SUPPLEMENT MATERIAL

Methods

Animals and diets

ApoE$^{-/-}$ mice on C57BL/6J background were kindly provided by Dr. Maeda (UNC, Chapel Hill, NC). Bmper$^{+/-}$ mice, previously generated in our laboratory on a C57BL/6J genetic background[1], were crossed with ApoE$^{-/-}$ mice to generate Bmper$^{+/-}$;ApoE$^{-/-}$ mice. We used the Bmper$^{+/-}$ mice instead of Bmper$^{-/-}$ mice because Bmper$^{-/-}$ mice die at birth[1]. All adult mice were fed with the standard chow or a high-fat/high-cholesterol diet (Western diet) (Harlan Laboratories, Indianapolis, IN) for 20 weeks. Body weight of mice was monitored before and after they were fed with different diets. Blood serum was obtained every four weeks. All mouse experimental procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Committee for the Use of Animals in Research.

Lipid analysis

Mice were fasted for 18 hours before blood sampling. Less than 200 μl of blood was collected through sub-mandibular bleeding using a lancet. The total cholesterol level was measured enzymatically with a commercially available kit (Infinity kits, Thermo Scientific, Waltham, MA).

Lesion quantification

The mice were euthanized and perfusion fixed with 10% buffered formalin via the left ventricle for 5 minutes. The lesions located in the aorta and aortic sinuses were analyzed using Oil Red O staining. To measure lesions in the aorta, the whole aorta, including the ascending arch, thoracic and abdominal segments, was dissected, gently cleaned of adventitial tissue and stained with Oil Red O following the previously described method[2]. The surface lesion area was quantified with ImageJ software and is presented as a percentage of the total surface area of the whole aorta. To measure the lesions in the aortic sinuses, the

heart and proximal aorta were excised, and the apex and lower half of the ventricles were removed. The remaining sample was embedded in OCT (Tissue-Tek, Fisher Scientific, Pittsburgh, PA) and frozen on dry ice. Starting from the appearance of the aortic valve, serial frozen sections at 5-μm thickness were collected until the aortic valves were completely sectioned following the previously described protocol[2]. Sections were stained with eosin and Oil Red O. The slides were imaged by light microscopy, and the atherosclerotic lesion area located in aortic sinus area was quantified with ImageJ and averaged over a 280 μm region.

Calcification quantification

Deposited calcium in the aorta was detected by staining with von Kossa. The 5-μm cryosections of aortic sinus were prepared as described above and subjected to the von Kossa staining procedure. The calcification area from each section was quantified as a percentage of the total vessel cross-sectional area using ImageJ software.

ELISA measurements

Blood samples were drawn from mice after consuming the high fat diet or standard chow for 4 weeks. Soluble VCAM (sVCAM) and soluble ICAM (sICAM) were measured in plasma in triplicate using an enzyme-linked immunosorbent assay (ELISA) method (R&D Systems, Minneapolis, MN).

Reagents

Recombinant human Bmp4 and Bmper protein and antibodies recognizing Bmper and Bmp4 were obtained from R&D Systems. VCAM1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) for Western blotting and Chemicon for immunofluorescence (Millipore, Billerica, MA). The ICAM antibody was purchased from Cell Signaling Technology (Danvers, MA) and used for western blotting experiments. An additional ICAM1 antibody (purchased from Chemicon) was used for immunofluorescence experiments.

The pSmad1,5,8 antibody was purchased from Cell Signaling Technology and used for both western blotting and immunofluorescence experiments.

