A Role for the Endothelium in Vascular Calcification

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ABSTRACT

Rationale: Vascular calcification is a regulated process that involves osteoprogenitor cells and frequently complicates common vascular disease such as atherosclerosis and diabetic vasculopathy. However, it is not clear if the vascular endothelium has a role in contributing osteoprogenitor cells to the calcific lesions.

Objective: To determine if the vascular endothelium contributes osteoprogenitor cells to vascular calcification.

Methods and Results: In this study, we use two mouse models of vascular calcification, mice with gene deletion of matrix Gla protein (MGP), a BMP-inhibitor, and Ins2Akita/+ mice, a diabetes model. We show that enhanced bone morphogenetic protein (BMP) signaling in both types of mice stimulates the vascular endothelium to contribute osteoprogenitor cells to the vascular calcification. The enhanced BMP signaling results in endothelial-mesenchymal transitions and the emergence of multipotent cells, followed by osteoinduction. Endothelial markers co-localize with multipotent and osteogenic markers in calcified arteries by immunostaining and fluorescence-activated cell sorting. Lineage tracing using Tie2-Gfp transgenic mice supports an endothelial origin of the osteogenic cells. Enhancement of MGP expression in Ins2Akita/+ mice, as mediated by an Mgp transgene limits the generation of multipotent cells. Moreover, MGP-depleted human aortic endothelial cells in vitro acquire multipotency rendering the cells susceptible to osteoinduction by BMP and high glucose.

Conclusions: Our data suggest that the endothelium is a source of osteoprogenitor cells in vascular calcification that occurs in disorders with high BMP activation such as deficiency of BMP inhibitors and diabetes.

Keywords: Vascular calcification, vascular endothelium, matrix Gla protein, bone morphogenetic protein, endothelial-mesenchymal transition, differentiation, osteogenesis

Nonstandard Abbreviations and Acronyms:
ALK Activin-like kinase receptor
ALP Alkaline phosphatase
BMP Bone morphogenetic protein
Cbfal Core binding factor alpha 1
CD Cluster of differentiation
EndMT Endothelial-mesenchymal transition
Flk-1 Fetal liver kinase 1
FOP Fibrodysplasia ossificans progressiva
Gla Gamma-carboxyglutamic acid
HAEC Human aortic endothelial cells
IgG Immunoglobulin G
MGP Matrix Gla protein
pSMAD Phosphorylated SMAD
SMAD Homolog of the drosophila protein, mothers against decapentaplegic (MAD) and the C. elegans protein SMA
SMC Smooth muscle cells
SSEA Stage-specific embryonic antigen
tg transgenic
Tie2 Tyrosine-protein kinase receptor TIE-2
vWF von Willebrand factor
wt wild type
INTRODUCTION

Vascular calcification is a frequent complication of vascular disease such as diabetes mellitus, renal disease and atherosclerosis, and is associated with an increased risk of cardiovascular and all-cause mortality. It is a regulated process with strong similarities to bone formation driven by osteochondrogenic progenitor cells. Vascular medial cells functioning as adult mesenchymal stem cells or smooth muscle cells (SMCs) transdifferentiating into multipotent cells are believed to be the major contributors of such progenitor cells.

Interestingly, endothelial cells (ECs) have been reported to contribute osteoblastic cells in fibrodysplasia ossificans progressiva (FOP), a rare genetic disorder linked to mutations in the activin receptor-like kinase 2 (ALK2), a receptor for bone morphogenetic protein 4 (BMP4). FOP is characterized by the development of soft tissue masses outside major vessels, in which ECs undergo endothelial-mesenchymal transitions (EndMT) and contribute cells to the ectopic ossification. Furthermore, prostate tumor endothelial cells have been shown to undergo mesenchymal-like transitions and osteochondrogenic differentiation, mitral valve leaflets contain ECs with osteogenic potential, and high glucose can induce transdifferentiation into chondrocyte-like cells in human aortic ECs. However, it is not known whether the arterial endothelium can act as a source of osteoprogenitor cells in vascular calcification, a frequent complication of vascular disease.

BMP signaling is known to promote vascular calcification, and others and we have reported that limiting vascular BMP signaling decreases both atherosclerotic lesion and diabetic medial calcification. Endothelial expression of BMP2 and 4 is highly responsive to pathological stimuli such as disturbed flow, increased oxidative stress, inflammation, and hyperglycemia, which may contribute to calcification. In diabetes, there is also a differential induction of BMP2 and 4 in the vascular wall; BMP2 is induced in the vascular media where it may have an osteoinductive effect, whereas BMP4 is preferentially induced in the endothelium. Activation of BMP signaling leads to an induction of BMP inhibitors, which provide negative feedback regulation. Matrix Gla protein (MGP), which directly binds and inhibits BMP2, 4 and 7, is readily induced in response to BMP activation in the vascular wall. Loss of MGP causes excess BMP activity, extensive media calcification and early death due to vascular rupture, whereas the presence of a Mgp transgene increases the MGP induction in response to BMP, enhancing the regulation of the BMP activity.

Here, we demonstrate that endothelium may be a source of multipotent osteoprogenitor cells in vascular calcification that occurs in the setting of high BMP activity in mice lacking MGP and in diabetic mice with high vascular BMP expression.

METHODS

See Supplemental Material for further details on fluorescence-activated cell sorting (FACS) analysis, immunostaining, transmission electron microscopy (TEM), and analytical procedures.

Animals.
Mgp mice on C57BL/6J background were obtained from Dr. Cecilia Giachelli (University of Washington, Seattle) with the permission of Dr. Gerard Karsenty (Columbia University, New York), and have been backcrossed more than 10 times. Ins2Akita/+ mice (strain C57BL/6-Ins Akita/J, stock # 003548), which are heterozygous for a mutation in one allele of the insulin-2 gene, were obtained from the Jackson Laboratory. Mgp/wt mice, generated in our laboratory on a C57BL/6J background, were crossed with Ins2Akita/+ mice to generate Mgp/wt,Ins2Akita/+ mice. Heterozygous Mgp/wt mice were used...
because the phenotype was apparent in \textit{Mgp}^{tg/wt} mice, and a low birth rate made it difficult to obtain hemizygous \textit{Mgp}^{tg} mice \cite{22}. \textit{Tie2-Gfp} transgenic (tg) mice (strain Tg(TIE2GFP)287Sato/J, stock # 003658), which express Green Fluorescent Protein (GFP) under the control of the endothelial-specific \textit{Tie2} promoter, were obtained from the Jackson Laboratory. Genotypes were confirmed by PCR \cite{2, 22, 23}, and experiments were performed with generation F4–F6. All mice were fed a standard chow diet (8604 Teklad Rodent Diet, Harlan Laboratories). \textit{Mgp}^{−/−} and \textit{Mgp}^{−/−};\textit{Tie2-Ggp}tg mice were used for experiments at 4 weeks of age, whereas \textit{Ins2}^{Akita/−}, \textit{Mgp}^{tg/wt};\textit{Ins2}^{Akita/−} and \textit{Ins2}^{Akita/−};\textit{Tie2-Ggp}tg mice were used at 35-40 weeks of age. Only male mice with the \textit{Ins2-Akita} mutation were used for experiments, and littermates were used as controls. The investigation conformed to the \textit{Guide for the Care and Use of Laboratory Animals} published by the US National Institutes of Health (NIH Publications No. 85-23, revised 1996), and had been reviewed and approved by the Institutional Review Board of the University of California, Los Angeles.

