SUPPLEMENTAL DATA

METHODS

Vector Constructions

The BMP-responsive luciferase reporter gene (BRE-Luc) [1] was obtained from Dr. Peter ten Dijke, Leiden University Medical Center, Leiden, the Netherlands. The N-terminally FLAG-tagged MGP epression construct (pN-FLAG-hMGP) vector has been described previously [2], The expression constructs for the mutated human MGP-proteins N-FLAG-MGP-P64G (where proline-64 is mutated to glycine-64) and N-FLAG-MGP-4GlaG (where glutamate residues 56, 60, 67 and 71, which are subject to gamma-carboxylation, are mutated to glycines) have been described elsewhere [3].

LC-MS/MS

BAEC were transfected with an expression construct for N-FLAG-MGP or empty control vector. Cells lysates were prepared 24 hours after transfection, and immunoprecipitated with anti-FLAG antibodies. The immunoprecipitated complexes were analyzed by SDS-PAGE gels and stained with SYPRO Ruby. In-gel digestion of proteins was performed as previously described [4, 5]. Briefly, protein-containing gel plugs were dehydrated in acetonitrile and dried completely in a Speedvac. Samples were reduced and alkylated with 10 mM dithiotreitol and 10 mM TCEP solution in 50 mM NH_4HCO_3 (1 h at 37 °C) and 100 mM iodoacetamide (45 min at 56 °C in the dark), respectively. Gels were washed with 50 mM NH_4HCO_3, dehydrated with acetonitrile and dried down in a Speedvac. Gel pieces were rehydrated in digestion buffer containing 50 mM NH_4HCO_3, and 20 ng/mL of trypsin (37 °C, overnight). Peptides were extracted by 0.1% trifluoroacetic acid in 50% acetonitrile solution, dried down and resuspended in liquid chromatography buffer.

Protein identification was accomplished by LC-ESI-MS/MS using an LTQ linear ion trap mass spectrometer (Thermo Electron) with a dedicated Surveyor pump system equipped with a reversed phase HPLC column (75 µm × 10 cm, BioBasic C_{18}, 5 µm particle size, New Objective,

Woburn, MA). The flow rate was 5 µL/min for sample loading and 250 nL/min for separation. Mobile phase A was 0.1% formic acid, 2% acetonitrile in water, and mobile phase B was 0.1% formic acid, 20% water in acetonitrile. A shallow gradient was used for analyses: linear gradient from 5% B to 40% B over 75 min, then to 100% B over 19 min, and finally keeping constant 100% B for 10 min. The ion transfer tube of the linear ion trap was held at 200°C; the normalized collision energy was 35% for MS/MS and MS^3; and the spray voltage was set at 1.9 kV. Spectra were acquired in data-dependent mode and searched against the human database using SEQUEST. All proteins were identified on the basis of 2 or more peptides. Criteria for positive identification include: Xcorr values of >3.0 (+1), >4 (+2), and >5 (+3); >2 sequenced peptides; and deltaCN >0.1. All spectra used for identification were manually inspected to ensure that the most abundant peaks were assigned.

Immunoprecipitation and Immunoblotting

Co-immunoprecipitation and immunoblotting of BMP-4 and N-FLAG-MGP were performed as described previously [2, 3]. Co-immunoprecipitation of HSP70 and N-FLAG-MGP or BMP-4 was performed using anti-HSP70 antibodies (2 µg/ml, Stressgen), and immunoblotting was performed using anti-FLAG antibodies for N-FLAG-MGP (2.5 µg/ml; Sigma-Aldrich, St. Louis, MO) or anti-BMP-4 antibodies for BMP-4 (0.4 µg/ml, R&D Systems). Alternatively, immunoprecipitation was performed using anti-FLAG or anti-BMP-4 antibodies, and immunoblotting was performed using anti-HSP70 antibodies (0.1 µg/ml).

