SUPPLEMENTAL MATERIAL

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ONLINE SUPPLEMENT

Detailed Method Section

Animals

Mgp$^{+/-}$ 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. *Cdh5*^{Cre} (B6.Cg-Tg(Cdh5-cre)7Mlia/J) and *Sox2*$^{flox/flox}$ (Sox2tm1.1Lan/J) 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). Diisopropylfluorophosphate (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*$^{-/-}$ 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; Mm03053810_s1 for mouse), Kruppel-like factor 4 (Klf4) (Hs01053049_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 (Hs00153510_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; Mm04208233_g1 for mouse), elastase 1 (Hs00608115_m1 for human; Mm00712898_m1 for mouse), elastase 2 (Hs00975994_g1 for human; Mm01168928_g1for 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; 200ng/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, 200ng/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). ß-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)(bothfrom 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 aortic 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. ^{3}H-Thymidine was added at 1 μCi/mL for 4 days, and ^{3}H-thymidine incorporation was determined as previously described (13).

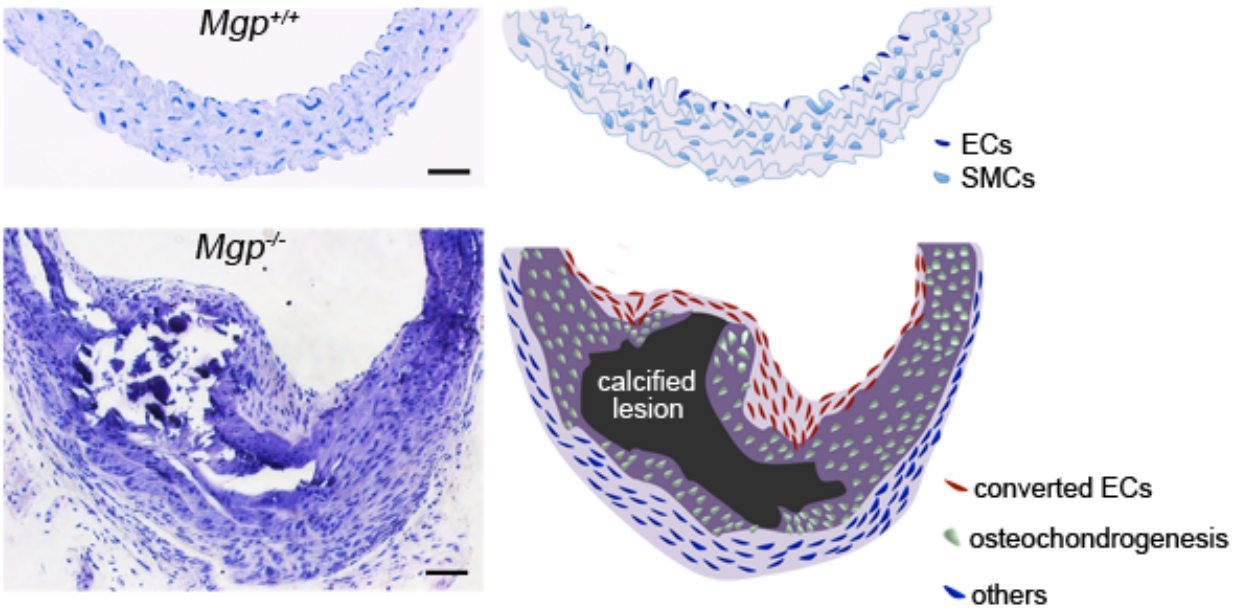
Analysis of colony forming units
HAECs were transfected and treated as indicated. Analysis of colony forming units

Was performed as previously described (14).