\textbf{Cell culture and SiRNA transfection.}

Human aortic endothelial cells (HAECs) were prepared and cultured as described \cite{15, 24}. Transient transfections of HAEC with siRNA were performed with Lipofectamine\textsuperscript{TM}2000 (Invitrogen) using 60 nM siRNA as described \cite{15}. Three separate siRNAs to each protein (Silencer\textsuperscript{®} predesigned siRNA, Ambion) and scrambled siRNA with the same nucleotide content were tested. The siRNA that provided the most efficient inhibition (90–95%), as determined by real-time PCR and immunoblotting or immunostaining, was used for experiments. Silencer\textsuperscript{®} predesigned siRNAs were obtained for MGP, \textit{Cbfa1}, and SM22\textit{α}.

Treatments were initiated 12-24 hours after the start of transfection, after removal of the transfection agent. For treatment, BMP2, BMP4, Noggin (all from R&D Systems), glucose, and osteogenic medium were added as indicated in the text.

\textbf{FACS.}

FACS was performed as detailed in Supplemental Methods.

\textbf{RNA analysis.}

RT-PCR and real-time PCR were performed as described \cite{15}. GAPDH was used as a control gene. Primers and probes for CD31, VE-cadherin, Flk-1, Sox2, Nanog, Oct3/4, \textit{Cbfa1}, and Osterix were obtained from Applied Biosystems as part of TaqMan Gene Expression Assays.

\textbf{Immunoblotting.}

Immunoblotting was performed as described \cite{25, 26}. Equal amounts of tissue or cellular protein were used. Tissues were collected at 4 weeks for \textit{Mgp}^{−/−} mice and controls, and at 35-40 weeks for mice with the \textit{Ins2}^{Akita} mutation and controls. Blots were incubated with specific antibodies to CD31 (300 ng/ml; Cell Signaling Technology); VE-cadherin (400 ng/ml; Santa Cruz Biotechnology); Flk-1 (200 ng/ml; Santa Cruz Biotechnology); Sox2 (200 ng/ml; Cell Signaling); Nanog (400 ng/ml; BD Pharmaningen and eBioscience); Oct3/4 (200 ng/ml; R&D Systems); \textit{Cbfa1} (500 ng/ml; Oncogene Research Products); Osterix); SM22\textit{α} (200 ng/ml; Santa Cruz Biotechnology) and \textit{αSMA} (200 ng/ml; R&D Systems). β-Actin (1:5,000 dilution; Sigma-Aldrich) was used as loading control.

\textbf{Immunostaining.}

The tissues were collected at 4 weeks for \textit{Mgp}^{−/−} mice and 35-40 weeks for mice with the \textit{Ins2}^{Akita} mutation, and the proximal descending aorta was used for tissue sections (Supplemental Figure I, left). 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 \textit{Mgp}^{−/−} mice was very extensive and uniform (Supplemental Figure I, right). The tissue sections were fixed in 4% paraformaldehyde and processed as described \cite{9}. Cultured cells were grown in chamber slides and fixed in 4% paraformaldehyde for 30 minutes, permeabilized with 0.1% Triton X-100, and blocked with 1% goat serum and 1% bovine serum.
albumin in Tris-buffered saline, and incubated overnight. The cells were immunostained using the same protocol as the tissues.

Immunofluorescence was performed as described in Supplemental Methods 7. We used specific antibodies for CD31 (Millipore), vWF (Dako), Cbfa1 and Osterix (Oncogene Research Products), SM22α (Santa Cruz Biotechnology), αSMA and Oct3/4 (R&D Systems), Sox2 (Cell Signaling), Nanog (BD Pharmingen and eBioscience), GFP (Abcam), and MGP (Dr. Reidar Wallin, Wake Forest University). The nuclei were stained with 4',6-Diamidino-2-Phenylindole (DAPI) (Sigma-Aldrich) 24. Non-specific IgG was included as a primary antibody control in all experiments, where it showed no significant staining, which has been included in selected figures.

**TEM.**
Aortic tissue samples were analyzed by TEM as described in Supplemental Methods.

**Histochemical staining.**
Histochemical staining for alkaline phosphatase activity and mineral (Alizarin Red and Von Kossa) was performed as previously described 7.

**Analytical procedures.**
Samples were analyzed as described in Supplemental Methods.

**Statistical analysis.**
Data were analyzed for statistical significance by two-way analysis of variance with post hoc Tukey’s analysis using the GraphPad Instat® 3.0 software (GraphPad Software, San Diego, CA). P-values less than 0.05 were considered significant. All experiments were repeated a minimum of three times.

