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Basic Fibroblast Growth Factor Induces Cyclooxygenase-2 Expression in Endothelial Cells Derived from Bone[1]

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Although histological studies have suggested that endothelial cells in bone (BDECs) are associated with some osteolytic bone diseases, it is still unclear how BDECs contribute to bone remodeling. Here we examined the response of BDECs to basic fibroblast growth factor (bFGF, FGF-2) using primary and cloned murine BDECs isolated from the femurs of BALB/c mice. Treatment of primary and cloned BDECs with bFGF induced cyclooxygenase-2 (COX-2) mRNA and protein expression. Furthermore, bFGF promotes the production of prostaglandin E_2 (PGE_2), which is known to be a potent stimulator of bone resorption and to induce osteoclast formation. Because the secretion of PGE_2 was suppressed by COX-2 specific inhibitor NS-398 and by COX-2 antisense oligodeoxynucleotides, bFGF promotes the synthesis of PGE_2 in a COX-2-dependent manner. Therefore, endothelial cells in bone are associated with bone remodeling by controlling COX-2 expression and consequently PGE_2 production.

Bone is a dynamic organ in which resorption and formation continue throughout life (1). In normal bone remodeling, the formative and degradative processes are carefully controlled by systemic hormones and by locally produced soluble factors in the adult skeleton (2). Disturbances of the balance lead to a number of metabolic bone diseases. Bone remodeling and production of soluble factors are regulated by complex interactions among bone-forming osteoblasts, bone-degrading osteoclasts, and other cells present in the bone microenvironment (e.g., endothelial cells and fibroblasts). Owing to the close proximity of endothelial cells to osteoblasts and osteoclasts in bone tissue (3, 4), endothelial cells may play a crucial role in normal bone remodeling and pathological states such as osteoporosis, arthritis, and cancer metastasis to bone.

Prostaglandins are abundant in bone and are reported to be produced largely from the cells of the osteoblast lineage. Among PGs, PGE_2 is a potent stimulator of bone resorption *in vitro* and induces osteoclast formation in mouse bone marrow culture. PGs synthesis is regulated by cyclooxygenases (COXs), which catalyze the production of PGs from arachidonic acids (5). Two isoform of COXs are now identified. COX-1 is constitutively expressed, and COX-2 is inducibly expressed by a variety of mitogen and inflammatory stimuli (5–8). Basic fibroblast growth factor (bFGF) is known to be a potent modulator of endothelial cell function and to be produced at the time of neovascularization or tumor growth (9, 10). The expression level of COX-2 is promoted by bFGF in osteoblastic cells and SHE cells (11, 12). However, the effects of bFGF on endothelial cells in bone are still unclear.

We have previously established mouse bone-derived endothelial cell lines (BDECs) from femurs of BALB/c mice (13). In this study, we investigated the effects of bFGF on the expression of COX-2 in primary and cloned BDECs. We found that the endothelial cells in bone play an important role in bone remodeling by producing PGE_2.

MATERIALS AND METHODS

Cell culture conditions. The mouse bone marrow-derived endothelial cell line BM-3 was established in our laboratory, as described previously (13). BM-3 cells were cultured on gelatin-coated dishes

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Abbreviations used: BDECs, endothelial cells in bone; bFGF, basic fibroblast growth factor; PG, prostaglandin; COX, cyclooxygenase; RT-PCR, reverse-transcription polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay.

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(Iwaki, Tokyo, Japan). The mouse calvaria-derived osteoblast cell line, MC3T3-E1, was obtained from the Riken Cell Bank (Ibaraki, Japan). All cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Nissui Phamaceutical Co., Tokyo, Japan) supplemented with 2 mM L-glutamine (Gibco Laboratories, Grand Island, NY), 100 μg/ml of kanamycin (Meiji Seika Co. Ltd., Tokyo, Japan), and 10% heat-inactivated FBS (Gibco) (DMEM growth medium) at 37°C in a humidified atmosphere of 5% CO_2-95% air.

