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Review Article

Erythropoiesis, EPO, macrophages, and bone

Joshua T. Eggold [a,b], Erinn B. Rankin [a,b,c,*]

[a] Department of Radiation Oncology, Stanford University School of Medicine, Stanford, CA, USA
[b] Cancer Biology Program, Stanford University School of Medicine, Stanford, CA, USA
[c] Department of Obstetrics & Gynecologic Oncology, Stanford University School of Medicine, Stanford, CA, USA

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ABSTRACT

The regulation of erythropoiesis in the bone marrow microenvironment is a carefully orchestrated process that is dependent upon both systemic and local cues. Systemic erythropoietin (EPO) production by renal interstitial cells plays a critical role in maintaining erythropoietic homeostasis. In addition, there is increasing clinical and preclinical data linking changes in EPO and erythropoiesis to altered skeletal homeostasis, suggesting a functional relationship between the regulation of erythropoiesis and bone homeostasis. As key local components of the bone marrow microenvironment and erythropoietic niche, macrophage subsets play important roles in both processes. In this review, we summarize our current understanding of the cellular and molecular mechanisms that may facilitate the coordinated regulation of erythropoiesis and bone homeostasis.

Contents

1. Erythropoiesis

Red blood cells (RBCs) are anucleate cells that play a critical role in carrying oxygen to tissues. At a concentration of 5 million RBCs per microliter of blood, red blood cells are the most abundant cell type in the blood. In order to maintain RBC numbers in the blood, the bone marrow produces two million erythrocytes per second. At this level of production, intrinsic and extrinsic factors controlling RBC production, or erythropoiesis, must be carefully coordinated [1]. Clinically, dysregulation of erythropoiesis results in the development of anemia or polycythemia.

In adult mammals, erythropoiesis occurs almost exclusively within the bone marrow under homeostatic conditions. Erythropoiesis is a complex and dynamic process whereby mature red blood cells are produced from hematopoietic stem and progenitor cells. This hierarchal process begins with a common hematopoietic stem cell (HSC) that is multipotent and capable of forming all blood lineages while maintaining self-renewal capacity. In the classical model of hematopoiesis, it is thought that HSCs differentiate into a series of progenitor cell intermediates that undergo gradual fate restriction and commitment into mature blood lineages [2]. Recent advances in single cell tracing, RNA sequencing and transplantation have suggested an alternative model where lineage fate may be determined at an earlier stage than previously suggested. Single cell RNA sequencing on myeloid progenitor cells revealed that very few progenitors expressed transcription factors

* Corresponding author at: 269 Campus Drive, 1245A CCSR, Stanford, CA 94305, USA.
E-mail address: erankin@stanford.edu (E.B. Rankin).

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that regulate multiple cell fates. Instead, individual cells clustered into lineage-specific differentiation programs [3]. Additionally, single cell lineage tracing through the transplantation of barcoded progenitor cells into mice suggested that very few myeloid progenitor cells have the ability to form erythroid and myeloid lineages suggesting that myeloid progenitors are a mixture of committed progenitors rather than a homogenous population of cells with multi-lineage potential [4]. Rodriguez-Fraticelli et al. utilized a native, non-transplant transposon lineage tracing model to support an earlier lineage fate commitment, but also suggested that the erythroid lineage shares a common progenitor more closely with the lymphoid and myeloid lineages than the megakaryocyte lineage under steady state conditions [5]. In contrast, Tusi et al. utilized single-cell transcriptomics, fate assays and theory to support the hierarchical view of hematopoiesis demonstrating a continuous differentiation trajectory of the erythroid lineage from multipotent progenitors (MPPs) to progenitors with either erythroid-basophil/mast-megakaryocytic progenitor to early erythroid progenitors and committed erythroid progenitors [6]. The differences in these studies may be due to the choice of surface markers or differences in the fate plasticity of progenitors *in vitro versus in vivo*. Overall these studies indicate that HSC differentiation is a highly dynamic and adaptable process that merits additional study as new resources and tools become available. Despite the complexities, once cells reach the step of erythroid terminal differentiation (ETD), their fate has been better characterized. In the bone marrow, erythroid progenitors differentiate through a series of stages including proerythroblasts, basophilic and polychromatophilic erythroblasts, orthochromatophilic erythroblasts, and finally reticulocytes by enucleation (Fig. 1). These distinct stages of erythropoiesis have been defined morphologically, based on a gradual decrease in cell volume, increasing chromatin condensation and increasing hemoglobinization as well as by the expression of the cell surface molecules CD71 and Ter119 [7].

