Serine Protease Activation Essential for Endothelial-Mesenchymal Transition in Vascular Calcification

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ABSTRACT

Rationale: Endothelial cells have the ability to undergo endothelial-mesenchymal transitions (EndMTs), by which they acquire a mesenchymal phenotype and stem-cell like characteristics. We previously found that EndMTs occurred in the endothelium deficient in matrix Gla protein (MGP) enabling endothelial cells to contribute cells to vascular calcification. However, the mechanism responsible for initiating EndMTs is not fully understood.

Objective: To determine the role of specific serine proteases and sex determining region Y-box 2 (Sox2) in the initiation of EndMTs.

Methods and Results: In this study, we used in vivo and in vitro models of vascular calcification to demonstrate that serine proteases and Sox2 are essential for the initiation of EndMTs in MGP-deficient endothelium. We showed that expression of a group of specific serine proteases was highly induced in endothelial cells at sites of vascular calcification in Mgp null aortas. Treatment with serine protease inhibitors decreased both stem-cell marker expression and vascular calcification. In human aortic endothelial cells, this group of serine proteases also induced EndMTs, and the activation of proteases was mediated by Sox2. Knockdown of the serine proteases or Sox2 diminished EndMTs and calcification. Endothelial-specific deletion of Sox2 decreased expression of stem-cell markers and aortic calcification in MGP-deficient mice.

Conclusions: Our results suggest that Sox2-mediated activation of specific serine proteases is essential for initiating EndMTs, and thus, may provide new therapeutic targets for treating vascular calcification.

Keywords:
Endothelium, progenitor cell, calcification, matrix Gla protein, serine protease.
Nonstandard Abbreviations and Acronyms:

- AVMs: Arteriovenous malformations
- BMP: Bone morphogenetic protein
- Cbfa1: Core binding factor alpha 1
- CD: Cluster of differentiation
- DFP: Diisopropylfluorophosphate
- EndMT: Endothelial-mesenchymal transition
- ELA: Elastase
- ELISA: Enzyme-linked immunosorbent assay
- FOP: Fibrodysplasia ossificans progressiva
- Gla: Gamma-carboxyglutamic acid
- HAEC: Human aortic endothelial cells
- KLF4: Kruppel-like factor 4
- KLK: Kallikrein
- MGP: Matrix Gla protein
- Sox2: Sex determining region Y-box 2
- Sca1: Stem Cell Antigen 1
- SEM: Scanning electron microscopy
- pSMAD: Phosphorylated SMAD
- SMAD: Homolog of the drosophila protein, mothers against decapentaplegic (MAD) and the C. elegans protein SMA
- SMC: Smooth muscle cells
- SMA: Alpha-smooth muscle actin
- TEM: Transmission electron microscopy
- Twist1: Twist-related protein 1
- vWF: von Willebrand factor
- wt: Wild type
INTRODUCTION

Transition of endothelial cells into mesenchymal cells (endothelial-mesenchymal transition, EndMT) allows endothelial cells (ECs) to acquire plasticity and to participate in embryonic development and disease progression. During embryonic development, the microenvironment or specific factors induce EndMT, driving the endothelium to engage in normal tissue differentiation. In disease, the occurrence of EndMTs may convert endothelium into a multipotent state that contributes cells toward undesirable differentiation such as cardiac and renal fibrosis, cancer progression, fibrodysplasia ossificans progressiva (FOP), pulmonary hypertension, and vascular calcification.

Vascular calcification is a frequent and severe complication of vascular disease, which increases morbidity and mortality in diabetes and atherosclerosis, and is associated with an increased risk of congestive heart failure, myocardial infarction, systemic hypertension, and chronic kidney disease. Previously considered to be a passive process of mineral precipitation, vascular calcification is now known to be an active process that involves ectopic bone formation.

METHODS

See Online Data Supplement for detailed Methods section.

Animals.

Mgp+/− mice on the C57BL/6J background were obtained from Dr. Cecilia Giachelli, University of Washington, with the permission of Dr. Gerard Karsenty, Columbia University. Cdh5Cre (B6.Cg-Tg(Cdh5-cre)7Mlia/J) and Sox2flox/flox (Sox2tm1.1Lan/J) mice were obtained from the Jackson Laboratory. Genotypes were confirmed by PCR, and experiments were performed with generations F4-F6. Littermates were used as wild type controls. All mice were fed a standard chow diet (Diet 8604, HarlanTeklad Laboratory). The studies were reviewed and approved by the Institutional Review Board and conducted in accordance with the animal care guideline set by the University of California, Los Angeles. The investigation conformed to the National Research Council, Guide for the Care and Use of Laboratory Animals, Eighth Edition (Washington, DC: The National Academies Press, 2011). Diisopropylfluorophosphate (DFP) (Sigma-Aldrich) and serpina1 (Origene) were injected via tail vein or retro-orbital injection (20-50 ng/g, daily) as in previous studies. Injections in Mgp−/− mice were started at 2 weeks of age, and continued for 2-4 weeks.

Tissue culture and siRNA transfections.

Human aortic endothelial cells (HAECs) were cultured as previously described. For treatment of HAECs, BMP-4 (40 ng/ml, R&D system), glucose (22 nmol/L, Sigma-Aldrich), DFP (300 ng/ml), serpina1 (300 ng/ml), elastase 1 (50 ng/ml, Abnova), elastase 2 (50 ng/ml, Abcam), and kallikrein 1, 5 and 6 (all 10 ng/ml, Abnova) were added as indicated in the text. Transient transfections of HAECs with siRNA (Silencer® predesigned siRNA, Ambion) were performed with Lipfectamine™2000 (Invitrogen) using 60 nM siRNA. The amount of siRNA was optimized per the manufacturer's instructions.
separate siRNAs and scrambled siRNA with the same nucleotide content were tested. When compared with unrelated control siRNA and scrambled siRNA, the specific siRNAs resulted in an 80–95% decrease in mRNA and protein levels as determined by real-time PCR and immunoblotting, respectively. The siRNA that provided the most efficient inhibition (90–95%) was used for all experiments. Silencer® predesigned siRNAs were obtained for MGP, SMAD1, SMAD5, SMAD8, Sox2, elastase 1 and 2, and kallikrein 1, 5 and 6. The same total amount of siRNA was added when transfections with multiple siRNAs were performed.

**RNA analysis.**
Real-time PCR analysis was performed as previously described. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control gene. Primers and probes for mouse Sox2, Kruppel-like factor 4 (Klf4), snail family zinc finger 2 (Slug or Snail2), spinocerebellar ataxia type 1 (Scal), cluster of differentiation (CD)10, CD44, CD71, CD90, c-kit (or CD117), N-cadherin, and all elastases and kallikreins were obtained from Applied Biosystems as part of Taqman® Gene Expression Assays.

**Immunoblotting.**
Immunoblotting was performed as previously described. Equal amounts of cellular protein or tissue lysates were used. Blots were incubated with specific antibodies to elastase 1 (200 ng/ml; Santa Cruz Biotechnology), elastase 2 (200 ng/ml; Abgent), kallikrein 1 and 6 (both 200 ng/ml; Sigma-Aldrich), kallikrein 5 (300 ng/ml; Acris Antibodies), c-kit (200 ng/ml; Cell Signaling Technology), Scal (200 ng/ml; Merck Millipore), CD10 (1:100; ThermoFisher), CD44 and CD90 (both 200 ng/ml; Abcam), CD71 (1:200; ThermoFisher), pSMAD1/5/8 (200 ng/ml; Santa Cruz Biotechnology), Sox2, Klf4, Slug and pSMAD2/3 (all 400 ng/ml; Cell Signaling Technology) and total SMAD (400 ng/ml; Santa Cruz Biotechnology). ß-Actin (1:5000 dilution; Sigma-Aldrich) was used as loading control.

