Objective: Arterial calcification and osteoporosis are associated in postmenopausal women. RANK (the receptor activator of nuclear factor κB), RANKL (RANK ligand), and osteoprotegerin are key proteins in bone metabolism and have been found at the site of aortic calcification. The role of these proteins in vasculature, as well as the contribution of estrogen to vascular calcification, is poorly understood.

Methods and Results: RANKL induced the calcification inducer bone morphogenetic protein-2 by human aortic endothelial cells (HAECs) and decreased the calcification inhibitor matrix Gla protein (MGP) in human aortic smooth muscle cells (HASMCs), as quantified by real-time PCR and Western blot analysis. RANKL also induced bone-related gene mRNA expression and calcium deposition (Alizarin red staining) followed by the osteogenic differentiation of HASMCs. Estrogen inhibited RANKL signaling in HAECs and HASMCs mainly through estrogen receptor α. Apolipoprotein E–deficient mice fed with Western high-fat diet for 3 months presented atherosclerotic calcification (Oil red and Alizarin red staining) and osteoporosis (microcomputed tomographic analysis) after ovariectomy and increased expression of RANKL, RANK, and osteopontin in atherosclerotic lesion, as detected by in situ hybridization. Estrogen replacement inhibited osteoporosis and the bone morphogenetic protein osteogenic pathway in aorta by decreasing phosphorylation of smad-1/5/8 and increasing MGP mRNA expression.

Conclusions: RANKL contributes to vascular calcification by regulating bone morphogenetic protein-2 and MGP expression, as well as bone-related proteins, and is counteracted by estrogen in a receptor-dependent manner. (Circ Res. 2010;107:466-475.)

Key Words: RANKL  vascular calcification  BMP-2  MGP  estrogen
Estrogen regulates bone metabolism by inducing osteoclast apoptosis and osteoblast expression of OPG, and estrogen also inhibits vascular lesion progression, promotes favorable alterations in lipoprotein metabolism, and controls adhesion molecules, contributing to antiinflammatory responses in vasculature. In the controlled Women’s Health Initiative Study, postmenopausal women aged 50 to 59 years treated with long-term estrogen therapy had lower levels of coronary artery calcification than those who received placebo. However, the relationship between estrogen deficiency and arterial calcification has not been fully clarified, with only a recent study confirming the positive correlation of the incidence of both pathologies in an animal model.

In the present study, we focus in the crosstalk of RANK system and estrogen as the common pathway regulating aortic calcification and osteoporosis.

Methods
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

In Vitro Experiment
Materials
PMA (phorbol 12-myristate, 13-acetate), 1β-estradiol (E4389), and ICI-182,780 (I4409) were obtained from Sigma (St Louis, Mo). Recombinant human soluble RANK and bone morphogenetic protein (BMP)-2 were purchased from Peprotech EC (London, UK). Anti-human OPG-neutralizing antibody was acquired from Cell Sciences (Canton, Mass). Anti-human RANK, RANKL, and RUNX2/CBFA1 (runt-related transcription factor 2/core binding factor α1) antibodies and recombinant human Noggin Fc chimera were purchased from R&D Systems (Minneapolis, Minn). Anti-human BMP-2, matrix Gla protein (MGP) (N-20) and anti-β-actin antibody were acquired from Santa Cruz Biotechnology (Santa Cruz, Calif). Anti-phospho-smad-1/5/8 antibody was obtained from Cell Signaling Technology (Beverly, Mass). We purchased the ERα agonist 4,4′-[4-(propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT) from Tocris Bioscience (Ellisville, Mo).

Cell Culture
Human aortic vascular smooth muscle cells (HASMCs), HAECs, and normal human osteoblasts (OBs) were obtained from Lonza Walkersville Inc (Walkersville, Md). THP1 cells were purchased from American Type Culture Collection (Rockville, Md) and were activated into macrophage by PMA (phorbol 12-myristate, 13-acetate), as described previously. For detailed cell culture procedures, refer to the Online Methods section.

Relative Quantification of mRNA by Real-Time PCR
The detailed procedure is described in the Online Methods section.

Western Blot Analysis
Western blot analysis was performed as described previously. Briefly, total protein (10 μg) was isolated using lysis buffer, size-fractioned by SDS-PAGE, and transferred to Immobilon-P membrane purchased from Millipore (Bedford, Mass). The blotted protein was incubated with antibodies after indicated treatment. The detailed procedure is described in the Online Methods section.

Calcification Induction
Calcified nodule formation was induced in HASMCs by the Osteogenic Induction Medium (Lonza). The detailed preparation of HASMCs is described in the Online Methods section.

Detection of Calcified Nodule
Cells were washed with HEPES buffer, and fixed with 4% PFA during 45 minutes at 4°C. The cells were washed again with water, stained with Alizarin red, and the nodules were visualized in the light microscope.

