Vascular calcification is a significant contributor to cardiovascular morbidity and mortality. We recently reported that cartilage oligomeric matrix protein (COMP) is pivotal for maintaining the homeostasis of vascular smooth muscle cells (VSMCs). Whether COMP affects the process of vascular calcification is unknown.

**Objective:** We aimed to test whether COMP modulates vascular calcification.

**Methods and Results:** VSMC calcification in vitro was induced by calcifying media containing high inorganic phosphate or calcium. In vivo medial vessel calcification was induced in rats by 5/6 nephrectomy with a high-phosphate diet or by periadventitial application of CaCl₂ to the abdominal aorta. COMP protein level was markedly reduced in both calcified VSMCs and arteries. COMP deficiency remarkably exacerbated VSMC calcification, whereas ectopic expression of COMP greatly reduced calcification. Furthermore, COMP knockdown facilitated osteogenic markers expression by VSMCs even in the absence of calcifying media. By contrast, COMP overexpression significantly inhibited high phosphate- or high calcium-induced VSMC osteochondrogenic transition. Induction of osteogenic marker expression by COMP silencing was reversed by a soluble form of bone morphogenetic protein (BMP)-2 receptor IA, which suggests a BMP-2–dependent mechanism. Our data revealed that COMP bound directly to BMP-2 through the C terminus, inhibited BMP-2 receptor binding, and blocked BMP-2 osteogenic signaling, indicating COMP inhibits osteochondrogenic transition of VSMCs at least partially through inhibiting BMP-2.

**Conclusions:** Our data strongly suggest that COMP is a novel inhibitor of vascular calcification. The imbalance between the effects of COMP and BMP-2 may provide new insights into the pathophysiology of vascular calcification. (Circ Res. 2011;108:917-928.)

**Key Words:** vascular smooth muscle cells ■ calcification ■ COMP ■ BMP-2

Vascular calcification is a common complication of chronic kidney disease, atherosclerosis, and diabetes mellitus. It is directly related to cardiovascular morbidity and mortality. Previously viewed as an inevitable, passive, and degenerative process, vascular calcification is increasingly being considered a complex and regulated process, with great similarities to skeletal mineralization. These findings have led to the important question of which cell types give rise to the skeletal elements of calcified arteries and what mechanisms regulate vascular calcification. To date, substantive studies have demonstrated that vascular smooth muscle cells (VSMCs) retain multipotential capability and can transform into osteo-/chondrocytic-like cells and express genes that are typically expressed by osteoblasts and chondrocytes during osteogenesis. Several factors facilitate osteochondrogenic transition of VSMCs and vascular calcification, including high calcium-phosphate products, oxidative stress, bone morphogenetic proteins (BMPs) (BMP-2, -4, -6), and vitamin D. Alternatively, loss of inhibitors of mineralization, such as matrix Gla protein (MGP) and osteopontin, also predispose vascular calcification. A finely tuned balance between inducers and inhibitors likely controls whether calcification occurs under pathological conditions. Other important mechanisms including apoptosis, mineral imbal-

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ance, or inflammation have also been involved in the pathogenesis of dystrophic calcification. However, the underlying molecular and cellular mechanisms of arterial calcification are still not fully understood.

Cartilage oligomeric matrix protein (COMP) is a 524-kDa pentameric noncollagenous glycoprotein expressed in all types of cartilage, vitreous of the eye, tendons, and VSMCs. Immunohistological staining of articular cartilage has revealed a developmentally regulated localization of COMP to the hypertrophic zone of the growth plate and in the superficial layer of the articular cartilage surface, but little detection in mineralized bone. In addition, COMP is recently identified as a normal component of the human artery wall and secreted by VSMCs. Of interest, COMP was also detected in areas of microcalcification in human atherosclerotic lesions but not in calcific foci within the lesions. The importance of COMP for chondrogenesis and skeletal development has been underscored by the identification of COMP mutations in two different inherited chondrodysplasias and osteoarthrotic phenotypes: pseudoachondroplasia and multiple epithelial dysplasia. In contrast, little is known about the function of COMP in arteries.

Recently, our studies suggested that COMP is essential for maintaining the quiescent/contractile phenotype of VSMCs. Ablation or degradation of COMP led to phenotypic transition and disturbance of VSMC homeostasis. Considering the similarities between bone mineralization and vascular calcification, the distinct localization pattern of COMP in arteries.
skeletal and calcified vascular tissues, the importance of COMP in chondrogenesis and in VSMC phenotype transition, we hypothesized that COMP could be an important regulator of VSMC mineralization and vascular calcification. Therefore, we used in vitro and in vivo models to investigate COMP as a novel endogenous modulator of VSMC calcification. We also investigated the involvement of BMP-2 in the effect of COMP on the osteogenic transition of VSMCs.

**Methods**

Animal care and use of 5/6 nephrectomy and CaCl₂ abdominal artery injury model in male Sprague–Dawley rats were in accordance with institutional guidelines. Aortic ring organ calcification was induced as described previously.²⁰,²¹ Human radial artery specimens were collected from uremic patients during arterial venous fistular operation by the approval from the Local Research Ethics Committee.

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

**Results**

COMP Protein Level Is Decreased in Calcifying VSMCs and Arteries

A high concentration of inorganic phosphate (Pi) or calcium (Ca²⁺) contributes directly to ectopic vascular calcification, although perhaps by different mechanisms.²² Therefore, we induced mineralization by osteogenic media containing 10 mmol/L β-glycerophosphate (β-GP) for BASMCs or by 5 mmol/L CaCl₂ for A7r5 cells. In vitro mineralization was verified by Alizarin red S staining, Ca accumulation and measurement of calcium deposition shown in Online Figure I (A and B). As shown in Figure 1A, full-length COMP (110 kDa) was markedly reduced at 3, 5 and 7 days in BASMCs after β-GP stimulation, which was paralleled by an increase in COMP degradation fragment (approximately 55 kDa). In accordance, a similar COMP protein reduction was observed in CaCl₂-induced calcifying A7r5 cells at various stages, whereas COMP mRNA level was not changed (Figure 1B; Online Figure II).

To further verify this observation in vivo, we used a chronic renal failure (CRF) rat model and an abdominal aortic injury rat model to mimic the process of vascular calcification. The first model is based on the epidemiological and clinical observation that calcification of the medial layer of arteries is prevalent in CRF and may contribute to the increased incidence of cardiovascular disease in this population.²⁴,²³ Rats in the CRF model underwent 5/6 nephrecto-
mization and received a high-phosphate diet to accelerate the process of vascular calcification. Based on Online Table II, CRF rats showed a significant increase in plasma level of blood urea nitrogen, creatinine, and phosphorus. Vascular calcification was successfully induced in the CRF rats, as evidenced by calcium deposition and von Kossa staining, with no calcification in sham-operated rats (Online Figure III). Western blot analysis revealed the COMP expression greatly reduced in abdominal arteries of CRF rats compared with sham-operated rats (Figure 1C). Similarly, in the CaCl₂-induced abdominal aortic calcification model (Online Figure IV), COMP expression was greatly reduced, whereas the COMP degradation fragment was concomitantly increased as compared with control rats (Figure 1D). In contrast, COMP mRNA level was not significantly altered (Online Figure V).