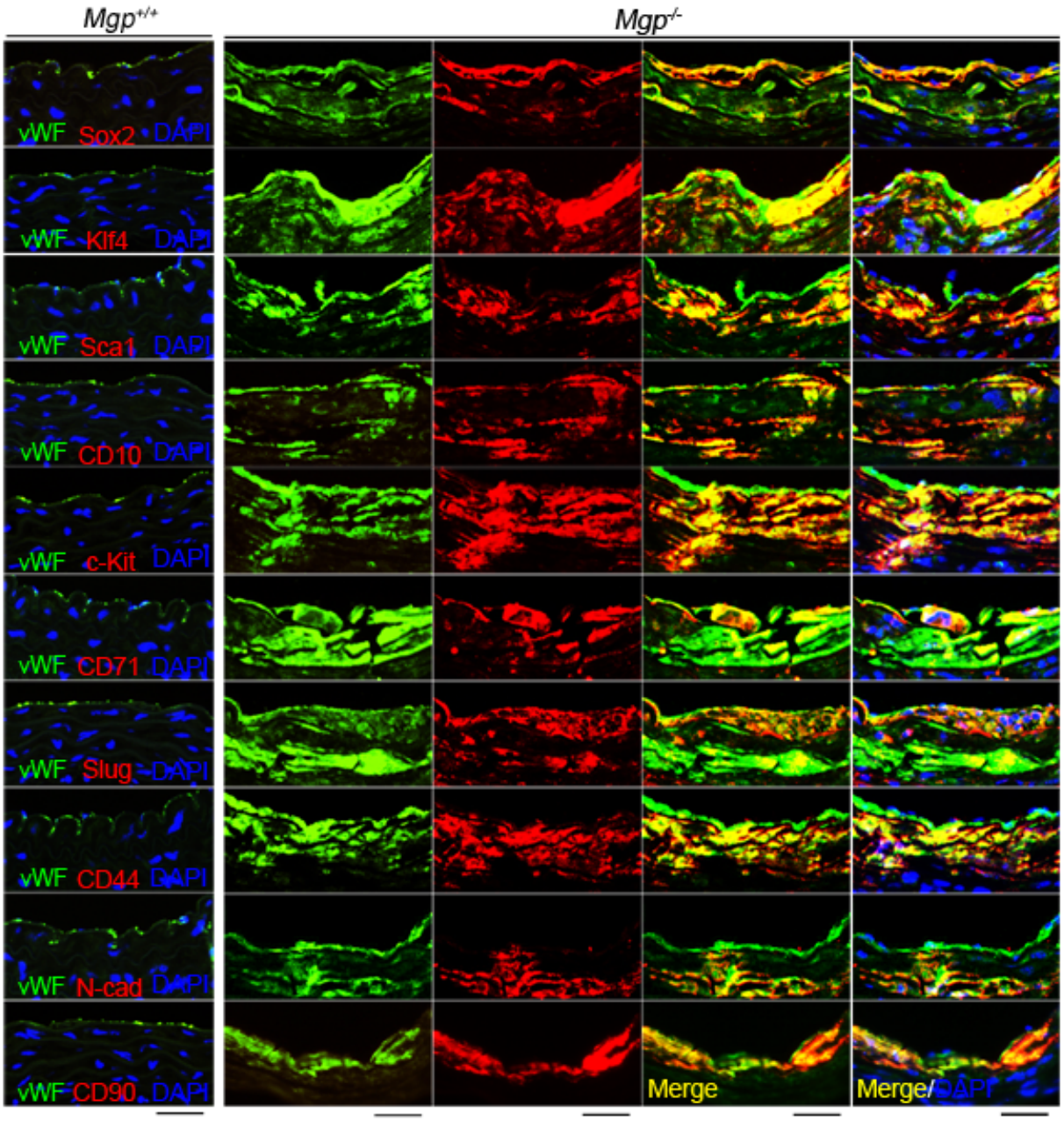
Lentivirus infection
Lentiviral vectors containing Sox2 open reading frame (ORF) under the cytomegalovirus (CMV) promoter were obtained from Applied Biological Materials Inc. (Richmond, BC). The cell infection was performed as per the manufacturer's protocol.

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% O_sO_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 samles 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.