Small Interfering RNA Transfection
MGP expression was silenced with the transfection of Silencer small interfering (si)RNA (siRNA ID: 122163) (Applied Biosystems, Foster City, Calif) with Lipofectamine 2000 (Invitrogen, Carlsbad, Calif) as the transfection agent. siRNA transfection was performed for 24 hours preceding subsequent stimulations in HASMCs.

Animal Experiments
Animal Preparation
Female apolipoprotein (Apo)E-deficient mice on a C57/Bl6 background were obtained from The Jackson Laboratory (Bar Harbor, Me).

Experimental Design
Female ApoE-deficient mice (n=45) were ovariectomized or sham-operated and treated with 20 μg/kg per day 17β-estradiol (E2) or saline and fed with normal or Western high-fat (HF) diet for 3 months. The detailed animal experiment design is described in the Online Methods section.

Histological Analysis of Atherosclerotic Lesions
Lipid deposition was detected by Oil red staining. Calcium deposition was visualized by Alizarin red staining, and the area was traced and quantified using BZ-II Analyzer (Keyence, Tokyo, Japan). The detailed procedure is described in the Online Methods section.

In Situ Hybridization
In situ hybridization was performed as described previously. Sections were made at 8-μm thickness, and the cDNAs used for generation of Dig-labeled mouse riboprobes were osteopontin.
Each bar shows the target gene level relative to 18S level.

**Histological Analysis of Proximal Tibia**

Osteoclasts were detected in proximal tibia by tartrate resistance acid phosphatase (TRAP) staining as described previously.22 Osteoclasts were detected in proximal tibia by tartrate resistance acid phosphatase (TRAP) staining as described previously.22 Histological Analysis of Proximal Tibia Osteoclasts were detected in proximal tibia by tartrate resistance acid phosphatase (TRAP) staining as described previously.22

**Microcomputed Tomography**

The tibias were collected and fixed with 4% PFA. Bone density measurements were performed by dual-energy x-ray absorptiometry (x-ray computed tomography system, SMX-100CT-SV; Shimazu, Osaka, Japan) and software TRI/3D-BON (RATOC System Engineering Co Ltd, Tokyo, Japan) as described previously.23

**Statistical Analysis**

All values are expressed as means±SEM. ANOVA with subsequent Fisher’s protected least significant difference test was used to determine the significance of differences in multiple comparisons.

**Results**

**RANK, RANKL, and OPG System in Vascular Cells**

The expression levels of RANK, RANKL, and OPG in human primary vascular cells were assessed by real-time PCR and Western blot analysis. RANK was observed in HAECs, HASMCs, but not, as expected, in OBs; RANKL expression was mainly observed in OBs, also detected in HASMCs, and at lower level in HAECs. OPG mRNA was mainly expressed in HASMCs (Figure 1A), and the protein level was confirmed in HAECs and HASMCs (Figure 1B). Overall, these data suggest that because of receptor RANK expression, vascular cells may respond to stimulation with RANKL, and can regulate the RANK stimulation by expressing OPG.

**Molecular Mechanisms of Vascular Calcification**

Because BMP-2 is a potent inducer of ectopic calcification, and an important factor in aortic calcification,24 we next measured the level of BMP-2 under RANKL stimulation in vascular cells. Basal expression of BMP-2 mRNA was detected in HAECs and macrophages, less in HASCs and THP1 (monocyte) (Online Figure I). Consistently, BMP-2

