Estrogen-Related Receptor γ Plays a Key Role in Vascular Calcification Through the Upregulation of BMP2 Expression

Ji-Hyun Kim,* Yeon-Kyung Choi,* Ji-Yeon Do, Young-Keun Choi, Chae-Myeong Ha, Sun Joo Lee, Jae-Han Jeon, Won-Kee Lee, Hueng-Sik Choi, Keun-Gyu Park, In-Kyu Lee

Objective—Vascular calcification which refers to ectopic mineralization in vascular cells is associated with several conditions, such as chronic kidney disease, atherosclerosis, and diabetes mellitus. Estrogen-related receptor (ERR)γ is a member of the orphan nuclear receptor superfamily, which plays diverse roles in regulating homeostatic and metabolic processes. However, the role of ERRγ in vascular calcification has not been investigated to date. The aim of the present study was to examine the role of ERRγ in vascular calcification.

Approach and Results—Vascular calcification was induced by treating rat aortic vascular smooth muscle cells with calcification medium. ERRγ expression in vascular smooth muscle cells was induced during calcification medium–induced vascular calcification. Adenovirus-mediated overexpression of ERRγ in vascular smooth muscle cells resulted in the upregulation of the expression of osteogenic genes, including runt-related transcription factor 2, osteopontin, and Msx2, and the downregulation of α-smooth muscle actin. Adenovirus-mediated overexpression of ERRγ induced bone morphogenetic protein 2 (BMP2) expression, leading to increased phosphorylation of the intracellular BMP2 effector proteins SMAD1/5/8. Collectively, these data suggested that ERRγ promotes dedifferentiation of vascular smooth muscle cells to an osteogenic phenotype during the vascular calcification process. Inhibition of endogenous ERRγ expression or activity using a specific siRNA or the selective inverse agonist GSK5182 attenuated vascular calcification and osteogenic gene expression in vitro and in vivo.

Conclusions—The present results indicate that ERRγ plays a key role in vascular calcification by upregulating the BMP2 signaling pathway, suggesting that inhibition of ERRγ is a potential therapeutic strategy for the prevention of vascular calcification.

Key Words: BMP2 | ERRγ | vascular calcification | vascular smooth muscle cells

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Bone morphogenetic proteins (BMPs) are members of the transforming growth factor β family and were originally identified by their capacity to induce ectopic bone formation.5,6 BMPs play a role in the differentiation of VSMCs and in determining the phenotypic profile of osteoblasts and chondroblasts.5 Increased BMP signaling activity promotes vascular calcification, and increased expression of BMP ligands has been detected in calcified vascular lesions in patients with diabetes mellitus or chronic kidney disease.9–11 Among >20 BMP ligands identified to date, BMP2 was shown to inhibit VSMC proliferation and drive both osteogenic and chondrogenic differentiation of mesenchymal stem cells.12,13 Binding of a BMP ligand to a specific type II receptor activates a type I receptor, resulting in the phosphorylation and nuclear translocation of SMADs 1, 5, and 8 (SMAD1/5/8), which act as transcription factors.
factors, promoting osteogenic gene expression. In a recent study, pharmacological inhibition of BMP signaling inhibited atheroma development and vascular calcification in high fat diet–fed low-density lipoprotein receptor−/− mice, suggesting a potential benefit of BMP inhibition in the treatment of vascular calcification. The development of novel agents against these targets with the capacity to modulate signaling pathways would be beneficial for the treatment of patients with atherosclerosis and vascular calcification.

Estrogen-related receptors (ERRs) are closely related to estrogen receptors, sharing high homology in the DNA-binding domain, although they do not bind estrogen. The estrogen receptor–related receptor subfamily consists of 3 members, ERRα, ERRβ, and ERRγ (NR3B1–3), which bind to classic estrogen response elements and to extended half-site core sequences (TNAAGGTCA; ERR response element [ERRE]) as either monomers or dimers. ERRα is strongly expressed throughout osteoblast differentiation and regulates osteopontin expression through a noncanonical ERRα response element. Previously, we reported that ERRγ is expressed in osteoblast progenitors and negatively regulates BMP2-induced osteoblast differentiation and bone formation. However, the role of ERRγ in vascular calcification remains to be determined.