Cell culture and siRNA transfection

HUVECs (human umbilical vein endothelial cells) were purchased from Lonza and cultured in endothelial basal medium (EBM; Lonza, Allendale, NJ) supplemented with hydrocortisone, bovine brain extract, epidermal growth factor and 2% fetal calf serum. The cells from passages 4-8 were used for experiments. The stealth siRNA duplexes were obtained from Invitrogen (Grand Island, NY). The siRNAs against mouse Bmper are a mixture of the duplexes of 5'-GAAUUUCAGCCAGAAGGAAGCAAAU-3' and 5'-GGAGAGAUGUGGUCCUCUAUCAAUU-3'. The siRNA against mouse Bmp4 is a duplex of 5'-GCAUGUCAGGAUUAGCCGAUCGUUA-3'. The control siRNA is the Stealth^{TM} RNAi negative control duplex (Cat. No. 12935-300) and was purchased from Invitrogen. The siRNAs were transfected into HUVECs according to the manufacturer's recommended protocol for Nucleofection (Amaxa; the HUVEC protocol). Briefly, for each sample, $2x10^5$ HUVECs were transfected with 300 pmol siRNA. The experiments with Bmp4 or Bmper siRNA-transfected HUVECs were performed one day or four days later, respectively. The siRNAs resulted in more than 70% knockdown of the protein levels of Bmp4 and Bmper.

Shear stress assays

HUVECs were post-confluent for 48 hours before the performance of fluid shear stress experiment to decrease the background signals. Laminar shear stress assay was described previously[3]. Briefly, confluent cells in 10-cm dish were exposed to shear stress using the cone and plate flow chamber system for eight hours at 20 dyne cm^{-2} for laminar shear stress or ±5 dyn cm^{-2} for oscillatory shear stress experiments.

Immunoblotting

Cells were harvested in lysis buffer (1% Triton X-100, 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L Na_3VO_4 and 0.1% protease inhibitor mixture; Sigma) and clarified by centrifugation at 16,000 *g*. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes.

Immunofluorescence

The aortic arch segments were dissected out and gently cleaned of the adventitia. The aortic fragments located at the greater curvature (GC) and lesser curvature (LC) were separated and fixed in 3.7% formaldehyde for 10 minutes at room temperature. The aortic fragments were sequentially treated with 70% ethanol for 30 minutes and 5% hydrogen peroxide in methanol. Then, the segments were washed with water for 5 minutes. For the phospho-Smad 1, 5, 8 antibody, the samples were soaked in boiling citric acid buffer (10 mmol/L, pH 6.0) for 9 minutes to expose the antigens. Next, the aortic fragments or 5-μm cryosections of the aortic root were blocked with 5% heat-inactivated goat serum for 1 hour and then incubated overnight with primary antibodies against ICAM1, VCAM1, CD31 or CD68 diluted in the blocking solution. After three washes in TBS, cells were incubated in the dark with a second antibody conjugated with Alexa Fluor 488 or 568 (Molecular Probes, Eugene, OR) in blocking solution for 90 minutes at 37 °C. After 3 washes in TBS, the fragments were counterstained with DAPI for phospho-Smad1, 5 and 8 staining. The *en face* images of the endothelial layer and the cross-sectional images of the aortic root were visualized by confocal laser scanning microscopy.

Immunohistochemistry

The aortic arch segments were dissected out and gently cleaned of the adventitia. The aortic fragments located at the greater curvature (GC) and lesser curvature (LC) were separated and fixed in 3.7% formaldehyde for 10 minutes at room temperature. After incubating tissue in a sucrose gradient, aortic samples were embedded in OCT compound and submitted for frozen sectioning. For the anti-Bmper and Bmp4 antibody, the samples were soaked in boiling citric acid buffer (10 mmol/L, pH 6.0) for 10 minutes to

expose the antigens. Next, the 5-μm cryosections of the aortic root or aortic arch were blocked with 5% heat-inactivated rabbit serum for 1 hour and then incubated overnight with primary antibodies against Bmper or Bmp4 antibodies diluted in the blocking solution. After three washes in TBS, samples were incubated in a second antibody. For Bmper staining, we utilized amplification processes including the serial incubation with ABC (Vector Labs, Burlingame, CA, USA) and tyramide signal amplification reagent (Waltham, MA, USA) in blocking solution for 30 minutes for each at 37 °C. After 3 washes in TBS, the sections stained with Bmper and Bmp4 antibodies were developed with DAB. The images were recorded using the bright field microscopy with 10x and 20x objective lens.

