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Review article

Bidirectional ephrin signaling in bone

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Abstract

The interaction between ephrin ligands (efn) and their receptors (Eph) is capable of inducing forward signaling, from ligand to receptor, as well as reverse signaling, from receptor to ligand. The ephrins are widely expressed in many tissues, where they mediate cell migration and adherence, properties that make the efn-Eph signaling critically important in establishing and maintaining tissue boundaries. The efn-Eph system has also received considerable attention in skeletal tissues, as ligand and receptor combinations are predicted to mediate interactions between the different types of cells that regulate bone development and homeostasis. This review summarizes our current understanding of efn-Eph signaling with a particular focus on the expression and functions of ephrins and their receptors in bone.

Keywords: Ephrins; Signaling; Bone; Osteoblast; Osteoclast

1. Introduction

Ephrin ligands (also known as efn) are a family of proteins that serve as the ligands of ephrin receptors (Eph), which represent the largest known subfamily of receptor tyrosine kinases (RTKs). Both efn ligands and Eph receptors are membrane bound proteins that exert many important functions in a variety of tissues during development and adulthood. In bone, a number of efn ligands and Eph receptors are known to be expressed in many cell types and have been shown to play important roles in communication between osteoblasts and osteoclasts that regulate their differentiation [reviewed in Ref. [1]]. In addition to propagating Eph receptor mediated forward signaling, one unique property of the efn ligands is that they all have the capacity to initiate a "reverse" signal that is distinct from the "forward" signal associated with activation of their corresponding receptors. Efn-Eph signaling is also known to interact with other growth factor signaling pathways that have been implicated in skeletal development [2–4] and maintenance [5,6]. In this review, we will focus on expression patterns of efn ligands and Eph receptors in bone and their mechanism of action in regulating skeletal development and homeostasis.

2. Ephrin ligand and receptor families

The efn ligands are comprised of two subfamilies, the GPI-anchored efnA family and the transmembrane efnB family. Eph receptors (EphA and EphB) belong to the RTK superfamily. In general, efnA ligands bind to EphA receptors while, with a few exceptions such as EphA4 and A5, efnB ligands bind to EphB receptors. However, the affinities between ligand and receptor pairs can vary depending on the ligand or receptor (Table 1). Interaction of the efnA ligand with its receptor displays a "lock-and-key" binding, while efnB ligand binding proceeds through conformational changes that result in an "induced fit" with its receptors [7]. The promiscuity of binding suggests that there is

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Table 1
Mammalian ephrin ligand-receptor binding specificity.

Ligand		efnA						efnB		
		A1	A2	A3	A4	A5	A6	B1	B2	B3
Receptor	EphA1	++	–	–	–	–	–	–	–	–
	A2	++	+	+++	B	B	B	–	–	–
	A3	+	+++	++	++	+++	B	+	+	B
	A4	+++	++	++	B	+++	B	–	++	B
	A5	++	++	++	B	+++	B	–	–	–
	A6	B	B	B	B	B	B	–	–	–
	A7	++	+++	++	B	B	B	–	–	–
	A8	B	++	++	B	+++	B	B	B	B
	A10	ND	ND	ND	ND	ND	ND	ND	ND	ND
	EphB1	++	–	++	++	–	ND	++	++	B
	B2	–	–	–	–	–	ND	+++	+++	B
	B3	ND	–	ND	ND	ND	ND	+++	+++	++
	B4	ND	–	ND	ND	ND	ND	+	+++	B
	B6	ND	ND	ND	ND	ND	ND	B	B	B

Binding affinities determined by ligand-receptor binding assays [reviewed in 72].
+: low, Dissociation Constant (K_d) greater than 10 nM.
++: medium, K_d 1 nM–10 nM.
+++: high, K_d less than 1 nM.
–: no binding detected.
B: ligand-receptor binding observed, but no data on binding affinity [19,72].
ND: no data on binding [72].
EphA9 and EphB5 are avian. EphA10 is not yet characterized for ligand binding.

considerable biological redundancy in efn-Eph functions within the ephrin family. The complexity of signaling is also dependent on ligand and receptor expression in *cis* versus *trans* signaling, which can involve clustering of ligands and receptors into higher order structures within and between cells to further modulate signaling [8,9]. Interactions with other intracellular signaling pathways also occur. Signaling is terminated through the cleaving of the extracellular ligand-receptor domains or the endocytosis of the complex [reviewed in Ref. [10]]. Efn-Eph signaling is therefore complex and exerts multitude functions beyond simple expression and binding of cognate ligands and receptors.

The interaction of ephrin ligands and receptors can activate a bidirectional signal in which receptor (forward) or ligand (reverse) signaling activate downstream signaling cascades to produce various outcomes [reviewed in Refs. [11,12]]. Forward signaling is generally mediated through phosphorylation and activation of the receptor protein-tyrosine kinase activity. While reverse signaling is known to be mediated through the PDZ (postsynaptic density 95/discs-large/zona occludens-1) binding domain in efnB ligands, it is not well characterized in the efnA ligands, which lack an intracellular domain. EfnA ligand reverse signaling is therefore thought to proceed through associations with non-ephrin intracellular partners. These efn-Eph bidirectional signaling mechanisms can mediate complex functions within and between tissues, and are therefore of great interest in tissue development and repair.

3. Role of efn-Eph signaling in tissue development

Efn-Eph signaling is predicted to be an important mediator of tissue development and patterning, where efn-Eph interactions define the spatial boundaries between tissues and maintain segment boundaries. The efn-Eph system have been established to regulate both cell adherence and cell migration during development, and can act as attractants and repellants between cells within and between tissues, but these functions can depend on the cell context in which they are expressed [reviewed in Ref. [10]].