The aim of the present study was to clarify the role of ERRγ in vascular calcification. The expression of ERRγ and the involvement of the BMP2 signaling pathway were examined during the process of vascular calcification. The effect of inhibition of endogenous ERRγ expression by siRNA or the ERRγ inhibitor GSK5182 on vascular calcification was examined in vitro and in vivo.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Ad</td>
<td>adenovirus</td>
</tr>
<tr>
<td>BMP2</td>
<td>bone morphogenic protein-2</td>
</tr>
<tr>
<td>CM</td>
<td>calcification medium</td>
</tr>
<tr>
<td>ERRγ</td>
<td>estrogen-related receptor</td>
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<tr>
<td>ERRE</td>
<td>ERR response element</td>
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<tr>
<td>Runx2</td>
<td>runt-related transcription factor 2</td>
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<tr>
<td>VSMCs</td>
<td>vascular smooth muscle cells</td>
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</table>

Figure 1. Estrogen-related receptor (ERR)-γ expression in vascular smooth muscle cells (VSMCs) is increased by calcification medium (CM). Primary rat VSMCs were cultured for the indicated times in CM. A–B, Vascular calcification was assessed by Von Kossa staining (A) and determination of calcium content (B). C–D, Induction of ERRγ mRNA and protein was detected by reverse transcriptase polymerase chain reaction (RT-PCR; C) and western blotting (D), respectively. Levels of ERRγ were normalized against those of β-actin. Values are expressed as the mean±SEM (n=5). Scale bar =200 μm. **P<0.01 and ***P<0.001 vs control.

Figure 2. Induction of osteogenic genes by overexpression of estrogen-related receptor (ERRγ) is mediated by bone morphogenetic protein 2 (BMP2) signaling in VSMCs. A–B, Vascular smooth muscle cells (VSMCs) were infected with adenovirus (Ad)-GFP (control) or Ad-ERRγ. At 2 days after adenovirus infection, VSMCs were incubated in the presence of calcification medium (CM) for 5 days. A, Expression levels of BMP2 and osteogenic markers were determined by reverse transcriptase polymerase chain reaction (RT-PCR). B, Expression levels of BMP2 and phosphorylation of SMAD1/5/8 were determined by Western blotting. C, Luciferase reporter assays testing the putative ERRγ binding sites in the Bmp2 gene promoter in A7r5 cells were performed in the presence of ERRγ plasmid or pcDNA3 (empty). Equal amounts of pcDNA3 or pcERRγ were transfected for 2 days. A plus (+) means 100 ng of pcERRγ; A (++) means 200 ng pcERRγ. D–E, Primary rat VSMCs were cultured 2 days after Ad-GFP or Ad-ERRγ infection for the indicated times in CM. Vascular calcification was assessed by Von Kossa staining (D) and determination of calcium content (E). A plus (+) means 50 MOI of Ad-GFP or Ad-ERRγ; A (+) means 100 MOI or Ad-GFP or Ad-ERRγ. Values are expressed as the mean±SEM (n=5). Scale bar =200 μm. **P<0.01 and ***P<0.001 vs control.
ERRγ Expression Is Increased in VSMCs by Calcification Medium

To determine the role of ERRγ overexpression in the process of VSMC calcification, cells were cultured in calcification medium (CM) for 5 days. CM-induced VSMC calcification was confirmed by morphological changes (nodule formation) and increased extracellular calcium deposits in VSMCs, as determined by von Kossa staining and quantification of calcium content (Figure 1A and 1B). During the process of VSMC calcification, the mRNA and protein levels of ERRγ increased progressively compared with those of the control (Figure 1C and 1D). These data indicate that the induction of ERRγ expression during VSMC calcification may play a role in the calcification process.

