HHS Public Access

Author manuscript

Curr Osteoporos Rep. Author manuscript; available in PMC 2015 December 01.

Published in final edited form as:

Curr Osteoporos Rep. 2014 December ; 12(4): 428–432. doi:10.1007/s11914-014-0236-x.

Osteoblasts: a Novel Source of Erythropoietin

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Abstract

Osteoblasts are an important cellular component of the bone microenvironment controlling bone formation and hematopoiesis. Understanding the cellular and molecular mechanisms by which osteoblasts regulate these processes is a rapidly growing area of research given the important implications for bone therapy, regenerative medicine, and hematopoietic stem cell transplantation. Here we summarize our current knowledge regarding the cellular and molecular crosstalk driving bone formation and hematopoiesis and will discuss the implications of a recent finding demonstrating that osteoblasts are a cellular source of erythropoietin.

Keywords

Erythropoietin; Osteoblast; Hypoxia; Angiogenesis; Bone remodeling; Osteogenesis

Introduction

Bone plays an essential role in the structure and movement of the body as well as the protection of vital organs. In adult mammals, the bone is also the primary site for hematopoiesis where hematopoietic stem cells (HSCs) are maintained and developing hematopoietic cells of the myeloid and lymphoid lineages are retained until they mature and are released into the vasculature [1]. Endochondral ossification and the establishment of hematopoiesis in the bone marrow are coordinated processes that involve the replacement of a cartilaginous matrix by bone and bone marrow. Endochondral bone formation is initiated by chondrocytes that establish a cartilaginous matrix template at the avascular growth plate followed by the invasion of blood vessels, osteoclasts, and osteoblast precursor cells into the avascular cartilage to establish a primary ossification center [2]. Osteoblasts, stromal cells, and endothelial cells then populate the vascularized bone marrow and produce chemokines and growth factors that recruit and maintain hematopoietic stem cells [3]. Understanding the cellular and molecular cross talk in bone marrow microenvironment controlling bone

Compliance with Ethics Guidelines

Human and Animal Rights and Informed Consent All studies by C. Wu, A. J. Giaccia, and E. B. Rankin involving animal and/or human subjects were performed after approval by the appropriate institutional review boards. When required, written informed consent was obtained from all participants.

Conflict of Interest C. Wu, A. J. Giaccia, and E. B. Rankin declare that they have no conflicts of interest.

formation and hematopoiesis is a rapidly growing area of research given the important implications for bone therapy, regenerative medicine and HSC transplantation. This review will summarize our current knowledge regarding the cellular and molecular crosstalk driving bone formation and hematopoiesis and will discuss the implications of a recent finding demonstrating that osteoblasts are a cellular source of erythropoietin (EPO).

Osteogenesis and Angiogenesis Coupling in Bone Formation

It is well established that osteogenesis and angiogenesis are intricately linked processes necessary for bone formation. Genetic models in which osteogenesis is impaired through loss of runt-related transcription factor 2 (Runx2) or osterix (Osx) results in defective vascular invasion and bone formation [4–6]. Similarly, manipulation of angiogenesis in bone by either inhibition or overexpression of vascular endothelial growth factor (VEGF) results in a significant decrease and increase in bone formation respectively [7–9]. Together, these studies support the notion that osteogenesis and angiogenesis are coupled during bone development.

Blood vessels deliver necessary oxygen, nutrients, hormones, growth factors, as well as cellular components including osteoblasts to support bone growth. Recent studies have demonstrated the vasculature plays an important role in directing the migration and differentiation of osteoblast precursor cells. Lineage tracing studies demonstrated that osteoblast precursor cells, but not mature osteoblasts, co-invade with blood vessels into developing bone to give rise to cells of the osteoblastic lineage and stromal cells [2]. Moreover, osteoblast precursors associate with a specific subset of endothelial cells characterized by high levels of CD31 and Endomucin ($CD31^{hi}Endomucin^{hi}$) that not only direct their migration, but also stimulate their differentiation through notch signaling [10•, 11•].