Statistical analysis

Data are shown as the mean ± SE for 3 to 4 separate experiments. Differences were analyzed with two-way ANOVA and post-hoc analyses such as Student's t-test when needed. Values of $P \leq 0.05$ were considered statistically significant.

Figure legends

Figure S1. The changes in body weight and total cholesterol level of Bmper/ApoE mice after 20 weeks on the standard chow (CH) or high fat diet (HF). A, The change in body weight after 20 weeks was calculated and demonstrated as a fold-change of body weight at Week 20 over Week 0. The numbers listed below the graph are the number of mice used for this experiment. B, The total serum cholesterol level was measured at Week 20. *, $P<0.05$, compared to mice with the same genotype but fed with the control diet. #, $P<0.05$, compared to mice fed with the same diet but with the $ApoE^{-/-}$ genotype.

Figure S2. sICAM1 and sVCAM1 plasma levels increased in Bmper$^{+/-}$ mice after consuming the high fat diet for 4 weeks. Blood was collected, and the protein levels of soluble ICAM1 (A) and VCAM1 (B) were measured at Week 4. n=3.

Figure S3. Bmper and Bmp4 expression was increased in mice fed a high fat diet. The cryo-scetions of aortic root were stained with anti-Bmper (A) and Bmp4 (B) antibodies. All the mice were on the ApoE$^{-/-}$ background. Scale bar: 200 μm.

Figure S4. Bmper haploinsufficiency leads to aggravated atherosclerotic plaque formation in aortic arch area. Mice were fed a high fat diet (HF) or standard chow (CH) for twenty weeks. The aortas were dissected out and stained with Oil Red O. The lesions on the surface of each greater and lesser curvature of aorta arch were quantified as a percentage of the total area of GC and LC. *, $P<0.002$, compared to that lesions located in GC of the same mouse. #, $P<0.05$, compared to Bmper$^{+/+}$ mice fed with the same diet. All the mice were on the ApoE$^{-/-}$ background.

Figure S5. Bmper protein level was increased in the LC compared to GC. (A) The regions of GC and LC located in aortic arch of mouse aortas were processed for staining with Bmper antibody. Scale bar: 200 μm. The arrows represent the positive staining of Bmper protein. Scale bar: 200 μm. (B) The vessel lysates obtained from the GC and LC region of mice were subjected to the Western blotting with anti-Bmper antibody. Each sample was obtained from two mice. *, $P<0.05$, compared to the sample of the GC in the same mouse. #, $P<0.05$, compared to the GC region of Bmper$^{+/+}$ mice. n=3.

