The therapeutic potential of hematopoietic stem cells in bone regeneration

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Abstract

The repair process of bone fractures is a complex biological mechanism requiring the recruitment and *in situ* functionality of stem/stromal cells from the bone-marrow (BM). While BM mesenchymal stem/stromal cells have been widely explored in multiple bone tissue engineering applications, the use of hematopoietic stem cells (HSCs) has been poorly explored in this context. A reasonable explanation is the fact that the role of HSCs and their combined effect with other elements of the hematopoietic niches in the bone healing process is still elusive. Therefore, in this review we intend to highlight the influence of HSCs in the bone repair process, mainly through the promotion of osteogenesis and angiogenesis at bone injury site. For that, we briefly describe the main biological characteristics of HSCs, as well as their hematopoietic niches, while reviewing the biomimetic engineered BM niche models. Moreover, we also highlighted the role of HSCs in translational *in vivo* transplantation or implantation as promoters of the bone tissue repair.

Impact statement

The ability of bone to natural self-heal depends on the size and stabilization level of the tissue fracture, and it is impaired in several pathophysiological conditions. Considering that the available treatment options have demonstrated limited regenerative performance, the hematopoietic stem cells (HSCs) cocultured in different tissue engineering strategies have emerged as a powerful tool to promote effective bone regeneration and healing. Here, we reviewed the most important biomimetic bone-marrow hematopoietic niches and showed the regenerative potential of these cells, both *in vitro* and in translational *in vivo* transplantation/implantation approaches. This knowledge encourages the development of new HSC-related bone regenerative therapies.

1. Introduction

The high incidence of bone defects places the bone as the second most transplanted tissue after blood transfusion[1]. Although bone tissue has the peculiar ability to fully regenerate and restore its biomechanical function, about 10% of fractures fail to heal properly[2,3]. In these cases several surgical interventions are required, which potentiates the risk of infection, pain, and disability[4]. Currently, even the gold standard treatment option for bone regeneration, namely bone autografts, has shown some drawbacks[5–7]. In the last decade, the demonstration that HSCs can act through a variety of mechanisms to promote repair and tissue regeneration has dramatically broadened their clinical utility for the repair and regeneration of several non-hematopoietic tissues[8–138]. Considering that the development of effective bone therapy strategies continues a challenge, researchers in bone tissue regeneration have shown great interest in exploring the use of HSCs[14–16].

The BM specialized microenvironments hold distinct cellular and non-cellular components where osteogenesis and hematopoiesis occur[17–20]. The dynamic BM microenvironment and the interactions between its components have been explored as novel therapeutic targets to facilitate the bone's regenerative capacity. Although most stem cell-based therapy approaches

aiming bone regeneration have focused on the role of BM-derived MSCs (BM-MSCs) and their interaction with endothelial cells (ECs), recently the plethora potential use of HSCs alone[10,21–26] or in combination with other cells from the BM[27–29] has been recently proposed and investigated for bone healing strategies. The understanding of the cellular and molecular crosstalk involved in the HSCs regulation and cell fate, in both health and bone disorder, is of fundamental importance to precisely clarify, control, modulate and find new HSC-therapeutic strategies aiming towards bone regeneration. In such context, advanced models that mimic the BM-HSCs niches have been developed to explore its biological complexity and precisely control key components that facilitate the regenerative process[30–33]. However, the understanding of such privileged environment is still elusive.

2. The hematopoietic stem cells and their bone-marrow niches

Self-renewal and pluripotency properties ensure the maintenance of functional hematopoiesis during the lifetime[34–36]. This hierarchical process is represented in **figure 1.** The primitive long-term HSCs (LT-HSCs) self-renew and maintain the HSC pool, while the short-term HSCs (ST-HSCs) differentiate towards the multipotential progenitors (MPPs). The MPPs give rise to common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs), originating all the diverse mature and functional hematopoietic cell types *in vivo*[37,38]. The HSCs and their progenitors are subdivided according to presence or absence of specific cell membrane markers (CD34, CD38, CD90, and CDR45)[38,39]. These cells are dynamic with a nonrandom spatial orientation within their BM niches, the endosteal and the perivascular niche. These niches comprehend a complex network of cell-cell contact and interaction with non-cellular components, all necessary for the HSC maintenance, proliferation, activation, differentiation, and migration[18,34,40–42]**(Figure 2).**

