Yao Y, Jumabay M, Ly A, Radparvar M, Cubberly MR, Boström KI. *Vascular Endothelium Contributes Osteoprogenitor Cells to Vascular Calcification*

ONLINE SUPPLEMENT
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} /J, stock # 003548), which are heterozygous for a mutation in one allele of the insulin-2 gene [1, 2] were obtained from the Jackson Laboratory. *Mgp*$^{tg/wt}$ mice, generated in our laboratory on a C57BL/6J background [3], 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*$^{tg/tg}$ mice [3]. *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 [3-5], 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-Ggp*tg mice were used for experiments at 4 weeks of age, whereas *Ins2*$^{Akita/+}$, *Mgp*$^{tg/wt}$;*Ins2*$^{Akita/+}$ and *Ins2*$^{Akita/+}$*;Tie2-Ggp*tg 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 [6, 7]. Transient transfections of HAEC with siRNA were performed with Lipofectamine™2000 (Invitrogen) using 60 nM siRNA as described [6]. 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 [8]. 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/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). 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 [9]. 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 *Ins2*$^{Akita/+}$ mice, and 3 aortas from *Mgp*$^{-/-}$ mice were required to obtain enough cells for analysis.

RNA analysis

RT-PCR and real-time PCR were performed as described [6]. 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.

Immunoblotting

Immunoblotting was performed as described [10, 11]. Equal amounts of tissue or cellular protein were used. Tissues were collected at 4 weeks for *Mgp*$^{-/-}$ mice and controls, and 35-40 weeks for mice with the *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 Pharmingen and eBioscience); Oct3/4 (200 ng/ml; R&D Systems); Cbfa1 (500 ng/ml; Oncogene Research Products); Osterix); SM22α (200 ng/ml; Santa Cruz Biotechnology) and αSMA (200 ng/ml; R&D Systems). ß-Actin (1:5,000 dilution; Sigma-Aldrich) was used as loading control.

Immunostaining

The tissues were collected at 4 weeks for *Mgp*$^{-/-}$ mice and 35-40 weeks for mice with the *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 *Mgp*$^{-/-}$ mice was very extensive and uniform (Supplemental Figure I, right). Tissue sections were processed and stained as previously described in detail [11, 12]. Tissue sections were fixed in 4% paraformaldehyde and processed as previously described. 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 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) [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.

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 4C 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 [13].

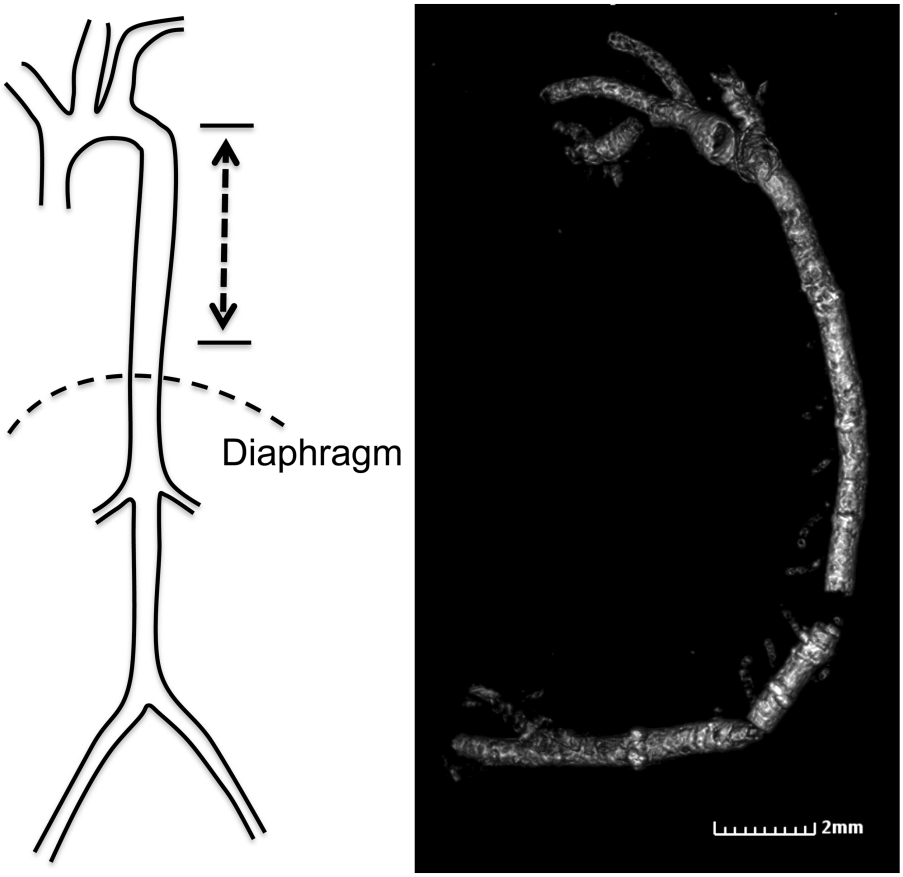
Analytical Procedures

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.

