Yao Y, Bennett BJ, Wang X, Rosenfeld ME, Giachelli C, Lusis AJ, Boström KI. *Inhibition of Bone Morphogenetic Proteins Protects against Atherosclerosis and Vascular Calcification*

ONLINE SUPPLEMENT
Detailed Method Section

Animals and Diets

Apoe$^{-/-}$ mice on a C57BL/6J background were purchased from Jackson Laboratory (Bar Harbor, ME). MGP$^{tg/wt}$ mice, previously generated in our laboratory on a C57BL/6J background [1] were crossed with Apoe$^{-/-}$ mice to generate MGP$^{tg/wt}$;Apoe$^{-/-}$ mice. We used the MGP$^{tg/wt}$ mice since the phenotype was apparent in MGP$^{tg/wt}$ mice, and a low birth rate of MGP$^{tg/tg}$ mice made it difficult to obtain MGP$^{tg/tg}$ mice [1]. MGP$^{+/-}$ mice on C57BL/6J background [2] were obtained from Dr. Gerard Karsenty (Columbia University, New York, NY) and crossed with the Apoe$^{-/-}$ mice to generate MGP$^{-/-}$;Apoe$^{-/-}$ mice. Genotypes were confirmed by PCR [1, 3], and experiments were performed with generation F4-F6. All mice were fed a standard chow diet (Diet 8604, Harlan Teklad, Laboratory, Madison, WI). At 8 to 10 weeks of age, littermate Apoe$^{-/-}$ and MGP$^{tg/wt}$;Apoe$^{-/-}$ mice were either continued on standard chow or switched to a high-fat/high-cholesterol diet (Western diet) (Research Diets, New Brunswick, NJ, diet #D12108, containing 21% fat [w/w], 1.25% cholesterol [w/w]) for 16 weeks. Littermate MGP$^{-/-}$;Apoe$^{-/-}$, MGP$^{+/-}$;Apoe$^{-/-}$, MGP$^{-/-}$, and Apoe$^{-/-}$ and wild type mice were maintained on standard chow. The studies were reviewed by the Institutional Review Board and conducted in accordance with the animal care guidelines set by the University of California, Los Angeles.

Lipid and Phosphate Analyses

Mice were fasted for 16 hours and anesthetized via exposure to isoflurane before blood was collected through the retroorbital sinus; plasma was stored at –80°C. Plasma triglyceride, cholesterol, high-density lipoprotein cholesterol (HDL-C), and free fatty acids were measured as previously described [4]. Serum phosphate levels were measured using a QuantiChrom™ Phosphate Assay kit as per manufacturer's instructions (BioAssay Systems, Hayward, CA).

Lesion Quantification

The mice were euthanized and perfusion fixed with 10% buffered formalin via the left ventricle for 4 minutes. The heart and proximal aorta were excised and the apex and lower half of the ventricles were removed. The remaining specimen was embedded in OCT (Tissue-Tek, Fisher Scientific), frozen on dry ice, and stored at –80°C until sectioning. Serial cryosections were prepared through the ventricle until the aortic valves appeared. From then on, every fifth 10-μm section was collected on poly-D-lysine–coated slides until the aortic sinus was completely sectioned. Sections were stained with hematoxylin and Oil Red O, which specifically stains lipids. Slides were examined by light microscopy and atherosclerotic lesion area was quantified with computer assisted image analysis (Image-Pro Plus, Media Cybernetics, Bethesda, MD) as previously described [5], and averaged over 40 sections [6].

En Face Analysis of Aortas

The aorta, including the ascending arch, thoracic, and abdominal segments, was dissected, gently cleaned of the adventitia, and stained with Sudan IV [5]. The surface lesion area was quantified with computer assisted image analysis (Image-Pro Plus, Media Cybernetics) as previously described [5].

Calcification Quantification

Calcified areas can be seen clearly in the hematoxylin stained sections, which was verified by Von Kossa calcium stain. The calcification area from each analyzed section was quantified in μm^2 using computer assisted image analysis (Image-Pro Plus, Media Cybernetics).