2.1. Constituents of the Endosteal niche

The endosteal niche is functionally responsible for the regulation of bone formation and resorption[33,43–45]. Localized at the inner surface of the bone cavity, this niche ensures the

maintenance of the HSCs numbers and trans-marrow migration[18,34,46,47]. The LT-HSCs are found quiescent in this niche and once activated they self-renew to sustain hematopoiesis during lifetime[9,48]. Osteoblasts control HSC stemness, and quiescence by N-cadherin-mediated adhesion[49.] These cells secrete a range of cytokines, such as stem cell factor (SCF), thrombopoietin (TPO), osteopontin (OPN), angiopoietin-1 (Ang-1), CXC-chemokine ligand 12 (CXCL12), CXCL-4, and interleukin (IL)-6[32,45,50]. Together with osteoclasts, these cells are responsible for bone remodeling. Once stimulated, osteoclasts secrete enzymes that cleave osteoblast-expressed niche molecules. Consequently, HSCs and HPCs are released from the niche and mobilized to the periphery from BM[51]. This osteoclast-osteoblast close association suggests a delicate and controlled balance of the HSC-regulating cytokines which is responsible to support the endosteal niche and required for bone repair. BM-MSCs can differentiate towards osteoblasts, chondrocytes, and adipocytes. These cells support HSC regulation through the production of interleukins, TPO, SCF, macrophage colony-stimulating factor, Flt3 ligand, Ang-1, and CXCL12[33]. Furthermore, BM-MSCs produce and create a network of extracellular matrix (ECM) proteins including proteoglycans, fibronectin, collagen, laminin, and thrombospondin[52]. These components modulate the HSC behavior by promoting cell homing, viability, self-renewal, expansion, differentiation, and mobilization of the HSCs between their different niches[32,53,54].

2.2. Constituents of the perivascular niche

The perivascular niche is placed adjacent to the blood vessels and secrete angiocrine factors required for the survival, maintenance, and self-renewal of the HSCs/HPCs[55]. The ST-HSCs and MPPs are the main hematopoietic cell population resident. Dormant HSCs, lymphoid and myeloid progenitors have been identified in the central marrow, around arterioles and sinusoids, closer to stromal cells[56,57]. This niche is implicated within ST-HSCs mobilization from the BM to the peripheral blood (PB), where they can originate MPPs to form the multiple hematopoietic cell lineages and might directly contribute to the recovery of damaged tissues[18,34,52]. Sinusoidal and arteriolar ECs secrete SCF, granulocyte colony-stimulating

factor, CXCL-12, pleiotrophin, and Ang-1[32,50,53]. They also express Notch ligand Jagged-1, CD44 ligands, and numerous adhesion molecules, as well as selectins, vascular cell adhesion molecule 1, type IV collagen, laminin, and integrins. These factors can be associated with regulation, homing, adhesion, and HSC transmigration[52]. Mesenchymal-derived stromal cells, namely CXCL12-abundant reticular (CAR), leptin receptor-positive ($LepR^+$), and $nestin^+$ cells are found in the surface of arterioles or sinusoids. These cells, together with endothelial, Schwann, and sympathetic neuronal cells secrete CXCL12 and SCF, essential factors for HSC homing and retention in the BM[13,18,34]. BM-MSCs-derived adipocytes have been considered as negative regulators of the HSCs, although recent data show that SCF produced by these cells is essential for hematopoietic regulation and regeneration[58,59]. Furthermore, oxygen, reactive oxygen species, and calcium gradients have been proposed as important HSC physiological regulators involved in the balance between HSC proliferation and differentiation[52,60,61].

Despite the efforts of intensive investigation to better characterize and understand the HSC-niche components, their interactions, and localization, a deep understanding of the mechanisms involving the HSC regulation and cell fate, in both health and bone disorder, is still crucial to identify key components that can facilitate and induce the bone regenerative process.

3. HSCs as powerful candidates aiming towards bone regeneration

After a bone fracture, loss of skeletal integrity, disruption of the bone vasculature, hematoma, and inflammation, occur locally[6,62]. The vascular restoration of bone forecasts its repair[63–65]. In fact, high vascularization of bone provides key cell players, including osteolineage cells, MSCs, HSC/HPCs, endothelial progenitor cells (EPCs), and ECs, for a proper bone formation, remodeling, repair, and homeostasis. Although these events compromise the hematopoietic niches, they also stimulate the HSCs to switch from quiescent to proliferative and differentiation state to quickly recover the hematopoiesis system, which is an essential HSC property to repair the bone tissue[10].

