Cytokine & Growth Factor Reviews xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Cytokine & Growth Factor Reviews

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

Mini review

The unbearable lightness of bone marrow homeostasis

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ARTICLE INFO

Article history:
Available online xxx

Keywords:
Bone marrow
Bone remodelling
Bone physiology
Mesenchymal stem cells
Hematopoietic stem cells

ABSTRACT

The anatomical and functional dimensions of bone marrow topography have been at the forefront of modern bone and immunological research for many years and remain a source of complexity and perplexity due to the multitude of microhabitats within this microenvironment. In fact, research has uncovered fascinating functional aspects of bone marrow residents, and the bone marrow niche has been identified as the foremost reservoir of a variety of cells including hematopoietic, skeletal and endothelial stem/progenitor cells. The physical interactions of the marrow residents, combined with the release of cytokines and growth factors, organize well-defined operative compartments, which preserve bone and immune cell homeostasis. In a simplistic view, both the hematopoietic and bone marrow stromal (mesenchymal) stem/progenitor cell populations dwell at the interface between the endosteum and the bone marrow area (endosteal niche) and in the perivascular space (vascular niche). Indeed, the tantalizing hypothesis of bone marrow regulatory dependency on these niches is supported by current research insofar as the increase in the number of osteoblasts results in a concomitant increase in the hematopoietic population, indicating that the osteoblasts and the endosteal niche are key components of HSC maintenance. On the other hand, impaired function of the vascular niche compromises the endosteal niche's ability to support hematopoiesis. These fascinating discoveries indicate that there are strong ties between bone marrow inhabitants within the confines of the bone marrow itself. When these ties fail, niche-niche communication suffers and results in reduced bone formation, enfeebled hematopoiesis and unrestrained HSC migration through blood circulation. This study focused on the extraordinary homeostatic equilibrium and function of both bone and immune cells within the spatially defined microenvironment of bone marrow. But how important is the anatomically outlined scenery in which the bone marrow entity supports and hosts the hematopoietic elements?

1. Introduction

The vertebrae, hips, ribs, skull and long bone cavities host a remarkable multicellular network in terms of complexity, integrity, functionality and dependency: bone marrow. The bone marrow microenvironment is committed to accommodating the hematopoietic and the bone stem/progenitor cells in a symbiotic, multifaceted setting. The hematopoietic stem cell (HSCs) population is a heterogeneous cell assortment including long-term (LT) and short-term (ST) components [1]. Under normal conditions the majority of the HSCs within the bone marrow are dormant or slowly cycling. According to Cheshier et al. [2] 75% of LT self-renewing HSCs remain in the G0 state. This state maintains HSC homeostasis through the control of self-renewal, proliferation and differentiation of the HSCs and progenitors [3]. The HSCs give rise to lymphoid progenitors, which differentiate to become immune cells and myeloid progenitors, which mature into osteoclasts, macrophages, neutrophils, basophils, eosinophils, megakaryocytes, erythrocytes and dendritic cells [4].

The bone marrow stromal microenvironment encompasses multiple cell types including bone-lining osteoblasts, endothelial cells, reticular adventitial cells, neuronal and muscle stem cells and mesenchymal stem and progenitors components. The bone marrow-derived mesenchymal stem cells (MSCs) consist of a self-renewing marrow population with multilineage potential, physically present in concomitance with the HSCs. The MSCs have recently been portrayed as perivascular cells with well-defined roles in microvessel wall formations and in the HSC architectural

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http://dx.doi.org/10.1016/j.cytogfr.2014.12.004
1359-6101/

niche framework [reviewed in 5]. The MSCs possess a plasticity grade and, although they are not a pluripotent cell population (their stemness properties are still an open debate, which tip the balance in favor of a peculiar non stem/progenitor cell behavior), can provide bone marrow functional cells, including osteoblasts, chondroblasts, fibroblasts, adipocytes and endothelial cells [4,6]. The far-reaching MSC features are responsible for the assembly and organization of the hematopoietic, skeletal and perivascular niches within the marrow cavity, and, thus, MSCs have been designated as the preeminent niche manufacturers. Schofield's pioneer study [7] gave prominence to the niche concept and spurred interest in stem cell research and its spatial dimension, giving rise to fascinating, as well as, in some cases, contradictory standpoints. The most important result of this prior research is the structural and functional singularity of the bone marrow niches and the value of each niche. From this point of view, both MSC and HSC niches share distinct anatomical districts with significant functional differences and complexities. This scenario involves the mature lymphoid components, the endosteal cells (including osteocytes, osteoblasts, osteoclasts and macrophages) and the sinusoids, which are demarcated by endothelial cells and adventitial reticular cells (Fig. 1). In an elegant study, Sacchetti et al. [8] use the CD146 marker, a cell adhesion molecule of the immunoglobulin superfamily, to distinguish MSCs from the other osteogenic and non-osteogenic progenitors. The CD146^{+} stromal cells, on the one hand, generate osteoblasts, which prepare the endosteal niche, and, on the other hand, differentiate into sinusoidal adventitial reticular cells, giving rise to the sinusoidal wall structure and organization. Both the endosteal and sinusoidal regions define strategic bone marrow areas, which contribute to HSC homing and maintenance [8,9]. The niche milieu presents two types of interaction: 1) an adhesive interaction between stem/mature cells within the niches [10]; 2) indirect interactions through cytokines/chemokines and other molecular mediators [reviewed in 11,12]. The interchanges between these mediators/cell-cell interactions guarantee stem cell activities within the niches and maintain bone marrow homeostasis. Accordingly, the bone-lining osteoblasts synthesize a vast number of cytokines, which can expand the number of LT-HSCs approximately 2–4-fold and support the hematopoietic stem/progenitor lineage [13,14]. Moreover, an increase in the osteoblast population reflects an increase in LT-HSCs [14] and, correspondingly, osteoblasts cotransplanted with HSCs considerably improve engraftment. As indicated by El-Badry et al. [15], the bone progenitor cells or the osteoblasts may be crucial components of the stromal cell population and facilitate engraftment of marrow stem cells in an allogeneic environment. Intriguing studies prove that not only can HSCs differentiate into MSCs but myeloid cells can also develop