Co-immunoprecipitation and immunoblotting of HSP70 and non-tagged MGP was performed with volumes of cell lysate or medium that were 5-10 times larger than for co-immunoprecipitation of HSP70 and FLAG-tagged MGP. Co-immunoprecipitation of HSP70 and MGP was performed using anti-HSP70 antibodies (2 µg/ml, Stressgen), and immunoblotting was performed using anti-MGP antibodies (20 µg/ml; Alexis Biochemicals, San Diego, CA) Alternatively, immunoprecipitation was performed using anti-MGP antibodies (10 µg/ml), and immunoblotting was performed using anti-HSP70 antibodies (0.1 µg/ml).

SMAD immunoblotting was performed as previously described [6]. Blots were incubated with specific antibodies to pSMAD1/5/8 (400 ng/ml, Cell Signaling Technology, Danvers, MA), pSMAD2/3 (400 ng/ml, Cell Signaling), total SMAD (400 ng/ml, Santa Cruz Biotechnology, Santa Cruz, CA).

Crosslinking

Serum-free conditioned media from HEK293 cells transfected with N-FLAG-MGP constructs were used in the crosslinking experiments as previously described [3]. The conditioned medium (80 µl; approximately 50 ng/ml) was mixed with BMP-4 (10 ng) or HSP70 (60-100 ng), chemically crosslinked with disuccinimidyl suberate (DSS) and the cross-linked products were analyzed by immunoblotting as previously described [3].

Immunocytochemistry

Immunocytochemistry of cultured BAEC using anti-MGP antibodies was performed as described previously [2].

Proliferation and Tube Formation Assays

To determine proliferation, cells were seeded in 24-well plates at a density of 100,000 cells/well, and ^{3}H-thymidine incorporation was performed as previously described [7]. To assess tube formation, Matrigel™ Matrix (BD Biosciences, Bedford, MA) was diluted 1:3 in conditioned medium from BAEC transfected with N-FLAG-MGP expression construct (final MGP level approximately 40 ng/ml) [2], and with added BMP-4, HSP70 or both; 300 µl was added per well in 12-well plate, and incubated at 37 °C for 30 min to allow polymerization. BAEC were suspended in the same medium at a density of 5 x 10^4 cells/well, and 400 µl of the cell suspension was added to each well. Photos were obtained after 6 hours.

RESULTS

Depletion of HSP70 did not affect expression and secretion of MGP

Since HSP70 is known to act as a chaperone protein [8], we determined if depletion of HSP70 affected expression and secretion of MGP, which is known to be a target gene of the unfolded protein response [9]. HAEC were transfected with HSP70 siRNA (0-100 nM), which reduced HSP70 expression to 20-30% of control cells as determined by real-time PCR and immunoblotting (Suppl. Fig. IA, B). The results showed that this level of depletion of HSP70 did not affect transcription or translation of MGP as determined by real-time PCR and immunocytochemistry, respectively (Suppl. Fig. IC, D). We also determined if depletion of HSP70 affected MGP secretion. Since HAEC are difficult to transfect with expression vectors, we used HEK293 cells to transfect the N-FLAG-MGP expression construct with or without prior transfection of HSP70 siRNA (60 nM). The media were collected and analyzed by immunoblotting using anti-FLAG antibodies. No significant differences were found in media levels of N-FLAG-MGP (Suppl. Fig. IE), suggesting that the depletion of HSP70 did not affect MGP secretion.

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FIGURE LEGENDS

Supplemental Figure I

Depletion of HSP70 using siRNA does not affect transcription, translation or secretion of MGP.

(A, B) HSP70 was depleted to 20-25% of control levels using siRNA as determined by real-time PCR (A) and immunoblotting (B).

(C-E) Depletion of HSP70 did not impair transcription of MGP as determined by real-time PCR (C), translation of MGP as determined by immunocytochemistry (D), or secretion of transfected N-FLAG-MGP from HEK293 cells as determined by immunoblotting with anti-FLAG-antibodies (E).

Supplemental Figure II

Extracellular HSP70 enhances BMP-4-induced tube formation.

BAEC were transfected with the N-FLAG-MPG, -MGP-P64G or –MGP-4GlaG construct or empty vector, and treated with BMP-4 and/or HSP70 as indicated. Tube formation was determined on Matrigel™. Photos were obtained after 6 hours.

