyl alcohol (PVA) can replace serum albumin for the culture of HSCs.[65] Through optimized SCF and TPO concentrations and the use of a fibronectin matrix, this albumin-free *in vitro* culture method can support the long-term expansion of mouse HSCs for more than 2 months.[19] However, the albumin-free *in vitro* culture method for human HSCs needs further improvement.[66]

The biochemical environment of HSCs in the niche is complicated; therefore, to simplify the model and/or to confirm the expression of specific cytokines, conditioned medium is often used for *in vitro* cultures of HSCs. Breems *et al.* utilized culture medium in which stromal cells were cultured to promote the proliferation of HSCs.[67] This method is similar to that of coculturing, which will be introduced in Section 3.2. Moreover, although the blockade of unwanted cytokines needs to be taken into consideration, it increases the uncertainty of the niche culture components.

3.2 Simulating cell–cell interaction through coculture

HSCs make close intercellular connections with nurse cells in the bone marrow niche. The physical and chemical mechanisms of the interactions between these cells are still not fully understood. Coculture of these cells is the basis of an *in vitro* engineering model in which an HSC niche is simulated but not particularly similar to a natural niche. However, using nurse cells as the feeder cells or feeder layer of HSCs for coculture is a direct and simple biochemical bionic means.

In the bone marrow niche, MSCs interact with HSCs and their progenitor cells to form a symbiotic environment, protect the phenotype of the HSCs, and promote HSC self-renewal. MSCs secrete SCF, Flt3L, IL-6, and CSF-1 cytokines, which can promote the expansion of HSCs *in vitro* and support long-term culture.[68] Under the same cytokine conditions *in vitro*, the expansion of $CD34^+/CD38^-$ cells in a population with MSCs as feeder cells was 45-fold greater after 10 days than that of a group without MSCs as feeder cells.[55]

Genetically engineered MSCs can be used as feeder cells to improve the efficiency of *in vitro* HSC amplification. As a cytokine that regulates the mobilization, homing and expansion of HSCs, stromal cell-derived factor-1 (SDF-1) can regulate the cell cycle of HSCs by combining with SCF or TPO and thus enhance the survival of HSCs *in vitro*.[69] Therefore, Ajami *et al.* used transfection technology to prepare MSCs capable of overexpressing SDF-1 and SCF (the soluble form and membrane form, respectively). The results showed that the maximum expansion factor of $CD34^+$ cells reached 4.73 ± 0.26-fold after seven days.[70]

UVECs can also be used as feeder layers to assist the expansion of HSCs *in vitro*. UVECs express some of the same surface markers and transcription molecules as HSCs, such as CD34, CD31, Runx1 and GATA-2, to support the expansion and differentiation of HSCs *in vitro*.[71,72] Therefore, Li *et al.* cocultured UVECs as feeder cells with HSCs in a culture medium containing SCF, TPO and Flt3L and tested the HSC stemness and HSC *in vitro* expansion. The results showed that the feeder-based coculture further promoted the expansion of the HSCs.[72] Huang *et al.* also used the cocultivation model of UVECs and HSCs to construct a comprehensive and balanced pool of long-term HSC cultures which effectively maintained the HSC phenotype *in vitro*.[46]

There are two ways of coculturing HSC: direct coculture and indirect coculture.[73] In the direct method, cells are in direct contact or adhere together. This method is simple with no technical barriers but is impossible to evaluate the degree of the specific cell interaction. The indirect method involves separating cells in the same culture space through physical isolation methods such as semipermeable membranes. This method avoids labelling cell populations to detect the effects of soluble cytokines, but is not suitable for studies in which physical contact triggers induction.

3.3 Simulating cell-matrix interactions by matrix coating platforms and nanostructured substrates

Inspired by the natural niche of HSCs, artificial niches (such as collagen fibres and fibronectin fibrils) were constructed to mimic and engineer the nano-structured ECM that supports cell behaviour on the basis of their own morphology and mechanical properties. HSCs can be induced by changes in substrate elasticity, morphology, and ligand lateral spacing in the culture environment.[74,75] Instead of using growth factors and cytokines to induce HSCs, the physical characteristics of the material such as substrate elasticity and substrate nanostructures is adjusted to stimulate HSCs and promote *in vitro* HSC expansion.

A platform based on tropoelastin-coated substrates first used by Holst *et al.* showed that matrix elasticity and tensile integrity influenced the growth of HSCs.[76] Choi *et al.* tested a type I collagen- and fibronectin-functionalized polyacrylamide surface, and photo-crosslinked laminin by sulfo-SANPAH and found that the fibronectin maintained the population of HSCs, the collagen promoted the proliferation of HSCs, and the laminin promoted the differentiation of HSCs into a red blood cell lineage.[77] Changes in the morphology of the substrate also affect the fate of stem cells. For example, a microcavity with a diameter no larger than 15 μm can contribute to an increase in the number of HSCs.[78] Nanopillars and nanogratings have also been shown to have an effect on the fate of HSCs.[79,80]

The arginine–glycine–aspartic acid (RGD) peptide is key to the ECM guidance of stem cell adhesion, differentiation and proliferation.[47] Raic *et al.* confirmed that RGD contributes to the maintenance of the HSC stemness.[81] Kratzer *et al.* used RGD to induce the differentiation of HSCs into T cells and provided a strategy for artificially inducing T cells.[82,83] RGD is

commonly used as a functional peptide in hydrogels to improve the supportive ability of hydrogels for the expansion of HSCs. In addition to RGD, the connecting segment (CS)-1 motif of fibronectin has been confirmed to support the proliferation of HSCs.[83]

The nanoscale lateral distance between coupled ligands has been shown to be a factor that mediates the interaction between cells and ECM molecules.[6] Ligands at a specific distance, which is determined by the type of ligand, facilitate the clustering of integrins to form a signalling complex. For peptide ligands derived from small fibronectin, the critical adhesion distance between HSCs is less than 45 nm. However, fibronectin-derived and OPN-derived protein domains can support long-distance cell adhesion.[84] Winkler *et al.* used an adhesive RGD peptide and DLL1 to construct a functional surface and proved that ligand density and clustering are key to promoting the proliferation of HSCs.[85]

Indeed, different artificial niche methods can be combined with each other, exerting a synergistic effect on the promotion of HSC amplification. However, pure matrix coating platforms and nanostructured substrates are flawed as they lack multi-dimensionality, one key aspect of ECM mimics. As a result, the culture model of cells is not spatially consistent with the natural niche. Therefore, a 3D culture method based on biomaterials has been developed and valued.