The calcified areas per animal were added and multiplied with 10, corresponding to the thickness of the sections, which was 10 μm. The total volume in μm^3 was used as the measurement of calcification for calcification. Total calcium in lyophilized aortic tissue was determined as previously described [7].

Immunoblotting

Immunoblotting was performed as previously described [8]. Equal amounts of cellular protein or culture medium were used. Blots were incubated with specific antibodies to pSMAD1/5/8 or pSMAD2/3 (both 400 ng/ml; Cell Signaling Technology, Danvers, MA), ICAM-1 (100 ng/ml; Cell Signaling Technology), total SMAD, BMP-2, BMP-4, ALK1, ALK2, Chordin, VCAM-1 or E-selectin (all 200 ng/ml; Santa Cruz Biotechnology, Santa Cruz, CA), IL-6 (1 μg/ml; R&D Systems, Minneapolis, MN), CV2 (400 ng/ml; R&D Systems), CD68 (1 μg/ml; Dako, Carpinteria, CA), HSP70 (1 μg/ml; Assay Designs, Ann Arbor, MI), or Noggin (400 ng/ml; Abcam, Cambridge, MA). ß-Actin (1:5000 dilution; Sigma) was used as loading control. For optimal detection of VEGF in culture media, VEGF was first immunoprecipitated with anti-VEGF antibodies (Santa Cruz Biotechnology), as previously described [9] and then analyzed by immunoblotting using specific antibodies to VEGF (200 ng/mL; R&D Systems). Densitometry using NIH Image J64 (http://rsb.info.nih.gov/nih-image/) was performed to compare protein levels.

Validation of Antibodies

The specificity of all antibodies was verified prior to use for immunoblotting and immunostaining (Supplemental Fig. I). For positive controls, commercially available antigens were obtained for BMP-2, BMP-4, VEGF, Noggin, IL-6 (all from R&D Systems), and HSP70 (Assay Designs). Mouse macrophages were used for CD68, and BMP-4 treated HAEC were used for ALK2, ALK1, ICAM1, VCAM-1, pSMAD1/5/8, pSMAD2/3 and total SMAD. We previously showed that siRNA to ALK2, ALK1, SMAD1 and SMAD2 transfected into HAEC depletes the signal on immunoblotting using the above antibodies [10]. In addition, the specificity of the anti-ALK1 antibodies was previously confirmed using HA-tagged ALK1 and anti-HA antibodies [11]. ICAM-1 and VCAM-1 are well known adhesion molecules in endothelial cells [12].

RNA Analysis

Total RNA was isolated using the RNeasy kit as per the manufacturer's instruction (Qiagen, Valencia, CA). Real-time PCR assays were performed as previously described [9] using an Applied Biosystems 7700 sequence detector (Applied Biosystems, Foster City, CA). Briefly, 2 μg of total RNA was reverse-transcribed with random hexamers using an MMLV Reverse Transcription Reagents kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Each amplification mixture (20 μl) contained 25 ng of reverse-transcribed RNA, 8 μM forward primer, 8 μM reverse primer, 2 μM dual-labeled fluorogenic probe (Applied Biosystems), and 10 μl of Universal PCR mix Quantitect probe RT-PCR kit (Qiagen). PCR thermocycling parameters were 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All samples were analyzed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression in parallel in the same run. Results of the real-time PCR data were represented as *C*t values, where *C*t is defined as the threshold cycle of PCR at which amplified product was first detected. To compare the different RNA samples in an experiment, we used the comparative *C*t method [13, 14] and compared the RNA expression in samples to that of the control in each experiment. The primers and probes were constructed so that the dynamic range of both the targets and the GAPDH reference were similar over a wide range of dilutions (1:1–10,000). PCR was performed as quadruplicates for each sample. The results were expressed as mean $\pm$ S.D. for the relative expression levels compared with the control, and minimum values of four independent experiments were performed. Pre-designed primers and probes for mouse BMP-2, BMP-4,

MGP, ALK1, ALK2 and VEGF were obtained from Applied Biosystems as part of Taqman® Gene Expression Assays.