Overexpression of ERRγ in VSMCs Induces Osteogenic Gene Expression Via Upregulation of BMP2 Signaling

To determine the role of ERRγ upregulation in the process of VSMC calcification, the effects of adenovirus-mediated overexpression of ERRγ (Ad-ERRγ) on osteogenic gene expression were examined. The results of semiquantitative reverse transcriptase polymerase chain reaction showed that Ad-ERRγ dose-dependently upregulated the expression of osteogenic genes, including Bmp2, Runx2, Spp1, Msx2, and Bglap, and downregulated the expression of Acta2 (Figure 2A). The upregulation of BMP2 by Ad-ERRγ was confirmed by Western blot analysis (Figure 2B). A luciferase reporter assay performed using reporter plasmids encoding Bmp2-Luc showed that ERRγ upregulated BMP2 gene expression at the transcriptional level (Figure 2C). To determine the potential effect of ERRγ on BMP2 downstream signaling, the phosphorylation status of SMAD1/5/8 was assessed in cells overexpressing ERRγ. The results showed that Ad-ERRγ increased the phosphorylation of SMAD1/5/8 in a dose-dependent manner (Figure 2B). Moreover, CM-induced vascular calcification and calcium deposition were further enhanced in Ad-ERRγ–infected VSMCs compared with Ad-GFP–infected cells (Figure 2D and 2E). However, overexpression of ERRγ did not induce vascular calcification in the absence of CM, although induction of osteogenic gene expression was observed (Figure 1 in the online-only Data Supplement). Taken together, these findings demonstrate that ERRγ promotes dedifferentiation of VSMCs to an osteogenic phenotype, thereby exacerbates vascular calcification via activation of BMP2 and its downstream signaling under calcification conditions.

ERRγ Increases BMP2 Promoter Activity Through Both Direct Binding to its Response Elements and Indirect Binding to CREB Binding Elements

To identify the ERRγ binding site on the Bmp2 promoter, we constructed 2 Bmp2 reporter gene promoters containing putative ERR response element 1 (put-ERR1) and put-ERR2 with serial 5′ deletions, namely D1 (−775 to +165 bp) and D2 (−195 to +165 bp; Figure 3A, upper panel). A transient transfection assay in C2C12 cells showed that the activity of the wild-type Bmp2 promoter (−2712 to +165 bp) was increased by ≈5-fold by

**Results**

**Figure 3.** Estrogen-related receptor (ERR)γ directly binds to the Bmp2 promoter in C2C12 cells. **A**, Constructs showing serial deletions of the mouse Bmp2 promoter linked to the basic luciferase reporter vector (upper) and results of luciferase assays performed after transfection of cells with Bmp2 promoter constructs and treatment with pcERRγ or control (lower). Equal amounts of pcDNA3 or pcERRγ were transfected for 2 days. A plus (+) means 200 ng of pcERRγ. **B**, ChIP assay showing binding of ERRγ to the Bmp2 promoter in C2C12 cells after 2 days infection with 100 MOI of GFP (control) or ERRγ adenovirus. **C**, Electrophoretic mobility shift assay (EMSA) of the ERRγ binding to the Bmp2 promoter showing binding of ERRγ to the Bmp2 promoter. **D**, Sequence of wild-type (WT) and mutated putative ERRE sites in the Bmp2 promoter (upper) and results of luciferase assays performed after transfection with WT and mutant plasmids (lower). Cells were also transfected with equal amounts of pcDNA3 (empty) or pcERRγ for 2 days. A plus (+) means 200 ng of pcERRγ. **E**, Bmp2 promoter reporter activity in C2C12 cells, co-transfected with WT and mutant −150/−165-Luc in which CREs were mutated. Cells were also transfected with equal amount of pcDNA3 (empty) or pcERRγ for 2 days. A plus (+) means 200 ng of pcERRγ. Values are expressed as the mean±SEM (n=5). **P<0.01 vs indicated group.
ERRγ. Deletion of put-ERRE2 (D1 construct) did not decrease ERRγ-stimulated Bmp2 promoter luciferase activity, whereas removal of both put-ERRE1 and put-ERRE2 (D2 construct) markedly attenuated the ERRγ stimulation of reporter activity (Figure 3A, lower), indicating that put-ERRE1 is essential for ERRγ binding. To further examine the binding of ERRγ to put-ERRE1 on the Bmp2 promoter, we performed a ChIP assay in C2C12 cells. The results showed that ERRγ binds to put-ERRE1 (Figure 3B). Moreover, a direct interaction between ERRγ and ERRE1 but not ERRE2 was confirmed by electrophoretic mobility shift assay analysis (Figure 3C and Figure II in the online-only Data Supplement). A mutagenesis study showed that mutation of put-ERRE1 markedly attenuated the effect of ERRγ on Bmp2 promoter activity (Figure 3D). However, ERRγ-induced Bmp2 promoter activation was still detected after deletion of 2 put-ERRE motifs or mutation of put-ERRE1, suggesting that an indirect mechanism may contribute to promoter stimulation by ERRγ. CREB is known to transactivate Bmp2 through CRE in the Bmp2 promoter,20 and we found a putative CRE-binding site on D2 −195 Bmp2 promoter. Therefore, we checked whether CRE contributes to ERRγ-stimulated Bmp2 promoter activation after deletion of both ERRE1 and ERRE2. The results showed that when the CRE of the D2−195 Bmp2 promoter was mutated, ERRγ no longer stimulated Bmp2 promoter activity, whereas it stimulated the wild-type CRE control (Figure 3E).