During embryonic development, efnB2 patterns the somites and regulates neural crest cell migration [13,14]; these structures contribute to the formation of a diverse set of tissues, including skeletal tissues. Efn-Eph regulation of neurogenesis has been one of the better studied areas of tissue development [reviewed in Ref. [15]], and several ephrin ligands and receptors expressed in neural tissues have been identified in bone. Studies in knockout mice identified efnB-EphB forward [16] and reverse [17] signaling as key in regulating axon fasciculation and guidance, respectively. The efnA family also regulates axon guidance in chick embryos. In this case the relative cell surface levels of efnA ligand and EphA receptor expression determine the migration of axon growth cones, with forward signaling generally inhibiting and reverse signaling generally promoting the growth cone survival [8]. It is probable that the efn-Eph regulation of neural tissue development might also be important for bone development, as the periosteum, which mediates bone growth and repair, is a highly innervated tissue.

Ephrin regulation of the vasculature has also received considerable attention, and is especially important in bone, a normally highly vascular tissue that must establish the blood supply during development and re-establish it under the hypoxic conditions that result from tissue injury. The ossification process itself requires the entry of osteogenic cells to the

nascent bone through the vasculature. Ephrins regulate the formation of networks of embryonic blood vessels through reciprocal repulsive cell–cell signaling [18,19]. As in neural tissues, several members of the efn A and B families of ligands and receptors expressed in bone are also expressed in endothelial cells, suggesting that they regulate communication between bone cells and endothelial cells in the developing skeleton. For example, EfnB1 and EphB2 participate in sprouting and epithelial–mesenchymal interactions [18], and efnB2 [20] and EphB4 expression marks the boundaries of the arterial and venous endothelium, respectively [reviewed in Ref. [21]]. Global knockout mice lacking EphB2 exhibit severe defects in angiogenesis of both arteries and veins and do not survive, implicating this ligand in the endothelial and mural cell motility and adhesion in the cardiovascular system [22]. *In vitro*, efnB1 expression is induced in response to stimulation by vascular endothelial growth factor (VEGF) in mesenchymal stem cells [23], suggesting that cells with osteogenic potential can mediate angiogenesis. EphB1 signaling to efnB1 promotes integrin-mediated cell motility and attachment in Chinese Hamster Ovary (endothelial) cells [24]. The number of ephrin ligands and receptors expressed in bone and in the developing vasculature and the importance of angiogenesis in bone development and repair suggest that the efn-Eph signaling is critical in establishing and maintaining vascularity in bone.

4. Role of efn-Eph signaling in tissue repair and pathology

Ephrin ligands and receptors are expressed during tissue repair, with expression best characterized in ischemia and optic nerve spinal cord injuries. Ephrin ligands and receptors also function in inflammatory conditions [reviewed in Ref. [25]]. EfnB1 and EphB1 enhance lymphocyte migration in rheumatoid arthritis [26], which could contribute to the inflammatory reaction in this pathologic condition, suggesting that they can also mediate a normal tissue post-injury inflammatory response. EfnB1 and efnB2 mediate PECAM-1 functions in endothelial cells, destabilizing endothelial cell junctions and promoting the motility of monocytes expressing

Table 2
Efn ligand and Eph receptor expression in skeletal tissues.

Ephrin		Skeletal tissue expression		Reference
efn (ligand)	Cell/Tissue type	Approach	Effect	
efnA1	Osteoblast precursors/osteoclast precursors	RT-PCR	Enhanced osteoclastogenesis	[35]
efnA2	Osteoblast precursors	RT-PCR	Increased EphA2 osteoclastogenesis	[35]
efnA4	Osteoblast precursors	RT-PCR		[35]
efnA5	Osteoblast precursors	RT-PCR		[35]
efnB1	Osteoblast precursors/osteoclast precursors	RT-PCR		[5]
	Limb prechondrogenic condensations	IHH		[2]
efnB2	Osteoblast precursors/osteoclast precursors	RT-PCR, Western Blot		[5]
	Embryonic calvarial sutures	lacZ expression		[73]
	Osteoblasts/osteocytes/osteoclasts	Microarray/RT-PCR/IHH	Response to PTH, PTHrP	[38]
	Osteoblasts/osteocytes/osteoclasts	IHH, IGF-1R KO		[39]
	OA gene expression	*In vitro* activation		[49]
Eph (receptor)	**Cell/Tissue type**	**Approach**	**Effect**	
EphA1	Osteoblast/osteoclast precursors	RT-PCR		[35]
EphA2	Osteoblast/osteoclast precursors	RT-PCR, Western Blot	Reduced efnA2 osteoblastogenesis	[35]
EphA3	Osteoblast precursors	RT-PCR		[35]
	Bone marrow MSC	Microarray/RT-PCR	Response to hypoxia	[74]
EphA4	Osteoblast/osteoclast precursors/osteoclasts	RT-PCR		[5,35]
	Chicken limb bud	IHH		[34]
EphA7	Osteoblast precursors	RT-PCR		[35]
	HoxA13 KO limb mesenchyme	IHH, ISH		[33]
EphB1	Calvarial osteoblasts	RT-PCR		[5]
	Embryonic calvarial sutures	lacZ-knockin		[73]
EphB2	Osteoblast precursors	RT-PCR		[5]
	Embryonic rib perichondrium, calvarial sutures	lacZ-knockin		[73]
	Limb mesenchyme	IHH		[2]
EphB3	Osteoblast precursors	RT-PCR		[5]
	efnB1 mutant palate	IHH		[41]
	Limb mesenchyme	IHH		[2]
EphB4	EphB4 transgenic osteoblast precursors	RT-PCR		[5]
	Fracture hypertrophic chondrocytes, osteoblasts	Col-I transgenic	Increased fracture callus bone	[48]
	OA subchondral bone	IHH, RT-PCR		[49]
EphB6	Osteoblast precursors	RT-PCR		[5]

IHH, immunohistochemistry; ISH, *in situ* hybridization; KO, knockout; MSC, mesenchymal stem cells; OA, osteoarthritis; RT-PCR, reverse transcription-polymerase chain reaction.

Table 3
Models of skeletal efn ligand and Eph receptor function.