Immunohistochemistry and Immunofluorescence

Tissue sections were fixed in 4% paraformaldehyde and processed as previously described [6]. 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 MicroImaging Inc., Thornwood, NY, USA). To eliminate the possibility of false colocalization 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.

For immunostaining, we used specific antibodies to MGP (provided by Dr. Reidar Wallin, Wake Forest University, Winston-Salem, NC), pSMAD1/5/8, pSMAD2/3, ICAM-1 (all from Cell Signaling Technology), total SMAD, BMP-2, BMP-4, ALK1, ALK2, Chordin, VCAM-1, E-selectin (all from Santa Cruz Biotechnology), vWF (Invitrogen, Carlsbad, CA), VEGF, IL-6, CV2 (all from R&D Systems), CD68 (Dako), Noggin (Abcam), and HSP70 (Assay Designs).

Enzyme-Linked Immunosorbent Assay (ELISA)

Serum levels of BMP-2 and -4 were determined by Quantikine® ELISA (R&D Systems) as per manufacturer's instructions, using 100 µl of serum per assay.

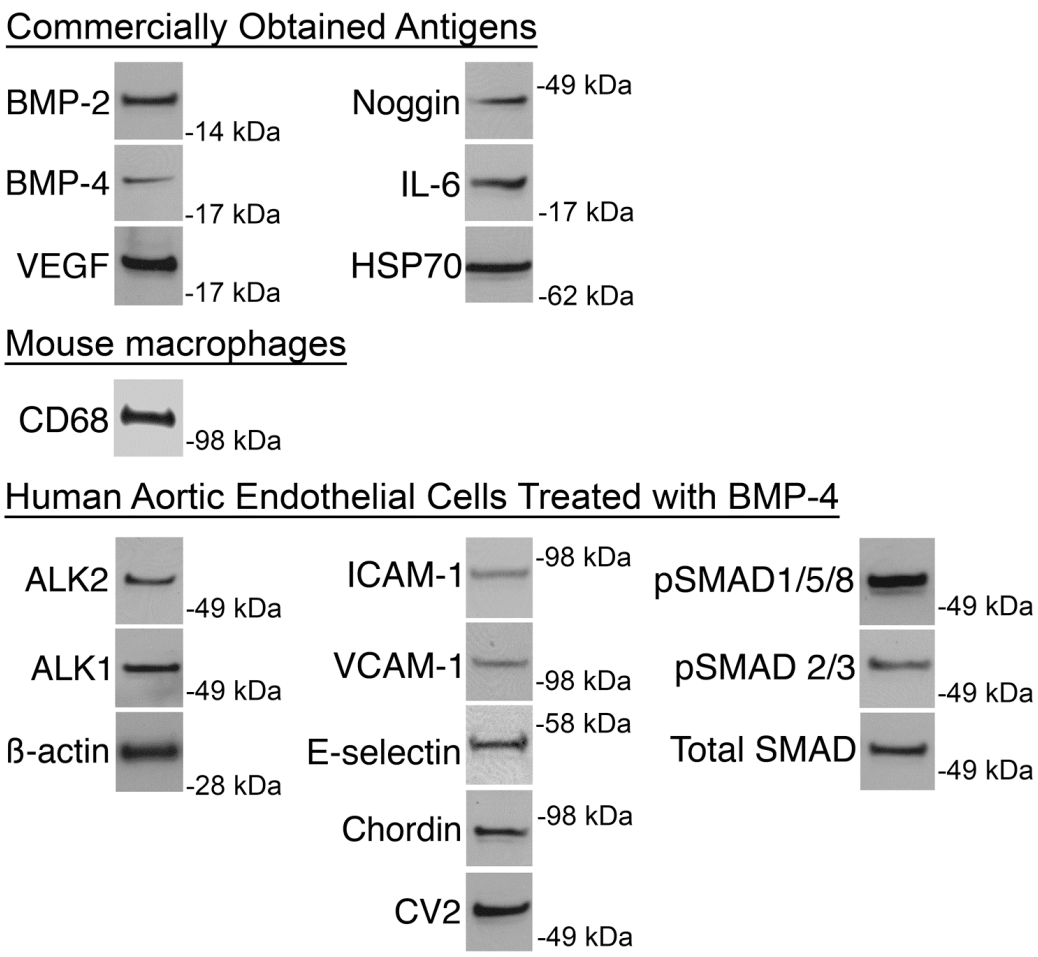
Complete Blood Count (CBC)