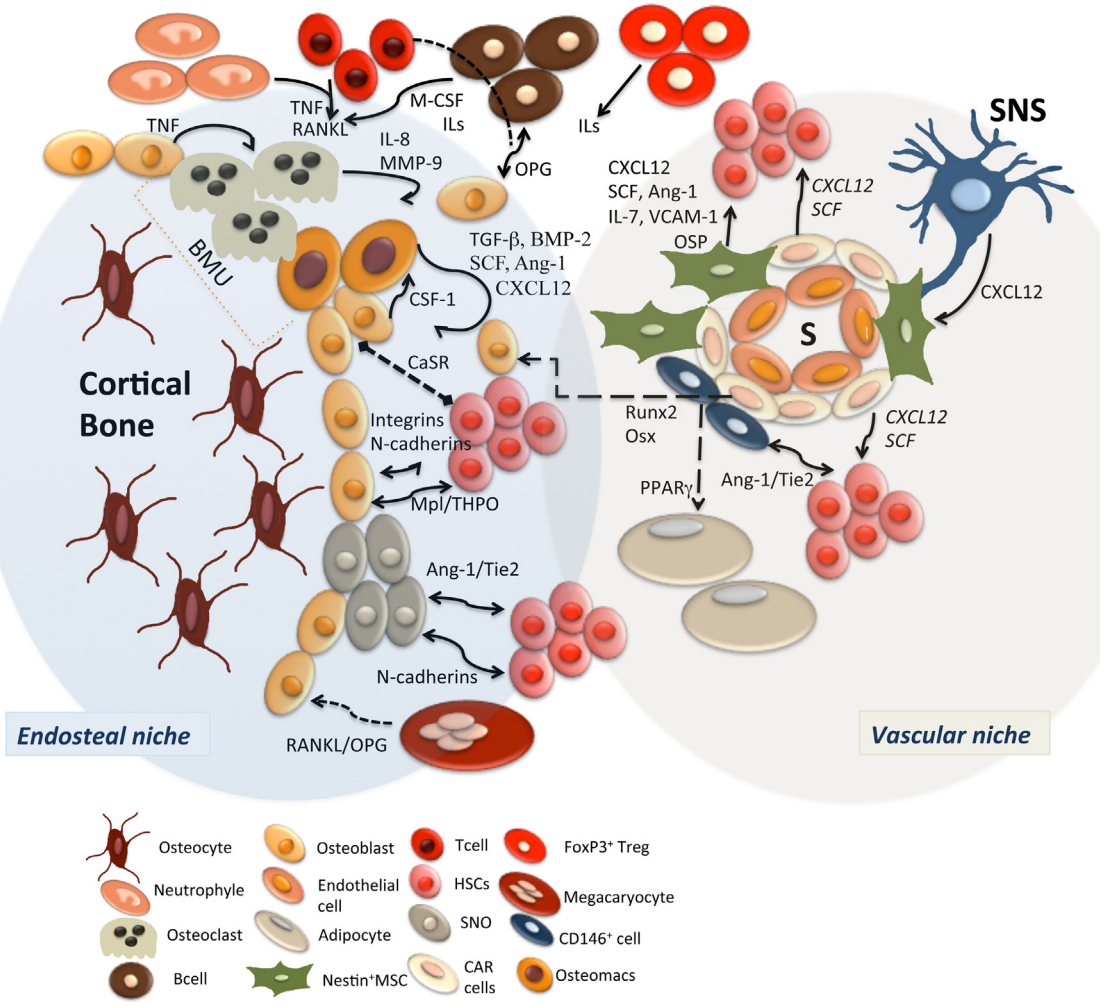

Fig. 1. Major interactions between the endosteal and the vascular niche. The hematopoietic stem cells and the mature immune cells exchange a wide number of molecular mediators with the mesenchymal stem cells and the bone elements sustaining a physiological stand within the bone marrow. The niche dimension and the inhabitant cell types are characterized by elective affinities with the skeletal and immune system that permit a homeostatic regulation into and beyond the bone marrow topography. The disruption of these niche liaisons drives to a pathological tableau including inflammatory bone diseases, hematopoietic defects and systemic disorders. S: sinusoid; SNS: sympathetic nervous system.

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a mesenchymal capability [reviewed in 16], which fundamentally alters the landscape of how we understand the bone marrow cavity. From this point onward, the conception of how the niche microenvironment manipulates the bone marrow dimension has been at the forefront of innovative research on MSC related therapies, HSC engraftment techniques and autoimmune disease treatments.

2. Does bone marrow ontogeny encompass more than one functional niche?

Over the last decade research has identified a number of interactions within the bone marrow and their protagonists, fueling the investigation of the spatial relations between these homeostatic components. The anatomically defined areas that bring into play the MSC and the HSC metabolites have not always been considered anatomically and functionally interdependent. Although the endosteum can even be at a 5-cell distance from the sinusoids [17], these two anatomical compartments have been considered either as distinct niches with exclusive functions or subcompartments of a single niche. In this view, Méndez-Ferrer et al. [18] have denoted that within bone marrow there is a unique niche shaped of heterotypic stem-cell pairs, although several sets of evidence support the existence of a multi-niche tableau. In addition, HSCs reside at the endosteal surfaces, which provide signals for their homing and regeneration (endosteal niche). Thus, a body of evidence suggests that the trabecular bone area is the primary site for HSC homing [14]. A paradigm of the multi-niche concept originates from the fact that the HSCs were also found close to the walls of sinusoids, the blood vessels of the bone marrow cavity (vascular niches) [19–21] (Fig. 2A, B). Kiel et al. [17,22] propose that a minority of HSCs localize near the endosteal surface (<20%) and a majority of HSCs (>60%) are confined to the sinusoid. The same authors have also suggested that HSCs cannot localize to the endosteum without being perivascular, and potentially influenced by factors secreted by endosteal cells [23]. Likewise, it has been shown that the endosteal niche maintains HSC quiescence [14,24,25] whereas the vascular niche supports stem/progenitor cell homeostasis and regulates megakaryopoiesis [26]. The physiological significance of these findings delineate a complex microarchitecture in bone marrow. The distinct anatomical compartments include the candidate niches, which act as coordinators of the bone marrow microenvironment.

2.1. Spindle-shaped N-cadherin^{+}CD45^{-} osteoblastic cells

In point of fact, specialized cell-residents have been identified with a niche-related function. A population of osteoblasts adjacent to the bone surface, called spindle-shaped N-cadherin^{+}CD45^{-} osteoblastic (SNO) cells, coalesce with the bone-lining osteoblasts to provide the HSCs a niche within the bone marrow. Zhang et al. [14] show that the SNO cells enrich both the trabecular bone surface and the endosteal surface of the long bones. SNO cells support HSCs (in particular by maintaining the LT-HSCs quiescent/slow cycling status) by virtue of N-cadherin and β-catenin cell–cell interactions. It has also been suggested that the increased number of SNO cells points to an increased number of HSCs, and therefore SNO cells have been identified as key components of the niche [14]. However, contradictory studies affirm that N-cadherin interactions do not have a fundamental role in HSC maintenance; HSCs express low levels of N-cadherin and the lack of N-cadherin in knockout mice does not influence the ability of HSCs to sustain either hematopoiesis over time or bone marrow cellularity [23,27]. Considering the data reviewed above it remains questionable whether HSC retention, at the endosteum level, by an N-cadherin-independent mechanism depends on direct contact with osteoblasts or on indirect interactions between various bone marrow cell populations. To address this issue, Arai and Suda [28] pointed to the fact that, since HSCs adjacent to the osteoblastic niche have greater N-cadherin levels compared to those situated in the vascular niche [14], the various N-cadherin expression levels exert a watchdog role on HSC localization among the niches.