Ephrin	Model	Biological effects	Reference
efn (ligand)			
efnA1	Global KO	Vertebrae number, vertebrae transformation, kinked tail vertebrae	[75]
efnA2	*In vitro*	Increased osteoclastogensis, decreased osteoblastogenesis	[35]
efnA4	Mutant	Cranial development	[76]
efnB1	Mutant	Craniofacial development	[41]
	PGK KO	Perinatal lethal, rib, sternum, carpal patterning	[2]
	Global KO	Perinatal lethal, polydactyly	[3]
	Neural crest cell KO	Craniofacial development	[3]
	PDZ mutant	Craniofacial development	[3]
	OB KO	Calvarial development, decreased bone size, mineralization	[36]
	Myeloid KO	Increase OC differentiation, resorption	[37]
	In vitro	Osteoblasts in response to alendronate	[55]
	In vitro	Human MSC chondrogenic/osteogenic differentiation (with efnB2)	[40]
	Col-I transgenic	Bone formation response	[43]
efnB2	OB KO	Decreased osteoblast differentiation, increased apoptosis	[53]
	OC KO	Decreased osteoclast differentiation	[5]
	In vitro	Human MSC osteogenic differentiation	[40]
	In vitro activation	OA gene expression	[50]
Eph (receptor)			
EphA1	Global KO	Kinked tail vertebrae	[77]
EphA2	*In vitro*	Osteoclast/osteoblast precursors	[35]
	Global KO	Kinked tail vertebrae	[78]
EphA4	*In vitro*	Osteoclast/osteoblast precursors	[35]
	In vitro	Reduced osteoclast activity	[47]
	Global KO	Craniosynostosis	[44]
EphB1	Global KO	Reduced calvarial bone	[73]
	In vitro	Osteoblasts in response to alendronate	[55]
EphB2/EphB3	PGK KO/truncation	Cleft palate	[42]
EphB3	*In vitro*	Osteoblasts in response to alendronate	[55]
EphB4	*In vitro*	Growth plate prehypertrophic chond, OB, OC differentiation	[39]
	Col-I transgenic	Promotes fracture callus endochondral ossification, inhibits remodeling	[48]
	IHH, RT-PCR	*In vitro* efnB2 activation of OA subchondral bone	[50]

Chond, chondrocyte; IHH, immunohistochemistry; KO, knockout; MSC, mesenchymal stromal cell; PDZ, postsynaptic density 95/discs-large/zona occludens-1 domain; OA, osteoarthritis; OB, osteoblast; OC, osteoclast; PGK, phosphoglycerate kinase; RT-PCR, reverse transcription-polymerase chain reaction.

EphB2 [27], evidence that implicates efn-Eph signaling as a normal component of the inflammation that occurs in post-injury tissues.

Importantly, the involvement of efn-Eph signaling in cell motility and adhesion also implicates dysregulation of efn-Eph signaling in cancer progression and tumor growth [reviewed by Refs. [28,29]], including bone-related cancers such as multiple myeloma and osteosarcoma [reviewed in Ref. [30]]. EphB2 can mediate cell migration or adenoma cell proliferation to carcinoma, depending on whether the intracellular signaling is phosphatidylinositol 3-kinase (PI3-K)-mediated or cyclin D1-mediated, respectively [31]. The recently described EphA10, although also expressed in several normal tissues, is expressed in triple negative breast cancers [32]. These observations establish the efn ligands and their receptors as critical mediators of cell motility in pathologic conditions, including those of skeletal tissues.

5. Efn-Eph expression in bone

While the expression of efn ligands and their receptors have been more extensively studied in the context of nervous system development, efn-Eph expression in bone development remains only partially characterized. Ephrin ligands and their receptors are expressed in embryonic tissues that form the skeleton (Table 2). Specifically, efnB1 is expressed in the prechondrogenic mesenchymal condensations and efnB2 is expressed in the embryonic calvarial sutures. EfnB1, EphB2 and EphB3 are expressed at the tips of the ribs where they would be expected to regulate rib development. With respect to Eph receptors, EphB1 and EphB2 are also expressed in the embryonic calvarial sutures, and EphA3 and EphA7 are expressed in the limb mesenchyme [33]. EphB4 is expressed in the chondrocytes, osteoblasts and osteoclasts of the growth plate. EphA4 is expressed in the prechondrogenic limb mesenchyme of the chicken [34]. These associations suggest that these ligands and receptors might be binding partners that regulate the development and homeostasis of both intramembranous and endochondral bone.

Studies at the cellular level have identified multiple efn ligands and Eph receptors expressed by cells of osteoblast and osteoclast lineage, as well as their precursors [5,35]. EfnB1, efnB2 and EphB receptors are expressed throughout osteoblast development, but more restricted in osteoclast precursors.

Studies involving conditional disruption of efnB1 in osteoblasts [36] and osteoclasts [37] have revealed that efnB1 expressed in both osteoblasts and osteoclasts regulates skeletal development by influencing bone formation and bone resorption processes, respectively.

The expression of both ligands and receptors is predicted to be under the control of osteoregulatory agents; for example, parathyroid hormone (PTH) and parathyroid hormone related protein (PTHrP) are known to regulate efnB2 expression in osteoblasts. The treatment of mice with PTH and PTHrP, both established enhancers of bone formation, resulted in an increase in efnB2 gene expression in mouse bone marrow stromal cells, suggesting that this ligand promotes PTH-mediated bone formation [38]. Other osteogenic growth factors have been associated with ephrin regulation. Studies of bone cells isolated from osteocalcin cre-driven (i.e., osteoblast-specific) insulin-like growth factor (IGF)-I receptor (IGF-1R) knockout mice demonstrated that inhibition of efnB2 and EphB4 communication impairs IGF-I-mediated differentiation of osteoblasts and osteoclasts in co-cultures. Col-II cre-driven (i.e., chondrocyte-specific) IGF-IR knockout mice exhibited a decreased expression of efnB2 and EphB4 *in vivo*. Inhibition efnB2-EphB4 signaling in a chondrocyte cell line *in vitro* performed in these studies identified a reduced expression of collagen markers of chondrocyte differentiation. Taken together, these results indicate that EphB4 and efnB2 expression in growth plate chondrocytes, and EphB4 and efnB2 expression in growth plate osteoblasts and osteoclasts, respectively, promote bone cell development and endochondral bone growth [39].