Thus, various in vitro and in vivo VSMC calcification models revealed a general reduction in COMP protein level. Combined with our recent observation that COMP is necessary to maintain the contractile phenotype of VSMCs, COMP may negatively regulate the process of VSMC calcification.

**COMP Deficiency Aggravates VSMC Calcification In Vitro**

To further investigate the potential causal role of COMP in VSMC mineralization, we first knockdown COMP by small interfering (si)RNA and test whether COMP knockdown alone was sufficient to drive calcification in the absence of calcifying medium. Specific knockdown of COMP was verified at both the mRNA and protein levels (Online Figure VI). As determined biochemically and histologically, COMP silencing did not cause detectable spontaneous calcification during 12-day culture period (Online Figure VII, A and B). Nevertheless, when exposed to high calcium, COMP knockdown greatly exacerbated calcium deposition by 50% in VSMCs as compared with scramble siRNA (Figure 2A and 2B). In contrast, the deterioration effect of COMP repression was gradually circumvented by supplementation with increasing amounts of exogenous purified COMP. Additionally, primary thoracic VSMCs from COMP−/− mice showed greatly increase of susceptibility to calcification in response to high-phosphate stimulation (Figure 2C through 2E). These in vitro data reinforce our hypothesis that COMP may act as an inhibitor of VSMC calcification.

**Ectopic Expression of COMP Ameliorates VSMC Calcification In Vitro**

We next asked whether overexpression of COMP could rescue the mineralization of VSMCs. We treated VSMCs with β-GP or CaCl₂ to induce mineralization. Adenovirus (Ad)-COMP was applied, and COMP overexpression was verified as indicated in Online Figure VIII (A and B). As shown in Figure 3A and 3C, calcium deposition was increased ~16-fold by 10 mmol/L β-GP or 7-fold by 5 mmol/L calcium, as compared with untreated control cells. Ectopic
infection with Ad-COMP greatly reduced the extent of VSMC calcification by 41% or 43% with the 2 treatments, whereas GFP adenovirus had no effect. The observation was further verified by Alizarin red S staining (Figure 3B and 3D), which indicated that the inhibitory effect of COMP on VSMC calcification did not differ by high calcium or phosphate stimulation but rather was a general phenomenon.

Ectopic Expression of COMP Retards Vascular Calcification In Vivo
To further validate the protective effect of COMP on VSMC calcification, we adopted a rat aortic-ring organ culture model.20,21 The viability of the aortic ring explants was verified by MTT assay after 6 days' culture (data not shown). As indicated in Figure 4A and 4B, calcification of aortic ring was markedly induced by 3.8 mmol/L PO₄³⁻ stimulation for 6 days, as demonstrated by calcium deposition and von Kossa staining. Conversely, Ad-COMP-infected aortic explants (Online Figure VIII, C) showed much less calcification than Ad-GFP-infected explants. We further evaluated the protective effect of COMP in an in vivo medial calcification model induced by perivascular application of 0.2 mol/L CaCl₂ to the infrarenal abdominal aortas of rat. Ad-GFP infection did not alter the calcification deposition in the abdominal aorta. In contrast, Ad-COMP greatly suppressed the aortic calcification by 63% (Figure 4C; n=5, P<0.05). The intensity of von Kossa staining was significantly lower in Ad-COMP-infected aortas than in Ad-GFP-infected aortas (Figure 4D). Thus, COMP overexpression may inhibit both high Pi- or CaCl₂-induced vascular calcification.

COMP Negatively Regulates Osteochondrogenic Transition of VSMCs
Previously, we showed that COMP is necessary to maintain a quiescent/contractile phenotype of VSMCs, and ablation of COMP facilitates a synthetic phenotype in response to injury.18 Therefore, we tested whether COMP could interfere with osteogenic transition of VSMCs. Interestingly, the expression of the osteochondrogenic markers Runx2, Msx2, Sox9 and BMP-2 were markedly higher in VSMCs with siRNA knockdown of COMP than with scramble siRNA treatment (Figure 5A; Online Figure IX, A). In parallel, the level of the contractile marker genes smooth muscle (SM) α-actin, SM22α and calponin was concomitantly decreased in VSMCs (Figure 5B; Online Figure IX, A), which suggests that VSMCs lacking COMP render a spontaneous osteochon-
drogenic transition even in the absence of calcifying microenvironment. Of note, the mRNA level of the known regulators of calcification, MGP, osteopontin and osteocalcin were not altered (Figure 5A). In accordance, COMP overexpression significantly circumvented the upregulation of Runx2, Msx2 and BMP-2 in high Ca\(^{2+}\)/H\(_{11001}\) induced calcified VSMCs at both mRNA level (Figure 5C) and protein level (Online Figure IX, B). Reciprocal protein alteration of Runx2/BMP-2 and SM\(_{H9251}\)-actin/SM22\(_{H9251}\), as well as enhanced ALP activity were also observed in cultured rat aortic rings 3 days after 3.8 mmol/L PO\(_{4}^{3-}\) stimulation, which was greatly rescued by COMP overexpression (Figure 5D; Online Figure X). We also monitored the onset of apoptosis because the latter has been reported to contribute to aortic ring calcification. At day 3, no obvious apoptosis appeared as evidenced by cleaved caspase-3 and Bax activation whereas osteogenic transition already occurred (Online Figure XI, A and B). At day 6, cleaved caspase-3 and Bax were significantly induced and TUNEL-positive cells were increased on phosphate stimulation. Of note, COMP overexpression partially inhibited high-phosphate induced apoptosis. To minimize the interfering of apoptosis, we applied 3.0 mmol/L PO\(_{4}^{3-}\) stimulation to the aortic rings (Online Figure XII, A and B). Six days after culture and stimulation, calcium deposition within aortic rings were significantly increased, although to a less extend compared with 3.8 mmol/L PO\(_{4}^{3-}\). No apoptosis was detected by the end of culture. In contrast, markedly enhanced Runx2/BMP-2 and decreased SM\(_{H9251}\)-actin/SM22\(_{H9251}\) were observed in aortic rings which can be rescued by excessive COMP. Similarly, perivascular administration of Ad-COMP markedly rescued the upregulated Runx2 level and reciprocally downregulated SM\(_{H9251}\)-actin/SM22\(_{H9251}\) levels in the CaCl\(_{2}\)-injured calcified vascular wall (Figure 5E). Collectively, the above data reinforce our hypothesis that COMP inhibits the osteogenic conversion of VSMCs and subsequent VSMCs calcification.

