Bidirectional Signaling through EphrinA2-EphA2 Enhances Osteoclastogenesis and Suppresses Osteoblastogenesis

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Bone is remodeled constantly throughout life by bone-resorbing osteoclasts and bone-forming osteoblasts. To maintain bone volume and quality, differentiation of osteoclasts and osteoblasts is tightly regulated through communication between and within these two cell lineages. Previously we reported that cell-cell interaction mediated by ephrinB2 ligand on osteoclasts and EphB4 receptor on osteoblasts generates bidirectional anti-osteoclastogenic and pro-osteoblastogenic signals into respective cells and presumably facilitates transition from bone resorption to bone formation. Here we show that bidirectional ephrinA2-EphA2 signaling regulates bone remodeling at the initiation phase. EphrinA2 expression was rapidly induced by receptor activator of NF-kB ligand in osteoclast precursors; this was dependent on the transcription factor c-Fos but independent of the c-Fos target gene product NFATc1. Receptor EphA2 was expressed in osteoclast precursors and osteoclasts. Overexpression experiments revealed that both ephrinA2 and EphA2 in osteoclast precursors enhanced differentiation of multinucleated osteoclasts and that phospholipase C C2 may mediate ephrinA2 reverse signaling. Moreover, ephrinA2 on osteoclasts was cleaved by metalloproteinases, and ephrinA2 released in the culture medium enhanced enhanced osteoclastogenesis. Interestingly, differentiation of osteoclasts lacking EphA2 was enhanced along with alkaline phosphatase, Runx2, and Osterix expression, indicating that EphA2 on osteoblasts generates anti-osteoblastogenic signals presumably by up-regulating RhoA activity. Therefore, ephrinA2-EphA2 interaction facilitates the initiation phase of bone remodeling by enhancing osteoclast differentiation and suppressing osteoblast differentiation.

Bone remodeling maintains bone mass constant during adulthood (1, 2). Resorption of old mineralized bone by osteoclasts is followed by new bone formation by osteoblasts. These processes, consisting of the initiation, transition, and termination phases, are tightly regulated by communication between osteoclasts and osteoblasts (3). Bone resorption is excessive in the most common skeletal diseases such as osteoporosis, but molecular mechanisms that balance bone remodeling are only partially understood.

Osteoclasts are multinucleated cells (MNCs) responsible for bone resorption. They originate from the fusion of hematopoietic precursor cells of the monocyte/macrophage lineage. Osteoclasts express the two major membrane-bound proteins required for osteoclast differentiation, macrophage-colony stimulating factor (M-CSF), and the receptor activator of NF-kB ligand (RANKL). Soluble forms of M-CSF and RANKL allow us to generate osteoclasts from M-CSF-dependent macrophages (MDMs) in cultures. Downstream signaling pathways ultimately activate critical osteoclastogenic transcription factors such as c-Fos (4) and NFATc1 (5–7). NFATc1 is a target gene product of c-Fos (8) and activates gene expression of tartrate-resistant acid phosphatase (TRAP), calcitonin receptor, and ephrinB2.

Ephrin ligands and Eph receptor tyrosine kinases are crucial for migration, repulsion, and adhesion of cells during neuronal, vascular, and intestinal development (9, 10). Both ephrins and Ehs are membrane-bound proteins, which generate bidirectional signaling by interacting with each other. Signaling through ephrins is called “reverse signaling,” whereas signaling through Ephs is called “forward signaling.” Ephrins fall into the following two classes, based on their structural homologies: ephrinAs (ephrinA1–A5), which are glycosylphosphatidylinositol-anchored proteins, and ephrinBs (ephrinB1–B3), which have the transmembrane and cytoplasmic domains. Ehs are also divided into two classes, EhsAs (EphA1–A10), which mainly interact with ephrinAs, and EhsBs (EphB1–B6), which mainly interact with ephrinBs.

We previously demonstrated that ephrinB2 on osteoclasts mediates inhibitory signals for osteoclast differentiation, which may be crucial for bone remodeling. It is thus important to elucidate the signaling mechanism of ephrinB2 in osteoclasts. The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1.