**RESULTS**

*Endothelial origin of osteogenic cells in Mgp−/− calcified aortas.*

To determine whether the endothelium contributes osteoprogenitor cells to vascular calcification, we first compared the aortic endothelium in wild type and Mgp−/− mice, a well-known model of vascular calcification 19. These mice are known to be tachycardic 19 with a very high pulse wave velocity in the aorta at rest 27, presumably from the dramatic increase in aortic calcium 8, and endothelial dysfunction as evidenced by minimal inflammatory response to hyperlipidemia 8 and the formation of arteriovenous malformations 17. In Mgp−/− mice, the endothelium was highly abnormal as visualized by phase contrast and transmission electron microscopy; a mixture of cells largely replaced normal ECs, including chondroblast-like cells (Figure 1A, the magnification is the same in all panels). Occasionally, EC-like cells were detected that appeared to have detached from the internal elastic lamina (IEL) and were surrounded by abnormal matrix (Figure 1A, bottom panels). The abnormalities were associated with increased aortic expression of EC lineage markers CD31, VE-cadherin and fetal liver kinase 1 (Flk-1), as determined by real-time PCR and immunoblotting (Figure 1B). Furthermore, CD31 and von Willebrand factor (vWF) expression was detected deep in the calcified media (Figure 1B), and immunostaining showed co-expression of CD31 and vWF, respectively, with osteogenic markers core binding factor alpha 1 (Cbfa1) and Osterix (Figure 1C). The Mgp−/− aortas contained about 28.8% cells that double-stained for CD31 and Cbfa1, as determined by FACS after dispersion of aortic cells (Figure 1D) and exclusion of CD45+ cells, which may represent CD31+ leukocyte populations. The efficiency of the CD45 pre-sorting was checked with FACS (Supplemental Figure II). Cells that co-expressed CD31 and a bone marker also appeared to penetrate into the media (Figure 1E).
To confirm the endothelial origin of the osteogenic cells, we performed lineage tracing as described 3. We crossed Tie2-Gfp transgenic (tg) reporter mice with Mgp+/+ mice to obtain Mgp−/−;Tie2-Gfp tg mice, in which GFP is expressed under the control of the endothelial-specific Tie2 promoter. Immunostaining revealed GFP-positive cells in the calcified aortic tissue of the Mgp−/−;Tie2-Gfp tg mice (Figure 1F). Furthermore, staining with anti-Cbfa1 and Osterix antibodies revealed GFP-positive cells that co-expressed Cbfa1 and Osterix (Figure 1G). GFP was only detected in the aortic endothelium in the Tie2-Gfp tg control mice.

Multipotent marker expression in Mgp−/− endothelium.

MGP is an efficient inhibitor of BMP4 16, which can activate the ALK2 receptor and promote EndMT in ECs 3. Therefore, we examined expression of multipotent markers Sox2, Nanog and Oct3/4 in the Mgp−/− aortas. The results revealed an increase of all 3 markers by immunoblotting (Figure 2A, left). The multipotent markers were detected in the cell nuclei by immunostaining, whereas the EC marker CD31 was found in the cell membranes of the same cells (Figure 2A, right). We also examined multipotent markers in the aortas of Mgp−/−;Tie2-Gfp tg mice. The results confirmed the increase in Sox2, Nanog and Oct3/4 expression by immunoblotting (Figure 2B, left). Sox2, Nanog and Oct3/4 were localized in the nuclei of GFP-expressing cells of Tie2-positive lineage by immunostaining (Figure 2B, right). Cells that only stained for the stem cell markers were noted in both Mgp−/− and Mgp−/−;Tie2-Gfp tg aortas (Figure 2A, B) and may represent SMC-like or other multipotent cells. FACS after aortic cell dispersion and exclusion of CD45+ cells showed that about 32.1% co-stained for CD31 and Sox2 (Figure 2C). Together, the results from Mgp−/− and Mgp−/−;Tie2-Gfp tg mice suggested that MGP-deficiency promotes multipotency in ECs.

Time course of multipotent and osteogenic marker expression in Mgp−/− aorta.

To better understand the time course of the aortic changes, we collected aortas from Mgp−/− mice on postnatal day (P)2, 4, 6, 8, 10, 15, 20, and 30, and examined the aortic tissues by H&E staining, real-time PCR and immunostaining. The H&E staining showed mild abnormalities on P8, and gross calcification on P15-30, which appeared to start on the endothelial side of the vessel wall (Figure 3A, see Supplemental Figure III for higher magnification). Real-time PCR showed that expression of EC markers CD31 and VE-cadherin increased as early as P4 in the Mgp−/− mice, and peaked at P15 (Figure 3B). The expression of Sox2, Nanog and Oct3/4 increased on P4-6 and peaked on P15 similar to the EC markers, whereas the expression of Cbfa1 and Osterix increased on P8 and had not reached a clear peak on P30 (Figure 3B). The expression of all markers was unchanged in the wild type mice. The time course of MGP expression in wild type mice was similar to that of the EC and multipotency markers in the Mgp−/− mice, whereas the BMP4 expression was similar in both mice (Figure 3B). MGP was not detected in the Mgp−/− mice as expected. Immunostaining revealed mild CD31 expression in the media of Mgp−/− mice on P6, which increased through P30 (Figure 4A). Sox2 and Osterix expression appeared on P8 and P10, respectively, and increased through P30 (Figure 4B, C). No significant change in CD31 expression was detected in the wild type endothelium, and Sox2 or Osterix were not detected. Overall, the data support that CD31 and the multipotency markers increase prior to the bone markers, suggesting a temporal relationship. Interestingly, the finding that the expression pattern of CD31 and Sox2 resembles that of MGP in the wild type mice suggests that MGP is required during this time period to promote EC differentiation.

Endothelial origin of osteogenic and multipotent cells in aortas of diabetic Ins2Akita/+ mice.

We previously showed increased aortic BMP activity in diabetic Ins2Akita/+ mice associated with aortic osteogenesis and calcium accumulation 9. These mice are known to develop diabetic cardiomyopathy but heart rate and blood pressure remains largely unchanged 28,29. In addition, there were
no significant differences in serum phosphate and total cholesterol between wild type and $\text{Ins}^2_{\text{Akita/+}}$ mice (Supplemental Table I).

To determine whether the endothelium was a source of multipotent cells in the $\text{Ins}^2_{\text{Akita/+}}$ mice, we first demonstrated expression of CD31 and vWF in areas of calcification in the vascular media (Figure 5A). Immunostaining revealed co-expression of CD31 and vWF with Cbfal and Osterix in the calcified areas (Figure 5B), and FACS analysis showed that 19.2% of the aortic cells double-stained for CD31 and Cbfal after exclusion of CD45+ cells (Figure 5C). The $\text{Ins}^2_{\text{Akita/+}}$ mice were then crossed with the $\text{Tie2-Gfp}\tg$ mice for lineage tracing. When examining the aortas of the $\text{Ins}^2_{\text{Akita/+}};\text{Tie2-Gfp}\tg$ mice, we detected GFP-positive cells in the endothelium and the media (Figure 5D), which co-expressed Cbfal and Osterix (Figure 5E), suggesting that the osteogenic cells were EC-derived. The aortic expression of Sox2, Nanog, and Oct3/4 was also increased in both $\text{Ins}^2_{\text{Akita/+}}$ and $\text{Ins}^2_{\text{Akita/+}};\text{Tie2-Gfp}\tg$ mice, as determined by immunoblotting (Figure 6A), and immunostaining (Figure 6B, C). FACS analysis showed that 21.5% of the cells co-expressed CD31 and Sox2 after exclusion of CD45+ cells (Figure 6D).