2.2. Thrombopoietin/Mpl interactions

Quiescence of LT-HSCs is a crucial condition that preserves and protects the stem cell pool whenever it occurs [29]. LT-HSCs express the thrombopoietin (THPO) receptor, called Mpl, and the bone lining cells at the endosteum level produce THPO. THPO was initially acknowledged as a key regulator of megakaryocyte and platelet metabolism [30]. Further earlier studies, using Thpo−/− and Mpl−/− mice, showed impaired HSC maintenance and cell cycle kinetics [31–33]. Later, the THPO/Mpl signalling interaction was proposed as an important pathway for the maintenance of LT-HSCs within bone marrow [25,34]. THPO/Mpl interaction modulates HSC cycle progression and preserves LT-HSCs quiescence through regulation of cell-cycle mediators such as $p57^{kip2}$, $p19^{Ink4d}$, Myc and various Homebox transcription factors involved in HSC self-renewal [25,33]. Moreover, THPO induces the expression of cell adhesion molecules, such as β1-integrin, supporting the thesis of direct niche-niche interactions [25]. Although impaired Myc function induces overexpression of integrins and N-cadherins in HSCs, resulting in a reduced ability to proliferate and uncontrollable cell adhesion, a THPO/Mpl-induced Myc decrease induces the regulation of cell adhesion molecules and HSC anchoring [35]. Keeping in mind that β1-integrin plays a critical role in the colonization of hematopoietic organs by HSCs during embryogenesis and postnatal bone marrow hematopoiesis [36], the functional dimension of the THPO/Mpl signalling becomes explicable. Insofar as Mpl^{+} HSCs are localized in the endosteal area adjacent to the THPO-producing osteoblasts, these observations clearly indicate the involvement of the endosteum cells in HSC metabolism.

2.3. Tunica endothelial cell kinase 2 receptor/angiopoietin-1 signalling

Supporting the concept that the endosteal niche orchestrates bone marrow homeostasis, Arai et al. [24] have demonstrated the importance of tunica endothelial cell kinase 2 receptor (Tie2)/angiopoietin-1 (Ang-1) signalling in HSC homeostasis and its critical role in the self-renewal ability of HSCs. Ang-1 is well known as the key component of angiogenesis and contributes to the remodelling of the slow flow sinusoids within the bone marrow [37]. It is also accepted that Tie2 is expressed on endothelial cells as well as on HSCs [38,39] and its ligand Ang-1 is produced by CD146^{+} osteoprogenitors [8] and by osteoblasts [40]. Tie2^{+} HSCs are quiescent, exert anti-apoptotic activities and are localized adherent to the bone-lining osteoblasts. The Tie2/Ang-1 binding supports tight adhesion of HSCs to the niche through a N-cadherin/β1-integrin-dependent mechanism, which results in HSC maintenance and survival [24]. As previously reported regarding THPO/Mpl signalling, Tie2/Ang-1 interaction facilitates HSC adhesion to the niche and provides additional support for the importance of HSC-bone cell communication at the endosteal level. Another study [8] shows that the endosteal and perivascular niches are functionally interdependent. Specifically, *via* Ang-1 production, CD146^{+} sub-endothelial cells and CD146^{+} stromal cells regulate the vascular remodelling and the organization of the vascular structure, which provide molecular and mechanical support for HSC homing [8].

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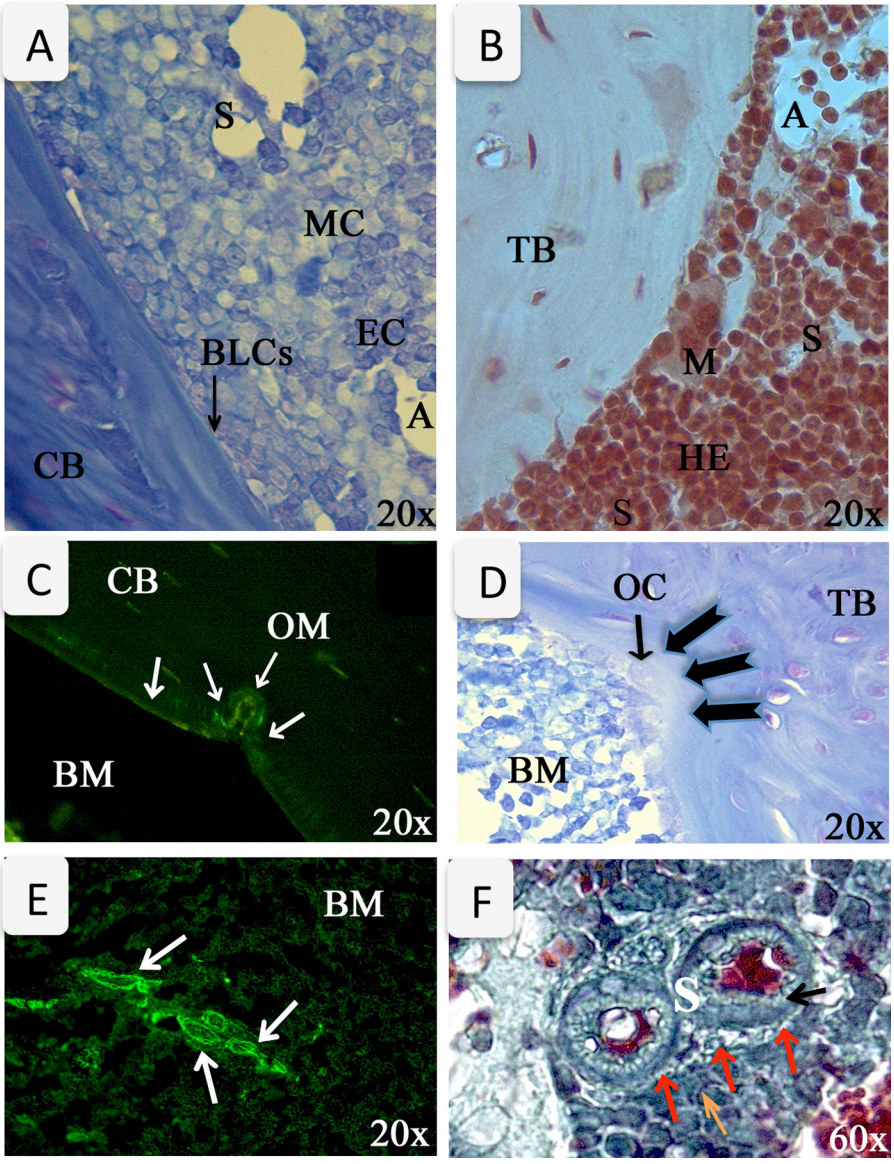

Fig. 2. Representative images of cortical and trabecular bone and bone marrow areas in mice. (A) Cortical bone and bone marrow stained with toluidine blue. Note the bone lining cells adjacent to the endosteum (black arrow) surrounded by the hematopoietic cell population. Erythroid elements present small round dense nuclei; granulocytes are characterized from larger bean-shaped nuclei; megakaryocytes possess multilobulated nuclei (B) Trabecular bone and bone marrow stained with Congo red. Note the sinusoids (perivascular niche) close to the trabecular endosteum. (C) OsteoMacs immunofluorescence using the F4/80 antibody. The OsteoMacs are localized at the endosteum and the BMU area of the cortical bone. (D) Trabecular bone and bone marrow stained with toluidine blue. Note the BMU area with the so-called "howship's lacuna" formation (bigger black arrows) and the osteoclast (smaller black arrow) action within this compartment during the bone remodelling process. (E) Mesenchymal perivascular cells immunofluorescence using the α-Sma specific antibody. Note the close proximity of the labeled cells with the sinusoids and the hematopoietic elements (white arrows). (F) Sinusoid and perivascular area stained with toluidine blue. Note the mesenchymal stromal cells (red arrows) confined with the endothelial cells (black arrow) and the hematopoietic population (orange arrow) organizing the vascular niche.
CB: cortical bone; TB: trabecular bone; EC: erythroblastic cells, MC: myeloid cells, BLCs: bone lining cells; S: sinusoids; A: adipocytes; HE: hematopoietic elements; M: megakaryocyte; OC: osteoclast; OM: osteomacs; BM: bone marrow. Slides were imaged using a Zeiss Axioplan fluorescence microscope.

