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PERSPECTIVES

EPHs and Ephrins: Many Pathways to Regulate Osteoblasts and Osteoclasts

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Abstract

The EPH/Ephrin family of receptor tyrosine kinases has been shown recently to play important roles in communication between osteoblasts and osteoclasts, and within the osteoblast lineage that regulates the differentiation of these cells. This *Perspective* provides a brief overview of EPH/Ephrin signaling from work carried out in other cell and organ systems, and reviews the current literature on the actions of these kinases in bone. The EPH/Ephrin family provides multiple signaling pathways that themselves may be important within bone cells. They also interact with other pathways known to regulate osteoblast and osteoclast activity (*e.g.*, FGFs, metalloproteinases, integrins and connexins). EPH/Ephrin signaling pathways demonstrate a high level of promiscuity within the family, and are particularly interesting by virtue of the ability of the EPH/Ephrin interaction to simultaneously induce signal transduction within two cells. At this stage, there are few studies of the actions of EPH/Ephrin signaling within bone cells. Although we now know a few members of the EPH/Ephrin family that modify both osteoblast and osteoclast differentiation, we do not know how they elicit their effects in bone, which pathways are most important, and which other regulatory pathways are influenced by the EPH/Ephrin family within the skeleton. *IBMS BoneKEy*. 2010 September;7(9):304-313.
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What Are EPHs and Ephrins?

The EPH family of receptor tyrosine kinases and their ligands, ephrins (*EPH* *r*eceptor-*in*teracting proteins), are involved in a wide spectrum of physiological and pathological processes, including axon guidance, synapse plasticity, vascular development and tissue remodeling (1). They constitute the largest subfamily of receptor tyrosine kinases, and their functions have been shown to be important in development (including skeletal development) (2), memory (3), insulin secretion (4), bone remodeling (5), and cancer (6).

EPHs and Ephrins are divided into two classes based on structural homology. EphrinA ligands are anchored to the cell membrane by glycophosphatidylinositol (GPI) linkage but lack an intracellular domain. In contrast, ephrinB ligands have intracellular regions containing phosphorylation sites and a Src homology (SH2) domain capable of intracellular signaling. B-class EPHs and Ephrins also each contain a C-terminal PDZ domain (6). This protein interaction domain acts as a molecular anchor to the cytoskeleton and a stabilizing scaffold for large molecular complexes (7). Within both classes, the distinction of which is a receptor and which is a ligand is based on structure rather than function, since both are capable of intracellular signaling. To distinguish between the two possible modes of signaling, "receptor" (*i.e.*, EPH)-mediated signaling is termed forward signaling. In contrast, "ligand" (*i.e.*, Ephrin)-initiated signaling is called reverse signaling. This ability of EPH/Ephrin-initiated signaling to occur in two directions (and, indeed, in two different cells) is termed "bidirectional signaling." Even the A-class ephrins, which do not possess an intracellular domain, are capable of reverse signaling by interaction with transmembrane proteins capable of eliciting intracellular signals (8).

In humans there are five EphrinA ligands, ten EPHA receptors, three EphrinB ligands and six EPHB receptors (6;9). EPH/Ephrin

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family signaling is remarkable by virtue of the high level of promiscuity between the 8 ligands (EphrinB1-3 and EphrinA1-5) and 16 receptors (EPHA1-10 and EPHB1-6) in the family. Each ligand is capable of binding to more than one receptor and *vice versa*, with the exception of the receptor EPHB4 that interacts only with EphrinB2. For a listing of all known interactions, see (10). Binding between receptor and ligand is generally specific within the subtype, but there are some exceptions, including binding of B-class ephrins by EPHA4 and binding of ephrinA5 by EPHB2.