6. Role of ephrins in skeletal development

Identification of the molecular pathways that regulate bone formation is important to understand bone regeneration and to develop therapeutic applications for bone injuries and diseases. Recent studies that target disruption of efnB ligands and their receptor genes to skeletal tissues *in vivo*, together with *in vitro* studies, now demonstrate a significant role for the efn ligands and their receptors in bone development (Table 3). For example, efnB1 and efnB2 interactions with the EphB receptors mediate the diverse processes of marrow-derived mesenchymal stromal cell attachment and spreading, and chondrogenic and osteogenic differentiation, respectively [40].

The initial demonstration of efnB1 effects on bone formation was derived from a mutant efnB1 mouse, which displayed craniofacial deformities in skeletal patterning. Mice with a disruption of efnB1 expression exhibited similar characteristics to cleft palate seen in patients with efnB1 mutations [2,3], where efnB1 specifies the position of the frontal coronal suture. The efnB1 knockout models have also identified ligand functions in the regulation of limb development, with efnB1 dysregulation producing polydactyly in a knockout mouse strain [2,3]. These studies established that efnB1 regulates skeletal patterning by defining tissue boundaries, particularly those of the mesenchymal-perichondral tissues during the development of the digits and the ribs, where it mediates the splitting of mesenchymal condensations to individual ribs or digits [3].

Changes in EphB3 expression in this efnB1 mutant identified EphB3 as a possible binding partner for efnB1 regulation of craniofacial development [41]. However, the single disruption of EphB1, B2, B3 or A4 receptors, the major receptors of efnB1 signaling, did not produce calvarial or other skeletal defects, although the combined deficiency of both EphB2 and EphB3 in a knockout/mutant mouse line resulted in a cleft palate [42] similar to the skeletal patterning defect observed in the efnB1 knockout [2]. These results suggest a degree of functional redundancy among Eph receptors in this tissue.

Although presenting a dramatic phenotype for the study of bone development, the global efnB1 knockout was also embryonic lethal. With respect to conditional knockouts, Wnt-cre-driven deletion of efnB1 in neural crest cells also produced the craniofacial phenotype. Additionally, the palatal defect was observed in an embryonic PDZ signaling mutant, establishing efnB1 reverse signaling functions for this phenotype [3]. Because efnB1 mediates several aspects of skeletal development, viable conditional knockouts with efnB1 deficiency in a specific skeletal tissue present better models for bone metabolism studies in adult subjects.

Recent findings in conditional knockout mouse models have further demonstrated an important role for ephrins in bone metabolism. The osteoblast-specific knockout of efnB1 in mice resulted in a reduced body length, smaller bone size, and reduced bone formation at three weeks of age [2]. A comparison of the bones in osteoblast-specific efnB1 knockout mice and wild-type mice by dynamic histomorphometry established that efnB1 expression promoted bone growth at the periosteum [36]. Conversely, transgenic mice that overexpress efnB1 in osteoblasts under the control of the Col-I promoter displayed an enhanced periosteal bone formation in response to mechanical loading [43]. These data indicate that efnB1 functions regulate bone formation at the periosteum during skeletal development and in response to bone injury, and that manipulating efnB1 expression to promote bone formation similar to that observed in development might indeed be applicable for fracture therapy.

In addition to the different RTK pathways associated with forward efnA and B ligand signaling to EphA and B receptors, different cell surface and growth factor family and signaling molecule proteins have been demonstrated to interact with EphA and EphB receptors. These cell surface interactions provide additional complexity to EphA and EphB receptor signaling pathways. Proteins interacting in *cis* with Eph receptors include claudin-4 with EphA2, “a disintegrin and metalloproteinase domain-containing protein” (ADAM)10 with EphA3, the fibroblast growth factor receptor (FGFR) family with EphA4 and E-cadherin with EphB3. Protein interactions in *cis* have been experimentally demonstrated for connexin43 and γ-secretase with efnB1 and B2, metalloproteinase with efnB2 and the N-methyl-D-aspartate receptor (NMDAR) with efnB2 and B3. Cell surface protein interactions in *trans* have also been demonstrated between efnB1 and claudins 1 and 4 [reviewed in Ref. [12]].

7. Ephrin forward and reverse signaling in bone

Ephrin signaling promotes bone and cartilage formation, and regulates bone formation and bone remodeling to maintain skeletal homeostasis. Because bone cells express ligands and receptors of the efnA and B families (Table 2), the activation of forward and/or reverse signaling and subsequent regulation of cell function is probably frequent, given the cell-to-cell contact between bone cells. Bidirectional ephrin signaling between osteoblasts and osteoclasts that coordinates their development has been referred to as "coupling", although a conclusion of activation or differentiation as a direct result of forward and reverse signaling between an interacting efn-Eph pair must also consider the possibility that the cellular response does not utilize this efn-Eph pair.

7.1. EfnA-EphA forward signaling

In addition to intracellular interactions signaling through the tyrosine kinase domain, forward signaling to EphA receptors can involve a variety of interactions with ephrin and non-ephrin proteins that introduce additional layers of complexity to ephrin signaling [1].

Knockout mouse studies have demonstrated that EphA receptor signaling regulates bone formation. The inhibition of EphA4 signaling in EphA4 knockout mice demonstrates that EphA4 results in craniosynostosis. Forward signaling through EphA4 normally excludes osteogenic precursor cell migration to the coronal suture of the calvaria. The failure to limit this migration in the EphA4 knockout mouse, as well as the disruption of EphA4 mediation of Twist1 functions, produces the calvarial suture fusion in this condition [44].