**COMP Inhibits Osteogenic Signaling of BMP-2**

Among various inducers of vascular calcification, BMP-2 is a potent stimulator of both orthotopic bone formation and osteoblastic differentiation of VSMCs. Hence, we asked whether COMP modulated osteogenic transition of VSMCs by interfering with BMP-2. As shown in Figure 6A, the addition of a soluble form of bone morphogenetic protein receptor-type 1A (BMPR-IA) (1 mg/L) almost completely abolished the COMP-silencing induction in expression of the osteochondrogenic markers Runx2, Msx2, and Sox9, which suggests a BMP-2-dependent mechanism. Interestingly, enhanced BMP-2 mRNA level evoked by COMP knockdown
was also greatly repressed by the soluble BMPR-IA. Of note, exogenous BMP-2 significantly unregulated endogenous BMP-2 mRNA expression in VSMCs. The data indicates that the induction of BMP-2 mRNA by COMP silencing was mainly attributable to lack of inhibition by COMP over extracellular BMP-2 (Figure 6B). Indeed, COMP silencing dramatically enhanced BMP-2–induced Smad1/5/8 phosphorylation and Runx2/Msx2 expression compared with that of scramble siRNA (Figure 6C). In accordance, COMP overexpression markedly suppressed BMP-2–induced Smad1/5/8 phosphorylation as well as downstream Runx2, Msx2 and osteocalcin expression (Figure 6D through 6F), suggesting COMP inhibits osteoblastic transition at least in part by interfering with BMP-2 signaling.

### COMP Binds to BMP-2 In Vitro and In Vivo

To further explore the relationship between COMP and BMP-2, the HEK293 cell line stable transfected with COMP underwent coimmunoprecipitation (Co-IP) assay. Recombinant human BMP-2 (500 μg/L) was added to the culture medium and incubated for 12 hours. As shown in Figure 7A, a specific COMP band was present in the immunoprecipitated complex brought down by anti–BMP-2 antibody but not by control IgG. Co-IP assay with anti-COMP antibody revealed that COMP also precipitated BMP-2. In addition, proteins from normal VSMCs and rat aortas underwent Co-IP with specific BMP-2/COMP antibodies or control IgG. The specific interaction between COMP and BMP-2 was observed as shown in Figure 7B. Next, we used an in vitro solid-phase binding assay to characterize the interaction between COMP and BMP-2. As shown in Figure 7C (left), use of anti-COMP antibody showed COMP bound to recombinant human BMP-2 but no detectable band with BSA. Accordingly, the use of anti–BMP-2 antibody showed increasing BMP-2 bound to purified COMP (right panel). Thus, COMP is capable of binding to BMP-2 directly because both COMP and BMP-2 were used as purified recombinant proteins.
Figure 6. COMP inhibits BMP-2 osteogenic signaling. A, Quantitative real-time PCR analysis of osteogenic gene expression in VSMCs transfected with scramble or COMP siRNA in the presence or absence of soluble BMPR-IA (1 mg/L). B, Relative expression of endogenous BMP-2 mRNA in VSMCs in response to treatment with increasing amounts of exogenous BMP-2 for 24 hours. *P<0.05 compared with control. C, Western blot analysis of BMP-2–induced Smad1/5/8 activation and downstream Runx2/Msx2 expression in VSMC. A7r5 cells were transfected with scramble or COMP-specific siRNA. Forty-eight hours later, Smad1/5/8 activation was detected in VSMCs stimulated by recombinant human BMP-2 (500 μg/L) for an additional 30 minutes. Expression level of Runx2 and Mss2 were assessed in VSMCs stimulated by BMP-2 for 24 hours. D, Western blot and quantitative analysis of BMP-2–induced Smad1/5/8 activation. A7r5 cells were infected with Ad-GFP or Ad-COMP 48 hours before challenge with recombinant human BMP-2 (500 μg/L) for 30 minutes. E and F, Relative mRNA (E) and protein (F) level of BMP-2–induced osteogenic transcriptional factors Runx2, Msx2, and osteocalcin (OCN). A7r5 cells were infected with Ad-GFP or Ad-COMP for 48 hours before treatment with recombinant BMP-2 (500 μg/L) for 3 hours (for mRNA) or 24 hours (for protein), respectively.
BMP-2 Binds Directly to the C Terminus of COMP

To further characterize the binding motif of COMP attributable to BMP-2 interaction, yeast 2-hybrid prokaryotic expression system was performed by cotransforming the yeast strain MAV203 with pPC86 plasmids encoding full-length mouse COMP and pDBleu plasmids encoding the N terminus, EGF-like domain, type III repeat domain, or the C terminus of mouse COMP. As shown in Figure 7D, β-galactosidase activity revealed that only the C terminus of COMP bound to BMP-2 in yeast. The interaction between the COMP C-terminal domain and BMP-2 was also confirmed by Western blotting with anti-COMP antibody.

The binding activity of COMP domains to BMP-2 was also determined by GST pull-down assay. Purified fusion protein of GST–N terminus, GST–EGF, GST–C terminus, and GST–type III of COMP were immobilized on GSH-sepharose beads and incubated with purified BMP-2. Proteins trapped by GST fusion protein were examined by immunoblotting with anti–BMP-2 antibodies. As shown in Figure 7E, the C-terminal domain of COMP binds to BMP-2 proportionally with the increase of COMP concentration.

The competitive binding of unlabeled COMP to 125I-labeled BMP-2 receptor binding was also determined by solid phase binding assay. The effect of increasing concentrations of unlabeled COMP on the binding of 125I-labeled BMP-2 by VSMC membrane–bound receptors was determined. Unlabeled BMP-2 (0.462 nmol/L) competitively inhibited 125I–BMP-2 receptor binding, whereas unlabeled COMP (6.5 nmol/L) significantly abolished the effect. The fitted curve was obtained by use of GraphPad Prism 5.0.