The regulation of erythropoiesis in the bone marrow microenvironment is dependent upon systemic and local cues that control the differentiation, proliferation, and survival of erythroid progenitors. Accumulating clinical and preclinical data suggest that there may be a coordinate regulation of erythropoiesis and bone homeostasis in the bone marrow. In this review, we will summarize our current understanding of the regulation of erythropoiesis and discuss how these cellular and molecular factors may influence bone homeostasis.

2. Clinical correlations between erythropoiesis and bone homeostasis

Clinical studies in anemic and polycythemic patients suggest a functional link between erythropoiesis and bone homeostasis. Both anemic and polycythemic patients are at risk for bone disease. Patients with chronic hemolytic anemia, such as thalassemia patients suffer from low bone mass, fractures, and bone pain [8,9]. Eighty percent of patients that develop anemia associated with sickle cell disease also experience low bone mass density [10]. Moreover, anemia is common in elderly patient populations and is associated with an increased risk for bone fractures and low bone mineral density [11–13]. Patients with polycythemia vera are also at increased risk for developing osteoporotic fractures [14]. While these clinical studies provide a correlation between erythropoietic disorders and bone disease, the mechanisms by which anemic and polycythemic patients develop bone disease are unknown. Bone disease has been recapitulated in murine models of thalassemia and polycythemia providing model systems where the mechanisms can be investigated [15,16]. It is tempting to speculate that under conditions of erythropoietic demand, the bone marrow microenvironment and hematopoietic niches may be coordinately remodeled to support the enhanced erythropoietic output, which will ultimately affect bone homeostasis.

3. Erythropoietin

Accumulating evidence suggests that the glycoprotein erythropoietin (EPO) may be a factor responsible for the coordinate regulation of erythropoiesis and bone homeostasis. EPO is essential for the regulation

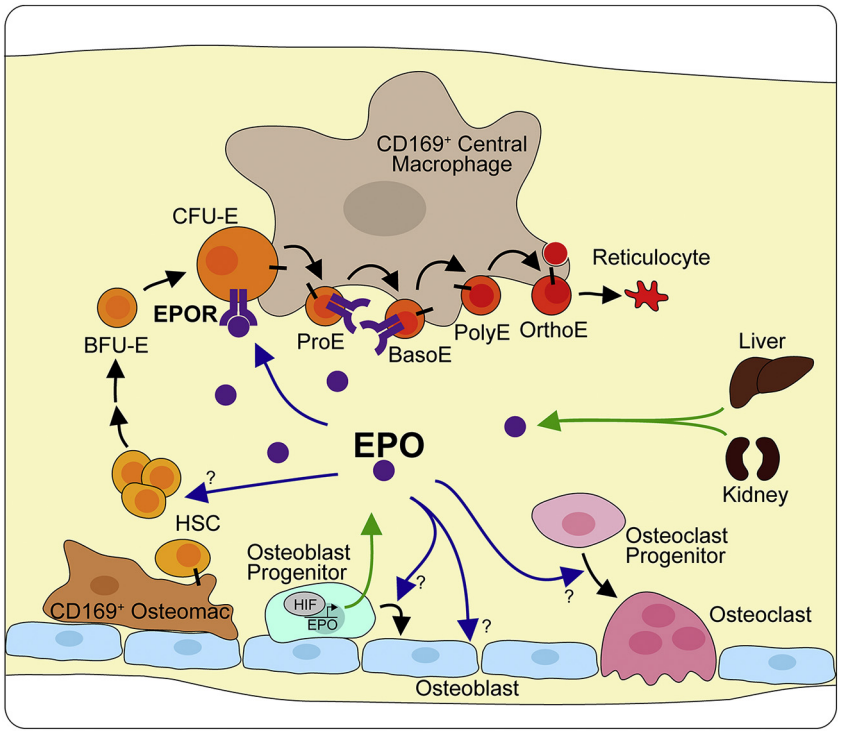

Fig. 1. EPO and macrophages affect erythropoiesis and bone homeostasis within the bone marrow microenvironment. The kidney, liver, and osteoblast progenitors can all supply EPO to the local bone marrow environment. Canonical EPO signaling promotes the differentiation of erythroblasts supported by CD169^{+} central macrophages. Studies demonstrate that EPO can also directly or indirectly modulate bone formation and homeostasis through the behavior of osteoblasts, osteoclasts, and HSCs. CD169^{+} osteomacs support the bone microenvironment through their interactions with osteoblasts and HSCs.