3 The abbreviations used are: MNC, multinucleated cell; ALP, alkaline phosphatase; GPI, glycosylphosphatidylinositol; MDM, M-CSF-dependent macrophage; MMP, matrix metalloproteinase; M-CSF, macrophage-colony-stimulating factor; OPG, osteoprotegerin; PLC, phospholipase C; RANKL, receptor activator of NF-kB ligand; TRAP, tartrate-resistant acid phosphatase; TRE, TPA (12-tetradecanoylphorbol 13-acetate)-response element; GFP, green fluorescent protein; OB, osteoblast; PTH, parathyroid hormone; RT, reverse transcription; qRT, quantitative RT; KO, knock-out; WT, wild type; ADAM, a disintegrin and metalloproteinase.

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whereas EphB4 on osteoblasts mediates stimulatory signals for osteoblast differentiation (11). Recently, it was reported that parathyroid hormone (PTH) and PTH-related peptide induce ephrinB2 expression in osteoblasts. Therefore, ephrinB2-EphB4 interaction among osteoblasts might contribute to the anabolic effect of PTH and PTH-related peptide (12). Class A ephrin-Eph members are also expressed in bone. For example, EphA4 may function in chondrocytes and osteoblasts (13). In this study, we focus on the ephrinA and EphA families expressed on osteoclasts and osteoblasts. We show that ephrinA2-EphA2 interaction within osteoclast precursors or between osteoclast and osteoblast precursors enhances osteoclastogenesis while inhibiting osteoblast differentiation. These data reveal that class A ephrins and Ephs regulate the initiation phase of bone remodeling.

EXPERIMENTAL PROCEDURES

In Vitro Differentiation of Osteoclasts and Osteoblasts—Spleen or bone marrow cells were isolated from C57BL/6J mice or from mice lacking c-Fos (Fos KO) (4) and were cultured for 6 h to overnight in α-minimal essential medium containing 10% fetal bovine serum to harvest nonadherent cells. For stromal cell-free osteoclast formation, nonadherent cells were plated at a density of $1 \times 10^6$ cells per well in 6-well plates, $1 \times 10^7$ cells per well in 24-well plates, or $2 \times 10^7$ cells (unless otherwise indicated) per well in 96-well plates in α-minimal essential medium with 10% fetal bovine serum containing 10 ng/ml recombinant human M-CSF (R & D Systems) for 3 days. These M-CSF-dependent macrophages (MDMs) were used as osteoclast precursors. Osteoclast differentiation was induced for 3–4 days in the presence of 10 ng/ml each of recombinant human M-CSF and recombinant mouse RANKL (R & D Systems). For co-cultures with osteoblasts, nonadherent cells were seeded at $10^3$ cells/96 wells with $10^4$ cells/96-well osteoblasts and cultured in the presence of $10^{-8}$ M 1,25-dihydroxyvitamin D$_3$ and $10^{-7}$ M dexamethasone. Differentiated osteoclasts were fixed with 4% paraformaldehyde and with ethanol/acetone (50:50) and then were stained for TRAP activity using a kit (Sigma) with 20 mT tartrate. MDMs were treated with calcineurin inhibitor FK506 (Calbiochem), which blocks nuclear factor of activated T-cell activation, or a broad spectrum matrix metalloproteinase (MMP) inhibitor BB94 (British Biotech Pharmaceuticals) where indicated. For osteoblast differentiation, calvarial osteoblasts were isolated from wild-type and EphA2-deficient neonatal mice (14) and expanded in α-minimal essential medium with 10% fetal bovine serum. Osteoblast differentiation was induced in the presence of 30 μg/ml ascorbic acid, 10 mM β-glycerophosphate, and 50 ng/ml BMP-2 (R & D Systems). Alkaline phosphatase (ALP) and calcium staining were described previously (15). ALP activity and calcium (Ca$_{2+}$) were measured using kits (LabAssaySTM ALP and calcium C, Wako Pure Chemical Industries) according to the manufacturer’s protocols.

Conventional and Quantitative RT-PCR Analysis—Total RNA isolation, cDNA synthesis, and quantitative RT-PCR (qRT-PCR) were performed as described previously (11). Sequences of RT-PCR primers are listed in supplemental Table 1 or as described previously (11). RT-PCR and qRT-PCR primers for ephrinA2, ephrinB2, NFATc1, and EphA2 were purchased from Applied Biosystems.