Knockdown of ERRγ Attenuates VSMC Calcification

Our results showing that ERRγ expression is induced during VSMC calcification and ERRγ overexpression promotes osteogenic gene expression led us to investigate the effect of siRNA-mediated ERRγ silencing on CM-induced VSMC calcification. As shown in Figure 4A and 4B, inhibition of endogenous ERRγ expression by siRNA prevented CM-induced upregulation of osteogenic genes, such as Bmp2, Runx2, Spp1, Msx2, and Bglap, and phosphorylation of SMAD1/5/8. Von Kossa staining showed that VSMC calcification induced by CM was markedly attenuated by siRNA silencing of ERRγ and that the attenuation could be rescued by overexpression of ERRγ (Figure 4C). Quantification of calcium levels revealed
that siERRγ decreased calcium deposition, which was rescued by overexpression of ERRγ (Figure 4D). Taken together, these data indicate that ERRγ has a pivotal role in CM-induced vascular calcification.

Pharmacological Inhibition of ERRγ Activity Attenuates VSMC Calcification

We next evaluated whether inhibition of ERRγ activity by its inverse agonist, GSK5182, could prevent vascular calcification. Consistent with the results of ERRγ siRNA, GSK5182 downregulated osteogenic marker gene expression in a dose-dependent manner and upregulated Acta2 expression (Figure 5A). GSK5182 inhibited CM-induced phosphorylation of SMAD1/5/8, as shown by Western blotting (Figure 5B), and successfully prevented CM-induced VSMC calcification (Figure 5C and 5D). The inhibition of ERRγ activity by GSK5182 was confirmed by transient transfection with a Bmp2 promoter luciferase reporter plasmid (Figure 5E).

GSK5182 Prevents Vascular Calcification Ex Vivo and In Vivo

To examine the inhibitory effect of GSK5182 on calcification ex vivo, mouse abdominal aorta was cut into rings and cultured in vitro with CM. Consistent with previous results, Von Kossa staining and immunohistochemical staining for BMP2 showed that GSK5182 significantly inhibited CM-induced VSMC calcification and calcium deposition and downregulated BMP2 expression (Figure 6A–6C). The effect of GSK5182-mediated ERRγ inhibition on vascular calcification was examined in vivo using an established vascular calcification animal model, in which mice are injected subcutaneously with high doses of cholecalciferol (Vit-D3) for 3 consecutive days. Daily intraperitoneal injection of GSK5182 (10 mg/kg/d) significantly attenuated vascular calcification in the aorta and decreased calcium deposition (Figure 6D and 6E).

Discussion

The results of the present study showed that ERRγ expression was induced during the process of vascular calcification, and overexpression of ERRγ upregulated BMP2 transcription, leading to activation of downstream signaling. Moreover, siRNA-mediated knockdown of endogenous ERRγ or inhibition of ERRγ activity by the selective inverse agonist GSK5182 attenuated vascular calcification by downregulating osteogenic gene expression and decreasing calcium deposition in vitro and in vivo.