**Figure 2. Stimulation with RANKL increased the calcification inducer BMP-2 expression only in HAECs. A**, Basal BMP-2 protein level in HAECs, HASMCs, THP1, and activated monocytes (macrophage) (Mac) assessed by Western blot analysis. **B**, BMP-2 mRNA quantification by real-time PCR after 6 hours of stimulation with RANKL (RL) (10 ng/mL) and anti-OPG (0.1 μg/mL) in HAECs (left graph). The right graph shows the BMP-2 protein quantification by ELISA after 24 hours of stimulation with RANKL and anti-OPG. **P**<0.05 vs no treatment (No treat). **P**<0.05 (n=6), **C**, BMP-2 protein level after 24 hours of stimulation with RANKL and anti-OPG in HAECs. **D**, OC, ALP, and OPN expression level quantified in HASMCs by real-time PCR after 1 and 3 days of BMP-2 (50 ng/mL) stimulation replaced every day. **P**<0.05 vs no treatment; 3 days (n=6). **E**, RANKL-induced BMP-2 in HAEC stimulated osteogenic pathway in HASMCs. Cbfa1, MXS2, OC, and ALP mRNA expression in HASMCs after 24 hours of incubation with conditioned medium of HAECs stimulated with RANKL and anti-OPG during 2 days. Addition of Noggin (100 μg/mL) in HASMCs cancelled the osteogenic-related gene expression induced by the condition medium in duplicated experiments. **P**<0.05 vs no treatment.
was detected at protein level mainly in HAECs and macrophages (Figure 2A). RANKL (10 ng/mL) increased the expression of BMP-2 mRNA in HAECs (2.5-fold) (Figure 2B). Because HAECs endogenously expressed OPG, we further studied the role of OPG in BMP-2 expression. As shown in Figure 2B, neutralizing anti-OPG antibody (anti-OPG) (0.1 μg/mL) significantly enhanced RANKL-induced BMP-2 expression. Increase in BMP-2 was confirmed at the protein level in the supernatant of HAECs treated with RANKL for 24 hours (4.6- and 5.2-fold with anti-OPG) (Figure 2B) and by Western blot analysis of total cell protein (Figure 2C). Of note, RANKL did not induce BMP-2 expression in monocytes and HASMCs even under the presence of neutralizing anti-OPG antibody (data not shown). BMP-2 (50 ng/mL) indeed induced the expression of osteogenic genes such as osteocalcin (OC), alkaline phosphatase (ALP), and OPN in HASMCs after 3 days stimulation (Figure 2D). The conditioned medium of HAECs stimulated with RANKL in the presence of anti-OPG during 2 days induced the expression of transcription factors related to osteogenesis (cbfa1, msh homeobox 2 [MSX2]), osterix, and the bone-related genes OC, and ALP in HASMCs after 3 days stimulation in the presence of neutralizing anti-OPG antibody, whereas RANKL alone did not induce (Figure 3B and Online Figure II). In addition, the continuous stimulation with RANKL increased the number of calcified nodules, as assessed by Alizarin red staining, *P<0.05 vs no treatment (n=4). D, This effect might be explained by RANKL additional increase in ALP and decrease in MGP mRNA level, with no effect on BMP-2 expression after 3 days of incubation, compared to medium-treated group (Med) group, *P<0.05 vs medium-treated group. Scale bar, 50 μm.

Effect of Estrogen on Calcification in RANKL-Stimulated HAECs and HASMCs

We further examined the effect of estrogen on RANKL-induced vascular calcification and found that estrogen, in a concentration equivalent to pharmacological levels found in target tissue (3.67 pmol/mL),27 significantly attenuated RANKL-induced BMP-2 mRNA expression in HAECs (Figure 4A), whereas estrogen significantly increased MGP expression in HASMCs (Figure 4B). The estrogen receptor inhibitor ICI-182,780 abrogated those estrogen effects (Figure 4B and 4C).
We verified the relevance of the MGP increase induced by estrogen in inhibiting RANKL-BMP osteogenic pathway. Pretreatment with estrogen during 24 hours inhibited the protein expression of osteogenic transcription factor cbfa1 induced by BMP-2 in HASMCs after 24 hours of stimulation. Knockdown of MGP by siRNA cancelled the protective effect of estrogen pretreatment, as shown by an increase in cbfa1 expression (Figure 4D).

Taken together, the results suggest that estrogen can dually block the RANKL-induced osteogenic pathways through an estrogen receptor–dependent manner.

Estrogen Receptor Isoforms in Vascular and Bone Cells

We next evaluated which estrogen receptor (ER) isoform regulated the estrogen response against vascular calcification and osteoporosis. The ERα mRNA level was higher than ERβ in HASMCs, and they were expressed at the same level in HAECs and OBs (Figure 5A). The addition of selective ER agonists inhibited the RANKL-induced BMP-2 protein increase in HAECs, with a stronger effect of the ERα agonist PPT, compared to ERβ agonist DPN (Figure 5B). In HASMCs, only PPT significantly increased MGP (Figure 5C). In osteoblasts, PPT decreased RANKL and increased OPG protein expression after 24 hours of stimulation, without significant changes in DPN-treated group (Figure 5D). These results show that estrogen simultaneously inhibits osteogenic pathway in vascular cells and decreases RANKL/OPG ratio in osteoblasts by acting mainly through ERα.

The Mouse Model for Vascular Calcification and Osteoporosis

To examine the molecular mechanism underlying osteoporosis and vascular calcification in vivo, we developed the mouse model for vascular calcification in vivo, we developed the mouse model for vascular calcification.
model for vascular calcification and osteoporosis using ApoE-deficient mice. Although it was reported that old ApoE-deficient mice developed calcified lesions in the innominate arteries by 75 weeks of age, we modified the ApoE-deficient mice model with estrogen deficiency by ovariectomy (OVX) and treatment with Western high-fat diet (HF) for 3 months.

