Upregulation of a Disintegrin and Metalloproteinase With Thrombospondin Motifs-7 by miR-29 Repression Mediates Vascular Smooth Muscle Calcification

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Objective—Vascular calcification significantly increases cardiovascular morbidity and mortality. We recently reported that the deficiency of cartilage oligomeric matrix protein (COMP) leads to vascular mineralization. We characterized the COMP-degrading metalloproteinase, a disintegrin and metalloproteinase with thrombospondin motifs-7 (ADAMTS-7). Here, we tested whether ADAMTS-7 facilitates vascular calcification.

Methods and Results—ADAMTS-7 expression was markedly upregulated in calcifying rat vascular smooth muscle cells (VSMCs) in vitro, calcified arteries of rats with chronic renal failure in vivo, and radial arteries of uremic patients. Silencing of ADAMTS-7 markedly reduced COMP degradation and ameliorated VSMC calcification, whereas ectopic expression of ADAMTS-7 greatly enhanced COMP degradation and exacerbated mineralization. The transcriptional activity of ADAMTS-7 promoter was not altered by high phosphate. We used bioinformatics and quantitative polymerase chain reaction analysis to demonstrate that high-phosphate upregulated ADAMTS-7 mRNA and protein via miR-29a/b repression, which directly targeted the 3′ untranslated region of ADAMTS-7 in VSMCs. MicroRNA (MiR)-29a/b mimic markedly inhibited but miR-29a/b inhibitor greatly enhanced high-phosphate–induced ADAMTS-7 expression, COMP degradation, and subsequent VSMC calcification. ADAMTS-7 silencing significantly diminished miR-29a/b repression–exaggerated VSMC calcification.

Conclusion—Our data reveal a novel mechanism by which ADAMTS-7 upregulation by miR-29a/b repression mediates vascular calcification, which may shed light on preventing cardiovascular morbidity and mortality. (Arterioscler Thromb Vase Biol. 2012;32:2580-2588.)

Key Words: calcification ■ smooth muscle cell ■ protease ■ microRNA ■ animal model of human disease remodeling

Vascular calcification, namely aberrant deposition of calcium phosphate crystals in the vessel wall, contributes directly to cardiovascular morbidity and mortality among patients with chronic renal failure (CRF), atherosclerosis, and diabetes mellitus.¹ Compelling evidence has demonstrated that vascular calcification is a highly regulated form of biomineralization resembling bone modeling, rather than simply passive calcium deposition.² These processes involve coordinated regulation of inducers and inhibitors of calcification, but the precise mechanisms are poorly defined.³-⁵

Metalloproteinases, including matrix metalloproteinase (MMP), a disintegrin and metalloproteinase or a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), play pivotal roles in both bone formation and arterial/cardiac remodeling by degrading matrix or nonmatrix substrates.⁶-⁸ However, the importance of metalloproteinases in the pathogenesis of vascular calcification has not been recognized. A few animal studies have suggested that activation of MMPs leads to vascular calcification in chronic kidney disease, whereas broad-spectrum MMP inhibition or MMP-2/9 deficiency prevents CaCl₂-induced aortic calcification and elastic fragmentation.⁹⁻¹¹ In addition, limited human studies have indicated that upregulation of MMP-2/9 in the arterial vasculature contributes to stiffening and vasomotor dysfunction in diabetes mellitus and chronic kidney disease.¹²,¹³ Nevertheless, little is known about other areas of metalloproteinase involvement and how critical these proteinases are for vascular mineralization. Identifying the key
ADAMTS-7 Is Upregulated in Calcified VSMC and Vessels

To test whether ADAMTS-7 is involved in VSMC calcification, we first examined the expression of ADAMTS-7 in various calcification models. Clinical observations have suggested that medial artery calcification is a common complication in CRF. Therefore, we examined ADAMTS-7 expression in radial artery specimens from uremic patients who underwent arterial venous fistula operation. The expression of ADAMTS-7 was greater in CRF radial arteries than in control arteries (Figure 1A). Furthermore, we analyzed ADAMTS-7 level in a rat CRF model. CRF rats showed a significant increase in the plasma level of blood urea nitrogen and creatinine (Table in the online-only Data Supplement). Vascular calcification was successfully induced in rats with 5/6 nephrectomy plus calcitriol, as evidenced by calcium deposition and von Kossa staining (Figure I in the online-only Data Supplement). Similarly, ADAMTS-7 expression was upregulated in the calcified arteries of CRF rats (Figure 1B). In addition, mineralization of VSMC was successfully induced in rat VSMCs with osteogenic media containing 2.6 mmol/L inorganic phosphate, as evidenced by Alizarin red S staining and increased calcium deposition (Figure II in the online-only Data Supplement). High phosphate significantly induced ADAMTS-7 mRNA expression as early as 6 hours (Figure 1C), which remained elevated up to 12 days (Figure 1E). In accordance, the protein level of ADAMTS-7 was enhanced at 24 hours and increased up to 12 days (Figure 1D and 1F). These observations were verified in human VSMCs (Figure III in the online-only Data Supplement). Alternatively, calcification of VSMCs was induced by 5 mmol/L CaCl₂ (Figure IV in the online-only Data Supplement). Both ADAMTS-7 mRNA and protein levels were consistently upregulated with 5 mmol/L CaCl₂ (Figure V in the online-only Data Supplement). In accordance, ADAMTS-7 expression was also greatly enhanced in the CaCl₂-induced abdominal aortic calcification model (Figure VI in the online-only Data Supplement). Together, these data suggest the relevance of ADAMTS-7 in vascular calcification.