Since EPHs and Ephrins are membrane-bound, interactions between them generally require cell-cell contact, and this must be considered when making conclusions about *in vivo* biology from *in vitro* experiments. EPHs and Ephrins can also bind in a *cis* configuration (on the same cell membrane), a pattern of binding thought to silence bi-directional signaling (11). There is also evidence of proteolytic cleavage of both EPHs and Ephrins, which can produce both extracellular and intracellular fragments that may initiate or interfere with signaling events (6).

EPH/Ephrin signaling requires the dimeric EPH/Ephrin complex to form tetramers, thereby enabling phosphorylation of either receptor (forward) or ligand (reverse). These tetramers then aggregate to form large clusters that vary in size (12). The large clusters are actively transported along actin filaments while still bound to both cell membranes, and are moved to an appropriate region of the cell. This clustering and localization appears to be required for physiological effects (13).

EPHs and Ephrins, either when bound to each other, or acting alone, also participate in cross-talk with a wide range of other signaling pathways, including some known to be important in the skeleton. For example, activated FGFR can bind in *cis* (same cell) directly to EphrinB1 and induce its phosphorylation in *Xenopus* blastomeres (14), but can also bind EPHA4 in *trans* in mammalian cells and activate downstream signaling (15). Interactions of EPHs and Ephrins with other pathways known to be important for bone include both agonistic and antagonistic interactions with SDF-1/CXCR4 signaling, and modification of integrin-mediated cell adhesion [see Arvanitis and Davy for a full review (11)].

EPH/Ephrin interaction leads to activation of a number of intracellular signaling pathways known to regulate osteoblast and osteoclast function, including interactions with Src, c-cbl and a number of integrins (11;16;17). Consistent with this, EPH/Ephrin signaling causes changes in cell morphology, adhesion, migration and invasion by modifying actin cytoskeleton dynamics (1). Despite these activities having been well-studied, and known to be important for normal osteoblast and osteoclast function, the role of these actions in the physiological effects of EPH/Ephrin signaling has not yet been reported in bone (1).

Which Cells Within Bone Express Which Ephrins?

Within bone, EPH and Ephrin expression has been described in both osteoblasts and osteoclasts, as well as in their precursors. In murine osteoblasts, mRNA for EphrinB1, EphrinB2, and EphrinsA1, A2, A4 and A5, as well as EPHB2-4, EPHB6, EPHA2-4 and EPHA7 have been detected (5;18;19). Immunohistochemical studies have also shown that EphrinB2 and EPHB4 are expressed by both osteoblasts and osteocytes, particularly in lamellar bone (*i.e.*, bone that is remodeled), rather than in newly-formed woven bone (18).

Identification of protein expression in pure osteoclast populations is problematic, but EphrinB2 has been identified in mature osteoclasts by immunohistochemistry of tissue sections (5;18). Furthermore, EphrinB1 and EPHA4 mRNA have been detected in osteoclast-rich cultures, but there is no evidence for expression of any EPHB receptors in the mature osteoclast (5). EphrinA2 and EPHA2 mRNA have been detected in osteoclast precursors (19). To date, the cellular distribution of EPHs and Ephrins in human bone has not been reported.

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Regulation of Osteoclast Formation by EPH/Ephrin Signaling

EphrinB2 expression in osteoclast precursors increases as osteoclast differentiation progresses *in vitro* (5). In other cell types it has been shown that EphrinB2 can initiate signaling when interacting with EPHA4 or EPHB1-EPHB4 (10); while all these receptors are expressed by the osteoblast lineage, only EPHA4 is expressed by the osteoclast lineage (5). When clustered receptors EPHB4, EPHA4 or EPHB2 were added to osteoclast cultures in the absence of osteoblasts, calcitonin receptor mRNA levels (a marker of mature osteoclasts) were reduced (5). This finding, and further work in the same paper, indicates that EphrinB2 reverse signaling within osteoclast precursors inhibits their differentiation into osteoclasts, and that the EphrinB2 PDZ domain is required for this to occur. It is likely that this mechanism explains the inhibitory effect of EPH receptor addition, but it is also possible that addition of exogenous non-signaling receptors may so saturate EphrinB2 that it is unable to interact with EPHA4 and initiate forward signaling.