Figure 7. COMP associates with BMP-2 and inhibits BMP-2 receptor binding. A, Co-IP assay in HEK293 cell line stably transfected with COMP. Recombinant human BMP-2 (500 ng/L) was added and incubated for 12 hours. Left, Whole-cell lysates of HEK293 cells were immunoprecipitated with anti–BMP-2 antibody or control IgG before protein A agarose beads, then analyzed by Western blot (WB) with anti-COMP antibody. Right, HEK293 cell extracts were immunoprecipitated with anti-COMP antibodies or control IgG and analyzed by Western blot with anti–BMP-2 antibody. B, Co-IP assay of rat VSMCs and aortas. Left, VSMCs lysates or vascular extracts were incubated with anti–BMP-2 antibody or control IgG, then protein A agarose, and then analyzed by Western blot with anti-COMP antibody. Right, VSMCs lysates or vascular extracts were immunoprecipitated with anti-COMP antibodies or control IgG and analyzed by Western blot with anti–BMP-2 antibody. C, Solid phase binding assay. Various amounts of recombinant human BMP-2 or purified COMP underwent 10% SDS-PAGE and were incubated with purified COMP or human recombinant BMP-2. Anti-COMP or anti–BMP-2 antibodies were used for subsequent detection. BSA served as negative control. D, BMP-2 selectively binds to the C-terminal domain of COMP. Top, Schematic structure of COMP constructs used to map those domains (N-terminal, EGF-like, type III, and C-terminal) that bind to BMP-2. Presence or absence of binding between COMP domains and BMP-2 is indicated with + or −, respectively. Bottom, β-Galactosidase activity was used to test the interaction between the C-terminal domain of COMP and BMP-2. Three independent yeast transformants for each pair of plasmids were transferred onto a nitrocellulose membrane, and β-galactosidase activity was measured. E, GST pull-down assay. Purified fusion protein of GST–N terminus, GST–EGF, GST–C terminus, and GST–type III of COMP were immobilized on GSH-sepharose beads and incubated with purified BMP-2. Proteins trapped by GST fusion protein were examined by immunoblotting with anti–BMP-2 antibodies. Purified BMP-2 (first lane) was used as a positive control. Arrow indicates BMP-2 band. F, Effect of increasing concentrations of unlabeled COMP on the binding of 125I-labeled BMP-2 by VSMC membrane–bound receptors. 125I-labeled BMP-2 was incubated in the absence or presence of unlabeled COMP with VSMC membranes at 4°C for 24 hours. Values are expressed as percentages of specific binding determined in the absence or presence of unlabeled COMP. Each point is the average of triplicate determinations. The fitted curve was obtained by use of GraphPad Prism 5.0. G, COMP retarded the competitive inhibition of unlabeled BMP-2 to 125I-labeled BMP-2 receptor binding. Unlabeled BMP-2 (0.462 nmol/L) competitively inhibited 125I–BMP-2 receptor binding, whereas unlabeled COMP (6.5 nmol/L) significantly abolished the effect. Each point is the average of triplicate determinations. *P<0.05.

BMP-2 and pDBleu plasmid encoding the N terminus, EGF repeat domain, type III repeat domain, or the C terminus of mouse COMP. As shown in Figure 7D, β-galactosidase activity revealed that only the C terminus of COMP interacted with BMP-2 in yeast. The interaction between the COMP C-terminus and BMP-2 was also confirmed by in
vitro GST pull-down assay. In accordance with the yeast 2-hybrid results, only the purified GST-C terminus pulled down BMP-2 (Figure 7E).

**COMP Inhibits BMP-2 Receptor Binding**

Because our data indicated that COMP directly bound to BMP-2 through the C-terminal domain and inhibited BMP-2–induced Smad activation, we wondered whether COMP interfered with BMP-2 signaling by preventing BMP-2 from binding to membrane BMP-2 receptors. We isolated bulk membranes from rat VSMCs for analysis of specific binding of BMP-2 by a radioligand receptor binding assay. We examined uptake of 125I-labeled BMP-2 by increasing amounts of plasma membranes of VSMCs. The binding of 125I-labeled BMP-2 to the receptor-enriched VSMC plasma membranes was inhibited by unlabeled COMP in a concentration-dependent manner (Figure 7F). Furthermore, competitive inhibition of unlabeled BMP-2 (0.462 nmol/L) to 125I-BMP-2 binding in the receptor-enriched VSMC plasma membranes was retarded in the presence of 6.5 nmol/L COMP (Figure 7G), so COMP inhibited BMP-2 receptor binding.

**Reciprocal Alteration of COMP and BMP-2 in Uraemia Radial Arteries of Patients With End-Stage Renal Disease**

To determine the potential clinical relevance of COMP in vascular calcification, we collected radial artery specimens from uremic patients who underwent arterial venous fistula operation (CRF; n=6) and from coronary heart disease (CHD) (n=8, as control) patients who underwent coronary artery bypass grafting, without complications of diabetes and chronic kidney disease. As shown in Figure 8, BMP-2 and Runx2 expression were greatly enhanced in the uremic arteries than in control CHD radial arteries. Reciprocally, the level of full-length COMP was significantly reduced in uremic than in CHD radial arteries, concomitant with an increase in the COMP fragment, which further suggests an imbalance between BMP-2 and COMP may contribute to vascular calcification in end stage renal failure patients.

**Discussion**

Vascular calcification is an increasingly important clinical problem, particularly in the context of renal failure, diabetes, and atherosclerosis, and correlates with poor cardiovascular outcomes.4 Accumulating evidence has pointed to an actively regulated, cell-mediated process of vascular calcification with competition between factors promoting calcification and inhibitors of mineralization. Our studies demonstrate that COMP, a macromolecule located both in cartilage and vessels, may act as a novel endogenous inhibitor of vascular calcification. Likewise, COMP suppressed the osteochondrogenic transition of VSMCs at least in part by interfering with BMP-2.

The cellular origins of calcified blood vessels are still controversial. Although some observations point to the activation and differentiation of nascent resident pericytes or circulating stem cells, a number of studies have indicated that the presence of osteochondrogenic cells in the vascular wall is the end result of the phenotypic change of VSMCs.9,26 Unlike most cell types undergoing terminal differentiation, VSMCs retain substantial phenotypic plasticity in response to injurious stimuli in the local microenvironment.7 For example, an elevated phosphate level induces cultured VSMCs to undergo an osteochondrogenic phenotype change characterized by reduced expression of SMC markers (SM22α, SM-α-actin) and increased expression of osteochondrogenic markers (Runx2, osteopontin, osteocalcin, and alkaline phosphatase).10 Nevertheless, the underlying mechanisms that control VSMC transdifferentiation to osteochondrogenic cells are still elusive.8 The known regulatory factors include...
alkaline phosphatase, BMP-2, Runx2, osterix, and phosphate, among others.2,26 Herein, we showed COMP to be involved in VSMC osteogenic phenotypic switching by demonstrating that COMP ablation renders the transition of VSMCs to osteogenic-like cells. Reciprocally, COMP overexpression reversed the osteogenic transition in vitro and in vivo. These observations are in agreement with our recent report that COMP is essential to maintain the contractile phenotype of VSMCs.18 Loss of expression or integrity of COMP under a calcifying microenvironment will lead to subsequent vascular calcification.

There are other possibilities of COMP inhibiting VSMC calcification. For example, COMP binds calcium, and so it could either potentially interact with mineral crystals and influence mineralization by modulating crystal formation or chelate/sequester calcium in the culture medium. The first one needs to be further elucidated. The latter is very unlikely thought because it would require roughly 20 g/L COMP to chelate 1.8 mmol/L calcium within the growth medium, which is more than 10^5 times the amount of COMP used (0.2 mg/L) according to the calcium-binding properties of COMP.17 Secondly, apoptosis is another key factor attributes to dystrophic calcification.20,27 In 3.8 mmol/L PO_4^3− ex vivo vessel culture model, the inhibition of apoptosis was observed in Ad-COMP–infected vessels but not in that of Ad-GFP–infected vessels at 6 days after high-Pi stimulation, indicating a potential negative modulation of COMP on vascular apoptosis. However, it’s also conceivable that the inhibition of calcification by COMP indirectly leads to reduced apoptosis resulting from the absence of nanocrystals which can induce VSMC apoptosis if uptaken. This needs to be further elucidated. Additionally, the perivascular application of CaCl_2 to induce vascular injury and in vivo calcification involves complex mechanisms including inflammation, oxidative stress, apoptosis etc, and the precise mechanism of COMP inhibition of vascular calcification in vivo therefore needs to be further explored.

