Bone morphogenetic protein 4 and Smad1 mediate extracellular matrix production in the development of diabetic nephropathy

Short running title: BMP4/Smad1 in diabetic nephropathy


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Abstract

Diabetic nephropathy is the leading cause of end-stage renal disease. Diabetic nephropathy is pathologically characterized by the accumulation of extracellular matrix in the mesangium, of which the main component is α1/α2 type IV collagen (Col4a1/a2). Recently, we identified Smad1 as a direct regulator of Col4a1/a2 under diabetic conditions in vitro. Here, we demonstrate that Smad1 plays a key role in diabetic nephropathy through bone morphogenetic protein 4 (BMP4) in vivo. Smad1-overexpressing mice (Smad1-Tg) were established, and diabetes was induced by streptozotocin. Non-diabetic Smad1-Tg did not exhibit any histological changes in the kidney; however, the induction of diabetes resulted in approximately 1.5-fold greater mesangial expansion, consistent with an increase in glomerular phosphorylated Smad1. To address regulatory factors of Smad1, we determined that BMP4 and its receptor were increased in diabetic glomeruli and that diabetic Smad1-Tg and WT mice treated with a BMP4-neutralizing antibody exhibited decreased Smad1 phosphorylation and approximately 40% less mesangial expansion than those with control IgG. Furthermore, heterozygous Smad1-knockout mice exhibited attenuated mesangial expansion in diabetic condition. In conclusion, our data indicate that BMP4/Smad1 signaling is a critical cascade for the progression of mesangial expansion and that blocking this signal could be a novel therapeutic strategy for diabetic nephropathy.
Diabetic nephropathy is a life-threatening complication of diabetes mellitus and is the leading cause of end-stage renal disease (1). The structural features of diabetic nephropathy include the thickening of the glomerular basement membrane (GBM) and mesangial matrix expansion (2, 3). Mesangial matrix expansion is pathologically important because it leads to glomerulosclerosis, accompanied by various tubulointerstitial damages and subsequent nephron loss (4, 5). In addition, the severity of mesangial matrix expansion is clinically important because it is closely associated with the decline of the glomerular filtration rate (GFR) (6).

Mesangial matrix expansion is characterized by increased amounts of extracellular matrix (7), particularly α1/α2 type IV collagen (Col4a1/a2) (8). Although various peptides or growth factors are shown to mediate the regulation of this key component, the protein responsible for its direct regulation remains to be determined.

Because various injuries of epithelial, endothelial, and mesangial cells converge on the accumulation of Col4a1/a2 in the mesangium, mesangial cells presumably play a central role for the regulation of Col4a1/a2, even if they are not the primary target of injury (9). Therefore, we attempted to elucidate the direct regulation of Col4a1/a2 under diabetic conditions and demonstrated that Smad1 can transcriptionally regulate Col4a1/a2 in the presence of advanced glycation end-products (AGEs) in mesangial cells (10).
Smad1 is an intracellular molecule originally cloned as a signal transducer of the TGF-β superfamily (11). In response to these stimuli, Smad1 is phosphorylated at the C-terminal SSXS motif, followed by accumulation in the nucleus where it regulates the transcription of specific target genes (12). In vivo, Smad1 is essential for the development of the kidney (13), although it is not detected in adult murine glomeruli (14). Previously, we reported that Smad1 is induced and phosphorylated by AGEs and binds to the promoter of Col4a1/a2, thus up-regulating its transcriptional activity in mesangial cells (10). We also found that Smad1 is highly expressed in human diabetic glomeruli and that glomerular expression of Smad1 is closely correlated with the severity of mesangial matrix expansion in a rodent model of diabetic nephropathy (15). However, the functional role of Smad1 in diabetic nephropathy in vivo remains unknown.

To this end, we attempted to demonstrate that transgenic mice overexpressing Smad1 can accelerate mesangial matrix expansion under diabetic conditions. We also tried to identify a regulatory factor of Smad1 and focused on bone morphogenetic proteins (BMPs) because they are potent stimulators of Smad1. Notably, we demonstrated that BMP4 is increased in diabetic glomeruli. Therefore, to prove the involvement of BMP4 in diabetic nephropathy, we treated diabetic mice with an anti-BMP4 antibody and demonstrated that the neutralization of BMP4 prevented the phosphorylation of Smad1, the accumulation of Col4a1/a2, and mesangial expansion both in diabetic Smad1-Tg mice and their littermates.
Research Design and Methods

Generation of $SMAD$- overexpressing mice

All animal experiments in this study were performed in accordance with the institutional guidelines, and the Review Board of Kyoto University granted ethical permission. The $pCAGGS$-$SMAD1$ vector was constructed by inserting human $SMAD1$ cDNA into the mammalian expression vector $pCAGGS$ (kindly provided by Dr. Miyazaki, Osaka University). Although a previous report indicated that this promoter could be transactivated in glomerular epithelial cells (16), another report showed that the transgene under this promoter was expressed ubiquitously in glomeruli (17). Therefore, it could still work in mesangial cells. Transgenic mice expressing $SMAD1$ were generated as described (18). Integration of the transgene into host genome was confirmed by Southern blot analysis of DNA using a $^{32}$P-labelled $SMAD1$ cDNA fragment as a probe (Fig. 1A).