of red blood cell mass in response to changes in tissue oxygenation. EPO induces erythropoiesis through the stimulation of the EPO receptor (EPOR) on erythroid precursor cells in the bone marrow to stimulate survival, proliferation, and differentiation, thus enhancing the oxygen-carrying capacity of blood (review in Ref. [17]). Upon EPO binding, EPOR forms a homodimer capable of signaling through the JAK2, MAPK, and PI3K kinases [18]. EPO/EPOR signaling promotes erythroid development in large part through the activation of the JAK2/STAT3/STAT5 signaling pathway. Inactivation of JAK2 results in the development of anemia whereas constitutive JAK2 mutations leads to increased red blood cell mass and polycythemia [19,20]. Lack of EPO during murine development results in embryonic lethality at E13.5 as a result of cardiac failure and anemia [21,22]. Clinically, dysregulated *EPO* expression results in the development of anemia when serum EPO levels are inadequately low or polycythemia as a result of EPO overproduction. *EPO* expression is tightly regulated by developmental, tissue-specific, and physiological cues [17]. The primary physiological source of EPO transfers from the fetal liver during development to the kidney in adult mammals [23]. The primary physiological stimulus of increased *EPO* gene transcription is tissue hypoxia, which can induce up to a 1000-fold increase in circulating serum EPO levels [17]. In response to anemia, renal peritubular interstitial cells produce EPO driven by HIF-2 [24]. We have recently demonstrated that under conditions of constitutive HIF stabilization, osteoblasts are also able to produce EPO to promote erythropoiesis [25]. These findings suggest that osteoblastic EPO may be exploited clinically for the treatment of anemia using PHD inhibitors that stabilize HIF-1 and HIF-2. However, the role of osteoblastic EPO in physiological conditions is still under investigation.

In addition to regulating erythropoiesis, EPO has also been implicated in the regulation of bone homeostasis. A variety of studies have observed that exogenous EPO treatment can increase bone volume and repair. EPO treatment increased bone volume and biomechanical properties in murine femoral fracture repair models [26,27]. Similarly, EPO stimulated bone formation in cranial defect and scaffold models [28,29]. In neonatal and adult mice, exogenous EPO treatment (6000 U/kg) was sufficient to induce bone formation [30]. In a rabbit spinal fusion model, daily subcutaneous injection of 250 IU/kg EPO beta for 20 days was sufficient to increase bone formation after 6 weeks [31]. Moreover, erythropoietin treatment has been shown to increase bone healing in porcine osteochondral and cranial defect models [32,33]. In many of the repair models, exogenous EPO treatment and bone formation is associated with increased vascular density and angiogenesis [26,28,31,34]. It is well established that angiogenesis plays a key role in bone formation and healing [35–40]. In the bone microenvironment, blood vessels ($CD31^{hi}Endomucin^{hi}$) carry oxygen, nutrients, growth factors, and osteoprogenitor cells to sites of developing bone [39,40]. However, the cellular and molecular mechanisms by which EPO promotes angiogenesis in the bone marrow microenvironment remain unknown. Of note it has been previously shown that endothelial cells express EPOR and that germline deletion of EPO or EPOR results in angiogenic defects during development, suggesting that EPO may stimulate bone marrow endothelial angiogenesis [41]. Moreover, the contribution of angiogenesis to EPO-mediated bone formation remains unknown. Alternate mechanisms by which EPO promotes bone formation have been proposed. For example, EPO has been reported to directly stimulate osteogenesis by targeting mesenchymal stromal and hematopoietic stem cells. EPO treatment of both human mesenchymal and mouse bone marrow stromal cells was sufficient to promote osteoblastic differentiation [30,33,42]. Constitutive HIF signaling in osteoblasts leads to their production of EPO as discussed above, as well as increases in trabecular bone volume associated with increased vascularization, although the role for osteoblastic HIF-driven EPO production in bone homeostasis remains under investigation [25]. Additionally, EPO has been shown to indirectly stimulate osteoblastic differentiation by stimulating HSCs to secrete bone morphogenetic protein [30].