Hypoxia is an important physiologic stimulus coupling osteogenesis and angiogenesis in the bone. During bone formation, oxygen gradients are established that promote the formation of new blood vessels to deliver oxygen, nutrients, osteoclasts, and osteoblast precursor cells to the growing bone tissue [12]. The primary molecular mediators of hypoxic signaling are the hypoxia inducible transcription factors HIF-1 and HIF-2. The alpha subunits of HIF-1 and HIF-2 are rapidly degraded in the presence of oxygen through the coupled actions of prolyl hydroxylase enzymes (PHDs 1–3) and the VHL E3 ubiquitin ligase complex [13–15]. In response to hypoxia or oxygen tensions below 5 %, the HIF-1 and HIF-2 alpha subunits are stabilized and translocate into the nucleus where they heterodimerize with their constitutively expressed binding partner ARNT and activate gene expression programs that mediate cellular adaptation to hypoxic stress including angiogenesis, erythropoiesis, and glucose metabolism [16]. The importance of hypoxia and hypoxic signaling in bone formation is underscored by the finding that deletion of HIF in chondrocytes, osteoblasts, and postnatal bone endothelium significantly inhibits endochondral bone formation [10•, 17, 18]. One of the primary mechanisms by which HIF signaling is thought to regulate chondrocyte and osteoblastic control of skeletal development is through the regulation of VEGF expression. As described above, VEGF is a key factor driving blood vessel invasion into the cartilaginous mold and is a well-established transcriptional target of HIF that is

upregulated in hypoxic chondrocytes and osteoblasts [17, 18]. Given the central importance of hypoxia and HIF signaling in bone development, future studies are needed to further elucidate the molecular mechanisms by which HIF signaling in the bone marrow microenvironment couples osteogenesis and angiogenesis and how HIF activity may be manipulated for bone therapy.

Osteogenic and Angiogenic Niches for Hematopoiesis

The coupling of osteogenesis and angiogenesis is not only important for bone formation, but is also important in establishing the hematopoietic stem cells (HSC) niche and hematopoiesis within the bone marrow. A direct link between bone formation and hematopoiesis was first reported in the late 1960s during ectopic bone marrow transplant studies in which bone formation and resorption preceded hematopoietic repopulation [19]. More recently, it was shown that key factors involved in bone formation, including osterix and vegf, are required for hematopoietic repopulation in ectopic bone marrow transplants [3]. Osterix is a transcription factor expressed by immature osteoprogenitor cells and mature osteoblasts during endochondral ossification, suggesting that cells of the osteoblastic lineage may be involved in the regulation of hematopoiesis [6, 20]. In support of this concept, osteoblasts are required for the developmental switch of hematopoiesis from the liver to the bone marrow, and also to maintain hematopoiesis in the bone marrow of adult mice [21, 22]. Conditional ablation of osteoblasts in adult mice demonstrated an early loss of B lymphocyte and erythroid progenitors as well as hematopoietic stem cells demonstrating that osteoblasts can affect multiple hematopoietic lineages. Multiple groups have confirmed a supportive role for osteoblasts in the regulation of HSCs, B-lymphocytes, and erythrocytes [23, 24]. However, whether osteoblasts regulate HSCs through direct or indirect mechanisms remains unclear. Recent data suggest that osteoblasts may indirectly regulate HSCs by modulating the vascular cell niche (for a recent review on this topic please see [25]). Despite recent advances in identifying osteoblasts as an essential cellular component of the bone marrow for skeletal and hematopoietic development and homeostasis, little is known regarding the mechanisms by which osteoblasts coordinate these processes.

Osteoblasts are a Cellular Source of EPO

Studies aimed at elucidating the role of osteoblastic hypoxia inducible factor (HIF) signaling in bone homeostasis and hematopoiesis revealed that EPO is a direct HIF target in osterix expressing cells. Conditional inactivation of VHL or PHDs 1–3, the primary negative regulators of HIF-1 and HIF-2, in Osterix-Cre expressing cells resulted in a HIF-2 dependent activation of EPO in bone that lead to the development of polycythemia in adult mice [26••]. Conversely, genetic inactivation of HIF-2 in osterix expressing cells resulted in a significant decrease in EPO expression in neonatal hindlimbs [26••]. These findings were the first to demonstrate that HIF signaling in cells of the osteoblastic lineage regulate EPO expression in bone under physiologic and pathophysiologic conditions. While hypoxia and activation HIF transcriptional activity are the primary physiologic stimuli for EPO expression, this finding was unexpected given the tight regulation of EPO by developmental and tissue specific factors. During development, the physiological source of EPO switches from the fetal liver to the kidney where it is estimated that peri-tubular interstitial fibroblasts

in the kidney are responsible for 70 %–90 % of total EPO production in adult mammals [27]. In most other cell types, EPO expression is tightly repressed.