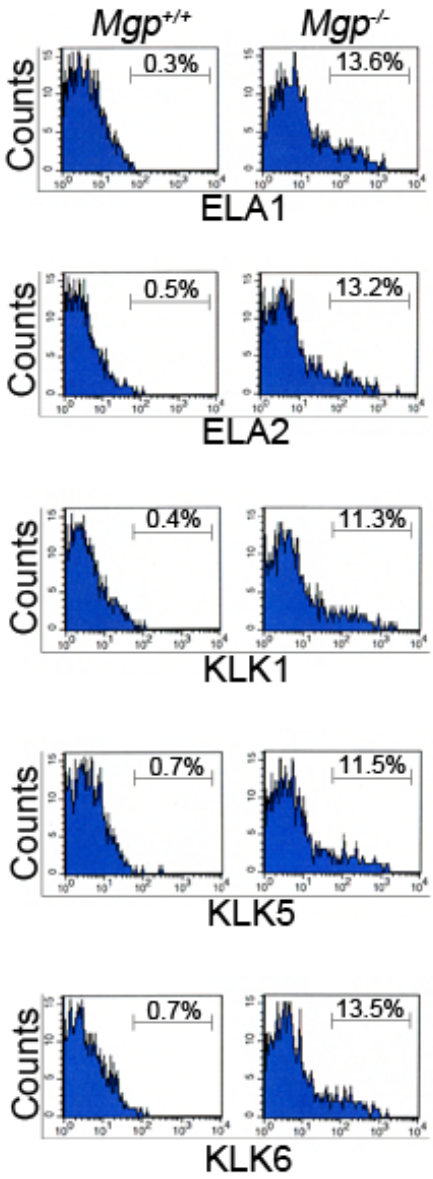
Online Figure I
Histology of the aortic wall of $Mgp^{-/-}$ mice.
Histology of the aortic wall of $Mgp^{+/+}$ (left, upper) and $Mgp^{-/-}$ (left, bottom) mice. The schematic drawing illustrates differences in the layout of cell layers in the aortic tissues of $Mgp^{+/+}$ (right, upper) and $Mgp^{-/-}$ (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.

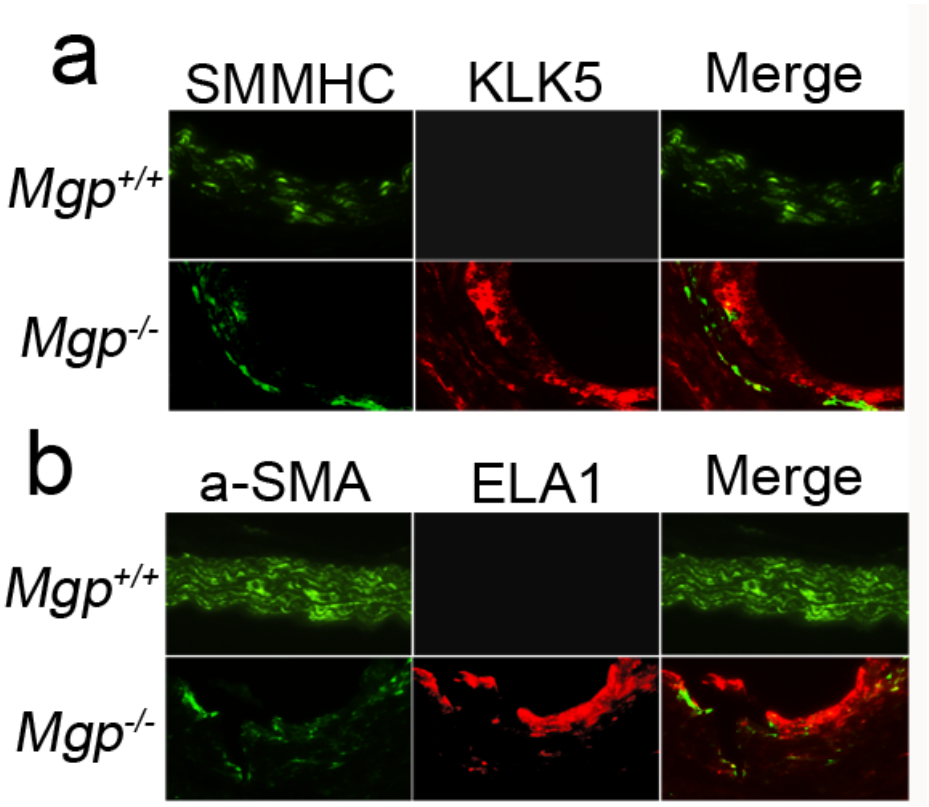
Co-expression of the endothelial marker vWF with Sox2 and Slug is also shown in Figure 1. Scale bars, 100 μm.