3.4 Constructing a hypoxic niche

Compared with niches of cells in other tissues, the bone niche of HSCs is a hypoxic environment, which ensures that HSCs stay in a quiescent state with an unchanging phenotype.[49,86] Quiescent HSCs maintain low oxidative stress to prevent self-depletion or differentiation and a high glycolysis rate to generate energy through anaerobic metabolism, which differs from the metabolism of progenitor cells or differentiated offspring cells.[87] An important transcription factor in this regulatory pathway, hypoxia-inducible factor-1alpha (HIF-1α), affects HSCs through the heat shock protein GRP78 and its ligand Cripto.[88] Without the expression of HIF-1α, HSCs are released from the quiescent state of the cell cycle, gradually age and lose their transplantation capacity. The excessive stability of HIF-1α (biallelic loss of von Hippel-Lindau protein) arrests the cell cycle and causes a loss of HSC transplantation capacity.[49,89]

Kiani *et al.* cocultured MSCs with overexpressed HIF-1α with HSCs and found that the MSCs effectively contributed to HSC phenotype maintenance and inhibited the differentiation of HSCs.[90] Capitano *et al.* reduced the oxygen stress response to restore the number and function of HSCs in aged mice.[91] Kobayashi *et al.* created a low-oxygen environment to achieve a long-term (one month) quiescent state for HSCs *in vitro*.[48] For the construction of a hypoxic culture environment, researchers mainly use a CO_2/O_2 incubator to adjust the culture environment to $5\%CO_2/1–5\%O_2$ or $5\%CO_2/95\%N_2$ for flushing the HSCs in a sealed culture chamber.[48,91,92] Research on the hypoxic niche mainly focuses on the maintenance of the *in vitro* HSC phenotype or the regulation of the resting state. The use of the hypoxic niche to promote the high-quality expansion (maintaining the phenotype while inducing high amplification magnification) of HSCs *in vitro* needs further study. Fig. 2 shows a schematic diagram of the methods by which biological mimics are used to create an HSC artificial niche.

3.5. Improvements: scaffold-based system and dynamicisation of biochemical stimuli

Biomaterials such as collagen, tropoelastin, fibronectin or other ECM-derived materials are always used as substrates to fabricate artificial niches in a facile way. This static suspension cell culture method addresses the initial problems of cell-matrix pathway triggering,[76,77,93] but unfortunately, fails in the biological and physiological cases and leads to obvious distortions. The growth mode of cells in the static suspension model is different from that in the human body, resulting in obvious differences in morphology, differentiation, cell–cell contact, and cell–matrix contact to those of cells growing under physiological conditions *in vivo*. As a result, there are difficulties in translating the static suspension model into clinical use or pharmacological and physiological use. The cells in a 3D culture model keep their natural forms and functions and proliferate in the same way as *in vivo* proliferation. 3D models provide a platform to study the interconnection of cells in a complex system with physiologically relevant physical structures that allow cells to grow in multiple

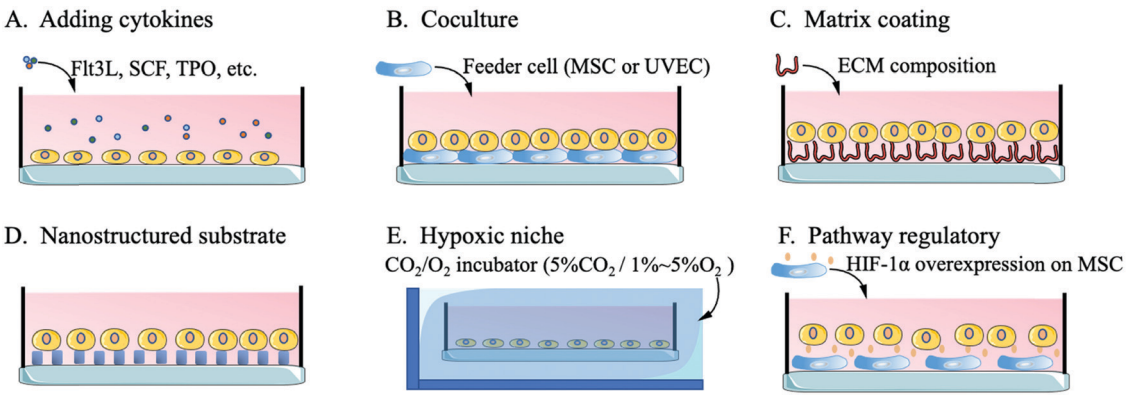

Fig. 2 Biochemical bionics to engineer artificial niches.

directions.[94] The 3D model reduces the contact area between the cell and the substrate to maximize the stability of the original morphology of the cell and help maintain the cell function *in vitro*. HSCs are more active and more responsive to external factors in the 3D model compared to static suspension models.[95] A functional 3D model, which avoids the ethical and economic issues, can be a substitute for animal models.

All biochemical stimulations are designed to mimic the original niche characteristics of HSCs and maintain the phenotype and amplification status of HSCs as much as possible. However, this desired outcome can hardly be yielded from *in vitro* culture of HSCs under traditional cultural conditions *via* free suspension.[96] It is hypothesized that this failure is attributed to the irregular and discontinuous dosage and dosing frequency along with the conventional operation of medium change.[96,97] The development of a 3D culture of HSCs *via* the traditional method of free suspension is hindered by the inevitable operations of centrifuging and re-suspending steps for medium change every time, which is labour intensive and causes damage to the cultured cells. In contrast, a dynamic 3D culture by means of equipment modulation can address these problems. Medium change can therefore be controlled and performed in a real-time and dynamic manner through programmed automation. In this way, the microfluidic administration of exogenous maintenance additives can better mimic the natural administration *in vivo*; thus, with the dynamic culture system, proliferation and differentiation resistance can be promoted simultaneously *in vitro* for quality and efficient expansion of HSCs.

3.5.1 Scaffold-free and scaffold-based systems. 3D culture models can be divided into scaffold-based and scaffold-free systems. A detailed diagram of scaffold-based and scaffold-free systems is shown in Fig. 3.

A scaffold-free system refers to a construct in which cells do not rely on external materials or other cells because they can provide their own self-supporting structures for cell adhesion, growth, and diffusion by forming tissue-like micro-tissue spheres. There are three main categories of scaffold-free systems: non-adhesive surfaces, hanging drops and equipment (spinner flasks or perfusion bioreactors). When culturing on a non-adhesive surface, cells do not attach to the surface but accumulate to form a cell sphere, thus developing a self-assembling 3D culture. In the hanging drop method, the cell culture environment is replaced by hanging drops that promote the self-assembly of cells into microspheres by gravity. The formation of the microstructure ball in the hanging drop method can be easily controlled and highly consistent from batch to batch. Moreover, the hanging drop method can be used to coculture different cell types, ensuring signal exchange between cocultured cells.[98] In the spinner flask method, a stirrer agitates the culture medium in a flask, which drives the cells into a circular motion and promotes the aggregation of cells, forming microstructures. The perfusion bioreactor system can continuously replenish the new culture fluid while draining the old culture fluid to maintain the optimal growth state of the cells and reduce the accumulation of environmental toxins.[99] The equipment used in the spinner flask/bioreactor system can improve the cell culture density, quality, and utilization.[10,100]