Using transgenic mouse lines expressing GFP under the control of a 180 kb EPO gene locus, Obara and colleagues discovered that a GATA box located within the EPO promoter is responsible for the repression and cell type specific regulation of EPO in specific populations of epithelial cells within the kidney, liver, lung, and thymus [28]. In bone, EPO expression was detected in neonatal hindlimbs however, EPO expression was not detectable in adult bone [26••]. The mechanisms responsible for the temporal regulation of EPO in bone remain unknown. One possibility is that similar to the liver, EPO expression in bone is inactivated postnatally through GATA mediated repression. Another possibility is that the Osterix positive cells expressing EPO in bone are depleted postnatally. Mizoguchi et al recently discovered that osterix marks distinct waves of primitive and definitive stromal progenitors during bone marrow development [29•]. Notably, it was observed that osterix positive progenitors in the fetal bone marrow contribute to nascent bone tissue and transient stromal cells that are replaced in the adult bone marrow [29•]. Thus, it is tempting to speculate that EPO may be expressed by osterix primitive stromal progenitors.

EPO and Hematopoiesis

The primary function ascribed to EPO is in the regulation of erythropoiesis. EPO mediated activation of the EPO receptor (EPOR) on erythroid progenitor cells stimulates JAK2, STAT5, PI3 kinase/Akt, and MAP kinase signaling pathways to promote cellular survival, proliferation, and differentiation [27]. Consistent with an important role for EPO in the regulation of erythropoiesis, genetic inactivation of EPO in mice results in embryonic lethality at E13.5 as a result of cardiac failure and anemia [30]. In contrast, overproduction of EPO results in the development of polycythemia [27]. Indeed persistent EPO production by osteoblasts was associated with a significant increase in erythroid progenitors in the bone marrow and spleen leading to the development of severe polycythemia in adult mice [26••]. These findings demonstrate that osteoblastic EPO has the ability to directly stimulate erythropoiesis in the bone marrow microenvironment and may have important implications for the treatment of renal anemia (for a recent review see [31]).

EPO and Bone Formation

In addition to regulating erythropoiesis, EPO has also been implicated in the regulation of bone formation and repair. One of the first experimental connections between erythropoiesis and bone formation was observed in rats where bleeding was shown to significantly increase mineral apposition rate, osteoblast number, and serum levels of osteogenic growth peptide [32]. Subsequent studies demonstrated that EPO alone is sufficient to increase bone volume and repair. EPO treatment enhanced both bone volume and biomechanical properties in multiple murine femoral fracture repair models [33–35]. Similarly, EPO stimulated BMP2-induced bone formation in cranial defect and scaffold models [36, 37]. Studies investigating the effects of EPO on bone are not limited to mice. 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 [38]. Additionally, EPO treatment increased bone healing in porcine

osteochondral and cranial defect models [39, 40]. These studies demonstrate an osteogenic role for EPO and indicate a potential therapeutic role for EPO in bone healing.

The role of EPO in skeletal development remains largely unknown. Shiozawa and colleagues demonstrated that exogenous EPO treatment (6000 U/kg) is sufficient to induce bone formation in neonatal and adult mice [41]. In addition, OSX-VHL mice with constitutive production of EPO in bone exhibited excessive accumulation of trabecular bone in the metaphyseal and diaphyseal regions of the long bones [26••]. While these studies demonstrate that elevated levels of EPO are associated with increased bone volume in bone remodeling studies, future studies are needed to determine the role of EPO in skeletal development.

The mechanisms by which EPO stimulates bone formation and repair remain unclear. Many of the studies described above reported that EPO-mediated bone formation and healing was associated with increased vascular density and angiogenesis. EPO treatment was shown to increase endothelial sprouting from metatarsal bones of E17.5 embryos, induce the vasculature in bone in vivo, as well as stimulate endothelial cell proliferation in vitro [33–36]. These findings are consistent with previous reports that EPO signaling regulates angiogenesis [27]. Most notably, genetic inactivation of EPO and EPOR in mice results in angiogenic defects during embryonic development [30, 42]. Given the important role of angiogenesis in bone formation and healing it is hypothesized that EPO-mediated bone formation may be at least in part through the regulation of angiogenesis.