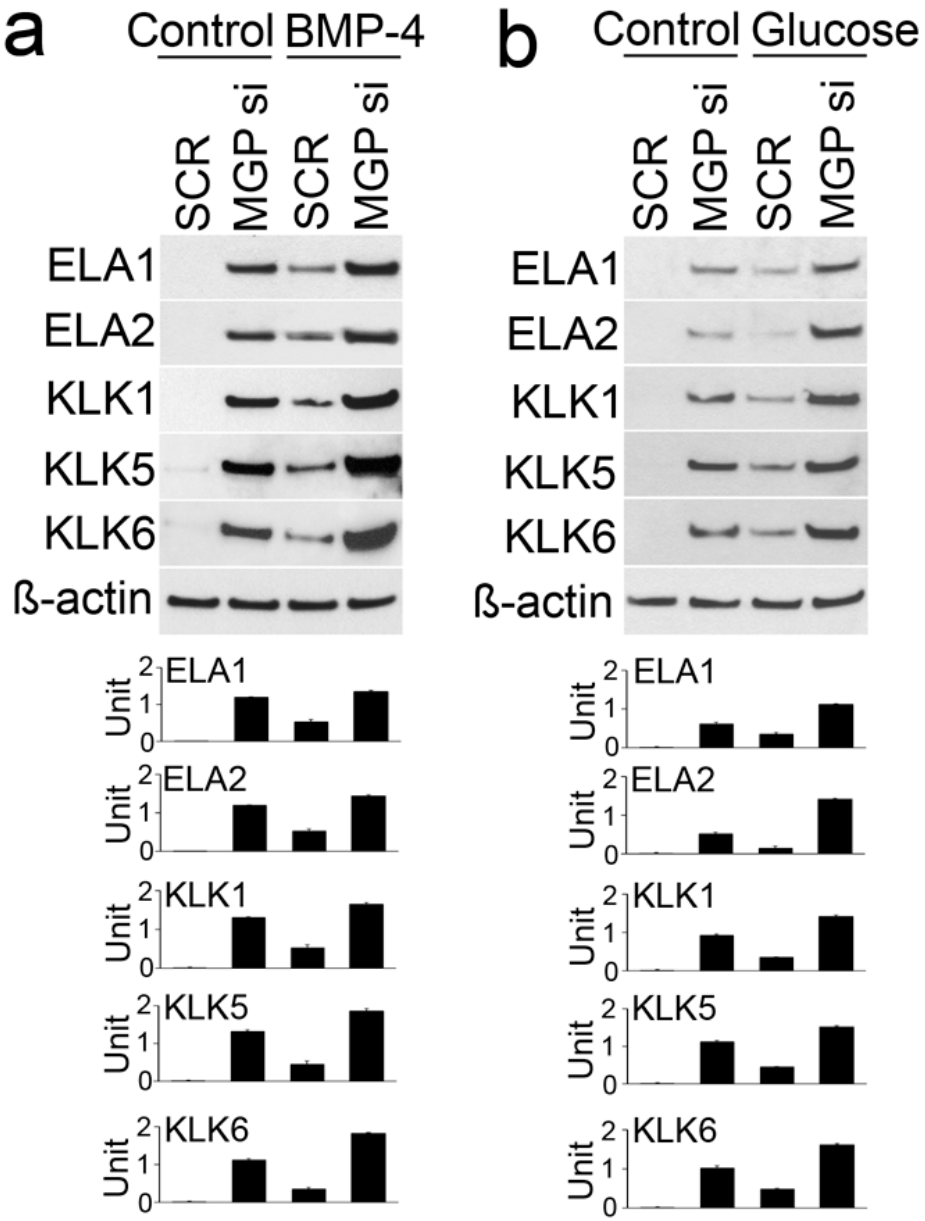
Online Figure III

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.