The $CD34^+$ cells population comprehend a plethora of cellular phenotypes, including EPCs and osteo precursors cells[66–70]. Several studies report the use of $CD34^+$ cells either from PB, BM, or UCB to enhance fracture repair in animal and human models, through angiogenesis and osteogenesis[21,66,71,72] **(Figure 3).** In the last decades, the ability of $CD34^+$ cells to differentiate into ECs and osteoblasts were pointed out, suggesting a possible overlap between endothelial and osteoblast precursor cells[16,68]. Importantly, it is not clear yet whether HSCs are able to differentiate into ECs or osteoblasts, or a fraction of the $CD34^+$ cells population could be instead accountable for that. Common BM progenitors with hematopoietic/endothelial and hematopoietic/osteoblastic differentiation potential may exist and be implicated in such endothelial and osteogenic phenotypic cells. Consequently, the mechanisms by which the bone healing potential of $CD34^+$ is addressed have been extensively investigated, but it still requires additional clarities.

3.1. The angiogenic potential of HSCs

The contribution of the $CD34^+$ cells for the restoration of bone vasculature at injury sites is indubitably evident. So far, these cells are believed to induce vascularization through endothelial differentiation and paracrine stimulation. With the discovery of EPCs in adults, the PB, BM and UCB-derived EPCs have gained attention for neovascularization therapies [73–76]. It strongly suggests that in response to ischemia and cytokines, corresponding to the early/acute phase of bone healing, EPCs are recruited from BM into PB and are then mobilized to the fracture site, where they differentiate into mature ECs[77–79]. Histological studies have uncovered the occurrence of neovascularization at local fracture independent of vasculogenesis from BM-EPCs. This finding suggests that BM-EPCs may also have a paracrine effect on resident ECs and EPCs, resulting in angiogenesis and vasculogenesis orchestrated by the respectively resident cells[71]. Accordingly, transplanted $CD34^+$ cells have shown to secrete vascular endothelial growth factor (VEGF), whose inhibition with a soluble antagonist showed both angiogenesis/vasculogenesis and intrinsic osteogenesis suppression, emphasizing the contribution of the paracrine mechanism[66].

3.2. The osteogenic potential of HSCs

Similarly, an osteo precursors population enriched in $CD34^+$ cells and osteogenic paracrine mechanisms are believed to be accountable for the osteogenic potential of the $CD34^+$ cells. PB and BM-derived $CD34^+$ cells osteoblastic differentiation is reported in *in vitro* studies, when cultured in the presence of osteogenic medium containing dexamethasone, resulting in mineral matrix secretion and increase in alkaline phosphatase (ALP) activity[23,69]. PB-$CD34^+$ cells were found to express osteoblastic genes such as osteocalcin, collagen type I and bone ALP[23,80]. Interestingly, PB-$CD34^+$ cells stained positive for the osteocalcin protein are reported to become plastic adherent and form mineralized nodules when cultured in osteogenic medium[80]. Evaluation of patients' blood samples 10 and 20 days after bone fracture have also showed an increase in the number of circulating osteocalcin positive-stained cells.

Co-culture of PB-$CD34^+$ cells with BM-MSCs have revealed significant improvements in bone formation *in vitro* and *in vivo* compared to PB-$CD34^+$ transplanted alone. This correlates with the secretion of growth factors and cytokines from the co-cultured cells as strong osteogenic paracrine stimulators[27]. HSC-derived BMP-2 and BMP-6 were identified as key players in this paracrine comunication[29].

Considering all these evidences, it is clear that $CD34^+$ cells exert an exceptional effect in the process of bone healing by promoting adequate conditions to angiogenesis and osteogenesis occur. Although, the precise cellular and molecular mechanisms are still not entirely clarified; similarly, the identity and characterization of the specific $CD34^+$ cells accountable for the outcomes remains poorly discerned.

