Boström KI, Jumabay M, Matveyenko A, Nicholas SB, Yao Y. *Activation of Vascular Bone Morphogenetic Protein Signaling in Diabetes Mellitus*

ONLINE SUPPLEMENT
Detailed Method Section

Animals

The generation of rats transgenic for human islet amyloid polypeptide (HIP rats) has been previously described [1]. The $Ins2^{Akita/+}$ mice (heterozygous for a mutation in one allele of the insulin-2 gene) [2, 3] and the db/db mice (homozygous for the spontaneous mutation $Lepr^{db}$) [4], both on C57BL/6J background, were obtained from the Jackson Laboratories (Bar Harbor, ME). $MGP^{tg/wt}$ mice, generated in our laboratory on a C57BL/6J background [5], were crossed with $Ins2^{Akita/+}$ mice to generate $MGP^{tg/wt}$;$Ins2^{Akita/+}$ 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 [5]. Genotyping was performed by PCR as previously described [5]. The rats were housed individually and fed Rodent Diet 8604 (50% carbohydrate, 24% protein, and 4% fat; Harlan Teklad, Madison, WI) ad libitum; the mice were fed standard chow (Diet 8604, Harlan Teklad, Laboratory, Madison, WI) also ad libitum. All animals were subjected to the standard 12-h light-dark cycle. 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.

Analytical Procedures

Plasma glucose was measured by the glucose oxidase method (Beckman Glucose Analyzer 2; Beckman Coulter, Fullerton, CA). Serum phosphate levels were measured using a QuantiChrom™ Phosphate Assay kit as per manufacturer's instructions (BioAssay Systems, Hayward, CA). Total calcium in lyophilized aortic tissue was determined as previously described [6].

Cell Culture and SiRNA Transfection

Bovine aortic endothelial cells (BAEC), human aortic endothelial cells (HAEC), human aortic smooth muscle cell (HASMC) and bovine calcifying vascular cells (CVC) were prepared and cultured as previously described [7-11]. For treatment, cells were seeded at a confluency of 50-80%, and treatments were added to the media 20-24 hours later. Transient transfections of HAEC were performed with Lipofectamine™2000 (Invitrogen) using 60 nM siRNA as previously described [7]. Briefly, the amount of siRNA was optimized as per the manufacturer's instructions. Three separate siRNAs (Silencer® predesigned siRNA, Ambion) 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 experiments, and treatments were usually initiated 24 hours after the start of the transfection. Silencer® predesigned siRNAs were obtained for BMP-2, BMP-4, ALK1, ALK2, ALK3, ALK6, BMPRII, MGP, Noggin, and VEGF.

RNA Analysis

Real-time PCR analysis was performed as previously described [12, 13], and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as control gene [12]. Primers and probes for human, rat and mouse BMP-2, BMP-4, MGP, Noggin, ALK1, ALK2, ALK3, ALK6, BMPRII, and VEGF were obtained from Applied Biosystems (Foster City, CA) as part of Taqman® Gene Expression Assays.

Immunoblotting

Immunoblotting was performed as previously described [13, 14]. Equal amounts of cellular

protein or culture medium were used. For optimal detection of VEGF in culture media, VEGF was first immunoprecipitated with anti-VEGF antibodies (Santa Cruz Biotechnology), as previously described [12] and then analyzed by immunoblotting using specific antibodies to VEGF (200 ng/mL; R&D Systems). For other proteins, blots were incubated with specific antibodies to pSMAD1/5/8, pSMAD2/3 (both diluted to 400 ng/ml; Cell Signaling Technology, Danvers, MA), total SMAD, BMP-4, ALK1, ALK2 (all diluted to 400 ng/ml; Santa Cruz Biotechnology, Santa Cruz, CA), BMP-2, ALK3, ALK6, osteopontin, osterix (all diluted to 200 ng/ml; Santa Cruz Biotechnology), Noggin (diluted to 400 ng/ml; R&D Systems), BMPRII (diluted to 200 ng/ml; R&D Systems), and core binding factor alpha 1 (Cbfa1, diluted to100 ng/ml; Calbiochem). ß-Actin (1:5000 dilution; Sigma-Aldrich) was used as loading control. The specificity of all antibodies was verified prior to use for immunoblotting and immunostaining as previously described [13].

Immunohistochemistry and Immunofluorescence

Tissue sections were processed and stained as previously described in detail [13, 15]. 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.

We used specific antibodies to MGP (provided by Dr. Reidar Wallin, Wake Forest University, Winston-Salem, NC), pSMAD1/5/8, pSMAD2/3 (both from Cell Signaling Technology), total SMAD, BMP-2, BMP-4, ALK1, ALK2, ALK3, ALK6 (all from Santa Cruz Biotechnology), Noggin, BMPRII (both from R&D Systems).

