Biotechnology Journal
www.biotechnology-journal.com

Hematopoietic Niche – Exploring Biomimetic Cues to Improve the Functionality of Hematopoietic Stem/Progenitor Cells

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The adult bone marrow (BM) niche is a complex entity where a homeostatic hematopoietic system is maintained through a dynamic crosstalk between different cellular and non-cellular players. Signaling mechanisms triggered by cell-cell, cell-extracellular matrix (ECM), cell-cytokine interactions, and local microenvironment parameters are involved in controlling quiescence, self-renewal, differentiation, and migration of hematopoietic stem/progenitor cells (HSPC). A promising strategy to more efficiently expand HSPC numbers and tune their properties ex vivo is to mimic the hematopoietic niche through integration of adjuvant stromal cells, soluble cues, and/or biomaterial-based approaches in HSPC culture systems. Particularly, mesenchymal stem/stromal cells (MSC), through their paracrine activity or direct contact with HSPC, are thought to be a relevant niche player, positioning HSPC-MSC co-culture as a valuable platform to support the ex vivo expansion of hematopoietic progenitors. To improve the clinical outcome of hematopoietic cell transplantation (HCT), namely when the available HSPC are present in a limited number such is the case of HSPC collected from umbilical cord blood (UCB), ex vivo expansion of HSPC is required without eliminating the long-term repopulating capacity of more primitive HSC. Here, we will focus on depicting the characteristics of co-culture systems, as well as other bioengineering approaches to improve the functionality of HSPC ex vivo.

1. Introduction

In the early 1960s, Till and McCulloch showed, through injection of marrow cells from healthy mice into sublethally irradiated animals, the presence of blood-forming stem cells in the bone marrow (BM),[1] which would then be referred to as hematopoietic stem cells (HSC). HSC, which comprise only about 0.005–0.01% of the BM cell population,[2] have the potential to give rise to the entire hematopoietic lineage (**Figure 1**), while retaining their self-renewal potential, a key characteristic to maintain a homeostatic hematopoiesis.[3]

Hematopoietic cell transplantation (HCT) was pioneered by Thomas and colleagues[4] with the first successful transplant performed in 1957 involving identical twins, one of whom with leukemia. Then, in 1968, Good and his team treated an infant with an immune deficiency with a BM transplant from his HLA-matched sister.[5] Since then, HCT occupies a frontline position as a therapeutic option for hemato-oncological diseases. Nevertheless, problems associated with lack of matched donors, inefficient engraftment of HSC into the BM, infections, and graft-versus-host disease (GvHD), which occurs when donor T lymphocytes recognize as foreigners the patient human leukocyte antigen (HLA) molecules,[6] are still major issues that need to be addressed.

Although the initial HCT procedures performed have relied on the use of cells harvested by biopsy from the BM, currently most transplants exploit the use of mobilized peripheral blood (mPB) cells due to its easier collection and faster engraftment after transplantation.[7] However, HCT are limited by the lack of HLA-matched donors. The use of umbilical cord blood (UCB) as alternative HCT cell source is particularly interesting to address such limitation, since it presents more immature T cells, with less stringent HLA matching,[8] an important characteristic to minimize the incidence of GvHD.[9,10] Nevertheless, UCB transplantation is still associated with delayed platelet and neutrophil engraftment. On the other hand, a single UCB unit contains a limited number of $CD34^+$ cells, a cell fraction known to encompass HSC, and that seems to correlate with the success of the transplant.[11] Low $CD34^+$ cell doses dramatically limit the success of cellular engraftment in adult patients and high weight children (over 50 kg). To

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DOI: 10.1002/biot.201700088

overcome this, different strategies have been performed, namely the use of more than one UCB unit[12] with eventual combination of ex vivo expanded UCB CD34$^+$ cells.[13,14] In particular, transplantation of expanded UCB cells in combination with unmanipulated UCB cells (non-expanded) could contribute to improve cell engraftment.[15] In this context, it has been suggested that, whereas the expanded UCB unit initially contributes to a faster hematopoietic repopulation activity, it is the non-expanded unit that allows the transplantation of HSC with long-term engraftment ability.[15] Indeed, ex vivo culture of HSC is frequently associated with loss of long-term repopulating activity.[16] In a clinical setting, this could result in graft failure upon transplantation due to incapacity to sustain hematopoiesis as more primitive HSC rapidly become exhausted. The ability of HSC to retain their "stemness" in culture is therefore a pivotal characteristic to preserve lifelong production of the hematopoietic system (**Figure 2**).

In this *Review*, we aim to depict different known players involved in the regulation of the hematopoietic system, with particular emphasis on the ex vivo expansion of HSC and progenitors (together referred to as HSPC). Moreover, and although historically the study of the hematopoietic niche has been limited to 2-D cell cultures, recent studies exploring the incorporation of stromal cells in 3-D culture configurations and/or relying on biomaterial-based HSPC culture platforms will be focused regarding their ability to replicate ex vivo hematopoietic niche features.

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2. The BM Microenvironment

In the "stem cell niche" model proposed by Schofield in 1978,[17] stromal cells present in the BM are suggested to provide essential cues for the function of HSC, namely to limit entry into the cell cycle, preventing their exhaustion and DNA replicative errors. While osteoblastic cells from the endosteal niche were the first cells shown to influence HSC frequency in vivo,[18] recent data suggest that the endosteal region is indeed important for

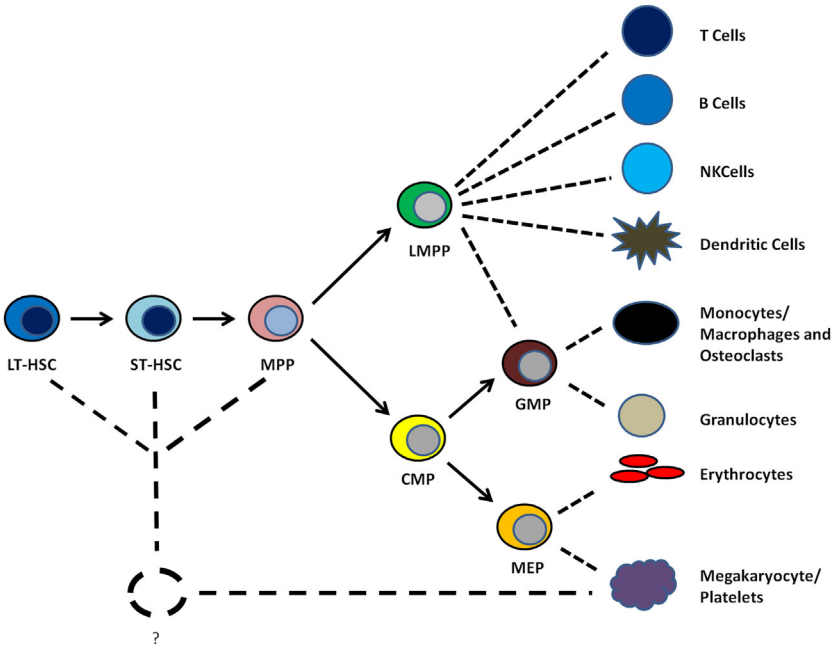

Figure 1. Current model of lineage determination in the human hematopoietic hierarchy. A long-term hematopoietic stem cell (LT-HSC), which is able to self-renew for the lifetime of the host, gives rise to a short-term HSC, which can only self-renew for a limited period of time. ST-HSC, in turn, give rise to multipotent progenitors (MPP), that can either originate myeloid-restricted progeny (CMP) or lymphoid-primed multipotent progenitors (LMPP) that have full lymphoid potential and some myeloid potential. CMP then commit to either granulocyte/macrophage progenitors (GMP) or megakaryocyte/erythrocyte progenitors (MEP). In this model, megakaryocytic lineage is closely tied to the fate of multipotent cells.[155] Terminally differentiated cells are shown on the right and lineage relationships are depicted with arrows.