Aortic calcification could be detected by Alizarin red staining only in mice with estrogen deficiency (OVX) and HF diet but was not observed in HF diet alone, regardless of the presence of atherosclerosis (Figure 6A). Quantification of the atherosclerotic lesion showed that calcified areas were significantly increased in the OVX/HF group, despite no significant difference in the atherosclerotic area compared to only HF group (Figure 6B).

Similarly, tartrate resistance acid phosphatase staining, which reflects osteoclast number, was significantly increased in the tibia of ovariectomized mice (Online Figure IV, A). Furthermore, the bone mineral density was significantly decreased by OVX: decrease in the number of trabeculas and increase in the space between them, as quantified by microcomputed tomography (Online Figure IV, B). The animal model exhibited typical features of the postmenopausal women with both aortic calcification and osteoporosis. Of note, we could not detect osteoclast-like cells in the calcified plaque of our animal model (data not shown).

In situ hybridization in aortas of HF/OVX group showed strong expression of the osteogenic marker OPN inside the atherosclerotic lesion, which suggests the intima calcification (Figure 6C). In sagittal sections, expression of RANK and RANKL mRNA were mainly found at medial layers and at outer membrane of atherosclerotic lesions, with detectable expression in endothelium, as indicated by arrows in Figure 6C.

Quantitative real-time PCR analysis demonstrated that OVX increased RANK, RANKL, and OPG expression (Figure 7A) in aorta. Estrogen deficiency also increased the BMP-2 expression in aorta, whereas in combination with HF diet, it increased the expression of calcification marker ALP. MGP level significantly decreased after OVX and/or HF diet (Figure 7B).

**Prevention of Vascular Calcification and Osteoporosis by Estrogen Replacement**

We replaced E2 simultaneously to the bilateral OVX and treated the mice with HF diet during 3 months. The estrogen was continuously administrated by osmotic mini pump in a concentration of 20 μg/kg per day. This dose was enough to prevent tartrate resistance acid phosphatase activity and osteoporosis induced by OVX (Online Figure IV, A and B). Importantly, estrogen replacement significantly decreased the atherosclerotic plaque and calcification induced by HF diet and estrogen deficiency, as shown by Oil red and Alizarin red staining (Figure 6A). Consistently, estrogen replacement attenuated OVX-induced increase in RANK, RANKL, OPG, BMP-2, and ALP mRNA levels, while simultaneously increasing MGP expression in vasculature, as quantified by real-time PCR (Figure 7A and 7B). In addition, estrogen decreased the phosphorylation of smad-1/5/8 in aorta of HF/OVX mice as early as 2 weeks from the start of the animal experiment (Figure 7C), which suggests that estrogen may also reduce the BMP proosteogenic signaling in vivo.

**Discussion**

Epidemiological studies relate incidence of aortic calcification with low bone mineral density in postmenopausal women. Similarly, OPG-deficient mice develop severe osteoporosis...
and vascular medial calcification. In the absence of OPG, RANKL induces exaggerated osteoclast formation, followed by osteoporosis. In this study, we report that in the vasculature, RANKL induces osteogenesis by acting on 2 important molecules of the calcification process: BMP-2, an inducer of calcification, and MGP, a calcification inhibitor. In addition, estrogen acts mainly through ERα to counteract these effects of RANKL stimulation.

MGP inhibits calcium deposition, and it binds to BMP-2, preventing the promotion of calcification. MGP has been detected at the site of calcification in vivo, and an increase in MGP expression following calcification in vitro has also been reported; however, it may reflect a compensatory mechanism to inhibit calcification because analysis of MGP-deficient mice showed calcification in aorta and its branches, and rats treated with warfarin, a vitamin K antagonist that interferes in the MGP processing, presented increased levels of vascular calcification. Moreover, it was reported that the amount of MGP alone does not determine the calcification incidence, but the ratio of MGP to BMP-2 is what regulates mineral deposition and osteogenic differentiation: low levels (≤1-fold) or high levels (≥15-fold) favor calcification, whereas intermediates levels inhibit the process. Our results showed that RANKL increased BMP-2 (Figure 2C and 2D) and decreased MGP expression (Figure 3A). Therefore we postulate that RANKL stimulation induces the low ratio of MGP to BMP-2 in vessel wall, which strongly induces calcification.

Importantly, it has been reported that systemic mineral imbalance caused by nephrectomy and calcitriol treatment induced RANKL expression in vasculature and consequent BMP-4 expression in vascular smooth muscle cells, which
resulted in medial calcification. Therefore, we speculate that some effects attributed in our study to BMP-2 may also be attributable to BMP-4 and that the RANKL system may contribute to vascular calcification by regulating BMP expression in both hypercholesterolemic and nonhypercholesterolemic condition.