2.4. OsteoMacs

Discoveries surrounding macrophage functionality within the bone marrow have attributed different roles to them based on their spatial localization. Macrophages resident at the bone marrow stroma are often interrelated with erythroid precursors to support erythropoiesis [41]. Macrophages situated at the periosteal and endosteal surfaces, called OsteoMacs, provide bone-forming signals during bone apposition [42,43] (Fig. 2C). In line with the above, macrophages express a variety of osteoinductive factors, including transforming growth factor-β (TGF-β), bone morphogenetic protein (BMP)-2 and 1,25-dihydroxyvitamin D_3 [44–46]; conversely, osteoblasts sustain macrophage growth and proliferation through colony-stimulating factor-1 (CSF-1) production [47]. Depletion of macrophages *in vivo* indicates a complete loss of endosteal OsteoMacs, loss of neighboring osteoblasts, decrease in osteoblast-produced cytokines including Ang-1, stem cell factor (SCF; also known as kit-ligand) and chemokine CXC-ligand 12 (CXCL12) and induces HSC mobilization [48]. The conclusions regarding the critical role of this immune component in bone physiology and hematopoietic stem cells metabolism reveal a codependence scenario between OsteoMacs and osteoblasts.

2.5. Chemokine CXC-ligand 12 and CXCL12-abundant reticular cells

Recently, Ding and Morrison [49] suggested that HSCs reside adjacent to the perivascular niche whereas early lymphoid

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progenitors inhabit the endosteal niche. Data concerning the significance of the perivascular region within the bone marrow indicate that HSCs localize near reticular cells that express high levels of CXCL12 [9,18,50]. CXCL12 is expressed by perivascular stromal cells, as well as by endothelial cells and osteoblasts [50,51]. The binding of CXCL12 to its physiological receptor CXCR4 provides signals for HSC maintenance and homing, as well as for B cell, plasmacytoid dendritic cell and natural killer (NK) cell maturation [50,52–54]. The CXCL12-abundant reticular (CAR) cells, which are mesenchymal progenitors, have the capacity to differentiate into osteoblasts and adipocytes, to participate in the osteogenic process at the endosteum level and to organize the hematopoietic microenvironment [8,12,55]. Based on their anatomical location, CAR cells orchestrate HSCs, lymphoid and erythroid progenitor cell metabolism *via* cytokines production, such as the SCF and the CXCL12, which are essential for hematopoietic niche maintenance and retention in the bone marrow. CAR cell ablation reduces the number of HSCs by about 50% in the marrow and increase splenic HSCs. For this reason, CAR cells have been characterized as one of the major niche-signal providers involved in HSC and hematopoietic progenitor homeostasis [55]. An interesting approach from Omatsu et al. [55] suggests that CAR cells exert a regulatory activity on HSCs. CAR population can maintain HSCs in an undifferentiated state or can inhibit the myeloid differentiation of HSCs. Furthermore, the same authors argue that CAR cells could produce adipogenic and osteogenic markers such as PPARγ, RUNX2 and Osterix enhancing MSC differentiation. It is also mentioned that CAR cells and/or osteoblasts regulate monocyte migration from the bone marrow into the circulation during an inflammatory stimulus [56]. This observation is consistent with results following the conditional deletion of *Cxcl12* in osteoprogenitors that lead to HSC migration toward the blood and spleen [55] and influence the chemotaxis of these hematopoietic elements.

2.6. Calcium-sensing receptor

Chemotaxis of selective bone marrow populations is controlled by ionic calcium through the calcium-sensing receptor (CaSR), which is a G-protein-coupled receptor expressed in osteoblasts but also in hematopoietic cells. Variations in calcium ion concentration play specific roles in bone modelling and remodelling, whereas calcium reaches levels of 40 mM near active osteoclasts [57]. *In vitro* findings have shown that local calcium concentration regulates cell morphology through cell–cell or cell–matrix interactions, leading to an augmented Ang-1 and connexin43 expression in osteoblasts [58]. Adams et al. [59] show that HSCs preferentially localize near cells releasing extracellular calcium and the CaSRs upholding the HSCs in close juxtaposition to the endosteal surface and, consequently, to the regulatory niche-maintaining factors. The influence of CaSR on HSC homeostasis is also evident in CaSR knockout mice, which are characterized by hypercalcaemia, bone marrow hypocellularity, absence of paratrabecular hematopoietic clusters and engraftment defects [59]. Indeed, on the one hand, CaSR−/− mice exhibit reduced ability to bind the extracellular matrix molecules expressed from endosteal cells, such as collagen I [59], and, on the other hand, calcium/CaSR signalling promotes CXCR4/CXCL12 expression and, thus, HSC homing and retention [60]. These data identify a molecular network that exceeds the regulatory microenvironment of the single niche and unifies the endosteal and perivascular elements within a unique homeostatic scenario. The calcium ion presence at the endosteum coupled with the ability of CaSRs, CAR cells and osteomacs to signal for HSCs adhesion and homing strongly support this view.

2.7. Stem cell factor and matrix metalloproteinases

Research in the field has provided additional elements to support the hypothesis that the perivascular stromal cells and endothelial cells are two key functional components for the maintenance of HSCs through SCF production. The SCF, expressed by bone marrow fibroblast, osteoblasts, *Cxcl12*-expressing perivascular stromal cells, endothelial cells and *Nestin*-expressing mesenchymal stem cells [18,55,61], has been proposed as an important mediator for the preservation of HSC dynamics [62]. In accordance, Heissing et al. [63] have suggested that matrix metalloproteinase-9 (MMP-9) exerts a fundamental role on SCF release, and, consequently, SCF production facilitates bone marrow recovery under myelosuppression or stress conditions through accelerating hematopoietic reconstruction. Early studies on matrix metalloproteinases revealed the implication of MMP-9 in cytokine production, which triggers angiogenesis and osteoclast recruitment [64–66]. Bone marrow hematopoietic cells and MSCs express MMP-9 and, noteworthy, in MMP-9−/− mice a severe drop in hematopoietic clusters at the endosteal and vascular areas has been observed. In addition, exogenous SCF administration in MMP-9−/− animals restores bone marrow hypocellularity, accelerates stem cell differentiation and hematopoietic reconstitution after bone marrow ablation [63]. These considerations underscore the physiological significance of MMP-9-induced release of SCF to support hematopoiesis in a steady-state scenario or after myelosuppression. The evidence that cytokines, such as CXCL12, vascular endothelial growth factor (VEGF) and granulocytes colony stimulating factor (G-CSF), enhance MMP-9 production [63], provides additional criteria for defining the niche functions.