Preparation of primary BDECs. Isolation of the primary endothelial cells in bone marrow was performed by the method described previously (13). Briefly, femurs and tibias of BALB/c mice (Charles River Japan Inc., Kanagawa, Japan) were isolated and washed with phosphate-buffered saline (PBS). After removing the smooth muscle and periosteum, the bone marrow was washed out of the femurs and tibias with PBS. The cells were collected and resuspended in DMEM growth medium containing 598 μg/ml of D-valine (Sigma, St. Louis, MO). The cells were plated on gelatin-coated dishes and cultured for about 2 weeks.

RT-PCR. mRNA was extracted using a QuickPrep mRNA isolation kit, according to the manufacturer's instruction (Pharmacia, Uppsala, Sweden). RT-PCR was carried out using a GeneAmp RNA PCR core kit (Perkin–Elmer, Norwalk, CT) (14). The sense and antisense primers of mouse glyceraldehyde-3-phosphate dehydrogenase (mG3PDH) were purchased from Clontech Laboratories, Inc. (Palo Alto, CA). Murine COX-1 sense (5′-TGCATGTGGCTGTGGATGTCATCAA-3′) and antisense (5′-CACTAAGACAGACCCGTCATCTCCA-3′) primers were designed according to the cDNA sequence. Murine COX-2 sense (5′-ACTCACTCAGTTTGTTGAGTCATTC-3′) and antisense (5′-TTTGATTAGTACTGTAGGGTTAATG-3′) primers were designed according to the cDNA sequence (15). The amplified fragments were detected in the expected sizes by ethidium bromide staining in a 1.8% agarose gel plate.

Nuclear run-off assay. BM-3 cells were seeded at a density of 1×10^5 cells/well onto six-well gelatin-coated dishes and grown to 50-80% confluence in DMEM growth medium. MC3T3-E1 cells were seeded at the same density onto six-well dishes and grown in DMEM growth medium. After incubation for 18–24 h, the cells were rinsed twice with PBS. Then 2 μg of reporter plasmid DNA (TIS10L; generously provided by Dr. Harvey Herschman, University of California, Los Angeles, CA) (16, 17) was introduced into cells using 8 μg of Lipofect Amine (Gibco), according to the manufacturer's instructions. After incubation for 5 h, medium was replaced with DMEM growth medium. After incubation for additional 18 h, the cells were washed twice with PBS and then cultured in serum-free DMEM medium containing 0.1% bovine serum albumin (BSA; Sigma) with 10 ng/ml of bFGF (Calbiochem, Cambridge, MA). Luciferase activity was measured using luciferase assay system as per the manufacturer's instructions (Promega, Madison, WI). Luciferase activity in the lysate was measured using MicroLumat LB96P luminometer (Berthold, Bad Wildbad, Germany). Activities were normalized by measuring protein concentrations in the cell lysates with a BCA protein assay kit (Pierce, Rockford, IL).

Western blot analysis. Western blot analysis was performed as described previously (18). Briefly, BM-3 cells were cultured in serum-free DMEM medium containing 0.1% BSA and incubated with or without bFGF. The cells were harvested and solubilized with lysis buffer containing Nonidet P-40 and 0.2% SDS. Then the cell lysates (50 μg/lane) were applied to a 4–20% gradient polyacrylamide gel. The electrophoresed proteins were transblotted onto a nitrocellulose membrane. After blocking, the membranes were incubated with an anti-mouse COX-1 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or an anti-mouse COX-2 monoclonal antibody (Transduction Laboratories, Lexington, KY). The membranes were then incubated with appropriate peroxidase-conjugated second antibody and developed with enhanced chemiluminescence mixture (Amersham, Buckinghamshire, UK).

PGE_2 ELISA. BM-3 cells were plated on 6-well gelatin-coated dishes (2×10^5 cells/well) and grown to confluence. After washing twice with PBS, the cells were incubated in DMEM medium containing 0.1% FBS with appropriate concentrations of bFGF. In some experiments, BM-3 cells were pretreated with COX inhibitors, 0.1 μM indomethacin, 0.2 μM dexamethasone, or 1 μM NS-398, 3 h before adding bFGF. The appropriate concentration and time of inhibitors was determined according to previous reports (19–23). In some experiments, 15 μM COX-2 antisense phosphorothioate oligodeoxynucleotide (5′-GCAGAGCAGCACCGA-3′) and sense phosphorothioate oligodeoxynucleotide (5′-TCGGTGCTGCTCTGC-3′) (24) were transiently transfected into BM-3 cells using Lipofect Amine 18–24 h before adding bFGF. The medium was filtered and frozen at −80°C until use. PGE_2 concentration in the culture medium was evaluated using the enzyme immunoassay kit, according to the manufacturer's instructions (Cayman Chemical Co., Ann Arbor, MI).