RANKL alone was not able to induce calcification in HASMCs, but it induced bone-related gene expression (Figure 3B) and increased the number of calcified nodules of HASMCs cultured with osteogenic-inducible medium (Figure 3C), which was accompanied by decreased MGP and increased ALP expression (Figure 3D).

Notably, estrogen acted mainly through ERα and inhibited the RANKL-induced BMP-2 expression (Figure 5B) and induced MGP expression (Figure 5C) in vascular cells. Early coronary calcification has been associated with loss of ERα in one man lacking functional ERα, and, in contrast, ERβ expression correlated with calcium content, plaque area, and calcium-to-plaque ratio. The associations of ERβ with atherosclerotic calcification and ERα with vascular protective effects of estrogen highlight the complexity of estrogen action in vasculature, especially because many factors may modulate the ERα to ERβ ratio. For example, increase in calcification in testosterone-treated ApoE-deficient mice was associated with an androgen receptor–mediated pathway for osteogenesis and to a decrease in ERα expression in the calcified vessel. Our findings suggested that ERα mediates the estrogen-protective effect against calcification, and further studies are required to clarify the opposite action of ERβ in vascular calcification.

The detection of RANK and RANKL mRNA by in situ hybridization in the vessel wall and in the plaque (Figure 6C) suggested vascular responsiveness to RANKL in the calcification context. RANKL and BMP-2 expression increased in the total mRNA of aorta from estrogen deficient and/or high fat treated mice (Figure 7A), and estrogen replacement decreased the expression of RANKL system back to basal levels, and significantly increased the expression of MGP (Figure 7B). Importantly, serum OPG and RANKL have been reported to not account for the association between osteoporosis and vascular calcification in postmenopausal women under hormone therapy. Based on our findings, we postulate that vascular calcification and osteoporosis resulted from RANK stimulation by locally expressed RANKL and not by circulating RANKL.

Vascular calcification has been induced in vitro by mineralocorticoid receptor activation in a mechanism independent of BMP-2 signaling, and estrogen deficiency was also related to vascular calcification in an ovarioectomized and HF diet–treated rabbit model. However, in contrast to our mouse model, it was reported a decrease in RANKL protein level in calcified aorta, and the calcification was not related to BMP-2 increase in vasculature. Time course differences in the RANKL expression may explain the discrepancy, because in that rabbit model, the decrease in RANKL expression was observed after 15 months, whereas in our model, the increase in RANKL was detected after 3 months. Moreover, the atherosclerotic lesion was induced by double endothelial denudation, in contrast to the presence of intact endothelium in the atherosclerotic plaque of our animal model (Figure 6C).

It may also explain the discrepancy in BMP-2 expression between the 2 studies, because our findings showed that endothelial cells are the main source of BMP-2 in vasculature. Importantly, our findings suggest that RANKL mediates calcification by inducing BMP-2 in HAECs and regulating MGP and osteogenesis in HASMCs; therefore, the autocrine and paracrine mechanisms activated by RANKL in both cells actively contribute to calcification in vasculature. Calcification results from complex mechanisms and the RANKL–BMP-2 axis may not be the unique pathway regulating this process in different conditions.

One limitation of our study is that the benefit of estrogen in preventing vascular calcification could not be confirmed in vivo because estrogen prevented atherosclerotic plaque development in the first place (Figure 6A). However, 2 weeks after OVX and HF diet, we were able to find an increase in phosphorylation of phospho–smad-1/5/8 in aorta but not in the estrogen-replacement group, which suggest that despite its limitation estrogen may reduce the BMP pro-osteogenic signaling in our model. In bone, the estrogen deficiency led to increased osteoclast number and osteoporosis (Online Figure IV, A and B). RANK-RANKL system has been widely studied in bone, in which osteoblasts express RANKL, which regulates osteoclast differentiation and activation. Estrogen promotes bone formation by increasing OPG expression in osteoblast and also by inducing osteoclast apoptosis via ERα. We confirmed that estrogen replacement decreased the osteoclast burden (Online Figure IV, A) and increased bone formation (Online Figure IV, B) and that ERα mediated the estrogen-induced OPG expression and RANKL decrease in osteoblast, as shown in Figure 5D. Taken together, we postulate that estrogen may be the key modulator of RANK-RANKL signaling in the development of both aortic calcification and osteoporosis.