Immunoblot Analysis—Proteins prepared from osteoclastogenic or osteoblastogenic cultures were separated on 4–12%
SDS-polyacrylamide gels (NOVEX) and transferred onto Hybond nitrocellulose membranes (Amersham Biosciences). Anti-ephrinA2 (R & D Systems), anti-EphA2 specific to intracellular domain (clone D7, Upstate), anti-EphA2 specific to extracellular domain (R & D Systems), anti-vimentin (Chemicon), anti-PLCγ2 (Cell Signaling Technology), anti-phospho-PLCγ2 (Cell Signaling Technology), anti-RhoA (Santa Cruz Biotechnology), and anti-actin antibodies (Santa Cruz Biotechnology) were used as primary antibodies. Membrane and nonmembrane proteins were isolated using a kit (Calbiochem) according to the manufacturer’s protocol. The active RhoA was precipitated with rhotekin-RBD GST beads (Cytoskeleton, Inc.).

Immunofluorescence—Femurs were dissected from C57BL/6j mice and decalcified using 10% EDTA (pH 7.6). They were immersed in 20% sucrose and embedded in OCT compound (Sakura Finetechnical). Embedded tissues were cut into 14-μm longitudinal sections. Anti-ephrinA2 (Santa Cruz Biotechnology and R & D Systems), anti-EphA2 (R&D Systems), anti-cathepsin K (Fuji Chemical), and anti-osteocalcin (Alexis Biosciences) antibodies were used as primary antibodies. Then anti-goat Alexa568, anti-rabbit Alexa546, anti-rabbit Alexa647 and anti-mouse Alexa647 antibodies (Molecular Probes) were used as secondary antibodies. Sections were mounted in Vectashield® mounting media containing DAPI (Vector Laboratories). Images were captured using a laser scanning confocal microscope (LSM510 META, Carl Zeiss).

Luciferase Reporter Assay—RAW264.7 cells were plated at 5 × 10⁴ cells/well in 48-well plates, and 0.2 μg of the luciferase construct, 0.2 μg of a pBabe activator plasmid, and 0.02 μg of β-actin-RENilla internal control were co-transfected using Lipofectamine LTX (Invitrogen). A 1.6 kb fragment containing the ephrinA2 promoter and the entire 5’-untranslated region fragment was PCR-amplified from mouse genome using the primers, 5’-AGCATGCAAATGAGGCCTGGTGATG-3’ and 5’-GAGTCTGAGGGTGCAGAGGGCTTCC-3’, and then cloned into pGL3 vector. pGL3 containing 5’ TRE (TPA-response element) and pBabe expressing c-Fos or c-Jun were tethered via a flexible peptide linker were used as secondary antibodies. Sections were mounted in Vectashield® mounting media containing DAPI (Vector Laboratories). Images were captured using a laser scanning confocal microscope (LSM510 META, Carl Zeiss).

Infection of Cells with Retroviral Vectors—Fetroviruses were produced from Plat-E cells (17) and were used to infect cells in the presence of 8 μg/ml Polybrene (Sigma) and 10 ng/ml M-CSF for 72 h.

Plasmids—An ephrinA2 cDNA was PCR-amplified from RAW264.7 macrophages treated with RANKL for 24 h. The forward primer contained a BamHI and the ATG codon, 5’-GGATCCACATGGCGCCGCAGCCGTGCCG-3’, and the reverse primer contained a stop codon and an XhoI, 5’-CGGTCACTGGCTGAGCCGGCAGCCG-3’. The PCR product was cloned into retroviral vector pMX-IRES-EGFP. pMX-Fos-IRES-EGFP, pMX-caNFATc1-IRES-EGFP (8), pShuttle-EphA2, pShuttle-EphA2-K646M, pEGFP-EphA2-ΔC (18), pCLXSN-EphA2-GLZ, pCLXSN-EphA2-Myr-GLZ, and pCLXSN-EphA2-Myr-GLZ-K646M (19) were described previously.

Bone Resorption Assay—Bone slices were prepared as described (11). The slices were placed at the bottom of each well, and cells were cultured on top of the slices. The resulting cells were removed in 50 mM NH₃OH at 4 °C. The slices were stained with wheat germ agglutinin/lectin/horseradish peroxidase (Sigma) (20) for 1 h and developed in diaminobenzidine solution (Dako). The bone slices were scanned, and the resorbed area per bone slice area was calculated using ImageJ (National Institutes of Health).