RESULTS

Effect of bFGF on COX-2 mRNA expression in BDECs. Osteoblast-lineage cells are reported to regulate bone remodeling by secreting PGE_2 in response to bFGF (11). We, therefore, examined the change of COX-2 mRNA expression in primary mouse BDECs and in cloned mouse bone marrow-derived endothelial BM-3 cells by RT-PCR analysis (Fig. 1). As shown in Fig. 1A (upper panel), the expression level of COX-2 mRNA in the primary BDECs was rapidly induced by 10 ng/ml of bFGF within 1 h (Fig. 1A, lane 2). Then the amount of COX-2 mRNA declined slightly in a time-dependent manner. In contrast, the expression levels of COX-1 and G3PDH were not changed by the bFGF treatment (Fig. 1A, middle and lower panels). The increase in COX-2 mRNA expression was similarly observed in BM-3 cells (Fig. 1B).

When primary BDECs were treated with various concentrations of bFGF, the amount of COX-2 mRNA increased in a dose-dependent manner (Fig. 1C, upper panel). The maximum COX-2 mRNA expression was observed when primary BDECs were treated with 1–10 ng/ml of bFGF. The expression levels of COX-1 and G3PDH mRNA were not affected by bFGF treatment (Fig. 1C, middle and lower panels). BM-3 cells exhibited similar responses to bFGF (data not shown).

To further quantify the transcriptional regulation of COX-2 by bFGF, we performed nuclear run-off assay. BM-3 and MC3T3-E1 cells were transiently transfected with COX-2 promoter-luciferase fusion gene containing 963 bases of 5′ flanking sequence (TIS10L) (16, 17). The addition of bFGF immediately increased the luciferase activity in TIS10L-transfected BM-3 and MC3T3-E1 cells (Fig. 2). This activity increase maximized at 3 h and was maintained for more than 24 h in BM-3 cells (Fig. 2A). In MC3T3-E1 cells the activity rapidly decreased (Fig. 2B).

bFGF stimulation of COX-2 protein expression and PGE_2 production. To confirm that bFGF stimulated COX-2 protein expression in BDECs, we performed Western blot analysis using an anti-COX-2 monoclonal

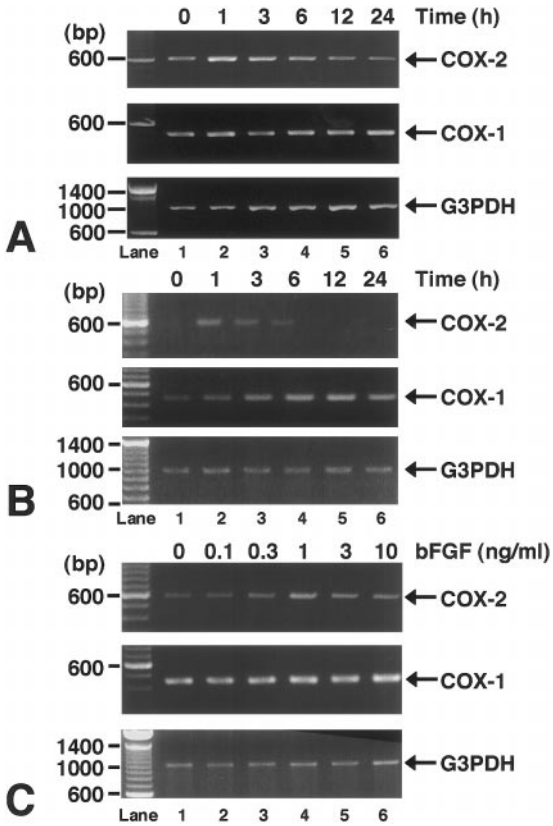

FIG. 1. Induction of COX-2 mRNA expression in primary BDECs and in BM-3 cells by bFGF treatment. (A and B) The primary BDECs (A) and BM-3 cells (B) were pretreated with serum-free DMEM for 12 h and then incubated with 10 ng/ml of bFGF for the indicated time. (C) The primary BDECs were pretreated with serum-free DMEM for 12 h and then incubated with the indicated concentrations of bFGF for 1 h. The mRNA of each sample was extracted, and RT-PCR was carried out using COX-2, COX-1, and G3PDH primer sets. The amplified products of COX-2 (583 bp), COX-1 (449 bp), and G3PDH (983 bp) were stained with ethidium bromide, electrophoresed in a 1.8% agarose gel plate, and visualized by UV illumination. The molecular size marker was the 100-bp DNA ladder. Similar results were obtained in three independent experiments.