Unlike B-class ephrins, overexpression of EphrinA2 or EPHA2 in osteoclast lineage cells impaired their differentiation into osteoclasts *in vitro* (19). This was not altered when the overexpressed EPHA2 kinase domain was mutated, suggesting that it is reverse signaling through EphrinA2 within the osteoclast lineage that stimulates osteoclast differentiation.

The EphrinB2/EPHB4 interaction within groups of osteoblasts also appears to modify their expression of RANKL, which is one of two key factors expressed by osteoblast-lineage cells that are required for osteoclast formation. Treatment of cultured osteoblasts with sEPHB4, an inhibitor of EphrinB2 signaling, increased their expression of RANKL (20). This finding is consistent with low osteoclast numbers in EPHB4-overexpressing mice (5).

Since EphrinB2 must interact with an EPH receptor for reverse signaling to be activated, the question of which cell bears the physiologically relevant EPH needs to be resolved. While it has been suggested that direct interaction of osteoblastic EPHB4 with osteoclastic ephrinB2 is the key interaction (5), EPH expressed on the surface of other cells may also initiate reverse signaling in osteoclast precursors. For example, EPHA4-expressing osteoclast precursors or mature osteoclasts may provide negative feedback through EphrinB2 within the osteoclast lineage, including the possibility of autocrine *cis* signaling within the osteoclast to limit precursor fusion. Osteoclast precursors *in vivo* would also interact with a range of EPH-expressing cells in hemopoietic tissues that may control their differentiation. Indeed, hemopoietic stem cell differentiation itself is defective in EPHB4-deficient embryonic stem cells, an effect likely to influence the availability of osteoclast precursors (21). Another possibility by which ephrins may control osteoclast formation *in vivo* is that the interaction of EphrinB2-bearing osteoclast precursors with EPHB4-expressing cells in the blood vessel wall (22) may allow egress of osteoclast precursors from the bloodstream to the bone surface. The role of EphrinB2 in angiogenesis and endothelial cell sprouting (23;24) may also influence the extent of vascularization, thereby modulating the availability of osteoclast precursors through capillaries at sites of bone remodeling (25). Changes in T-cell signaling, known to contribute to osteoclast formation in physiological and pathological bone remodeling for osteoclastogenesis (26), may also play a role, since T-cell adhesion is modified by the EPHA/EphrinA family (27) and EphrinB1 regulates T-cell development (28). Surprisingly, although EphrinB2 regulates osteoclast formation *in vitro,* no osteoclast phenotype was observed when EphrinB2 was deleted in the osteoclast lineage *in vivo* (5), suggesting compensatory mechanisms from other cell types.

Regulation of Osteoblast Differentiation by EPH/Ephrin Signaling

EPH/Ephrin signaling also modifies the ability of osteoblastic cells to differentiate and mineralize. Osteoblast differentiation *in*

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vitro was stimulated by treatment with clustered EphrinB2 or EphrinB2 overexpression (5), an effect independent of the EphrinB2 PDZ domain, which may indicate involvement of EPHB4 (or EPHB2) forward signaling. There is also some *in vitro* evidence for an inhibitory role of osteoblastic EPHA2 signaling in osteoblast differentiation (19). EPHA2 knockout calvarial osteoblasts and EphrinB2-Fc-treated osteoblasts both demonstrate enhanced osteoblast differentiation *in vitro* and reduced RhoA activation (5;19). This is consistent with a previous report indicating that RhoA inactivation stimulates osteoblast differentiation *in vitro* (29). However, the influence of EPH/Ephrin signaling on RhoA activation is complex; RhoA activation is inhibited by EPHA2 in mammary gland development (30) while in most other cellular contexts EPHA2 and EPHB4 signaling activates RhoA (31-34). It is also likely that other mechanisms, including integrin-mediated signaling and gap junction communication, may be involved in the effects of EphrinB2 and EPHA2 on osteoblast differentiation.