Flow Cytometry—MDMs were stained with anti-ephrinA2 (R & D Systems) and anti-goat Alexa647 (Molecular Probes) antibodies. Stained cells were analyzed using FACSCalibur (BD Biosciences). For cell sorting, retrovirus-infected GFP-positive MDMs were enriched by MoFlo (Beckman Coulter).

RESULTS

Expression of EphrinA2 and EphA2 in Osteoclast and Osteoblast Precursors—We first analyzed expression of all known members of the ephrinA and EphA families during osteoclast and osteoblast differentiation by RT-PCR. In osteoclasts, ephrinA2 was induced 1 day after RANKL addition, and its receptors EphA2 and EphA4 were also detected in the osteoclast lineage, EphA4 being limited to mature osteoclasts (Fig. 1A). Osteoblasts expressed multiple ephrinAs and EphAs (Fig. 1A). By qRT-PCR analysis, induction of ephrinA2 was detected 10 h after the RANKL addition following c-Fos expression (Fig. 1B). Curiously, EphA2 expression was transiently reduced when ephrinA2 was induced during the early phase of osteo-
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To examine whether c-Fos could regulate ephrinA2 expression, we prepared MDMs from c-Fos KO splenocytes. By qRT-PCR analysis, we found that RANKL-induced ephrinA2 expression was abolished in Fos KO MDMs, suggesting that ephrinA2 is a direct or indirect transcriptional target of c-Fos (Fig. 2A). Next, we prepared wild-type MDMs and treated them with increasing concentrations of the calcineurin inhibitor FK506, which blocks NFATc1 activation. The presence of FK506 did not affect RANKL-induced ephrinA2 (Fig. 2B, left panel), whereas FK506 suppressed ephrinB2 expression in a dose-dependent manner, as expected (11) (Fig. 2B, right panel). Consistently, ephrinA2 expression in Fos KO MDMs under osteoclastogenic conditions was rescued by retrorional gene transfer of c-Fos but not by that of NFATc1 (Fig. 2C, left panel). As expected, both c-Fos and NFATc1 restored expression of Caler (encoding calcitonin receptor), a target gene of NFATc1, in Fos KO MDMs (Fig. 2C, right panel). Furthermore, the 1.6-kb fragment containing the ephrinA2 promoter and a promoter containing consensus AP-1-binding sites (5′× TRE) were both activated by a tethered AP-1 dimer, c-Fos−c-Jun in which “−” indicates a polypeptide linker (16) (Fig. 2D). These results demonstrate that ephrinA2 expression is c-Fos-dependent and NFATc1-independent.

Reverse Signaling through EphrinA2 Enhances Osteoclastogenesis—To examine the function of ephrinA2 in osteoclast differentiation, we infected MDMs with an ephrinA2-expressing retroviral vector. Higher numbers of TRAP-positive MNCs were produced for ephrinA2-infected MDMs than empty vector-infected controls, and the effect was more prominent when infected cells were enriched by cell sorting before inducing differentiation (Fig. 3A). Consistently, ephrinA2-expressing MDMs resorbed a larger area of bone surface compared with controls (Fig. 3B); this reflected enhanced differentiation because the bone resorption activity per cell remained unchanged as judged by re-plating experiments of mature osteoclasts (data not shown). To stimulate reverse signaling through ephrinA2 into osteoclasts in the absence of forward signaling, we transfected a wild-type (WT) and EphA2

oclast differentiation (Fig. 1B). By contrast, ephrinB2, NFATc1, and TRAP were gradually increased and reached maximum levels over 60 h (Fig. 1B). Similar patterns of ephrinA2 and EphA2 expression were observed at protein levels (Fig. 1C). Furthermore, both ephrinA2 and EphA2 were detected in osteoclasts and osteoblasts on the bone surface in vivo (Fig. 1, D–O). These data suggest that ephrinA2 on cells in the osteoclast lineage can interact with EphA2 on osteoclasts in addition to EphAs on osteoblasts.

c-Fos-dependent, NFATc1-independent Expression of EphrinA2—The AP-1 component c-Fos and its target NFATc1 are both essential transcriptional factors for osteoclastogenesis.
mutants lacking the cytoplasmic region (∆C) or lacking kinase activity of intracellular region (K646M) into MDMs. As expected, these EphA2 proteins were found in a membrane fraction of MDMs (Fig. 3C). The ectodomain of ∆C and K646M enhanced osteoclast differentiation as WT did, suggesting that EphA2 induces reverse signaling into osteoclast precursors and positively regulates osteoclastogenesis (Fig. 3D).