Long known to be essential for differentiation of osteoblastic cells, BMP-2 is increasingly being recognized as a mediator of vascular calcification.25 BMP-2 may promote vascular calcification by increased phosphate uptake and inducing an osteogenic phenotype modulation in SMCs by binding to the receptors BMPR1 or BMPR2, which in turn phosphorylates the regulatory Smad1/5/8 and upregulates downstream key osteogenic transcription factors, including Runx2 and Msx2.25,29 In the present study, we demonstrated that COMP physically interacts with BMP-2 through the C-terminal domain of COMP, prevents BMP-2 receptor binding, suppresses Smad1/5/8 phosphorylation, and inhibits the expression of the downstream transcriptional factors Runx2 and Msx2. COMP therefore may modulate osteogenic transition of VSMCs by antagonizing BMP-2. The activity of BMP-2 is also antagonized by MGP, an inhibitor of arterial calcification, which was interfered by heat shock protein 70.30 Combined with our observations, the complex protein–protein interaction within the matrix can actively modulate the process of calcification. Along with the progress of vascular calcification, COMP level was gradually decreased and the inhibitory effect of COMP on BMP-2 was gradually lost, which in turn enhanced BMP-2 expression. The imbalance of inducers and inhibitors of mineralization ultimately results in ectopic vascular calcification.

In summary, we revealed COMP as a novel endogenous inhibitor of vascular calcification. COMP exerts its inhibitory effect at least partially by directly binding to BMP-2 through its C-terminal domain and interfering with BMP-2 osteogenic signaling. Targeting COMP may open up new avenues for the prevention of vascular calcification in renal failure, diabetes and atherosclerosis.

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Disclosures
None.

References
Novelty and Significance

**What Is Known?**

- Vascular calcification is positively associated with and directly contributes to cardiovascular morbidity and mortality.
- Osteochondrogenic cells in the calcified vascular wall arise mainly from a phenotypic change in vascular smooth muscle cells (VSMC).
- Cartilage oligomeric matrix protein (COMP) is a matricellular protein essential for maintaining the quiescent or contractile phenotype of VSMCs.

**What New Information Does This Article Contribute?**

- COMP is an endogenous inhibitor of vascular calcification.
- COMP inhibits the osteochondrogenic transition of VSMCs.
- COMP directly binds to bone morphogenetic protein (BMP)-2 and therefore inhibits osteogenic signaling by BMP-2 during vascular calcification.

Vascular calcification is a major risk factor for the development of cardiovascular disease. It has been recently recognized as a process similar to skeletal mineralization. The osteochondrogenic cells in the calcified vascular wall mainly arise from a phenotypic change in VSMCs. However, the mechanism regulating the osteogenic transition of VSMC remains elusive. Herein, we demonstrate for the first time that cartilage oligomeric matrix protein (COMP) suppresses osteogenic transition of VSMC at least partially through direct binding to BMP-2. The imbalance between inducers (BMP-2) and inhibitors (COMP) of mineralization ultimately results in ectopic vascular calcification. COMP therefore is an endogenous inhibitor of vascular calcification. Our study advances the present knowledge of mechanisms by which vascular calcification is regulated, and it suggests that targeting COMP may be a promising therapeutic approach for ameliorating vascular calcification.
Cartilage Oligomeric Matrix Protein Inhibits Vascular Smooth Muscle Calcification by Interacting With Bone Morphogenetic Protein-2

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Supplement Material

Materials

Antibodies against COMP and BMP-2, Runx2 and SM22α were purchased from Abcam (Cambridge, UK). Antibodies against smooth muscle (SM) α-actin and calponin were from Sigma-Aldrich (St. Louis, MO). The antibodies against pSmad 1/5/8 and cleaved caspase-3 were from Cell Signaling Technology (Boston, MA) and that against GAPDH was from Meridian Life Science (Saco, ME). The antibody against β-actin, Msx2, and Bax were from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human BMP-2 was from ProSpec–Tany TechnoGene (Rehovot, Israel). Recombinant human BMP receptor 1A (BMPR-IA) was from R&D Systems (Minneapolis, MN). $^{45}$Ca$^{2+}$ was from PerkinElmer (Billerica, MA).

Cell Culture

Primary bovine aortic smooth muscle cells (BASMCs) were isolated from adult bovine aortic explants as described previously $^1$ and maintained in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; containing 4.5 g/L glucose, 10 mmol/L sodium pyruvate, 100 U/mL penicillin, and 100 g/mL streptomycin) at 37°C in a humidified atmosphere containing 5% CO$_2$. BASMCs at passages 3 to 6 were used for all experiments. Calcification of BASMCs was induced by calcifying media containing 10 mM $\beta$-glycerophosphate ($\beta$-GP) for 7 days with media changes every 2 days. $^2$ Alternatively, the rat smooth-muscle embryonic thoracic aorta cell line A7r5 was purchased from the American Type
Culture Collection (Manassas, VA), and mineralization of A7r5 was induced by 5 mM CaCl$_2$ challenge for 12 days with media changes every 2 days.$^3$ Primary mouse thoracic VSMCs were isolated by collagenase digestion as described previously.$^4$

**Animal Model of Chronic Renal Failure**

Male Sprague-Dawley rats weighing 220 to 250 g were used in these experiments. All studies followed the guidelines of the Animal Care and Use Committee of Peking University. Chronic renal failure (CRF) was induced by subtotal (5/6) nephrectomy, namely, the excision of two-thirds of the left kidney, then complete right nephrectomy 1 week later. Sham-operated rats underwent similar surgical procedures but with only removal of the renal envelope. To accelerate the process of aortic calcification, on the date after completing renal ablation, animals received a standard high-phosphate diet (1.7% Pi) for the duration of the study.$^5$ Sham-operated rats received standard rat chow (0.6% Pi). The schematic experimental design is given in online Figure IIIA.

Twelve weeks after the 5/6 nephrectomy, rats were sacrificed, and plasma levels of blood urea nitrogen, creatinine, calcium, and phosphate were measured with commercially available kits (Biosino Bio-Technology and Science, Beijing). The carotid and abdominal arteries were dissected for calcium deposition assay, von Kossa staining and protein extraction.