EfnA-EphA signaling regulates angiogenesis, an important aspect of bone formation and repair. *In vitro* inhibition of EphA2 expression and functions promote endothelial cell migration and angiogenesis in response to VEGF but not fibroblast growth factor (FGF)2, growth factors both expressed in bone, suggesting potential modulation of angiogenesis by ephrin and other growth factor families in the bone vasculature [45].

EphA receptor signaling has been associated with several different malignancies [reviewed in Ref. [28]], including the metastasis of prostate cancer to bone, although this dysregulation is not well studied. Studies also indicate that EphA2 forward and reverse signaling functions can even display ligand-independent functions in mediating opposite directions of the epithelial mesenchymal transition (EMT), with ligand-independent signaling promoting the mesenchymal–epithelial transition (MET) and ligand-independent signaling promoting EMT [reviewed in Ref. [46]]. Although EphA2 is expressed during bone cell differentiation, ligand-independent EphA receptor signaling has not been characterized in bone. Forward signaling in the ephrinA family is discussed in other reviews [1,46].

7.2. EphA-efnA reverse signaling

EfnA ligands and EphA receptors also mediate communication between osteoblasts and osteoclasts in bone cell development through forward and reverse signaling. *In vitro* studies have demonstrated that efnA2 expression was induced by NF-κB in osteoclasts, consistent with RANK regulation of osteoclast development, and was c-Fos dependent, indicating that efnA2 promoted the development of osteoclast precursors. Because EphA2 expression also suppressed osteoblast differentiation in co-culture with osteoclasts, efnA2-EphA2 signaling between osteoclasts and osteoblasts, respectively, would be expected to promote osteoclast differentiation and enhance bone remodeling over bone formation prior to efnB-EphB regulation of this circuit [35]. *In vivo*, the deletion of EphA4 in osteoclasts promoted osteoclast activity and bone remodeling, reduced cortical and trabecular bone volume and mineralization, thus establishing its importance as a negative regulator of osteoclast activity [47]. Mechanistically, EphA-efnA reverse signaling is not well characterized, but EphA4 might act by sequestering the efnA2 ligand to modulate its signaling in osteoclast development [35]. Alternatively, because EphA4 is one of the EphA receptors that can bind efnB ligands, its binding could mediate reverse signaling through efnB1 or efnB2 in pre-osteoblasts, osteoblasts and osteoclasts, much as EphB receptors mediate reverse signaling through efnB ligands (Table 1).

7.3. EfnB-EphB forward signaling

As with the EphA receptors, forward signaling to EphB receptors can also involve a variety of intracellular interactions that mediate the interactions of ephrin and non-ephrin proteins and introduce additional layers of complexity to ephrin signaling [1].

EfnB1 and EphB receptors are expressed in bone and the vasculature (Table 2), and might regulate bone and vascular cell communication. EfnB1, efnB3 and EphB2, EphB3 and EphB4 regulate the formation of the vasculature. EfnB2 pericyte/vascular smooth muscle cell knockout mice exhibit defective vessel development due to defective cell motility, migration and cell matrix adhesion [22]. Along with EphB4 expression in venous cells, efnB2 defines the arterial and venous boundaries of the developing vasculature [18–20]. Because the vasculature is critical to developmental ossification and reestablishing the blood supply is so important for bone repair, further investigation is needed to establish ephrin communication between the bone and vasculature.

With respect to bone repair, Col-I (osteoblast)-specific overexpression of EphB4 in mice increases fracture callus bone formation and inhibits remodeling, although this regulation has not been fully characterized [48]. Forward signaling through the EphB receptors has also been associated with pathologic skeletal conditions. EphB4 expression was increased in osteoarthritis (OA) chondrocytes but reduced in response to efnB2 treatment [49]. Further studies demonstrated that the *in vitro* application of efnB2 to osteoarthritic (OA) chondrocytes [50] and resorption-promoting subchondral bone osteoblasts inhibited the expression of the inflammatory genes interleukin (IL)-1β and IL-6, as well as the matrix degrading matrix metalloproteinase (mmp) genes

mmp-1, mmp-9 and mmp-13. These results indicate that a therapeutic approach for alleviating cartilage degradative enzyme functions in OA that regulates the expression of these two ephrins in OA chondrocytes, and in what the authors refer to as "proresorption" osteoblasts, might indeed be utilized to inhibit abnormal resorption in OA [50].

7.4. EphB-efnB reverse signaling

Reverse signaling through efnB1 was demonstrated to be important in bone development in an efnB1 signaling-deficient mutant. The global efnB1 knockout mouse exhibited a wide range of skeletal phenotypes attributed to impaired forward signaling, but a mutation that deleted the efnB1 PDZ domain produced a mouse with defective neural crest cell migration, an effect attributed to reverse signaling through efnB1 [3]; this approach resolved efnB1-mediated forward and reverse signaling in skeletal development. Several EphB receptor candidates are expressed in osteoblast development that could mediate reverse signaling through efnB1. Other studies established that reverse signaling through EphB2 and mediated by PI3-kinase was enhanced in TGFβ3-dependent palate fusion in the chicken [51], demonstrating skeletal defects similar to the reverse signaling functions of efnB1 deficiency. In agreement with these conclusions, the application of EphB2 increased Osterix expression and bone formation marker expression in an osteoblast-specific efnB1 knockout mouse [36].

EfnB2 signaling also regulates cartilage remodeling. In cartilage, efnB2 forward and reverse signaling might proceed through the sequestration of efnB2 expression in chondrocytes, regulating the expression of the cartilage remodeling enzyme "a disintegrin and metalloproteinase domain-containing protein with thrombospondin motifs" (ADAMTS) 4 in Osterix-expressing hypertrophic chondrocytes [52]. This study postulates that the efnB2-dependent production of cartilage degrading enzymes such as ADAMTS4 that are required for osteoblast and osteoclast attachment to the cartilage is impaired, which inhibits remodeling during endochondral bone formation.