To determine whether osteoblasts could enhance osteoclastogenesis through EphA2 on osteoclasts, we co-cultured EphA2-expressing MDMs with calvarial osteoblasts (OB). Overexpression of EphA2 in osteoclasts significantly increased TRAP-positive MNCs in a manner that depended on osteoblast cell number (Fig. 3E). Consistently, osteoblasts lacking EphA2 were less able to induce osteoclastogenesis (Fig. 3F). Reduction in RANKL, or increase in the decoy receptor osteoprotegerin, could explain the reduced osteoclast-inductive activity, and a ratio of RANKL/osteoprotegerin was decreased in osteoblasts lacking EphA2 than WT osteoblasts by qRT-PCR (data not shown). However, RANKL addition into coculture medium did not relieve suppressed osteoclast-inductive activity of osteoblasts lacking EphA2. These data suggest that osteoclast differentiation is enhanced through EphA2 reverse signaling.

Forward Signaling through EphA2 Also Enhances Osteoclastogenesis—It is known that ephrinA2 is cleaved in trans by membrane ADAMs, especially a disintegrin and metalloproteinase (ADAM) 10 (21, 22). Cells in the osteoclast lineage express ADAM10 and other ADAMS during differentiation (data not shown) (23, 24). To determine whether ADAMs cleave EphA2 on osteoclasts, we used fluorescence-activated cell sorter to analyze cell surface EphA2 in the presence or absence of BB94, a widely used inhibitor of MMPs and ADAMs. The amount of RANKL-induced ephrinA2 measured as mean fluorescence intensity was moderately but significantly increased by BB94 treatment (Fig. 4A, right panel; *p = 0.005). EphrinA2 was not detected on MDMs treated with M-CSF alone (Fig. 4A, left panel). These data indicate that MMPs cleave a fraction of cell surface ephrinA2 on osteoclast precursors. Next we determined the effect of BB94 on osteoclast differentiation. The number of giant osteoclasts was slightly but significantly increased by BB94 treatment when cells were seeded at high cell densities (Fig. 4B). These results suggest that osteoclast surface proteins such as ephrinA2, which are cleaved by MMPs in the absence of BB94, enhance osteoclastogenesis in a cell-cell contact-dependent manner. To examine potential functions of released ephrinA2 after cleavage, osteoclastogenesis was induced in the presence of culture supernatants of MDMs overexpressing ephrinA2 (Fig. 4C). The supernatant enhanced formation of giant osteoclasts, suggesting that soluble ephrinA2 stimulates EphAs on osteoclast precursors and enhances osteoclast differentiation via forward signaling. We therefore determined the effect of forward signaling through EphA2 in the absence of reverse signaling. We infected MDMs with retroviruses expressing wild-type EphA2, two constitutively active forms of the EphA2 cyto-
plasmic region (GLZ, the GCN4 leucine zipper was added to the amino terminus of the EphA2 cytoplasmic region; and Myr-GLZ, a myristoylation sequence was added to the amino terminus of GLZ), and a kinase-dead mutant of Myr-GLZ (Myr-GLZ-K646M) (19) (Fig. 4D). We found that overexpression of GLZ and Myr-GLZ but not Myr-GLZ-K646M enhanced giant osteoclast formation as efficiently as wild-type EphA2 (Fig. 4E). These data demonstrate that EphA2 positively regulates osteoclastogenesis through forward signaling. Collectively, both reverse and forward signaling of ephrinA2-EphA2 interaction result in enhanced osteoclastogenesis. It is known that both glycosylphosphatidylinositol-anchored proteins and EphA4 signaling can activate phospholipase Cγ (PLCγ) (27, 28). Furthermore, PLCγ2 positively regulates osteoclastogenesis (29–31). To determine whether ephrinA2 or EphA2 on osteoclasts regulates PLCγ2, we analyzed expression of PLCγ2 and phosphorylated PLCγ2 in MDMs expressing ephrinA2, EphA2, EphA2-ΔC (to stimulate reverse signaling through ephrinA2), and EphA2-GLZ (to activate forward signaling through EphA2) using immunoblot analysis. 48 h after RANKL addition, PLCγ2 and phosphorylated PLCγ2 expression were increased in ephrinA2-, EphA2-, and EphA2-ΔC-expressing cells but not in GLZ-expressing cells (Fig. 4F). These results suggest that ephrinA2 reverse signaling up-regulates PLCγ2 expression.