Standard CBC was determined using a HemaTrue Hematology Analyzer (Heska Lab Systems, Loveland, CO) as per manufacturer's instructions.

Statistical Analysis

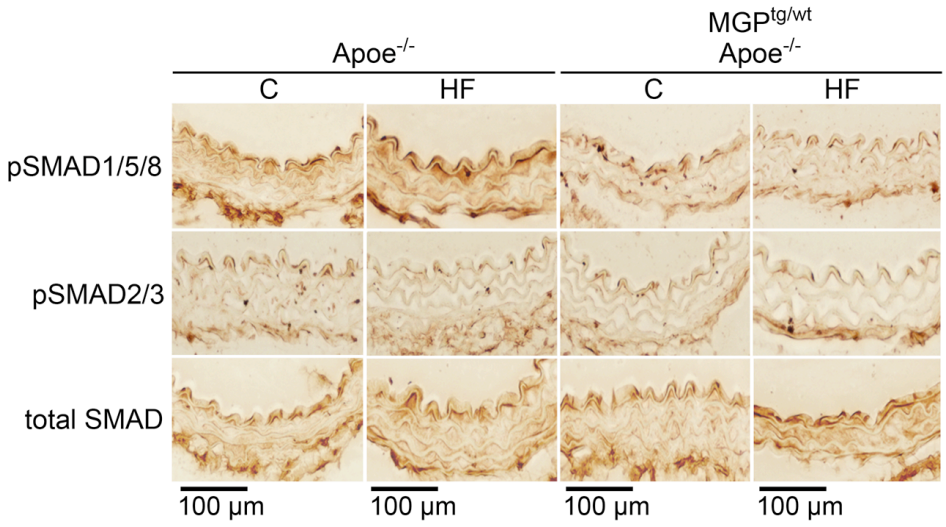
Data were analyzed for statistical significance by ANOVA with post hoc Tukey's analysis, or by the Mann-Whitney rank sum test or the unpaired Student t-test for unequal variance. The analyses were performed using the GraphPad Instat® 3.0 software (GraphPad Software, San Diego, CA). P-values less than 0.05 were considered significant.