Large prevention trial in the United States failed to confirm the protective effect of estrogen alone, or estrogen combined with progesterin hormone therapy on coronary heart diseases. However, subgroup analyses of the Women’s Health Initiative study have shown that women in whom hormone therapy was initiated at a younger age (50 to 59 years) and earlier after menopause tended to have reduced risk of coronary heart disease and total mortality, and an ancillary study showed a significant reduction in coronary artery calcium (Agatston) score in younger (50 to 59 years) women randomized to conjugated estrogen compared to placebo, indicating a reduced calcified plaque burden. The data reported herein are consistent with these protective effects of estrogen observed as vascular calcification decrease in postmenopausal women and further suggest that the beneficial effect of estrogen in vascular calcification may be explained by inhibition of RANK-RANKL signaling and expression, thus implying a crosstalk of estrogen and RANKL in the arterial wall. The underlying mechanism remains to be elucidated, and ovarioectomized ApoE-deficient mice provide a useful model to examine interventions for both osteoporosis and vascular calcification.
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Disclosures
None.

References


### Novelty and Significance

**What Is Known?**
- Epidemiological studies relate aortic calcification with low bone mineral density in postmenopausal women, and pathological studies show the presence of bone-related proteins and cells at the site of aortic calcification.
- Unopposed RANKL (receptor activator of nuclear factor κB ligand) activity in mice lacking the decoy receptor osteoprotegerin (OPG) induces early onset of osteoporosis and vascular calcification.
- Clinical trials have indicated that estrogen-replacement therapy in postmenopausal women decreases the burden of osteoporosis and arterial calcification.

**What New Information Does This Article Contribute?**
- Stimulation with RANKL in vascular cells leads to an imbalance of pro- and anticalcification factors: increased expression of bone morphogenetic protein (BMP) by endothelial cells and decreased expression of matrix Gla protein (MGP) by vascular smooth muscle cells (VSMCs).
- Estrogen counteracts the effect of RANKL toward calcification by inducing MGP expression in VSMCs and inhibiting RANKL-induced BMP expression in endothelial cells in an estrogen receptor (ER)-α-dependent manner.
- Estrogen replacement decreases the activation of BMP-osteogenic pathway in ovariectomized and HF diet–treated ApoE-deficient mice.

Osteoporosis and vascular calcification are present in OPG-deficient mice and frequently coincide in women with estrogen deficiency. Estrogen-replacement therapy is effective in reverse osteoporosis, and clinical trials show its beneficial effect in vascular calcification. Similarly, RANKL inhibition by the human monoclonal antibody denosumab ameliorates osteoporosis in postmenopausal women and decreases calcium deposition in mice. Here, we propose the molecular mechanism underpinning vascular calcification under RANKL activation and estrogen deficiency. We report an increase in RANKL and calcification in vasculature of mice with estrogen deficiency. We report for the first time that RANKL increases the ratio of calcification inducer BMP/calcification inhibitor MGP in vascular cells and may contribute to calcified nodule formation in VSMCs by inducing master transcription factors responsible to osteogenesis. We also demonstrate that estrogen interferes with RANKL signaling by decreasing the ratio of BMP/MGP in vascular cells in an ERα-dependent manner. Importantly, estrogen replacement reversed the osteoporosis and decreased the activation of BMP-osteogenic pathway. Our work shows that estrogen may be beneficial for vascular calcification by inhibiting RANKL expression and signaling in vascular cells and by the crosstalk between estrogen and RANKL in the arterial wall.
Estrogen Inhibits Vascular Calcification via Vascular RANKL System: Common Mechanism of Osteoporosis and Vascular Calcification

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SUPPLEMENTAL MATERIAL

Estrogen Inhibits Vascular Calcification Via Vascular RANKL System:
Common Mechanism of Osteoporosis and Vascular Calcification

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Materials and Methods

In Vitro Experiment

Cell Culture

Human aortic vascular smooth muscle cells (HASMC) were obtained from Lonza Walkersville, Inc. (Walkersville, USA), and cultured in SMC growth medium (Smooth Muscle Cell Basal Medium/SmBM supplemented with SmGM-2 SingleQuots: 25 ml FBS, 0.5 ml insulin, 1 ml hFGF-2, 0.5 ml gentamicin, 0.5 ml hEGF; Lonza, USA). Human aortic endothelial cells were obtained from Lonza, and cultured in EGM-2 medium (Endothelial Basal Medium-2/EBM-2 supplemented with EGM-2 SingleQuots: 10 ml FBS, 0.2 ml hydrocortisone, 2 ml hFGF, 0.5 ml VEGF, 0.5 ml IGF-1, 0.5 ml ascorbic acid, 0.5 ml hEGF, 0.5 ml GA-1000, 0.5 ml heparin; Lonza, USA). Normal human osteoblasts were obtained from Lonza, and cultured in OGM medium (Osteoblast Basal Medium/OBM supplemented with 50 ml FBS, 0.5 ml GA-1000, 0.5 ml ascorbic acid; Lonza, USA). THP1 cells were obtained from American Type Culture Collection (Rockville, MD), and cultured in RPMI-1640 (GIBCO BRL, Eggenstein, Germany) supplemented with 10 % FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. All the cells were cultured at 37°C with 5 % CO2. THP1 were activated into macrophage by PMA as described previously¹.