*Enhanced MGP expression limits multipotency in aortas of $\text{Ins}^2_{\text{Akita/+}}$ mice.*

Our previous study showed that enhanced MGP expression limited BMP activity and aortic calcification when $\text{Ins}^2_{\text{Akita/+}}$ mice were crossed with MGP transgenic ($\text{Mgp}^{\text{tg/wt}}$) mice. We examined whether the enhanced MGP expression also limited multipotent marker expression in $\text{Ins}^2_{\text{Akita/+}};\text{Mgp}^{\text{tg/wt}}$ mice. The results revealed reductions in Sox2, Nanog, and Oct3/4 as determined by real-time PCR and immunoblotting (Figure 6E, F), supporting that the enhanced aortic expression of MGP also reduced multipotency in the diabetic aortas. Immunostaining showed that increased MGP limited CD31 expression to the endothelium (Figure 6G).

*Endothelial cells acquire multipotency and susceptibility to osteoinduction after depletion of MGP.*

To examine the effect of loss of MGP on multipotency in vitro, HAECS were transfected with MGP siRNA or scrambled control siRNA (SCR). More than >99.5% of the HAECS expressed CD31 by FACS (data not shown). The siRNA transfection decreased MGP protein levels to <10-20% of normal levels as shown by immunostaining after 20-24 hours (Supplemental Figure IV). We also combined the siRNA transfection with treatment of the cells with BMP4 (40 ng/ml), Noggin (a BMP inhibitor) (200 ng/ml), or BMP4 and Noggin, added 12 hours after the transfection. The results showed that expression of Sox2, Nanog, and Oct3/4 increased after MGP depletion, as determined by immunoblotting (Figure 7A, lanes 1-2), BMP4 further increased expression of Sox2, Nanog and Oct3/4, whereas Noggin abolished the marker expression (Figure 7A, lanes 3-6). Added together, BMP4 and Noggin were similar to control (Figure 7A, lanes 7-8). We confirmed that BMP4 increased multipotency in MGP-depleted HAECS by FACS analysis. Co-expression of the stem cell markers SSEA-3 and SSEA-4, and CD31 was observed in MGP-depleted HAECS (Figure 7B, left). BMP4 increased the population of SSEA-3+/CD31+ cells from 18.9% to 57.1%, and the population of SSEA-4+/CD31+ cells from 22.8% to 60.1% (Figure 7B, right).

To test for osteogenic differentiation in MGP-depleted HAECS, we treated with osteogenic medium, BMP2 (200 ng/ml), or a combination of osteogenic medium and BMP2 for 4 days starting the day after transfection. Alternatively, we replaced the osteogenic medium with glucose (22 nM) in order to mimic hyperglycemia. Expression of Sox2, Nanog and Oct3/4 increased after MGP depletion, but decreased when the osteoinduction was strongly promoted by the combination of BMP2 and osteogenic medium or glucose, as determined by immunoblotting (Figure 7C & E, top 3 panels). Expression of the osteogenic markers Cbfal and Osterix, as well as the early smooth muscle cell (SMC) markers SM22$\alpha$ and $\alpha$-SM actin ($\alpha$SMA) increased in all three conditions (Figure 7C & E, bottom panels). Interestingly, expression of SM-myosin heavy chain (SM-MHC), a late SMC marker, was not detected in any of the samples (Figure 7C & E, bottom panels). Alkaline phosphatase (ALP) activity, an early osteogenic
marker, and calcium accumulation, a late osteogenic marker, increased in the MGP-depleted HAECs after 7 and 14 days of treatment, respectively, as determined by ALP and mineral staining (Alizarin Red and Von Kossa) (Figure 7D & F). Altogether, the results are consistent with the in vivo experiments, and support that endothelial MGP depletion may cause multipotency and osteoinduction.

**SM22α expression is not required for expression of osteogenic markers in endothelial cells.**

Speer et al. recently reported that osteochondrogenic cells in Mgp−/− aortas transdifferentiate from vascular SMCs based on lineage tracing using SM22α-Cre;R26R-LacZ mice. Sun et al. similarly used SM22α-Cre mice to specifically delete Cbfa1 (Runx2) in SM22α-expressing cells, and concluded that SMC-derived Cbfa1 regulated vascular calcification. Although SM22α is considered an early marker of SMCs, it is also expressed in myofibroblasts, pericytes, and after EndMT. To determine if SMC markers were induced in the MGP-deficient ECs, we co-stained aortas from Mgp−/− and Ins2Akita/+ mice with antibodies to CD31 and SM22α or αSMA. The results showed co-expression of CD31 and SM22α or αSMA in both aortas (Figure 8A). We then tested if SM22α was required for osteoinduction in HAECs in vitro, and conversely, if Cbfa1 was required for expression of early SMC markers. HAECs were transfected with SCR or MGP siRNA, and MGP siRNA was co-transfected with SM22α or Cbfa1 siRNA. The cells were treated with osteogenic medium and BMP2 as before, and expression of Cbfa1, Osterix, SM22α, αSMA and SM-MHC was determined by immunoblotting. The depletion of Cbfa1 did not affect expression of SM22α or αSMA, and depletion of SM22α did not affect expression of Cbfa1 or Osterix (Figure 8B). No expression of SM-MHC was detected in any of the samples, suggesting that the cells do not undergo late SMC differentiation. The results suggested that expression of SM22α is not required for the ECs to undergo osteogenic differentiation.

**DISCUSSION**

In this report, we demonstrate that the vascular endothelium acts as a source of multipotent cells that may contribute to vascular calcification in states of high BMP activity, such as lack of the BMP-inhibitor MGP and hyperglycemia. Vascular calcification could therefore be considered an acquired stem cell disorder in these settings.

It has previously been shown that BMP4 binds to the ALK2 receptor, which allows for EndMT and the generation of cells that are able to undergo osteoinduction. FOP, characterized by ectopic soft tissue calcification, is caused by mutations in ALK2 that render the receptor constitutively active. In our study, ALK2 is activated in the aortic endothelium of both Mgp−/− and the Ins2Akita/+ mice, in the Mgp−/− mice due to lack of BMP4 inhibition, and in the Ins2Akita/+ mice due to induction of BMP4 and ALK2. However, the resulting EndMT and multipotent cells appear to be restricted to the artery wall in these mice, whereas FOP lesions are found outside the major vessels. In the diabetic mice, the increase in BMP4/ALK2 activity overwhelms the available BMP inhibition, which can be enhanced by increasing the expression of MGP through a transgene. The increase in MGP led to a limitation of vascular calcification in the previous study, and was consistent with the decrease in the expression of multipotency markers seen in the current study. EndMT have also been reported in ECs derived from the mitral valve leaflets, and in HAECs in vitro, which together with our data support an important role for the endothelium in the development of cardiovascular calcification.