**Immunofluorescence.**
Tissue sections were fixed in 4% paraformaldehyde and processed as previously described. Immunofluorescence was performed as previously described. We used specific antibodies for CD31 (Merck Millipore), VWF (Dako), ß-catenin, Osterix and elastase 1 (all from Santa Cruz Biotechnology), elastase 2 (Abgent), kallikrein 1 and 6 (Sigma-Aldrich), kallikrein 5 (Acris Antibodies), Sox2, Klf4, c-kit, Slug (all from Cell Signaling Technology), Scal (Merck Millipore), CD10 and CD71 (both from ThermoFisher), N-cadherin (Life technology), CD44 and CD90 (both from Abcam). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich).

**Flow cytometric analysis.**
Fluorescence-activated cell sorting (FACS) analysis was performed as described. The cells were stained with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, or Alexa Fluor 488 (AF-488)-conjugated antibodies against Sox2, VE-cadherin, elastase 1 and 2, kallikrein 1, 5, and 6. Nonspecific fluorochrome- and isotype-matched IgGs (BD Pharmingen) served as controls.

**Expression profile.**
The total mRNA was extracted from aortic tissue and examined by Mouse Ref-8v 2.0 Expressin Bead Chip kit (Illumina). The array data was analyzed by using GenomeStudio software (Illumina). The expression profiles of all serine proteases were extracted from array data, and the heat map was generated by using GenomeStudio.

**Quantification of aortic calcium.**
The aortic calcium was measured by using calcium assay kit (Bioassay) as previous described.
Enzyme-linked immunosorbent assay (ELISA).
The levels of elastases and kallikreins in plasma were examined using ELISA kits, elastase 1 (Antibodies-online), elastase 2 (MyBiosource), kallikrein 1 (Abcam), kallikrein 5 (Biocompare) and kallikrein 6 (Enzo). The assays were performed as per the manufacturer’s protocols.

Lentivirus infection.
Lentiviral vectors containing Sox2 open reading frame (ORF) under the cytomegalovirus (CMV) promoter were obtained from Applied Biological Materials Inc. The cell infection was performed as per the manufacturer’s protocol.

Transmission electron microscopy and scanning electron microscopy.
Transmission electron microscopy and scanning electron microscopy were performed as described 11.

Statistical analysis.
Data were analyzed for statistical significance by ANOVA with post hoc Tukey’s analysis. The analyses were performed using GraphPad Instat®, version 3.0 (GraphPad Software). Data represent mean ± SD. P<0.05 was considered significant, and experiments were performed a minimum of three times.

RESULTS
Lack of MGP causes endothelial-mesenchymal transition.
To investigate EndMTs in MGP deficient endothelium, we examined lesions of aortic calcification in 4-week-old Mgp−/− mice. We observed multiple layers of EC-like cells that appeared to have penetrated into the medial tissues and proliferated in the areas adjacent to an osteochondrogenic zone (Online Figure I). The internal elastic lamina (IEL) was undetectable and EC-like cells were oriented as if migrating from the luminal side toward the calcifying lesion (Online Figure I). We further examined the lesions using transmission electron microscopy (TEM) and scanning electron microscopy (SEM) at different time points from postnatal day 5 (P5) to P30. TEM indicated that degradation of IEL, which is in close contact with endothelium, began in the Mgp−/− aorta as early as P5 (Figure 1a). At P15, fractures in the IEL were apparent and Mgp−/− aortic ECs lost their normal morphology. At P30, the IEL was completely degraded, and a mixture of cells had replaced the normal aortic endothelium (Figure 1a). Abnormalities in the Mgp−/− aortic endothelium were also observed by SEM, which showed ruptures in the endothelium at P10 that became progressively worse at P15 and P30 (Figure 1b).

To examine whether EndMTs drove the Mgp−/− endothelium into a multipotent state, we analyzed the expression of the stem-cell markers Sox2, Klf4, c-kit, Sca1, CD10, CD44, CD71, CD90, and N-cadherin, and the typical mesenchymal marker Slug (also known as Snail2) in isolated Mgp−/− aortic ECs at P14 and P28. We found that expression of all of these markers was upregulated (Figure 1c). We also immunostained the calcified aortic tissues using specific antibodies for these markers and for vWF, an endothelial marker. All of the stem-cell and mesenchymal markers were strongly induced in vWF positive cells, which were detected in the aortic medial layer and calcified lesional areas (Figure 1d and Online Figure II) suggesting that EndMTs occurred in the Mgp−/− endothelium during the calcifying process.
Identification of serine proteases in EndMTs and calcification.

Because of the highly degraded IEL in Mgp<sup>−/−</sup> aortic tissues, we examined whether serine proteases were induced in these tissues. By comparing gene expression profiles of aortic tissues from wild type and Mgp<sup>−/−</sup> mice at 4 weeks of age, we identified 5 serine proteases, elastase 1 and 2, and kallikrein 1, 5, and 6, which were highly induced in Mgp<sup>−/−</sup> aortas (Figure 2a). Real-time PCR and immunoblotting of Mgp<sup>−/−</sup> aortic tissue confirmed the induction of these 5 serine proteases (Figure 2b-2d).

We also analyzed elastases and kallikreins in plasma by ELISA, which showed similar protease levels in Mgp<sup>−/−</sup> and Mgp<sup>+/+</sup> mice (data not shown). Among isolated Mgp<sup>−/−</sup> aortic cells, the fraction of elastase- and kallikrein-positive cells was 11-14% (Online Figure III). We performed immunohistochemistry to localize the elastase- and kallikrein-positive cells in Mgp<sup>−/−</sup> aortic tissue. Elastase- and kallikrein-positive cells co-localized with the endothelial marker vWF (Figure 3a). The flow cytometric analysis also showed co-expression of elastases and kallikreins with the endothelial marker VE-cadherin in isolated Mgp<sup>−/−</sup> aortic cells (Figure 3b), providing evidence that elastases and kallikreins were induced in Mgp<sup>−/−</sup> aortic endothelium. By way of confirmation, co-localization was not observed between kallikrein 5 and smooth muscle myosin heavy chain, nor between elastase 1 and alpha-smooth muscle actin (SMA) (Online Figure IV).

We treated Mgp<sup>−/−</sup> mice with the serine protease inhibitors DFP and serpin1 for two weeks starting at 2 weeks of age. We found that 80% and 50% of Mgp<sup>−/−</sup> mice were rescued by DFP and serpin1, respectively, after 6 weeks of treatment (Figure 4a). Aortic calcification in Mgp<sup>−/−</sup> mice, as visualized by Alizarin Red staining, was dramatically decreased after the DFP and serpin1 treatments (Figure 4b). Total aortic calcium was similarly decreased (Figure 4c), suggesting that serine protease inhibitors limited the calcifying process. To determine whether DFP and serpin1 affected EndMTs, we analyzed the expression of stem-cell and mesenchymal markers in aortic tissues by real-time PCR and immunoblotting. We found a significant decrease in these markers in Mgp<sup>−/−</sup> aortic tissues after DFP and serpin1 treatments (Figure 4d), suggesting that serine protease inhibitors also limited EndMTs. Together, our results suggest that serine proteases play important roles in transitioning ECs to a multipotent state allowing for osteochondrogenic differentiation.