ADAMTS-7 Mediates VSMC Calcification

Previously, we demonstrated that COMP was degraded in calcified vessels, and COMP prevents VSMC calcification by interfering in COMP degradation and BMP-2 signaling. As compared with adenosine-green fluorescent protein infection, ADAMTS-7 overexpression by adenosine infection (adenosine-ADAMTS-7; Figure VIIA in the online-only Data Supplement) led to excess COMP degradation, paralleled by increased BMP-2, p-Smad1/5/8, and runt-related transcription factor 2/msx homeobox 2 levels, and then increased calcium deposition (Figure 2A–2C). In contrast, the silencing of ADAMTS-7 by small interfering RNA (Figure VIIIB in the online-only Data Supplement) ameliorated COMP degradation and BMP-2 osteogenic signaling. Here, we used gain- and loss-of-function study to test whether ADAMTS-7 mediates VSMC calcification by interfering in COMP degradation and BMP-2 signaling. In accordance, the protein level of ADAMTS-7 was enhanced at 24 hours and increased up to 12 days (Figure 1D and 1F). These observations were verified in human VSMCs (Figure III in the online-only Data Supplement). Alternatively, calcification of VSMCs was induced by 5 mmol/L CaCl₂ (Figure IV in the online-only Data Supplement). Both ADAMTS-7 mRNA and protein levels were consistently upregulated with 5 mmol/L CaCl₂ (Figure V in the online-only Data Supplement). In accordance, ADAMTS-7 expression was also greatly enhanced in the CaCl₂-induced abdominal aortic calcification model (Figure VI in the online-only Data Supplement). Together, these data suggest the relevance of ADAMTS-7 in vascular calcification.

Materials and Methods

Animal care and use of 5/6 nephrectomy and CaCl₂ arterial injury model in male Sprague-Dawley rats were in accordance with institutional guidelines. Aortic ring organ calcification was induced as described previously. Small interfering RNA against ADAMTS-7 was transfected in vitro by use of oligofectamine. The adenosine for full-length rat ADAMTS-7 was constructed and 2.5 × 10⁶ plaque-forming units of adenovirus-ADAMTS7 dissolved in 30% pluronic gel solution was perivascularly delivered to the rat carotid arteries 3 days before organ culture. The microRNA (miRNA) expression was detected by use of the Exiqon miR assay kit (Denmark) according to the manufacturer’s protocol. Double-stranded RNA oligos representing mature sequences that mimic endogenous pre-miR-29a/b and negative control mimic miRNA (GenePhama, Shanghai) were transfected into cells by use of Oligofectamine (Invitrogen). Similarly, single-stranded RNA oligos designed to inhibit endogenous miR-29a/b (anti-miR-29a/b and negative control anti-miR (GenePhama) were transfected. For ex vivo studies, 10 μg of anti–miR-29a or anti–miR-29b, dissolved in 30% pluronic gel solution, was perivascularly delivered to rat carotid arteries 3 days before high-phosphate–induced aortic ring calcification. In CaCl₂-induced regional calcification, 10 μg mimic or inhibitor of miR-29a/b, dissolved in 30% pluronic gel solution, was perivascularly delivered to rat carotid arteries right after CaCl₂ treatment and washing. Human radial artery specimens were collected from uremic patients during arterial venous fistula operation and from patients with coronary heart disease who underwent coronary artery bypass grafting after getting the approval from the Local Research Ethics Committee. The procedures used were in accordance with the institutional guidelines. All the patients gave their informed consent for radial artery biopsy before surgery.

An expanded and detailed materials and methods section is available in the online-only Data Supplement.

Results
but ADAMTS-7 knockdown reduced the extent of calcification in high-phosphate–induced human VSMCs (Figure VIIA and VIIB in the online-only Data Supplement) and CaCl₂-induced rat VSMCs (Figure VIIC and VIID in the online-only Data Supplement). To further confirm the finding, we investigated the ex vivo rat aortic ring model cultured with high phosphate. Indeed, excess ADAMTS-7 exaggerated aortic ring calcification and its knockdown decreased the calcification (Figure 3).

**ADAMTS-7 Is a Target of miR-29a/b in VSMCs**

To explore the mechanism of ADAMTS-7 upregulation during vascular calcification, we first cloned a 1.45-kb promoter region flanking the 899 bp upstream to 550 bp downstream of the transcriptional starting site into the PGL3 luciferase vector. The vector was transfected into VSMCs, and transcriptional activity was monitored with high-phosphate stimulation. It is intriguing that high phosphate alone did not enhance ADAMTS-7 transcription, and further serial deletions of the promoter region produced no transcriptional activity (Figure IX in the online-only Data Supplement), which indicates a potential post-transcriptional regulatory mechanism.