Mechanistically, BMP4 is known to induce expression of MGP in ECs, which provides negative feedback inhibition for BMP2 and 4 and regulates BMP-induced events in the vascular wall. Such activities may include the promotion of multipotency, EC proliferation and differentiation, and...
osteogenic induction 13, 17, 35-37. BMP4 alone was sufficient to stimulate osteogenic induction in human umbilical vein ECs and human cutaneous microvascular ECs 3, whereas MGP depletion, preferably in combination with BMP treatment, induced osteogenic differentiation in the HAECs in our experiments. The optimal balance between BMP4 treatment and MGP depletion required for osteoinduction may vary between different types of cultured ECs, and is not yet fully elucidated.

We used the Tie2-Gfp transgene for endothelial lineage tracing in an approach similar to that used by other investigators 3. Expression of Tie2 and VE-cadherin, both commonly used for lineage tracing and excision in the endothelium, has been detected in small subpopulations of hematopoietic cells, and in areas of endocardial-mesenchymal transformation in the embryonic atrioventricular canal and outflow tract 38, 39. Although it is impossible to exclude, it is less likely that hematopoietic cells, such as monocytes, would directly transition to osteoprogenitor cells in these studies, especially in the Mgp-/- aortas where the expression of inflammatory and monocyte markers is minimal or undetectable 8. Furthermore, even if the exclusion of CD45+ cells prior to FACS analysis of aortic cells from Mgp-/- and Ins2Akita/+ may have removed a small number of CD45+CD31+ leukocytic cells, 20-30% of the analyzed cells still co-expressed CD31 and Cbfa1 or Sox2. Finally, our lineage tracing was accompanied by co-staining of EC and multipotency or osteogenic markers, which gave similar results, supporting our conclusions. Overall, this supports that osteogenic cells can be derived from the vascular endothelium.

The goal of our study is to determine whether ECs can give rise to osteogenic cells. Thus, an analysis of the origin of the ECs is beyond the scope of this study. The ECs may be derived locally or from EC progenitors from the bone marrow. Indeed, Cho et al. recently showed that bone-marrow derived cells easily gained access to atherosclerotic aortic wall in Apoe-/- mice 40, and Naik et al. estimated that bone marrow-derived cells account for ~20% of Cbfa1-positive cells in the calcified atherosclerotic vessels of Apoe-/- mice 41. However, none of these investigators explored whether the bone marrow-derived cells differentiated into ECs or EC-like cells.

It has been proposed that osteoblastic cells in the media are derived from SMCs based on lineage tracing using SM22α-LacZ transgenic mice and Cbfa1 (Runx2) deletion in SM22α-expressing cells 2, 30. The osteoblastic cells could also be derived from a new type of multipotential vascular stem cells recently identified in the blood vessel wall, which become proliferative and undergo SMC and osteochondrogenic cell differentiation after vascular injury 42. In our studies, MGP-depletion in the HAECs induced expression of both early SMC markers (SM22α and αSMA) and osteogenic markers (Cbfa1 and Osterix). However, no expression of the late SMC marker SM-MHC was detected. Furthermore, depletion of both MGP and SM22α still allowed for the induction of the osteogenic markers, suggesting that SM22α is not required for osteoinduction in these cells. Thus, it is possible that osteoprogenitor cells derived from the endothelium do not express SM22α and would not be detected when the SM22α-promoter is used for lineage tracing. Alternatively, SM22α-expressing cells in the vascular wall, known to contribute to vascular calcification, may be the result of prior EndMT in the vascular endothelium.

Induction of multipotency by BMP in endothelium is an important physiological mechanism during development and after injury. Such activation would provide a local source of stem cells that could promote growth or healing of the vasculature itself, or tissue-specific cell differentiation depending on local cues. It would be consistent with the concept of “stemness” being a function of the local context 43. Overall, our data support that diseased endothelium with excess BMP activity may contribute osteoprogenitor cells to vascular calcification.
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DISCLOSURES
None.

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14. Chang K, Weiss D, Sue J, Vega JD, Giddens D, Taylor WR, Jo H. Bone morphogenetic protein antagonists are coexpressed with bone morphogenic protein 4 in endothelial cells exposed to...


FIGURE LEGENDS

**Figure 1:** Endothelium contributes cells to aortic calcification of Mgp<sup>−/−</sup> mice. (A) Aortic wall (confocal microscopy, top 2 panels), and aortic endothelium () from wild type (Mgp<sup>+/+</sup>) and Mgp<sup>−/−</sup> mice. DIC: differential interference contrast. EM: electron microscopy. Magnification for EM, 3.7 x10<sup>3</sup>. (B) Aortic expression of endothelial markers CD31, VE-cadherin (VE-cad), Flk-1 and vWF in Mgp<sup>+/+</sup> and Mgp<sup>−/−</sup> mice determined by real-time PCR, immunoblotting and immunostaining. ***, p <0.001. (C) Immunostaining of aortic tissues from Mgp<sup>+/+</sup> and Mgp<sup>−/−</sup> mice showed co-expression of endothelial markers CD31 and vWF (bottom) and osteogenic markers Cbfa1 and Osterix (OSX) in the Mgp<sup>−/−</sup> mice. (D) Co-expression of CD31 and Cbfa1 in enzymatically dispersed CD45-negative aortic cells from Mgp<sup>+/+</sup> and Mgp<sup>−/−</sup> mice, as determined by FACS. (E) Cells co-expressing CD31 and that have penetrated into the medial layer in Mgp<sup>−/−</sup> aorta. (F) Visualization of GFP (top) and immunostaining with anti-GFP antibodies (bottom) in aortic tissues of Tie2-Gfp<sup>tg</sup> and Mgp<sup>−/−</sup>;Tie2-Gfp<sup>tg</sup> mice. (G) Immunostaining of aortic tissues from Tie2-Gfp<sup>tg</sup> and Mgp<sup>−/−</sup>;Tie2-Gfp<sup>tg</sup> mice showed co-expression of GFP with Cbfa1 and OSX in the Mgp<sup>−/−</sup>;Tie2-Gfp<sup>tg</sup> mice. Scale bars, 100 µm. DAPI (blue) was used to visualize nuclei. Non-specific IgG control showed no staining. Vessel lumen faces upwards in the photos unless otherwise indicated.