Online Figure I



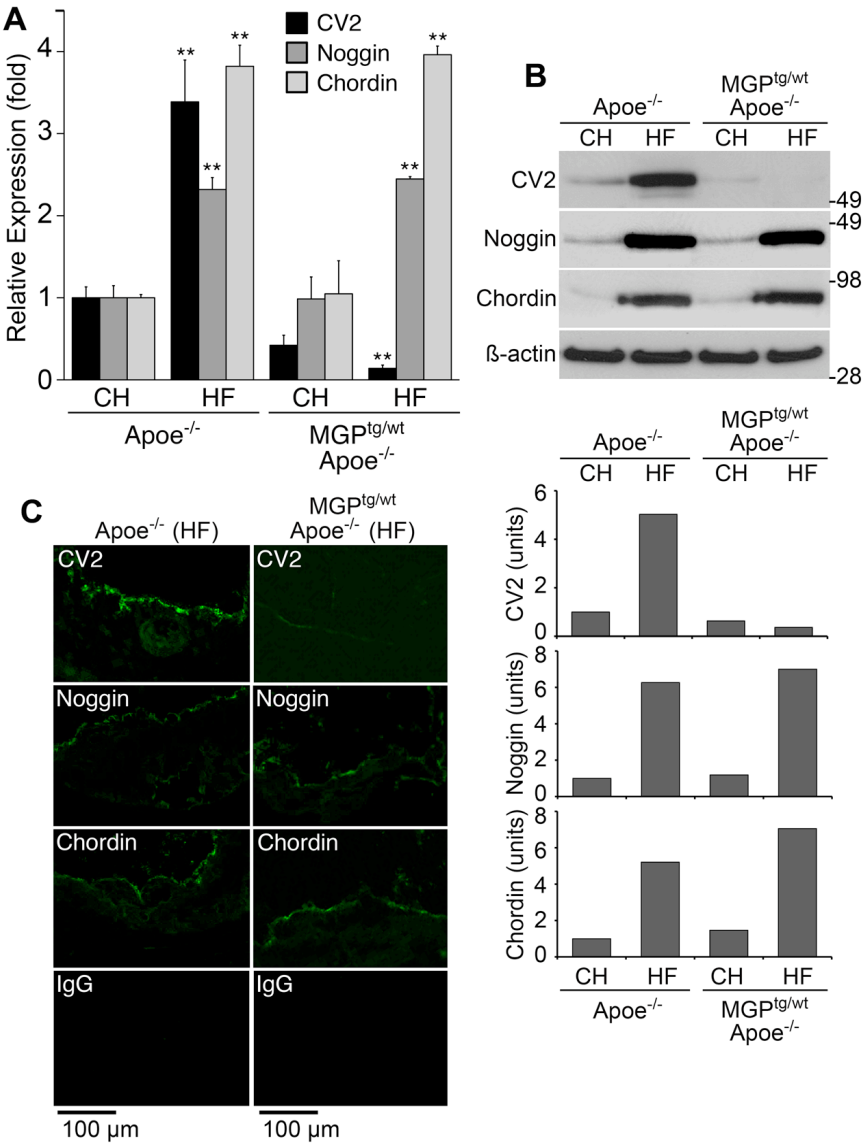
Supplemental Figure I
Positive controls for antibodies used for immunoblotting and immunostaining
Immunoblotting was performed using positive controls obtained as indicated in the figure. The locations of a relevant weight marker is indicated in each blot.

Online Figure II



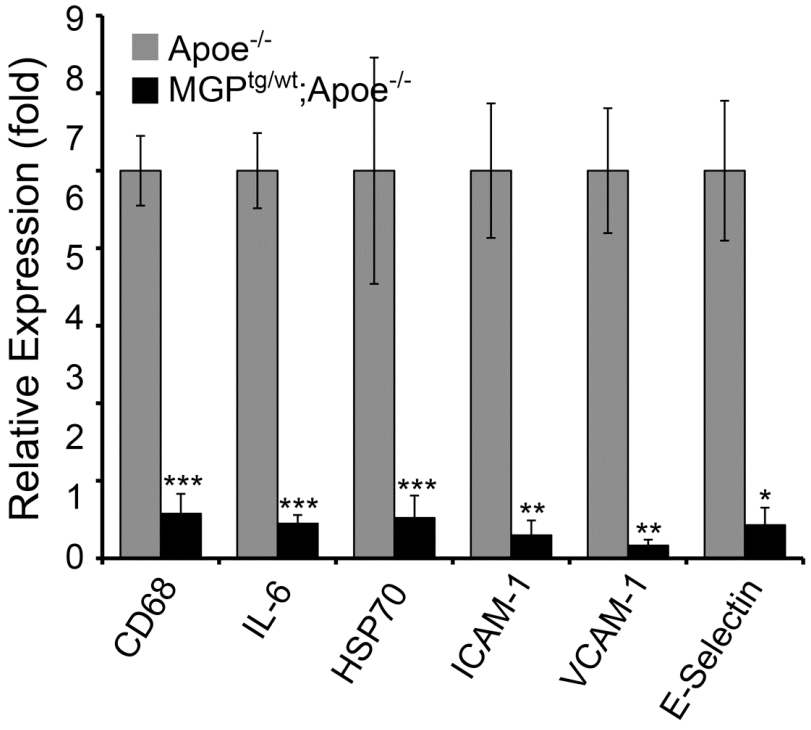
Supplemental Figure II
Fat-feeding enhances and increased MGP expression limits aortic BMP-signaling.
Apoe$^{-/-}$and MGP$^{tg/wt}$;Apoe$^{-/-}$mice fed chow (CH) or Western (HF) diet were examined. (G) BMP activity in aortic wall without lesions as determined by pSMAD1/5/8 immunohistochemistry. PSMAD2/3 and total SMAD are shown for comparison.