Complexes of serine proteases induce EndMTs.

To determine if the complexes of elastases and kallikreins induce EndMTs, we used HAECs, more than 99.5% of which were CD31 positive<sup>11</sup>. We depleted MGP by siRNA transfection, which decreased MGP expression to <10% of normal levels after 20 to 24 hours<sup>30</sup>. We combined siRNA transfection with treatment of the cells with BMP-4 (40 ng/mL) or high glucose (22 nmol/L), which strongly induces BMP expression in ECs<sup>11,22</sup>. The results showed that expression of elastase 1, 2 and kallikrein 1, 5, 6 increased with both MGP depletion and the two treatments (Online Figure V). Combined transfection of siRNA to SMAD1, 5 and 8, however, abolished the induction (Figure 5a), suggesting that elastases and kallikreins were induced by BMP signaling. We also found that expression of stem-cell and mesenchymal markers were induced in MGP-depleted HAECs (Figure 5b). We then transfected MGP-depleted HAECs treated with BMP-4 and glucose with siRNA to elastase 1, 2 and kallikrein 1, 5, 6. Depletion of the elastases and kallikreins abolished the induction of stem-cell and mesenchymal markers in MGP-depleted HAECs, and addition of BMP-4 or glucose failed to restore the expression (Figure 5c). Addition of all of elastase 1, 2 and kallikrein 1, 5, 6 induced the expression of stem-cell and mesenchymal markers in HAECs after 24 hours of treatment (Online Figure VI). We tested the addition of individual elastases, kallikreins and various combinations; only the combination of all five proteases affected the marker expression (data not shown). We examined the progenitor capacity of HAECs undergoing EndMTs. We transfected HAECs with MGP siRNA and treated with glucose and BMP-4, or treated with serine proteases. We examined the proliferative activity, and found increased proliferation in MGP-
depleted HAECs. The increase was enhanced by BMP-4 and glucose (Online Figure VIIa). We examined colony-forming unit fibroblast (CFU-F) ability by alkaline phosphatase staining, and found that the CFU-F was higher in MGP-depleted or serine proteases treated HAECs (Online Figure VIIb). We also treated cells with adipogenic, cardiomyogenic or neurogenic medium. After 7 days of treatment, we examined the expression of adipogenic markers (peroxisome proliferator-activated receptor gamma, PPARgamma and CCAAT/enhancer-binding protein beta, C/EBPbeta), cardiomyocyte markers (NK2 homeobox 5, Nkx2.5, and Troponin I), and neuronal markers (nestin and neuroD) by real-time PCR. All these markers were increased in the MGP-depleted or serine proteases treated HAECs (Online Figure VIIc-d). The results suggested that HAECs gained multipotency after MGP-depletion or treatment with serine proteases. Together, the results suggested that EndMTs are activated by complexes of elastases and kallikreins induced by BMP signaling in the endothelium.

**Induction of Sox2-mediated mesenchymal transitions.**

To identify factors that mediate activation of EndMTs by elastases and kallikreins, we focused on the highly induced stem-cell markers that included both transcription factors and cell surface receptors. We depleted the expression of Sox2, Klf4, c-kit, Slug, Sca1, CD10, CD44, CD71 and CD90 individually in HAECs (using specific siRNA), and treated with elastase and kallikrein. Immunoblotting showed that depletion of Sox2 abolished the induction of all other markers (Figure 6a). We then used Sox2-expressing lentivirus to restore Sox2 in HAECs depleted of MGP, elastases, and kallikreins, and treated the cells with BMP-4 and high glucose. Immunoblotting showed a re-emergence of stem-cell and mesenchymal markers in HAECs after restoration of Sox2 (Online Figure VIII). We also restored Sox2 in MGP-depleted HAECs treated with DFP and serpina1, which similarly led to the re-emergence of stem-cell and mesenchymal markers (Figure 6b). These results suggested that Sox2 mediated EndMTs after depletion of MGP.

To examine the effects of elastases, kallikreins, and Sox2 on osteogenic differentiation in MGP-depleted HAECs, we depleted MGP, elastases, and kallikreins, and then treated the cells for 4 days with osteogenic medium alone, osteogenic medium with BMP-2 (200 ng/mL) and high glucose (22 mmol/L), or a combination of osteogenic medium with either BMP-2 or glucose starting the day after siRNA transfection. Immunoblotting showed that with all four treatments, expression of the osteogenic markers Cbfa1 and Osterix was increased when MGP was depleted, but when elastases and kallikreins were knocked down, the expression of osteogenic markers was abolished (Figure 6c). Depletion of Sox2 also inhibited induction of Cbfa1 and Osterix in MGP-depleted HAECs in the same type of experiment (Figure 6d). Mineral staining (Alizarin Red and Von Kossa) confirmed that depletion of elastases and kallikreins or Sox2 limited calcium accumulation in MGP-depleted HAECs after 7 and 14 days of treatment (Figure 5e-f).

**Endothelial-specific deletion of Sox2 decreases aortic calcification.**

We examined if Sox2 co-localized with proteases and Cbfa1 in vivo using immunofluorescence. Elastase 1, 2 and kallikrein 1, 5, 6 all co-localized with Sox2 and Cbfa1 in aortic endothelium of Mgp-/- mice at 4 weeks of age (Figure 7a and Online Figure IX). We pre-sorted VE-cadherin+CD45- aortic ECs from aortic tissues; CD45+ cells, which may represent CD31+ leukocyte populations, were excluded. Flow cytometric analysis showed that more than 30% of aortic ECs co-expressed elastases and kallikreins together with Sox2 (Online Figure X). These results suggested that the induction of Sox2 expression in ECs is caused by induction of elastases and kallikreins.

To determine if a cell-specific decrease of Sox2 in endothelium would limit EndMTs and vascular calcification, we crossed Cdh5Cre and Sox2Flox/Flox mice with Mgp-/- mice to selectively reduce Sox2 by Cre-mediated deletion in ECs by Cdh5 (VE-cadherin)-driven Cre expression. We examined the aortas of Cdh5CreSox2Flox/+-Mgp-/- mice at 4 weeks of age and found that expression of Sox2 was decreased...
in the aortic endothelium (Figure 7b). Immunoblotting showed that expression of stem-cell and mesenchymal markers were significantly decreased in the aortic endothelium of Cdh5\textsuperscript{Cre}\Sox2\textsuperscript{Flx/wt}Mgp\textsuperscript{−/−} mice (Figure 7c). Total aortic calcium was significantly reduced (Figure 7d) and Alizarin Red staining of whole aortic tissues showed a decrease in aortic mineralization in Cdh5\textsuperscript{Cre}\Sox2\textsuperscript{Flx/wt}Mgp\textsuperscript{−/−} mice (Figure 7e). This suggested that EndMTs and vascular calcification are limited by endothelial Sox2 reduction.

We further examined the expression of elastase 1, 2 and kallikrein 1, 5, 6 in aortas of Cdh5\textsuperscript{Cre}\Sox2\textsuperscript{Flx/wt}Mgp\textsuperscript{−/−} mice by real-time PCR. We found decreased expression of all the five serine proteases (Figure 7f), suggesting that reducing Sox2 in Mgp\textsuperscript{−/−} endothelium decreased the induction of serine proteases.