A scaffold-based system involves a hydrogel scaffold which is generated by natural or synthetic biomaterials and can be further tailored. In addition, biomaterials secreted by cells can be used as scaffold materials, such as decellularized ECM scaffolds or decellularized bone marrow scaffolds. Cells can also be used to construct organoids or microsphere scaffolds to provide an additional anchor for target cells.[101] The design and use of scaffolds requires consideration of the structure, pore size, morphology, mechanical properties, surface characteristics and degradation products.[102] Scaffold-based systems have advantages over scaffold-free systems in mimicking the complex and functional ECM environment which is highly relied on for HSC expansion, or controlling the positioning of transplanted cells.[95,100] Scaffolds provide a controlled environment for cell growth while supporting long-term survival or proliferation of cells *in vitro*. They are also able to carry growth factors, maintain their protein activity and improve cellular capture of growth regulatory signals. The use of a scaffold-based system can improve the competitive advantage of donor HSCs and increase the success rate of HSCT.[33]

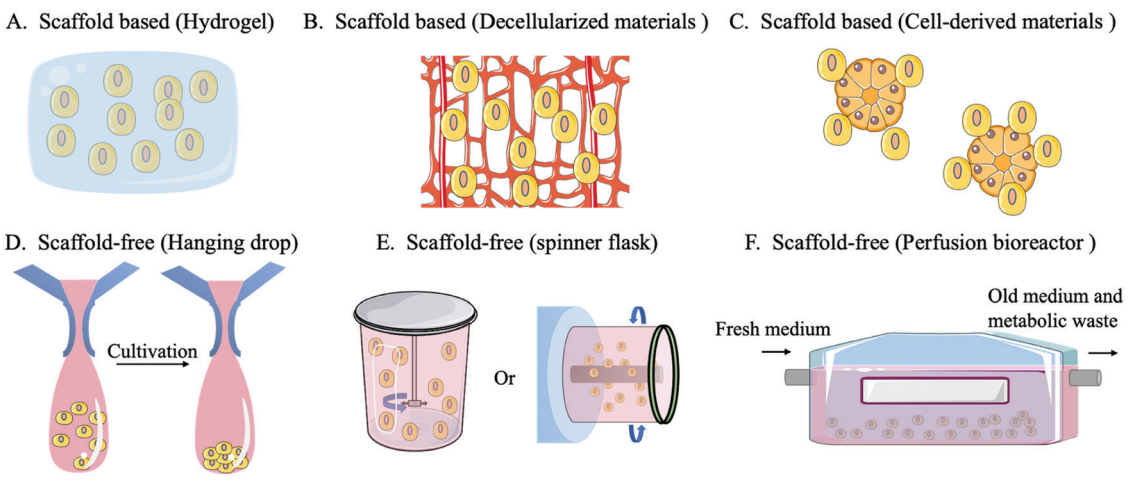

Fig. 3 A sketch of the 3D culture model including scaffold-based and scaffold-free systems.

3.5.2 Improvement strategies for biochemical stimuli. Cytokines enhance HSC proliferation, but also largely facilitate HSC differentiation at the same time, which is unacceptable for maintaining HSC cell stemness *in vitro*.[103] Furthermore, cytokines are unstable in *in vitro* culture environments and are rapidly inactivated in aqueous liquid, leaving HSCs unable to receive sufficient biological signals to maintain a proliferative or quiescence state.[104,105] Mhanna *et al.* then used sulphated scaffolds to improve the capture of cytokines in the culture system.[106] By sulphating alginate to varying degrees, Mhanna *et al.* found that an increase in the sulphation degree of hydrogels resulted in an increase in the binding capacity for cell growth factors and maintaining cell stemness.[106] Comparing heparin, dextran sulfate, λ-carrageenan, and chondroitin sulfate as binding and stabilizing agents for growth factors, Sun *et al.* found that sulfated polysaccharide scaffolds helped stabilize cytokines.[107] This suggests that the sulphated polysaccharide scaffold contributed to maintaining cytokine activity and further cell stemness (Fig. 4).[108]

For coculture, a direct three-dimensional approach is no better than static suspension coculture. Because MSCs or UVECs can adhere to the dish, they have the advantage of subsequent cell isolation on culture plates. If the HSCs and nursing cells are embedded in the scaffold, subsequent cell isolation or detection would be tedious.[109,110] The phase separation method is therefore used, *i.e.* MSCs and HSCs are encapsulated in different hydrogels, which makes the detection and separation easier.[111,112]

In addition, the disadvantages of isolation or characterization can be circumvented by culturing nursing cells into cell spheres and using the size difference between HSCs and cell microspheres.[113] Hur *et al.* used mononuclear blood cells to construct cellular microspheres that provided physical support for HSCs and included cytokine modulation such as introduction of SDF-1 and CXCR4. The presence of a large ECM component in the microspheres also greatly facilitated the proliferation of HSCs.[114] However, heterogeneous cell populations can have other potential effects on growth stimulation and activity maintenance.[115] Furthermore, potential signalling pathways are difficult to detect and fully demonstrate in the laboratory; therefore, using cells present in the natural HSC niche as microsphere scaffolds is effective in preventing the effects of too many