2.8. Nestin-expressing MSCs

Nestin is an intermediate filament protein expressed in various progenitor cells and endothelial cells [67]. Nestin-expressing MSCs are a special bone marrow cell population that can differentiate into mesenchymal lineages contributing to skeletal remodelling. These cells present a perivascular distribution adjacent to the bone and neighboring with HSCs and adrenergic nerve fibers. Indeed Nestin$^+$ cells show a close physical association with HSCs and express genes that regulate the hematopoietic progenitor niche such as *Cxcl12*, c-kit ligand, angiopoietin-1, interleukin 7, vascular cell adhesion molecule-1 and osteopontin. Likewise, it has been established that HSCs rapidly home near Nestin$^+$ cells and the depletion of these cells results in a reduction of HSCs within the bone marrow [18].

In conjunction with Nestin$^+$ cell properties, some studies have shown the critical role of the sympathetic nervous system in bone marrow stem cell homeostasis. Nestin$^+$ MSCs express the β3-adrenergic receptor and play an indirect role in sympathetic nerve regulation of HSC mobility [18]. In fact, β-adrenergic receptors signal through the sinusoidal osteoprogenitor cells and influence HSC egression from the bone marrow *via* Cxcl12 suppression [68]. Another approach by Yamazaki et al. [69] has upheld that nonmyelinating Schwann cells (glial cells) laying in proximity to blood vessels in the marrow not only express stem cell niche genes but also regulate the TGF-β/Smad signal, which is characterized as one of the critical niche signals involved in HSC homeostasis. The discoveries surrounding Nestin$^+$ MSCs and glial cell-functions dictate a coordinated and balanced regulation of marrow inhabitants, which act in a controlled spatial manner to finally preserve HSC maintenance and MSC homeostasis.

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3. Niche *vs.* niche: an "unbearable" hypothesis or a peculiar niche regulation?

It is well documented that MSCs, as well as HSCs, inhabit the endosteal and central regions of the bone marrow, with singular proliferative and immunomodulatory activities [70]. MSCs, localized at the sinusoids, have been portrayed as perisinusoidal stromal cells and attributed with specific functional roles within the bone marrow [8,71]. Explicit structural sections provide regulatory signals to maintain the stem cell pool and the niche size. Several studies have revealed that the various niches dwell in two distinct anatomical compartments: the sinusoidal and the endosteal compartments [21,72,73]. Analogously, skeletal and hematopoietic progenitors, which allocate at the same microenvironment within the long bones, are in competition for the marrow space, and further, the osteolineage cells orchestrate a variety of stimuli and interactions that control the niche dimensions [61,14,74,21]. For instance, bone calcium ions, which accumulate at the endosteum, influence the HSC niche expansion *via* calcium sensing receptors expressed by the hematopoietic progenitor cells [59]. In addition, the fact that osteopontin, a sialoprotein produced by osteoblasts, interacts with HSCs, enhances their migration toward the endosteal district and limits the number of stem cells [75–77], strongly supports the above niche-dimension hypothesis. The spatial and temporal relationships between the niches clearly illustrate the composite regulatory network of bone marrow. Thus, the bone residents (including HSCs, MSCs and the mature bone and hematopoietic cells) exchange molecular mediators, which sustain their homing, self-renewal, proliferation, differentiation and shape the niche confines. This niche dualism and competition can also shift toward a basal or a pathological bone metabolism through the control of the inflammatory signals and the bone remodelling process.

4. Bone remodelling under bone marrow components ascendancy

Physiological bone remodelling is the result of the coordinated efforts of bone-forming cells, the osteoblasts, and the bone-resorbing cells, the osteoclasts [4]. As mentioned, OsteoMacs have specific functions in bone deposition and mineralization and thus are defined as the third player in bone remodelling [78]. Since the remodelling process occurs at the trabecular and cortical surfaces of the bone, a substantial overlap of molecules and regulatory mechanisms, shared between bone and immune cells, emerges. The receptor activator of nuclear factor-kappa B (RANK) ligand (RANKL), a Tumor Necrosis Factor (TNF) superfamily member that is expressed by osteoblasts, plays a key role in osteoclastogenesis. The RANKL binding to its RANK receptor at the surface of osteoclast precursors prompts receptor trimerization and the activation of signalling cascades which drives osteoclast formation and migration at distinct areas called basic multicellular units (BMU). Within the BMU area, the osteoclasts begin resorption of the old or damaged bone [79]. Following the resorptive phase, the osteoblasts recruited to the BMU secrete collagenous and non-collagenous proteins for extracellular matrix deposition and bone formation [80] (Fig. 2D).

The osteoclast activity is not only crucial for bone homeostasis but also extends to the maintenance and mobilization of immature hematopoietic progenitors. Notably, stem cell anchorage molecules, such as SCF and osteopontin, have lower expression after RANKL stimulation. Instead, in PTPε-knockout mice, characterized by defective osteoclast (and macrophage) functions, RANKL had impaired effects on HSC progenitor mobilization [81]. Mature osteoclasts also produce interleukin (IL)-8 and the proteolytic enzyme MMP-9, both factors involved in progenitor bone marrow cell mobilization [63,82]. In addition, pre-osteoclasts express CXCR4 that respond to CXCL12 and in turn increase MMP-9 production [83]. Taking into account that osteoclast precursors are attracted by CXCL12 signals [83], CXCR4 ablation results in compromised osteoclast recruitment within the BMU area and reduced bone-resorption and MMP-9 expression. Kollet et al. [81] propose an archetypal paradigm of the multiple actions of osteoclasts. Once osteoclasts reach the BMU area, MMP-9 (which are able to degrade all components of the ECM as the other MMPs family proteins) and cathepsin K (a type I collagen protease) exert a bone-resorbing action, and, furthermore, incite CXCL12 degradation and bone marrow progenitor cell mobilization. Thus, osteoclasts perform a dual function: degrading the endosteal niche components and enhancing egression of HSC progenitors to the circulation due to the disruption of their anchorage potential [81]. It is noteworthy how osteoclasts, both in undifferentiated and mature stages, coordinate bone marrow component kinetics, even though earlier studies identified the granulocyte colony-stimulating factor (G-CSF) as the principal stem cell mobilization factor [84]. On the contrary, it has been proposed that osteoclast-less or impaired osteoclast functioning does not necessarily prohibit HSC progenitor mobilization, and the osteoclast-induced HSC-egression role remains controversial and case specific [85]. Interesting speculation by Purton and Scadden [86] suggests that osteoclast-induced cell mobility results in a bone stroma-surveillance mechanism which guards against the overpopulation of the bone marrow area or/and fine tunes the apparatus that coordinate hematopoiesis. The clinical significance of the above findings is in line with the development of the transplantation methodologies based on mobilizing agent treatments.