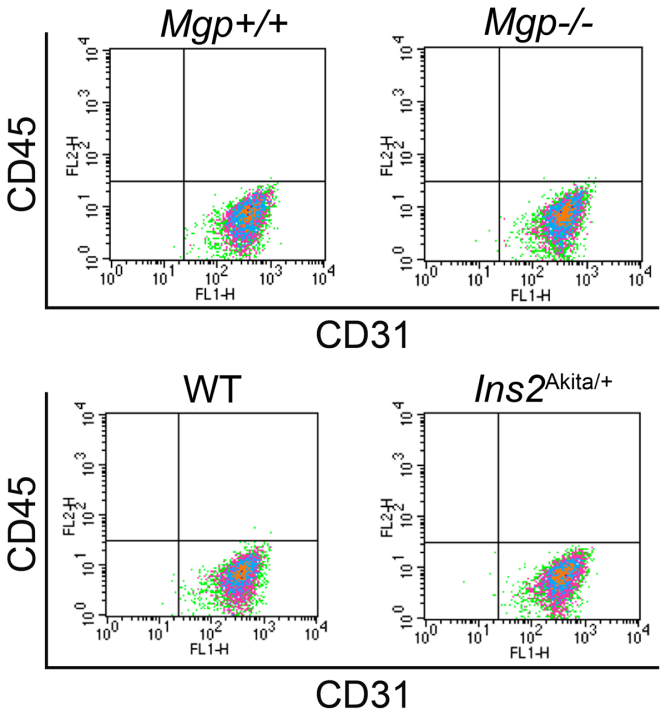
Statistical analysis

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 RESULTS

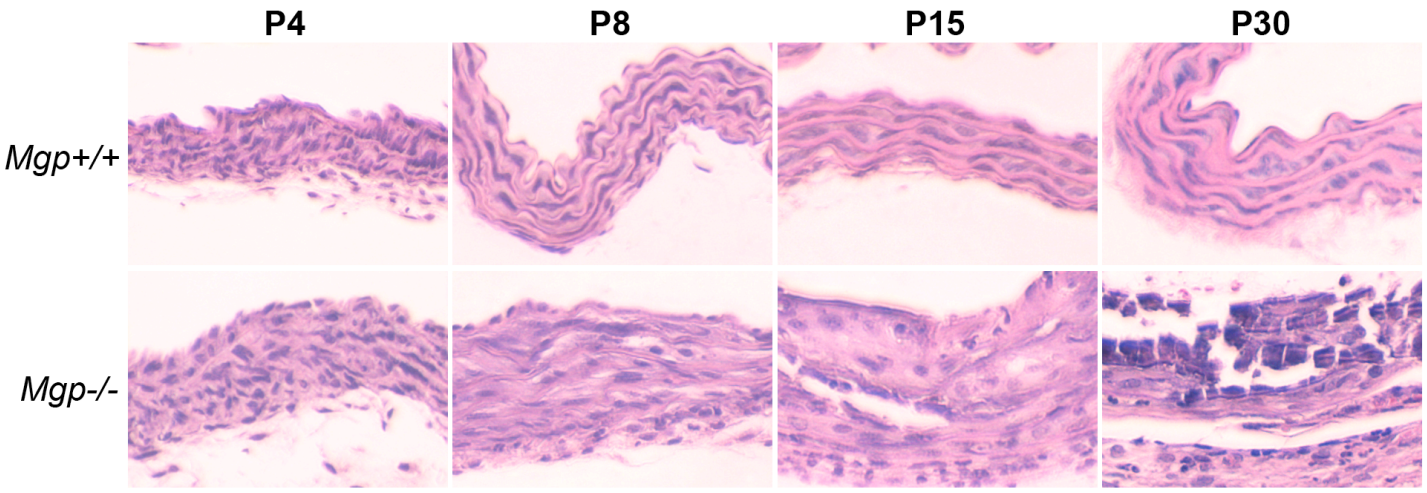


Supplemental Figure I
Area used for sectioning of aortas.
(Left) Schematic representation. (Right) Photo from Micro-CT of *Mgp*$^{-/-}$ aorta demonstrating the extent of the aortic mineralization. Only the mineral is detected by the micro-CT.



Supplemental Figure II

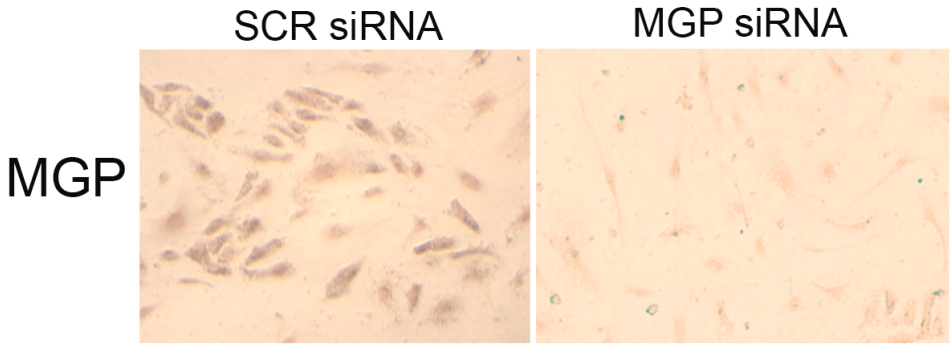
Enzymatically dispersed aortic cells from *Mgp*$^{+/+}$ and *Mgp*$^{-/-}$ mice (top) and WT and *Ins2*$^{Akita/+}$ mice (bottom) were pre-sorted to remove CD45-expressing cells using anti-CD45-antibodies and FACS. The efficiency of the pre-sorting was checked bytesting for the presence of cells with 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 $Mgp^{-/-}$ mouse aorta.

Higher magnification of aortas shown in Figure 3. The aortas were collected between postnatal day (P) 4-30 from $Mgp^{+/+}$ and $Mgp^{-/-}$ 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 *Ins2*$^{Akita/+}$mice (C57BL6/J background), 40 weeks of age. Only male *Ins2*$^{Akita/+}$mice were used.

Mice	**Glucose** (mg/dl)	**Phosphate** (ng/ml)	**Total Cholesterol** (mg/dl)
Wild type	116.5±13.4	0.826±0.008	144.8±7.7
Ins2$^{Akita/+}$	358.5±10.6***	0.832±0.023	143.9±24.3

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

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