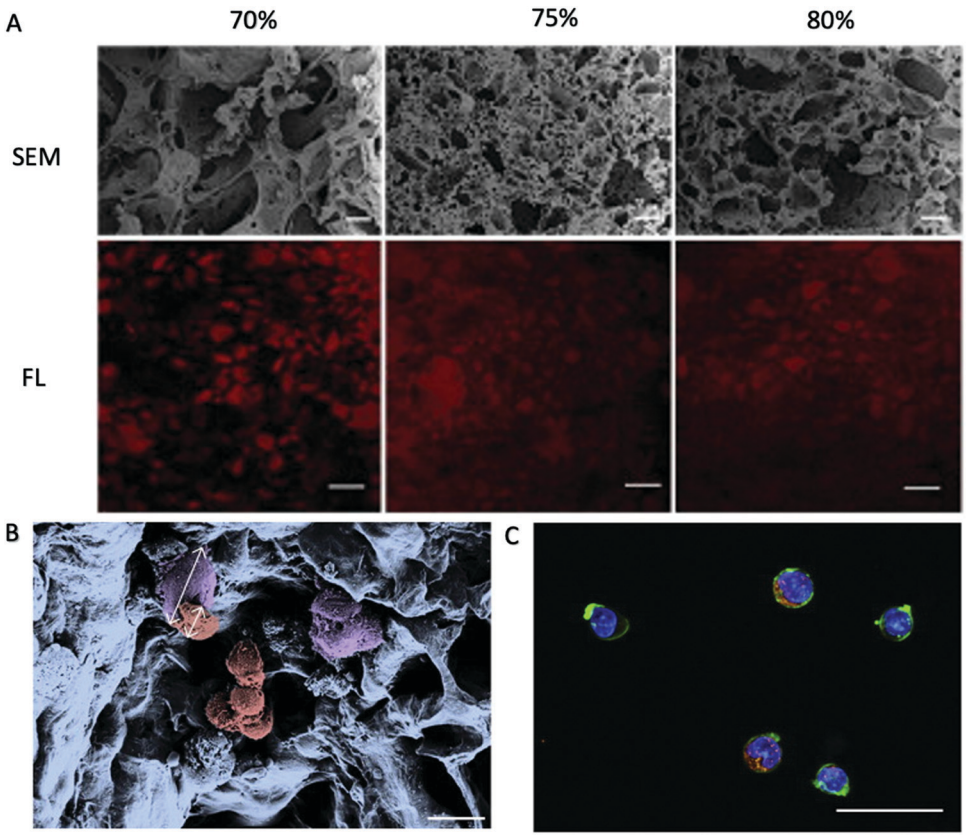

Fig. 4 PEG-DA porous hydrogels prepared using NaCl as a pore-forming agent.[81] (A) Cross-sections of hydrogels fabricated with different porogen content as indicated on top were analyzed for their porosity. Scanning electron micrographs (SEM) reveal the density and distribution of the pores. Fluorescence micrographs (FL) of hydrogels with Alexa Fluor 647-labeled BSA in red as the tracer molecule reveal interconnectivity of the pores. Scale bars, 20 μm (SEM) or 100 μm (FL). (B) Pseudo coloured SEM of a porous hydrogel seeded with primary MSC and HPCs. The cell types can be distinguished by morphology and size as depicted by the arrows. Purple: adherent MSC, ~28 μm in size; red: HPCs, ~12 μm in size. Scale bar, 20 μm. (C) FL of a porous hydrogel seeded with primary MSC and HPCs. Blue: DAPI/cell nuclei and green: phalloidin/cytoskeleton visualize both cell types. Red: Alexa 568/CD34 identifies HPCs. Scale bar, 20 μm. Reproduced with Copyright Permission, 2014 Elsevier.

unknown factors. Isern *et al.* used stromal stem cells, which constitute part of the same bone marrow niche as HSCs, to construct cell microspheres and found that the microspheres were the most effective in the long-term culture of HSCs (40-fold higher than the group without cell microspheres after four weeks).[116] However, the mechanisms and secreted products within the cellular microspheres require further investigation (Fig. 6).

3D matrix coating platforms are mainly achieved by decellularized scaffolds. Decellularized materials are constructed from allogeneic or xenogeneic tissues upon the removal of internal cells through physical or chemical methods and are nonimmunogenic or low-immunogenic. Decellularized scaffolds are naturally complex in internal interconnection structures, and are difficult to isolate simply using porogen, salting or freeze-drying methods. The decellularized scaffold originated from a natural biological source, supporting its use as a platform for whole-organ reconstruction. Therefore, decellularized materials are advantageous in simulating the HSC niche. The most common physical method of lysing cells is rapid and repeated freeze-thawing, which generates ice crystals in the intracellular fluid, resulting in the rupture of the cell wall. Chemical reagents or enzymes are then used to remove the remaining cell contents.[117] Alternatively, rapid changes in pressure can be used to lyse cells while washing with detergent to completely remove the cellular components.[118]

Bianco *et al.* decellularized bovine bone marrow and used it as an *in vitro* culture platform for HSCs and found that the decellularized scaffold was able to support the adhesion and expansion of HSCs while maintaining the phenotype of HSCs *in vitro* for a long time with the help of the cytokines secreted by stromal cells. Surprisingly, the transplanted HSCs preferentially adhered to areas corresponding to connective tissue surrounding the vascular region, recreating the natural vascular niche of HSCs *in vitro*.[117] Nakamura *et al.* used HHP and sodium dodecyl sulfate (SDS) detergent to decellularize cancellous bones. After being implanted into mice, the scaffold was found to be recellularized, stimulating the continuous homing of HSCs and promoting ectopic haematopoiesis.[119] However, the use of SDS can cause damage to the vascular structure; therefore, decellularized scaffolds prepared by enzymatic digestion are recommended. In addition to bone marrow sources, decellularized scaffolds can be prepared on the basis of the ECM. Decellularized ECM scaffolds can promote the adhesion of HSCs and induce $CD34^+$ cells to proliferate more than fourfold within seven days.[120] Soffer-Tsur *et al.* used freezing and enzymatic digestion to decellularize the ECM and construct porous hydrogels for culturing HSCs.[121] Li *et al.* used a decellularized Wharton jelly matrix to construct a porous hydrogel scaffold and similarly demonstrated its unique advantage as a decellularized scaffold in promoting the maintenance of the phenotype of HSCs, enhancing cell self-renewal and maintaining the differentiation potential.[122]

The hypoxic niche is not closely integrated with other engineering strategies.[86,123] Stem cell research requires a holistic view of HSC regulation, which requires a multi-parameter approach, and also aids in the development of new techniques to analyse the conditions of the HSC niche *in vivo*.

4. Microscale technologies

4.1 Microspheres

Microspheres are superior to other particle geometries for biomedical applications as they have a consistent shape and physical properties that ensure delivery stability and a large surface area that increases the efficiency of the coating and reduces the risk of immunization.[125] Microspheres can be used as drug or cytokine carriers for therapeutic or tissue regenerative purposes.[126] Liu *et al.* used poly(lactic-*co*-glycolic acid) (PLGA) microspheres as carriers of cytokines that are related to the

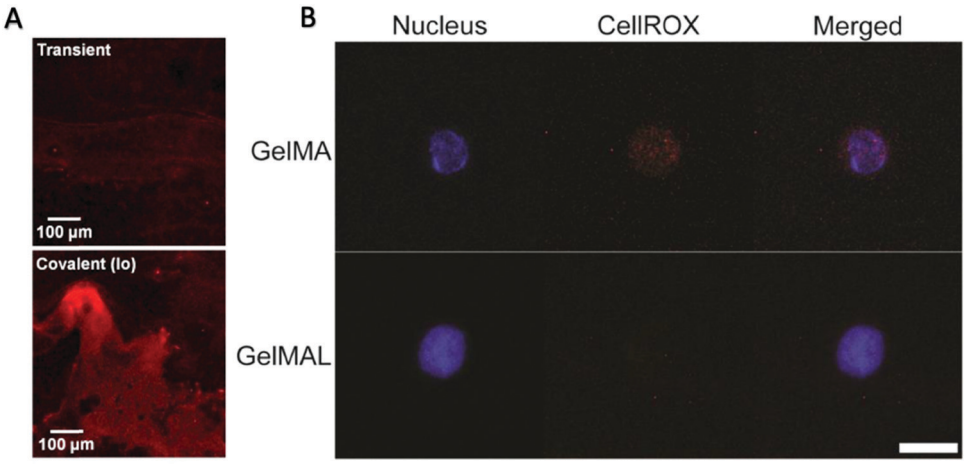

Fig. 5 (A) Characteristic fluorescence image of histology sections taken of the GelMA hydrogel network after 7 days, showing SCF (AlexaFlour 633) retained within the GelMA network.[58] Reproduced with Copyright Permission, 2015 Elsevier. (B) Representative images of an HSPC encapsulated in 5 wt% GelMA or GelMAL. The nucleus is stained with Hoechst (blue). ROS are stained with CellROX (red).[195] Images are auto-thresholded to Max/Min using ZEN lite (Zeiss Microscopy). Scale bar is 10 μm. GelMAL = maleimide-functionalized gelatin. Reproduced with Copyright Permission, 2021 Elsevier.