Online Figure III



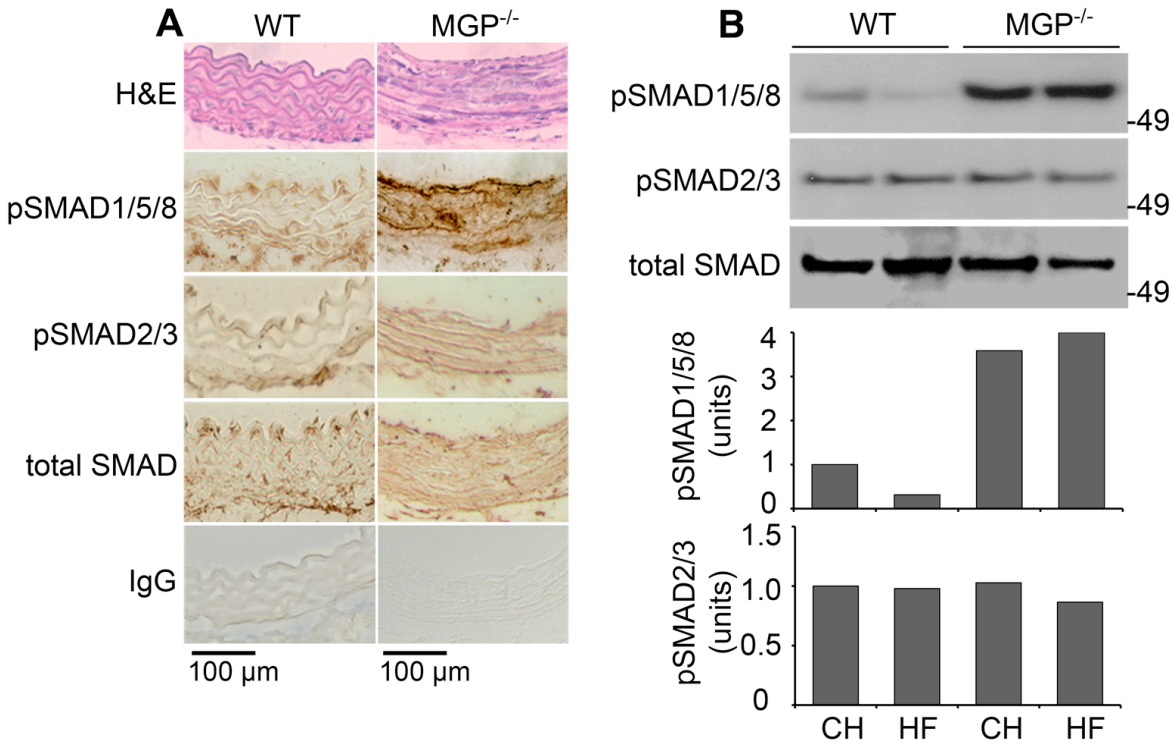
Supplemental Figure III
Expression of BMP inhibitors in Apoe$^{-/-}$and MGP$^{tg/wt}$;Apoe$^{-/-}$mice fed chow or Western diet.
(A-C) Aortic expression of CV2, Noggin and Chordin in Apoe$^{-/-}$and MGP$^{tg/wt}$;Apoe$^{-/-}$mice fed chow (CH) or Western (HF) diet, as determined by real-time PCR (A), immunoblotting with densitometry (B), and immunofluorescence (C).
Asterisks indicate statistically significant differences. **<0.01, ***<0.001, Tukey's test*.