A number of independent lines of evidence have confirmed that osteoblastic EPHB4 is required for the stimulatory effect of EphrinB2 on osteoblast differentiation. These include *in vitro* studies that have neutralized the EphrinB2 effect by addition of EPHB4 as a competitive inhibitor of receptor binding (5), and reduced mineralization by cultured osteoblast lineage cells treated with soluble EPHB4 (18) or a peptide capable of specifically inhibiting the EphrinB2/EPHB4 interaction (20). The latter two studies have indicated that inhibition of the EphrinB2/EPHB4 interaction does not influence transcription factors required for osteoblast commitment, such as Osterix or Runx2, but reduces the expression of late markers of osteoblast differentiation, including osteocalcin, DMP-1 and sclerostin. The ability of this interaction to modify gene expression by mature osteoblastic cells and osteocytes, and the expression of both components in mature osteoblasts and osteocytes in bone remodeling (18), suggests a role for ephrinB2-EPHB4 interaction as a juxtacrine regulator of osteoblast function within osteoblast teams already differentiated and acting on the bone surface. This may be regulated by paracrine factors within the BMU, such as parathyroid hormone (PTH)-related protein (PTHrP), as discussed below. Other factors that may regulate EphrinB2 or EPHB4 signaling or expression levels have yet to be identified. Whether the EphrinB2-EPHB4 interaction takes place by forward signaling through EPHB4 or reverse signaling through EphrinB2 remains to be determined.

EphrinB2 production is rapidly upregulated by PTH and PTHrP in osteoblasts *in vivo* and in whole bone *in vitro* (18). Both PTH and PTHrP stimulate osteoblast activity and osteoclast formation through a common receptor (PTH1R) expressed in differentiated osteoblasts (18). Osteoblastic PTHrP is required for normal bone formation during remodeling (35), indicating an important physiological process to which EphrinB2 signaling may contribute. Regulation of EphrinB2 by PTHrP has been confirmed by reduced EphrinB2 levels in genetically-altered mice lacking the midregion, nuclear localization signal, and C-terminus of PTHrP (*i.e.*, residues 67-137) (36).

The regulation of late-stage osteoblast markers by the EPHB4/EphrinB2 interaction may provide a mechanism by which PTH1R ligands stimulate bone formation. An alteration in the effect of PTH on EphrinB2 mRNA levels in β-arrestin knockout mice, which do not respond normally to PTH (37), is further evidence suggesting a relationship between the two. Since intermittent PTH treatment is used as a therapeutic agent for osteoporosis (38), its influence on EphrinB2 expression suggests a pathway that may be critical for this action. It will be important to define whether the influence of PTH or PTHrP on bone formation depends on Ephrin family members, and is something we are seeking to do.

EphrinB1 has also been shown to regulate osteoblast function. Mice engineered for osteoblast-specific deletion of EphrinB1 exhibit a cell lineage-autonomous reduction in osteoblast activity and BMD without any

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detectable change in osteoclast formation or activity (39). EphrinB1 signals by interacting with EPHB1-3, and both EPHB2 and EPHB3 are expressed by osteoblasts. One issue that has not been explored in this, or in the EPHB4-overexpressing mouse, is the possibility of altered expression and signaling of other Ephrin/EPH family members when EPHB4 or EphrinB1 levels are modified in the osteoblast, and this may have an impact. For example, would there be increased signaling of EphrinB2 through EPHB2 in an EphrinB1-deleted osteoblast due to reduced competition for the receptor?