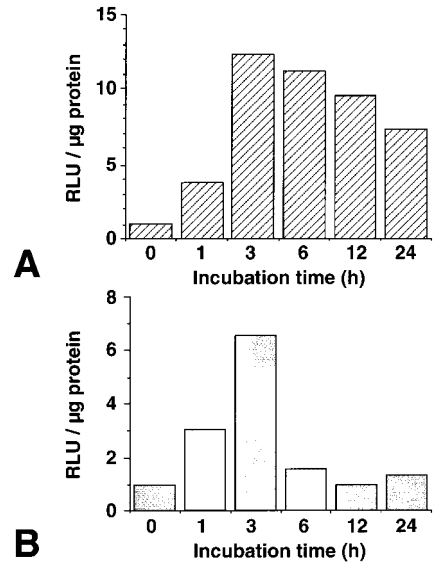

FIG. 2. Up-regulation of COX-2 promoter activity by incubation with bFGF in BM-3 and MC3T3-E1 cells. BM-3 (A) and MC3T3-E1 (B) cells were transiently transfected with the vector containing the luciferase reporter gene (TIS10L). After incubation for 5 h, the cells were incubated with serum-free DMEM for 12 h. The cells were then incubated with 10 ng/ml of bFGF. At the indicated time points, cells were harvested, and luciferase activity in the cell lysate was measured, as described under Materials and Methods. The luciferase activity was represented by the relative light unit (RLU; activity/μg of protein). Repeated experiments gave similar results.

antibody. After pretreatment with serum-free medium for 12 h, BM-3 cells were incubated with 10 ng/ml of bFGF for 1, 3, 6, 12, 24, and 48 h (Fig. 3A). The expression level of the COX-2 protein increased in a time-dependent manner, and it continued for 24 h, while that of the COX-1 protein was not changed by bFGF. When BM-3 cells were treated with various concentrations of bFGF for 6 h, the maximum COX-2 protein expression was observed at 1–30 ng/ml of bFGF. Consistent with RT-PCR analysis (Fig. 1), the expression level of COX-1 protein was not affected by bFGF treatment (Fig. 3).

Because COX-2 was associated with the production of PGs, we measured the concentrations of PGE_2 in BM-3 cell culture medium using an ELISA kit specific for PGE_2. Among the prostaglandins, PGE_2 is a potent stimulator of bone resorption (25–29). bFGF increased PGE_2 secretion from BM-3 cells, and the maximum concentration of PGE_2 in BM-3 cell culture medium was observed after 24 h of bFGF treatment (Fig. 4A). bFGF promoted PGE_2 synthesis in a dose-dependent manner (Fig. 4B).

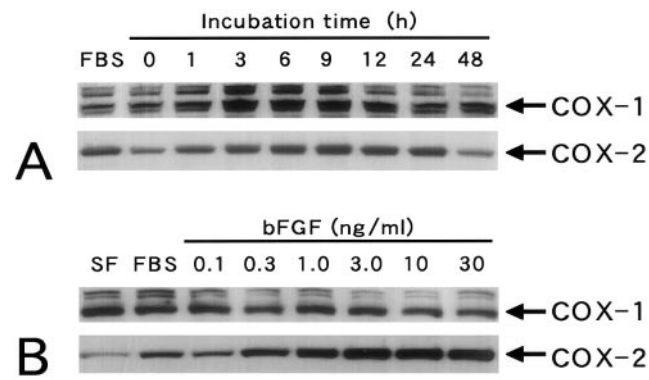

FIG. 3. Effects of bFGF on the COXs protein expression. (A) BM-3 cells were pretreated with serum-free DMEM for 12 h and then incubated with 10 ng/ml of bFGF for the indicated time points. (B) BM-3 cells were pretreated with serum-free DMEM for 12 h and then incubated with the indicated concentrations of bFGF for 6 h. The cell lysates were electrophoresed and blotted onto a nitrocellulose membrane. The membrane was developed with an anti-mouse COX-1 polyclonal antibody (upper panels) or an anti-mouse COX-2 monoclonal antibody (lower panels), as described under Materials and Methods. BM-3 cells cultured in DMEM growth medium (10% FBS) were used as the positive control. Repeated experiments gave similar results.