The physical competitor of RANKL action is a member of the TNF receptor superfamily, called osteoprotegerin (OPG), which operates as a decoy receptor for RANKL. As an osteo-anabolic/anti-RANKL factor, OPG prevents osteoclast activation and controls bone loss [87–89]. In this context, transgenic and knockout animals have been employed to interpret the role of the RANKL/OPG axis. With reference to the *Opg* gene functions, it has been reported that *Opg* deficiency results in bone disorders characterized by decreased bone density and increased osteoclast activity due to an overwhelming RANKL function [4], while OPG overexpression results in severe osteopetrosis in mice [90] caused by the failure of osteoclast formation *via* complete RANKL inhibition. Polymorphisms in the *Opg* gene in humans are associated with an increased incidence of fractures due to a lower mineral density [4,91]. It is also noteworthy that disruption or functional mutations in *Rankl* or *Rank* result in autosomal recessive osteopetrosis both in mice [92–96] and humans [97,98]. In addition, 15-base pair tandem duplications in the RANK gene were observed in patients diagnosed with expansile skeletal hyperphosphatasia [99]. On the other hand, when RANKL overwhelms the effects of OPG, as occurs in RANKL transgenic mice [100] and in postmenopausal osteoporosis [101], the imbalance in bone remodelling results in bone resorption. As a consequence, the primary mechanism of osteoclast regulation and bone remodelling has been ascribed to the RANKL/OPG ratio.

Likewise, the Wnt signalling pathway plays an essential role in bone formation, and the osteo-anabolic effects of the canonical Wnt cascade culminates with β-catenin stabilization, nuclear translocation and gene transcription activation including c-myc and OPG [102]. It is well known that canonical Wnt signalling decreases CXCL12 bone marrow concentrations and facilitates osteoblasts commitment and differentiation with concurrent RANKL/OPG ratio regulation [103]. Thus, the coordinated action of CXCL12, RANKL and OPG, in part arranged by the Wnt secreted proteins, designate the bone remodelling process and the mobilization of the participants within the BMU areas. Still, the

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augmented OPG expression observed in CXCR4−/− mice reveals the relevance of CXCR4 in osteoclastogenesis and osteoclast recruitment for the initiation of bone resorption. Based on these findings, CXCL12/CXCR4 signalling in osteoblasts has been recently portrayed as playing a preeminent role regarding MSCs and osteoclast precursor pool size and the recruitment of osteoclasts and osteoblasts in the bone remodelling sites [104]. Indeed, either suppression of the osteoblast lineage or osteoblast-specific CXCR4 deletion accounts for deregulated bone marrow CXCL12 expression and thus impaired bone-remodelling modulation [104,105].

Antithetically, CXCL12 overexpression in the synovial space and the bone tissue of patients with rheumatoid arthritis (RA) aggravates bone disruptive episodes through osteoclasts recruited to the inflamed locus [106]. In point of fact, the key role of CXCL12 in bone and cartilage destruction in pathological scenarios such as osteoporosis, arthritis and metastatic bone cancers is under broad investigation (reviewed in 107).

The CXCL12 accomplishment in bone marrow physiology and bone remodelling progression has recently been correlated with a bioactive sphingolipid metabolite, the sphingosine-1-phosphate (S1P). S1P is also synthesized by osteoblast and osteoclasts and both cell types express its receptors, $S1P_1$ and $S1P_2$ [108]. The balance between CXCL12 in the bone marrow and the S1P in the circulation has been portrayed as one of the principal coordinators of stem cell mobilization. Although S1P concentrations are higher in the blood, bone marrow concentrations generate an operational plateau that implicates osteoblast and osteoclast motility, survival, differentiation and adhesion [reviewed in 109]. Simultaneously, the S1P/CXCL12 molecular interchanges tune the bone remodelling through chemotactic instructions and signalling cascade activation such as Rac $S1P_1$-induced and the Rho $S1P_2$-induced mechanisms [110]. On the other hand, S1P overabundance in synovial fluids, as previously described for the CXCL12, meets the pathological RA scenery and drives pro-inflammatory cytokines/chemokines production [111].

5. Mature hematopoietic cells coordinate bone marrow homeostasis

Accumulating evidence suggests that both mediators of the adaptive immune system, B and T cells, can carry out osteoinductive or osteodestructive features and preserve bone homeostasis. In point of fact, these mature hematopoietic cells produce 20% RANKL in basal conditions, whereas in an inflammatory context (RA or periodontal tissue diseases) the percentage rises to 50% for B cells and 90% for T cells [112,113]. In accordance thereto, Manabe et al. [114] report that B cells are a major source of endogenous RANKL in bone marrow, and B-lymphoid lineage cells, and in earlier developmental stages, may have the potential to differentiate into osteoclasts once treated with the osteoclastogenic M-CSF and soluble RANKL *in vitro*. M-CSF binds to its receptor c-Fms on the surface of osteoclast precursors and signals similarly to RANKL/RANK promoting osteoclast survival, activation and differentiation [115]. Furthermore, it is well established that T cells play a central role in the mechanism of ovariectomy (ovx)-induced bone loss. The estrogen deficiency induces T-cell tumor necrosis factor (TNF) expression and, in turn, the induction of osteoclastogenesis [116]. These findings were further sustained by the fact that ovx fails to induce catabolic effects in bone in T cell deficient nude mice [116–118]. It is also remarkable that the dendritic cells may influence RANKL production in T cells and augment bone loss within an inflammatory scenario [119,120]. Within a synergistic plateau, T cells may regulate the B cell-production of various molecules involved in bone turnover. B cells can enhance or overwhelm osteoclastogenesis when stimulated by Th2 or Th1 cytokines, respectively [121]. The anti-inflammatory cytokines produced by Th cells such as interferon (IFN)-α, IFN-β, IFN-γ, IL-4, IL-5, IL-10, IL-12, IL-13 and IL-18, as well as TGF-β and PTH, have been classified as RANKL signalling inhibitors [122,123]. Conversely, IL-1, IL-3, IL-6, IL-7, IL-11, IL-17, as well as prostaglandin (PG) E2 and TNF, have been characterized as pro-inflammatory moderators leading to pathological osteoclastogenesis *via* RANKL expression [12,124]. Moreover, several cytokines (*i.e.* TNF and IL-1) can act with RANKL within a combined scenario to directly aggravate bone resorption [125]. Another T-helper cell subset with an osteoclastogenic feature is Th17 which produces IL-17, and is involved in matrix metalloproteinases synthesis and bone matrix degradation [126]. Recent findings emphasize the fact that the anti-inflammatory $Foxp3^+$ regulatory T cells (Treg) could converse into Th17 cells in autoimmune arthritis. The Foxp3 expression furnishes the suppressive ability of the Treg cells. Komatsu et al. [127], considering the inflammatory microenvironment of arthritis, have observed that $CD25^{lo}Foxp3^+CD4^+$ T cells downregulate Foxp3 expression and undergo transdifferentiation into Th17 cells. Th17 express high levels of RANKL resulting in osteoclastogenic induction and enhanced inflammation. Treg plasticity is a considerable illustration of bone marrow component ductility under pathologic conditions such as in RA. Opposing views have posited that RANK/RANKL interaction plays a crucial role in peripheral $CD4^+CD25^+FoxP3^+$ Treg homeostasis, while RANKL-stimulated Treg expansion could convoy anti-inflammatory responses [128]. Although several findings identify T cells as one of the principal bone marrow regulators in basal or in pathological conditions, emerging research has highlighted the combined effort of the T and B cells within bone cavities. In addition to T-cells, OPG expression has also been detected in B-lymphocytes, which participate in B cell hematopoiesis and the maintenance of bone mass. Of interest, mature B cell OPG-secretion accounts for about 40% of the total OPG production composing a regulatory homeostatic network between B cells, MSCs and osteoblasts [reviewed in 129]. Li et al. [130] assert that under basal conditions B-cell RANKL secretion remains at low levels. The same authors state that activated T cells and CD40/CD40L interaction serve as regulatory stimuli in osteoclastogenesis stimulating B cell-OPG production within a physiological tableau. Indeed, CD40/CD40L knockout mice have been attributed an osteoporotic phenotype with a significant decrease in bone marrow OPG levels [131]. Not to mention that in pathological conditions B-cell derived OPG plays a watchdog role in osteoclast maturation and prevents bone destruction [130]. This provides a comprehensive picture of how the coadjuvancy of these two immune populations coordinates bone marrow components. The complexity of bone marrow homeostatic steadiness can be evaluated if we consider the fact that the marrow inhabitants of the hematopoietic lineage could exert pro- or anti-inflammatory actions based on the clinical milieu. Although activated T cells have the capacity to secrete osteoclastogenic cytokines, such as RANKL and TNF [132,133], it has also been asserted that they conjointly exert anti-osteoclastogenic effects through cytokines secretion such as IL-4 [133].