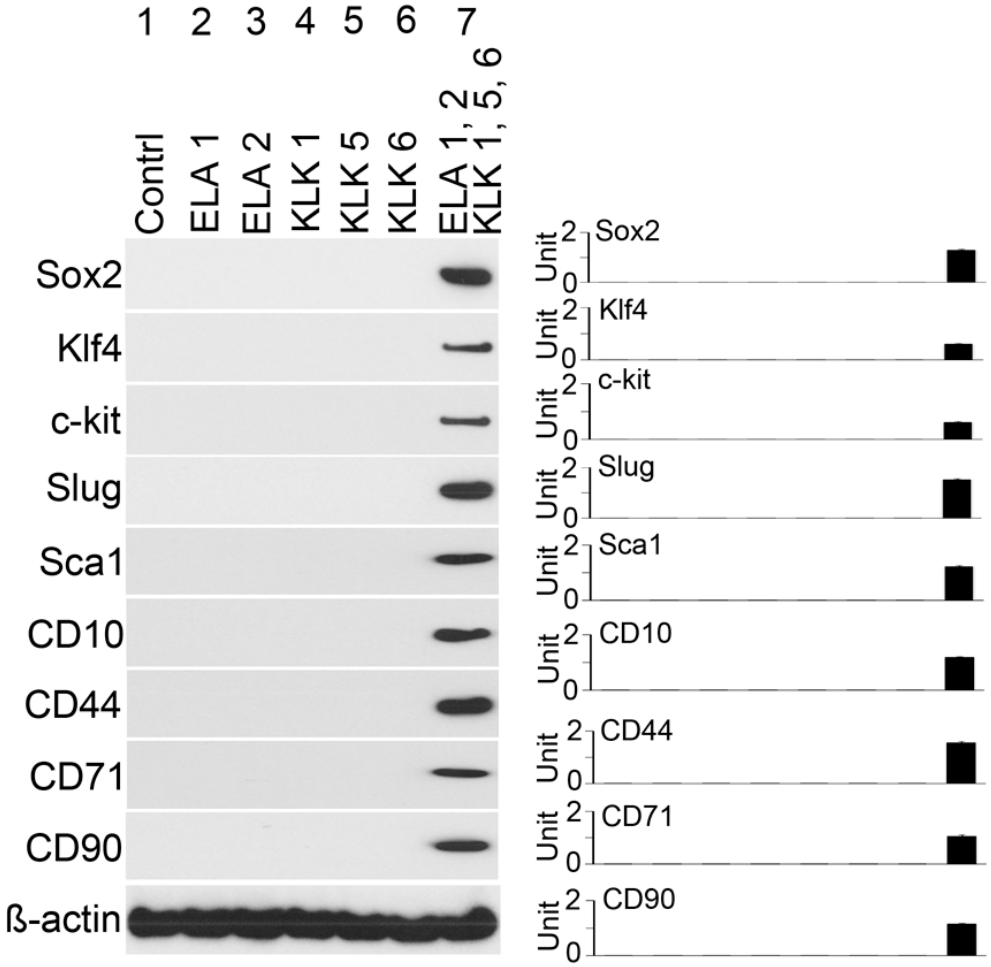
Online Figure IV
Absence of co-localization between proteases and smooth muscle cell markers
Co-localization was not observed for kallikrein 5 and smooth muscle myosin heavy chain (SMMHC) (a), nor was co-localization observed for elastase 1 and alpha-smooth muscle actin (a-SMA) (b*).*



Online Figure V

Elastases and kallikreins induced in HAECs.