Generation of inducible Smad1-Tg mice

To generate inducible Smad1 transgenic mouse lines, we used the tamoxifen-regulated Cre-loxP system (TaconicArtemis GmbH, Germany). This system consists of two transgenes. The first transgene is the inducible Smad1 expression cassette,
pMacII-floxed GFP pA-BMP4 using expression vector pMacII, consisting of a cytomegalovirus enhancer and mouse β-actin promoter (Supplemental figure 2A). The second transgene is a construct for the expression of a fusion protein of mutated murine estrogen receptor (Mer) and Cre recombinase (MerCreMer; MCM), with the pCAGGS vector (Supplemental figure 2A). MCM cDNA was a kind gift from Prof. M. Reth (19). Each of these two transgenes was microinjected into the pronuclei of C57BL/6J fertilized eggs to create a transgenic mouse line. For induction of SMAD1 gene expression, 8-week-old transgenic mice were fed a diet containing tamoxifen citrate (400 mg/kg).

**Induction of diabetes**

Diabetes was induced in 8-week-old mice by injecting Streptozotocin (STZ) (50 mg/kg) (Sigma, St. Louis, MO) intraperitoneally for 5 consecutive days. Control animals received 0.1 mmol/L sodium citrate buffer (pH 4.5) alone.

**Tissue preparation**

The right kidney was divided into fragments, each of which was fixed with Carnoy’s solution, neutral buffered formalin, 2% glutaraldehyde or was frozen immediately in OCT compound.

**Antibody preparation**
The rhBMP4 peptide was purchased from R&D systems (Minneapolis, MN). For immunization with the peptide, 6-week-old mice were immunized subcutaneously with 50-µg conjugated peptide once a week during a 4-week period, followed by an interval of 2 weeks. Three days after final immunization, spleen cells were harvested for production of hybridomas to rhBMP4. Monoclonal antibodies were generated using established procedures.

**Protocol for the treatment with a BMP4-neutralizing antibody in mice**

Ten milligrams/kilogram of neutralizing anti-BMP4 antibody was subcutaneously injected into each group of mice once every two weeks from 24 weeks until 36 weeks after the induction of diabetes. As a negative control, mouse IgG (MP Biomedicals-Cappel, OH) was injected at the same time points.

**Renal histology and morphometric analyses**

Two-micrometer sections embedded in paraffin were collected through the largest axial section and were stained with periodic acid silver methenamine (PASM) stain. To quantify mesangial expansion, all tissues were sectioned and stained by one professional pathology technician (H.U.) to control the thickness of sections and the intensity of silver staining of the individual slides. Sections were further coded and read by an observer (M.A.) blinded to the experimental protocol applied (15).
Electron microscopy and measuring glomerular basement membrane thickness

Portions of the cortex were fixed in 2% glutaraldehyde and post-fixed in 1% osmic acid. After embedding, ultrathin sections were stained (20). The average GBM thickness was measured using IMAGE Pro PLUS (Media Cybernetics, Bethesda, MD).

Immunohistochemistry

Kidneys were processed as previously described (15). The primary antibodies used in this experiment are listed in supplemental table 3. For immunostaining of Smad1, a mouse monoclonal antibody was used as described (21).

Immunofluorescence staining and morphometric analysis of glomerular type IV collagen expression

Immunofluorescence staining was performed as described (15). FITC labeled or biotinylated secondary antibody followed by avidin-labeled Alexa 594 (Molecular Probe, Carlsbad, CA) was applied. For immunofluorescence staining of type IV collagen, sections (2 µm) of formalin-fixed paraffin-embedded tissue blocks were used. The anti-type IV collagen antibody used in this experiment reacted mainly with mesangium, which includes α1/α2 type IV collagen, but not with the GBM, which includes α3/α4/α5 type IV collagen. The immunoreactivity of type IV collagen was quantified as described
Isolation of glomeruli

Glomeruli were isolated by Dynabeads® (22). For the quantification of alpha smooth muscle actin (αSMA), laser-manipulated microdissection was performed because some of the glomeruli isolated by Dynabeads had the afferent and/or efferent arteriole still attached, which contained abundant αSMA (23).