Neutrophil granulocytes are also implicated in RANKL production and osteoclastogenic activity in inflammatory circumstances [134]. Another protagonist in the bone marrow microenvironment is megacaryocyte, which has the potential to drive bone formation or resorption through RANKL or OPG production [135]. The discrepancies concerning the anabolic or catabolic effects of the immune cells on bone could be due to the different clinical conditions and the compromised niche-to-niche communication within the bone marrow. The impaired cross-talk between the bone marrow stromal and hematopoietic components results in pathologic conditions like chronic inflammatory diseases, such as RA [136,137] and chronic arthritis [138], whereas chronic immune

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activation disrupts the dynamic equilibrium of bone homeostasis [125].

6. Growth factors and hormone accomplishment within bone marrow

Bone marrow research has brought to the fore the functional relationship between the niches and the regulatory molecular network of cytokines, growth hormones and receptors. Modern developments in understanding the accomplishment of immune components in bone homeostasis have established that T cells play a critical role in regulating the bone-remodelling outcomes of PTH, an important calcium-phosphate modulator. Namely, T cells respond to intermittent PTH treatment and enhance osteoblast differentiation through a Wnt10b/Wnt osteoblastogenic related mechanism [139–141]. The PTH osteogenic activity and its consequential capacity to expand the bone niche space have also been correlated with the increase of the HSC pool and HSC maintenance. In point of fact, PTH-induced specific mediators on bone-lining cell and on HSC, such as N-cadherin, Wnt/β-catenin and Notch/Jagged1, promote LT-HSC engraftment toward the niche [61,142]. Mendez-Ferrer et al. [18] have associated HSC expansion after PTH administration with an increment in Nestin$^+$ MSCs, highlighting the quintessential role of the various bone marrow components, including the adventitial reticular cells and the sympathetic neuronal fibers, in HSCs and MSCs homeostasis. Parallel studies using genetically modified mice as regards the PTH receptor (PPR) established that PPR represents a crucial modulator of the bone marrow ontogeny and, therefore, of the fate of HSCs and MSCs [74]. Conversely, continuous PTH administration switches the metabolic scenario toward a catabolic stance. For instance, T cells mediate PTH-induced bone loss in hyperparathyroidism through the CD40L/CD40 signalling network [143]. Our previous research brought to light the relevance of PTH on osteoblast metabolism and the close liaison of fibroblast growth factor-2 (FGF-2) in this process [144]. The effects of FGF-2 on the skeletal and hematopoietic stem cells have been a subject of controversy. From one point of view, FGF-2 is acknowledged as an anabolic bone agent that enhances bone formation by stimulating MSC proliferation and differentiation in a stage-dependent manner [145,146]. More than that, FGF signalling regulates the hematopoietic developmental events during either early or late differentiation phase [147,148]. Recent findings by Zhao et al. [149] reveal that FGF receptor-1 (FGFR1)-mediated response supports HSC proliferation and facilitates HSC egression to the circulation, both unavoidable conditions for post-injury hematopoietic recovery. These observations have revealed the concurrence of the FGF pathway as part of the hematopoietic stress response and have given prominence to FGFR1 attendance, acting as a moderator of NF-kB, CXCL12/CXCR4, AKT and MAPK signalling within the bone marrow. According to a divergent standpoint, FGF-2 abrogates the hematopoietic activity and induces compensatory extramedullary hematopoiesis. These adverse FGF-2 effects occur at a molecular level, due to the disruption on the CXCL12/CXCR4 axis to Ang-1-decreased and osteopontin-increased expression, contingencies that result in a restricted stem cell pool and compromised MSC-supported HSC homing [8,150,151]. Keeping in mind that a high-level of seric FGF-2 incites anemia, osteomalacia [152], and clonal myeloid disorders correlated with severe hematopoietic defects [153], it is reasonable to deduce that the benefits of this growth factor on HSC and MSC homeostasis depend on intramedullary FGF-2 production levels and serum concentrations. To circumvent the deleterious effects of high doses of FGF-2, Meng et al. [151] have developed an erythroid-specific promoter of FGF-2, which controls FGF-2 concentrations in serum and in the marrow cavity, and, consequently, leads to trabecular bone formation. In our previous studies, using gain-of-function and loss-of-function approaches, we set out the FGF-2 interrelationship on bone morphogenetic protein (BMP)-2 osteogenic activity and we underscored the importance of the crosstalk between both factors in the osteoblast metabolism [154–156]. BMP signalling plays multiple roles in bone, bone marrow vascularization and MSC homeostasis [157]. Therefore, BMP receptor type IA (BMPRIA) mutations generate dissimilar phenomena in function of the area/niche. Of note, in Bmpr1a mutant mice, there is an abnormal bone formation and trabecular bone-like area alteration, as well as increased numbers of SNO cells and LT-HSCs [14]. Recently, a mouse model with BMP-2 abrogation in early osteoblasts displayed compromised micro-capillary architecture, decreased α-smooth muscle actin (α-SMA)$^+$ MSCs (Fig. 2E, F), CD31 and CD146 cell pool, coupled with decreased Ang-1 production, and reduced levels in vascular endothelial growth factor A (VegfA) within the bone marrow [157]. Considering that the VegfA has been characterized as a significant moderator in blood vessel formation and in osteoblast precursor maturation [158], and that the α-SMA$^+$ cell lineage has been involved in angiogenesis during bone formation [159], it is fathomable that the BMP-2 signal transduction mechanism can provide extra criteria for the above-mentioned manipulation of the bone marrow microenvironment.