Alkaline Phosphatase (ALP) Assay and Quantification of Calcium Deposition

Bovine CVC were cultured for up to 8 days in conditioned media from endothelial cells, which were changed every 2 days. Noggin (300 ng/ml, R&D Systems) was added where indicated. ALP activity and calcium accumulation were quantified as previously described [10, 14] after 2 and 8 days, respectively.

Proliferation Assay

BAEC transfected with siRNA were seeded in 24-well plates at a density of 100,000 cells per well, and allowed to attach for 4–6 hours. ^{3}H-Thymidine was added at 1 µCi/ml for 4 days, and ^{3}H-thymidine incorporation was determined as previously described [12].

Tube Formation Assay

Matrigel™ Matrix (BD Biosciences, Bedford, MA) was diluted 1:3 in medium from HAEC treated with high glucose, and 300 µl was added to each well of a 12-well plate and incubated at 37^{o}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.

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.

Media Thickness and Histochemical Staining of Aortic Sections

The media thickness was measured in hematoxylin-stained sections by light microscopy with a calibrated eyepiece micrometer. Five thickness measurements equally spaced along the ventral surface of the aorta were made on each tissue section, and 15 measurements were used to calculate an average thickness for each of five aortic tissue segments as previously described [16]. Von Kossa staining (30 minutes, 5% silver nitrate) and Alizarin Red S staining [17] were performed to visualize calcification in 10 µm-sections. Alcian Blue staining (0.05% Alcian Blue GX in 0.025 M acetate buffer solution, containing 0.025M $MgCl_2$, final pH 5.8, for 18 hrs) was performed to visualize cartilage proteoglycans.

Statistical Analysis

Data were analyzed for statistical significance by two-way analysis of variance with post hoc Tukey's analysis. 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. All experiments were repeated a minimum of three times.

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Supplemental Table I. Serum levels of glucose and phosphate in wild type and diabetic mice (C57BL6/J background), 20 weeks of age.

Mice	**Glucose** (mg/dl)	**Phosphate** (ng/ml)
Wild type	109.3±25.1	0.836±0.004
$Ins2^{Akita/+}$	336.7±53.6***	0.837±0.010
db/db	327.8±45.4***	0.837±0.011

Asterisks indicate statistically significant differences compared to wild type. ****<0.001, Tukey's test* (n=4).

Supplemental Table II. Serum levels of glucose and phosphate in wild type and HIP rats, aged 3-18 months.

Rats	**Glucose** (mg/dl) 3 months	**Glucose** (mg/dl) 6 months	**Glucose** (mg/dl) 12 months	**Glucose** (mg/dl) 18 months
Wild type	79.0±11.5	99.0±7.4	90.3±12.8	72.0±8.5
HIP	104.7±19.9*	139.3±38.5**	292.1±99.8***	263.0±15.6***
Rats	**Phosphate** (ng/ml) 3 months	**Phosphate** (ng/ml) 6 months	**Phosphate** (ng/ml) 12 months	**Phosphate** (ng/ml) 18 months
Wild type	0.837±0.027	0.834±0.024	0.837±0.031	N.D.
HIP	0.839±0.019	0.833±0.022	0.835±0.014	N.D.

Asterisks indicate statistically significant differences between wild type and HIP rats. **<0.05, **<0.01, ***<0.001, Tukey's test* (n=6 for glucose, n=4 for phosphate).