In addition to stimulating endothelial cells and angiogenesis, EPO has also been reported to stimulate the activities of mesenchymal stromal and hematopoietic stem cells to support osteogenesis. A direct role for EPO in inducing osteoblastic differentiation has been suggested as EPO treatment induced an osteoblastic phenotype in both human mesenchymal and mouse bone marrow stromal cells [41, 43, 44]. In addition, an indirect role for EPO in stimulating HSCs to induce osteoblastic differentiation has been reported. Shiozawa et al demonstrated that EPO/EPOR signaling on HSCs activates downstream JAK/STAT signaling to stimulate the secretion of bone morphogenetic protein and bone formation [41]. However, it is important to note that the mechanisms by which EPO regulates the activities of mesenchymal, stromal cells, and HSCs are unclear as the status of EPO receptor (EPOR) expression on these cells is highly controversial. The specificity of the commercial EPOR antibodies used in these studies to detect EPOR on stromal cells and HSCs have been called into question [45]. Furthermore, lineage-tracing studies in which EPO-R-Cre mice were crossed to Rosa26 YFP reporter mice indicate that neither LKS+CD150+CD48-HSCs, mesenchymal, or osteoblastic enriched populations from mouse bone marrow express EPO-R-Cre [46]. Thus, future studies genetically targeting EPOR within specific cellular subsets within the bone marrow microenvironment are needed to determine the mechanisms by which EPO regulates bone remodeling and repair.

Conclusions

Increasing evidence supports a role for EPO signaling in bone remodeling and repair. The majority of studies in which exogenous EPO was administered in vivo have demonstrated a

role for EPO in promoting bone repair and remodeling (Table 1). In addition, a recent study demonstrated that persistent production of EPO by osteoblasts is associated with increased trabecular bone volume in adult mice. While the mechanisms by which EPO enhances bone volume remain unknown, the effects of EPO on bone have been associated with increased osteogenesis, osteoclastogenesis, and angiogenesis. It will be important in future studies to identify which cell types within the bone marrow microenvironment directly contribute to EPO-mediated bone remodeling and repair.

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Table 1

Effects of EPO on bone

Source	Model	Phenotype	Reference
Osteoblast (OSX-VHL)	Remodeling (mouse)	Increased trabecular bone volume associated with increased angiogenesis and erythropoiesis.	Rankin et al.
EPO (4500; 6,000 U/Kg)	Remodeling (mouse)	Increased bone volume in neonatal and adult mice associated with increased osteoblasts and erythropoiesis.	Shiozawa et al.
EPO (300 U/Kg)	Remodeling (mouse)	Modest decrease in bone volume.	Singbrant et al.
EPO (5000 U/Kg)	Repair (mouse)	Increased torsinal stiffness, callus density, and mineralized bone.	Holstein et al.
EPO (40 ng)	Repair (mouse)	Increased cartilaginous callus formation and bone healing associated with increased angiogenesis.	Wan et al.
EPO (1000 U)	Repair (mouse)	Increased BMP-2 induced bone regeneration in a cranial defect model associated with enhanced angiogenesis.	Sun et al.
EPO (500 IU)	Repair (mouse)	Increased bone volume in an bridging calvarial defect model.	Nair et al.
EPO (500 IE/Kg)	Repair (mouse)	Increased bone volume and repair in an femoral segmental defect model associated with increased angiogenesis.	Holstein et al.
EPO (500 U/Kg)	Repair (mouse)	Increased callus formation in a closed femoral fracture model.	Garcia et al.
EPO (250 IU/Kg)	Repair (rabbit)	Increased bone fusion in a posterolateral spinal fusion model associated with enhanced angiogenesis.	Rolfing et al. (2011)
EPO (900 IU)	Repair (porcine)	Modest increase in bone formation in a calvarial defect model.	Rolfing et al. (2013)
EPO (900 IU)	Repair (porcine)	Increase in bone formation when combined with bone marrow concentrate in a osteochondral defect model.	Betsch et al.

EPO erythropoietin