EphA2 Signaling Suppresses Osteoblast Differentiation—Next, we hypothesized that RANKL-induced ephrinA2 on osteoclast precursors could act on osteoblasts by stimulating EphA2 on osteoblasts. To determine the function of EphA2 on osteoblasts, calvarial osteoblasts were isolated from EphA2-deficient newborn mice and were cultured under osteoblastogenic conditions. Staining for ALP activity and calcium deposition revealed that osteoblasts lacking EphA2 differentiate more efficiently than wild-type controls (Fig. 5A). As expected, expression of EphA2 was undetectable in EphA2 KO osteoblasts (Fig. 5B). Expression levels of osteoblast differentiation markers, ALP, Runx2 and Osterix, were increased in EphA2-deficient osteoblasts compared with wild-type osteoblasts (Fig. 5B). We have previously shown that GTP-RhoA negatively regulates osteoblastogenesis in mice (11). In osteoblasts lacking EphA2, we found that enhanced osteoblastogenesis is accompanied by a decrease in GTP-RhoA, suggesting that signaling through EphA2 into osteoblasts suppresses osteoblast differentiation by activating RhoA (Fig. 5C).

**DISCUSSION**

Accumulating evidence indicates that ephrins and Epha influence cell proliferation and fate determination (25). In this study, we found that interaction between osteoclastic ephrinA2 and either osteoclastic or osteoblastic EphA2 regulates differentiation of these cell types in a different way from ephrinB2-EphB4 interaction.

Our data revealed that ephrinA2 mRNA was rapidly induced after RANKL addition following c-Fos induction, with expression peaking by 24 h. This early induction of ephrinA2 during osteoclast differentiation is c-Fos-dependent but NFATc1-independent. Because ephrinB2 is expressed at a later stage of osteoclast differentiation and the induction is dependent on both c-Fos and NFATc1 (11), the c-Fos-NFATc1 transcriptional cascade differentially regulates expression of ephrinA2 and ephrinB2. Luciferase reporter assay demonstrated that the 1.6-kb upstream region of ephrinA2 gene was activated by c-Fos~c-Jun. Although there are a few potential c-Fos/AP-1-binding sites in this region, it is not known whether c-Fos directly binds to the ephrinA2 or not. Further analysis is neces-
We propose that the osteoclast precursor senses its neighboring osteoclast precursors as well as osteoblasts through ephrinA2-EphA2 interactions. Osteoclast precursors mutually enhance differentiation toward cell-cell fusion, and ephrinA2 reverse signaling into osteoclasts may also be stimulated by EphA-expressing osteoblasts. In summary, at the initiation phase of bone remodeling, ephrinA2-EphA2 interaction promotes bone resorption and concomitantly suppresses osteoclastogenesis (Fig. 6). This is in contrast to the transition phase of bone remodeling from bone resorption to bone formation, when ephrinB2-EphB4 interaction inhibits osteoclastogenesis with concomitant promotion of bone formation (Fig. 6). We conclude that bone remodeling is distinctly regulated by ephrin-Eph of both classes A and B.

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FIGURE 6. Schematic presentation of ephrin-Eph interactions during bone remodeling. At the initiation phase of bone remodeling, ephrinA2-EphA2 interaction enhances osteoclastogenesis and inhibits osteoblastogenesis. At the transition phase, ephrinB2-EphB4 interaction inhibits osteoclastogenesis and enhances osteoblastogenesis (11). Note that the effects of ephrin-Eph on osteoclast and osteoblast differentiation are opposite between classes A and B.
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