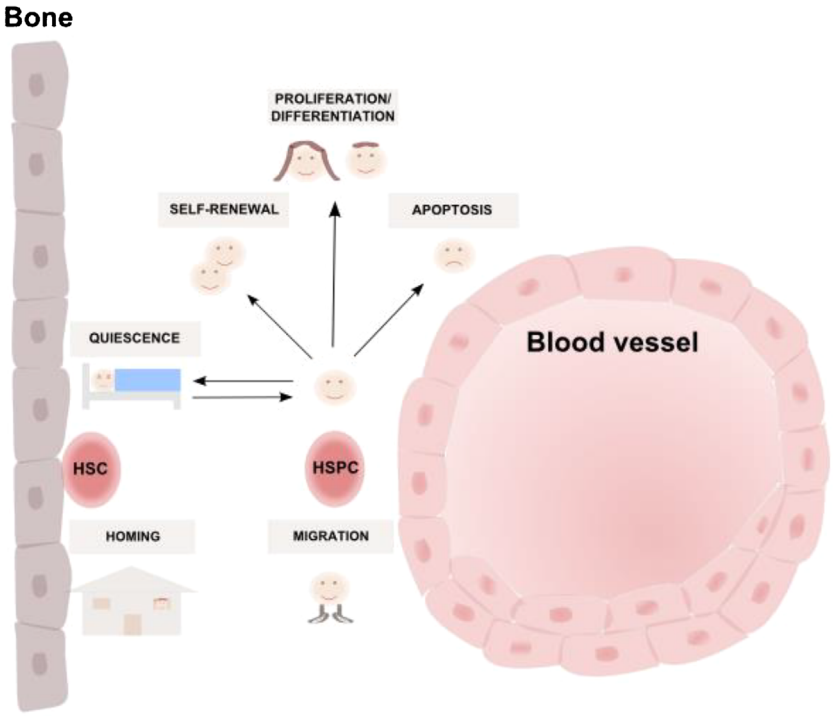

Figure 2. In an adult individual, HSPC are located in the BM, where they interact with the hematopoietic niche, which is thought to be responsible to promote a tight balance between quiescence, self-renewal and proliferation/differentiation of HSPC. The more hypoxic endosteal region ($\leq$1% O_2) of the BM is thought to harbor more quiescent, non-proliferative HSPC, whereas the vascular niche ($\approx$6% O_2)[21,156] is frequently associated with a more proliferative state of HSPC. HSPC can migrate from the endosteal niche toward the BM sinusoids and be eventually released into circulation.

hematopoiesis, but through regulation of restricted progenitors.[19] It is thought that HSC reside and are tightly regulated mostly in a perivascular niche, in close contact with sinusoids distributed throughout the BM.[20] Indeed, although HSC are thought to reside in a hypoxic niche in the BM (0.1–6% O_2 levels),[21–23] namely in the endosteal region, this area is actually known to be highly vascularized, with most HSC being perivascular. The existence of an oxygen gradient throughout the BM has led researchers to explore ex vivo culture systems established at different oxygen tensions. Particularly, it has been shown that culture at more physiological oxygen tensions, such as 10% O_2 (rather than atmospheric levels – 21% O_2), closer to the values found on the hematopoietic niche environment, could contribute to higher expansion levels of UCB HSPC in co-culture with BM mesenchymal stem/stromal cells (MSC), namely of the primitive $CD34^+CD90^+$ subset.[24] In another study, although higher levels of expansion of UCB-derived $CD34^+$ and $CD34^+CD133^+$ were observed at 20% O_2, higher maintenance of severe combined immunodeficiency (SCID)-repopulating cells (SRC) were reported at 3% O_2.[25]

BM comprises a heterogeneous population of stromal cells, whose expression of stem cell factor (SCF), vascular cell adhesion molecule-1 (VCAM-1), Jagged-1, C-X-C motif chemokine 12 (CXCL12) (also known as stromal cell-derived factor-1 alpha [SDF-1α]), and angiopoietin, is involved in HSC maintenance and adhesion to the niche cells[26–29] (**Figure 3**). Some of the best characterized HSPC-stromal cell adhesions are mediated by very late antigen-4 (VLA-4) receptors expressed on $CD34^+$ cells and VCAM-1 expressed by marrow stromal cells,[30,31] being an important player of homing and mobilization processes.[32] Similarly, Tie2, and the Notch ligand Jagged-1 are also important molecules for the homing ability of HSC through close adjacent interactions with niche cells.[18,33] Particularly, the interaction between SDF-1α and its receptor C-X-C chemokine receptor type 4 (CXCR4) is important to retain HSC in their niche and can contribute to maintain the HSC pool.[34] Other factors to be considered in HSC regulation by the BM microenvironment include the overall 3-D arrangement of the niche.[35]

The interaction between extracellular matrix (ECM) components and cell surface receptors on HSC, such as hyaluronic acid and its receptor CD44 (Figure 3), is also part of the trafficking and homing mechanisms occurring in the hematopoietic niche.[36,37] The relevance of ECM components was evidenced by the higher expansion levels observed when UCB $CD34^+$ stem/progenitor cells were co-cultured with an acellular matrix derived from a human BM stromal line.[38]

Therefore, both cellular and extracellular components are key parts of the hematopoietic niche. Attempts to recreate it without fully understanding the individual role and the interplay of ECM, cellular components and cytokines involved in the hematopoietic milieu still limits the development of a functional hematopoietic microenvironment in vitro. Indeed, the requirement of uncommitted primitive cells to repopulate the BM is often unmet since HSC, outside their niche, tend to differentiate into different hematopoietic cell lineages, become senescent or proliferate, leading to exhaustion of the stem cell pool. The tight regulation between HSC self-renewal and

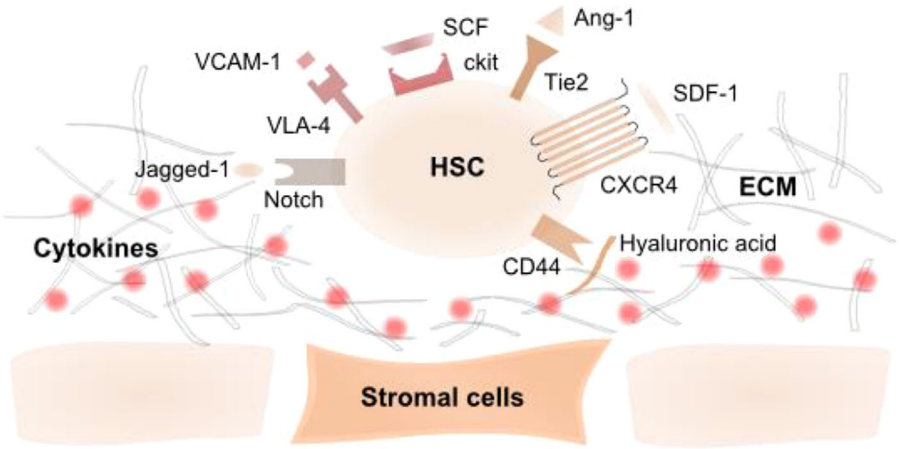

Figure 3. An orchestra of signals provided by either soluble cues (such as cytokines) or through the establishment of physical interactions between HSC-niche cells or HSC-ECM is responsible to regulate the fate of HSC. Stromal cells and HSC engage in cell-cell and cell-ECM contacts through several receptors, with some of the best studied adhesion molecules being the axis CXCR4/SDF-1α, Tie2/Angiopoietin, ckit/SCF, VLA-4/VCAM-1, Notch/Jagged, and CD44/hyaluronic acid, an ECM component.

differentiation that happens in vivo has inspired researchers to develop in vitro biomimetic hematopoietic niches aiming to promote not only expansion but also homeostasis of the hematopoietic system.

3. The Hematopoietic Supportive Capacity of the Native Niche and Niche Biomimetic Components

Stromal-based co-cultures of HSPC have been established to provide biological cues that liquid cultures, in the absence of stromal cells, could hardly replicate. Nevertheless, in most cases where stromal cells were explored as part of the strategy to expand HSPC ex vivo, either the addition of recombinant cytokines or genetic manipulation of the stromal cells, namely to increase secretion of specific biological factors, was still required to prevent apoptosis and stimulate proliferation of HSPC.[39] Importantly, whereas some studies support the idea that cell-cell contact between HSPC and stromal cells is essential to promote the expansion of hematopoietic cells,[37–41] others suggest that direct contact is not required,[43–45] with soluble biological factors assuming the key role on governing HSPC fate. More likely, however, it is the interplay between cytokines, ECM, and stromal cells that allow niche cues to efficiently regulate the fate of HSPC.

3.1. Soluble Factors – The Role of Cytokines and Small Molecules

Choosing an appropriate culture medium is crucial to determine the fate of ex vivo cultured HSPC. In addition to being capable to potentiate the function of HSPC, and, particularly, if clinical applications are envisioned, culture conditions should be able to comply with good manufacturing practices (GMP), without the use of xenogeneic components more prone to increase the risk of transmitting infections and trigger immunological reactions. On the other hand, culture formulations would benefit from being fully defined, therefore limiting variability and enhancing the control over the final cell product.