Online Figure IV



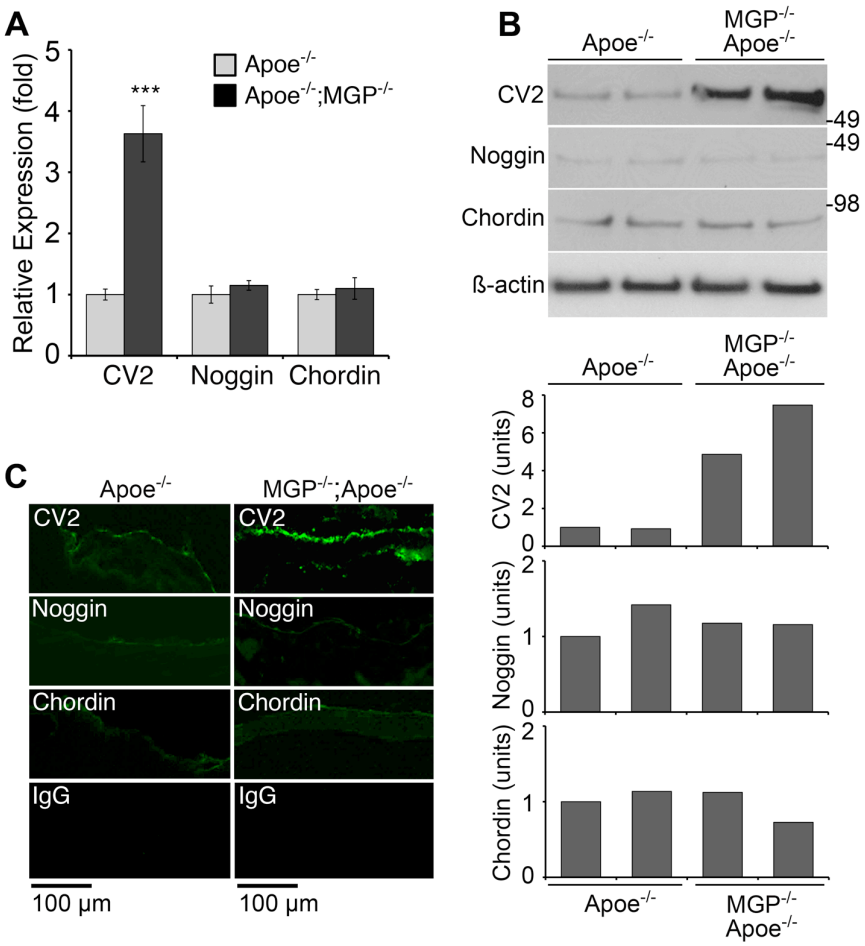
Supplemental Figure IV
Increased MGP expression suppresses inflammatory activity
Aortic expression of CD68, IL-6, HSP70, ICAM-1, VCAM-1 and E-selectin in $Apoe^{-/-}$and $MGP^{tg/wt}$;$Apoe^{-/-}$mice a Western (HF) diet, as determined by real-time PCR and normalized to lesion area. The lesion areas used were derived from the *en face* analysis; 8.8% was used for the $Apoe^{-/-}$ mice and 4.2% was used for the $MGP^{tg/wt}$;$Apoe^{-/-}$mice.
Asterisks indicate statistically significant differences. **<0.01, ***<*0.001, Tukey's test*.

Online Figure V



Supplemental Figure V
Increased aortic BMP-signaling and expression of ALK1 and VEGF in MGP$^{-/-}$ mice.
(A,B) BMP activity in aortas of Apoe$^{-/-}$ mice as determined by immunofluorescence (A) and immunoblotting with densitometry for pSMAD1/5/8 (B).
Structural changes in aortas of MGP$^{-/-}$;Apoe$^{-/-}$ mice are visible in the H&E-stained sections (A, top).