**Figure 2:** Multipotent marker expression in Mgp<sup>−/−</sup> and Mgp<sup>−/−</sup>;Tie2-Gfp<sup>tg</sup> endothelium. (A) Aortic expression of Sox2, Nanog and Oct3/4 in Mgp<sup>+/+</sup> and Mgp<sup>−/−</sup> mice detected by immunoblotting (left) and immunostaining (right). ß-actin was used as control. (B) Aortic expression of Sox2, Nanog and Oct3/4 in Tie2-Gfp<sup>tg</sup> and Mgp<sup>−/−</sup>;Tie2-Gfp<sup>tg</sup> mice determined by immunoblotting (left) and immunostaining (right). ß-actin was used as control. (C) Co-expression of CD31 and Sox2 in enzymatically dispersed CD45-negative aortic cells from Mgp<sup>+/+</sup> and Mgp<sup>−/−</sup> mice, as determined by FACS. Scale bars, 50 µm. Non-specific IgG control showed no staining. Vessel lumen faces upwards in the photos.

**Figure 3:** Time course of aortic changes in Mgp<sup>−/−</sup> mouse aorta. (A) Aortas were collected between postnatal day (P) 2-30 from Mgp<sup>+/+</sup> and Mgp<sup>−/−</sup> mice as indicated, and stained with H&E. (B) Time course of aortic expression in Mgp<sup>+/+</sup> and Mgp<sup>−/−</sup> aorta (P2-30) of MGP, BMP4, EC markers VE-cadherin (VE-cad) and CD31, osteogenic markers Cbfa1 and Osterix (OSX), and multipotency markers Nanog, Sox2 and Oct3/4. The expression was compared to that on P2. Scale bars, 100 µm.

**Figure 4:** Time course of aortic changes in Mgp<sup>−/−</sup> mouse aorta. (A-C) Aortas were collected between postnatal day (P) 2-30 from Mgp<sup>+/+</sup> and Mgp<sup>−/−</sup> mice as indicated. They were immunostained for (A) CD31, (B) co-stained for CD31 and Sox2, and (C) co-stained for CD31 and Osterix (OSX). DAPI (blue) was used to visualize nuclei. Vessel lumen faces upwards or to the right in the photos.

**Figure 5:** Endothelium contributes cells to aortic calcification of Ins2<sup>Akita/+</sup> mice. (A) Aortic expression of endothelial markers CD31 and vWF in Ins2<sup>Akita/+</sup> mice visualized by immunostaining. (B) Immunostaining of aortic tissues from wild type (WT) and Ins2<sup>Akita/+</sup> mice showed co-expression of endothelial markers CD31 (left) and vWF (right) and osteogenic markers Cbfa1 and Osterix (OSX) in the Ins2<sup>Akita/+</sup> mice. (C) Co-expression of CD31 and Cbfa1 in enzymatically dispersed aortic cells from WT and Ins2<sup>Akita/+</sup> mice, as determined by FACS. (D) Aortic expression of GFP in Tie2-Gfp<sup>tg</sup> and Ins2<sup>Akita/+</sup>;Tie2-Gfp<sup>tg</sup> mice by immunostaining with anti-GFP antibodies. (E) Immunostaining of aortic tissues from Tie2-Gfp<sup>tg</sup> and Ins2<sup>Akita/+</sup>;Tie2-Gfp<sup>tg</sup> mice showed co-expression of GFP with Cbfa1 and OSX in the Ins2<sup>Akita/+</sup>;Tie2-Gfp<sup>tg</sup> mice. Scale bars, 100 µm. DAPI (blue) was used to visualize nuclei. Non-specific IgG control showed no staining. Vessel lumen faces upwards in the photos.
Figure 6: Endothelial origin of multipotent cells in aortas of diabetic Ins2^{Akita/+} mice. (A) Aortic expression of Sox2, Nanog and Oct3/4 in wild type (WT), Ins2^{Akita/+}, Ins2^{Akita/+};Tie2-Gfp and Ins2^{Akita/+};Tie2-Gfp mice. ß-actin was used as control. (B) Co-expression of CD31 with Sox2, Nanog and Oct3/4 in aortas of Ins2^{Akita/+} mice detected by immunostaining. (C) Co-expression of GFP with Sox2, Nanog and Oct3/4 in aortas of Tie2-Gfp and Ins2^{Akita/+};Tie2-Gfp mice detected by immunostaining. (D) Co-expression of CD31 and Sox2 in enzymatically dispersed CD45-negative aortic cells from WT and Ins2^{Akita/+} mice, as determined by FACS. (E-G) Enhanced MGP expression limits aortic expression of Sox2, Nanog and Oct3/4 in Ins2^{Akita/+} mice, as determined by (E) real-time PCR, (F) immunoblotting (ß-actin was used as control), and (G) immunostaining in WT, Mgp^{wt/wt}, Ins2^{Akita/+}, and Ins2^{Akita/+};Mgp^{wt/wt} mice. Scale bars, 50 μm. DAPI (blue) was used to visualize nuclei. Vessel lumen faces upwards in the photos.

Figure 7: Depletion of MGP allows for multipotency and osteoinduction in human aortic endothelial cells (HAECs). (A) Expression of multipotent markers Sox2, Nanog and Oct3/4 in HAECs after transfection of scrambled control siRNA (SCR) or MGP siRNA only (lane 1, 2), or MGP siRNA transfection with BMP4 treatment (lane 3, 4), siRNA transfection with Noggin treatment (lane 5, 6), or siRNA transfection with BMP4 and Noggin treatment (lane 7, 8), as determined by immunoblotting. (B) Co-expression of CD31 and SSEA-3, or CD31 and SSEA-4 after transfection of scrambled control siRNA or MGP siRNA without additional treatment (left), and with BMP4 treatment (right), as determined by FACS analysis. (C) Expression of multipotent markers Sox2, Nanog and Oct3/4, osteogenic markers Cbfa1, Osterix (OSX), early SMC markers SM22α, α-SM actin (αSMA), and late SMC marker SM-myosin heavy chain (SM-MHC) (top) after transfection of scrambled control siRNA or MGP siRNA and treatment with control (C), BMP2 (B), osteogenic media (O) or both (B+O), as determined by immunoblotting. (D) Staining for alkaline phosphatase (ALP), and mineral (Alizarin Red and Von Kossa staining) in HAECs treated as described in (c). (E) Expression of multipotent markers Sox2, Nanog and Oct3/4, osteogenic markers Cbfa1, OSX, SM22α, αSMA, and SM-MHC (top) after transfection of scrambled control siRNA or MGP siRNA and treatment with control (C), BMP2 (B), high glucose medium (G) or both (B+G), as determined by immunoblotting. (F) Staining for ALP and mineral in HAECs treated as described in (E).