**Mutual regulation of Sox2 and Twist1 in EndMTs in vascular calcification.**

Twist1 is known to induce osteogenesis and play important roles in EndMTs and in epithelial-mesenchymal transitions 33-35, where Twist1 and Sox2 were also shown to regulate each other 36. To determine if Twist1 plays a role in EndMTs in Mgp\textsuperscript{−/−} aortas, we examined the expression of Twist1 in presorted VE-cadherin+CD45- aortic ECs from 4-week-old Mgp\textsuperscript{−/−} mice by real-time PCR, and found it to be increased (Figure 8a). We co-stained Twist1 with the endothelial marker vWF, which showed that Twist1 co-localized with vWF in Mgp\textsuperscript{−/−} aortas (Figure 8b). We also examined expression of Twist1 in MGP-depleted HAECs, and similarly found it to be increased. The induction of Twist1 was enhanced by treatment of BMP-4 and glucose, and abolished by depletion of Sox2 (Figure 8c). We then depleted Twist1 in MGP-depleted HAECs, treated with BMP-4 and glucose, and examined the stem-cell markers Sox2, Klf4 and c-kit. The depletion of Twist1 decreased induction of Sox2, Klf4 and c-kit (figure 8d), suggesting that Twist1 and Sox2 are able to mutually regulate each other during the initiation of EndMTs in MGP-depleted HAECs.

Furthermore, we depleted MGP and Twist1, and treated the cells for 4 days with osteogenic medium with either BMP-2 or glucose starting the day after siRNA transfection. The results showed that depletion of Twist1 abolished the induction of Cbfa1 and Osterix in MGP-depleted HAECs (Figure 8e). Altogether, the results suggested that Twist1 cooperated with Sox2 and played a role in initiating EndMTs leading to calcification.

**DISCUSSION**

Our results suggest that lack of MGP, a BMP inhibitor, results in the induction of endothelial elastases and kallikreins. The proteolytic activation of the elastases and kallikreins is instrumental in initiating EndMTs via a process mediated by Sox2, and allows for the emergence of multipotency and ultimately vascular calcification. Other studies have shown that elastases and kallikreins play important roles in vascular development and diseases, such as remodeling of the brachial artery and pulmonary hypertension 37-39. Our results expand the role of serine proteases to the transition of ECs into stem-cell like cells, suggesting that tight regulation of these enzymes is important for the maintenance of endothelial stability and differentiation.

In our experiments, all five serine proteases had to be combined to induce mesenchymal stem-cell markers. Any combination with fewer serine proteases failed to initiate EndMTs. Elastase 1 and 2, and kallikrein 1, 5 and 6 all have chymotrypsin-like specificities, but because of different structures, the cleavage of various protein substrates differs 40, 41. Under MGP-deficient conditions, it is possible that these proteases modify the endothelial microenvironment allowing the ECs to adopt a different cell fate.
Vascular calcification is a common complication of diabetic vasculopathy and atherosclerosis, and levels of elastases and kallikreins are elevated in diabetic patients as well as in atherosclerotic lesions. Our observations point toward new ways of explaining vascular calcification in these conditions and may lead to new strategies for limiting calcification.

Endothelial Sox2 was instrumental in the activation of EndMTs in HAECs. As an essential factor for development and somatic cell reprogramming, Sox2 acts as a key factor in cell fate determination. Previous studies have shown that Sox2 plays a role in epithelial-mesenchymal transition in neural crest development and cancer. It has also been reported that fibroblasts exhibit mesenchymal phenotypes after infection with Sox2 lentiviral vectors. Our findings broaden the functions of Sox2 to also include activation of EndMTs in the aortic endothelium.

Our observation that serine proteases induce Sox2 also provides new information applicable to development. Expression of Sox2 is sustained along the ectoderm, where organs emerge from interactions between the epithelium and the mesenchyme, and proteolytic activity appears to be induced in tissue development. Based on our data, it is possible that serine proteases regulate Sox2 to modulate mutual transitions of the endothelium and the mesenchyme to achieve correct cell differentiation as well as coordination between tissue-specific elements and the vasculature.

In our study, BMP-4 acted as an inductive factor of EndMTs in MGP deficiency, which is consistent with results from other investigators. The BMPs elicit their activity through ligand-initiated heterodimerization of BMP type II receptors (BMPRII) and type I receptors referred to as activin receptor-like kinase (ALK) 1, 2, 3, and 6. Activation of the type I receptor by BMPRII leads to phosphorylation of a series of SMAD proteins, which mediate the gene regulation. Our previous studies in HAECs showed that the same glucose treatment used to induce EndMTs also enhanced expression of BMPRII. Interestingly, it has been shown that EndMTs associated with pulmonary arterial hypertension occur when the BMPRII levels are reduced. Therefore, we also examined the expression of stem-cell markers in Mgp-/- lungs, and the effect of MGP-deficiency, BMP-4, glucose, and serine proteases on EndMTs in cultured human pulmonary artery ECs (HPAECs). To our surprise, we found no evidence of enhanced stem-cell marker expression or EndMTs in the Mgp-/- lungs or the HPAECs (unpublished observations), strongly suggesting that the pulmonary arteries differ in their response to MGP deficiency. This is further supported by the finding that MGP-deficiency causes arteriovenous malformations (AVMs) rather than vascular calcification in the pulmonary circulation. Indeed, tissue-specific ECs from various organs have been reported to have unique signatures of gene expression, and to differ in their responses to stimulus. In addition, we have observed different responses to MGP-deficiency in the pulmonary and cerebral vascular beds. VEGF is enhanced in the pulmonary vasculature, whereas Notch signaling is enhanced in cerebral vasculature, even though in both cases, MGP-deficiency leads to AVMs.

The response to loss of BMPRII may be difficult to predict, since BMPRII has been shown to be a target of multiple pro-inflammatory factors, and absence of BMPRII might allow for enhanced BMP6/7 signaling through recruitment of the activin type II receptor. Another complicating factor is that different BMP and TGFβ ligands affect EndMTs differently. Whereas BMP-4 enhances EndMTs, BMP-2 and -7 have been reported to reverse EndMTs induced by TGFβ1. TGFβ1 elicits its activity through ALK5, which is induced by BMP-4 in HAECs, suggesting that TGFβ1-mediated EndMTs might still be regulated by BMP-4 depending on the type of endothelium. In addition, excess BMP-2 and -7 might compete with BMP-4 for binding to the same BMP receptors, thereby altering the balance that promotes or reverses EndMTs.

Previous studies have shown increased alpha-SMA expression in ECs undergoing EndMTs in pulmonary hypertension. In addition, we showed increased expression of alpha-SMA and SM22alpha...
in EC-like cells in the media of Mgp<sup>−/−</sup> aortas, further supporting that these early SMC markers are expressed in transitioning ECs. Speer et al. demonstrated by lineage-tracing that SM22alpha positive cells contribute to osteochondrogenic differentiation in Mgp<sup>−/−</sup> aortas, although it is not clear if a fraction of these cells might have been derived from the endothelium.

Smooth muscle cells (SMCs) reside in the vascular media, which is separated from the endothelium by the IEL. As the IEL is degraded, the SMCs are likely to be exposed to endothelial signaling, matrix alterations, and other effects of increased serine protease activity. This may affect the SMC phenotype and contribute to vascular calcification.