MicroRNAsmiRNAs are, in general, 22-nt, noncoding small RNAs that ensure enhancement of mRNA degradation or suppression of protein translation by binding to the 3′ UTR of their target mRNA. Use of the bioinformatics databases TargetScan, PITA, MicroCosom, and PicTar predicted that ADAMTS-7 is a potential target of miR-29a/b. Sequence alignment of miR-29a or miR-29b sites in the 3′ UTR of ADAMTS-7 showed high conservation among human, mouse, rat, and canine (Figure 4A). To test whether miR-29a/b directly regulates ADAMTS-7, we constructed luciferase reporters containing the wild-type 3′ UTR of ADAMTS-7 or with deletion or mutation of the predicted miR-29 binding seed region (Figure 4B). VSMCs were cotransfected with miR-29a/b mimic or negative control molecules. The miR-29a/b mimic significantly inhibited the reporter luciferase activity, by ≈40%, as compared with the control molecule. In contrast, deletion or mutation of the predicted miR-29 binding seed region within the 3′ UTR of ADAMTS-7 or with deletion or mutation of the predicted miR-29 binding seed region (Figure 4B). VSMCs were cotransfected with miR-29a/b mimic or negative control molecules. The miR-29a/b mimic significantly inhibited the reporter luciferase activity, by ≈40%, as compared with the control molecule. In contrast, deletion or mutation of the predicted miR-29a/b binding site within the 3′ UTR of ADAMTS-7 abolished miR-29a/b inhibition. In addition, the miR-29a/b mimic markedly repressed ADAMTS-7 mRNA and protein expression in VSMCs, whereas anti-miR-29a/b increased the levels (Figure 4C and 4D). Therefore, miR-29a/b can negatively regulate ADAMTS-7 mRNA by targeting its 3′ UTR.

**MiR-29a and miR-29b Are Downregulated in Calcifying VSMCs**

Because ADAMTS-7 was negatively regulated by miR-29a/b and its expression was enhanced during vascular calcification,
we wondered whether miR-29a/b level was altered during the process. First, we analyzed miR-29a/b expression by quantitative reverse transcriptase-polymerase chain reaction. High phosphate inhibited miR-29a/b as early as 1 hour after stimulation; the repression was sustained up to day 6 and returned to normal on day 9 (Figure 5A and 5B). Second, because miRNAs targeting mRNA depend on the association of the miRNA/mRNA complex with Ago proteins to form microRNA-induced silencing complex, we investigated the expression of miR-29a/b in Ago2-immunoprecipitated microRNA-induced silencing complex. High phosphate significantly ameliorated miR-29a/b level at 3 and 12 hours as compared with that at time 0, but Ago2 protein expression was not altered (Figure 5C; Figure X in the online-only Data Supplement). Third, miR-29a/b expression was decreased in the calcified arteries of rats with 5/6 nephrectomy plus calcitriol and in the radial arteries of CRF patients (Figure 5D and 5E). Of note, miR-29a/b expression was exclusively repressed by high phosphate in human VSMCs, by CaCl₂ in rat VSMCs, and in CaCl₂-induced calcified rat carotid arteries (Figure XIA–XIC in the online-only Data Supplement).
To further explore the potential mechanism of miR-29 repression during VSMC calcification, we first detected nuclear factor (NF)-κB activation within 1 hour after high-phosphate stimulation. Phosphorylation of p65 was increased and IκBα protein level decreased as early as 10 minutes after high-phosphate stimulation (Figure XIIA in the online-only Data Supplement). In addition, inhibition of NF-κB activation by 50 multiplicity of infection of adenovirus-IκBα or 1 μmol/L MG-132 markedly reversed miR-29a/b repression with high phosphate (Figure 5F; Figure XIIB in the online-only Data Supplement). Therefore, high phosphate inhibited miR-29a/b mainly via NF-κB activation.

MiR-29a/b Inhibits VSMC Calcification by Suppressing ADAMTS-7

To explore whether miR-29a/b is truly involved in vascular calcification, we transfected VSMCs with mimic miR-29a/b before high-phosphate stimulation. The overexpression of miR-29a/b inhibited high-phosphate–induced ADAMTS-7 expression, COMP degradation, and BMP-2 signaling activation at day 6 (Figure XIIIA in the online-only Data Supplement), followed by reduced VSMC calcification at day 12 (Figure XIIIB and XIIIC in the online-only Data Supplement). Reciprocally, transfection of anti–miR-29a/b enhanced high-phosphate–evoked ADAMTS-7 expression with high phosphate (Figure 5F; Figure XIIIB in the online-only Data Supplement). Therefore, high phosphate inhibited miR-29a/b mainly via NF-κB activation.

Figure 3. A disintegrin and metalloproteinase with thrombospondin motifs-7 (ADAMTS-7) aggravates aortic ring calcification. A, Western blot analysis of ADAMTS-7 protein level in rat abdominal arteries transduced with adenovirus (Ad)-green fluorescent protein (GFP) or Ad-ADAMTS-7 at day 3 after infection. Rat aortas were then cut into aortic rings and cultured in DMEM or calcifying medium containing 3.8 mmol/L PO43− for 6 days. Quantification of calcium deposition in ADAMTS-7–transfected aortic rings at day 6. B, Western blot verification of ADAMTS-7 knockdown in rat carotid arteries with scramble or ADAMTS-7 small interfering RNA (siRNA) for 3 days. The aortas were cut into aortic rings and cultured in DMEM or calcifying medium for 6 days. Quantification of calcium deposition in aortic rings with ADAMTS-7 knockdown at day 6. Data are means±SEM from 3 independent experiments performed in triplicate. *P<0.05.

Discussion

Researchers have embraced the concept that vascular calcification is a multifaceted and actively regulated process rather than simple passive calcium deposition and degeneration.
Various molecular mechanisms have been elucidated, including abnormal calcium or phosphate metabolism, osteogenic reprogramming of VSMCs, apoptosis, oxidative stress and inflammation, and loss of inhibitors of mineralization.18–21 However, the understanding of vascular mineralization is far from ideal, and an effective preventive or therapeutic strategy for vascular calcification in atherosclerosis, CRF, and diabetes mellitus is still lacking. Here we reveal a novel underlying mechanism in vascular calcification that involves the miR-29/ADAMTS-7/COMP axis.