A growing body of evidence suggests that ERRγ is relevant to vascular pathophysiology, including angiogenesis and restoration of blood perfusion in ischemic tissues. Here, we provide another pathophysiological function for ERRγ in the process of vascular calcification. Because a previous study reported that ERRγ negatively regulates BMP2-induced osteoblast differentiation and bone formation, we initiated this study with the expectation that ERRγ would have beneficial effects on vascular calcification under calcification-prone conditions. However, the results showed the ERRγ has opposite roles in mineralization between VSMCs and osteoblasts. Upregulation of ERRγ expression during vascular calcification promoted dedifferentiation of VSMCs to an osteogenic phenotype and exacerbated CM-induced calcification through upregulation of BMP2 expression. However, we did not observe induction of vascular calcification after ERRγ overexpression under normal phosphate conditions, indicating that the action of ERRγ on vascular calcification requires calcification-prone conditions. ERRγ induced BMP2 transcription via direct binding to its response element in the BMP2 promoter and indirect induction of CREB, as shown by electrophoretic mobility shift assay and mutagenesis of promoter–reporter assay. BMP2 regulates osteoblast differentiation and bone formation through the induction of osteogenic transcription factors, such as Runx2 and Msx2. Runx2 regulates the transcription of genes associated with osteoblast differentiation, endochondral
bone formation, and neovascularization, and Msx2 is a critical regulator of intramembranous bone formation.\textsuperscript{24–27} BMP2 signaling is mainly mediated by SMAD1/5/8 phosphorylation and their modulation of target gene expression.\textsuperscript{28} Phosphorylation of SMAD1/5/8 and their subsequent nuclear translocation are critical steps in BMP2 signaling,\textsuperscript{29} and our results showed that Ad-ERR\textsubscript{γ} promoted the phosphorylation of SMAD1/5/8 via BMP2 signaling and, consequently, upregulated BMP2 target genes, such as Runx2, Msx2, and Bglap.

Pharmacological BMP2 inhibition has received much attention because BMP2 signaling plays a key role in the regulation of vascular calcification. In a previous study, the BMP antagonist noggin blocked osteoblastic differentiation and mineralization in the presence of high phosphate.\textsuperscript{30} Derwall et al reported that a small molecule BMP inhibitor or a recombinant BMP antagonist inhibited vascular calcification, atherosclerosis development, vascular inflammation, and atheroma development in atherogenic animals by suppressing the activation of SMAD1/5/8.\textsuperscript{31} In line with the previous observations, we showed that pharmacological inhibition of ERR\textsubscript{γ} activity by its inverse agonist GSK5182 decreased BMP2 signaling and attenuated CM-induced VSMC calcification ex vivo. These protective findings were supported by results obtained in our high-dose vitamin D\textsubscript{3}-induced vascular calcification animal model. Taken together, our data indicate that GSK5182 may reduce the risk of cardiovascular morbidity and mortality in patients with vascular calcification. The generation of ERR\textsubscript{γ} may be a promising therapeutic target for the treatment of vascular calcification. The generation of ERR\textsubscript{γ}-specific recombinant agonists, such as GSK5182, may have therapeutic applications in the prevention of vascular calcification.

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Disclosures

None.

References


Significance

Vascular calcification is a chronic condition and a major contributing factor to the morbidity and mortality of cardiovascular disease. Although many regulators of vascular calcification have been identified, the role of estrogen-related receptor γ (ERRγ) in this process remains unknown. In the present study, we showed that ERRγ expression is upregulated during vascular calcification, and ectopic ERRγ expression promoted bone morphogenetic protein 2 transcription and the activation of its downstream signaling pathway through the phosphorylation of the bone morphogenetic protein effector proteins SMAD1/5/8. Inhibition of ERRγ by the selective inverse agonist GSK5182 attenuated vascular calcification and downregulated osteogenic gene expression in vitro and in vivo. The results of the present study provide new insight into the importance of ERRγ in vascular calcification and suggest that ERRγ plays a critical role in the differentiation of vascular smooth muscle cells to an osteogenic phenotype. ERRγ inhibitors, such as its inverse agonist GSK5182, may have a potential therapeutic application in the prevention of vascular calcification.
Estrogen-Related Receptor γ Plays a Key Role in Vascular Calcification Through the Upregulation of BMP2 Expression

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**Supplemental Table I.** siRNA sequence or primer sequence for RT-PCR.