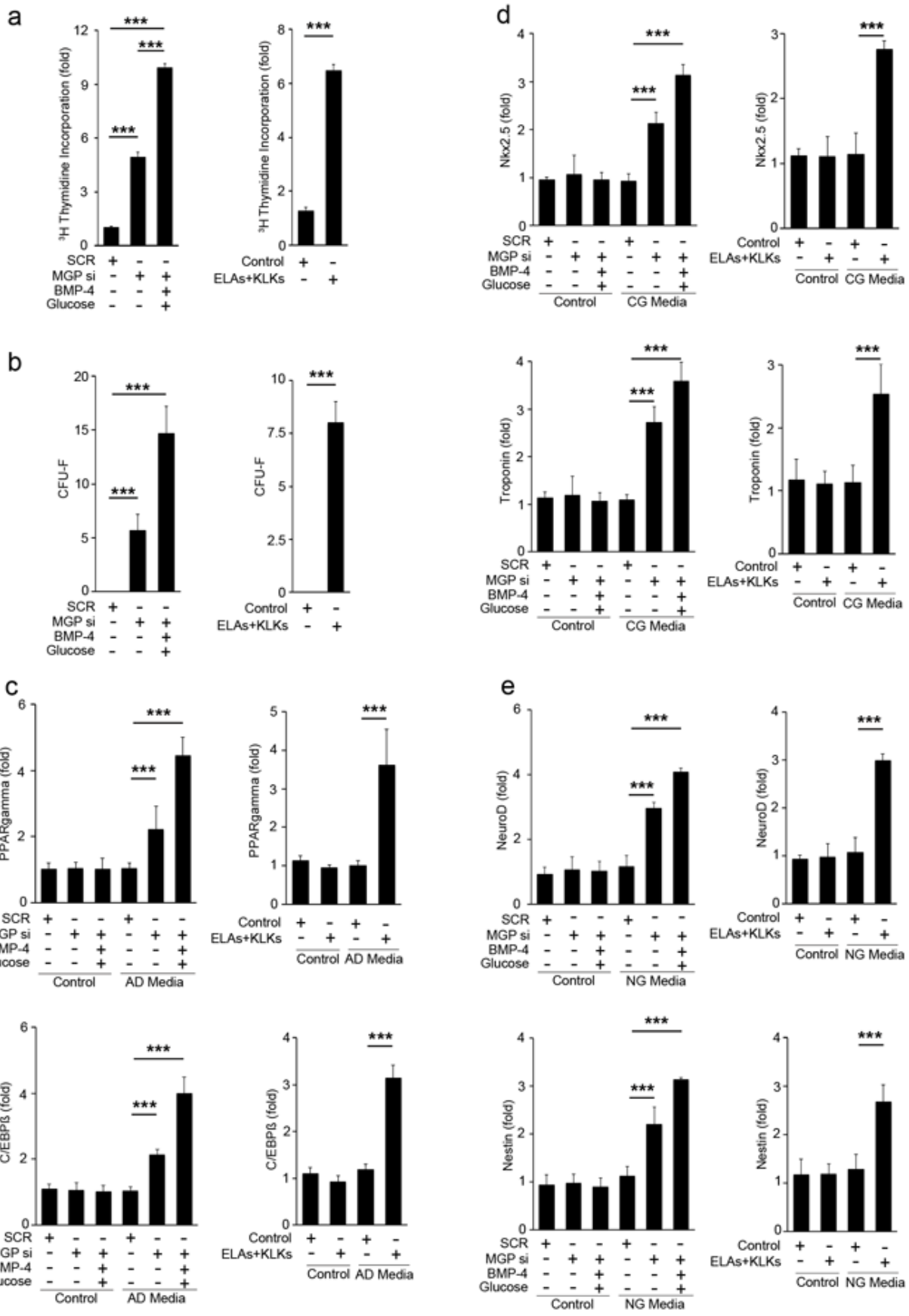
Elastase (ELA) 1, 2 and kallikrein (KLK) 1, 5, 6 were induced after depletion of MGP by siRNA (MGP si) in HAEC (a-b). The induction was enhanced by BMP-4 (a) or high glucose (b) as shown by immunoblotting with densitometry.