**CaCl$_2$-Induced Rat Abdominal Aortic Calcification**
Rats were placed under general anesthesia (300 mg/kg chloral hydrate), and the infrarenal abdominal aortas were treated with periadventitial application of 0.2 mol/L CaCl$_2$ for 10 min as described previously.$^6$ Control rats were treated with 0.2 mol/L NaCl. After 7 days, the abdominal aorta was excised for quantitative analysis of calcium content in aortic segments by use of a QuantiChrom Calcium Assay Kit (Biosino Bio-Technology and Science, Beijing) as described previously.$^2$ The sections from each aorta (7 μm each, 210-μm apart) were processed for von Kossa and hematoxylin eosin (H&E) staining.

**Aortic Ring Organ Calcification**

Aortas (from the renal to the iliac arteries) were removed in a sterile manner from rats. After the adventitia and endothelium were carefully removed, the vessels were cut into 2- to 3-mm rings and placed in high-Pi (3.8 mM PO$_4^{3-}$) or regular DMEM containing 10% FBS at 37°C in 5% CO$_2$ for 6 days, with medium changes every 2 days. The viability of the aortic ring was monitored by methylthiazoletertrazolium (MTT) assay as described previously.$^8$

**Quantification of VSMC Calcification**

VSMCs (BASMCs and the A7r5 cell line) were grown in 6-well plates and were treated with growth medium or calcifying medium. After removing culture medium and washing with phosphate buffered saline (PBS), VSMCs were treated with 0.6 N HCl overnight at 4°C. After removing the HCl supernant, the remaining
cell layers were then dissolved in 0.1 N NaOH and 0.1% SDS for protein concentration analysis. The calcium content in the HCl supernatant was colorimetrically analyzed by use of a QuantiChrom Calcium Assay Kit and normalized by protein concentration. For $^{45}$Ca accumulation assay, $1 \mu$Ci $^{45}$CaCl$_2$ was added 48 hr before cell harvesting. The medium was then removed and the cell layer was washed 5 times with PBS. The cells were dissolved in formic acid, then scintillation liquid containing 2, 5-diphenyloxazole and 1,4-bis(5-phenyl-2-oxazolyl) benzene, and radioactivity was measured by liquid scintillation counting (Beckman, Fullerton, CA, USA).

**Characterization of Calcifying Nodules by Alizarin Red S or von Kossa Staining**

For Alizarin red S staining, cells in 6-well plates were washed 3 times with PBS, then fixed with 10% formaldehyde for 10 min. Then cells were washed 3 times with PBS and exposed to 2% Alizarin Red S (aqueous, Sigma) for 30 min and washed with 0.2% acetic acid. Positively stained cells display a reddish/purple color. For von Kossa staining, aortic sections were incubated with 5% silver nitrate solution for 30 min, exposed to bright sunlight for 15 min, washed and treated with 5% sodium thiosulfate. Calcified nodules are stained as brown to black.

**Recombinant Adenovirus Construction**

The adenovirus for full-length mouse COMP (Pubmed No. NM_016685.2) (Ad-COMP) was constructed and amplified according to the manufacturer’s protocol.
An adenovirus carrying green fluorescence protein (Ad-GFP) was used as a negative control. For *in vivo* studies, a single exposure of $6 \times 10^8$ plaque forming units (pfu) of Ad-COMP dissolved in 30% pluronic gel solution was periadventitially delivered to the rat abdominal arteries immediately after CaCl$_2$ injury.

**Real-Time Quantitative PCR and Western Blot Analysis**

Real-time PCR amplification involved use of an Mx3000 Multiplex Quantitative PCR System (Stratagene Corp, La Jolla, CA) and SYBR Green I reagent normalized to that of the internal control β-actin. Primer sequences of target genes are in the online Table I.

For western blot analysis, extracts containing equal amounts of total protein from cells or rat arteries were resolved by 10% SDS-PAGE. The membranes were incubated with primary antibody and IRDye 700DX-conjugated secondary antibody (Rockland Inc, Gilbertsville, PA). The immunofluorescence signal was detected by the Odyssey infrared imaging system (LICOR Biosciences, Lincoln, NB).

**COMP siRNA Transfection**

Small interfering RNA (siRNA) against COMP was designed by use of the Block-iT™ RNAi Designer and chemically modified by the manufacturer (Invitrogen). Sequences corresponding to the siRNA of COMP were sense, 5’-AGAAACUUGAGCUGUGUUGAUAGCC-3’, and antisense,
5’-GGCUAUCAAGACAGCUCAAGUUUCU-3’. Transfection of rat VSMCs with the siRNA (50 nmol/L) \textit{in vitro} was by use of Oligofectamine (Invitrogen). A scramble Stealth RNAi duplex (catalog no. 12935, Invitrogen) served as a negative control.

**Recombinant Expression of COMP in HEK293 cells and COMP Purification**

The cDNA clone coding for full-length COMP was reconstituted as described previously and transfected into the HEK293 cell line by use of Lipofectamine 2000 (Invitrogen). The empty vector pCDNA3.1 was transfected into HEK293 cells as a control. Transfected cells were selected with 750 mg/L G418 for 2 weeks. The cells were then maintained in DMEM supplemented with 10% FBS and G418 at 37°C in 95% air, 5% CO$_2$ with 95% humidity. For COMP purification, stable transfected cells were incubated in serum-free opti-MEM (GIBCO) without G418 for 24 hr. The conditioned medium was applied to a heparin-agarose column (Sigma) equilibrated with Tris-buffered saline containing 2 mM CaCl$_2$ for binding overnight at 4°C by gentle agitation. After 5 washes, COMP was eluted with 0.75 M NaCl (buffered in 10 mM Tris, pH 7.5) containing 2 mM CaCl$_2$, and the purity was verified by silver staining and western blot analysis.

**Co-Immunoprecipitation**

Rat abdominal artery or VSMC lysates were incubated with anti-COMP (Abcam, ab42225) or anti-BMP-2 (Abcam, ab14933) antibodies before immunoprecipitation
with protein A agarose beads (Vigorous, Beijing). Precipitated proteins were resolved by 10% SDS-PAGE and then immunoblotted with anti-BMP-2 or anti-COMP antibodies. Rabbit normal IgG served as a negative control.

Solid Phase Binding Assay

Recombinant human BMP-2 or purified COMP underwent 10% SDS-PAGE and transblotting onto a nitrocellulose membrane. The membrane was then incubated with purified COMP or recombinant human BMP-2 in Tris-buffered saline (pH 7.4) for 2 hr before immunological analysis with primary anti-COMP or anti-BMP-2 antibodies, then IRDye 700DX-conjugated secondary antibodies. The immunofluorescence signal was detected by the Odyssey infrared imaging system (LICOR Biosciences, Lincoln, NB).