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Supplemental Figure I. A-B, VSMCs were infected with Ad-GFP (control) or Ad-ERRγ. At 2 days after adenovirus infection, mRNA and protein expressions were analyzed in the absence of CM. A, Expression levels of Bmp2 and osteogenic markers were determined by RT-PCR. B, Expression levels of BMP2 and phosphorylation of SMAD1/5/8 were determined by western blotting. C-D, At 2 days after adenovirus infection, primary rat VSMCs were cultured for the indicated times in the absence of CM. Vascular calcification was assessed by Von Kossa staining (C) and determination of calcium content (D). Values are expressed as the mean ± SEM (n=5). Scale bar = 200 μm.

Supplemental Figure II. Electrophoretic mobility shift assay (EMSA) of the ERRγ on ERRE2 DNA binding activity in the BMP2 promoter. C2C12 cells were infected with Ad-ERR or Ad-GFP for 2 days.
Supplemental Figure II

- + - -  Ad-GFP
- - + +  Ad-ERRγ
- - - +  Non-labeled probe
+ + + +  Biotin-labeled probe
Materials and Methods

Cell and aortic ring cultures

Rat aortic smooth muscle cells (VSMCs) were cultured using the transplant method as described previously. Cells were isolated from 4-week-old Sprague-Dawley (SD) male rats (KOATECH, Pyungtaek, Korea). The aorta was washed using sterilized PBS and sliced into 1–3 mm thick sections. Sections of the aorta were attached to dishes and cultured in low glucose Dulbecco Modified Eagle’s Medium (DMEM) (Gibco, Gaithersburg, MD, USA) supplemented with 20% FBS (Hyclone, Logan, UT, USA) for 3 weeks with daily medium changes at 37°C in 5% CO₂. Cells were maintained in DMEM containing 5.5 mM of glucose and 10% FBS. Cells at passage 6–8 were used in all experiments. Calcification was induced by calcification medium (CM) containing 10% FBS, 2.6 mM of inorganic phosphate, and 2 mM of CaCl₂ for 5 days. The rat aortic smooth muscle cell line A7r5 and mouse myoblast skeletal muscle cell line C2C12 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). For aortic ring culture, 5-week-old C57BL/6N mice (KOATECH) were sacrificed to obtain the aortas. Endothelial cells and adventitia were carefully removed by rolling after syringe insertion into the aorta. The clean aorta was cut into 3 mm-thick sections and stabilized in DMEM high glucose without serum for 24 hours. After stabilization, the medium was replaced by CM (DMEM high glucose containing 2.6 mM of inorganic phosphate with 10% FBS) for 6 days.

Recombinant adenovirus construction and small interfering RNA (siRNA) transfection

Adenoviral constructs for GFP (empty) or Flag-ERRγ adenovirus were cloned in our laboratory. Mouse ERRγ was inserted to pAdTrack-CMV vector and we created adenovirus for GFP or ERRγ using Ad-easy system (Stratagene, La Jolla, CA). All viruses were purified by CsCl₂ gradient centrifugation method. Cells were transfected with 50 nM of scrambled RNA or siRNA using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA according to the manufacturer's protocol. The siRNA used for silencing ERRγ is shown in Supplemental Table I.

Semi-quantitative RT-PCR

RNAs were purified from rat VSMCs using the QIAzol reagent (Qiagen, Maryland, USA) according to the manufacturer’s protocol. cDNAs were synthesized from mRNAs using the RevertAid First Strand cDNA Synthesis Kit (Fermentas/Thermo Fisher Scientific, Vilnius, Lithuania) and analyzed by semi-quantitative RT-PCR. Primers for RT-PCR are listed in Supplemental Table I. The relative levels of each mRNA were normalized to β-actin expression.
Western blot analysis