Several studies have explained the spatio-temporal osteogenic action of BMP-2 and its clear-cut role in MSC differentiation into osteoblast progenitors. The destiny of preosteoblasts, among other bone marrow stimuli, is controlled by TGF-β1, a potent regulatory cytokine produced by multiple cell types [reviewed in 160]. Indeed, TGF-β1 exerts controlling effects on the bone remodelling process and has both stage-dependent osteoclastogenic activities (as a result of NF-kB and RANK induction), and osteoprotective functions regarding the imbalance of the OPG/RANKL axis *via* OPG upregulation [161]. TGF-β produced by glial cells also behaves as a perivascular niche modulator supporting HSC maintenance [69]. The TGF-β response is in accordance with its concentrations levels within the bone marrow. Low TGF-β levels support Th17 maturation and consequently osteoclastogenesis, while elevated levels of this factor sustain Treg-induced MSCs-mediated osteoblastogenesis [162]. The TGF-β activity presents a molecular circuit wherein more influential factors such as FGF-2, PTH and the prostaglandins (PGs) have been engaged into a synergistic module, to become part of the canvas of marrow signalling interactions. As regards the involvement of PGs within the bone marrow, our research, along with that of others, has shown the involvement of these lipid mediators in bone cells as well as in skeletal and hematopoietic progenitors. Particularly, PGE2 and PGF2α exert a bifold dose/stage-depended action on osteoblast and osteoclast metabolism in cell and organ cultures [163,164]. The PGF2α mitogenic and survival effects on osteoblasts were mediated by FGF-2 [165], underlining the importance of FGF signalling in the skeletal and, as previously mentioned, on hematopoietic cells. Concurrently, other findings have focused attention on the ability of PGE2 to facilitate either HSC engraftment *via* CXCR4 upregulation or HSC egression into the circulation and to provide anti-apoptotic signals on HSCs *via* surviving production [166]. PGE2 can also amplify the PTH effects in the marrow cavity with dissimilar outcomes, based on the clinical profile of the bone and the hematopoietic components [167]. Thus, based on the above-described factors, it is explicable that the molecular mechanisms within the Daedalean bone marrow entity assume distinct bias in basal or pathological (inflammatory) conditions.

7. Complexities and perplexities of bone marrow topography

The findings that the stromal elements have a direct impact on HSCs homeostasis have established over the last two decades a

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unique paradigm for the potency of the bone marrow niche. The various specialized cell clusters are delineated in distinct structural areas with conspicuous commitments, such as the bone marrow adaptability to support HSC homing, egression and cycling, as well as MSC differentiation and engagement to the endosteal and perivascular area.

The importance of every single component in the bone marrow ontogeny is conditioned by complexities and perplexities caused by the considerable interactions amongst the distinct anatomical compartments within the axial and long bone cavity. These complexities arise out of the fine-tuned hematopoietic mechanisms that sustain HSCs and blood cell maturation, as well as the bone marrow microenvironment, which tends to preserve the HSC pool at the stable number of 1.1×10^4 cells in all mammalian systems [168]. Bone formation and humoral immune system maturation is based on strong ties between the niche components. For instance, osteoblasts by RANKL and OPG secretion coordinate B cell development but only in the precise spatial limits of the "hematopoietic workroom." Outside these "B cell maturation borders," RANKL and OPG carry out different functions (*i.e.* osteoblastogenic/osteoclastogenic activities) [reviewed in 169]. But how do these molecules respect the topographic limitations and act in a niche/context specific way without being dissipated into other functional compartments with, in some cases, deleterious consequences? Up to this point, a clear-cut answer has not been found.

In a thought-provoking study, Bianco [21] has addressed a captivating question regarding the contribution of the osteoblasts to HSC homing and maintenance. A perplexity arises out of the fact that osteoblasts, from the point of view of osteogenic functionality, have a rigorous spatio-temporal existence and thus a transient life at the endosteum level. Consequently, since the HSCs allocated at the endosteal niche have to deal with endosteal behavior, the whole "niche" dimension tends to depend on the limited life span of the osteoblasts. Alternatively, the osteoblast influences on HSCs homeostasis remain contingent to an extended regulatory scenario, which involves additional homeostatic players in the bone marrow, such as the MSCs, initially identified as stem cells and thus far considered multirole elements, such as contributors to the HSC niche physiology [reviewed in 5]. In addition, the extramedullary engraftment of the HSCs in the absence or impaired expression of key regulatory factors within the marrow area, such as the calcium-sensing receptor, resulted in perplexities regarding the specified "niche harbour" properties of the bone marrow. Indeed, Sacchetti et al. [8] have shown how bone marrow MSCs (perisinusoidal $CD146^+$ cells) can establish an effective hematopoietic habitat outside the boundaries of the bone marrow. In line with this, Serafini et al. [170] have shown in a wider scope that the heterotopic "ossicles" generated by transplantation of bone marrow-derived MSCs and by non-mineralized cartilage pellets formed by bone marrow stromal cells have reproduced a functionally extramedullary HSC niche. Due to the architectural and operational posture of these heterotopic "ossicles," it has become clear that a role of the MSC is to generate hematopoietic vascularized microenvironments and emulate the skeletal hematopoietic niche assembly.

Recently, it has been described another complex scenario, which bring into question the topographic niche limitations: the suppression of key factors involved in the endocondral ossification process, including osterix and VEGF, results in compromised niche formation and HSC retention [171]. Hence, the endocondral ossification mechanisms, which act within a predesignated local microenvironment, become an additional issue for the consideration of the niche ontogeny.

The above concerns, which are quite crucial for understanding the bone marrow ontogeny, are particularly important for determining the MSC magnitude and the HSC demands, in terms of microenvironmental constructural/homeostatic characteristics. Although modern findings have unveiled salient clues for defining the mosaic of the bone marrow ontongeny, there is still a long way to go. Undoubtedly, the *in vitro* approaches do not always meet the real scale of the bone marrow concern due to the lack of many significant niche regulators, and the *in vivo* research has to deal with systemic organism vicissitudes, and, therefore, the improvement of schemes/assays is warranted. Resolving the complexities and perplexities associated with bones and bone marrow remains a compelling task for further research to address.

8. Conclusion

The bone marrow reservoir accommodates an extraordinarily multipart microenvironment that tends to merge the singular features of the diverse cell populations in a labyrinthine homeostatic tableau. The progenitor/stem and mature components that inhabit the bone marrow cavity deal with a broad range of intramural and external molecules and preserve the characteristic plasticity, consistency and functionality of the bone marrow compartments. This dynamic hematopoietic and mesenchymal stem cell incubator, enclosed within the axial and long bones, in physiological conditions, could be identified as the body's ground control, involved, for example, in bone modelling and remodelling, immune cell egression and maturation and cooling down pro-inflammatory stimuli. The bone marrow regulatory activities go beyond its topography and meet the needs of other tissues and organs (*i.e.* muscles, adipose tissue and kidney) in terms of the release of cytokines/chemokines, the control of phosphate/calcium, the production of growth factors, their distribution through circulation, *etc.* It is also a tremendous cell breeding locus, where the disruption of the functionality of even one of its residents can lead to local or systemic pathological conditions, such as chronic inflammation, kidney failure, bone diseases and hematopoietic disorders.

Disclosure statement

The authors have nothing to disclose.

Acknowledgements

The authors are grateful for the financial support of Camerino University (FAR 2013)

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