ADAMTS comprise a family of 19 secreted enzymes that have been linked to various physiological processes, including development, angiogenesis, and coagulation. Aberrant expression or function of ADAMTS members have been implicated in many disease states such as arthritis, cancer, thrombocytopenic purpura, and cardiovascular disease.22–24 Recently, 2 independent genome-wide association studies identified ADAMTS-7 as a novel locus for coronary atherosclerosis, CRF, and diabetes mellitus is still lacking. Here we reveal a novel underlying mechanism in vascular calcification that involves the miR-29/ADAMTS-7/COMP axis.

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COMP is a 550-kD pentameric glycoprotein necessary for maintaining the VSMC contractile phenotype.27 COMP deficiency facilitates osteoelastic transdifferentiation of VSMCs, because the deficient COMP is incapable to inhibit the interacting BMP-2.24 BMP-2 promotes vascular calcification by its increased phosphate uptake and by modulating an osteogenic phenotype in smooth muscle cells by binding to the receptor, BMP receptor 1 or BMP receptor 2, which in turn phosphorylates the regulatory Smad1/5/8 and upregulates downstream key osteogenic transcription factors, including runt-related transcription factor 2 and msh homeobox 2. Of note, we observed COMP degradation in various vascular mineralization models.14 In the current study, we further demonstrated that the interference of ADAMTS-7 in calcifying VSMCs or vessels led to subsequent change in COMP degradation in parallel with the alteration of BMP-2, p-Smad1/5/8, and runt-related transcription factor 2/msh homeobox 2 levels. These observations reinforce the importance of a counterbalance between COMP and BMP-2 during vascular calcification. Interfering in ADAMTS-7 or COMP processing may shed light on preventing VSMC calcification.
Of note, purified COMP has been reported to be digested in vitro by several MMPs at higher concentrations than those in physiological or pathological conditions. Accumulating evidence also suggests the importance of MMP-2, -9, and -12 during the etiology of vascular calcification by degrading elastin or nonmatrix substrates such as transforming growth factor β. Nevertheless, we previously demonstrated that 4 C-terminal thrombospondin repeats of ADAMTS-7 are required and sufficient for COMP binding and degrading, which are lacking in the above-mentioned MMPs.

To date, COMP is the only identified substrate of ADAMTS-7 in blood vessels. However, we cannot exclude the existence of other potential substrates of ADAMTS-7 in smooth muscle cells or a COMP-independent mechanism of ADAMTS-7 in vascular calcification. Previously, we showed that ADAMTS-12, a homology of ADAMTS-7, can also bind and process COMP in vitro. Whether ADAMTS-12 contributes to vascular calcification remains elusive. Considering the complexity of the biological function of individual metalloproteinases, continued research is required to unravel the key enzyme(s) relevant in vascular calcification.

Here, we identified the novel regulator miR-29 in VSMC mineralization, which is downregulated during calcification and epigenetically targets ADAMTS-7 in VSMCs. The exclusive downregulation of miR-29a/b within current vascular calcification models suggests that it may act as a modulator during the process. Indeed, our data indicated that interference of miR-29a/b led to subsequent alteration in the expression of ADAMTS-7, BMP-2, p-Smad1/5/8, and runt-related transcription factor 2/msh homeobox 2 and COMP degradation to ultimately affect vascular calcification. These data highlight the importance of the miR-29/ADAMTS-7/COMP axis during the process of calcification. Compelling evidence suggests that the miR-29 family members directly target multiple genes, although their functional relevance in various disease states may not be identical. The miR-29 family may be pivotal for controlling osteoblast and osteoclast differentiation and function during bone remodeling by
targeting osteonectin and Wnt signaling.\textsuperscript{31,32} In addition, miR-29 may be a critical regulator of extracellular matrix factors including elastin, collagen, laminin, gamma 1, fibronectin, BMP-2, ADAM12, and NFKB inhibitor interacting Ras-like 2, which are closely related to trabecular meshwork reassembling, cardiac/pulmonary/liver fibrosis, or aortic aneurysm.\textsuperscript{33–35} Recently, RNA sequencing of a stable miR-29–transfected C2C12 cell line revealed ADAMTS-7 as 1 of the potential targets.\textsuperscript{36} In addition, miR-29 may affect cellular senescence or aging by regulating B-myc or p53.\textsuperscript{37,38} Our current study has extended our understanding of miR-29 in matrix turnover by providing evidence that by negatively regulating ADAMTS-7, miR-29 indirectly affects COMP degradation, thereby influencing vascular calcification. Because miR-29 is known to target many other genes, particularly those from the fibrotic/extracellular matrix pathway, decreased miR-29 level in the context of calcific stimulus would possibly lead not only to vessel calcification but also to vascular thickening and fibrosis. Recently, miR-125 was also reported to regulate VSMC calcification by targeting the osteoblast transcription factor osterix.\textsuperscript{39} An in-depth understanding of the roles of these regulatory RNAs in VSMC mineralization will be critical for developing new therapies for vascular calcification.