Studies indicate that the differential expression of efn ligands and Eph receptors and forward and reverse signaling between osteoblasts and osteoclasts might coordinate bone formation and bone resorption. EfnB2-EphB4 signaling between osteoclasts and osteoblasts has been postulated to coordinate osteoclast-mediated resorption with osteoblast-mediated bone formation *in vitro*, as well as *in vivo* in knockout and transgenic mouse models. Transgenic overexpression of EphB4 in osteoblasts resulted in increased bone formation, thus suggesting a role for forward signaling in bone formation, while osteoclast lineage deletion of efnB2 increased bone resorption, suggesting that efnB2 reverse signaling suppresses osteoclast formation, possibly by inhibiting RANKL expression [5]. When efnB2 deletion from osteoblasts was driven by Osterix-cre expression, osteoblast differentiation and bone formation were impaired and there was an increase in expression of the markers of apoptosis, indicating that efnB2 signaling mediated osteoblast survival [53]. Further evidence in support of this regulation was obtained in studies where the inhibition of EphB4 communication with efnB2 in osteoblasts impaired the anabolic effect of PTH on bone formation, restricting both osteoblast development and the support of osteoclastogenesis [54]. These observations also make efn-Eph signaling an obvious candidate for the development of therapeutic approaches in challenging situations such as osteoporosis, where bone formation and resorption are unbalanced. They also imply that impaired bone healing might benefit from efn/Eph-related therapy to promote bone formation.

Interestingly, *in vivo* treatment of mice with alendronate enhanced efnB1 expression in pre-osteoclasts, and enhanced EphB1 and EphB3 expression in osteoblasts co-cultured with osteoclasts from these mice. The inhibition of osteoblast development marker expression *in vivo* suggested that the effect was produced by forward signaling between the efnB1 ligand on osteoclasts, and that the EphB receptors on osteoblasts reduced osteoblast function to balance it with reduced osteoclast function [55]. EfnB1 was also demonstrated to reduce osteoclast functions in response to reverse signaling from EphB2 treatment *in vitro*, as it has in response to deletion in a myeloid-specific efnB1 knockout mouse *in vivo* [37].

Ephrin signaling can mediate other regulatory pathways that regulate bone homeostasis. For example, mechanical loading increases the periosteal bone formation in efnB1 transgenic mice, and increases EphB2 expression in bone [43]. The increased EphB2 expression in a mechanically loaded bone promoted Osterix expression through reverse signaling to efnB1 and further stimulated bone formation, implying that bidirectional signaling through EphB2 and efnB1 regulated this effect, and that EphB2-activated efnB1 reverse signaling might coordinate periosteal osteoblast proliferation and angiogenesis for bone homeostasis. EfnB2-EphB2 reverse signaling regulates the inflammatory differentiation of endothelial cells [27], suggesting that reverse signaling might mediate normal post-injury and pathological inflammation in bone.

8. Ephrin signaling in the stem cell niche

EphB2-efnB1 and EphB4-efnB2 signaling regulates communication between mesenchymal stromal cells (MSC) and hematopoietic cells. The inhibition of reverse signaling from EphB2 to efnB1 or efnB2 forward signaling to EphB4 in MSC inhibited T cell activation [56]. Other studies that examined stromal support of the hematopoietic niche demonstrated that stromal cells from Col-I-cre EphB4 transgenic mice supported hematopoietic growth factor production and bone marrow reconstitution [57]. These results support a role for ephrin signaling from bone cells in maintaining the hematopoietic stem cell niche of the bone marrow.

9. Efn-Eph intracellular signaling

Cell adherence, shape and motility depend on the intracellular signaling mechanisms activated by EphA and B

receptors in forward signaling, and efnA and B ligands in reverse signaling. The EphA and B receptors signal through the Rho and Ras GTPases that mediate actin dynamics [reviewed in Ref. [1]]. EphA-activated Rho GTPases mediate cell shape and movement though specific adapter proteins, while EphB-activated GTPases use a different set of adapter proteins to mediate actin filament elongation. Eph receptors also regulate the MAP kinase pathway through Ras GTPases. This pathway regulates transcription, proliferation and migration, as well as adhesion; its regulation by EphA and B receptors is generally negative. EphA receptors also regulate the Jak/Stat pathway, and EphB receptors regulate PI3-K-mediated proliferation.

The highly conserved cytoplasmic tail of the efnB ligands contains several tyrosine residues that can be phosphorylated when the extracellular domain of an efnB ligand interacts with an EphB receptor. However, this signaling pathway is subject to several types of modulation. Intracellular reverse signaling pathways of efnA ligands are glycosylphosphatidylinositol (GPI)-linked and are less well characterized. Accurate regulation of ephrin signaling and the cross-talk between the ephrin and growth factor intracellular signaling pathways that mediate cell proliferation, migration and invasion is critical in maintaining tissue homeostasis and avoiding the development of cancer [29].

Ephrin signaling regulates various aspects of bone homeostasis. Retroviral augmentation of EphA2-efnA2 reverse signaling *in vitro* increased NFATc1 and c-Fos transcription, suggesting that this signaling circuit promoted the differentiation of osteoclasts [35]. EphA2 forward signaling was proposed to inhibit osteoblast differentiation by reducing Osterix and Runx2 activity [35]. In combination with this EphA2 inhibition of osteoblast development, these studies suggest that efnA2-EphA2 signaling initiated a bone remodeling phase. The authors of this study propose that an EphA2-mediated increase in intracellular RhoA signaling might inhibit osteoblast differentiation. EfnB2-EphB4 forward signaling promotes osteoblast differentiation via a reduction in RhoA signaling that is thought to modify cell division, as well as the cytoskeletal properties and motility of osteoblasts [58]. Thus, both ephrinA and B family signaling is implicated in the regulation of osteoblast development.