4. Bioengineering strategies for recapitulation of HSC niches

Bioengineered BM niches allow a better understanding of the dynamic signaling between their elements[81]. Particularly, the hematopoietic niches have recently received special attention to clarify the role of HSCs/HPCs in bone regeneration **(Table 1)**. Importantly, it allows to reconstitute the mechanisms, or at least elucidate part of them, by which hematopoiesis, osteogenesis, and vasculogenesis reciprocally evolve. Additionally, such bioengineered

models are especially useful to address HSCs expansion. One of the major limitations of using HSCs for transplantation relates to the significative low number of cells that can be isolated from donors. This determines *ex vivo* expansion of the HSC numbers as an essential prerequisite for clinical and biomedical applications. However, the difficulty to maintain HSC self-renewal and stemness preservation in culture has been hampering its efficiently *ex vivo* expansion. In this context, artificial hematopoietic/ BM models have been widely explored to assess successful HSCs expansion, while crucial local environmental features required to modulate HSCs self-renewal, proliferation, and differentiation are recapitulated.

The latest improvements have been achieved by co-culturing HSCs with other hematopoietic niche cells, with or without cytokines exposure directly added to the culture medium or secreted by stromal supportive cells, and biomaterial-based approaches associated with several culture systems[40,66,82,83] **(Figure 4)**. Either hydrogels composed of alginate, Matrigel, Puramatrix, polyethylene glycol[84–89], scaffolds containing bone-like materials (ceramic, collagen, fibrin, β-tricalcium phosphate)[34,94,95,114] and bio-derived cancellous bone[31,90,91] as scaffolds have been applied in these strategies with the purpose to recreate the hematopoietic niches. Furthermore, other physiological relevant properties of the BM microenvironment, such as oxygen levels, have also been recapitulated[31,32,84–86,92–98].

4.1. Static culture systems

Aiming HSCs expansion and/or differentiation to mimic the BM-HSC niches, conventional engineering methods have used two-dimensional or three-dimensional (3D) static culture systems. 3D systems-based co-cultures of $CD34^+$ cells and BM-MSCs have shown to effectively recapitulate functional properties of BM niches allowing HSCs expansion and ECM molecules secretion[83,87,90]. Accordingly, in the presence of HSCs, MSCs express high levels of several molecules known from the native BM environment, such as ECM proteins, OPN, runx-2, and Ang-1, that *in vitro* enhances the biological outcome of HSCs. In such systems, crucial aspects have been uncovered regarding HSCs expansion, namely retention of a considerable number of primitive HSCs and formation of progenitors committed to myeloid

and lymphoid lineages. Most of co-cultured $CD34^+$ cells also expressed N-cadherin, at different levels. Although a dispute exists about the dependence on this adhesion molecule for the HSCs maintenance[99,100], functional blocking or genetic knockout of N-cadherin showed to originate a loss of primitive HSCs population[101], suggesting that at least in culture, this molecule is implicated in primitive HSCs anchorage within the stromal support. Furthermore, the establishment of the CXCR4/ CXCL12 axis headed the retention of the HSCs pool. Correspondingly, HSCs showed migration-dependence towards the high levels of the chemokine CXCL12 secreted by MSCs. Indeed, the addition of AMD-3100, a CXCR4 antagonist, triggered mobilization of the primitive HSCs from the co-culture matrix to the supernatant[83]. CXCL12 is reported to be implicated in the maintenance of HSCs quiescence[102–105]. Moreover, a large percentage of the retained HSCs in the co-culture revealed an expression of the protein p21 by qPCR assay[87], which is also recognized as an important regulator of HSCs quiescence[106]. Altogether, the co-culture with MSCs have proven to provide an adequate stromal support which, similarly to the native HSC niche, are able to foster a large pool of quiescent HSCs whereas expansion and self-renewal are ensured.

Heretofore, these types of 3D engineered hematopoietic niches in static systems have shown to mimic part of key features of the native BM niches, such as bone architecture, ECM secretion, osteogenesis stimulation by the HSC-MSC interactions and cell signaling molecules which ultimately support the maintenance of HSCs with preservation of the primitive phenotype, quiescent state, and multi-lineage differential potential.

4.2. Dynamic culture systems

To more accurately recapitulate the physiologic conditions of BM-HSC niches, bioengineered hydrogels and scaffolds have been designed through 3D *in vitro*-culture approaches combining advanced biomaterials and dynamic culture systems. In fact, such innovative systems are becoming a promising bioengineering tool to approximate the bench research to the *in vivo* physiology. The capability to control key physicochemical parameters such as

oxygen levels and mechanical forces would permit the conceiving of more complex BM models. Moreover, these approaches are also suitable for drug and toxicity testing[107,108].