In the current study, miR-29a/b level was repressed in high-phosphate–induced calcifying VSMCs or blood vessels with chronic kidney disease, which agreed with a previous report of miR-29a/b suppression in muscles from mice with chronic kidney disease.\textsuperscript{40} Decreased aortic expression of miR-29b was also reported in angiotensin II– and pancreatic elastase–induced murine models of abdominal aortic aneurysm.\textsuperscript{41,42} In contrast, markedly upregulated miR-29 was suggested in senescent VSMCs, in aging mice or in Marfan syndrome.\textsuperscript{38,41} Therefore, different mechanisms are involved in the regulation of miR-29 under various states. The mechanism accounting for downregulation of miR-29a/b during calcification is not yet determined. However, miR-29a/b is transcriptionally suppressed by transforming growth factor beta, c-Myc, hedgehog, and NF-NF.\textsuperscript{κ} Here, we have shown that NF-κB activation mediated high-phosphate–induced miR-29a/b repression, which agrees with our previous observation that mitochondrial NF-κB activation mediated by reactive oxygen species facilitated high-phosphate–induced VSMC calcification.\textsuperscript{43} In addition, the in vivo model we used in the current study involved complex mechanisms, including high phosphate, inflammation, oxidative stress, and apoptosis, so the precise mechanism of miR-29a/b inhibition of vascular calcification in vivo needs to be further explored.

In summary, we have indicated a novel regulatory role of the miR-29/ADAMTS-7/COMP axis during vascular calcification in vitro and in vivo. The axis may be a novel therapeutic target for vascular calcification.

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**Disclosures**

None.

**References**


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Supplement Material

Materials

Antibodies against ADAMTS7 and COMP were from Abcam (Cambridge, UK). Antibodies against GAPDH, β-actin and Ago2 were from Cell Signaling Technology (Boston, MA). Calcitriol was from Sigma (St. Louis. MO). Ad-IκBα was kindly provided by Professor Nanping Wang in Xi’an Jiaotong University.

Cell Culture

Primary rat VSMCs were isolated from adult rat aortic explants as described, and cells at passages 3 to 8 were used for all experiments. Primary mouse thoracic VSMCs were isolated by collagenase digestion as described. The rat smooth-muscle embryonic thoracic aorta cell line A7r5 and human thoracic aortic VSMCs (T/G HA-VSMC) were from the American Type Culture Collection (Manassas, VA). VSMCs were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO₂. Calcification of VSMCs was induced by incubation in calcifying media containing 2.6 mM phosphate or 5 mM CaCl₂ for 12 days with medium changes every 2 days.

Animal Model of CRF

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. CRF was induced by 5/6 nephrectomy. For sham-operated rats, only the renal envelope was removed. To accelerate the aortic calcification, on the day after completing renal ablation, calcitriol (0.08 μg/kg) was injected intraperitoneally 3 times a week for 12 weeks.\textsuperscript{5, 6} The sham group received vehicle injection. Rats were killed, and plasma levels of urea nitrogen, creatinine, calcium, and phosphate were measured (Biosino Bio-Technology and Science, Beijing). The abdominal arteries were dissected for calcium deposition assay, von Kossa staining and protein extraction.

### CaCl\textsubscript{2}-Induced Rat Aortic Calcification

The infrarenal abdominal aortas of rats underwent periadventitial application of 0.2 mol/L CaCl\textsubscript{2} for 15 min as described\textsuperscript{7} and were dissected 7 days post-injury. Alternatively, CaCl\textsubscript{2} was delivered periadventitiously to the rat carotid artery before application of miR-29 mimic or inhibitor.

### Aortic Ring Organ Calcification

Common carotid arteries were removed in a sterile manner from rats. The contralateral carotid was used as a control. After the adventitia and endothelium were removed, the vessels were cut into 2- to 3-mm rings and placed in high-phosphate medium (3.8 mM PO\textsubscript{4}\textsuperscript{3-}) or regular DMEM containing 10% FBS at 37ºC in 5% CO\textsubscript{2} for 6 days, with medium changes every 2 days.\textsuperscript{3} The viability of the aortic ring was monitored by methylthiazoletertrazolium (MTT) assay as described.\textsuperscript{8} For \textit{ex vivo}
interference, siRNA (ADAMTS7 or scramble) or miRNA (mimic or anti-miR-29a/b or control miRNA) was dissolved in 30% pluronic gel solution and perivascularly delivered to rat carotid arteries; 3 days later, carotid arteries were harvested.

**Quantification of VSMC Calcification**

Rat VSMCs were grown in 6-well plates and treated with growth medium or calcifying medium. After medium was removed and washed with phosphate buffered saline (PBS), VSMCs were treated with 0.6 N HCl overnight at 4°C. After the HCl supernatant was removed, the remaining cell layers were 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 the QuantiChrom Calcium Assay Kit and normalized by protein concentration.  

**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, fixed with 10% formaldehyde for 10 min, washed 3 times with PBS, exposed to 2% Alizarin Red S (Aqueous, Sigma) for 30 min and washed with 0.2% acetic acid. Positively stained cells displayed 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, then washed and treated with 5% sodium thiosulfate. Calcified nodules were stained brown to black.
Recombinant Adenovirus Construction

The adenovirus for full-length rat ADAMTS-7 (Ad-ADAMTS7) was constructed and amplified according to the manufacturer’s protocol (BD Biosciences Clontech, CA). An adenovirus carrying green fluorescence protein (Ad-GFP) or Ad-LacZ was used as a negative control. For ex vivo studies, a single exposure of $2.5 \times 10^9$ plaque forming units (pfu) of Ad-ADAMTS7 dissolved in 30% pluronic gel solution was periadventitially delivered to the rat carotid arteries 3 days before organ culture.\textsuperscript{10}

Quantitative Real-Time PCR and Western Blot Analysis

Real-time PCR amplification involved use of an Mx3000 Multiplex Quantitative PCR System (Stratagene, La Jolla, CA). The mRNA level was normalized to that of β-actin or GAPDH.