Assay of Protein–Protein Interactions by the Yeast 2-hybrid System

The fragments encoding the 4 functional domains of mouse COMP (i.e., the N terminus [NT; aa 20–83], epidermal growth factor (EGF) repeat domain [aa 84–261], type III repeat domain [aa 266–520], and C terminus [CT; aa 521–755; GenBank accession number AF257516] were amplified by PCR and cloned in frame into the Sall/NotI sites of pDBleu (pDB-COMP-NT, pDBCOMP-EGF, pDB-COMP-type III, and pDB-COMP-CT). cDNA inserts encoding the full-length human BMP-2 were cloned in-frame into the pPC86 vector to generate the indicated plasmids. Three independent colonies were analyzed for interaction in the yeast MAV203 strain of 2
proteins, of which the COMP fragment was fused to the Gal4 DNA binding domain and BMP-2 to the VP16 transactivation domain. β-galactosidase activity was tested as described.\textsuperscript{11}

\textit{In Vitro Glutathione S-transferase Pulldown Assay}

The bacterial expression vector pGEX-3X (Life Technologies) was used to produce recombinant glutathione S-transferase (GST) fusion proteins in \textit{Escherichia coli}. The cDNA fragments encoding the N terminus, EGF repeat domain, type III repeat domain, and C terminus of mouse COMP were subcloned into pGEX-3X to generate plasmids. For expression of GST fusion proteins, the appropriate plasmids were transformed into \textit{E. coli} DH5 (Life Technologies). Fusion proteins were affinity purified on GSH-agarose beads, and the GST moiety was removed from the GST fused protein as described previously.\textsuperscript{12}

For examining the binding motif of COMP to BMP-2 \textit{in vitro}, GSH-Sepharose beads (50 μl) preincubated with purified GST-NT, GST-EGF, GST-type III, or GST-CT (0.5 μg) were incubated with 0.5 μg of recombinant human BMP-2 in 150 μl buffer acetoxymethyl ester (10 mM Tris-HCl, pH 7.9, 10% glycerol, 100 mM KCl, and 0.5 mg/ml BSA). The bound proteins were denatured in sample buffer and separated by 12% SDS-PAGE, and BMP protein was detected by western blot analysis with polyclonal anti-BMP-2 antibody. Purified BMP-2 served as a positive control.
Ligand Receptor Binding Assay

Recombinant human BMP-2 was radiolabeled to a high specific activity (85 μCi/μg) with 1.5 mCi of carrier free $[^{125}\text{I}]$NaI. Free $[^{125}\text{I}]$NaI was separated from $[^{125}\text{I}]$BMP-2 by dialysis against PBS. Assays for receptor binding of radioactive trace-labeled BMP-2 in the presence of increasing amounts of BMP-2 or COMP were performed at 4 °C as previously described. 13 Briefly, bulk membrane was isolated from VSMCs by a mixture of 150 mM NaCl, 0.1% Triton X-100, 50 mM Tris-Cl and 1 mM EDTA, pH 7.4, supplemented with protease inhibitor cocktail, homogenized with a 27-G needle fixed to a 1-ml syringe, centrifuged at 3,000 g for 10 min, then additionally centrifuged at 12,000 g for 10 min. The supernatant was then centrifuged at 130,000 g for 30 min, and the membrane extracts in precipitation was suspended in buffer A (mixture of 150 mM NaCl, 10 mM MgCl$_2$, 0.5% SDS, 1% Triton X-100, 50 mM Tris-Cl, and 1 mM EDTA, pH 7.4, supplemented with protease inhibitor cocktail).

For competition binding studies, membrane extracts were incubated at 4°C for 24 hr with PBS containing $[^{125}\text{I}]$BMP-2 in the presence of unlabelled BMP-2 (0.0004-59.07 nM) or COMP (0.003-22.34 nM). Non-bound protein was discarded by incubation with 25% polyethylene glycol-4000 for 30 min at room temperature, followed by centrifugation at 3500 rpm for 15 min. Membrane extracts were transferred to polystyrene tubes, and radioactivity was measured with a gamma counter. Each concentration point was determined in triplicate.
Patients and Radial Artery Analysis

A 2- to 3-mm circumferential segment of radial artery was excised from each of 8 uremic patients who underwent arterial venous fistular operation (CRF, chronic renal failure; n=8) and from coronary heart disease (CHD, n=6, as control) patients who underwent coronary artery bypass grafting, without diagnosed complications of diabetes and chronic kidney disease. Approval from the Local Research Ethics Committee was granted for human tissue use, and the procedures used were in accordance with institutional guidelines. All the patients gave their informed consent for radial artery biopsy before surgery. The vessel was then dissected free of fat and tissue was prepared for western blot analysis or histomorphological study. The reason we chose radial arteries from uremic patients is based on the clinical observation that medial artery calcification is a common complication in CRF, and the extent and histoanatomic type of vascular calcification are predictors of subsequent vascular mortality. Additionally, in patients with CRF, medial calcification has been documented in coronary arteries, particularly in peripheral arteries (epigastric and radial arteries). The reason we chose radial arteries from CHD patients who underwent coronary artery bypass grafting as control is due to two reasons; first, radial arteries have became a reasonable alternative to the saphenous vein and left internal thoracic artery graft in coronary artery bypass grafting. So it’s relatively easy to harvest human tissue and exclude the bias of COMP distribution in different vessels. Secondly, in contrast to calcification in CRF, which usually occurs at tunica media of smaller muscular and distal arteries, atherosclerotic calcification (if there is any in
atherosclerotic lesion of CHD patients) occurs at sites of tunica intima of atherosclerotic plaques in large elastic and proximal sites of arterial tree.\textsuperscript{16}

**Fluorometric Terminal deoxynucleotidyl transferase-mediated dUTP Nick-End Labeling (TUNEL) Assay**

Frozen tissue sections were stained with TUNEL reagent (Promega, Madison, WI) for in situ apoptosis detection. In brief, cryosections were incubated with permeabilization solution containing Proteinase K (20 $\mu$g/ml) at room temperature for 15 minutes, washed in PBS, then incubated with a rTdT reaction mixture containing fluorescein-12-dUTP at 3’-OH DNA ends, rTdT Enzyme (terminal transferase) for 60 min in humidified chamber at 37°C following the manufacturer’s instructions. Nuclei were stained with Hoechst. Confocal imaging system with the appropriate argon beam lasers was applied. For quantification of the number of TUNEL-positive cells, six sections with each of ten high-power fields at 400× magnification were averaged. The percentage of TUNEL-positive nuclei was calculated.

**Alkaline Phosphatase Activity**

Alkaline phosphatase (ALP) activity was measured colorimetrically as the hydrolysis of p-nitrophenyl phosphate with the use of ALP assay kit (Jiancheng Bioengineering Co., Nanjing, China). Results were normalized to the levels of total protein. Aortic rings were homogenized with 1% Triton X-100 in 0.9% saline on ice and centrifuged in a microfuge at 8000 g for 5 min. Supernatant was removed for
ALP and the protein concentration assay.