References

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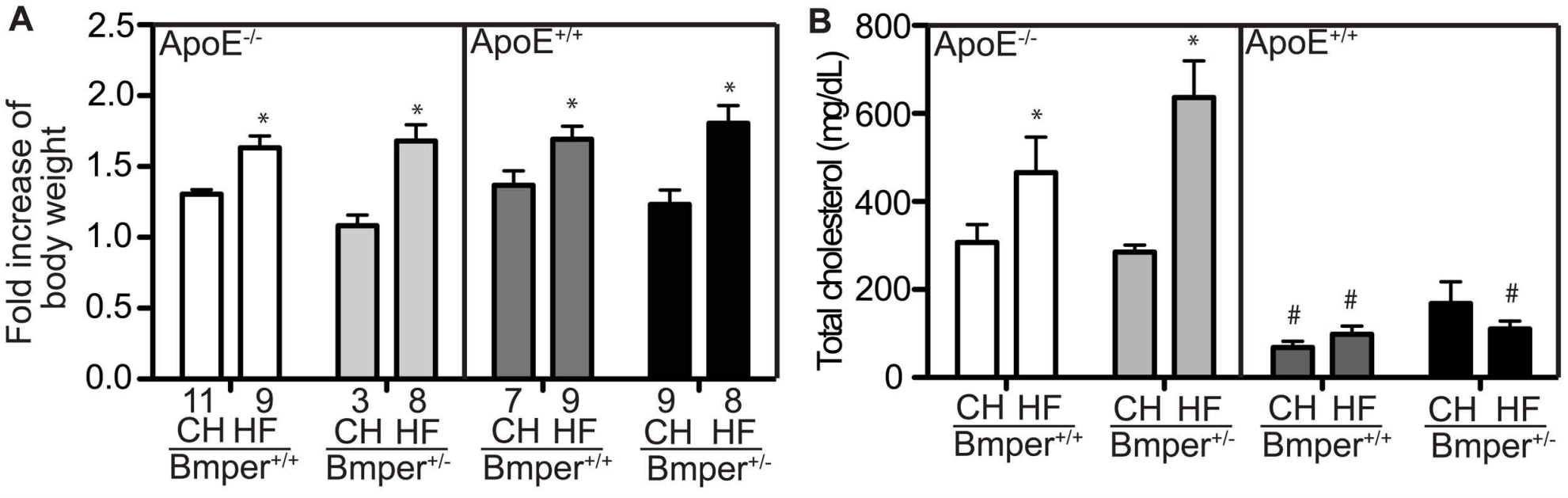
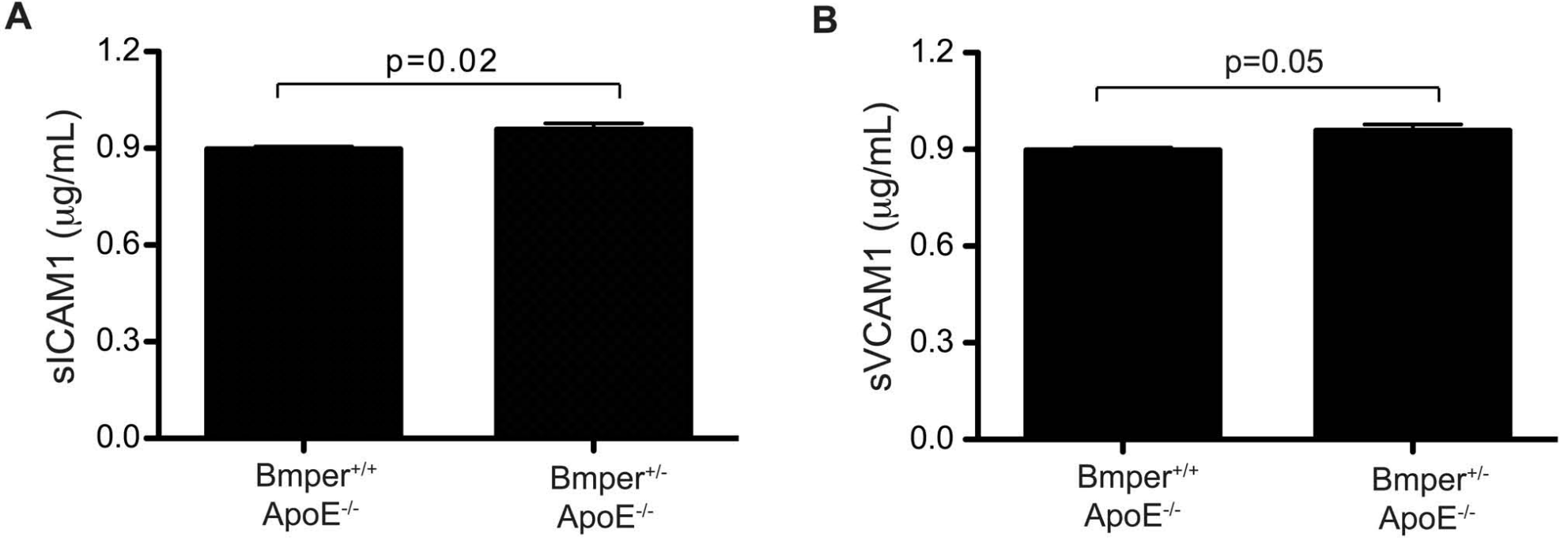
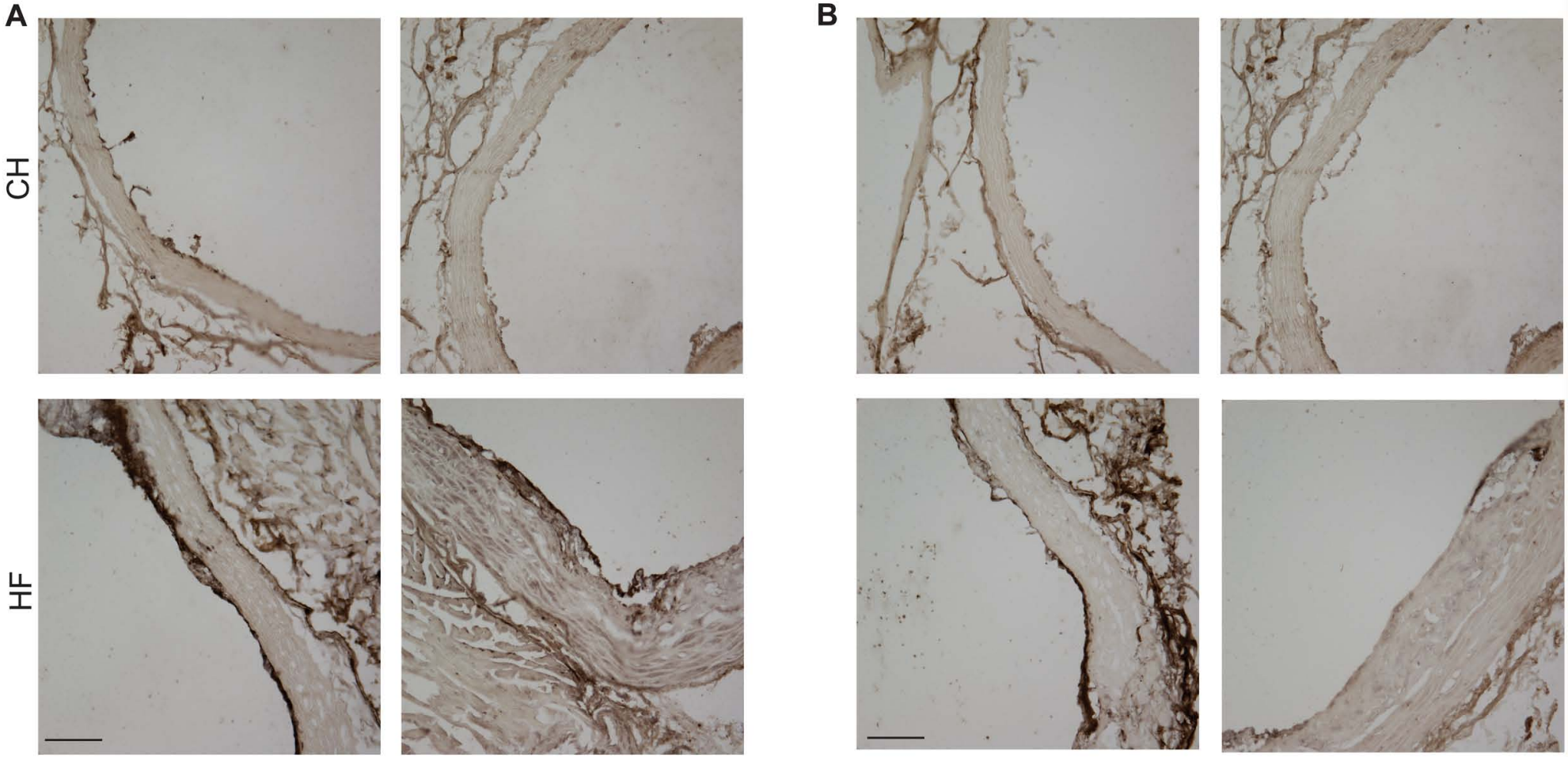
2. Paigen B, Morrow A, Holmes PA, Mitchell D, Williams RA. Quantitative assessment of atherosclerotic lesions in mice. *Atherosclerosis.* 1987;68:231-240.
3. Chen Z, Rubin J, Tzima E. Role of PECAM-1 in arteriogenesis and specification of preexisting collaterals. *Circ Res.* 2010;107:1355-1363.