Figure 8: Osteoinduction in MGP-depleted human aortic endothelial cells does not depend on SM22α expression. (A) (Top) Immunostaining of aortic tissues from Mgp^{+/+} and Mgp^{−/−} mice showed co-expression of endothelial marker CD31 and early SMC markers SM22α and αSMA in the Mgp^{−/−} mice. (Bottom) Immunostaining of aortic tissues from wild type (WT) and Ins2^{Akita/+} mice showed co-expression of CD31 with SM22α and αSMA in the Ins2^{Akita/+} mice. Scale bars, 100 μm. (B) HAECs were transfected with scrambled control siRNA (SCR) (lane 1), or Mgp siRNA with either SCR (lane 2), Cbfa1 siRNA (lane 3), or SM22a siRNA (lane 4). Expression of Cbfa1, Osterix (OSX), SM22α, α-SM actin (αSMA), and SM-myosin heavy chain (SM-MHC) was determined by immunoblotting. ß-actin was used as control. Scale bars, 100 μm. DAPI (blue) was used to visualize nuclei. Vessel lumen faces upwards in the photos.
Novelty and Significance

What Is Known?

- Vascular calcification is a frequent complication of diabetic vasculopathy and atherosclerosis.
- Vascular calcification is a regulated process driven by osteochondrogenic differentiation of vascular medial cells.
- Bone morphogenetic proteins (BMPs) promote vascular calcification, whereas Matrix Gla protein (MGP), a BMP inhibitor, protects against calcification.

What New Information Does This Article Contribute?

- The vascular endothelium contributes osteoprogenitor cells to vascular calcification in diabetic mice and MGP-null mice.
- Enhanced BMP signaling in endothelial cells (due to the MGP knockout or enhanced BMP expression in diabetes) results in endothelial-mesenchymal transitions and the emergence of multipotent cells, which are susceptible to bone induction.
- MGP prevents the generation of multipotent cells from the vascular endothelium.

Vascular calcification is a regulated process that involves osteoprogenitor cells and frequently complicates vascular disease. Nevertheless, the role of the vascular endothelium in this process is poorly understood. We report that the endothelium can directly contribute osteoprogenitor cells to the vascular calcification. Using mouse models and cultured endothelial cells, we demonstrate that increased BMP signaling, either due to lack of the BMP-inhibitor MGP or enhanced BMP expression, stimulates the endothelial-mesenchymal transition and the emergence of multipotent cells. These multipotent cells are susceptible to bone induction in the setting of disorders such as diabetic vasculopathy. These findings identify the endothelium as a new source of osteoprogenitor cells in vascular calcification and suggest that multipotency or “stemness” in the vascular wall is a function of the local context.
Figure 1

A

B

CD31

VE-cad

Flk-1

β-actin

CD31

DAPI

vWF

DAPI

IgG

DAPI

C

D

Mgp+/+

Mgp−/−

CD31

Cbfa1

CD31

OSX

vWF

Cbfa1

vWF

OSX

IgG

E

H&E

CD31/DAPI

CD31/OSX

F

Tie2-Gfptg

Mgp−/−Tie2-Gfptg

GFP

Cbfα1

GFP

OSX

αGFP

DAPI
Figure 2

**A**

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**C**

![Fluctuation of Sox2 and CD31](image)
Figure 4
Figure 6

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B

WT

Ins2<sup>Akita</sup>

C

Tie2-Gfp tg

Ins2<sup>Akita</sup> + Tie2-Gfp tg

D

WT

Ins2<sup>Akita</sup>

E

Expression (fold)

***

Sox2

Nanog

Oct3/4

F

WT

Mgp<sup>+/wt</sup>

Ins2<sup>Akita</sup>

Ins2<sup>Akita</sup> + Mgp<sup>+/wt</sup>

G

WT

Ins2<sup>Akita</sup>

Ins2<sup>Akita</sup> + Mgp<sup>+/wt</sup>

CD31

DAPI
Figure 7

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Figure 8
A Role for the Endothelium in Vascular Calcification
Yucheng Yao, Medet Jumabay, Albert Ly, Melina Radparvar, Mark R. Cubberly and Kristina I Bostrom

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Detailed Method Section

Animals

Mgp\(^{+/–}\) mice on C57BL/6J background were obtained from Dr. Cecilia Giachelli (University of Washington, Seattle) with the permission of Dr. Gerard Karsenty (Columbia University, New York), and have been backcrossed more than 10 times. Ins2\(^{Akita+}\) mice (strain C57BL/6-Ins\(^{Akita}\), stock # 003548), which are heterozygous for a mutation in one allele of the insulin-2 gene, were obtained from the Jackson Laboratory. Mgp\(^{tg/wt}\) mice, generated in our laboratory on a C57BL/6J background, were crossed with Ins2\(^{Akita+}\) mice to generate Mgp\(^{tg/wt},\)Ins2\(^{Akita+}\) mice. Heterozygous Mgp\(^{tg/wt}\) mice were used because the phenotype was apparent in Mgp\(^{tg/wt}\) mice, and a low birth rate made it difficult to obtain hemizygous Mgp\(^{tg/0}\) mice. Tie2-Gfp transgenic (tg) mice (strain Tg(TIE2GFP)287Sato/J, stock # 003658), which express Green Fluorescent Protein (GFP) under the control of the endothelial-specific Tie2 promoter, were obtained from the Jackson Laboratory. Genotypes were confirmed by PCR and experiments were performed with generation F4–F6. All mice were fed a standard chow diet (8604 Teklad Rodent Diet, Harlan Laboratories). Mgp\(^{+}\) and Mgp\(^{−}\), Tie2-Ggptg mice were used for experiments at 4 weeks of age, whereas Ins2\(^{Akita+/−}\), Mgp\(^{tg/wt}\), Ins2\(^{Akita+/−}\), and Ins2\(^{Akita+/−}\), Tie2-Ggptg mice were used at 35-40 weeks of age. Only male mice with the Ins2-Akita mutation were used for experiments, and littermates were used as controls. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publications No. 85–23, revised 1996), and had been reviewed and approved by the Institutional Review Board of the University of California, Los Angeles.