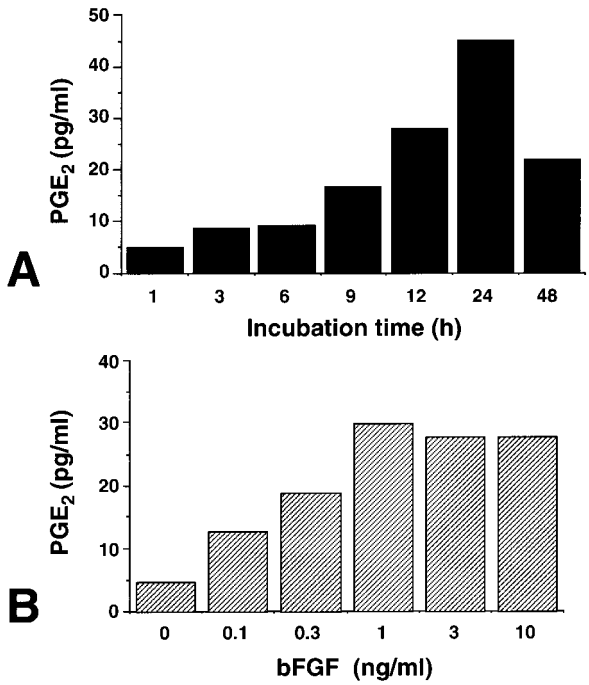

FIG. 4. Induction of PGE_2 production by bFGF in BM-3 cells. (A) BM-3 cells were pretreated with serum-free DMEM for 12 h and then incubated with 10 ng/ml of bFGF for the indicated times. (B) BM-3 cells were incubated with the indicated concentrations of bFGF for 6 h. The concentration of PGE_2 in the BM-3 cell culture medium was estimated by ELISA, as described in Materials and Methods. All data represent means of duplicate determinations. Repeated experiments gave similar results.

Involvement of COX-2 in bFGF-mediated PGE_2 production in BDECs. After pretreatment with serum-free medium for 12 h, BM-3 cells were incubated with three kinds of COX inhibitors 3 h before bFGF was added. Then the cells were incubated with 10 ng/ml of bFGF for 3 h. We used 0.1 μM indomethcin, nonselective COX-1/COX-2 inhibitor, 0.2 μM dexamethasone, an inhibitor of the transcription of the COX-2 gene and 1 μM NS-398, which inhibits the COX-2 enzymatic activity by binding to the COX-2 enzyme active site. Because the COX-2 specific inhibitor, NS-398, suppressed the secretion of PGE_2 from BM-3 cells (Fig. 5A), COX-2 might be involved in the bFGF-induced production of PGE_2.

To confirm the role, BM-3 cells were treated with COX-2 sense or antisense oligodeoxynucleotides before adding bFGF. The treatment of BM-3 cells with COX-2 antisense oligonucleotides specifically reduced the secretion of PGE_2 from BM-3 cells (Fig. 5B). The COX-2 sense oligonucleotides had marginal effects on PGE_2 production in BM-3 cells. These results further confirmed that bFGF-induced PGE_2 production in BDECs was mediated through the up-regulation of COX-2 protein expression.

DISCUSSION

PGs are abundantly produced from cells of the osteoblast lineage cells and are complex regulators of bone metabolism. Many osteoclast- and osteoblast-regulating hormones and cytokines, such as PTH, TGFs, IL-1, TNFs, and FGFs, are known to affect PG production from bone. Among PGs, PGE_2 is known to be a strong stimulator of bone resorption (25–29). Two types of COX (prostaglandin G/H synthetases) are associated with the PGs production. COX-1 is an enzyme that is expressed constitutively in many tissues. In contrast, the expression of COX-2 is induced by appropriate stimuli in fibroblasts, endothelial cells, macrophages, and osteoblastic cells (5–8). The reason for acute increase in PGE_2 in inflammatory conditions might depend on the rapid and transient increase in COX-2 expression.