The vast majority of the current HSPC culture methods explore the use of recombinant cytokines, either directly added to the culture medium, and being frequently associated with cellular exposure to supraphysiological cytokine concentrations,[46] and/or secreted by stromal supportive cells. Stromal cultures constitutively secrete several hematopoietic supportive cytokines, such as SCF, thrombopoietin (TPO), interleukin-6 (IL-6), IL-11, insulin-like growth factor-2 (IGF-2), pleiotrophin, angiopoietin-1, and osteopontin,[46–53] which has prompted studies on the role of these factors as alternatives to HSPC-stroma co-cultures on determining HSPC fate. Indeed, many of these cytokines have been successfully used in protocols for human HSPC expansion in liquid cultures (**Table 1**, reviewed in).[54] While most studies differ in terms of cytokine combinations and/or concentrations, many clinical and preclinical studies have generally relied on the use of the early-acting cytokines SCF, TPO, and Flt-3L.[55] These factors have been shown to be essential to favor HSPC expansion in vitro by preventing cell apoptosis[56] and supporting the self-renewal of primitive stem cells through prevention of telomere degradation.[57] Other cytokines such as IL-6, IL-11, IL-3, erythropoietin (EPO), and platelet-derived growth factor (PDGF) have also been tested in culture but results generally show that these factors trigger cell differentiation.[58,59]

Ligands such as the Notch ligand Delta-1, in an immobilized form, can also impact the activity of HSPC, resulting in increased total hematopoietic cell expansion and more rapid engraftment.[60,61] More recently, small molecules have emerged as interesting alternatives to regulate the fate of HSPC, accompanying the advances in virtual design of chemical structures and development of high-throughput screening methods. The capability of StemRegenin (SR1), for instance, a purine derivative, to increase the expansion of HSPC with functionally repopulating activity, was discovered after using the expression of CD34 as readout to screen small molecule libraries

Table 1. Comparison of different expansion protocols for human UCB HSPC using various cytokines, small molecules, and Notch canonical ligands.

	Input cells	Cytokines/molecules	Days of culture	Cell fold-expansion	SRC/LTC-IC	Ref.
Cytokines only	$CD34^+CD38^-$	SCF, Flt-3L, G-CSF, IL-3, IL-6	4	4 ($CD34^+CD38^-$ cells)	4 SRC (8–10 weeks)	[157]
	$CD34^+$	SCF, Flt-3L, IL-6, IL-6R chimera	3-6	6 ($CD34^+CD38^-$)	10-fold increase in % human $CD45^+$ BM cells (NOD/SCID)	[158]
	$CD34^+$	SCF, Flt-3L, TPO, IL-3, IL-6, sIL-6R	7	45.4 (TNC)	4.2 SRC (10–12 weeks)	[159]
	$CD34^+$	SCF, Flt-3L, TPO, IL-3	7	20 ($CD34^+$)	Same frequency of human $CD45^+$ BM cells compared to fresh cells (NOD/SCID)	[160]
	$CD34^+CD38^-Lin^-$	SCF, Flt-3L, TPO, IL-6, IL-3, GM-CSF, G-CSF	28	1280 ($CD34^+$)	12 LTC-ICs	[55]
Cytokines + small molecules	$CD133^+$	SCF, Flt-3L, TPO, IL-6, TEPA	3 weeks	89 ($CD34^+$)	Engraftment superior than unexpanded cells	[161]
	$CD34^+$	5-AzaD, TSA, FBS, SCF, TPO, Flt-3L, IL-3	9	5 ($CD34^+$)	9.6 SRC (8 weeks)	[162]
	$CD133^+$	SCF, TPO, FGF-1, IGFBP-2, ANGPTL5	10	220 (TNC)	20 SRC (8 weeks)	[163]
	$CD34^+$	StemRegenin1, SCF, TPO, Flt-3L, IL-6	3 weeks	670 ($CD34^+$)	17 SRC (16 weeks)	[63]
	$CD34^+$	Nicotinamide, SCF, TPO, Flt-3L, IL-6, FBS	21	700 ($CD34^+CD38^-$)	9 SRC (6 weeks)	[164]
	$CD34^+$	UM171, SCF, TPO, Flt-3L	12	90 ($CD34^+$)	13 SRC (20 weeks)	[165]
Cytokines + notch ligands	CD34+$CD38^-$	$Delta1^{extIgG}$, fibronectin, SCF, TPO, Flt-3L, IL-6, IL-7, GM-CSF, G-CSF	29	3300 ($CD34^+$)	18-fold increase in % of human $CD45^+$ cells	[61]
	$CD34^+CD38^-$	$Delta1^{extIgG}$, fibronectin, SCF, TPO, Flt-3L, IL-6, IL-3	21	184 ($CD34^+$)	6.2 SRC (8 weeks)	[60]
Stromal-based co-cultures	$CD34^+$	SCF, TPO, Flt-3L	7	15 ($CD34^+$)	–	[13]
	$CD133^+$	SCF, TPO, G-CSF	14	12 ($CD34^+$)	–	[166]
	$CD34^+$	SCF, Flt-3L, bFGF, LIF	26	35 ($CD34^+$)	–	[89]
	$CD34^+$	SCF, Flt-3L, bFGF, LIF	18	35 ($CD34^+$)	–	[42]
	MNC	SCF, IL-3, G-CSF	12	21.4 ($CD34^+$)	–	[167]
	$CD34^+$	SCF, Flt-3L, TPO, IL-3	10	50 ($CD34^+$)	>1% of human $CD45^+$ cells in BM (NOD/SCID)	[168]
	$CD34^+$ co-cultured with human UCB MSC	FBS, SCF, G-CSF, MGDF	12	4 ($CD34^+$)	5 LTC-ICs	[169]
	$CD34^+$ co-cultured with human UC	SCF, Flt-3L, TPO, IL-6	10	2.5 ($CD34^+CD38^-$ cells) when co-cultured with placental	4.4 and 22.8% of human $CD45^+$ cells in BM (12 weeks)	[170]

(*Continued*)

Table 1. (*Continued*)

Input cells	Cytokines/molecules	Days of culture	Cell fold-expansion	SRC/LTC-IC	Ref.
MSC or human placental MSC			MSC vs UC MSC	co-cultured with UC and placental MSC, respectively	
$CD34^+$ co-cultured with human BM MSC	SCF, Flt-3L, TPO, TEPA	10	110 ($CD34^+$)	–	[171]
$CD34^+$ co-cultured with human placental MSC	SCF, Flt-3L, TPO	14	13 ($CD34^+$)	–	[172]
$CD34^+CD133^+$ co-cultured with human BM MSC expressing ANGPTL5	SCF, FGF-1, TPO, IGFBP-2, ANGPTL5	11	60 ($CD34^+CD133^+$)	60 SRC (14 weeks)	[173]
$CD34^+$ co-cultured with human BM MSC at 1.5% O_2	SCF, TPO, G-CSF	10	32 ($CD34^+$)	>5-fold increase in % $CD45^+$ BM cells versus nonexpanded cells	[174]
$CD34^+$ co-cultured with human BM MSC	SCF, TPO, FGF-1, IGFBP-2, ANGPTL5	14	≈40 ($CD34^+$)	Threefold increase in % $CD45^+$ BM cells versus stromal-free expanded cells (NSG)	[39]
$CD34^+$ co-cultured with human BM MSC spheres	SCF, TPO, FGF-1	3–4 weeks	6–40 ($CD34^+$)	>12-fold increase in % $CD45^+$ BM cells versus stromal-free expanded cells (NSG)	[44]

SCF, stem cell factor; Flt-3L, Fms-like tyrosine kinase-3; G-CSF, granulocyte-colony stimulating factor; IL-3, interleukin-3; IL-6, interleukin-6; SRC, SCID repopulating cells; BM, bone marrow; NOD-SCID, nonobese diabetic/severe combined immunodeficiency; TNC, total nucleated cells; TPO, thrombopoietin; GM-CSF, Granulocyte, macrophage-colony stimulating factor; LTC-IC, long-term culture-initiating cell; TEPA, tetraethylenepentamine; 5-AzaD, 5-aza-2′deoxycitidine; TSA, trichostatin; FBS, fetal bovine serum; IGFBP-2, insulin-like growth factor binding protein-2; Angpt5, angiopoietin-5; IL-7, interleukin-7; FGF-1, fibroblast growth factor-1; LIF, leukemia inhibitory factor; MNC, mononuclear; UCB, umbilical cord blood; MSC, mesenchymal stem/stromal cells; MGDF, megakaryocyte growth and development factor; UC, umbilical cord; NSG, NOD/SCID gamma. Based on (Pineault and Abu-Khader, 2015).

composed by thousands of compounds. SR1 has already been tested in a phase I/II clinical trial (NCT01474681), promoting a marked expansion of $CD34^+$ cells and enhanced hematopoietic recovery, although long-term persistence of SR1-treated cells was not observed in 6 of 17 patients.[62] Nevertheless, the potency of SR1 is still dependent on cytokine-driven expansion cultures, as SR1 alone did not induce proliferation of human mPB $CD34^+$-isolated cells.[63]