RNA isolation

Total RNA was extracted from isolated glomeruli using the guanidinium thiocyanate-phenol-chloroform method (TRIzol reagent, Invitrogen) in 20 µl RNase free water or from microdissected glomeruli using PicoPure™ RNA Isolation Kit (ARCTURUS, CA) in 15 µl Elution buffer.

cDNA preparation and quantification by real-time RT-PCR

For RNA from isolated glomeruli, real-time RT-PCR was performed (24). Specific primers are listed in supplemental table 2, except Col1a2, which was synthesized commercially (ABI Primer & Probes; Applied Biosystems). For RNA from microdissected glomeruli, the primers and probes were obtained from Applied Biosystems.
Western blotting

Tissues were homogenized in RIPA buffer and subjected to immunoblotting (25). The anti-β-actin antibody (#4967), anti-Smad1 antibody reactive only to human or monkey (#9512) and anti-Smad1 antibody reactive to both human and mouse (#9743) were obtained from Cell signaling (Danvers, MA). The anti-phospho-Smad1/5/8 antibody was obtained from Chemicon (Millipore, Billerica, MA). The anti-αSMA antibody was obtained from SIGMA (St. Louis, MO). The anti-GAPDH antibody was obtained from BD Bioscience (San Jose, CA).

Plasmid constructs

The reporter plasmid that contained the Smad1 responsive element (3GC2-Lux) was kindly provided from Prof. Miyazono (University of Tokyo). The αSMA promoter reporter plasmid (SMA-Luc) contains 219 bp of the proximal 5′-flanking region of the αSMA gene subcloned into the luciferase reporter vector (Promega, Madison, WI). Expression vectors for wild type and mutant Smad1 have been described previously (26).

Cell cultures

Murine mesangial cells were established as described previously (25). After
12-hour incubation, cells were starved in DMEM containing 0.5% FCS, followed by the stimuli. Treatment with Dorsomorphin (Tocris bioscience, MO), anti-BMP4-neutralizing antibody, or control IgG antibody was performed 30 min before the stimulation.

**Plasmid transfection and reporter assay**

Mesangial cells or Cos7 cells (1.0 X 10^5/ml) were seeded into 12-well plates (Nunc). After 6 hours, the cells were transfected with 375 ng of SMA-Luc or 3GC2-Lux and 37.5 ng of pRL-CMV (Promega), along with Smad1-DVD, Smad1-ΔC expression vector, or the mock vector. Transfection was performed with FuGENE6 (Roche Diagnostics, Indianapolis, IN). Media was changed 12 hours after transfection to 0.5% FCS in DMEM. Twenty-four hours after medium change, the cells were harvested, and luciferase activity was measured (15).

**Immunostaining of cultured cells**

Mesangial cells (1.0 X 10^5/ml) were seeded in Chamber slides (Nalge Nunc, Denmark). Twenty-four hours after transfection and medium change, cells were fixed in 4% paraformaldehyde and treated with anti-Smad1 antibody at 1:100 (T-20, Santa Cruz Biotechnology, CA). An appropriate FITC-conjugated secondary antibody was used.

**Statistical analysis**
All analyses were performed using JMP11 (SAS Institute, Cary, CA). Normal distribution assumptions were verified using the Shapiro-Wilk test. For BmprII, Alk3, Smad1, and albuminuria, logarithmically normal distribution assumptions were verified. Analyses were performed using a multivariate analysis of variance (MANOVA) for the time course of albuminuria, and one-way or two-way ANOVA for other variables, followed by the Tukey’s HSD test for multiple comparisons. The data are presented as the mean ± SD. A p value of < 0.05 was considered significant.

Results

Establishment of Smad1-overexpressing mice

We constructed a transgene consisting of a fragment of the chicken β-actin promoter and human Smad1 cDNA (Fig. 1A). Transgenic founder lines carrying the human Smad1 transgene were identified by Southern blot analysis (Fig. 1B). Human SMAD1 mRNA was detected only in two founders (Tg#59 and Tg#60). Moreover, because of their poor fertilizing ability, only one line could be finally established from the male founder (Tg#59) (Fig. 1C). Western blot analyses for Smad1 revealed that Smad1 from the transgene was mainly expressed in skeletal muscle, heart, and testis and was slightly expressed in whole kidney, although endogenous Smad1 was expressed ubiquitously (Fig. 1D). On the other hand, Smad1 was increased approximately 50% in
Tg mice compared with their WT littermates in isolated glomeruli, (Fig. 1E). Immunohistochemical analysis revealed that Smad1 from the transgene was detected mainly in the mesangial area (Fig. 1F). To confirm that the Smad1 transgene was expressed in mesangial cells, primary cultures were established from glomeruli isolated from normal 4-week-old Smad1-Tg#59 mice. Smad1 from the transgene was detected in primary cultured mesangial cells from Tg#59 mice (Supplemental Fig. 1A). We also confirmed that no contamination of glomerular epithelial cells in this primary culture from Tg#59 mice by demonstrating that cell lysate from Tg#59 cells did not contain E-cadherin (Supplemental Fig. 1B). However, there were no significant histological changes in the kidney between Smad1-Tg#59 mice and their WT littermates. The glomerular density and glomerular surface area were also comparable between the two groups of mice (10.8 ± 1.9 versus. 11.3 ± 2.2 /µm², and 1980 ± 290 µm² versus 1910 ± 260 µm², respectively).