Basic fibroblast growth factor (bFGF; FGF-2) is a member of the FGF family that comprises nine members (30). bFGF has many biological effects during such physiological or pathological processes as neovascularization, tissue repair, growth of smooth muscle cells,

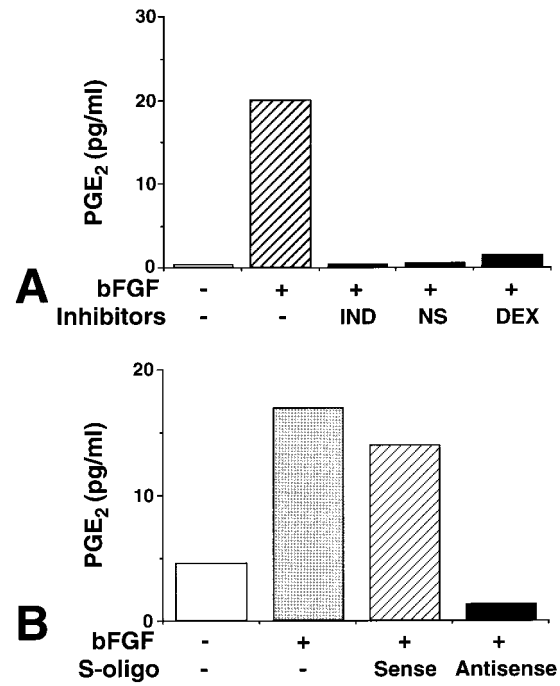

FIG. 5. Involvement of COX-2 in bFGF-induced PGE_2 production. (A) BM-3 cells were pretreated with serum-free DMEM for 12 h and then the cells were incubated with COXs inhibitors, indomethcin (IND), NS-398 (NS), or dexamethasone (DEX) 3 h before the addition of bFGF. (B) BM-3 cells were transiently transfected with 15 μM sense or antisense phosphorothioate oligodeoxynucleotide, as described under Materials and Methods. Then the transfected cells were stimulated with bFGF. BM-3 cell culture medium were collected after stimulation with 10 ng/ml of bFGF for 12 h. The PGE_2 concentration in the medium was estimated by ELISA, as described under Materials and Methods. All data represent means of duplicate determinations. Repeated experiments gave similar results.

wound healing, hematopoiesis and differentiation of the nervous system. bFGF, which also plays an important role in the differentiation and the function of the skeleton, is produced from osteoblast-lineage cells and stored in the extracellular matrix in association with heparin sulfate. bFGF stimulates new bone formation through regulating the proliferation and differentiation of osteoblast-lineage cells. In contrast, it is also known to stimulate bone resorption (6, 31). Thus bFGF plays some roles in bone metabolism.

Since the vascular endothelial cells in bone (BDECs) are thought to play an important role in bone metabolism (13, 32), we previously established BDECs from femurs of BALB/c mice by transformation with the SV40 virus (13). The established and primary BDECs promoted bone resorption *in vitro* by secreting bone-resorption inducing factors in response to several stimuli. These results indicate that bone endothelial cells are certainly involved in the bone remodeling. We here found that bFGF promoted the expression of COX-2 mRNA and protein in the SV40-transformed BM-3 cells. Because freshly prepared non-transformed primary BDECs also expressed COX-2 mRNA in response to bFGF, the expression of COX-2 mRNA in BM-3 cells might not due to SV40 transformation.

This is the first report proving that bFGF increased production of COX-2 and PGE_2 in BDECs, though bFGF was known to induce the expression of COX-2 in osteoblastic cells (11). Because several reports proved that endothelial cells in different organs had different characteristics (13, 33), it is important to examine the effects of systemic hormones and soluble factors on endothelial cells in bone microenvironments to clarify the role of endothelial cells in bone remodeling. Our present results strongly indicate that vascular endothelial cells in bone are the important component of bone metabolism, like osteoblasts and osteoclasts.

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