**Statistical Analysis**

All continuous data are presented as mean±standard error of the mean (SEM). Protein band density was normalized to the corresponding loading control and then to the mean of the corresponding control group. Comparisons between 2 groups involved paired Student t test (two-sided). Comparisons among more than 2 groups involved one-way ANOVA followed by Student-Newman-Keuls test for post-hoc comparison as appropriate to evaluate the effect of recombinant COMP adenovirus or siRNA knockdown on osteogenic marker gene expression or calcium deposition. Two-way ANOVA followed by Bonferroni test for comparisons between groups was performed in analyzing the effect of β-GP or CaCl₂ on COMP expression over time, the effect of COMP silencing on CaCl₂ induced calcium deposition in the absence or presence of various concentration of purified COMP. Statistical analyses involved use of GraphPad Prism 5.0 (GraphPad Software Inc, La Jolla, CA). A $P < 0.05$ was considered statistically significant.

**References**


Online Figure legends

**Online figure I:** Characterization of calcification in VSMCs. (A) $^{45}\text{Ca}^{2+}$ incorporation analysis and Alizarin red S staining of Bovine VSMCs stimulated by 10 mM β-GP. (B) Calcium deposition analysis and Alizarin red S staining of A7r5 cell line evoked by 5 mM CaCl$_2$. Results are expressed as mean±SEM from three independent experiments. *$P<0.05$ versus control. Scale bar, 20 μm.

**Online figure II:** Quantification of relative mRNA level of COMP in A7r5 stimulated by 5 mM CaCl$_2$. β-actin served as internal control.

**Online figure III:** (A) Schematic experimental design for rat chronic renal failure (CRF) model. Calcium deposition of carotid arteries (B) and von Kossa staining (C) of abdominal arteries indicated vascular calcification of rats treated by 5/6 nephrectomy and a high-phosphate diet. Values are mean±SEM (n=8 per group). *$P<0.05$ versus sham group. Scale bar, 20 μm.

**Online figure IV:** Verification of CaCl$_2$-induced local abdominal aortic calcification in rats. Calcium deposition (A) and Alizarin red S staining (B) of abdominal arteries at 7 days after 0.2 mol/L CaCl$_2$ or NaCl treatment. Results are mean±SEM (n=5 per group). *$P<0.05$ versus vehicle.

**Online figure V:** Quantification of relative mRNA level of COMP in abdominal
arteries treated by CaCl$_2$ compared to that of vehicle. β-actin served as internal control. Results are mean±SEM (n=5 per group). NS, not statistical significant.

**Online figure VI:** Identification of specific COMP knockdown by real-time PCR (A) and western blot analysis (B). *P<0.05, compared to scramble siRNA.

**Online figure VII:** Calcium deposition in COMP silencing VSMCs. (A) Quantitative analysis of calcium deposition in VSMCs specifically knockdown of scramble siRNA or COMP siRNA. (B) Alizarin staining of calcium nodule. NS, not statistical significant. Scale bar, 20µm.

**Online Figure VIII:** COMP overexpression by Ad-COMP *in vitro* and *in vivo*. Western blot analysis of COMP expression of (A) bovine vascular smooth muscle cells (BVSMC) infected with 5 or 10 multiplicity of infection (MOI) of Ad-COMP for 48 hr. (B) A7r5 cells infected by 50 or 100 MOI Ad-COMP for 48 hr, and (C) rat abdominal arteries infected with $6\times10^8$ pfu Ad-COMP for 72 hr in *vivo*.

**Online Figure IX:** Representative western blot and quantification analysis of Runx2 and BMP-2 expression in A7r5 cells transfected with scramble siRNA and COMP siRNA (A) or infected by 50 MOI Ad-COMP for 48 hours followed by treating 5 mM CaCl$_2$ for 12 days (B). Results are expressed as mean±SEM from three independent experiments. *P<0.05.
**Online Figure X:** Alkaline phosphatase (ALP) activity of rat abdominal aortic rings treated with 3.8 mM Pi for 3 or 6 days respectively. Results are mean±SEM (n=3 per group). *P<0.05.

**Online figure XI:** Detection of apoptosis in cultured rat aortic rings treated by 3.8mM PO₄³⁻ for 3 or 6 days respectively. (A) Representative western blot and quantification analysis of cleaved caspase-3 and Bax expression of aortic rings. (B) Representative immunofluorescence photomicrographs of TUNEL assay and the ratio of TUNEL-positive cells. Results are mean±SEM (n=4 per group). *P<0.05. Scale bar, 10 μm.

**Online figure XII:** Detection of calcification, phenotype transition and apoptosis in cultured rat aortic rings treated by 3.0 mM PO₄³⁻ for 6 days. Rat abdominal aortas were infected periadventitially with 6×10⁶ plaque-forming units of Ad-GFP or Ad-COMP. After 3 days, abdominal aortas were cut into rings and cultured in vitro in DMEM or calcifying medium containing 3.0 mM PO₄³⁻ for additional 6 days. (A) Quantification of calcium deposition. (B) Representative western blot and quantification analysis of osteogenic-related marker genes (Runx2, BMP-2), smooth muscle marker genes (α-actin, SM 22α), and apoptosis-related genes (cleaved caspase-3, Bax). Values are mean ±SEM (n=5 for each group), *P<0.05.
**Online Table I:** Specific primers for quantitative RT-PCR.

**Online Table II:** Body weight and serum biochemical measurements of rats with chronic renal failure (CRF). Values are mean±SEM (n=8 per group).* $P<0.05$ versus sham group.
Online Figure I

A

\[ {^{45}}\text{Ca}^2+ \text{ incorporation (}\times 10^4 \text{ CPM/mg)} \]

- Control
- \( \beta \)-GP

\( * \)

Alizarin red staining

B

\[ \text{Calcium deposition (\text{\textmu mol/mg)} } \]

- Control
- CaCl\(_2\)

\( * \)

Alizarin red staining

Control
\( \beta \)-GP

Control
CaCl\(_2\)
Online Figure II
Online Figure III
Online Figure IV
Online Figure V
Online Figure VI
## Online Figure VIII

### A

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### B

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### C

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*Note: The image includes grayscale bar graphs for each condition.*
Online Figure IX
A

Day 3

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Relative protein level of cleaved caspase-3

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Relative protein level of Bax

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Online Figure XI
Online Figure XI (continued)
Online Figure XII
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Table II Serum biochemical measurements of 5/6Nx rats with high phosphate diet.

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<tr>
<td>Cr (mg/ml)</td>
<td>0.916±0.0256</td>
<td>1.271±0.045*</td>
</tr>
<tr>
<td>Pi (mM)</td>
<td>2.082±0.0938</td>
<td>2.720±0.149*</td>
</tr>
<tr>
<td>Ca×Pi (mM²)</td>
<td>4.348±0.1866</td>
<td>6.607±0.3925*</td>
</tr>
</tbody>
</table>

BUN, blood urea nitrogen; Cr, creatinine; Ca, calcium; Pi, phosphorus.

*Mean±SEM (n=8); P<0.05 vs sham group.