Supplemental Figure III

Extracellular HSP70 enhances BMP-4-induced condensation formation in CVC.

CVC were treated with BMP-4 (50 ng/ml), HSP70 (50 ng/ml) and conditioned medium from BAEC transfected with the N-FLAG-MGP, -MGP-P64G or –MGP-4GlaG (approximately 50 ng/ml) construct or empty vector as indicated. Formation of condensations was examined after 2 days.

Supplemental Figure IV

HSP70 enhances BMP-induced ALP activity and mineralization in CVC.

CVC were treated with BMP-4 (50 ng/ml) or BMP-2 (300 ng/ml), HSP70 (50 ng/ml) and conditioned medium from BAEC transfected with the N-FLAG-MGP -MGP-P64G or –MGP-

4GlaG (approximately 50 ng/ml) construct or empty vector as indicated for 2 or 8 days. After 2 days, ALP activity was determined (A, C). After 8 days, accumulation of calcium was determined (B, D).

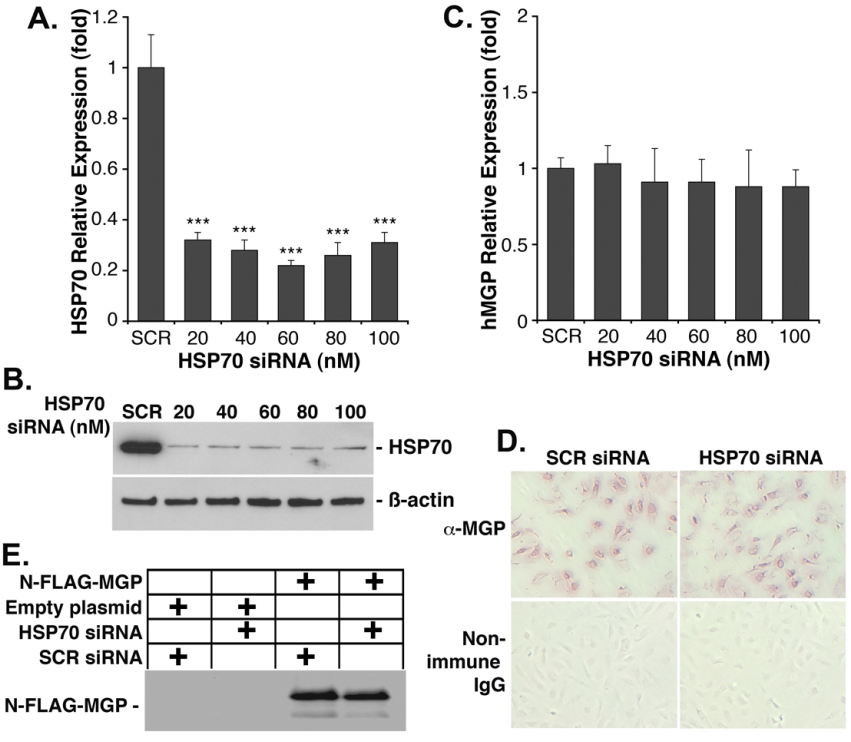
Asterisks indicate statistically significant differences compared to control (empty plasmid). *, $p<0.05$; **, $p < 0.01$; ***, $p < 0.001$; Tukey's test.