Microfluidic techniques are the utmost sophisticated systems that have been ultimately applied to produce bone-marrow-on-a-chip (BMoC) units for dynamic systems[107–109]**.** The benchmark BMoC was developed through the creation of new bone *in vivo,* following *in vitro* culture of living marrow on a microfluidic device[108]. Briefly, a poly(dimethylsiloxane) device with a cylindrical cavity filled with type I collagen holding bone-inducing demineralized bone powder, BMP2 and BMP4 was subcutaneously implanted in mice. The 8 weeks of implantation allowed the deposition of a newly formed cortical bone holding a marrow hematopoietic content very similar to that found on the mice natural BM. A distinctive aspect of this work is the cellular organization of $CD31^+$ vascular endothelial, the perivascular $nestin^+$ and $LepR^+$ cells in the construct in a fashion that resembled its localization within mice natural hematopoietic niches. This engineered BM showed ability to effectively maintain the hematopoietic system entirely functional when cultured *in vitro* in the microfluidic device, preserving HSCs self-renewing and multipotency, even without exogenous cytokine supplementation on the perfused culture medium. Subsequently, BMoC constructs have been developed through *in vitro* cultivation of bone scaffolds seeded with human-derived (h) co-cultured cells in microfluidic devices. A hydroxyapatite-coated zirconium oxide scaffold seeded with hBM-MSCs and hUCB-HPCs has also showed a structural and molecular microenvironment very similar to the native BM[109]. After four weeks of culture, such biomimetic BM retained not only HSCs in their primitive phenotype as well as HPCs with their multi-lineage differentiation potential, capable of granulocyte, erythrocyte, macrophage, and megakaryocyte colony formation.

The most recent bioengineered BM strategy combines an additional vascular channel composed of human umbilical vein endothelial cells (HUVECs). The endothelial channel served to feed the 3D co-culture of $hCD34^+$ cells and hBM-MSCs through perfusion[107]. Although the cells were not in direct contact, the authors did not explore the paracrine stimulation between endothelial and the co-cultured cells.

In recent years, a biomimetic perfusion bioreactor containing a porous hydroxyapatite scaffold functionalized with hUCB-HSCs and hBM-MSCs was purposed as an advantageous BM niche comparing to microfluidic systems[31]. The novelty of this system consists in the creation of a functional compartmentalization comprising a stromal ECM and a liquid-phase supernatant. Fundamentally,while HSCs, MPPs, and CMPs were exclusively found confined to the stroma, committed cells showed more equal distribution between the stroma and the supernatant.. Moreover, the hematopoietic cells and the BM-MSCs in the stroma were found to establish physical contacts within an organized ECM. The production of the inflammatory IL-6 and IL-8 by BM-MSCs increased substantially compared to the levels obtained before the addition of the HSCs. The number of HSCs and MPPs have not changed, although a significant increase in the number of committed progenitors were found. Consequently, IL-6 and IL-8 may have influenced the proliferation of the committed populations. Inflammation is a key regulator of bone repair[110] and it is known that HSCs undergo distinct cell fate choices under inflammatory-stimulation[64]. However, the direct effect of inflammatory cytokines on the biology and regulation of HSCs is not entirely clarified yet. Therefore, investigation on how inflammation regulates HSC fate and function both in normal and in bone fracture conditions is of utmost importance in the development of new bone regenerative strategies.

Altogether, these findings strongly suggest that the presence of HSCs potentiates the deposition of several ECM proteins, secretion of various growth factors, and cytokines with an improvement of *in vitro* vasculogenesis and osteogenesis processes. The hematopoietic beneficial properties evidenced by the bioengineered BM HSC-niches are very similar to the *in vivo* regenerative bone microenvironment. However, despite the remarkable advances in HSCs biomedical research in the last years, the high degree of complexity in the bone regenerative process leads to an endless possible combination of several cell-cell and cell-molecules, and thus multiple scenarios for the development of new HSC-related regenerative therapies still need to be explored. Other *in vitro* strategies can also be envisaged in the future to accommodate the complex ecosystem found in the niches, such as the use of advanced encapsulation systems[111].