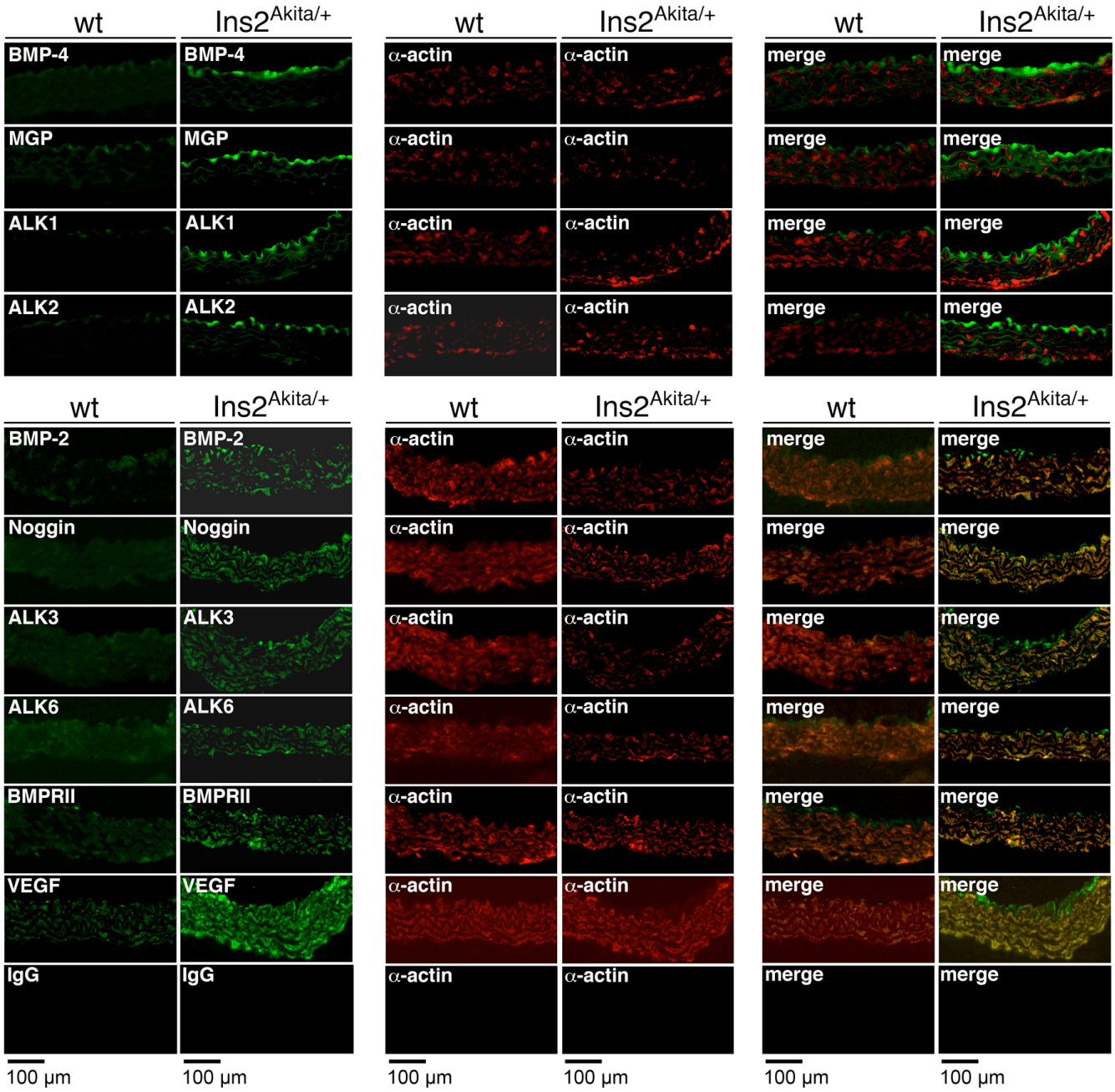
Supplemental Table III. Serum levels of glucose and phosphate in wild type and diabetic mice (C57BL6/J background) with or without the MGP transgene, 20 weeks of age.

Mice	**Glucose** (mg/dl)	**Phosphate** (ng/ml)
Wild type	120±11.3	0.826±0.005
$MGP^{tg/wt}$	121±3.6	0.826±0.014
$Ins2^{Akita/+}$	360±7.9***	0.828±0.018
$Ins2^{Akita/+}$; $MGP^{tg/wt}$	371.7±32.6***	0.826±0.014

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

Supplemental Figure I

A



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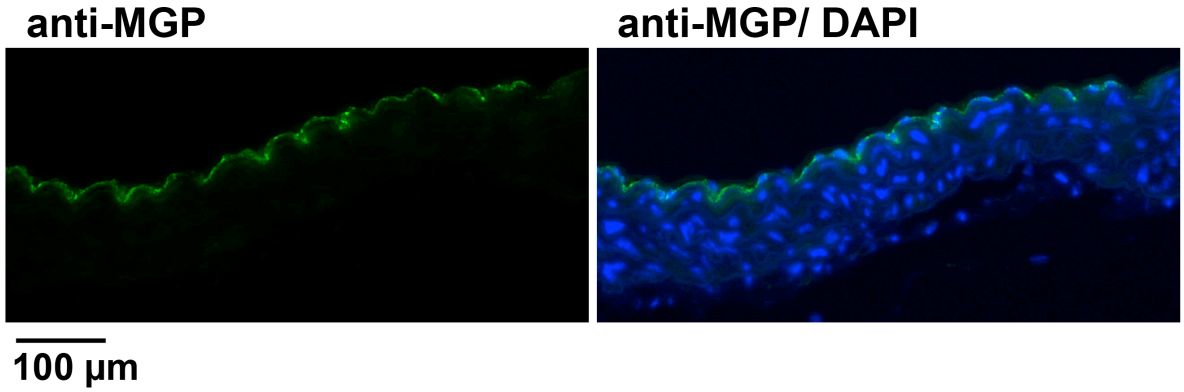