For the experiment with conditioned medium, HAEC were stimulated with or without RANKL (10 ng/ml) in the presence of anti-OPG antibody (0.1 μg/ml) during 2 days. HASMC were incubated with the conditioned medium of HAEC for 24 hours with or without the addition of the BMP-2 antagonist, noggin (100 μg/ml). After that, we collected mRNA from each group and quantified the mRNA expression of osteogenic markers.

Relative Quantification of mRNA by Real-Time PCR

In in vitro experiments, total RNA was isolated using the Agilent Total RNA Isolation Mini Kit (Agilent Technologies, Wilmington, USA) and the cDNA reaction was performed using the
High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, USA) according to the procedures recommended by the manufacturers. Each cDNA library was analyzed for the respective target gene and 18S rRNA expression by real-time PCR using the fluorescent TaqMan 5' nuclease assay. Oligonucleotide primers were purchased according to the identification RANK: Hs00187192, RANKL: Hs01092186, OPG: Hs00171068, BMP-2: Hs00154192, osteocalcin: Hs00609452, alkaline phosphatase: Hs00758162, osteopontin: Hs00959010, cbfa1: Hs00231692, MGP: Hs00179899, osterix: Hs01866874_s1, MSX2: Hs00751239_s1, ER1: Hs01046818_m1, ER2: Hs00230957_m1, and Eukaryotic 18S rRNA (18S): 4352930E (Applied Biosystems).

Total RNA was isolated from mice aorta by ISOGEN (Nippon Gene, Toyama, Japan), and the cDNA reaction was performed using the High-Capacity cDNA Archive kit (Applied Biosystems). The cDNA libraries were analyzed for RANK, RANKL, OPG, BMP-2, MGP, and 18S rRNA expression by real-time PCR using the fluorescent TaqMan 5' nuclease assay. The primers used were RANK: Mm00437135, RANKL: Mm01313944, OPG: Mm00435451, BMP-2: Mm01340178, MGP: Mm00485009, and 18S rRNA: 4352930E (Applied Biosystems).

The 5' nuclease assay PCRs were performed in a MicroAmp Optical 96-well reaction plate using the ABI PRISM 7700 Sequence Detection System. Levels of the target gene and 18S mRNA were quantified by comparison of the fluorescence generated by each sample with that of a serially diluted standard and the target gene expression was normalized by the level of 18S expression in each individual sample.

**Western Blot Analysis**

Western blot analysis was performed as previously described. Briefly, total protein (10 μg) was isolated using lysis buffer (50 mmol/L Tris-HCl, 2.5 mmol/L EGTA, 1 mmol/L EDTA, 10 mmol/L NaF, 0.1% sodium deoxycholate, 1% Triton X-100, 1 mmol/L PMSF, 1 mmol/L Na₃VO₄), and size-fractioned by SDS-PAGE and transferred to 0.45 μm pore size Immobilon-P membrane
purchased from Millipore (Bedford, MA). The blotted protein was incubated with antibodies against RANK, RANKL, OPG, BMP-2, CBFA-1, and β-actin after 24 hours of indicated treatment. For MGP detection, 20 μg of total protein was size-fractioned by 4-20% SDS-PAGE and transferred to 0.2 μm pore size Immobilon-P membrane.

**Calcification Induction**

HASMC were seeded in 24 well plates at 30 % confluence. In the next day the medium was changed to Osteogenic Induction Medium (Osteogenic Basal Medium supplemented with Osteogenic SingleQuots: dexamethasone, L-glutamine, ascorbate, pen/strep, MCGS, and β-glycerophosphate) (Lonza, USA) with or not 10 ng/ml RANKL, and 0.1 μg/ml neutralizing OPG antibody. The medium was changed every 2 days during 21 days for nodule formation, and every day during 3 days for mRNA extraction.

**siRNA Transfection**

MGP expression was silenced with the transfection of predesigned human MGP siRNA (Silencer: ID: 122163; NM_0009000)\(^3\), purchased from Applied Biosystems (Foster City, USA). The siRNA transfection was performed using 1 μg/ml Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as the transfection agent for 24 hours preceding subsequent stimulations in HASMC.

**Animal Experiments**

**Experimental Design**

After the female apolipoprotein E (ApoE)-deficient mice (n = 30) were anesthetized with intraperitoneal ketamine (80 mg/kg) and xylazine (10 mg/kg), bilateral ovariectomy or sham operation was performed, and we implanted an Alzet micro-osmotic pump model 1004 (Durect, Cupertino, USA) containing 17 β-estradiol (20 μg/ kg/ day)\(^4\) diluted in propylene glycol (Wako) or
saline. The mice were fed with normal diet (MF; Oriental Yeast Co., Tokyo, Japan) or western diet (MF plus 0.5 % [wt/wt] cholesterol and 10 % coconut oil; Oriental Yeast Co.), and maintained on this diet and estrogen replacement therapy for 3 months. The animal experiments were conducted according to the Guidelines for Animal Experiments at Osaka University Graduate School of Medicine.