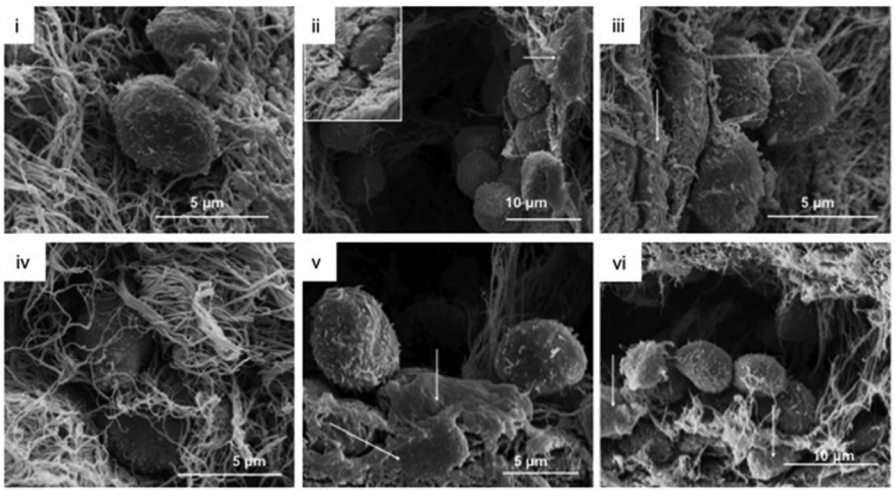

Fig. 6 Collagen gel topography and HPC morphology in the collagen gels were analyzed *via* SEM at day 14.[198] MSC were located in direct association to HPC (arrows, ii, iii, v, vi). Reproduced with Copyright Permission, 2012 Elsevier.

regulation of HSCs to enhance angiogenesis in damaged muscle.[127] In addition to be directly involved in the construction of the system, microspheres can be used for auxiliary characterization or be used as a crosslinker and reinforcer in the hydrogel. For example, Mahadik *et al.* used microfluids to simulate the bone marrow microenvironment *in vitro*, and later used fluorescent microspheres to construct gradient hydrogels, to help with the characterisation of hydrogel structures.[128] Wang *et al.* used methacrylamide microspheres as a cross-linker and reinforcer of hydrogels.[129]

Microspheres can be made as scaffolds for culturing cells, mimicking the *in vivo* environment and functioning to maintain cell phenotypes or increase the proliferation rate of cells.[130] Bello-Rodriguez *et al.* cultured MSCs onto collagen microspheres, covered the MSC encapsulated microspheres with a plasma coat, and then used this system to induce ectopic haematopoiesis in mice.[131] Colloidal crystal microspheres were used as templates and later wrapped with sodium silicate or acrylamide hydrogels to form a HSC *in vitro* culture scaffold with an inverted colloidal crystal structure. The microsphere scaffolds were coated with a layer of nanomaterial to improve cell adhesion and were proved to support the proliferation and differentiation of $CD34^+$ cells into B cells and to be essential for the production of B cells and antigen-specific antibodies.[132] Fathi *et al.* used alginate and gelatine to encapsulate HSCs into microspheres, after which they examined changes in the state of cell differentiation towards NK cells and found that the microsphere scaffold affected the telomere length in the cells.[133] Microspheres made using thermo-sacrificing materials such as gelatine can play the role of a porogen, as mentioned in Section 4.2, while microspheres made using materials that remain stable in the culture environment are used to construct micron-scale scaffolds and to verify the effect of micro-regulation on the fate of HSCs.

Typical methods used to produce microspheres include emulsion-solvent evaporation, sol-spray drying and electrospinning processes.[126] The oil/water emulsion method involves the use of agitators to float the material as microdroplets in another phase of liquid, after which impurities are removed by washing or evaporation.[125] The sol-spray drying is based on dispersing the material into tiny droplets of mist through an atomizer and removing the water in a stream of hot air to obtain a microsphere product.[134] The electrospinning involves electrostatic charges on the surface of polymeric fluids under a high voltage electric field to generate microdroplets.[135] However, electrospinning is more commonly used as a method of manufacturing nanofibres, which is explained in detail below.

4.2 Microporous structure

The scope of scaffolding applications in medicine has begun to shift from nondegradable synthetic materials and tissue grafts to degradable porous materials and regenerative medical tissues.[50] In cases of porous scaffold materials, the appropriate porosity determines the ability of a scaffold to induce tissue regeneration and functionalization.[136] The porous structure inside the scaffold results in a uniform distribution of cells, provides space for intercellular contact and stimulates cell proliferation and differentiation (when adapted to different cells). In addition, a porous scaffold is superior to a non-porous scaffold in terms of better nutrient and gas exchange, which is particularly important for tissue regeneration. Porous hydrogels with their macromolecular cross-linked network structure and additional pore space are uniquely suited to mimic the complex network structure of the ECM and are preferred for engineered artificial bone marrow analogues.[81]

The pore size of porous scaffolds can directly influence the behaviour and viability of cells. 5 μm is a suitable pore size for vascular regeneration, while 20 μm is suitable for cell growth. Pore sizes suitable for bone regeneration range from 100 to 350 μm, while tissue regeneration models require a pore size larger than 500 μm.[50] The interconnected space inside a porous scaffold directs cell growth inwards, a process known as spontaneous phase transfer cell culture (PTCC).[137] In cell-laden hydrogel constructs, the creation of microcavities introduces

microedges, which are gel-cavity interfaces, into the bulk cell-embedded hydrogel. As non-anchorage-dependent species, the embedded cells tend to proliferate into isogenic groups (such as colonies) in non-cell-adhesive bulk gel. Cells residing in the gel-cavity interface were loaded with asymmetric encapsulating stress from different directions, with the stress from the gel bulk side being significantly greater than that from the cavity side. The net force of these unbalanced stresses guides and drives the proliferative expansion of these cells across boundaries from the gel phase towards the cavity phase, causing outgrowth from the bulk gel into the intra-gel cavities. These trans-phase outgrowing cells fill the open space inside the cavities upon further development. In addition to the mechanical impetus, the cell outgrowth direction also aligns with the ascending gradient of medium diffusion and nutrient exposure from a relatively concentrated phase towards a diffuse and ultimately void phase (cavity) in the bulk gel.