Cells were lysed in lysis buffer containing 20 mM of Tris-HCl [pH 7.4], 5 mM of EDTA [pH 8.0], 10 mM of Na₄P₂O₇, 100 mM of NaF, 2 mM of Na₃VO₄, 1% NP-40, and protease and phosphatase inhibitors. Samples containing 30 μg of denatured protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% skim milk in TBS containing 0.1% Tween 20 (TBST) and incubated with each primary antibody overnight at 4°C. Primary antibodies and dilutions were as follows: anti-ERRγ (1:1000; R&D Systems, Minneapolis, MN, USA); anti-α-SMA (1:5000; Abcam, Cambridge, UK); anti BMP2 (1:1000; Novus Biologicals, Inc, Littleton, CO, USA); anti-phospho-Smad 1/5/8 (1:500; Cell Signaling Technology, Beverly, MA, USA); anti-Smad 1/5/8 (1:2000) and β-actin (1:5000) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Membranes were washed 3 times for 10 minutes with TBST and incubated with HRP-conjugated mouse (Santa Cruz Biotechnology) or rabbit (Cell Signaling Technology) secondary antibodies. The enhanced chemiluminescence reagent (Bionote, Hwasung, Korea) was used to detect HRP.

Relative quantification analysis

Relative mRNA expression, western blotting results, and immunohistochemical signals for BMP2 were analyzed using image J software (US National Institutes of Health).

Histological analyses

Mouse aortic rings were fixed in 4% paraformaldehyde and embedded in paraffin; deparaffinized tissue sections were stained with hematoxylin and eosin. For Von Kossa staining, cells or tissues were washed three times with PBS and fixed with 4% paraformaldehyde for 15 minutes or overnight. Fixed and paraffinized tissue sections were deparaffinized. Then cells and tissues were washed three times with PBS and one time with sterilized water, incubated with 5% silver nitrate solution and exposed to UV for 20 minutes until their color turned dark blue. After removing the silver nitrate solution, cells were washed three times with sterilized water and dried. For BMP2 immunohistochemical staining, paraffinized aorta sections were deparaffinized by xylene and ethanol. Endogenous peroxidase activity was blocked by 3% H₂O₂ for 15 minutes and sections were incubated with anti-BMP2 antibody (1:100; Abcam). Blocking of endogenous peroxidase and protein detection were performed using the Ultravision LP detection system HRP Polymer kit (Lab Vision, Fremont, CA, USA) according to the manufacturer’s protocol.

Calcium content analysis

Calcium deposition in the extracellular matrix of cells at each time point was measured by washing the cells with PBS, decalcifying them in 0.6N HCl overnight, and then determining the calcium contents in the supernatants colorimetrically using the QuantiChrom™ Calcium Assay Kit (Bioassay Systems, Hayward, CA, USA). The remaining cells were washed with PBS and solubilized with lysis buffer (0.1N NaOH and 0.1% SDS) and total protein contents was determined by bicinchoninic acid
(BCA) method (Pierce, Rockford, IL). Total calcium content was normalized to total protein content for each culture. For the determination of calcium contents ex vivo in aortic ring tissue, the sliced aortic ring tissues were decalcified with 0.6N HCl for 24 hours and calcium contents were determined according to the above procedure and normalized to the length (mm) of the sliced aorta. For determination of calcium contents of aortas in vivo, whole aorta tissues were washed with PBS and weighted. The whole aorta tissues were ground using the Precellys 24 tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) with 0.6 N of HCl, followed by incubation at 4°C for 1 day. Calcium deposition was determined by colorimetric analysis using the QuantiChrom™ calcium assay kit and normalized to micrograms CA/mg wet weight.