In contrast to osteogenic roles for EPO described above, EPO has also been reported to decrease bone volume in murine models. Singbrant et al. observed that administration of EPO at clinically relevant doses (300 U/kg) resulted in decreased trabecular bone volume associated with increased bone remodeling. In this model, inhibition of osteoclast activity with bisphosphonate therapy inhibited EPO-induced bone loss suggesting that osteoclasts may be responsible for this effect [43]. Other studies have observed a similar decrease in bone volume following exogenous EPO treatment [44,45]. Moreover, transgenic models of EPO overexpression in mice has been associated with bone loss, an increase in bone resorption and/or decrease in bone formation rate [15,45,46]. *In vitro* data support a role for EPO in osteoclast differentiation. EPO was shown to directly stimulate pro-osteoclast differentiation into mature osteoclasts [45]. Osteoclast differentiation led to a corresponding decrease in EPOR transcript levels, indicating that EPOR expression is limited to pre-osteoclasts rather than their mature progeny. This study also demonstrated that EPO stimulation resulted in the activation of pJak2 and pAKT, indicating that signaling is occurring through the Jak2 and PI3K pathways in response to EPO [45]. Other studies have seen similar increases in osteoclastogenesis upon EPO stimulation [28,30,42]. Interestingly, EPO does not appear to promote osteoclast activity. One study reported that EPO stimulation decreased the RANKL-mediated bone resorption of osteoclasts *in vitro* [47]. Future studies are needed to determine the role of EPO signaling in osteoclasts *in vivo*. A recent study demonstrated that EPO can stimulate the expression of the phosphaturic hormone fibroblast growth factor 23 (FGF23) by HSCs within the bone marrow, thus resulting in an increase in serum FGF23 and a decrease of serum phosphate [48]. This could be an additional mechanism by which elevated levels of EPO can impair bone mineralization, and also suggests that HSCs express functional EPOR. Lineage-tracing studies in which EPOR-Cre mice were crossed to Rosa26 YFP reporter mice have suggested that neither $LKS^{+}CD150^{+}CD48^{-}$ HSCs, nor mesenchymal, nor osteoblastic enriched populations from mouse bone marrow express EPOR-Cre [43]. However, this reporter system may overlook cell types with low expression due to imperfect recombinase efficiency. Whether EPO stimulates osteoclast progenitor, endothelial, mesenchymal stem cells, or hematopoietic stem cells through EPOR. Future studies using conditional gene targeting of EPOR *in vivo* within specific cell subsets of the bone marrow microenvironment are needed to determine whether EPO/EPOR signaling influences bone homeostasis or repair.

4. Macrophages

Hematopoiesis, erythropoiesis, and bone marrow homeostasis are all important processes occurring within the bone marrow microenvironment that have been shown to depend upon macrophages. Multiple subsets of BM macrophages that support bone maintenance, hematopoiesis, and erythropoiesis have been identified using different marker classifications [49–51]. Looking across numerous studies, the number of discrete BM macrophage populations and their potential plasticity remains unclear. The origin of BM macrophages is also a topic of debate (see [51] for a review); canonically they are derived from the monocyte HSC lineage, but certain tissue-resident populations may arise independent of HSC sources [52]. Jacome-Galarza and colleagues demonstrated that functional BM macrophages, dendritic cells, and osteoclasts could all be derived *in vitro* from a shared progenitor distinguished as $B220^{-}CD3^{-}CD11b^{-/lo}CD115^{+}CD117^{+}$ [53]. However, it has been difficult to extend *in vitro* studies to accurately infer the developmental origins of macrophages *in vivo*. Regardless of these challenges, macrophages play important roles in the bone marrow microenvironment, given that their depletion results in a variety of defects including bone mineralization, HSC retention, and impaired stress erythropoiesis [49,50,54].

A key BM resident macrophage population is the osteomac, or osteal macrophage. Osteomacs are found within the endosteal niche lining the

inner bone surface, where they often reside in close physical proximity to osteoblasts (Fig. 1). They support osteoblasts in the maintenance of bone homeostasis by promoting their differentiation and mineralization behavior [49]. Together these cells preserve the HSC niche and prevent the aberrant egress of HSCs into the blood [50,54]. Recently the interaction between CD82 on long-term HSCs and CD234 on osteomacs was found to be vital to their maintenance [55]. Osteomacs have been classified as F4/80$^+$CD11b$^+$CD115$^+$CD68$^+$CD169$^+$VCAM-1$^+$ and can be distinguished from inflammatory macrophages and osteoclasts by their lack of Mac2 and TRAP expression [50,54,56,57].