Intracellular signaling by which ephrins regulate osteoclast development and activity also utilizes non-ephrin molecular pathways. In the RAW264.7 pre-osteoclast cell line, Dishevelled2 (Dvl2) co-precipitation with efnB2 was increased by RANKL treatment and reduced by EphB4 treatment, indicating that Dvl2 could be a downstream effector of reverse signaling that associates with efnB2 and mediates cell migration [59]. It has been also recently shown that EphA4 regulates osteoclast activity by modulating β3-integrin functions [47], presumably by promoting attachment to the extracellular matrix. Thus, it is likely that a multiple forward and reverse intracellular signaling pathways are involved in mediating diverse efn-Eph effects in different types of bone cells.

An examination of efnB1 mutations in human craniofrontonasal syndrome revealed that a significant proportion of the mutations occurred in the C-terminal 33 amino acids of the intracellular PDZ motif [60]. A deletion of the PDZ binding motif at the C-terminal tail abolished efnB1 binding to PDZ proteins and impaired neural crest cell function and calvarial formation, indicating that the PDZ signaling domain was critical for correct bone patterning and formation [36]. With respect to reverse signaling through efn ligands, there is considerable flexibility for interactions with PDZ and non-PDZ proteins [61], and the potential for the formation of additional complex regulatory networks also exists between their signaling pathways [62]. In addition to tyrosine phosphorylation, there is evidence for the phosphorylation of serine residues of efnB1 by serine/threonine kinases to facilitate the binding of adapter proteins. At later steps in reverse signaling, efnB1 clusters recruit the PDZ domain-containing protein tyrosine phosphatase PTPN13, which dephosphorylates the efnB1 tyrosine kinase, thereby shifting signaling from phosphotyrosine-dependent signaling pathways to PDZ domain-dependent signaling pathways of interacting cytoplasmic molecules that presumably modulate efnB ligand signaling [63]. There are over 300 interactions of these PDZ-interacting molecules with PDZ that could regulate different cellular processes, including the wnt receptors frizzled 1, 4 and 7, the calcium mobilizer adenine purinoreceptor 1 (P2RY1), the cytoskeletal regulators syndecans 1, 2, 3 and 4, the tight junction protein claudin 1, and NMDAR2A and B [64].

The C-terminus of the efnB ligand contains a conserved binding motif (YYKV) to which PDZ-binding transcriptional co-activating factors can bind and interact with other transcription factors. One such transcriptional co-activating factor implicated in efnB ligand reverse signaling is TAZ (transcriptional co-activator with PDZ binding motif). The C-terminus of TAZ bears a PDZ binding motif that localizes TAZ to discrete nuclear foci essential for transcriptional activation, as well as binding to the cytoplasmic tails of transmembrane receptors and the actin cytoskeleton.

Studies on transcriptional regulatory mechanisms in bone cells have revealed an important role for TAZ in mediating osteogenic differentiation. For example, the increased expression of TAZ by bone morphogenetic protein (BMP)2 stimulation is associated with an elevation in the expression of osteocalcin, a marker of mature osteoblasts [65]. *In vitro* and *in vivo* studies strongly suggest that TAZ functions as a membrane/cytoskeleton-associated transcriptional regulator to coordinate specific osteogenic and adipogenic transcription factors and promote mesenchymal stem cell development to bone [66]. TAZ has a highly conserved transcriptional activation WW domain that functions as a transcriptional co-activator by binding to the PPXY motif present on a large number of transcription factors that include Runx2, PPARγ, Smad, MEF2B, Sox9, C/EBP, Oct4 and Pax3 [reviewed in Ref. [67]]. Observations that the Col-I (osteoblast)-specific overexpression of TAZ enhanced bone formation *in vivo* through the regulation of transcription factors suggest that efnB ligand reverse signaling produced this effect [68]. Thus, extracellular signals promoting bone formation are transmitted into the nucleus through nuclear trafficking with TAZ.

The "Na/H exchange regulatory factors" (NHERFs) are important mediators of intracellular signaling. NHERF1 contains two tandem PDZ domains at the N-terminus and has been shown to regulate trafficking and signaling of several G-protein coupled receptors, including the PTH receptor [reviewed in Ref. [69]]. NHERF1 is expressed in osteoblasts and has been associated with the regulation of bone formation, and the binding of the Na/Pi cotransporter and TAZ [70]. Targeted disruption of NHERF1 results in postnatal lethality, often accompanied by bone fractures due to 25–30% reduction in bone mineral density [71]. NHERF1 appears to be involved in the reverse signaling of efnB1 through the dephosphorylation of TAZ and its relocation to the nucleus where its osteogenic transcription factor target genes such as Osterix are expressed [36], constituting yet another pathway of TAZ mediation of efnB1 signaling.

10. Proposed models for the functions of ephrins in bone

Ephrin actions in bone are diverse, but require more complete characterization. They are critical in determining the skeletal pattern and in maintaining bone homeostasis. Based upon the current literature, we propose the following model for ephrin regulation of bone formation and resorption (Fig. 1). In this model, different combinations of efn ligand and Eph receptor binding partners regulate the development of osteoblasts, osteoclasts and chondrocytes. During the initial phases of development in the osteogenic and chondrogenic lineages, these interactions involve efnB1 and efnB2 binding with EphB1/EphB2 and EphB2/EphB4, respectively. Forward and reverse signaling involving EphB2 and EphB3 also coordinates angiogenesis with bone formation, possibly through endothelial cell expression. With respect to osteoclasts, EphA4 is inhibitory early in development, while efnA2 and EphA4 coordinate development with pre-osteoblasts. The balance between bone formation and resorption is maintained by the subsequent interaction of EphB4 in osteoblasts with efnB2 in osteoclasts, which regulates osteoblast-osteoclast development. In this way cartilage and bone formation are coordinated with remodeling and angiogenesis.