Supplemental Figure V

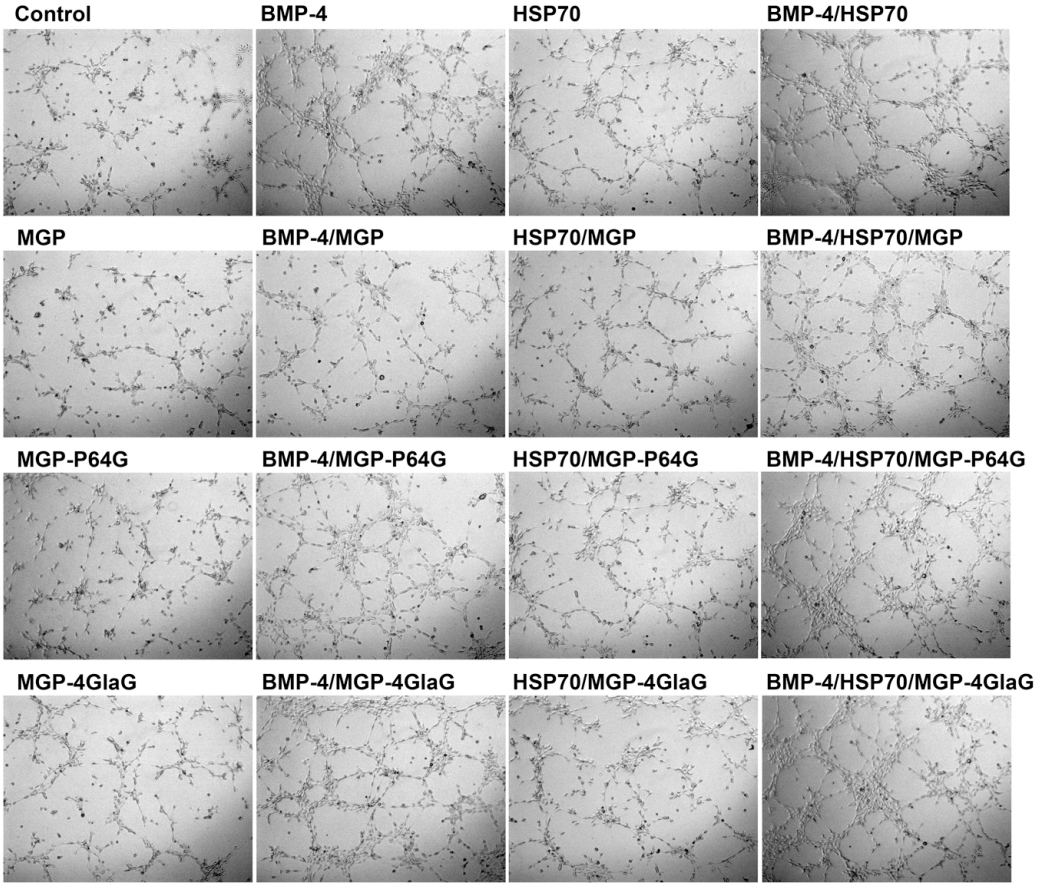
Staining for HSP70 and pSMAD1/5/8 is increased in corresponding areas of atherosclerotic lesions.

Immunohistochemistry for HSP70, pSMAD1/5/8 and control (secondary antibody only) in aorta from chow-fed Apoe-/- mice. Lu, lumen; Les, lesion.