Online Figure VI



Supplemental Figure VI
Expression of BMP inhibitors in Apoe$^{-/-}$and MGP$^{-/-}$;Apoe$^{-/-}$mice.
(A-C) Aortic expression of CV2, Noggin and Chordin in Apoe$^{-/-}$and MGP$^{-/-}$;Apoe$^{-/-}$mice, as determined by real-time PCR (A), immunoblotting with densitometry (B), and immunofluorescence (C).
Asterisks indicate statistically significant differences. ****<0.001, Tukey's test*.

Online Table I.
Body mass and lipid levels of Apoe$^{-/-}$, Apoe$^{-/-}$;MGP$^{tg/wt}$, and Apoe$^{-/-}$;MGP$^{-/-}$ mice.

After 16 weeks on Western diet	**Mice**	**Body Mass** (gram)	**Triglycerides** (mg/dl)	**Total Cholesterol** (mg/dl)	**HDL-C** (mg/dl)
male	Apoe$^{-/-}$	35.7±1.4	67.6±5.4	1534.2±219.9	14.4±3.2
	Apoe$^{-/-}$;MGP$^{tg/wt}$	35.4±1.6	63.5±30.0	1332±177.3	16.4±2.0
female	Apoe$^{-/-}$	27.9±1.3	63.8±27.2	1110.6±184	21.4±5.1
	Apoe$^{-/-}$;MGP$^{tg/wt}$	28.2±0.7	60.5±25.3	1376.2±205.0	22.4±10.2
After 18 weeks on chow diet	**Mice**	**Body Mass** (gram)	**Triglycerides** (mg/dl)	**Total Cholesterol** (mg/dl)	**HDL-C** (mg/dl)
male	Apoe$^{-/-}$	26.9±1.4	39.8±17.1	355.5±59.2	21±8.9
	Apoe$^{-/-}$;MGP$^{-/-}$ ***	17.2±7.0	33±11.8	428.8±53.9	20.6±7.1
female	Apoe$^{-/-}$	23.7±0.8	34.5±9.2	339.3±51.2	17.8±9.4
	Apoe$^{-/-}$;MGP$^{-/-}$ ***	15.1±1.4	39.8±16.1	366.8±52.0	21.8±6.4

Asterisks indicate statistically significant differences. ****<0.001, Tukey's test* (n=3 for each group).

Online Table II.
Serum phosphate levels of Apoe$^{-/-}$, Apoe$^{-/-}$;MGP$^{tg/wt}$, and Apoe$^{-/-}$;MGP$^{-/-}$ mice.

After 16 weeks on chow or Western diet	**Mice**	**Phosphate** (ng/ml)
chow	Apoe$^{-/-}$	0.782±0.027
	Apoe$^{-/-}$;MGP$^{tg/wt}$	0.783±0.031
Western diet	Apoe$^{-/-}$	0.779±0.022
	Apoe$^{-/-}$;MGP$^{tg/wt}$	0.786±0.022
After 18 weeks on chow diet	**Mice**	**Phosphate** (ng/ml)
chow	Apoe$^{-/-}$	0.785±0.017
	Apoe$^{-/-}$;MGP$^{-/-}$	0.786±0.018

No statistically significant differences (n=3 for each group).

Online Table III.
Complete blood count (CBC) of Apoe$^{-/-}$ and Apoe$^{-/-}$;MGP$^{-/-}$ mice.

Mice	WBC	Lym	Mono	Gran	Lym %	Mono %	Gran %
Apoe$^{-/-}$	5.27±0.32	4.17±0.23	0.53±0.06	0.57±8.9	79.57±0.72	8.50±0.62	11.93±0.15
Apoe$^{-/-}$;MGP$^{-/-}$	5.43±0.12	4.27±0.15	0.57±0.06	0.60±0.01	79.17±0.71	8.77±0.58	12.07±0.45

No statistically significant differences (n=3 for each group).

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