Traditionally, the porous hydrogels are made by solvent casting/particle leaching. The porogen with controllable particle size and the polymer solution are homogeneously mixed and solidified. The porous structure is then formed by melting and leaching the porogen through external changes such as in temperature, pH, and other conditions or by dissolving the porogen. Sodium chloride (NaCl) is often used to construct porous scaffolds for culturing HSCs.[81,138]

Pan *et al.* used NaCl as a porogen and adopted direct solvent casting to construct porous polycaprolactone (PCL) scaffolds.[139] However, this PCL material has poor hydrophilicity and biological activity and is reported to exhibit low porosity with a large pore size, which is unfavourable for cell adhesion.[140] Therefore, the PCL scaffold is not as effective as the fibrin or collagen scaffolds which exhibit high porosity and low fibre stiffness for promoting the expansion of HSCs *in vitro*. However, this limitation can be improved by adding modified silica into the scaffold. Yang *et al.* used vinyltrimethoxysilane to modify silica particles and then melted the particles within PCL. The silica/PCL scaffolds with 800 nm particles showed better performance in the surface roughness, hydrophilicity, and mechanical strength than PCL scaffolds, while the 100 nm silica/PCL scaffolds showed significantly better stem cell adhesion.[141]

Porous structures can also be generated using the stacking nanofibres made by electrospinning technology,[142] which enables controllable thickness, composition and porosity of the nanofibres. Porous scaffolds made from these types of nanofibres benefit from a large surface area, exhibit enhanced cell adhesion, and can further be functionalized by incorporating growth factors during the manufacturing process to better control the proliferation and differentiation of cells cultured in the scaffold. However, the electrospinning technology is limited in producing more complex 3D shapes.[143]

In many cases, polymeric materials are made into nanofibres to build porous hydrogels.[144] Polyether sulfone (PES) nanofibers have been shown to support $CD34^+CD45^+$ cell expansion while promoting the expression of CXCR4 and lymphocyte function-associated antigen-4 (LFA-4) in HSCs.[145,146] Sabaghi *et al.* found that aminated PES nanofibres had a greater effect on the differentiation of megakaryocyte progenitors than conventional cultures, which was more conducive to accelerating platelet recovery and improving the transplantation success rate.[147] Chua *et al.* also used electrospinning technology to construct polyethylene terephthalate (PET) nanofibre networks, followed by grafting poly(acrylic acid) onto the surface of the networks by photopolymerization and finally modifying the scaffolds by ammonification. The scaffolds with ethylene and butene spacers were found to have the greatest effect on $CD34^+CD45^+$ cell expansion (a 200- and 235-fold increase, respectively), while those with hexene spacers maintained the maximum purity of $CD34^+CD45^+$ cells (41.1%).[148] PET has also been used as a scaffold to study bone regeneration. For example, bone marrow stem cells and PET scaffolds were combined to repair Achilles tendon defects, enhance collagen regeneration and facilitate tissue healing.[149] However, it is worth noting that the fibronectin-conjugated PET scaffold had a more pronounced effect on the expansion of HSCs, cell adhesion and the differentiation potential of subsequent *in vitro* cultures of $CD34^+$ cells than a collagen-PET scaffold.[150]

Lyophilization, also known as freeze-drying, creates pores by generating thermodynamic instability within the material and causing phase separation through the rapid cooling and sublimation of specific solvents using vacuum technology.[151] Severn *et al.* constructed an *in vitro* differentiation niche for HSCs based on a polyurethane (PU) scaffold by freeze-drying and found that the PU scaffold increased cell differentiation to the reticulocyte stage and maintained $CD34^+$ cells during 28 days of culture but caused a high cell death rate.[151] However, polymerized high internal phase emulsions (polyHIPEs) can be prepared with pendant thiol (–SH), which enables easy attachment of the molecule, and is applicable to more kinds of cells than PU scaffolds.[152] Bai *et al.* constructed a star-like poly(carboxybetaine)-based porous hydrogel through lyophilization.[153] Within 24 days of culture, HSCs achieved 73-fold expansion in this zwitterionic hydrogel, which is better than a PEG hydrogel. The mechanism by which the zwitterionic hydrogel promotes the proliferation of HSCs may involve stimulating HSC self-renewal by inhibiting aerobic metabolism and inhibiting DNA-damaging reactive oxygen species (ROS) production.[153] This mechanism is similar to that of UM-171 disruption of the accumulation of ROS by regulating inflammatory signalling, thereby driving HSC self-renewal.[19]

Lyophilization can also be applied to natural materials such as heparin-chitosan scaffolds.[154] Compared to the 14% maintenance rate of a 2D monolayer, the 3D chitosan-heparin scaffold exhibited a 17% maintenance rate and a 22% maintenance rate in a 5% O_2 hypoxic environment.[154] Chitosan is commonly used to encapsulate HSCs together with sodium alginate or collagen.[133,155]

In addition, pores can be created by generating gas inside supporting materials under high pressure or by foaming agents.[50] For instance, Keskar *et al.* used the gas forming method to construct a poly(ethylene glycol) diacrylate (PEGDA) porous scaffold using sodium bicarbonate as a foaming agent to simulate the bone marrow niche.[156]

4.3 Microchannels

The construction of microchannels in hydrogel scaffolds creates a porous hydrogel derivative for various applications, and is used to further control and improve cell diffusion and transport in the hydrogel and enhance the mass transfer capacity of the scaffold.[157] Miller *et al.* used carbohydrate glass as a biocompatible sacrificial material to construct homogeneous channels within the hydrogel.[158] The creation of microchannels in a hydrogel can improve cell viability, promote cell spreading and direct cell extension.[159,160] The most effective areas of material exchange in the culture environment and the highest rate of waste metabolism are found close to the transport channels, allowing for the maintenance of cell viability and function. The combination of this transport system and the porous structure of the scaffold makes the hydrogel more physically compatible with the human bone marrow cavity environment and facilitates the functional maintenance of HSCs.

A hydrogel-independent microchannel system is also capable of serving as a bioreactor for the *in vitro* culture of HSCs. Kresnowati *et al.* used microchannels to optimize the HSC cultures, avoiding tedious experimental procedures such as regular culture medium replenishment and possible contamination. In this system, a uniform distribution of nutrients is maintained, which is beneficial for HSC viability due to the stabilized oxygen levels and the increased cellular yield of the bioreactor.[161].

Soft lithography micromoulding is often used for the construction of microchannels inside hydrogels. For example, soft lithography micromoulding is used to construct PLGA scaffolds that mimic microvessels.[162] The construction process involves the manufacture of a semicylindrical photoresist template using a thermal reflow technique, topography of the PLGA over the template and, finally, stitching of the PLGA to build a scaffold with an internal microchannel structure.

Reconstructing microstructures is essential for the *in vitro* culture of HSCs; therefore, microfabrication techniques for scaffold modification to optimize cell viability, morphology and function while incorporating activating factors to maximally mimic the characteristics of the HSC niche is worth investigating.