During development, expression of VE-cadherin is detected in ECs as well as in hematopoietic cells that arise from the hemogenic endothelium of the dorsal aortas. Therefore, we cannot rule out a contribution to the observed phenotype from altered Sox2 signaling in hematopoietic cells. However, we have previously shown that expression of inflammatory and monocyte markers is minimal or undetectable in Mgp<sup>−/−</sup> aortas. Thus it is less likely that hematopoietic cells, such as monocytes, would directly transit to or affect osteoprogenitor cells in the Mgp<sup>−/−</sup> aortas. Furthermore, even if the exclusion of CD45<sup>+</sup> cells prior to flow cytometric analysis of the Mgp<sup>−/−</sup> aortic cells removed CD45<sup>+</sup>C31<sup>+</sup> leukocytic cells, 20-30% of the analyzed ECs still co-expressed CD31 and Sox2. EC lineage tracing and co-staining of EC and multipotency or osteogenic markers in our previous studies further supported the endothelium as a source of osteogenic cells. Altogether, our results suggest an important role for Sox2 in initiating EndMTs in vascular calcification.

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DISCLOSURES
The authors have declared that no conflict of interest exists.

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FIGURE LEGENDS

**Figure 1.** Lack of MGP causes EndMTs in aortic tissue. (a-b) *Mgp<sup>+/+</sup>* and *Mgp<sup>−/−</sup>* aortic endothelium was examined by transmission electron microscopy (TEM) (a) and scanning electron microscopy (SEM) (b). Magnification for TEM, 3.7×10<sup>3</sup>. Magnification for SEM, 5×10<sup>2</sup>. IEL: Internal elastic lamina. Areas of IEL degradation are indicated by arrowheads. (c) Expression of stem-cell and mesenchymal markers in VE-cadherin-positive and CD45-negative presorted cells from *Mgp<sup>+/+</sup>* and *Mgp<sup>−/−</sup>* aortas at postnatal day (P) 14 and 28, as determined by real-time PCR. ***p<0.001. (d) Co-expression of the endothelial marker vWF with the stem-cell marker Sox2 and the mesenchymal marker Slug in calcified lesions of *Mgp<sup>−/−</sup>* aortas. Scale bars, 100 µm.

**Figure 2.** Activation of serine proteases in EndMTs and calcification. (a) Global gene expression profile of serine proteases in aortas from wild type (*Mgp<sup>+/+</sup>*) and *Mgp<sup>−/−</sup>* mice. (b-d) Expression of elastases (ELA) and kallikreins (KLK) in *Mgp<sup>+/+</sup>* and *Mgp<sup>−/−</sup>* aortas, determined by real-time PCR and immunoblotting with densitometry. ***p<0.001.

**Figure 3.** Induction of elastases and kallikreins in *Mgp<sup>−/−</sup>* aortic endothelium. (a) Immunostaining to detect co-localization of ELA 1, 2 and KLK 1, 5, 6 with the EC marker vWF. Scale bar: 100 µm. (b) Flow cytometric analysis of co-expression of KLK 1, 5, 6 and VE-Cadherin in *Mgp<sup>−/−</sup>* aortic cells.

**Figure 4.** Inhibition of serine proteases decreases EndMTs and vascular calcification. (a) Survival rate of *Mgp<sup>−/−</sup>* mice after treatment with DFP and serpina1 (n=8 per group). (b) Alizarin Red staining of *Mgp<sup>−/−</sup>* aortas after 2 weeks of treatment with DFP or serpina1 injections. (c) Aortic calcium in *Mgp<sup>−/−</sup>* aortas after injection of DFP (top) or serpina1 (bottom). ***p<0.001. (d) Expression of stem-cell markers in *Mgp<sup>−/−</sup>* aortas after treatment with DFP or serpina1 as determined by real-time PCR (top) and immunoblotting with densitometry (bottom). ***p<0.001.

**Figure 5.** Elastases and kallikreins induce EndMTs in HAECs. (a) Elastase (ELA) 1, 2 and kallikrein (KLK) 1, 5, 6 were induced after depletion of MGP by siRNA (MGP si) in HAEC. The induction was enhanced by BMP-4 or high glucose, and abolished by transfection of siRNA to SMAD1/5/8 as shown by immunoblotting with densitometry. (b) Stem-cell and mesenchymal marker were induced after MGP si depletion in HAECs. The induction was enhanced by BMP-4 or high glucose as shown by immunoblotting with densitometry. (c) Depletion of elastase 1, 2 and kallikrein 1, 5, 6 by siRNA (si) abolished the induction of stem-cell markers in MGP-depleted HAECs as shown by immunoblotting with densitometry. SCR: scrambled siRNA.

**Figure 6.** Sox2 mediates activation of elastases and kallikreins in the induction of EndMTs. (a) The expression of stem-cell markers was examined by immunoblotting with densitometry in HAECs treated with medium containing elastase 1, 2 (ELAs) and kallikrein 1, 5, 6 (KLKs), and transfected with siRNA (si) to each of the stem-cell markers individually. Ctr: control. SCR: scrambled siRNA. (b) HAECs were depleted of MGP by siRNA (si) and treated with high glucose and BMP-4, then treated with control (lane 1-3), DFP (lane 4-5), serpina1 (lane 6-7), or a combination of DFP, serpina1, and lentivirus (Lv) expressing Sox2 (lane 8-11). Expression of stem-cell markers was examined by immunoblotting with densitometry. (c-f) MGP-depleted HAECs were transfected with siRNAs (si) to elastase (ELA) 1, 2 and kallikrein (KLK) 1, 5, 6, or Sox2, and then treated with control (C), BMP-2 (B), glucose (G), or a combination. Expression of Cbfa1 and Osterix (OSX) was determined by immunoblotting with densitometry (c and d). Calcium accumulation was determined by Alizarin Red and Von Kossa staining (e and f). SCR: Scrambled siRNA.
Figure 7. Limiting Sox2 in endothelium decreases EndMTs and calcification in Mgp⁻/⁻ aortas. (a) Co-expression of elastase (ELA) 1, 2 and kallikrein (KLK) 1, 5, 6 with EC marker vWF and multipotency marker Sox2 in Mgp⁻/⁻ aortas as shown by immunostaining. Scale bars: 100 µm. (b) Decreased expression of Sox2 in aortas of Cdh5CreSox2²Flox/wtMgp⁻/⁻, as shown by immunoblotting with densitometry. (c) Decreased expression of stem-cell and mesenchymal markers in aortas of Cdh5CreSox2²Flox/wtMgp⁻/⁻, as shown by immunoblotting with densitometry. (d) Aortic calcium in Cdh5CreSox2²wtMgp⁻/⁻ and Cdh5CreSox2²Flox/wtMgp⁻/⁻ mice. ***p<0.001. (e) Alizarin Red staining of Cdh5CreSox2²wtMgp⁻/⁻ and Cdh5CreSox2²Flox/wtMgp⁻/⁻ aortas. (f) Decrease of expression of elastase (ELA) 1, 2 and kallikrein (KLK) 1, 5, 6 in the aortas of Cdh5CreSox2²Flox/wtMgp⁻/⁻, as shown by real-time PCR. ***p<0.001.