Moreover, chemically-synthesized small molecules could replace already naturally occurring compounds due to their enhanced stability and improved characteristics. NR-101, for instance, can constitute an alternative to TPO, leading to increased numbers of HSPC when compared to this cytokine, although showing comparable effects regarding its effect on megakaryocytopoiesis.[64] Interestingly, a cytokine-free culture of mice BM HSPC has been performed and shown to be capable to maintain HSPC ex-vivo aided by two small molecules, lithium, an activator of the Wnt/β-catenin pathway, and rapamycin, an inhibitor of the mammalian target of rapamycin (mTOR).[65] The study performed by Perry and colleagues also reinforces the notion that simultaneous modulation of several signaling pathways is required to improve cellular functions where authors demonstrated that only activation of both phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) and Wnt/β-catenin signaling by SCF and the small molecule CHIR99021, and not of any of these pathways in separate could result in expansion of mice HSPC while keeping its long-term functional capacity.[66]

Although we only mentioned a few small molecules, several other chemical compounds have been highlighted in recent literature communications due to their capability to impact the fate of HSC. Not only small molecules can contribute to increase HSC self-renewal and inhibit their differentiation, but also inhibit apoptosis and enhance HSC homing (reviewed in Ref. [67]). Small molecules could potentially work as substitutes of recombinant cytokines and unknown factors typically present in serum, contributing to the development of defined media and therefore increasing the reproducibility and cost-effectiveness of hematopoietic cell culture. In addition, beyond the use of chemical compounds to culture HSC, one of the major contributions of small molecules could be to unravel the mechanisms behind cellular fate decisions, a knowledge that would help researchers to better regulate and control the activity of HSPC ex vivo.

3.2. ECM Components

Several ECM components, such as fibronectin, collagens I and IV, laminin, and proteoglycans,[66–70] are distributed throughout the hematopoietic niche, and can be sensed by HSC through their interaction with integrins, contributing to regulate the fate of HSPC. Moreover, more than being an inert framework, ECM actively allows binding of growth factors produced by the niche cellular constituents, therefore favoring co-localization of cells and biological cues.[71] Exploiting the capability of ECM components to retain bioactive factors, Bladergroen, and co-workers tethered SDF-1α, a known cellular chemoattractant, to heparinized collagen scaffolds that were therefore used to recreate the hematopoietic niche in vivo.[72] Diffusion of nutrients, oxygen and cytokines in the ECM can also lead to gradients that might provide regulatory cues to HSPC.

Nevertheless, despite the contribution of ECM components to the hematopoietic niche, the presence of stromal cells seems to add extra value to the niche capability to promote self-renewal of HSPC. Gottschling and colleagues showed, for instance, that activation of β1-integrins, known to be crucial for the interaction of HSPC with the BM microenvironment, by ligands alone, such as fibronectin, was not sufficient to promote asymmetric cell divisions capable to ensure self-renewal of human HSC from mPB. In addition, the presence of MSC was actually key to induce self-renewing divisions of HSC.[73]

3.3. Stromal Co-Culture of HSPC

Several cell types have been shown to be part of the hematopoietic niche, as well as to support the activity of HSPC ex vivo, such as osteoblasts,[18] endothelial cells,[20] stromal cells such as mouse MS-5 cells,[74,75] CXCL12-abundant reticular (CAR) cells,[34] macrophages,[76] and MSC.[13,42,77]

Particularly, in the hematopoietic BM microenvironment, MSC are a major constituent, being in close association with HSPC[78] and are thought to have an important HSPC supportive function through their capability to secrete hematopoietic cytokines.[47,79] Although cytokine crosstalk between hematopoietic and mesenchymal cells has been pointed out by several authors,[78–80] MSC-HSPC contact also seems to be important to retain HSPC in their niche and favor maintenance of a more immature state.[52,73,81–83] Of notice, although MSC were initially identified in the BM,[84] these cells have been shown to be present in various tissues from either perinatal or adult sources.[85] Studies performed over a decade ago reported that co-transplantation of human MSC and HSPC in fetal sheep and immunodeficient mice models enhanced the long-term engraftment of hematopoietic cells in the BM through mechanisms that might not only rely on the homing of MSC to the BM but also on secreted cytokines.[79,86] Importantly, MSC have been proposed as an adjuvant cellular therapy to promote hematopoietic recovery in patients subjected to HCT, particularly to avoid GvHD,[87] and enhance cellular engraftment.[79] In addition, MSC have long been used to support the ex vivo expansion of HSPC.[13,88,89] Indeed, since the establishment of long-term cultures of BM stromal cells to be used as hematopoietic supportive feeder layers in Dexter-type cultures,[90] MSC have been conventionally cultured as 2-D adherent cells to tissue culture polystyrene (TCPS) and explored in HSPC co-culture systems.

Despite the fact that the majority of the studies on regulation of hematopoiesis are often restricted to 2-D cell culture, 3-D culture systems might better resemble the environment present in human hematopoietic niches. Moreover, the fact that MSC have been routinely isolated based on their plastic adherence to TCPS and expanded in vitro as 2-D monolayer cells results in heterogeneous stromal populations, which is thought to modulate their biological activity.[91,92] One of the main arguments behind the development of 3-D MSC co-culture systems of HSPC is that MSC isolated and grown as

TCPS-adherent monolayer cells might not provide the required cues to preserve more primitive HSC.

To address this limitation, 3-D co-culture systems of HSPC and stromal supportive cells have been designed to mimic a BM niche-like environment, mostly relying on scaffold-free 3-D spheroid culture of MSC or on biomaterial-based approaches (**Table 2**), as we will discuss in the following section.

4. 3-D Co-Culture of HSPC and MSC

2-D co-culture systems of HSPC and MSC have a limited capacity to capture the 3-D architecture and topographic cues characteristic of the BM niche. The organization of MSC as 3-D spheroids or supported by biomaterial constructs could be explored to provide distinct compartments to accommodate the cellular components and the ECM organization characteristic of the hematopoietic niche. Simultaneously, this organization is expected to impact the gene expression and secretion profile of MSC and to control the exposure of HSPC to soluble cues.[93–97] On the other hand, 3-D stromal supportive cultures of HSPC can also be designed to better resemble the physiological oxygen gradients of the hematopoietic niche. Importantly, biomimetic approaches relying on 3-D systems, besides bringing together the components of the hematopoietic niche (supporting stromal cells, ECM molecules), are also essential to mediate autocrine and paracrine signaling in contrast to standard 2-D cell culture platforms where secreted factors are rapidly diluted into the bulk medium.

4.1. MSC Cultured as 2-D Monolayer Versus 3-D Spheroids

Assembly of MSC into 3-D spheroids enhances cell-cell and cell-ECM interactions and impacts their secretion profile and phenotype.[94,98,99] On the contrary, in 2-D, cellular attachment to the substrate dominates over more physiological interactions with the surrounding cells and ECM. Importantly, the elasticity (measured by its Young's modulus) of MSC spheroids has been reported to be significantly lower in comparison to TCPS (0.1 kPa vs. 3 GPa, respectively),[98,100] mimicking the softer nature of in vivo microenvironments like the BM, whose Young's modulus is inferior to 0.3 kPa.[101]

3-D spheroids of MSC can be formed using either static or dynamic methods. Static methods rely on physical forces, such as in the pellet culture technique, which exploits centrifugal force to promote cell-cell interactions in the bottom of a tube,[102] or as in the hanging drop method, where cells aggregate at the bottom of a drop formed upon inversion of a plate.[103] Using non-adherent substrates (e.g., agarose), cell-cell interactions can also be favored, leading to the formation of cellular aggregates.[104] Particularly, the use of microfluidics-generated water-in-oil-in-water (w/o/w) double-emulsion droplets[105] and microwells could contribute to generate more reproducible and uniform spheroids. In this context, Futrega and colleagues have claimed to be the first to develop a high-throughput platform (i.e., microwells) to assemble more homogeneous multicellular spheroids of co-cultured human BM MSC and UCB $CD34^+$ cells. Nonetheless, an increase in the yield of the primitive $CD34^+CD38^-$ cells observed when hematopoietic cells were cultured in polydimethylsiloxane (PDMS) microwells was shown to be independent of MSC and did not improve the engraftment of the expanded human HSPC in Non-Obese Diabetic (NOD)/SCID gamma (NSG) mice [106].