4.4 Microfluidics

Microfluidics involves the precise control of fluids at the microscale, enabling the integration of the culture, isolation, detection, and analysis of cells at the micron scale and is a powerful tool for controlling the cellular environment.[128,163] HSCs are nonadherent cells, making them difficult to be monitored individually for long periods of time using conventional cell culture methods. Instead, Faley *et al.* used a microfluidic platform to implement a $CD34^+$ single-cell isolation assay system using passive capture for microscale drug response detection.[164] Han *et al.* similarly achieved the capture of individual HSCs with the help of microfluidic devices, enabling high-throughput analysis of the motility and behaviour of individual cells.[165]

In addition to the fluid state of the internal microenvironment of bone marrow, the cellular diversity and complex functions of living bone marrow cannot be simulated using traditional plate cultures or scaffolds. Therefore, to test the method of culturing live bone marrow *in vitro*, Torisawa *et al.* induced new bone formation *in vivo* in mice, removed the engineered bone after the eighth week and transplanted it to an *in vitro* culture platform based on microfluidic technology. Live bone marrow on the microfluidic platform independently produced cytokines that maintain haematopoietic function *in vitro* and help restore haematopoietic niche function. At the same time, the bone marrow *in vitro* maintains HSCs in their spatial location in the original niche, allowing HSCs to remain at their normal ratio.[166]

Microfluidic devices can also serve as new tools for the long-term detection of stem cell behaviour *in vitro* and for the study of stem cell niches, providing a new direction to break the current HSCT dilemma with respect to stem cell shortages. Sieber *et al.* used a zirconia scaffold to mimic the porous and rigid nature of cancellous bone microstructures, created a microenvironment similar to the bone marrow niche using microfluidics and found that 31.71% of the cells maintained a $CD34^+CD38^-$ phenotype after 28 days.[167] Carreras *et al.* used microfluidics to construct bilayer microdroplets with MSCs on the inside and HSCs on the outside, and based on this coculture model, they achieved sustainable expansion of $CD34^+$ cells *in vitro* for eight weeks.[111]

4.5 Bioprinting

The internal space and topology of scaffolds cannot be precisely designed for either by porogen or by electrospinning technology.[168] Photolithography requires moulds for fabricating internal microchannels, which is costly and cannot be built directly into the final scaffold.[169] However, the use of bioprinting in constructing the HSC artificial niche can circumvent these problems and efficiently create a flexible architecture of the internal space of the scaffold at a low cost.[168,170] In addition, bioprinting can easily modify the scaffold structure at the microscopic level, with a high degree of flexibility and consistency of the constructed scaffolds, which is essential for the tissue engineering of the HSC niche.[169]

There are three categories of bioprinting systems: bioplotting, inkjet printing and light-based printing.[169] Bioplotting, also known as extrusion printing, is based on extrusion equipment printing the hydrogel fibers, which are then deposited layer by layer to form a 3D structure.[171] This approach was used by Pati *et al.* to construct scaffolds suitable for long-term cell culture with PCL and the ECM.[172] Inkjet printing involves spraying material in the form of droplets, building 2D layers of material, and subsequently building scaffolds with complex internal structures in a superimposed manner.[173] Due to the advantages of inkjet printing in constructing 2D structures, this method is used to construct micropatterns on polymers and later control the stem cell adhesion geometry.[174]

There are two forms of light-based bioprinting: digital light processing (DLP) and laser-assisted printing.[170] DLP is a full-layer printing process in which the entire plane of an optical pattern is projected onto a photosensitive polymer solution, significantly reducing the manufacturing time and eliminating

connecting interfaces between materials.[175,176] The hydrogel material used in this method is mainly methacrylamide-functionalized gelatine (GelMA).[177] Laser-assisted printing enables direct shaping of the support material by laser beams without the use of a mask. This enables the construction of more complex scaffold structures in a single process step and can be applied from the submicron to the centimetre level.[178] Table 3 lists and compares four different types of bioprinting.

5. Construction and dissolution of hydrogel scaffolds

5.1 Hydrogels for haematopoietic stem cell culture

The 3D porous structure of a hydrogel has a suitable spatial structure and porosity, which is conducive to the adhesion, growth, and proliferation of stem cells. In addition, hydrogels have good biocompatibility and can be stably combined with stem cells. A high surface activity is conducive to the combination of the stem cells and scaffold, and promotes the proliferation and phenotype maintenance of HSCs.[180] The hydrogels show a similar mechanical strength to the ECM, and are easy to mould, which stabilize the growth space for HSCs. Implantable hydrogels are biodegradable and can self-degrade in the body. The degradation rate needs to be adjusted within an appropriate range to ensure the growth of other tissues is not affected while the growth of HSCs is supported.[181] Research on *in vitro* HSC cultures with a hydrogel focuses on the expansion of the HSCs. A variety of materials have been proved to significantly promote the expansion of HSCs.[3,10,100] However, whether the cells inside the hydrogel are accessible or not needs to be taken into consideration. This is because the main objective of *in vitro* expansion studies of HSC is to obtain transplantable stem cells. Therefore, materials that can be degraded with minimal damage to cells have been valued to minimize unavailable HSCs which are useless for HSCT. A typical example is the sodium alginate hydrogel that can be removed by sodium citrate. And to address the problem of lysis of polymeric hydrogels, researchers used the addition of peptides to the polymeric material to facilitate the removal of cells from the hydrogel by enzymatic dissolution at the end of the culture.[153] This introduction of peptide self-assembly properties into polymers, thereby imparting biological properties to polymer hydrogels, such as enzymatic degradation, can effectively eliminate the limitations imposed by the material itself (Table 4).[182]

5.2 Using ion exchange reactions to construct hydrogels

The ion exchange reaction is a process in which the cations or anions in the exchange agent are reversibly exchanged with ions of the same kind in the material solution.[183] Through the ion exchange reaction, the functional units of the material build up and form a cross-linked network structure, resulting in a hydrogel.[184]

Alginate is a polysaccharide isolated from seaweed with mild gelling properties in the presence of divalent ions (*e.g.*, calcium).[185] The limited range of the mechanical properties of alginate limits its application, but meets the requirements for use as a scaffold for HSC *in vitro* proliferation.[186] Alginate can be de-cross-linked, allowing the cells inside the scaffold to be collected for further analysis at the end of the culture period. Yuan *et al.* cultured HSCs with sodium alginate, and the percentage of $CD34^+$ cells increased from 2.60 ± 0.52% to 13.27 ± 2.65% after only 12 days of culture.[187] To maintain the porosity and permeability of the deposited structure of an alginate system while increasing the Young's modulus of the scaffold, Moore *et al.* used methylcellulose to modify sodium alginate and produce a bioink suitable for 3D printing. The combination of 6% methylcellulose and 2% alginate was found to best mimic the optional properties of bone marrow properties, such as the overall structure, oxygen availability and rheological properties.[188] Braham *et al.* then added an additional external mechanical stimulus, intermittent hydrostatic pressure (IHP), based on sodium alginate to simulate the mechanical environment of osteoblasts within the bone marrow. The combination of osteoblasts and IHP resulted in an increase in the migration rate of HSCs while maintaining a high survival rate.[189]

5.3 Using a photochemical reaction to construct hydrogels

Photoinitiators can be initiated by ultraviolet or visible light, participate in the chain polymerization reaction of monomers, and quickly form a highly cross-linked polymer network.[190] A common HSC *in vitro* culture scaffold constructed *via* photochemical reaction is the methacrylamide-functionalized hydrogel.