Figure 8. Mutual regulation of Sox2 and Twist1 in EndMTs in vascular calcification. (a) Increase of Twist1 expression in VE-cadherin+CD45- presorted cells from Mgp⁻/⁻ aortas, as shown by real-time PCR. ***p<0.001. (b) Co-localization of Twist1 with the EC marker vWF in Mgp⁻/⁻ aortas, as shown by immunostaining. Scale bars: 100 µm. (c) MGP-depleted HAECs were transfected with siRNAs (si) to Sox2, and then treated with combinations of BMP-4 and glucose. Expression of Twist1 was examined by real-time PCR. ***p<0.001. (d) MGP-depleted HAECs were transfected with siRNAs (si) to Twist1, and then treated with combination of BMP-4 and glucose. Expression of Sox2, Klf4 and c-kit was examined by real-time PCR. ***p<0.001. (e) MGP-depleted HAECs were transfected with siRNAs (si) to Twist1, and then treated with BMP-2 or glucose for 4 days. Expression of Sox2, Klf4 and c-kit was examined by real-time PCR. ***p<0.001. (f) Schematic working model for the induction of serine proteases and Sox2 in EndMTs in vascular calcification.
Novelty and Significance

What Is Known?

- Vascular calcification is a frequent complication of cardiovascular diseases.
- Endothelial-mesenchymal transitions (EndMTs) contribute to vascular calcification.
- Lack of matrix Gla protein (MGP), an inhibitor of bone morphogenetic proteins (BMP), induces EndMTs thereby promoting vascular calcification.

What New Information Does This Article Contribute?

- Specific serine proteases and sex determining region Y-box 2 (Sox2) are induced in endothelial cells at sites of vascular calcification in Mgp null aortas.
- Serine proteases are essential for the initiation of EndMTs by inducing Sox2 in MGP-deficient endothelium.
- Inhibition of serine proteases or suppression of Sox2 diminished EndMTs and vascular calcification.

Vascular calcification frequently complicates vascular diseases. EndMTs enable endothelium to contribute cells to vascular calcification. However, the initiation of EndMTs in vascular calcification is poorly understood. We report that Sox2-mediated activation of specific serine proteases is essential for initiating EndMTs. Using mouse models and cultured endothelial cells, we demonstrate that lack of the BMP-inhibitor MGP or enhanced BMP expression activates serine proteases and induces EndMTs. The activation of serine proteases is mediated by Sox2. These findings identify the serine proteases and Sox2 as the factors in EndMTs and vascular calcification and may provide therapeutic targets for treating vascular calcification.
Figure 3

(a) Immunofluorescent images showing localization of various proteins in different genotypes. Mgp<sup>+/+</sup> and Mgp<sup>-/-</sup> mice were analyzed for vWF, ELA1, ELA2, KLF1, KLF5, and KLF6 expression in the vasculature.

(b) Flow cytometry analysis of ELA1 and ELA2 expression in Mgp<sup>+/+</sup> and Mgp<sup>-/-</sup> mice. Scatter plots show the expression levels of ELA1 and ELA2 in the vasculature.

VE-Cadherin expression was also analyzed in both genotypes.
Figure 8

(a) Mgp<sup>−/−</sup> VE-Cad<sup>+</sup> vs Mgp<sup>−/−</sup> VE-Cad<sup>+</sup>

(b) vWF, Twist, Merge, Merge/DAPI

(c) Twist expression (fold)
- SCR
- MGP si
- BMP-4
- Glucose
- SCR
- Sox2 si

(d) Sox2 expression (fold)
- SCR
- MGP si
- BMP-4
- Glucose
- SCR
- Twist si

(e) K<sub>α</sub>4 expression (fold)
- SCR
- MGP si
- BMP-4
- Glucose
- SCR
- Twist si

(f) MGP
- BMP
- High Glucose and other factors
- Elastase 1, 2
- Kallikrein 1, 5, 6
- Sox2
- Twist
- EndMT
- Vascular calcification

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Serine Protease Activation Essential for Endothelial-Mesenchymal Transition in Vascular Calcification

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SUPPLEMENTAL MATERIAL

ONLINE SUPPLEMENT
Detailed Method Section

Animals
Mgp<sup>−/−</sup> mice on the C57BL/6J background (1) were obtained from Dr. Cecilia Giachelli, University of Washington, with the permission of Dr. Gerard Karsenty, Columbia University. Cdhl5<sup>Cre</sup> (B6.Cg-Tg(Cdh5<sup>cre</sup>)7Mlia/J) and Sox2<sup>flox/flox</sup> (Sox2<sup>tm1.1Lan/J</sup>) mice were obtained from the Jackson Laboratory. Genotypes were confirmed by PCR (2-4), and experiments were performed with generations F4-F6. Littermates were used as wild type controls. All mice were fed a standard chow diet (Diet 8604, HarlanTeklad Laboratory). The studies were reviewed and approved by the Institutional Review Board and conducted in accordance with the animal care guideline set by the University of California, Los Angeles. The investigation conformed to the National Research Council, Guide for the Care and Use of Laboratory Animals, Eighth Edition (Washington, DC: The National Academies Press, 2011). Disisopropylfluorophosphate (DFP) (Sigma-Aldrich, St. Louis, MO) and serpinA1 (Origene, Rockville, MD) were injected via tail vein or retro-orbital injection (20-50 ng/g, daily) as in previous studies (5, 6). Injections in Mgp<sup>−/−</sup> mice were started at 2 weeks of age, treated continued for 2-4 weeks.

Tissue culture and siRNA transfections

HAECs were cultured as previously described (7). For treatment of HAECs, BMP-4 (40 ng/ml, R&D Systems, Minneapolis, MN), glucose (22 nmol/L, Sigma-Aldrich), DFP (300 ng/ml), serpinA1 (300 ng/ml, Origene), elastase 1 (50 ng/ml, Abnova, Walnut, CA), elastase 2 (50 ng/ml, Abcam, Cambridge, MA), kallikrein 1, 5 and 6 (all 10 ng/ml, Abnova) were added as indicated in the text. Transient transfections of HAECs with siRNA (Silencer® predesigned siRNA, Life Technologies, Grand Island, NY) were performed with Lipofectamine™2000 (Life Technologies) using 60 nM siRNA. The amount of siRNA was optimized per the manufacturer's instructions. Three separate siRNAs and scrambled siRNA with the same nucleotide content were tested. When compared with unrelated control siRNA and scrambled siRNA, the specific siRNAs resulted in an 80–95% decrease in mRNA and protein levels as determined by real-time PCR and immunoblotting, respectively. The siRNA that provided the most efficient inhibition (90–95%) was used for all experiments. Specific Silencer® predesigned siRNAs were obtained for MGP (s8753), SMAD1 (s8394), SMAD5 (s8408), SMAD8 (s8417), Sox2 (s13295), elastase 1 (s4597) and 2 (s4600), kallikrein 1 (s7874), 5 (s24545) and 6 (s11276) and Twist1 (s14523). The same amount of siRNA was added when transfection with multiple siRNAs were performed.