Suppl. Figure I



Suppl. Figure II



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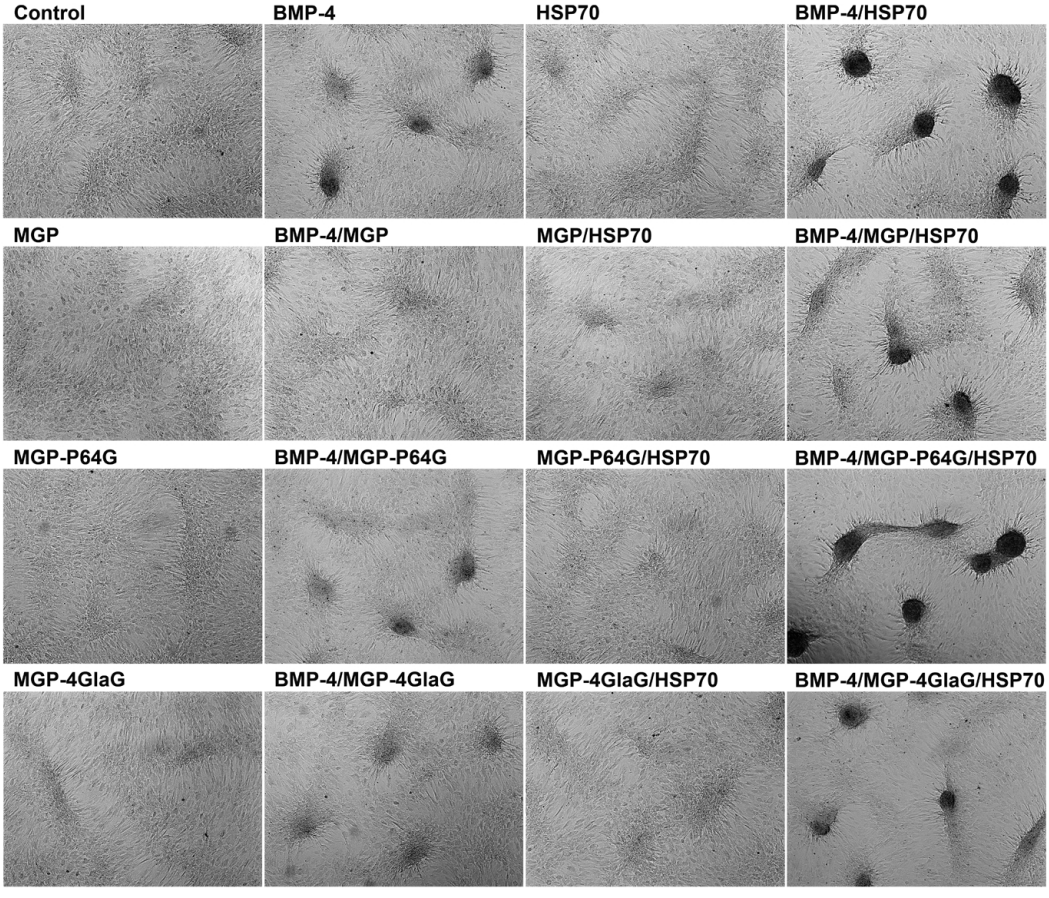