Dynamic methods have also been applied to culture MSC as 3-D spheroids. The rotating wall vessel, for instance, allows formation of spheroids in a low shear stress environment as cells are maintained in suspension in a continuous free fall through rotation about an x-axis.[107] Culture of spheroids in spinner flasks has also been accomplished by preventing cells in suspension from settling, while cell-cell interactions are facilitated by means of constant stirring generated by an impeller or magnetic bar.[108]

Although mostly relying on static methods, such as in the study performed by Futrega and co-workers, where a microwell platform was explored, several works have recently focused on the use of 3-D spheroids of MSC or of osteogenic committed stromal cells to support co-cultured HSPC.[44,109–112] In some of these studies, special care has been put on MSC isolation and selection methods as it has been proposed that isolation of MSC based on their adherence to TCPS might bias the potential behavior of these cells, namely regarding their capability to maintain an undifferentiated phenotype and to express HSC maintenance genes.[44,78,113]

Maintenance of the key hematopoietic niche supportive $Nestin^+$ MSC, for instance, has been reported to be rapidly lost when cells are cultured on 2-D TCPS.[78] Therefore, Isern and colleagues isolated human BM $CD45^-$ $CD105^+$ $Nestin^+$ MSC and promoted their expansion under non-adherent conditions as floating spheroids, preserving an immature phenotype that was capable to enhance the expansion of UCB $CD34^+$ cells through secretion of growth factors.[44] Interestingly, some of the more abundant secreted proteins were involved in ECM formation. Indeed, the secretion of soluble factors rather than membrane-bound factors is likely the main responsible factor that account for the capacity of stromal spheroids to expand a HSC-enriched population. This idea has been corroborated by the study performed by Pinho and co-workers with $PDGFR\alpha^+$ $CD51^+$ $Nestin^+$ spheres, who revealed that direct contact between human fetal BM $CD34^+$ cells and mesenspheres was not required for the expansion of the hematopoietic cells.[113]

Interestingly, de Barros and colleagues observed a stronger adhesion of UCB $CD34^+$ HSPC in osteo-induced MSC spheroids co-cultures relatively to undifferentiated spheroids.[109] Nevertheless, the presence of osteo-induced MSC within mixed spheroids, composed by osteo-induced and non-differentiated MSC, reduced the proliferation of hematopoietic cells.[109] The authors observed a preferential positioning of $CD34^+$ cells in mixed spheroids at the interface of the two stromal cells, whereas a randomly distribution was evidenced in non-differentiated spheroids. This highlights the role played by the 3-D organization of supportive cells on providing instructive cues regarding cell homing and migration processes. Osteo-induced MSC seem to be able to display some properties of the subendosteal microenvironment, secreting collagen I and osteopontin, two key components of the hematopoietic niche that contribute to the retention of $CD34^+$ cells within the BM environment. In addition, the organization of the cytoskeleton and ECM components within the 3-D spheroid structure

Table 2. HSPC co-cultured with 3-D organized stromal cells – scaffold-free MSC spheroids and biomaterial-based approaches.

	Stromal cells/biomaterial	Characteristics	Outcomes	Ref.
3-D spheroids	Murine MSC in microwells	High throughput method to maintain uniform-sized spheroids (100 MSC/spheroid)	Higher expression of hematopoietic niche factors and expansion factor of 2-fold in 3-D micromarrows in comparison to 2-D systems	[110]
	Human BM MSC in hanging drop model	Larger contact area between HSPC and 2-D monolayer MSC in comparison to 3-D spheroid MSC	Expansion of more primitive hematopoietic cells higher in 2-D than in 3-D	[111]
	Human $CD45^-$ $CD105^+$ $Nestin^+$-enriched non-adherent BM MSC	Importance of isolation (non-adherent MSC) and medium selection to better preserve undifferentiated BM MSC	Human BM mesenspheres with a more primitive phenotype promote higher expansion of UCB $CD34^+$ cells and enhanced long-term human hematopoietic engraftment in immunodeficient mice when compared with more differentiated mesenspheres	[44]
	Human $PDGFR\alpha^+$ $CD51^+$ $Nestin^+$ non-adherent BM MSC	Highly purified non-adherent BM MSC	Expansion of multipotent hematopoietic progenitors capable of engrafting immunodeficient mice	[113]
	Human BM MSC in microwells	High throughput method to maintain uniform-sized spheroids (25-400 MSC/spheroid)	2-D and 3-D MSC co-culture of HSPC improve hematopoietic expansion; the microwell platform alone increases the $CD34^+CD38^-$ cell yield although engraftment in NSG mice is not increased	[106]
	Murine stromal line M2-10B4	Specific signaling cascades involved in hematopoietic cell migration investigated	Migration of hematopoietic cells into 3-D spheroids independent of integrin-mediated signaling (VLA-4, VLA-5, lymphocyte function-associated antigen-1, CXCR4)	[112]
	Human non-osteo-induced and mixed (osteo- and non-induced) BM MSC	Mixed spheroids mimicking the ECM organization of the subendosteal niche	Osteo-induced BM MSC spheroids reduce proliferation of hematopoietic cells. Adhesion of $CD34^+$ cells stronger in mixed spheroids than in non-osteo-induced spheroids while higher expression of SDF-1α, a factor involved in homing processes, is observed in non-osteo-induced spheroids	[109]
Biomaterial-based approaches	PCL, PLGA, fibrin and collagen scaffolds in co-culture with human UC MSC	Comparison of biomaterials with different chemistries, porosities and fiber diameters	Fibrin scaffolds with UC MSC support render the highest expansion of hematopoietic cells, maintenance of primitive cells and engraftment in NSG mice; stromal co-culture significantly stimulate scaffold-supported HSC proliferation	[115]
	Decellularized cancellous bone seeded with osteogenic-induced MSC of human origin	Mimicry of BM environment (natural geometric structure and ECM components supported by MSC-induced osteoblasts)	Maintenance and expansion of HSPC (increased CFU and LTC-IC content in 3-D in comparison to 2-D stromal-supported systems)	[117]
	Human BM MSC and MSC-derived osteoblasts cultured in bio-derived bone scaffold	Natural spongy architecture of trabecular bones preserved and supported by stromal co-culture capable of expression of cytokines and ECM synthesis	Higher number of more primitive HSPC and expansion of $CD34^+$ cells with ability to reconstitute long-term hematopoiesis in vivo	[118]
	β-TCP/Matrigel® scaffolds seeded with human MSC	Multicomponent BM mimicry system (promoting hematopoietic-mesenchymal interactions and ECM remodeling) suited for transplantation	Recruitment of hematopoietic cells to the sites of ectopic transplantation in a murine model, vascular ingrowth and promotion of hematopoiesis	[119]
	Multilayered electrospun fiber scaffold with human BM MSC as feeder cells	High density stromal environment in a substrate elasticity favoring the control of the bioactive cues secreted by co-cultured MSC	Higher proliferative potential and multipotency of HSPC in 3-D biohybrid scaffold in comparison to 2-D systems	[122]
	Electrospun PLLA nanofiber scaffolds seeded with niche-like units isolated from murine BM	HSPC harvested in association with their natural microenvironment and cultured in BM biomimetic scaffold	Transplanted bioengineered scaffold with ability to interconnect with sinusoidal vessels, favoring long-term self-renewal of HSPC	[123]
	Microencapsulated murine stromal cells or human immortalized MSC	HSPC exposed to the soluble cues continuously provided by co-cultured microencapsulated feeder cells	Higher expansion of total hematopoietic cells and maintenance of primitive progenitor cells when HSPC culture is supported by microencapsulated feeder cells	[127]
	Microencapsulated rabbit BM MSC cultured in a rotating wall vessel bioreactor	HSPC exposed to the soluble cues continuously provided by co-cultured microencapsulated feeder cells in a dynamic system	Higher expansion of CFU, total and $CD34^+$ cells in rotating wall vessel bioreactor supported by microencapsulated cells when compared to bioreactor alone or static co-culture system	[128]

(*Continued*)

Table 2. (*Continued*)

Stromal cells/biomaterial	Characteristics	Outcomes	Ref.
Human placental MSC seeded on a Puramatrix™ hydrogel	Creation of a hypoxic gradient in the 3-D MSC hydrogel forming an ECM- and integrin-rich environment and evidencing higher expression of SDF-1α	3-D MSC foster a pool of quiescent HSC, with superior in vivo (mice) engraftment potential	[129]
Encapsulated osteoblast-like cells, human BM or UC MSC in macroporous PEG hydrogel	Biofunctionalized hydrogel mimicking the spongy architecture of trabecular bones	3-D MSC contribute to preserve more primitive HSC in comparison to 2-D conventional culture	[130]
Human BM or UC MSC encapsulated in a collagen hydrogel	Creation of distinct compartments regulating the fate of HSPC	Proliferation of HSPC occurs mostly in suspension whereas more primitive HSC are found within the collagen fiber network comprising BM MSC	[132]
Microcavities containing human BM MSC inserted in a microfluidic bioreactor	300 μm-sized microcavities promoting a 3-D distribution of HSPC within a MSC network and favoring cell-cell interactions	3-D microarray environment preserves more primitive HSC whereas monolayer co-cultures increases HSPC differentiation	[134]
MS-5 cells adhered to the surface of polymer particles	Modulation of gene expression in MS-5 cells relevant for hematopoiesis in 3-D co-culture system	Higher levels of hematopoietic progenitors adhered to the mouse stromal cells detected in the adherent layer of 3-D culture in comparison to 2-D monolayer culture, maintaining an equilibrium between proliferation and differentiation	[135]
Human BM MSC-seeded macroporous PEG hydrogel in a perfusion bioreactor	Perfused 3-D BM analog	Development of an in vitro model that mimics the hematopoietic niche under steady-state conditions or in an activated state, suitable to be used as a drug testing system	[146]
Hydroxyapatite coated zirconium oxide scaffold comprising human BM MSC inserted in a microfluidic device	Pre-culture of MSC on the ceramic scaffold promoting deposition of ECM and secretion of growth factors	Long-term culture of primitive HSPC	[147]
BM-on-a-chip	Engineered in vivo in a mouse and subsequently transferred to a microfluidic device	Complex tissue-level interactions mimicked through maintenance of 3-D spatial organization of niche components and in vivo-like HSPC proportions	[148]