The miRNA expression was detected by use of the Exiqon miR assay kit (Denmark) according to the manufacturer’s protocol. MiR-29a/b expression was examined with use of specific primers (miR-29a: 5’ uagcaccaucugaaaucgguua 3’; miR-29b: 5’ uagcaccuuugaaacaguguu 3’). Levels of miRs were normalized to that of RNU5G as a reference.

For western blot analysis, extracts containing equal amounts of total protein from cells or 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).
ADAMTS-7 siRNA Transfection

Small interfering RNA (siRNA) against ADAMTS-7 was designed by use of the Block-iT™ RNAi Designer and chemically modified by the manufacturer (Invitrogen). Primer sequences for the siRNA of ADAMTS7 were sense, 5’-CACAUCACCGUUGUGCGCCUUAUUA-3’, and antisense, 5’-UAAUAAGGCGCACAACGGUGAUGUG-3’. Transfection of VSMCs with siRNA (50 nmol/L) in vitro involved use of Oligofectamine (Invitrogen). A scramble Stealth RNAi duplex (Invitrogen) was a negative control. For ex vivo aortic ring studies, 15 μg of the siRNA dissolved in 30% pluronic gel solution was perivascularly delivered to rat carotid arteries 3 days before organ culture.10

Transfection of miR-29 Mimic or Inhibitor

Double-stranded RNA oligos representing mature sequences that mimic endogenous pre-miR-29a/b and negative control mimic miRNA (GenePhama, Shanghai) were transfected into A7r5 cells at 10 to 20 nM concentration by use of Oligofectamine (Invitrogen). Similarly, single-stranded RNA oligos designed to inhibit endogenous miR-29a/b (anti-miR-29a/b) and negative control anti-miR (GenePhama) were transfected. For ex vivo studies, 10 μg of the anti-miR-29a or anti-miR-29b dissolved in 30% pluronic gel solution was perivascularly delivered to rat carotid arteries 3 days before high-phosphate–induced aortic ring calcification. In CaCl2-induced regional calcification, 10 μg mimic or inhibitor of miR-29a/b
dissolved in 30% pluronic gel solution was perivascularly delivered to rat carotid arteries right after CaCl$_2$ treatment and washing.

**Luciferase Reporter Assays**

The 3’ UTR of ADAMTS7 bearing the predicted miR-29a/b binding site was PCR-amplified and subcloned in-frame into a pMIR-REPORT vector (Ambion) to generate the ADAMTS7-3’ UTR reporter construct. Mutagenesis of the predicted miR-29–binding “seed region” involved use of the primer sequences forward, 5’-GAG GTT CCG TGT ATT AGG ACA CGA GGA GCA-3’, and reverse, 5’-CCT AAT ACA CGG AAC CTC ATC CCT GCA CAA-3’, and deletion of the seed region involved the primer sequences forward, 5’-GGA TGA GGT TAT TAG GAC ACG AGG AGC A-3’, and reverse, 5’-GTC CTA ATA ACC TCA TCC CTG CAC AAT G-3’. The mimic or anti-miR-29a/b and pRSV-β-Gal were cotransfected with the wild type, truncated or mutated ADAMTS7-3’-UTR-pMIR reporter vector into HEK293 cell lines by use of jetPE$^{TM}$ (PolyPlus, France). The pRSV-β-Gal vector was used for normalization of transfection efficiency. Luciferase and β-galactosidase activities were measured as described.$^{11}$

**Argonaut Pull-down Assay**

VSMCs were trypsinized and lysed with lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40, 0.5% sodium deoxycholate, 100 U/ml RNase inhibitor, and a protease inhibitor cocktail). Final protein concentration of cell lysates was
adjusted to 2–3 mg/ml. Ago2-specific antibody was added into the cell lysate, with incubation and rotation at 4°C overnight. The immune complexes were pulled down by use of protein A/G Sepharose beads. After 3 washes, 1 ml Trizol reagent (Invitrogen) was added into each sample, and RNA was extracted. The purified RNA was analyzed by quantitative real-time(RT-PCR).\textsuperscript{11}

**Patients and Radial Artery Analysis**

A 2- to 3-mm circumferential segment of radial artery was excised from uremic patients undergoing arterial venous fistular surgery (for CRF) and as a control, patients with coronary heart disease (CHD) but showing no complications of diabetes and chronic kidney disease who were undergoing coronary artery bypass grafting. Approval from the Local Research Ethics Committee was granted for human tissue use, and the procedures were in accordance with institutional guidelines. All patients gave their informed consent for radial artery biopsy before surgery. The vessels were dissected of fat, and tissue was prepared for western blot analysis or histomorphology.

**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) and among more than 2 groups, one-way ANOVA followed by Student-Newman-Keuls test for post-hoc comparison as
appropriate to evaluate the effect of recombinant ADMATS7 adenovirus or siRNA knockdown on calcium deposition or two-way ANOVA followed by Bonferroni test. Statistical analyses involved use of GraphPad Prism 5.0 (GraphPad Software Inc, La Jolla, CA). A $P < 0.05$ was considered statistically significant.