Gilchrist *et al.* used lithium acylphosphinate as a photoinitiator to construct GelMA hydrogels and used them as an exogenous stimulus to increase the number of haematopoietic

Table 3 The pros and cons of bioprinting[169,170,175,179]

Type	Principle and resolution	Advantages	Disadvantages
Bioplotting	Extrusion equipment presses out the scaffold material in a line (50 μm)	Low cost and easy to operate	Causes damage to the cells, the process is slow, the nozzle easily clogs, and the structure of the built-up scaffold is simple
Inkjet printing	The scaffold material is ejected in the form of liquid droplets (50 μm)	Moderate build speed, low damage to cells, low equipment cost and simple operation.	Simple structure of the built-up scaffold, and the nozzle is easily clogged
DLP	Full-width printing through masks (1 μm)	High speed	High cost, complex operation, most of the initiators are toxic, and require additional mask design
Laser assisted	A direct laser-writing technology (<500 nm)	Supports submicron scale scaffolds	High equipment costs and high waste of raw materials

Table 4 The pros and cons of three methods in constructing hydrogels[10,51,185,198]

Type	Advantages	Disadvantages
Ion exchange reaction	Time efficient and low cellular damage	Inability to build complex inner structures
Photochemical reaction	Ability to build complex structures	Complex in terms of cellular release
Self-assembling	Low cellular damage and superiority in mimicking cell growth environments	Long gel formation time

progenitors and increased the progeny by 7.52 ± 3.65-fold over a seven-day culture period.[191] Interestingly, Gilchrist *et al.* found that MSC-mediated matrix remodelling may contribute to a dynamic culture environment and promote retention of quiescent HSC populations when GelMA is used as a coculture scaffold for MSCs and HSCs.[192] This outcome may have been due to MSC density-dependent upregulation and the altered mechanical properties of the hydrogel. For the maintenance of quiescence, Mahadik *et al.* found that in the GelMA hydrogel system, covalent SCF was effective in maintaining the phenotype of HSCs and preserving the quiescent state, while soluble SCF significantly enhanced the proliferation and differentiation of HSCs.[58] This finding has implications for HSC amplification and genealogical specification.

Zhang *et al.* then used lithium phenyl-2,4,6-trimethylbenzoylphosphinate as a photoinitiator and methacrylic anhydride (MA) to functionalize hyaluronic acid (HA) to construct HAMA hydrogels.[193] They enhanced the physical properties of the hydrogel using carbon nanotubes while supporting the scavenging ability of the culture system for oxides and peroxides. In addition, the proliferation ability and pluripotency of HSCs were improved by inhibition of the ROS pathway. HAMA and carbon nanotube scaffolds provide a new strategy for establishing artificial niches for HSCs with low ROS levels. At the end of the cell culture, the GelMA lysis solution can be used to lyse the hydrogel at the end of the culture to facilitate the removal of the cells, but the lysis time is not advantageous compared to sodium alginate hydrogels (Fig. 5).[194]

5.4 Self-assembling hydrogels

Some materials can self-assemble and form ordered structures without external intervention, which can then be applied to simulate ECM structures in the field of regenerative medicine.[196,197] Self-assembled hydrogels can be hydrolyzed enzymatically, reducing the damage to cells during hydrogel removal.

The main natural self-assembling materials applied for the *in vitro* expansion of HSC are collagen, fibrin and matrigel. Collagen is the main component of the ECM and is superior to synthetic materials in facilitating the amplification of HSCs.[140] It is reported that HSCs were able to migrate in collagen fibres assisted by spindle MSCs. And interestingly, bone marrow-derived MSCs enhanced the maintenance of the cellular $CD34^+CD38^-$ phenotype in the collagen scaffold, whereas cord blood-derived MSCs promoted the proliferation and differentiation of HSCs.[198] Collagen can also be used as a scaffold material together with synthetic materials. For example, collagen and PCL were combined to construct an artificial niche, achieving 20-fold proliferation of $CD34^+$ cells within ten days of culture.[199]

The fibrin network in the body mainly protects neutrophils, fibroblast infiltration and macrophages while combining collagen, fibronectin, elastin and laminin to form a repair structure.[200] Fibrin is superior to other extracellular matrices in mediating signalling between cells.[201] This superiority is reflected in the maintenance and expansion support of HSCs by fibrin scaffolds.[140] Garcia-Abrego *et al.* used fibrin to construct an artificial niche simulating foetal liver and found that a soft fibrin hydrogel (0.6 mg mL^{-1} fibrinogen) with an elastic modulus value of 0.78 ± 0.10 kPa best mimics the foetal liver in terms of hardness of the liver niche and allowed the proliferation of HSCs and differentiated progeny cells.[202] In contrast, the fibronectin in fibrinogenesis was used as a coating material to modify PES or poly-L-lactic acid.[203,204] Notably, the results showed that fibronectin-coated scaffolds facilitated the expansion of $CD34^+$ cells.

Matrigel is extracted from the ECM of protein-rich mouse tumours and has a complex composition that can be undesirable when studying specific signalling pathways but can be used as a scaffolding material to support the expansion of HSCs *in vitro*.[189] However, the promotion of cell differentiation by these matrix gels is more pronounced, leading to the most frequent matrix gel applications in cell regeneration studies.[205]

For natural self-assembling materials, their physicochemical properties can hardly be modified to mimic specific cell growth environments. In contrast, synthetic self-assembling biomaterials can be designed and modified to mimic specific cellular microenvironments and key elements of ECM components.[206] Raic *et al.* generated an ECM-like environment within the PEG-DA hydrogel to enhance the promotion of HSC expansion by MSCs *in vitro* by using RGD as an artificial ECM additive to increase the adhesion of PEG-based hydrogels with HSCs.[81] The addition of RGD functional peptide also provided self-assembly capabilities to PEG-DA hydrogels. This combination of the functional properties or natural structures of self-assembled peptides with biomaterials gives the material a completely new bioactive function.[207] In the future, a large number of molecular tools can be used to facilitate the pioneering design of synthetic biomaterials and to provide impetus for the development of clinical applications.

6. Conclusions

Various attempts for *in vitro* proliferation of HSCs using biochemical and physical strategies have been made to break through the barriers of existing stem cell science research and make progress in the regeneration of haematopoietic systems. HSC niche mimics engineered using biochemical bionics, biomimetic materials and microscale technologies, which is a multidisciplinary process, have been summarized

and elaborated in this review. Efforts to engineer human organ tissues by combining the semiconductor industry, biology and materials science are being made. Currently, effective methods for the *in vitro* expansion of HSCs are coculture systems, or the use of decellularized scaffolds to construct an *in vitro* ECM environment. However, the clinical translation of these methods is still hindered by their low cost-efficiency. The current systems are still unable to meet the need for rapid and substantial amplification of the HSCs while maintaining phenotypes in short term culture, and efficient long-term culture of HSCs. Moreover, the study of regulatory factors and signalling cascades in the preparation of HSCs by inducing differentiation of pluripotent stem cells remains unclear. It is also a long way from clinical application for ethical reasons. In the future, with the in-depth combination of bioprinting and stem cell research, a breakthrough in the field of haematopoiesis is expected. Furthermore, with the improvement of bioinks, it is possible to simulate the biochemical and complex physical environment of an ECM simply utilizing hydrogels.

Conflicts of interest

There are no conflicts to declare.

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