RNA Analysis
Real-time PCR analysis was performed as previously described (8). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control gene (8). Primers and probes for Sox2 (Hs01053049_s1 for human; Mm00353810_s1 for mouse), Kruppel-like factor 4 (Klf4) (Hs00153049_s1 for human; Mm03053810_s1 for mouse), snail family zinc finger 2 (Slug or Snail2) (Hs00161904_m1 for human; Mm00441531_m1 for mouse), Stem Cell Antigen 1 (Sca1) (Hs00165656_m1 human; m00726565_s1 for mouse), cluster of differentiation (CD) 10 (Hs001535310_m1 for human; Mm01285052_m1 for mouse), CD44 (Hs01075861_m1 for human; Mm01277161_m1 for mouse), CD71 (Hs00951083_m1 for human; Mm00441941_m1 for mouse), CD90 (Hs00264235_s1 for human; Mm00493682_g1 for mouse), c-kit (or CD117) (Hs00174029_m1 for human; Mm00445212_m1 for mouse), N-cadherin (Hs00983056_m1 for
human; Mm01162497_m1 for mouse), Twist1 (Hs01675818_s1 for human; Mm04028233_g1 for mouse), elastase 1 (Hs00608115_m1 for human; Mm00712898_m1 for mouse), elastase 2 (Hs00975994_g1 for human; Mm01168928_g1 for mouse), kallikrein 1 (Hs01086545_m1 for human; Mm03052649_s1 for mouse), kallikrein 5 (Hs01548153_m1 for human; Mm01203811_m1 for mouse) and kallikrein 6 (Hs00160519_m1 for human; Mm00478322_m1 for mouse) were obtained from Life Technologies as part of Taqman® Gene Expression Assays.

**Immunoblotting**

Immunoblotting was performed as previously described (9). Equal amounts of cellular protein or tissue lysates were used. Blots were incubated with specific antibodies to elastase 1 (sc-292230; 200 ng/ml; Santa Cruz Biotechnology), elastase 2 (AP12084a; 200 ng/ml; Abgent, San Diego, CA), kallikrein 1 (SAB2500569) and 6 (AV33888) (both 200 ng/ml; Sigma-Aldrich), kallikrein 5 (AP16633PU-N; 300 ng/ml; Acris Antibodies, San Diego, CA), c-kit (#3074, 200 ng/ml; Cell Signaling Technology, Boston, MA), Sca1 (MABD26, 200 ng/ml; Merck Millipore, Billerica, MA), CD10 (MS-728; 1:100; ThermoFisher, Waltham, MA), CD44 (ab51037) and CD90 (ab225) (both 200 ng/ml; Abcam), CD71 (MS-1096; 1:200; ThermoFisher), pSMAD1/5/8 (sc-12353, 200 ng/ml; Santa Cruz Biotechnology), Sox2 (#3579), Klf4 (#12173), Slug (#9585) and pSMAD2/3 (#8828) (all 400 ng/ml; Cell Signaling Technology) and total SMAD (sc-7153; 400 ng/ml; Santa Cruz Biotechnology, Dallas, Texas). β-Actin (A2228; 1:5000 dilution; Sigma-Aldrich) was used as loading control.

**Immunofluorescence**

The tissues were collected at 4 weeks for Mgp^−/− mice, and the proximal descending aorta was used for tissue sections. We did not detect any particular areas that consistently showed more calcification than others in the mice that were included in this study. The calcification in the Mgp^−/− mice was very extensive and uniform. Tissue sections were fixed in 4% paraformaldehyde and processed as previously described (10). For immunohistochemistry or immunofluorescence, sections were permeabilized with 0.5% Triton X-100 for 10 minutes, followed by 3 washes with wash buffer (WB, phosphate-buffered saline (PBS) containing 0.1% Tween-20). Non-specific antibody binding sites were blocked by incubating the sections for 30 minutes in blocking buffer (1% BSA, 2% goat serum and 0.5% Triton X-100 in PBS). Primary antibodies were diluted in antibody buffer (PBS containing 1% BSA, 0.5% Triton X-100), and sections were incubated for 60 minutes at room temperature, followed by several washes in WB. Alexa Fluor 488-conjugated (green fluorescence) or Alexa Fluor 594-conjugated (red fluorescence) secondary chick anti-goat or anti-rabbit antibodies (Molecular Probes, Eugene, OR) were applied to the sections and incubated for 30 minutes at room temperature. After several washes in WB and a brief equilibration of the sections with PBS, the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). A DAPI stock solution was diluted to 300 nM in PBS, and 300 µl of the diluted solution was added to the sections, making certain that they were completely covered. The sections were incubated for 1-5 minutes and rinsed several times in PBS. Images were acquired with an inverted Zeiss Axiovert 200 microscope (Carl Zeiss Micro Imaging Inc., Thornwood, NY). To eliminate the possibility of false co-localization caused by emission filter bleed through, only images showing signals that were clearly visible by eye through the microscope when using the appropriate filters for the respective antibodies were considered significant and included in the results.

We used specific antibodies for CD31 (04-1074; Merck Millipore), vWF (A0082; Dako, Carpinteria, CA), Cbfa1 (sc-22536), Osterix (Sc856), elastase 1 (sc-292230; Santa Cruz Biotechnology), elastase 2 (AP12084a; Abgent), kallikrein 1(SAB2500569) and 6 (AV33888) (Sigma-Aldrich), kallikrein 5 (AP16633PU-N; Acris Antibodies), Sox2 (#3579), Klf4 (#12173), c-
kit (#3074), Slug (#9585) (all from Cell Signaling Technology), Sca1 (MA.26; Merck Millipore), CD10 (MS-728) and CD71 (MS-1096)(both from ThermoFisher), N-cadherin (33-3900, Life technology), CD44 (ab51037) and CD90 (ab225) (both from Abcam). Non-specific IgG was included as a primary antibody control in all experiments, where it showed no significant staining, which has been included in select figures.

Flow Cytometric Analysis
Fluorescence-activated cell sorting (FACS) analysis was performed as described (11). Briefly, the cells were detached from the culture dish with 0.25% trypsin/EDTA, centrifuged at low speed, and stained with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, or Alexa Fluor 488 (AF-488)-conjugated monoclonal mouse anti-human antibodies against Sox2 (#3579; Cell Signaling Technology), VE-cadherin (560411; BD Pharmingen, San Jose, CA), elastase 1 (sc-292230; Santa Cruz Biotechnology) and elastase 2 (AP12084a; Abgent), kallikrein 1 (SAB2500569) and 6 (AV33888) (both from Sigma-Aldrich), kallikrein 5 (AP16633PU-N; Acris Antibodies). Nonspecific fluorochrome- and isotype-matched IgGs (BD Pharmingen) served as controls. Flow cytometer gates were set using unstained cells and the isotype-matched controls. Cells were gated by forward scatter (FSC) versus side scatter (SSC) to eliminate debris. A region was established to define positive PE/AF-488 fluorescence using a PE/AF-488-conjugated isotype-specific control. The number of cells stained positive for a given marker was determined by the percentage of cells present within a gate, which was established such that fewer than 2% of positive events represented nonspecific binding by the PE/AF-488-conjugated isotype-specific control. Minimums of 10,000 events were counted for each analysis. All FACS analyses were performed using a BD LSR II Flow Cytometer (BD Biosciences, San Jose, CA). FACS files were exported and analyzed using BD Cellquest Software v.3.3. Aortas were enzymatically dispersed using a modification of a previously described protocol (11). The mice were perfused with dispase, the aortas were dissected and cut to pieces, and incubated for 45 minutes prior to fixation, staining and FACS analysis. For each sorting, 3 aortas from Mgp−/− mice were required to obtain enough cells for analysis.

Expression profile
The total mRNA was extracted from aortic tissue and examined by the Mouse Ref-8 v2.0 Expression Bead Chip (Illumina, San Diego, CA). The array data was analyzed using GenomeStudio software (Illumina). The expression profiles of all serine proteases were extracted from array data, and the heat map was generated using GenomeStudio.