Cell Culture and SiRNA Transfection

Human aortic endothelial cells (HAECs) were prepared and cultured as described. Transient transfections of HAEC with siRNA were performed with Lipofectamine™2000 (Invitrogen) using 60 nM siRNA as described. Briefly, the amount of siRNA was optimized as per the manufacturer's instructions. Three separate siRNAs to each protein (Silencer® predesigned siRNA, Ambion) and scrambled siRNA with the same nucleotide content were tested. The siRNA that provided the most efficient inhibition (90–95%), as determined by real-time PCR and immunoblotting or immunostaining, was used for experiments. Silencer® predesigned siRNAs were obtained for MGP, Cbfa1, and SM22α. Treatments were initiated 3 hours after transfection, after removal of the transfection agent. For treatment, BMP4 (40 ng/ml) and BMP2 (300 ng/ml) (both from R&D Systems) were added as indicated in the text.

Flow Cytometric Analysis

Fluorescence-activated cell sorting (FACS) analysis as described. 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 CD31, CD45, SSEA-3 and SSEA-4 (1:500 for CD31 and CD45, and 1:200 for SSEA-3 and SSEA-4; BD Pharmingen and eBioscience). 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/AI-488 fluorescence using a PE/AI-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/AI-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). FACS files were exported and analyzed using BD Cellquest software v.3.3. Aortas were enzymatically
Transmission electron microscopy (TEM)
dispersed using a modification of a previously described protocol. 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, 2 aortas from wild type or \textit{Ins2}^{Akita/} mice, and 3 aortas from \textit{Mgp}^{−/} mice were required to obtain enough cells for analysis.

**RNA analysis**
RT-PCR and real-time PCR were performed as described. GAPDH was used as a control gene. Primers and probes for CD31, VE-cadherin, Flk-1, Sox2, Nanog, Oct3/4, Cbfa1, and Osterix were obtained from Applied Biosystems as part of TaqMan Gene Expression Assays.

**Immunofluorescence**
Immunofluorescence was performed as described\textsuperscript{10,11} on cryosections of wild type and \textit{Ins2}^{Akita/} mice. The tissue sections were fixed in 4% paraformaldehyde and processed as previously described. The tissue 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). 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), andsections 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 sample was incubated for 1-5 minutes and rinsed several times in PBS. Staining without primary antibodies served as controls. Images were acquired with an inverted Zeiss Axiovert 200 microscope (Carl Zeiss Micro Imaging Inc., Thornwood, NY, USA). 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 (Millipore), vWF (Dako), Cbfa1 and Osterix (Oncogene Research Products), SM22α (Santa Cruz Biotechnology), αSMA and Oct3/4 (R&D Systems), Sox2 (Cell Signaling), Nanog (BD Pharmingen and eBioscience), GFP (Abcam), and MGP (Dr. Reidar Wallin, Wake Forest University). The nuclei were stained with 4',6-Diamidino-2-Phenylindole (DAPI) (Sigma-Aldrich)\textsuperscript{24}. Non-specific IgG was included as a primary antibody control in all experiments, where it showed no significant staining, which has been included in selected figures.

\textit{Transmission electron microscopy (TEM)}
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 buffer and postfixed in a solution of 1% OsO4 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 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 then 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 lead citrate for 3 min at room temperature. Thin sections subsequently were examined with a 100CX JEOL electron microscope.

**Histochemical staining**

Histochemical staining for alkaline phosphatase activity and mineral (Alizarin Red and Von Kossa) was performed as previously described.

**Analytical Procedures**

Plasma glucose was measured by the glucose oxidase method (Beckman Glucose Analyzer 2; Beckman Coulter, Fullerton, CA). Serum phosphate was measured using the QuantiChrom™ Phosphate Assay kit as per manufacturer’s instructions (BioAssay Systems, Hayward, CA). Total serum cholesterol was measured using the Cholesterol E kit (Wako Diagnostics) as per manufacturer’s instructions.

**Statistical analysis**

Data were analyzed for statistical significance by two-way analysis of variance with post hoc Tukey’s analysis using the GraphPad Instat® 3.0 software (GraphPad Software, San Diego, CA). P-values less than 0.05 were considered significant. All experiments were repeated a minimum of three times.
Supplemental Figure I
Area used for sectioning of aortas.
(Left) Schematic representation. (Right) Photo from Micro-CT of $Mgp^{-/-}$ aorta demonstrating the extent of the aortic mineralization. Only the mineral is detected by the micro-CT.
**Supplemental Figure II**

Enzymatically dispersed aortic cells from $\text{Mgp}^{+/+}$ and $\text{Mgp}^{-/-}$ mice (top) and WT and $\text{Ins2}^{\text{Akita}+/+}$ mice (bottom) were pre-sorted to remove CD45-expressing cells using anti-CD45-antibodies and FACS. The efficiency of the pre-sorting was checked by testing for the presence of cells with co-expression of CD45 and CD31. No significant CD45 expression was detected by FACS. The CD31+CD45- cells were subsequently used to detect co-expression of CD31 and Cbfa1 (Figures 1D and 5C), and co-expression of CD31 and Sox2 (Figures 2C and 6D) by FACS.
Supplemental Figure III

Time course of aortic changes in \( \text{Mgp}^{-/-} \) mouse aorta.

Higher magnification of aortas shown in Figure 3. The aortas were collected between postnatal day (P) 4-30 from \( \text{Mgp}^{+/+} \) and \( \text{Mgp}^{-/-} \) mice as indicated, and stained with H&E.
Supplemental Figure IV
Depletion of MGP by siRNA.
HAEC were transfected by scrambled (SCR) or MGP siRNA. The cells were stained for MGP 24 hours after transfection. We were unable to obtain immunoblotting of the MGP protein due to lack of anti-MGP antibodies that work on immunoblots.
**Supplemental Table I.** Serum levels of glucose, phosphate, and total cholesterol in wild type and \( \text{Ins}^2 \text{Akita}^+ \) mice (C57BL6/J background), 40 weeks of age. Only male \( \text{Ins}^2 \text{Akita}^+ \) mice were used.

<table>
<thead>
<tr>
<th>Mice</th>
<th>Glucose (mg/dl)</th>
<th>Phosphate (ng/ml)</th>
<th>Total Cholesterol (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>116.5±13.4</td>
<td>0.826±0.008</td>
<td>144.8±7.7</td>
</tr>
<tr>
<td>( \text{Ins}^2 \text{Akita}^+ )</td>
<td>358.5±10.6***</td>
<td>0.832±0.023</td>
<td>143.9±24.3</td>
</tr>
</tbody>
</table>

Asterisks indicate statistically significant differences compared to wild type. ***<0.001, Tukey’s test (n=3).
REFERENCES