A model of intracellular signaling pathways of efnB ligand and EphB receptor communication that mediate bone formation and resorption between osteoblast and osteoclast precursors is presented in Fig. 2. Osteoblast differentiation is promoted by the expression of osteogenic genes in response to reverse signaling through efnB1 and forward signaling from osteoclast efnB2 through EphB4. Osteoclast differentiation is reduced by the inhibition of osteoclast gene expression in response to reverse signaling from osteoblast EphB4 through efnB2. The net result of these functions in this model is bone formation.

11. Conclusions and future directions

Although it has become very clear that efn-Eph signaling plays a key role in skeletal development and homeostasis, a number of key questions with respect to the increasingly complex ephrin regulation of bone cell communication. These questions involve the binding preferences within and between ephrin families of ligands and receptors, higher order clustering interactions between receptors and ligands within and between cells, the regulation of forward and reverse signaling in different contexts, and the interactions with other ephrin and non-ephrin extracellular and intracellular signaling pathway regulators.

Specifically:

1) Does the binding of an Eph receptor to its ligand occur by cell–cell interaction or through a cleaved receptor to the membrane anchored ligand, or vice versa?

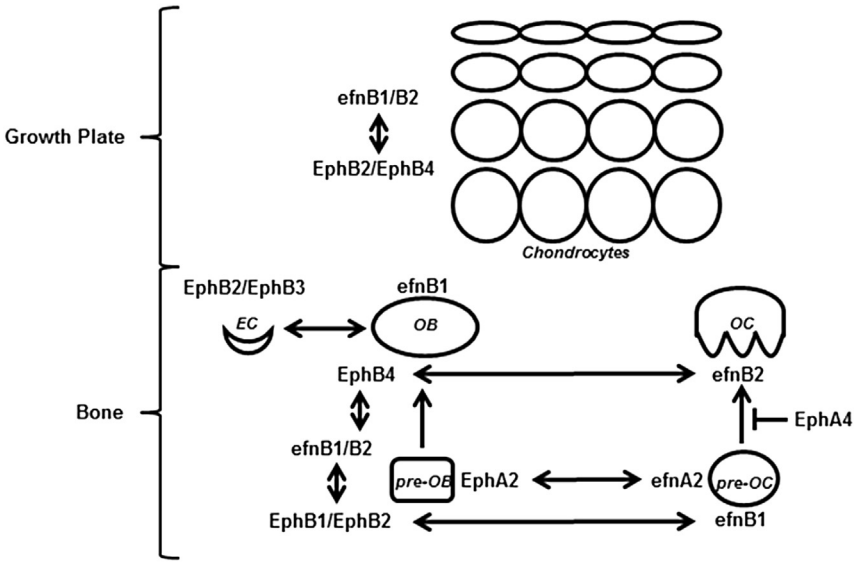

Fig. 1. A model for ephrin ligand-receptor regulation of bone cell interaction in bone development and homeostasis. Several ephrin ligands and receptors mediate growth plate development during bone growth. Interactions between osteoblasts (OB), osteoclasts (OC), endothelial cells (EC) and chondrocytes are mediated by different ephrin ligand-receptor combinations, coordinating bone cell development and activity.

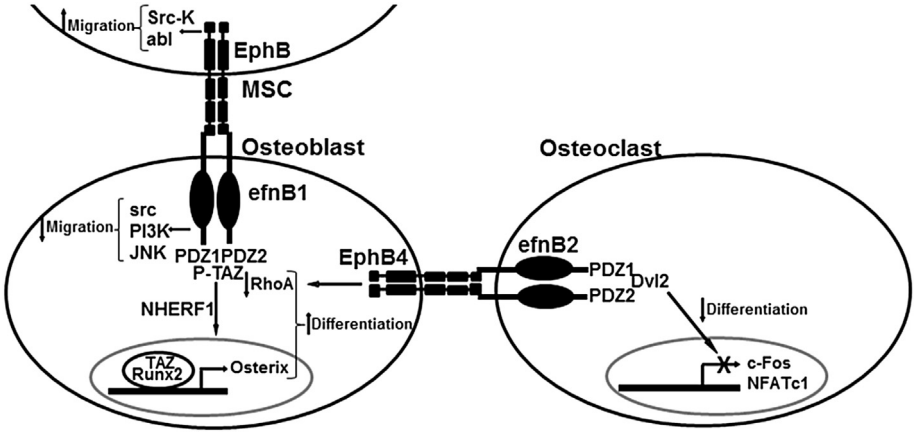

Fig. 2. Intracellular EphB/efnB signaling between osteoblast and osteoclast lineage cells in bone formation. Osteoblast development is mediated by forward signaling through EphB4 from efnB2 on osteoclasts and reverse signaling through efnB1 from EphB receptor on MSC. NERFH1 dephosphorylates TAZ for localization to the nucleus. Osteoclast development is mediated by reverse signaling through efnB2 from EphB4 on osteoblasts. Dvl2, Dishevelled2; MSC, mesenchymal stromal cells; NHERF, Na/H exchange regulatory factor; TAZ-P, phosphorylated TAZ.

2) Are both forward and reverse signaling initiated upon EphB receptor-efnB ligand interaction, or are the activation of forward or reverse signaling cell-type specific depending on the binding partners available in a given cell type?
3) Is reverse signaling activated via only efnB ligand interaction with its receptor, or does efnB ligand binding to other proteins or activation of kinases by other growth factors and G protein-coupled receptors activate efnB ligand reverse signaling?
4) Does receptor-ligand interaction mainly occur via osteoblast-osteoblast, osteoblast-osteoclast, osteoblast-osteocyte, or does it involve other cell types?
5) What are the interactions between Eph/efn signaling and bone growth factor signaling pathways?
6) Are the Eph/efn signaling pathways involved in bone repair similar to those of bone development and homeostasis?

The elucidation of these functions will facilitate the optimization of forward and reverse signaling strategies in the development of therapeutic applications of the ephrins for a wide variety of conditions in various tissues.

Conflicts of interest

The authors have no potential conflicts of interest to disclose.

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