Pi et al. Supplemental Figure I



Pi et al. Supplemental Figure II



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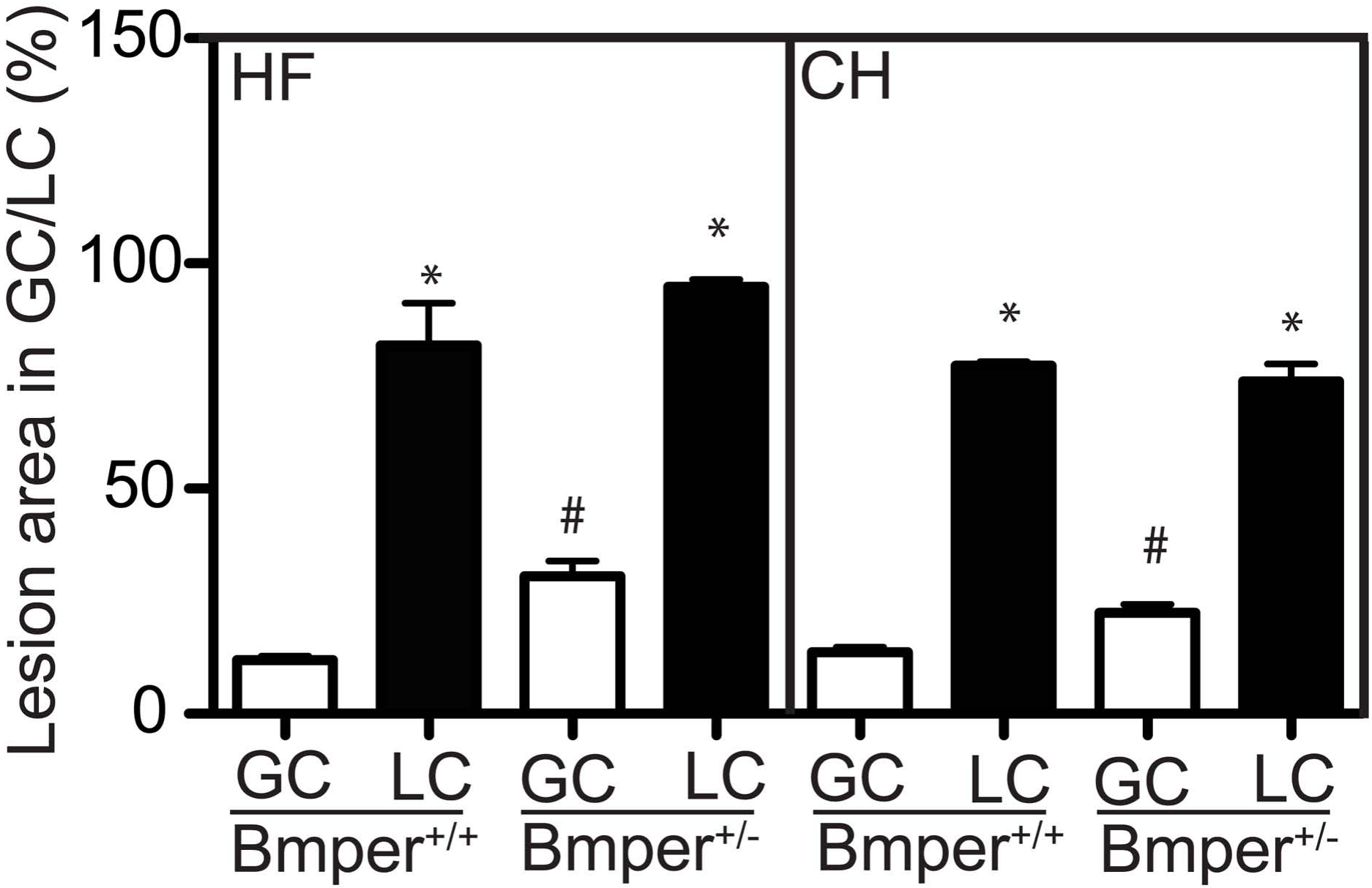
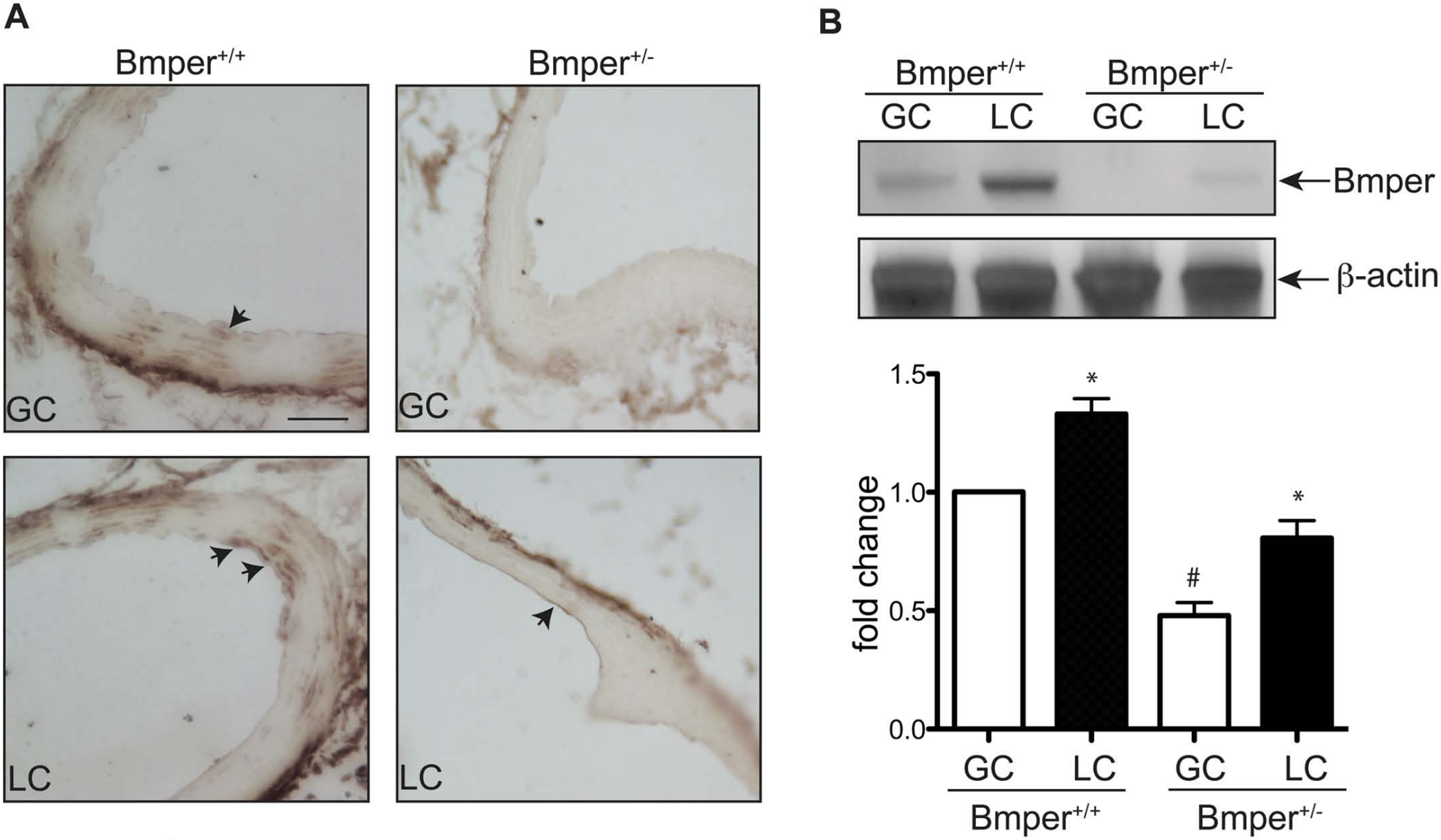


Pi et al. Supplemental Figire IV



Pi et al. Supplemental Figure V