The mechanism by which EPH/Ephrin interactions modify osteoblast activity is still fairly elusive. RhoA has been implicated, along with a direct interaction of EphrinB1 with two PDZ-domain-containing factors, NHERF1 (a sodium/hydrogen exchange regulator) and TAZ (a transcriptional co-factor known to interact with Runx2) (5;19;39). Each of these pathways has been noted to regulate Osterix expression, a factor involved in osteoblast commitment. In contrast, inhibition of EPHB4/EphrinB2 within the osteoblast lineage influences late markers of osteoblast differentiation (18;20). The possibility that Ephrins influence cell migration or gap junction communication, as in cranial suture closure (see below), has not been explored in the context of bone remodeling.

Ephrins in Calvarial Suture Formation

Suture closure in the developing skull is determined by carefully-timed movement of mesenchymal cells into the suture region and their differentiation into osteoblasts. Early closure of sutures is termed craniosynostosis, a common defect that requires surgical intervention, and EphrinB1 and EphrinA4 mutations have been identified in human craniofrontonasal syndrome and coronal craniosynostosis, respectively (40;41).

Mice deficient in Twist1, a transcription factor involved in osteoblast differentiation, exhibit coronal suture craniosynostosis, and this is associated with deficient expression of EPHA4, EphrinA2 and EphrinA4 in the suture (40), consistent with the finding of EphrinA4 deletion in patients with the same syndrome (40). The most recent work has shown that EPHA4 is required to delay migration of osteogenic cells into the suture until the appropriate stage of skull development (42), an effect that may involve EPH/Ephrin-mediated repulsion between the osteogenic and neural crest cells residing at the suture boundary.

Enhanced osteoblast differentiation has also been shown to play a role in the Twist1-deficiency calvarial phenotype (43). Does Twist1 also regulate osteoblast differentiation through EPH/Ephrin signaling? A role for EphrinA2 in this process may be supported by the enhanced alkaline phosphatase (ALP) activity of EPHA2-deficient osteoblasts (19), but an interaction between Twist1 and EPH/Ephrin signaling in the context of osteoblast differentiation has not yet been shown. Similarly, the role of EphrinB1 in suture formation may also include both an influence on osteoprogenitor migration and differentiation of these cells (39).

EphrinB1 signaling in skeletal development shares some similarities with that of EPHA4, as indicated by axial skeletal patterning defects including craniofrontonasal syndrome in female EphrinB1 heterozygous mice (44;45). It appears that this is not due to a defect in cell migration as observed in Twist1 mice, but is associated with impaired gap junction formation (46). Connexin 43 expression was impaired in the affected sutures, and connexin 43 overexpression partially rescued the calvarial defect (46). An EPHB2/EphrinB1 interaction appeared to be required for connexin 43 to establish normal gap junction communication. Since connexin 43 and gap junction formation are required for normal osteoblast function (47;48), the impaired bone formation phenotype observed in osteoblast-specific EphrinB1 null mice may also relate to this influence of Ephrin signaling.