**Histological Analysis of Atherosclerotic Lesions**

Mice were anesthetized with 80 mg/kg pentobarbital sodium intraperitoneally (Abbot Laboratories, Illinois, USA), the blood was collected, and aortas (thoracic and abdominal section) were dissected and immediately frozen at −80 °C. The samples were cut in small portions and embedded in OCT compounds (Tissue-Tek; Sakura Finetechncial Co., Tokyo, Japan) and sectioned (10 μm thickness). Lipid deposition was detected by Oil Red staining. Calcium deposition was visualized by Alizarin Red staining and the area was traced and quantified by BZ-II Analyzer (Keyence, Tokyo, Japan).

**In Situ Hybridization**

*In situ* hybridization was performed as previously described\(^5\), with slight modifications in the fixation process: The mice were infused with fixation solution (4% formaldehyde, 0.1 mol/L 4-morpholinepropanesulfonic acid, 2 mmol/L EGTA, and 1 mmol/L MgSO\(_4\)), the aortas were extracted and fixed for 4 hours at room temperature and stored at −20°C in methanol. Sections were made at 8 μm thickness, and the cDNAs used for generation of Dig-labeled mouse riboprobes were OSTEOPONTIN, PECAM, RANKL, and RANK.
References


Online Figure Legends:

**Figure I:** A) Basal BMP-2 expression in human aortic endothelial cell (HAEC), human aortic smooth muscle cell (HASMC), human monocyte (THP1) and activated monocyte (macrophage) measured by real-time PCR. (n=6). Each bar represents BMP-2 gene expression relative to 18S expression.

**Figure II:** Osterix, MXS2, and osteocalcin (OC) mRNA expression after 3 days incubation with RANKL (RL) (10 ng/ml), RL with neutralizing OPG (anti-OPG) (0.1 μg/ml), and incubation with only anti-OPG replaced every day. (n=6). Each bar shows the target gene level relative to 18S level. * P < 0.05 vs. No treat.

**Figure III:** Efficacy of the siRNA targeting MGP. MGP expression was dose dependently silenced after 24 h of the transfection of Silencer siRNA targeting MGP using Lipofectamine 2000 (1 μg/ml) as the transfection agent. (n=4). Western blotting of MGP was not satisfactory due to the high insolubility of this protein. However MGP was visible in 20 μg of protein lysates of HASMC, and MGP could not be detected after the siRNA transfection.

**Figure IV:** Osteoporosis detection in ApoE-deficient treated with high fat diet (HF), after ovariectomy (OVX) or sham operation (Sham) with or without estrogen (E2) replacement for 3 months. A) Estrogen replacement strongly reduced the osteoclast number in the growth plate of proximal tibia compared with HF/OVX group as shown by TRAP staining (40x). B) Estrogen decreases the osteoporosis rate in HF/OVX group as assessed by μCT analysis of the tibia: the groups were compared according to the number of trabecullas and the space between them. * P < 0.05 vs. No treat., # P < 0.05. (n=3).
**Online Figure I**

![Bar chart showing BMP-2 expression in various cell types.](chart)

**Figure I:** Basal BMP-2 expression in human aortic endothelial cell (HAEC), human aortic smooth muscle cell (HASMC), human monocyte (THP1) and activated monocyte (macrophage) measured by real-time PCR. (n=6). Each bar represents BMP-2 gene expression relative to 18S expression.
Online Figure II

**Figure II**: Osterix, MXS2, and osteocalcin (OC) mRNA expression after 3 days incubation with RANKL (RL) (10 ng/ml), RL with neutralizing OPG (anti-OPG) (0.1 μg/ml), and incubation with only anti-OPG replaced every day. (n=6). Each bar shows the target gene level relative to 18S level. *P < 0.05 vs. No treat.
Online Figure III

**A)**

![Graph showing MGP/18S mRNA comparison](image)

- 5 nM 10 nM 25 nM 50 nM

**B)**

![Western blot image](image)

**Figure III**: Efficacy of the siRNA targeting MGP. MGP expression was dose dependently silenced after 24 h of the transfection of Silencer siRNA targeting MGP using Lipofectamine 2000 (1 μg/ml) as the transfection agent. (n=4). Western blotting of MGP was not satisfactory due to the high insolubility of this protein. However MGP was visible in 20 μg of protein lysates of HASMC, and MGP could not be detected after the siRNA transfection.
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