Supplemental Figure I

A. Localization of BMP components in the aortic wall of Ins2 $^{Akita/+}$.
To examine the localization of the expression of the different BMP components in the aortic wall, we selected the Ins2$^{Akita/+}$ mouse and compared it to littermate controls at age 20 weeks. We co-localized expression of BMP-2, BMP-4, ALK1, ALK2, ALK3, ALK6, BMPRII, Noggin, MGP and VEGF with that of smooth muscle α-actin, a marker of the medial SMC, using immunofluorescence.
(Top) BMP-4, MGP, ALK1 and ALK2 (green) were predominantly detected in on the luminal side (up) in proximity to the endothelium, and did not co-localize with smooth muscle alpha-actin (red).
(Bottom) BMP-2, Noggin, ALK3, ALK6, BMPRII and VEGF (green) were detected throughout the vascular wall, and co-localized with alpha-actin (red).

B. High magnification of MGP immunofluorescent staining in wild type mouse aorta.
High magnification of MGP staining in wild type mice (aged 20 weeks), with and without DAPI nuclear stain, demonstrates that the MGP staining (green) is associated with the endothelial layer. Autofluorescence of the elastic lamellae was minimal with green fluorescence in most of our stained specimens, but visible with blue fluorescence.

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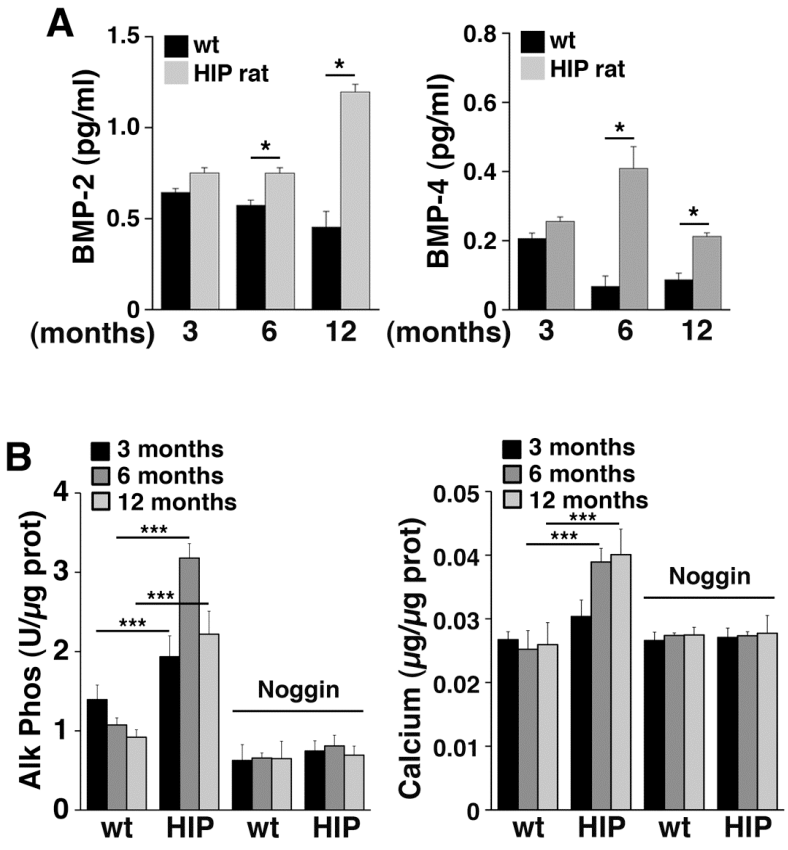
Supplemental Figure II



Supplemental Figure II
Increased serum levels of BMP-2 and BMP-4 in diabetic rats.

(A) Levels of BMP-2 (left) and BMP-4 (right) in serum from HIP rats aged 3-12 months, as determined by ELISA (n=6 animals in each group).

(B) Osteogenic differentiation in CVC incubated with serum from HIP rats and wt rats aged 3-12 months. The cells were treated with medium containing 10% serum from the HIP rats or controls, and alkaline phosphatase activity (left) and calcium accumulation (right) were determined in absence and presence of Noggin (300 ng/ml) after 2 and 8 days, respectively.

Asterisks indicate statistically significant differences compared to wt for the respective age. *<0.05, ***<0.001, Tukey's test*.