Closely related to the canonical macrophage, osteoclasts are multinucleated BM resident macrophages that lack expression of the classical macrophage marker F4/80, and are noted for their expression of receptor activator of NF-κB (RANK). Just as macrophages are reliant on the cytokine macrophage-colony stimulating factor (CSF-1), osteoclast development also depends on this molecule [58]. They play a key role in bone maintenance and homeostasis given their specialized function in bone resorption [51].

Another important BM macrophage is the central macrophage that forms the basis for the erythroblastic islands that form a niche for erythroblast differentiation. Erythroblast islands are distinct structures that support the differentiation of erythroid progenitors into reticulocytes, and they have been identified *in vivo* at every site of human erythropoiesis [59]. In the bone marrow niche, islands are distributed evenly throughout rather than clustered near vessels or other structures [60]. Each island is comprised of a single central macrophage surrounded by a ring of 5–30 erythroid cells in varying states of differentiation [61,62]. The central macrophage provides a stable niche for the developing erythroblasts by secreting stimulatory cytokines such as insulin-like growth factor-1 and BMP-4 [63,64]. They also furnish the differentiating erythroblasts with iron in the form of ferritin [65].

Cell-cell interactions between erythroblasts and their attendant central macrophage are crucial to the maintenance of the island. These interactions have been demonstrated to depend on erythroid macrophage protein (EMP or macrophage erythroblast attacher [MAEA]), intracellular adhesion molecule 4 (ICAM4) – $\alpha_v\beta_3$ integrin, and vascular cell adhesion molecule 1 (VCAM-1) – very late activation antigen 4 (VLA4) integrin $\alpha_4\beta_1$ [66–70]. During the final stages of their development, nascent reticulocytes undergo enucleation and their close association with the central macrophage allows the macrophage to efficiently clear the resultant pyrenocytes [71]. This phagocytic behavior is driven by the TAM-family member receptor tyrosine kinase MerTK (Fig. 1, [72]).

A recent study demonstrated that selective depletion of CD169$^+$ central macrophages decreased the frequency of erythroblast islands in the bone marrow [50]. Interestingly, this did not result in anemia under homeostatic conditions, indicating that erythroblasts can mature without the aid of central macrophages in healthy adult mice. However, the loss of CD169$^+$ macrophages impaired the stress erythropoietic response, revealing that central macrophages and erythroblast islands are necessary to achieve maximum efficient erythropoiesis in response to anemia [73,74]. Interestingly, osteomacs described above also express CD169. Recent studies have demonstrated that CD169 depletion using the CD169-diptheria toxin model does impair bone repair in tibial injury and in femoral fracture repair models, indicating that CD169$^+$ macrophages may have the potential to coordinate erythropoiesis and bone homeostasis [75].

5. EPO and macrophages

As described above, the expression of erythropoietin receptor outside of the erythroid lineage is a controversial subject [43,76]. However, an increasing body of evidence suggests that at least in certain contexts, other cell types including macrophages can functionally respond to EPO. Luo and colleagues found that LysM-Cre driven deletion of EPOR impaired the ability of peritoneal macrophages to clear apoptotic cells *in vivo*, resulting in an increased frequency of systemic autoimmunity due the accumulation of apoptotic cells [77]. Other studies by the same group demonstrated that inflammation-induced hypoxia leads to increased expression of EPOR in macrophages as well as elevated serum EPO, which cooperate to suppress inflammatory macrophage signaling and promote resolution [78,79]. EPO treatment has also been shown to increase the T-cell suppressive ability of peritoneal macrophages in an iNOS-dependent fashion [80]. In a mouse model of rhabdomyolysis-induced kidney injury, EPO provided a protective effect partially through modulating the macrophage response. EPO administration increased the expression of M2 markers by kidney macrophages *in vivo*, suppressed inflammatory signaling, and promoted M2 polarization of BMDMs *in vitro*. These effects were linked to pJak2 and pSTAT3 signaling [81]. EPO has also been studied in brain-resident glial macrophage populations. Multiple groups have demonstrated the EPO stimulation of microglia suppresses inflammatory signaling to promote an M2 phenotype and stimulates phagocytosis. EPO also provides neuroprotection against inflammation and cerebral ischemic and promotes oligodendrogenesis [82,83]. A recent study detected EPOR expression in Kupffer cells (KC), liver resident macrophages, and demonstrated that EPO administration increases KC proliferation and frequency within the liver. It also elevated their expression of CCL2 to assist their recruitment of Ly6C^{hi} monocytes to the liver after acetaminophen-induced injury [84]. Within the bone marrow, a study of multiple myeloma patients found an increased expression EPOR by their bone marrow macrophages [85]. Stimulation of these macrophages with EPO increased proangiogenic gene expression and secretion in a pJak2 and PI3K-dependent manner. The authors also demonstrated a functional increase the ability of EPO-stimulated macrophages to promote bone marrow endothelial cell migration in a scratch assay and angiogenesis in a chick embryo chorioallantoic membrane assay [85]. These studies suggest that EPO stimulation may promote the anti-inflammatory, pro-repair, and pro-angiogenic functions of macrophages, shifting them towards more M2-like behaviors. Future studies are needed to determine the functional role of EPOR signaling in bone marrow macrophage populations and the potential role in erythropoiesis and/or bone homeostasis.