5. Bone regenerative properties of HSCs in translational *in vivo* models

The potential of $CD34^+$ cells in bone regeneration is recognized in both animal and human *in vivo* models. HSCs are mainly transplanted/implanted monocultured or co-cultured with MSCs **(Table 2)**. The stromal support by the MSCs is believed to help the engraftment of HSCs at the host site, while the different cytokines and growth factors secreted by the co-cultured cells are recognized as key paracrine inducers of osteo-angiogenesis[46,112–114].

Xenotransplantation of human cells into mice is the approach foremost documented among the translational research *in vivo*. hPB-$CD34^+$ purified cells intravenously administered in nude rats with nonhealing femoral fractures showed stimulation of osteogenesis with adequate blood flow supply, providing an ideal local environment to fracture healing occur[66]. Nanofiber-expanded hUCB-$CD34^+$ cells administered via cardio-ventricular injection in an osteoporotic mice model showed that the $CD34^+$ cells home to the BM, improving bone deposition, mineral density, and micro-architecture[115]. Furthermore, the transplantation not only improved osteoblast functionality but also impaired differentiation and maturation of osteoclasts, reducing the *in vivo* osteoclast activity. These findings suggest a novel therapeutic potential of $CD34^+$ cells in reverting osteoporosis.

As mentioned, in co-culture systems with stromal cells, the bone regeneration outcomes are remarkably improved. The co-implantation of hBM-HSCs and hBM-MSCs seeded in 3D calcium phosphate (CP) scaffolds into immunocompromised mice revealed significant osteo-angiogenic improvements[16]. After four weeks of subcutaneous implantation, a vascular ingrowth was visible into the micropores of CP scaffolds and human osteocalcin expression increased in comparison to hBM-MSC implanted alone. When seeded on 3D Matrigel and supplemented with endothelial differentiation medium, the $CD34^+$ cells formed tubular intercellular structures. Additionally, when cultured on fibronectin-coated plates with endothelial differentiation medium, the $CD34^+$ cells formed attached colonies expressing positive immunofluorescent staining for acetylated low-density lipoproteins and von Willebrand factor, typical endothelial markers[75,116,117]. Of note, these results do not prove the

differentiation of HSC into endothelial cells, it rather evidences a $CD34^+$ endothelial differentiation potential, which could be due to the presence of EPCs. Orthotopic humanized bone scaffolds seeded with human osteoblasts and HUVECs implanted in the femur of immunocompromised mice and then BM transplanted with $hCD34^+$ cells originated an *in vivo* bone organ with all features of human bone[118]. Histomorphological analysis showed a new trabecular bone formation surrounded by an osseous cortex, human cellular and ECM components after 6 weeks of bone remodeling. Interestingly, after myeloablation treatment, the $hCD34^+$ transplanted cells were able to colonize the whole animal organism as well as differentiate into myeloid and lymphoid cells.

Allogenic implantation of hydroxyapatite cell-sheets of PB-HSCs co-cultured with BM-MSCs is also reported in calvarial critical-size defects in rabbits, showing a more efficient bone regeneration compared to only BM-MSCs (control). This reinforces the importance of the paracrine stimulators secreted by the co-cultured cells[27].

HSC implantation has also been performed in human models to assess its therapeutical potential in bone healing for mandible defects, and tibial or femoral nonunion fractures. Accordingly, patients with 6- to 8- continuity defects of the mandible received an *in situ* tissue-engineered graft with a combination of hBM-derived $CD34^+$ and osteoprogenitor cells, together with BMP-2 in an absorbable collagen sponge[72]. All patients that received 1012 ± 725 $CD34^+$ cells/mL achieved the primary endpoint of mature bone regeneration, whilst such outcome could only be observed in 40% of the patients that received 54 ± 38 $CD34^+$ cells/mL. The system combined with the most elevated concentration of HSCs was directly correlated with clinical regeneration of bone in craniomandibular reconstructions. The elevated regenerative potential of circulating $CD34^+$ cells associated with transplanted HSC, which uses these cells isolated from PB systemically transplanted with minimally invasive techniques, has become increasingly attractive. A phase 1/2 clinical trial performed systemic transplantation of autologous hPB-$CD34^+$ cells combined with conventional surgery in patients with tibial or femoral nonunion fractures[10]. Following transplantation, a favorable environment for fracture

healing via osteogenesis and angiogenesis/vasculogenesis was observed. Interestingly, radiological analysis of fracture healing after 1 year was achieved in 71.4% of the patients. These promising outcomes *in vivo* are of great value and encourage more studies with transplanted or implanted HSC alone or in combination with biomaterials and/or molecular signals to elucidate the efficacy of HSCs and their progeny for bone repair and healing.