MSC, Mesenchymal stem/stromal cell; BM, Bone marrow; HSPC, Hematopoietic stem/progenitor cell; UCB, Umbilical cord blood; PDGFRα$^+$, Platelet-derived growth factor receptor α$^+$; NSG, NOD/SCID gamma; VLA, Very late antigen; CXCR4–C-X-C chemokine receptor type 4; ECM, Extracellular matrix; SDF-1α, Stromal cell-derived factor-1α; PCL, Polycaprolactone; PLGA, Poly(lactic-co-glycolic acid); UC, Umbilical cord; CFU, Colony-forming unit; LTC-IC, Long-term culture-initiating cell; β-TCP, β-tricalcium phosphate; PLLA, Poly(lactic acid); PEG, Poly(ethylene glycol).

resembled the in vivo environment, in contrast to 2-D conventional cell culture systems, where stress fibers and cytoskeleton reorganization are induced.[114]

The advantages of 3-D systems composed by MSC spheroids were also partially unraveled by Cook and collaborators, who observed that MSC spheroids express higher levels of key hematopoietic niche factors (angiopoieitin-2, angiopoieitin-1, SCF, SDF-1α, Jagged-1) in comparison to their 2-D counterparts.[110] Moreover, although MSC cultivated as 2-D adherent monolayer cells or as 3-D spheroids supported similar expansion levels of total BM hematopoietic cells, higher expansion of more primitive HSC was observed in the presence of a 3-D HSPC/MSC organization. However, contradictory results were obtained by Schmal and colleagues, who stated that, despite enhanced expression of niche ECM components by 3-D MSC spheroids, expansion of primitive UCB CD34$^+$-isolated hematopoietic progenitors was favored by a 2-D monolayer arrangement of MSC.[111] These conflicting results might be, at least in part, explained by differences in MSC isolation protocols, culture media and species variation since the former study[110] used murine MSC and HSPC populations whereas the latter[111] obtained UCB HSPC and BM MSC from human donors.

4.2. Biomaterial-Based Approaches

Culture platforms exploiting 3-D cell organization can provide valuable tools to establish in vitro interactions that better resemble the signaling mechanisms present in vivo. Moreover, 2-D TCPS cell culture misses several cues present in the natural BM niche architecture, such as surface stiffness, porosity, and gradients of oxygen and bioactive factors.

Different biomaterial-based culture platforms, supported by the incorporation of stromal cells but also relying on cultures free of adjuvant cells have been explored to help unraveling the interactions between HSPC and their microenvironment. Importantly, tailoring the properties of biomaterials, namely by addressing their capability to present biochemical and biophysical cues to cultured HSPC, would likely broaden the capacity of researchers to impact cell fate in a more controlled manner.

4.2.1. Macroporous Scaffolds

Some biomaterial-based approaches have attempted to create scaffolds that resemble the macroporous structure characteristic

of the trabecular bone present in the BM. For instance, Ventura-Ferreira and colleagues explored a broad range of 3-D biomaterial scaffolds (polycaprolactone (PCL), poly(lactic-co-glycolic acid) (PLGA), fibrin and collagen) to expand UCB $CD34^+$ cells supported by umbilical cord (UC)-derived MSC.[115] The highest cell growth was obtained by the 3-D fibrin scaffold, a human-derived material that not only favored maintenance of a more primitive phenotype but also evidenced superior adhesion, migration, and engraftment efficiency of hematopoietic cells transplanted to NSG mice.[116]

Creation of an artificial osteoblastic niche was attempted by Tan and colleagues,[117] who seeded osteogenic-induced BM MSC onto a bio-derived bone scaffold. The 3-D organized osteoblastic scaffold rendered higher colony-forming units (CFU) progenitors and long-term culture-initiating cell (LTC-IC) numbers, both indicators of maintenance of more primitive HSPC from UCB $CD34^+$-isolated cells, relatively to 2-D control cultures. Similarly, in another study, a mixture of MSC and osteoblasts seeded on a bio-derived bone scaffold were observed to grow in the spongy architecture of the trabecular bone while fostering a higher number of more primitive hematopoietic cells in comparison to 2-D co-culture systems.[118] Interestingly, the interactions between human BM MSC and MSC-derived osteoblasts modulated the expression of cytokines and ECM synthesis, contributing to a more physiologically relevant hematopoietic environment.

Some biomaterial strategies, however, rely on the development of constructs rather than focusing on the ex vivo expansion of HSPC, which might facilitate the recruitment of endogenous cells toward the functionalized scaffold once implanted in vivo. Bladergroen and collaborators, for instance, loaded heparinized collagen scaffolds with SDF-1α and implanted these in vivo (mouse), leading to recruitment of HSPC.[72] On the other hand, Ventura-Ferreira and colleagues combined the use of a β-tricalcium phosphate (TCP) scaffold, evidencing bone-characteristic porosity, with bioactive cues, provided by ECM hydrogels and co-culture with MSC, to promote the recruitment of hematopoietic cells and enhance the scaffold vascularization once implanted in vivo in a mice model.[119]

4.2.2. Fibrous Meshes

Fibrous meshes, through their nanoscale dimensions and organization, can replicate important topographical cues provided by ECM in vivo. In this context, the electrospinning technique has emerged as a possible strategy to produce polymeric nanofiber meshes capable to provide nanotopographical and chemical cues to cells through functionalization of the nanofibrous structures. Covalently modified polyethersulfone (PES) nanofibers with amino groups were shown to support enhanced expansion of human UCB $CD34^+$ cells comparatively to TCPS.[120] The amino functional groups evidenced a superior behavior relatively to carboxylic or hydroxyl groups. Later, the same research group reported that, although expansion of UCB $CD133^+$-isolated cells was enhanced as surface amine density increased, optimal expansion was obtained at a moderate density of 20–80 nmol cm^{-2}.[121] This suggests that the chemistry of the ligand/functional group, as well as its density, is sensed by HSPC. Moreover, the topographical cues provided by the size and characteristic organization of aminated PES nanofiber meshes present an enhancing effect on HSPC adhesion and expansion of a higher number of more primitive HSPC compared to aminated PES films.[120]

Batnyam and colleagues further explored MSC-seeded fibrous scaffolds (using an electrospun polyether–polyurethane–elastomer) assembled in a multilayer construct to mimic the BM endosteum and support self-renewal of HSPC. This 3-D biohybrid scaffold facilitated high-density expansion of multipotent HSPC. The microscale architecture of the electrospun fiber scaffolds, more closely resembling natural ECM, together with its mechanical properties and ability to enhance expression of Jagged-1in co-cultured MSC (known to play a role in the self-renewal of HSPC through Notch-1 – in HSPC – and Jagged-1 – in stromal cells – interactions), were suggested to be relevant features of the 3-D system in comparison to TCPS-cultured HSPC and MSC.[122] Electrospun poly(lactic acid) (PLLA) nanofibrous scaffolds, seeded with niche-like units extracted from mice BM, were also shown to be suitable for implantation in irradiated mice and to contribute to the restoration of their hematopoietic system.[123] Contrarily to mice transplanted with scaffolds alone, scaffolds seeded with native niche-like units showed interconnection with sinusoidal vessels, likely due to the role played by the angiogenic factors secreted by the co-cultured stromal cells. Furthermore, PLLA has been described to present osteogenic properties,[124] being able to direct the fate of seeded MSC into the osteogenic lineage, which could therefore impact the activity of co-cultured HSPC.[109]

4.2.3. Hydrogel-Based Culture Approaches

Alginate, due to its capability to jellify under mild conditions and to allow easy retrieval of encapsulated cells, has been used to encapsulate and amplify HSPC from UCB either under static or dynamic conditions.[125] This allows alginate-encapsulated HSPC to be inserted in a high cellular density microenvironment, thus being more easily accessible to paracrine signaling.[126] Alternatively, stromal cells can be encapsulated in alginate microbeads to explore their paracrine support of HSPC.[127,128] Such an approach was followed by Sharma and colleagues, who showed the benefits of hydrogel encapsulated MSC over traditional MSC culture to support human BM $CD34^+$ HSPC.[129] 3-D cultured HSPC, supported by MSC encapsulated in a Puramatrix™ gel, not only retained a higher percentage of primitive cells but also evidenced superior engraftment potential in immunocompromised mice. The formation of a hypoxic gradient within the hydrogel (identified upon staining with hydroxyprobe) and differential secretion patterns of trophic factors by 3-D encapsulated MSC might have contributed to these observations.[129]