Conclusion

At this stage, it is possible to make a number of broad generalizations about

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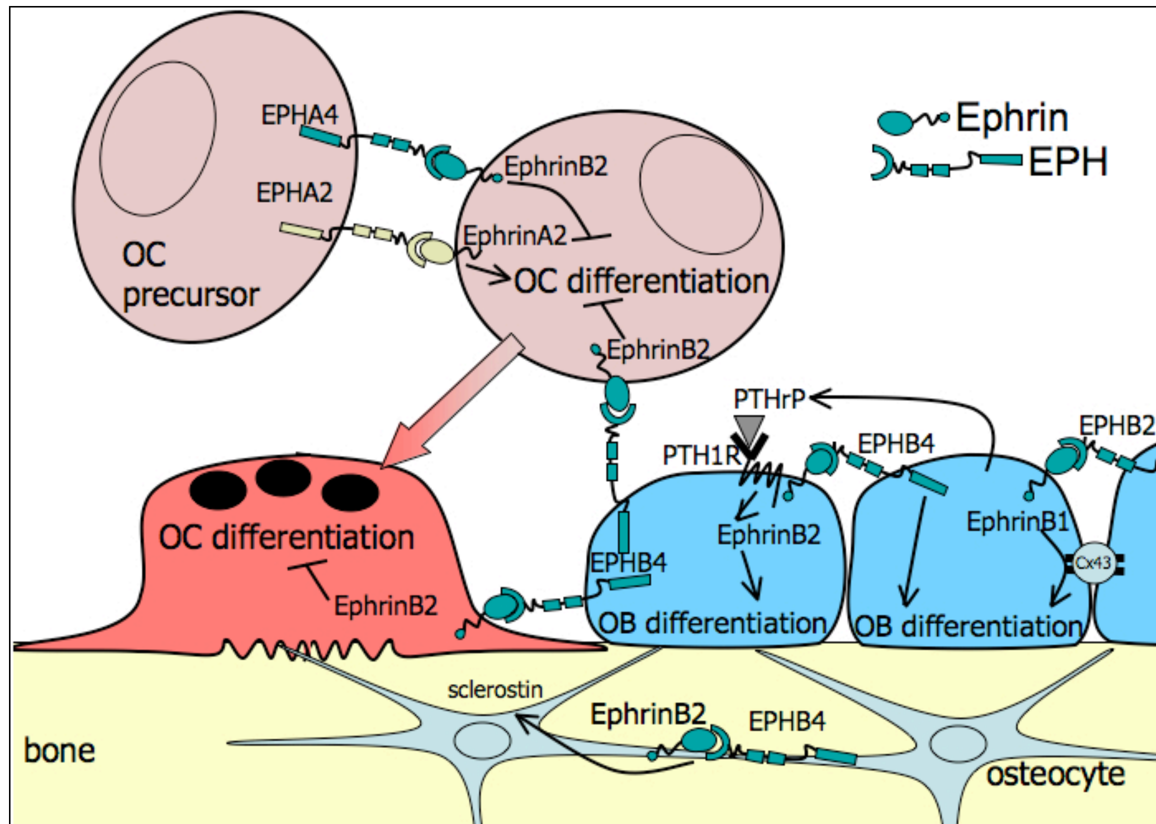

Fig. 1. Postulated EPH/Ephrin interactions in the bone remodeling unit. EphrinB2 signaling within osteoclast precursors (an EPHA4 interaction) and interaction of osteoclast precursors and osteoclasts with EPHB4-expressing osteoblasts limits osteoclast differentiation. In osteoblasts, paracrine PTHrP, acting through the PTH receptor (PTH1R), enhances EphrinB2 production. EphrinB2/EPHB4 interaction within the osteoblast lineage enhances osteoblast differentiation. EphrinB1/EPHB2 interaction within the osteoblast lineage also enhances osteoblast differentiation through a direct interaction of EphrinB2 with connexin 43 that promotes gap junction formation. Both EphrinB2 and EPHB4 are expressed by osteocytes embedded in the bone matrix, and interfering with this interaction inhibits sclerostin expression.

EPH/Ephrin signaling in osteoblast/osteoclast interactions in the bone remodeling unit, and these are illustrated in Fig. 1. In brief, EPH/EphrinB signaling inhibits osteoclast formation and stimulates osteoblast differentiation. In contrast, EPH/EphrinA signaling has the reverse effect on both cell types. However, we have only started to define EPH/Ephrin interactions in bone cells. As well as being unsure about which cell types are the key contributors to these interactions, we do not know how many members of this family play active roles in osteoblast and osteoclast function. We also do not know how their expression or function is regulated, nor do we know the full spectrum of intracellular signaling pathways influenced by EPH/Ephrin signaling or what changes in cytoskeletal organization or intercellular connections may result from these interactions. Finally, we do not know the influences of diseases of bone, including bone cancers and joint disease, on EPH/Ephrin signaling. Despite these gaps in our knowledge, it seems very likely that these factors play important roles in regulating both the formation and activity of osteoblasts and osteoclasts.

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