6. Alternative HSCs source for bone tissue engineering

Although the emphasis here was given to HSCs and HSPCs isolated from BM, PB, and UC, it is worthy to mention that pluripotent stem cells (PSCs) are an attractive cells source for tissue engineering (TE). Embryonic stem cells (ESCs) require very strict and time-consuming culture conditions and encompass an associated risk of cell tumorigenicity. Moreover, the use of hESCs is still debatable due to several ethical issues. Otherwise, induced iPSCs are a promising alternative source for cell-based therapies lacking ethical concerns. However, derivation of efficient cells from iPSCs with successful *in vivo* engraftment and resembling the functional properties of its native counterpart cells is still a challenge[119,120]. To the best of our knowledge, the use of ESC/iPSC-derived HSCs in the scope of bone TE strategies is still poorly reported. Given the difficulty to produce fully functional and engraftable HSCs, special attention is given to generate MSCs or monocyte/macrophages lineages (HSCs-derived cells) that in turn differentiate into osteoblasts and osteoclasts, respectively[121–123]. For example, an engineered scaffold co-cultured with hiPSC-MSCs and hiPSC-macrophages was able to support an accelerated bone formation characterized by a coordinated activity between osteoblasts and osteoclasts, both *in vitro* and *in vivo*[124].

In the same line, recent works have reported the use of iPSC-derived MSCs with satisfactory osteoblastic differentiation, secretion of bone proteins (e.g., BMPs and osteocalcin), and *in vitro* bone formation[123,125–127]. Moreover, a study reported a successful new bone formation after the transplantation of iPSC-derived MSCs into mini-pigs and no significant differences in the animals transplanted with autologous BM-MSCs was observed. Surprisingly, although animals were not prior subject to immunosuppression, no inflammatory reactions were observed, which

discloses the immunosuppressor properties of the iPSC-MSCs[127]. This property has also been reported in iPSC-MSCs cocultured with HSCs, where iPSC-MSCs showed to support HSCs proliferation and suppressed inflammatory reaction[128]. The use of iPSC-MSCs as stromal support for HSCs culture *in vitro* is also reported in the literature. However, in comparison to BM-MSCs, the iPSC-MSCs have shown a lower performance[129].

As a promising alternative for new HSC-related bone regenerative therapies, it would be interesting to explore the use of iPSC-derived HSCs in the scope of bone TE. Albeit the reprogramming process and culture conditions of these cells still require harsh developments, the engineering of biomimetic BM models combining iPSC-derived HSCs/MPPs and HSCs-derived cells is an open window for the research in the regenerative medicine field.

Conclusions

HSCs/HPCs isolated from BM, PB, and UCB have shown increasingly evidences to facilitate the bone regeneration, repair, and healing. Their capacity to promote a favorable bone regenerative microenvironment is associated with other cells and molecular component interactions, including stromal support, ECM proteins and paracrine communications. Considering that the exact mechanisms behind the hematopoietic contribution for bone repair is sparse and not clear yet, new advanced hematopoietic models which enhance and accelerate fracture repair and healing process are of significant clinical importance. Several *in vitro* approaches have been developed to mimic the BM niches. The reconstruction of such environments with several cell and component combinations associated with biomaterials and TE approaches enables the understanding of the complex HSC biology and regulation both in physiologic conditions and bone disease. Furthermore, given the importance of osteogenesis, vascularization, and inflammation in the bone regenerative process, additional studies are required to further characterize the specific role of hematopoietic cells, their progenitors as well as myeloid and lymphoid lineage cells during the different phases of the bone fracture regeneration. This knowledge could provide strategies for the development of new HSC-related bone regenerative therapies. Ultimately, the HSCs therapeutic potential demonstrated

in the translational *in vivo* models strongly encourages more *in vitro* and *in vivo* research focus on the application of these cells in the context of bone regeneration.

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Author Contributions

C.S.O. designed, collected data, wrote, and produced the images and tables of the manuscript. M.C. designed, collected data, wrote, and produced the images and figures of the manuscript. C.R.C. supervised all the work, and contributed to the conception, design, and revision of the manuscript. J.F.M. contributed to the conception, design, and revision of the manuscript. All authors read and approved the final version of the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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