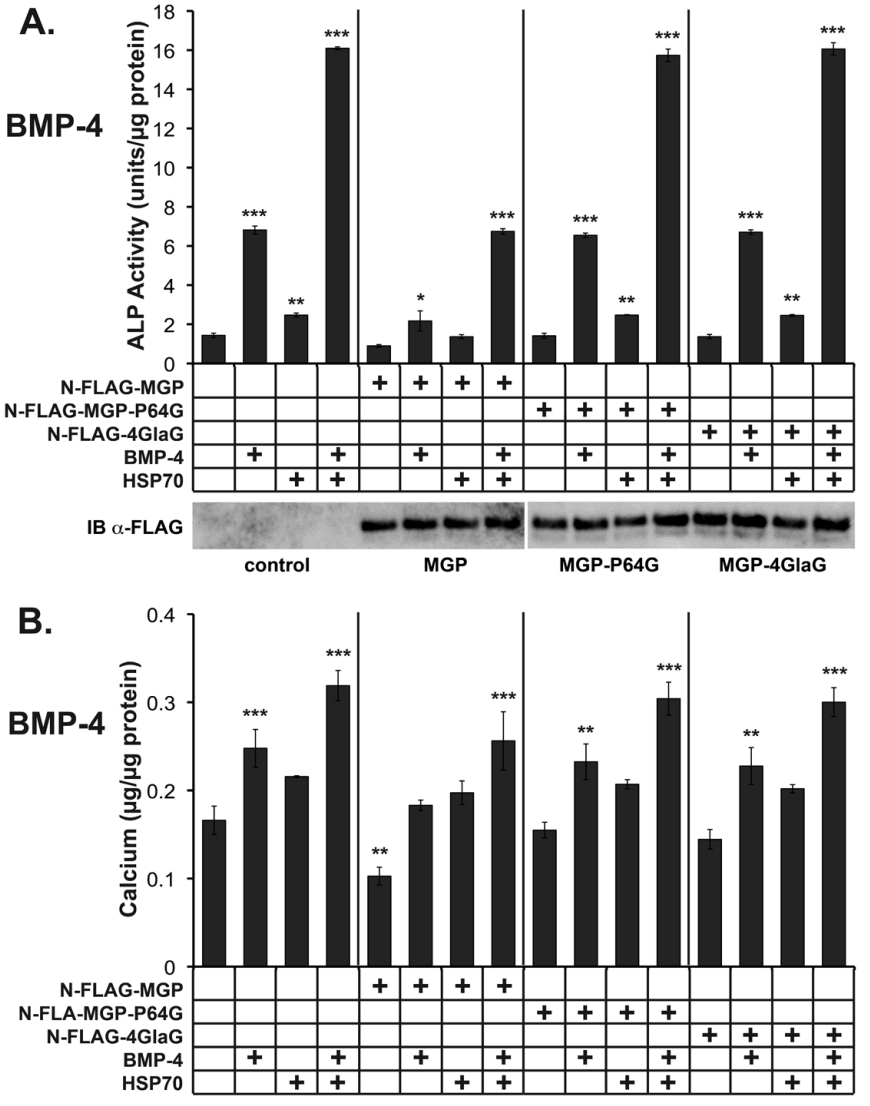
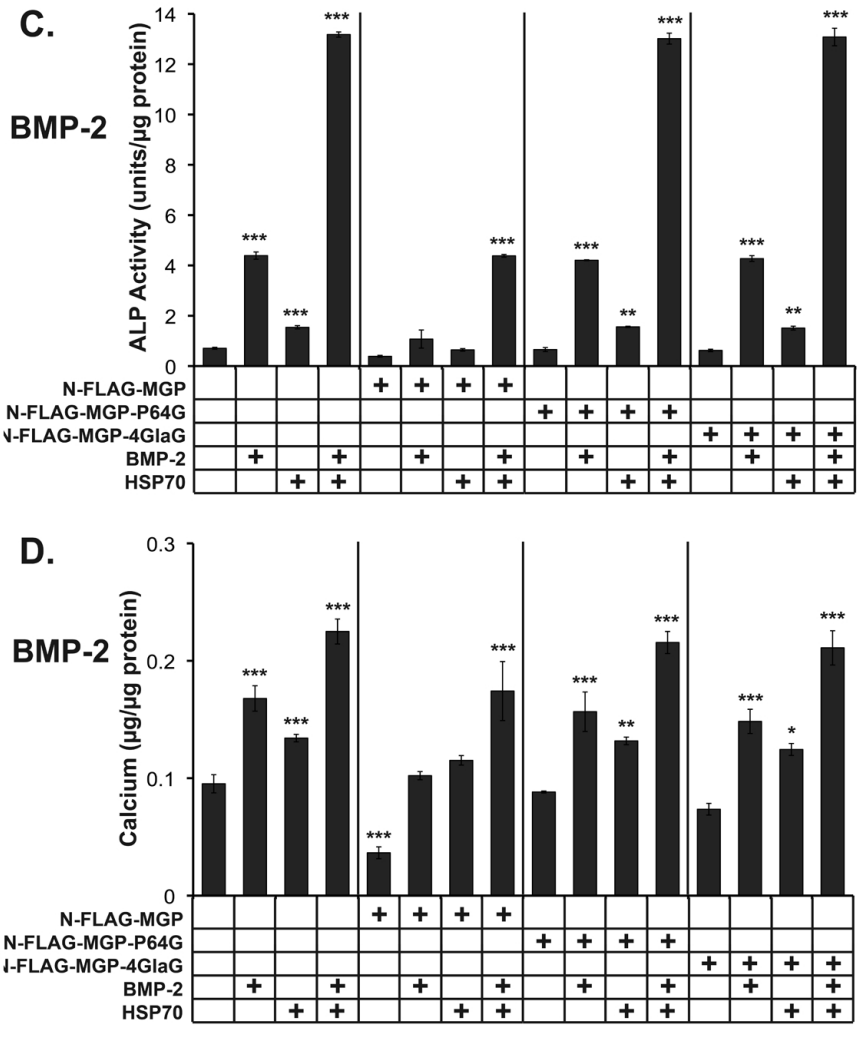


Suppl. Figure IV A&B



Suppl. Figure IV C&D



Suppl. Figure V