On the other hand, exploring the porosity created by salt leaching in poly(ethylene glycol) (PEG) hydrogels, the architecture of trabecular bones could be mimicked to promote co-culture of UCB $CD34^+$-isolated HSPC with MSC isolated from UC or BM.[130] PEG hydrogels were also used to develop a

microwell platform for assessing the response of single HSC to specific proteins. Reduced proliferation kinetics and increased asynchronous division of single HSC cultured in Wnt3a- or N-Cadherin-coated PEG microwells was shown to be correlated with long-term reconstitution of whole blood in serial transplantation experiments in mice.[131] Interestingly, Leisten and colleagues used collagen scaffolds comprising human MSC to create two distinct compartments with different effects on the fate of HSPC.[132] Whereas HSPC in suspension above the collagen gel were proliferative and evidenced a higher proportion of more committed progenitors, HSPC within the collagen gel showed higher levels of a more primitive phenotype.

4.2.4. Microcavities

The idea that constructing hematopoietic niches by applying bioengineering strategies can provide insights into the signaling pathways involved in HSPC fate regulation was corroborated by a recent study performed by Müller and colleagues, who found autocrine signals established in single-cell niches made on PDMS or hydrogel microcavities to be critical for stem cell quiescence.[133] The small-sized cavities allow secreted factors to accumulate more efficiently than in larger spaces, therefore attenuating autocrine signals over paracrine cues. Further exploiting a microcavity array, stromal cells were incorporated in a chip-based 3-D co-culture of UCB hematopoietic cells favoring cell-to-cell contact through β-catenin and N-cadherin intercellular junctions and therefore contributing to preserve the primitiveness of hematopoietic progenitor cells in comparison to monolayer co-cultures.[134]

4.2.5. Microparticles

3-D co-culture of UCB-derived $CD34^+$ cells and stromal cells adhered to the surface of polymeric microparticles has also been explored.[135] Whereas a 2-D culture of $CD34^+$ cells supported by a MS-5 mouse stromal layer resulted in exhaustion of hematopoietic cells upon 4 weeks of culture, in 3-D co-culture, hematopoietic progenitors in a resting state were detected in the adherent stromal layer. This suggests the ability of the 3-D culture system to establish a homeostatic balance between proliferation and differentiation of hematopoietic cells, similarly to what happens with the hematopoietic niche in vivo.

Although in this *"Biomaterial-based approaches"* section we described several advantages presented by the incorporation of biomaterials onto cell culture platforms, 2-D culture is still frequently preferred over 3-D culture systems to study and expand HSPC ex vivo. Not only 3-D approaches usually imply higher complexity, but challenges associated with uneven cell seeding, difficulty to retrieve cells from 3-D structures and poor control over spatial gradients of oxygen, available nutrients and cytokines are still hard to address. Both bioreactor culture of 3-D biomaterial-based cell constructs, namely with medium perfusion, and development of high-throughput methods, such as microfluidic devices and creation of microscale arrays, could potentially address those challenges.

5. Biomaterial-Supported Cultures of HSPC in Bioreactors

Several different types of bioreactors have been used to culture UCB hematopoietic cells,[14,136] including stirred tank suspension,[137] perfusion chamber,[138] fixed bed,[139] airlift, and hollow fiber reactors.[140] The most commonly described reactors attempting to expand HSPC rely on suspension cultures unable to maximize cell-cell and cell-ECM contact, two types of interactions known to be essential regulators of the HSPC fate. An interesting alternative to these systems would be the design of bioreactors coupled to the use of biomaterial approaches.

Following such strategy, Sullenbarger and colleagues were capable to continuously produce high numbers of functional platelets from UCB $CD34^+$ cells using a woven polyester fabric scaffold in a 3-D perfusion bioreactor system.[141] Further efforts attempting to address the need for platelet production exploited a bioreactor platform that incorporated biomimetic osteoblastic and vascular niches, capable to combine, at once, several features of the BM microenvironment. To that purpose, a collagen I hydrogel was developed to represent the endosteal niche while von Willebrand Factor (vWF)- and fibrinogen-coated silk microtubes, through which culture medium could be perfused, were used as representatives of the vascular niche. Megakaryocyte migration within this 3-D perfused system was observed together with the release of functional platelets.[142] More recently, the same group showed that increased production of platelets could be achieved upon coupling a bioreactor setup that allowed physiological shear rates to be used to more closely mimic the BM environment. To that purpose, incorporation of ECM components and control of the topography and stiffness of porous silk in co-cultures performed with endothelial cells rendered enhanced levels of platelet production ex vivo,[143] showing the relevance of the concomitant use of biomaterial tools with bioreactor approaches to build more functional 3-D tissue models of the BM niche.

Also exploiting a perfusion-based system, Schmelzer and colleagues developed a multi-compartment hollow-fiber membrane-based 3-D perfusion bioreactor for the culture of whole BM mononuclear (MNC) cells.[144] Macroporous spongeous hydroxyapatite scaffolds, mimicking the in vivo bone matrix, were embedded between hollow-fiber membrane layers in the bioreactor core. Cells cultured on these scaffolds, in a 3-D perfused system, enabled long-term maintenance of primary BM cells, namely HSPC. Similarly, a 3-D perfusion bioreactor with a porous heparin-functionalized chitosan scaffold demonstrated improved expansion of human UCB $CD34^+$ HSPC, exhibiting higher maintenance of more primitive progenitors than $CD34^+$ cells cultured under static conditions. Of notice, this perfused system, when operated at lower oxygen tensions (5% O_2), rendered higher percentages of more primitive cells.[145]

Furthermore, bioreactor-cultured HSPC could benefit from cues provided by co-cultured stromal cells. Indeed, co-culture of UCB hematopoietic cells with alginate microencapsulated MSC in a rotating wall vessel rendered higher expansion levels of total nucleated and $CD34^+$ cells than static co-culture or bioreactor culture alone while maintaining more primitive HSPC.[128] On the other hand, Rodling and colleagues explored HSPC and MSC-seeded PEG hydrogels on a perfused

model of the BM niche and suggested that a similar approach could be followed in drug screening and toxicity testing.[146] Microfluidic systems can also contribute to develop more efficient drugs, with higher specificity for treatment of hemato-oncological diseases. Contrarily to traditional animal testing systems, whose use poses ethical issues and limits the translation of obtained results to human patients due to inherent species-specific differences, and 2-D systems, which lack the complexity of in vivo microenvironments, in vitro 3-D co-cultured models of HSPC and supportive MSC in a microfluidic environment can help improving our capacity to successfully mimic the BM niche. Such an approach has been followed by Sieber and co-workers whose 3-D co-culture model, based on a hydroxyapatite coated zirconium oxide scaffold comprising human BM MSC inserted in a microfluidic device, was capable of supporting the long-term culture of primitive HSPC.[147] Equally exploiting a microfluidic device, BMP2- and BMP4-loaded collagen scaffolds were implanted subcutaneously into mice so that native cells and vasculature would migrate and develop on the chip-sized bone matrix, ultimately resembling a BM compartment that was built in vivo throughout 8 weeks after transplantation. Once explanted, this composite, that evidenced a hematopoietic cell composition close to that of natural BM, was transferred to an in vitro microfluidic device and cultured for 7 days.[148]

We envision that culture of HSPC supported by biomaterial constructs, once inserted in scalable bioreactor culture systems, could benefit from the biophysical and chemical cues provided by scaffolds seeded with supportive stromal cells or functionalized with bioactive factors, while taking advantage of the higher expansion/differentiation cell yields and enhanced control associated with culture in bioreactors.

6. Clinical Applications of HSPC – Ex Vivo Expanded HSPC in HCT

High doses of chemotherapy or radiation exposure are inflicted to patients suffering from disorders such as leukemias, lymphomas, multiple myeloma, among others. The harsh treatment applied to these patients destroys abnormal BM that would then benefit from HCT to reconstitute the hematopoietic system.