Online Figure VI

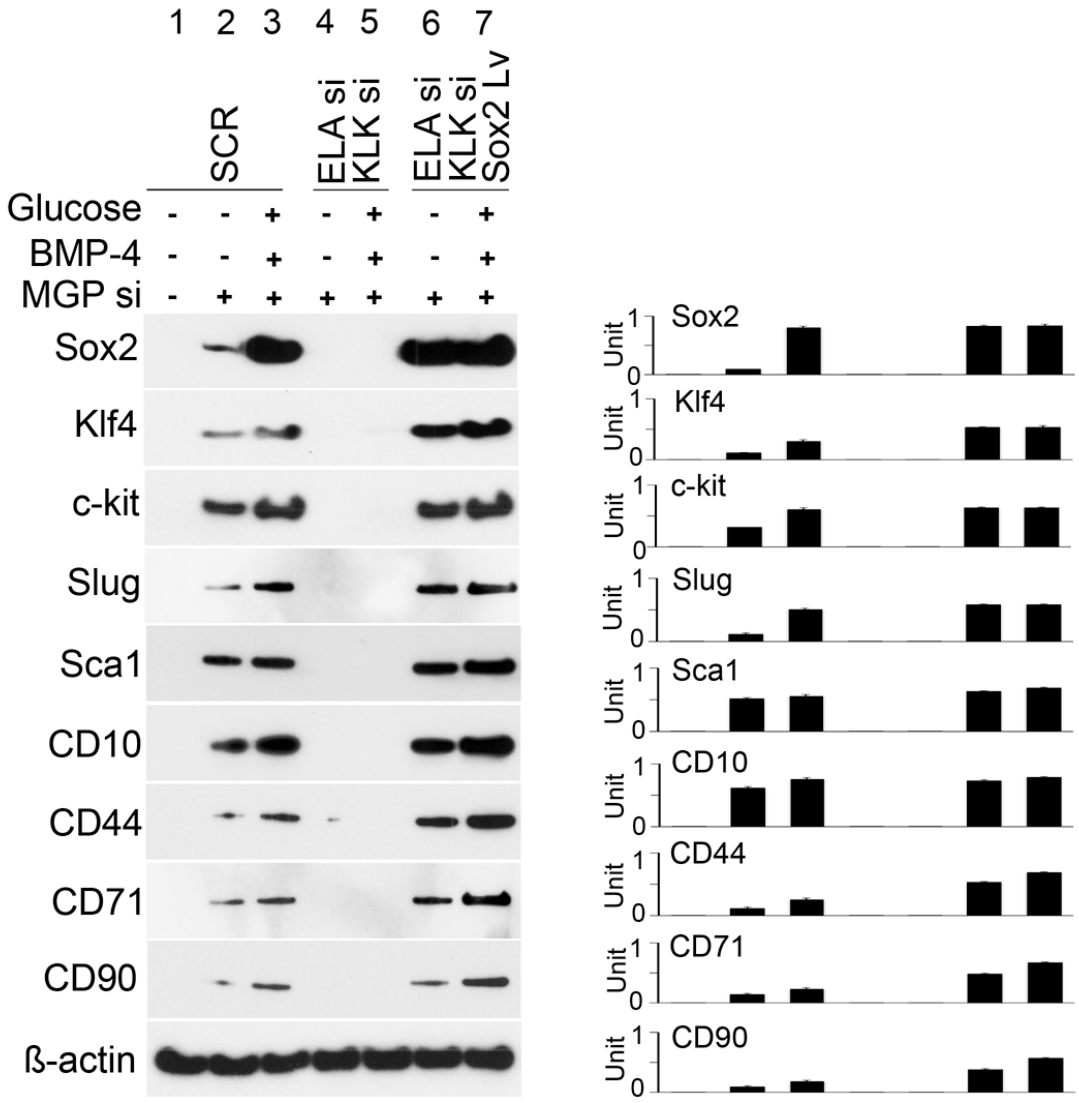
A complex of elastases and kallikreins induces stem-cell markers in HAEC.

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).


a
^{3}H Thymidine Incorporation (fold)
***
SCR
MGP si
BMP-4
Glucose
Control
ELAs+KLKs
b
CFU-F
c
PPARgamma (fold)
C/EBPß (fold)
AD Media
d
Nkx2.5 (fold)
CG Media
e
NeuroD (fold)
NG Media
Troponin (fold)
Nestin (fold)