**Reference**


Supplemental Figures/Tables/Legends

**Online Figure I.** Characterization of vascular calcification in rats with chronic renal failure (CRF). (A) Schematic experimental design for rat CRF with calcitriol accelerating the vascular calcification. Calcium deposition (B) and von Kossa and hematoxylin and eosin (H.E.) staining (C) of abdominal arteries in rats treated with 5/6 nephrectomy and calcitriol. Data are mean±SEM (n=5-7 per group). *P<0.05 vs sham group. Scale bar, 20 μm.

**Online Figure II.** Quantitative analysis of calcium deposition and Alizarin red S staining to characterize calcification in rat vascular smooth muscle cells (VSMCs) stimulated with high phosphate (Pi; 2.6 mM). Data are mean±SEM from 3 independent experiments. *P<0.05 vs. control. Scale bar, 20 μm.

**Online Figure III.** Representative western blot analysis of ADAMTS-7 expression in human thoracic aortic VSMCs stimulated with high phosphate (2.6 mM) for 3 days.

**Online Figure IV.** Quantitative analysis of calcium deposition and Alizarin red S staining to characterize calcification in rat VSMCs stimulated with 5 mM CaCl₂. Data are mean±SEM from 3 independent experiments. *P<0.05 vs. control. Scale bar, 20 μm.

**Online Figure V.** Relative mRNA (A) and protein expression (B) of ADAMTS-7 in
rat VSMCs at various times after stimulation with 5.0 mM CaCl$_2$. Data are mean±SEM from 3 independent experiments. *$P<0.05$ vs control.

**Online Figure VI.** Representative western blot and quantitative analysis of ADAMTS-7 protein level in abdominal arteries of CaCl$_2$-injured or NaCl-treated rats at day 7. n=3 per group. *$P<0.05$ vs con.

**Online Figure VII.** Relative mRNA level and representative western blot of ADAMTS-7 expression in rat VMSCs infected with 50 or 100 multiplicity of infection (MOI) of or Ad-GFP or Ad-ADAMTS-7 (A) or A7r5 cells transfected with 50 nM scramble or ADAMTS-7 siRNA (B) for 48 hr.

**Online Figure VIII.** Quantification of calcium deposition in (A) human thoracic aortic VSMCs infected with Ad-LacZ or Ad-ADAMTS-7 and stimulated with high phosphate (2.6 mM) for 12 days; (B) human thoracic aortic VSMCs transfected with scramble or ADAMTS-7 siRNA and stimulated with high phosphate (2.6 mM); (C) rat VSMCs infected with Ad-LacZ or Ad-ADAMTS-7 and stimulated with 5 mM CaCl$_2$ for 12 days; and (D) rat VSMCs transfected with scramble or ADAMTS-7 siRNA and stimulated with 5 mM CaCl$_2$ for 12 days. Data are mean±SEM from 3 independent experiments. *$P<0.05$.

**Online Figure IX.** Luciferase reporter assay of human ADAMTS-7 promoter. (A)
Schematic illustration of reconstructed ADAMTS-7 promoter flanking 899 bp, 681 bp and 134 bp upstream and 550 bp downstream of transcriptional starting site (+1). ATG, translational starting site. (B) Luciferase reporter assay of ADAMTS-7 promoters. Serial-deleted promoters of 1.45 kb (-899 to +550 bp), 1.23 kb (-681 to +550 bp), and 0.68 kb (-134 to +550 bp) were transient transfected into HEK293 cells before stimulation with lipopolysaccharide (LPS; 100 ng/ml) or high phosphate (2.6 mM). Data are mean±SEM from 3 independent experiments. *P<0.05 vs. control.

**Online Figure X.** Representative western blot of Ago2 expression in VSMCs challenged with high phosphate for 3 or 12 hr.

**Online Figure XI.** Quantitative RT-PCR analysis of mRNA level of miR-29a/b. (A) Human thoracic aortic VSMCs were treated with or without high phosphate (2.6 mM) for 3 days. (B) Rat VSMCs were treated with or without 5 mM CaCl₂ for 3 days. (C) Rat carotid arteries were sham or injured with CaCl₂, then after 7 days, miR-29a/b level was measured. RNU5G was an internal control. Data are means±SEM from 3 independent experiments. *P<0.05 vs. sham or control.

**Online Figure XII.** (A) Western blot analysis and quantification of phosphorylated protein level of p65 and IκBα in high-phosphate–stimulated VSMCs. (B) Quantification of miR-29a/b level with 1 μM MG-132 and high phosphate at 1 hr. Data are means±SEM from 3 independent experiments. *P<0.05.
Online Figure XIII. (A) Representative western blot and quantification of level of ADAMTS-7 protein, COMP protein degradation, BMP-2 protein, and total and phosphorylated Smad1/5/8, Runx2 and Msx2 protein in rat VSMCs transfected with control miR or mimic miR-29a/b for 48 hr before high-phosphate stimulation for an additional 6 days. Quantification of calcium deposition (B) and Alizarin Red S staining (C) in rat VSMCs transfected with control mimic miR or mimic miR-29a/b for 48 hr, then high phosphate for 12 days. *P<0.05.

Online Figure XIV. (A) Representative western blot and quantification in VSMCs transfected with control anti-miR or anti-miR-29a/b for 48 hr before high-phosphate stimulation for an additional 6 days. Quantification of calcium deposition (B) and Alizarin Red S staining (C) in rat VSMCs transfected with control anti-miR or anti-miR-29a/b for 48 hr before high-phosphate stimulation for 12 days. *P<0.05.