Luciferase reporter assay and plasmid constructs

C2C12 and A7r5 cells were used for transient transfection assays. Transient transfection was performed using Mirus TransIT-LT1 transfection reagent (Mirus Bio LLC, Madison, WI, USA) and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturers’ protocols. The cells were then harvested 48 hours after transfection and luciferase activity was measured. Luciferase activity was normalized to β-galactosidase activity. The data are representative of at least three to five independent experiments. The pcDNA3 vector expressing Flag-ERRγ was described previously2. The reporter plasmid encoding BMP2-Luc (+165 to −2712) was kindly donated by Ming Zhao (Vanderbilt University, Nashville, USA). BMP2-Luc (+165 to −2712) was used as the template for generating truncation/point mutant constructs. Truncated mouse BMP2 promoter constructs were cloned with the following primers: BMP2(−775)-F, 5′-CTGGTACCAGCGGCGGCTCCTTTAAAAGC-3′; BMP2(−195)-F, 5′-CTGGTACCAGCGGCGGCTCCTTTAAAAGC-3′; GLprimer2-R, 5′-CTTTATGTTTTTGGCGTCTTCCA-3′. Point mutation on the ERRE of the mouse BMP2 promoter was generated with the following primers: BMP2-pm-F, 5′-GAGCGCGATTTCAACCAGGATT-3′; BMP2-pm-R, 5′-AAACCGGGTGAATCGCGCT-3′.

Chromatin immunoprecipitation (ChIP)

C2C12 cells were infected by Ad-Mock or Ad-ERRγ adenovirus and cross-linked with 1% PFA for 10 minutes. After sonication, soluble chromatin was immunoprecipitated by anti-Flag antibody (Santa Cruz Biotechnology). Collected DNA was purified using the SolGent PCR purification kit (SolGent, Daejeon, Korea) and PCR was performed using the following primers: non-binding-F, 5′-TCTCGCCTTTCTGAACTGAGGATC-3′; non-binding-R, 5′-TGGAAATGAGTAGTGAACACGATGGG-3′; ERRE1-F, 5′-GTGGAGTGTATTTTGCGGCTT-3′; and ERRE1-R, 5′-CCAGTTTTGCAAAGCAAGACGGAG-3′.
**Electrophoretic Mobility Shift Assay (EMSA)**

EMSA was used for detecting DNA-protein binding and was performed using the Lightshift chemiluminescence EMSA kit (Thermo Fisher Scientific, MA, USA) according to manufacturer’s protocol. C2C12 cells were infected with Ad-GFP or Ad-ERRγ for 48 hours and nuclear extracts were obtained using buffer A (10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.05% NP40 (or 0.05% lgepal or Tergitol) pH 7.9) and buffer B (5 mM HEPES, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 26% glycerol (v/v), pH7.9). Nuclear extracts and oligomers were incubated for 1h at 4 °C with anti-ERRγ antibody, and loaded onto a 6% polyacrylamide-0.5×Tris base-boric acid-EDTA gel. Image acquisition was performed using a Image Quant LAS 4000 (GE Healthcare, Piscataway, NJ, USA). For ERRE1 and ERRE2 sites, the following oligomers were used as probes.

ERRE1 : 5’-GGCTCGGAGCGCGAGGTCACCCGGTTTGGCAAC-3’
ERRE2 : 5’-AAAATACCTTATTTGACCTCTAGAA-3’

**Animal model of vascular calcification**

The 6-week-old C57BL/6N mice were treated daily with vehicle or 10 mg/kg/day GSK5182 diluted in 30% polyethylene glycol via intraperitoneal (ip) injection. GSK5182 was synthesized by Seong Heon Kim (Daegu Gyeongbuk Medical Innovation Foundation). To induce calcification, mice were subcutaneously injected with 5.5×10⁵ IU/kg of cholecalciferol (Sigma Aldrich, St. Louis, MO, USA) diluted in 6.2 ml of sterilized water containing 70 μl of absolute EtOH (Merck Millipore, Darmstadt, Germany), 500 μl of cremophor A25 (Sigma Aldrich), and 250 mg of dextrose (Becton Dickinson, Franklin Lakes, NJ, USA) for 3 consecutive days (from day 4 to day 6). Aortas were collected on day 12. Mice were pair-fed, and provided daily with water during the experiment.

**Statistical analysis**

All values are presented as the mean ± SEM. Statistical analysis was performed using the Mann-Whitney for two group comparisons and the Kruskal-Wallis test for more three group comparisons. If the Kruskal-Wallis test showed a significant difference between the groups, comparisons between two independent groups for post-hoc analysis were done by employing the Mann-Whitney U-test using Bonferroni correction to adjust the probability. A value of p<0.05 was considered statistically significant.

**Study approval**

All animal procedures were approved by the Institutional Animal Care and Use Committee of Kyungpook National University.