Online Figure VII

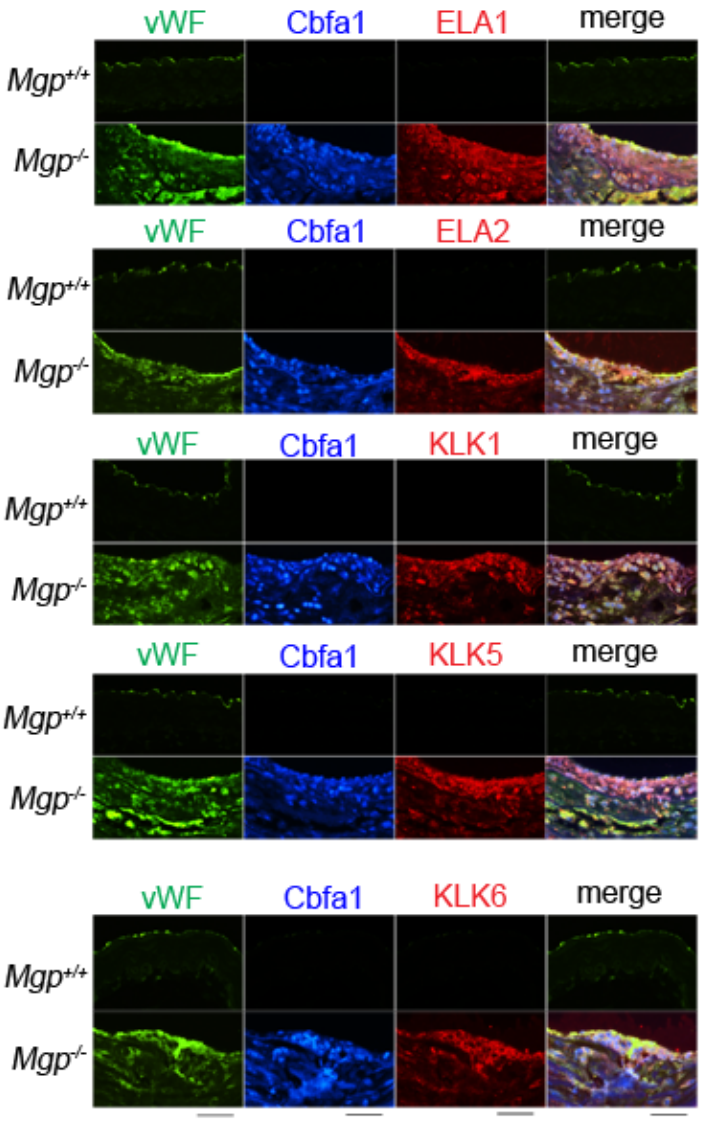
Multipotent potentials in MGP-depleted HAECs or HAECs treated with serine proteases. HAECs were transfected with MGP siRNA or treated with a complex of serine proteases (ELAs+KLKs). The cells were subsequently treated with BMP-4 and glucose for 24 hours before cell proliferation (a) and colony-forming 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/EBPß (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.



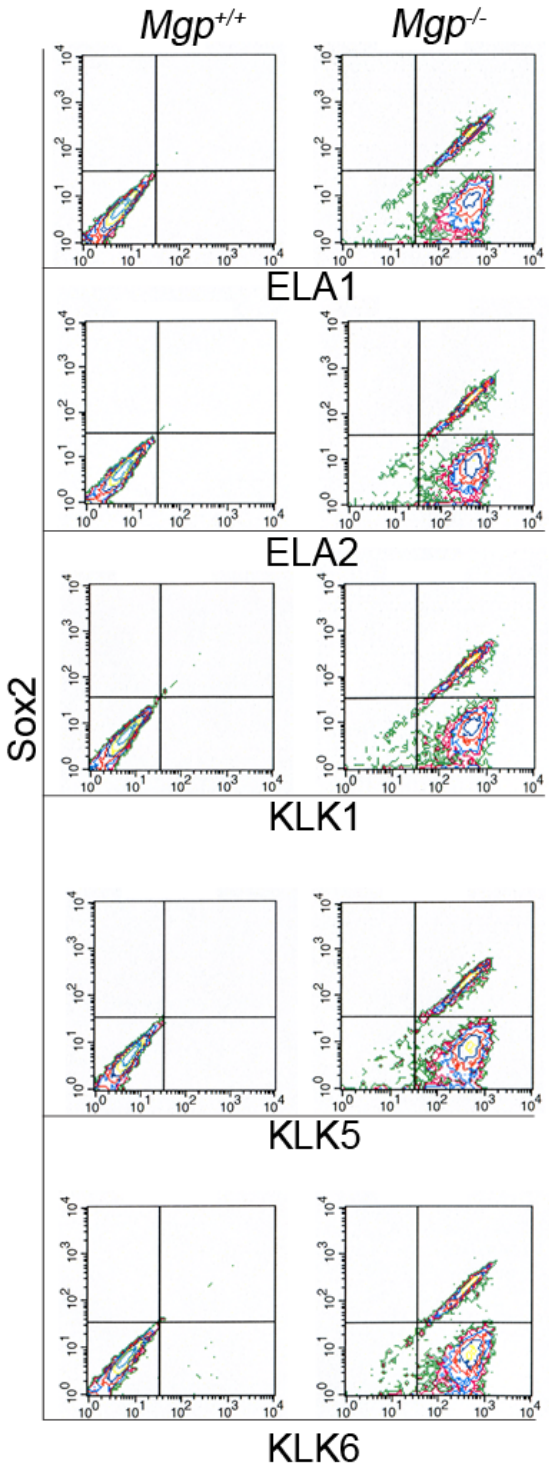
Online Figure VIII

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.



Online Figure IX
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.

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