Online Figure XV. Quantification of calcium deposition in VSMCs. Human VSMCs were transfected with mimic miR-29a/b (A) or anti-miR-29a/b (B) for 48 hr before high-phosphate (2.6 mM) stimulation for an additional 12 days. Rat VSMCs were transfected with mimic miR-29a/b (C) or anti-miR-29a/b (D) for 48 hr before 5 mM CaCl₂ stimulation for an additional 12 days. Data are means±SEM from 3 independent experiments. *P<0.05.
Online Figure XVI. (A-B) Representative western blot and quantification of ADAMTS-7 expression in rat carotid arteries periadventitiously transfected with various concentrations of control mimic miR or mimic miR-29a/b for 3 days. (C) Quantification of calcium deposition of rat aortic ring culture. Rat carotid arteries were treated periadventitiously with control mimic miR or mimic miR-29a/b. Three days later, aortas were cut into rings and cultured in DMEM or calcifying medium containing 3.8 mM PO4³⁻ for 6 days. *P<0.05.

Online Figure XVII. Confocal microscope analysis of fluorescent-labeled mimic or anti-miR-29a/b content and immunohistochemical detection of ADAMTS-7 expression in CaCl₂-injured rat carotid arteries. (A) Alexa Fluor 488-labeled mimic miR-29a/b or control miRNA in rat carotid arteries. Successful transfection was evidenced by green staining seen by confocal microscopy 7 days after injury. Lack of miRNA transfection was revealed by no or weak staining in the sham or injured carotid artery. Immunohistochemical staining of expression of ADAMTS-7 in consecutive cross sections. (B) Alexa Fluor 488-labeled anti-miR-29a/b or control miRNA in rat carotid arteries. Scale bar, 70 μm.

Online Table 1. Serum biochemical parameters of rats with chronic renal failure (CRF). Rats were treated with sham operation, calcitriol, 5/6 nephrectomization, or 5/6 nephrectomization plus calcitriol (n=5-7 per group). Data are mean±SEM.* P<0.05 vs. sham group.
A

Left kidney  Right kidney
2/3 removal  removal
Calcitriol 0.08 μg/kg
Autopsy

1W
12W

B

Calcium deposition (µg/mg)

sham  Calcitriol  5/6Nx  5/6Nx+Calcitriol

C

von Kossa

H.E.

Online Figure 1
Online Figure II
Online Figure IV

Calciun deposition (µmol/mg)

Control vs CaCl$_2$

Alizarin red staining

Control

CaCl$_2$
Online Figure V
Online Figure VI

ADAMTS7

GAPDH

Relative protein level

con  CaCl\(_2\)

*
Online Figure VII
Online Figure VIII
Online Figure X
A. Human VSMCs

B. Rat VSMCs

C. Rat carotids arteries

Online Figure XI
Online Figure XII
### A

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control</th>
<th>Pi</th>
<th>Pi+con mimic miR</th>
<th>Pi+mimic miR-29a</th>
<th>Pi+mimic miR-29b</th>
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<tbody>
<tr>
<td>ADAMTS7</td>
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<td>COMP</td>
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<td>p-Smad 1/5/8</td>
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<td>Msx2</td>
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<tr>
<td>GAPDH</td>
<td></td>
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</tr>
</tbody>
</table>

**Relative protein level of ADAMTS7**

- Control: 1
- Pi: 0.5
- Pi+con mimic miR: 1.5
- Pi+mimic miR-29a: 2
- Pi+mimic miR-29b: 2

### B

**Calcium deposition (μmol/mg)**

- Control: 1
- Pi: 3
- Pi+con mimic miR: 3
- Pi+mimic miR-29a: 3
- Pi+mimic miR-29b: 3

### C

**Online Figure XIII**

![Control](image1.png)
![Pi](image2.png)
![Pi+con mimic miR](image3.png)
![Pi+mimic miR-29a](image4.png)
![Pi+mimic miR-29b](image5.png)
Online Figure XV
Online Figure XVII
# Online Table I: Serum biochemical measurements of 5/6Nx rats with calcitriol.

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Calcitriol</th>
<th>5/6 Nx</th>
<th>5/6 Nx +calcitriol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bun (mM)</strong></td>
<td>7.336 ± 0.492</td>
<td>6.832 ± 0.294</td>
<td>9.532 ± 0.919 *</td>
<td>13.564 ± 0.537 *</td>
</tr>
<tr>
<td><strong>Cr (µM)</strong></td>
<td>75.6 ± 2.619</td>
<td>74.6 ± 2.943</td>
<td>94.2 ± 3.308 *</td>
<td>110.0 ± 2.273 *</td>
</tr>
<tr>
<td><strong>Pi (mM)</strong></td>
<td>2.003 ± 0.101</td>
<td>1.999 ± 0.830</td>
<td>2.150 ± 0.210</td>
<td>2.249 ± 0.132</td>
</tr>
<tr>
<td><strong>Ca × Pi (mM²)</strong></td>
<td>4.355 ± 0.198</td>
<td>4.101 ± 0.242</td>
<td>4.851 ± 0.443</td>
<td>5.121 ± 0.303</td>
</tr>
</tbody>
</table>

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

*Mean ± SEM(n=5-7); P<0.05 vs sham group.