6. Conclusions

Erythropoiesis plays an important role in the maintenance of red blood cell mass and the delivery of oxygen to tissues. This process occurs almost exclusively in the bone marrow microenvironment of adult mammals where both systemic and local cues regulate erythropoiesis. Clinical and preclinical studies suggest a coordinate regulation of erythropoiesis and bone homeostasis. Anemia and polycythemia are associated with bone disease in human patients and in murine models. Whether the changes in bone are mediated through EPO dependent and/or independent mechanisms remains unclear. To begin to address this question, Oikonomidou et al. compared bone phenotypes in EPO transgenic mice (tg6, high EPO) and in JAK2^{V617F} (low circulating EPO levels) murine models of polycythemia [15]. While the EPO tg6 mice exhibited the most severe bone phenotype, JAK2^{V617F} mice also had a significant decrease in bone volume and osteoblast numbers compared to control mice, indicating that there may be both EPO dependent and EPO independent changes in bone within these polycythemic models [15]. As discussed above, there is increasing evidence to support a role for exogenous EPO in the regulation of bone homeostasis. However, the notion that EPO stimulated-erythroid progenitors may produce factors that influence bone homeostasis has also been proposed [43,86]. The identification of specific factors produced by erythroid progenitors that functionally link erythropoiesis and bone homeostasis would greatly support this hypothesis.

Another important question in the field is how does exogenous EPO regulate bone formation and resorption. The effects of EPO on bone formation and resorption appear to be related to the experimental

condition and the amount of EPO administered. In models of bone regeneration, exogenous EPO treatment has been associated with enhanced bone formation and repair. In these studies, EPO treatment has been associated with increased angiogenesis and osteogenesis. In contrast, the majority of studies where EPO is administered or overexpressed in non-injury models, EPO has been associated with decreased bone volume and osteoclastogenesis. It is still unresolved whether osteoblasts and/or osteoclasts express a functional EPO receptor *in vivo*. Conditional gene targeting of EPOR in specific subsets of osteoblasts and osteoclasts is needed to determine the role of EPOR within these cells in EPO-mediated bone remodeling *in vivo*. Moreover, it is important to consider that at high doses EPO may bind and activate receptors other than EPOR. For example, Pradeep and colleagues identified ephrin-type B receptor 4 (EphB4) as an alternative EPO receptor utilized by cancer cells to promote tumor progression in response to exogenous EPO [87]. Previous studies have suggested that EPO-mediated bone formation and osteoblastic differentiation may occur through EphB4 [88].

Finally, macrophages are a key cellular component of the bone marrow microenvironment that control erythropoiesis and bone homeostasis. Future studies are needed to determine if specific macrophage populations contribute to bone disease observed in anemic and/or polycythemic conditions. Recent studies have shown that global depletion of macrophages using clodronate can normalize the erythroid compartment in $JAK2^{V617F}$ and B-thalassemia murine models [73,89]. Interestingly, $CD169^{+}$ macrophages have been identified in regulating erythropoiesis and in bone repair. However, the effects of $CD169^{+}$ macrophage depletion on bone mass within anemic and/or polycythemic models need to be investigated in future studies.

It is clear that erythropoiesis and bone homeostasis are finely regulated processes that are spatially, if not also functionally, linked. Under baseline conditions, the bone marrow must be carefully maintained to provide the proper hematopoietic and erythropoietic niches. When disease or other insults disrupt this proper balance, anemia, polycythemia, or osteoporosis may occur. It is crucial to increase our detailed mechanistic understanding of how erythropoiesis, EPO, and other signals affect the various cell types in the bone marrow microenvironment in order to alleviate these health concerns.

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