Even though it is now possible to treat adult patients by double UCB transplantation, this procedure is associated with slow hematopoietic recovery (e.g., of neutrophils and platelets) and increased incidence of GvHD, as well as higher transplant costs.[149] As previously mentioned, alternative strategies such as the ex vivo expansion of UCB stem/progenitor cells have been developed in the last decades. Some ex vivo expansion strategies have moved from preclinical studies, in animal models, to clinical trials in humans. Such an example is the phase I clinical trial where 10 patients were transplanted with a UCB unit that had been previously expanded for 16 days in the presence of an immobilized Notch signaling ligand, Delta-1$^{ex\text{-}IgG}$ (164-fold-increase of CD34$^+$ cells), along with cells from an unmanipulated UCB unit.[60] This resulted in a reduced median time to neutrophil engraftment, when compared to controls transplanted with cells from two unmanipulated UCB units. Furthermore, this procedure was considered to be safe, as there were no reports of engraftment failure or incidence of GvHD. However, only two patients demonstrated persistence of the expanded graft after 180 days, which is indicative that either the expanded cells lost their self-renewal potential or those were rejected by the T cell–containing unmanipulated unit. In another multicenter phase I clinical trial, 23 patients were infused with an unmanipulated graft, along with a partially HLA-matched graft, previously expanded with immobilized Delta-1$^{ext\text{-}IgG}$.[150] However, due to the complex logistics associated to expanding patient-specific UCB units, researchers at Fred Hutchinson Cancer Research Center in Seattle, USA, have been trying to develop an "off-the-shelf" product (already in phase II clinical trial), where patients receive an unmanipulated UCB graft and a Delta-1-expanded HLA-mismatched UCB unit.[151]

Based on the positive results observed with the ex vivo culture of UCB HSPC with a copper chelator, TEPA,[152] Gamida Cell developed StemEx®. With this product, a fraction of a single UCB unit is expanded for 21 days with cytokines and TEPA, and infused in patients along with the unmanipulated cell fraction. Gamida Cell launched a multinational phase II/III registration trial (NCT00469729, clinicaltrials.gov website accessed on 3rd May, 2017) and concluded that transplanting a StemEx® graft improves a number of important clinical endpoints.[153]

In a phase I clinical trial, NiCord®, which consists of an ex vivo expanded HSPC population that contains a previously frozen non-cultured T cell population from the same UCB unit, was infused with another unmanipulated UCB unit. Significantly earlier neutrophil and platelet recoveries were achieved for patients engrafting with this product.[154]

With the support of Mesoblast, the MD Anderson Cancer Center, Texas, USA, developed and brought to the clinic a MSC-UCB co-culture that is used in combination with hematopoietic cytokines to promote the expansion of UCB HSPC for 7 days, after which the expanded UCB cells are cultured with cytokines alone for another 7 days. In this study, 31 patients were infused with an expanded graft along with an unmanipulated graft, with favorable results in terms of hematopoietic recovery compared to historical controls who received cells from two unmanipulated UCB units.[15]

Based on the capability of SR1, a purine derivative, to expand functional HSPC ex vivo, patients in a phase I/II clinical trial were submitted to a double UCB transplantation procedure that consists of infusing a previously SR-1-expanded unit, an unmanipulated unit, and a T-cell containing fraction. Encouraging results in terms of neutrophil and platelet recovery have been reported in the context of this trial.[62]

The promise use of ex vivo expanded UCB HSPC in clinical trials is supported by the absence of infusional toxicity of these cells reported to date. Nevertheless, optimization of expansion strategies is still required to address some issues such as the delayed time for effective neutrophil engraftment and platelet recovery. In this context, exploiting proper hematopoietic niche biomimetic cues would likely contribute, namely concerning the use of adequate cytokine cocktails combined with accessory stromal cells, to potentiate the expansion of more primitive HSPC, as well as more differentiated progenies.

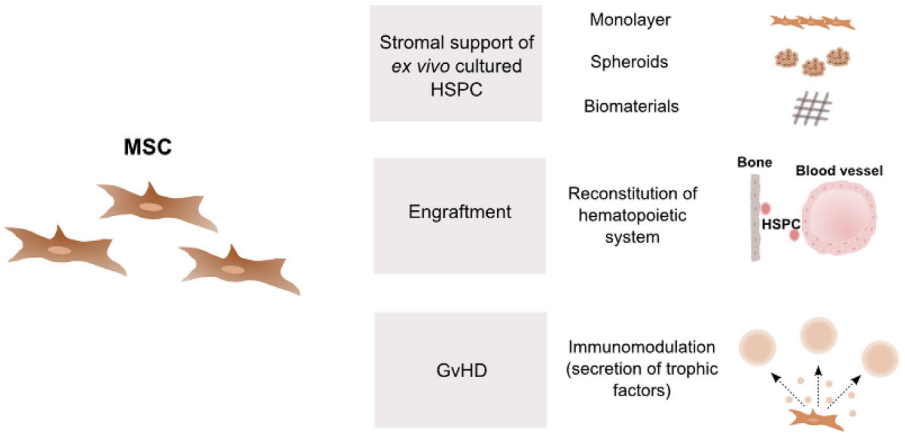

Figure 4. MSC can support the ex vivo culture of HSPC as they are capable to recapitulate important hematopoietic niche cues either through direct contact with HSPC or secretion of trophic factors. The hematopoietic supportive behavior of MSC can be explored through culture as adherent monolayer cells in TCPS, as 3-D organized spheroids or incorporated within biomaterial structures. Additionally, MSC can constitute a relevant therapeutic tool as a co-adjuvant in the context of HCT, by favoring increased engraftment in the BM or limiting GvHD, namely through secretion of immunomodulatory factors.

7. Conclusions and Future Perspectives

Culture of HSPC in flat TCPS or in liquid suspension solely sustained by addition of cytokines, with none or limited contact with other supportive cell populations, or biophysical cues provided by 3-D biomimetic organized cultures, might not help to recapitulate the niche cues required to maintain a homeostatic hematopoietic system. In addition, the high costs associated with the use of high concentrations of cytokines, that hardly resembles the physiological concentrations encountered by cells in their native environment, together with the short half-life of cytokines and their limited capability to account for the dynamic spatiotemporal crosstalk that take place in vivo highlights the importance of incorporating biomimetic cues to extend our control over the fate of ex vivo cultured HSPC.

3-D biomimetic hematopoietic niches would not only improve cellular outcomes but also help clarifying the regulation mechanisms involved on the control of HSPC fate. Uncovering ligand-receptor interactions present within the hematopoietic niche would certainly contribute to improve our understanding of the regulation of HSPC behavior. Our limited knowledge of the complexity of cellular, biological, and physical cues provided by the native hematopoietic niche still positions the use of stromal-supported cultures as an important strategy to sustain ex vivo HSPC cultures. Nevertheless, some recent approaches have attempted to incorporate biological cues, such as Notch ligands, within biomaterials capable to constitute an alternative to the signaling provided by co-culture with stromal cells.

Moreover, in addition to the relevant role of stromal cells in regulating the activity of HSPC, diffusion-limited 3-D cell formats might elevate the degree of control over flat 2-D culture systems. However, one of the major limitations of the various attempts to recreate the hematopoietic niche is that these fail to incorporate the vascular structure, composed by sinusoids and arterioles, known to be part of the BM.

Besides the importance of establishing proper culture configurations, adequate isolation methods of HSPC supportive cells, such as MSC, which are frequently isolated based on their plastic adherence and optimization of chemically defined medium formulations able to comply with GMP, will likely result in more controlled, reproducible, and clinically translatable ex vivo culture systems.

To date, although considerable effort has been performed to better understand the mechanisms governing the capability of the hematopoietic niche to sustain life-long hematopoiesis, the success of cell therapies is still hindered by the limited capacity of expanded HSPC to retain their primitiveness and failure of HSC engraftment. Although the use of co-adjuvant MSC in HCT settings seems to favor not only the maintenance of a more primitive HSC phenotype, but facilitate engraftment and contribute to prevent GvHD (**Figure 4**) (particularly relevant in allogeneic transplantation), controversial reports are still presented.

Overall, many of the factors identified to impact the expansion/differentiation of HSPC are actually natural regulators residing in the hematopoietic niche, which highlights the importance of increasing our knowledge of this complex 3-D microenvironment.

Acknowledgements

MHC and AMS acknowledge Fundação para a Ciência e a Tecnologia (FCT), Portugal, for granting PhD scholarships SFRH/BD/52000/2012 and SFRH/BD/52014/2012, respectively. Funding received by iBB-Institute for Bioengineering and Biosciences from FCT-Portuguese Foundation for Science and Technology (UID/BIO/04565/2013) and from Programa Operacional Regional de Lisboa 2020 (Project N. 007317) is acknowledged. The authors also acknowledge the funding received from Programa Operacional Regional de Lisboa 2020 through the project *PRECISE – Accelerating progress toward the new era of precision medicine* (Project N. 16394).

Conflict of Interest

The authors declare no commercial or financial conflict of interest.

Keywords

biomaterials, ex vivo culture, hematopoietic stem/progenitor cells (HSPC), mesenchymal stem/stromal cells (MSC), microenvironment

Received: May 31, 2017
Revised: October 27, 2017
Published online: December 28, 2017

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