Quantification of aortic calcium
The expression calcium was measured by using calcium assay kit (Bioassay) as previous described (12).

Enzyme-linked immunosorbent assay (ELISA)
The levels of elastases and kallikreins in plasma were examined by using ELISA kits, elastase 1 (Antibodies-online), elastase 2 (MyBiosource, San Diego, CA), kallikrein 1 (Abcam), kallikrein 5 (Biocompare, San Francisco, CA) and kallikrein 6 (Enzo, Farmingdale, NY). The assays were performed as per the manufacturers’ protocols.

Proliferation assay
HAECs were transfected and treated as indicated. 3H-Thymidine was added at 1 µCi/mL for 4 days, and 3H-thymidine incorporation was determined as previously described (13).

Analysis of colony forming units
HAECs were transfected and treated as indicated. Analysis of colony forming units
Was performed as previously described (14).

Lentivirus infection
Lentiviral vectors containing Sox2 open reading frame (ORF) under the cytomegalovirus (CMV) promoter were obtained from Applied Biological Materials Inc. (Richmond, BC). The cell infection was performed as per the manufacturer’s protocol.

Transmission electron microscopy and scanning electron microscopy
Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were performed as described (11). For TEM, dissected aortic tissues were immersed in a solution containing 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M PBS, pH 7.4, for 2 hr at room temperature, then incubated at 4°C overnight. On the next day, 0.5% of tannic acid was added to the tissues and incubated for an hour at room temperature. The tissue blocks were then washed five times in 0.1 M PBS and postfixed in a solution of 1% $\text{Os}_2\text{O}_4$ in PBS, pH 7.2–7.4. The combination of tannic acid/glutaraldehyde/paraformaldehyde followed by osmification increased the staining of the membranes. The samples were washed four times in 0.1 M Na acetate buffer, pH 5.5, block-stained in 0.5% uranyl acetate in 0.1 M Na acetate buffer, pH 5.5, for 12 hr at 4°C. The samples were dehydrated in graded ethanol (50%, 75%, 95%, 100%, 100%, 100%) 10 minutes each, passed through propylene oxide, and infiltrated in mixtures of Epon 812 and propylene oxide 1:1 and then 2:1 for two hours each. The tissues were then infiltrated in pure Epon 812 overnight. Embedding was performed in pure Epon 812 and curing was done in an oven at 60°C for 48 hr. Sections of 60 nm thickness (gray interference color) were cut on an ultramicrotome (RMC MTX) using a diamond knife. The sections were deposited on single-hole grids coated with Formvar and carbon and double-stained in aqueous solutions of 8% uranyl acetate for 25 min at 60°C and 26.6mM lead citrate for 3 min at room temperature. Thin sections subsequently were examined with a 100CX JEOL electron microscope.
For SEM, a 2.5-3% concentration of glutaraldehyde in PBS was used to fix the samples. The samples were dehydrated in graded ethanol (50%, 60%, 70%, 80%, 90% and 100%). The samples were dried in following steps: 1:2 solution of Hexamethyldisilizane (HMDS):100% ethanol for 20 minutes; 2:1 HMDS: 100% ethanol for 20 minutes; 100% HMDS for 20 minutes, then covered or capped loosely in a fume hood overnight. Sputter coating was applied to the samples, which were subsequently imaged by SEM.

Statistical analysis
Data were analyzed for statistical significance by ANOVA with post hoc Tukey’s analysis. The analyses were performed using GraphPad Instat®, version 3.0 (GraphPad Software, La Jolla, CA). Data represent mean ± SD. $P<0.05$ was considered significant, and experiments were performed a minimum of three times.
**Online Figure 1**

*Histology of the aortic wall of Mgp⁺/⁻ mice.*

Histology of the aortic wall of *Mgp⁺/⁻* (left, upper) and *Mgp⁻/⁻* (left, bottom) mice. The schematic drawing illustrates differences in the layout of cell layers in the aortic tissues of *Mgp⁺/⁻* (right, upper) and *Mgp⁻/⁻* (right, bottom) mice. Scale bars, 100 µm.
Online Figure II
Co-expression of the endothelial marker vWF with stem-cell and mesenchymal markers in calcified lesions of Mgp−/− aortas.
Co-expression of the endothelial marker vWF with Sox2 and Slug is also shown in Figure 1. Scale bars, 100 µm.
Online Figure III
Flow cytometric analysis of Mgp−/− aortic cells.
Elastase (ELA) 1, 2 and kallikrein (KLK) 1, 5, 6 positive cell populations from enzymatically dispersed Mgp−/− aortic cells were analyzed by flow cytometric analysis.
Online Figure IV
Absence of co-localization between proteases and smooth muscle cell markers
Co-localization was not observed for kallikrein 5 and smooth muscle myosin heavy chain (SMMHC) (a), nor was co-localization observed for elastase 1 and alpha-smooth muscle actin (a-SMA) (b).
Online Figure V

Elastases and kallikreins induced in HAECs.
Elastase (ELA) 1, 2 and kallikrein (KLK) 1, 5, 6 were induced after depletion of MGP by siRNA (MGP si) in HAEC (a-b). The induction was enhanced by BMP-4 (a) or high glucose (b) as shown by immunoblotting with densitometry.
Online Figure VI

A complex of elastases and kallikreins induces stem-cell markers in HAEC. Immunoblotting with densitometry showed expression of stem-cell markers in HAECs treated with elastase (ELA) 1 and 2 and kallikrein (KLK) 1, 5 and 6 individually (lane 2-6) or in combination (lane 7).
Online Figure VII

*Multipotent potentials in MGP-depleted HAECs or HAECs treated with serine proteases.*

HAECs were transfected with MGP siRNA or treated with a complex of serine proteases (ELAs+KLKs). The cells were subsequently treated with BMP-4 and glucose for 24 hours before cell proliferation (a) and colony-forming units (CFU-F) were determined (b). The cells were also treated with cardiogenic, adipogenic and neurogenic media, respectively, for 7 days. Expression of the adipogenic markers PPARgamma and C/EBPβ (c), the cardiomyocyte markers Nkx2.5 and Troponin I (d) and the neural markers neuroD and nestin (e) was determined by real-time PCR. ***P < 0.001.**
**Online Figure VIII**

Over-expression of Sox2 restores the expression of stem-cell markers.

The expression of stem-cell markers was examined by immunoblotting with densitometry in HAECs, which were depleted MGP by siRNA (si), treated with BMP-4 and glucose, and then transfected with siRNA to elastase 1, 2 (ELK si) and kallikrein 1,5,6 (KLK si) without (lane 4-5) and with (lane 6-7) infection of lentivirus expressing Sox2 (Sox2 Lv). SCR: scrambled siRNA.
Co-expression of elastases and kallikreins with Cbfa1 in Mgp^/- endothelium. Co-expression of elastase (ELA) 1, 2 and kallikrein (KLK) 1, 5, 6 with the EC marker vWF and the osteogenic marker Cbfa1 in Mgp^/- aortas as shown by immunostaining. Scale bars: 100 µm.
Online Figure X

*Flow cytometric analysis of Sox2 and elastases and kallikreins*

Flow cytometric analysis showed co-expression of Sox2 and elastases and kallikreins in VE-cadherin+CD45- aortic endothelial cells from aortas of *Mgp*+/+ and *Mgp*−/− mice.
REFERENCE