Because we could obtain only one line using this construct, we established another line of Smad1-Tg mice with inducible Smad1 expression using the tamoxifen-regulated Cre/LoxP system (Supplemental Fig. 2A). The transgene was expressed mainly in epithelial cells (Supplemental Fig. 2B-J) and partly in the mesangial area (Supplemental Fig. 2K). After induction by tamoxifen, Smad1 was significantly increased in various tissues, including the kidney (Supplemental Fig. 2L).
The effect of Smad1 overexpression on albuminuria and mesangial matrix expansion after the induction of diabetes

To examine the role of Smad1 overexpression in diabetic nephropathy, we induced diabetes by STZ. Body weights, HbA1c, and blood pressure in diabetic Smad1-Tg mice did not differ from their diabetic littermates (Table 1).

First, we analyzed mesangial matrix expansion of each group of mice 36 weeks after STZ treatment. Morphometric analysis revealed a significant increase in the mesangial matrix expansion of diabetic Smad1-Tg #59 mice (Fig 1H) and inducible Smad1-Tg #5 mice (Supplemental Fig 2M) compared with their diabetic littermates. Histologically, most glomeruli exhibited a widespread increase in PASM-positive material within the mesangium, termed diffuse lesion (Fig. 1G). Diabetic MCM Tg mice, which did not express the Smad1 transgene, exhibited similar mesangial expansion compared with their WT littermates (Supplemental Fig. 2M).

Next, we measured albuminuria in each group of mice. Both WT and Smad-Tg#59 diabetic mice exhibited more albuminuria than non-diabetic mice at 24 weeks, and these increases were sustained through 32 weeks during the experimental period (Fig. 1I). However, there was no difference in albuminuria levels between WT and Smad1-Tg#59 mice, although diabetic Smad1-Tg#59 mice tended to exhibit slightly more albuminuria compared with WT mice. Inducible Smad1-Tg#5 also exhibited a
tendency towards slightly extended albuminuria relative to their littermates or MCM-Tg mice 32 weeks after the induction of diabetes, but the trend was not statistically significant (Supplemental Fig. 2N).

**Effects of Smad1 overexpression on diabetic nephropathy**

Previously, we reported that Smad1 transcriptionally regulates Col4a1/a2 and other extracellular matrix proteins, such as type I collagen, *in vitro*. Because mesangial matrix expansion was accelerated in diabetic Smad1-Tg mice compared with their diabetic littermates, we quantified the glomerular expression of Col4a1/a2 and Col1a1/a2 in these mice. As shown in Fig. 2A, the glomerular expression of these molecules was increased in diabetic mice compared with those in non-diabetic mice. Moreover, the expression was significantly increased in diabetic Smad1-Tg mice relative to their diabetic littermates. Immunohistochemistry revealed that these molecules were accumulated mainly in the mesangial area (Fig. 2B). Alpha smooth muscle actin (αSMA) is another key molecule in diabetic glomerulopathy and is a marker of mesangial phenotypic changes. Therefore, we examined the glomerular expression of αSMA in each group of mice. In diabetic mice, the expression of αSMA was significantly increased after STZ treatment. Of note, Smad1-Tg mice exhibited significant expression of αSMA at the same stage (Fig. 2A). Immunohistochemistry revealed that increased αSMA was also
localized mainly in the mesangial area (Fig. 2B).

We further investigated the thickness of the GBM by electron microscopy. Diabetic Smad1-Tg mice exhibited significant GBM thickening relative to their diabetic littermates at the same stage (Fig. 2C and D). Glomerular expression of Col4a3, a major molecular component of the GBM, was also increased in diabetic Smad1-Tg in parallel with GBM thickening (Fig. 2E).

**Phosphorylation of Smad1 after the induction of diabetes in Smad1-Tg mice**

These data demonstrated that the overexpression of Smad1 per se did not exacerbate nephropathy. Smad1 is activated by the phosphorylation of its carboxyl terminus. Therefore, we quantified Smad1 activation by counting cells with positive staining of phosphorylated Smad1. Although glomerular expression of Smad1 was increased in Smad1-Tg mice relative to their littermates (Fig. 2F), phosphorylated Smad1 was barely detectable both in Smad1-Tg mice and their littermates before STZ treatment. After STZ treatment, however, Smad1 was phosphorylated and translocated into the nucleus both in Smad1-Tg mice and their littermates (Fig. 2G and 2H). Notably, nuclear translocation of phosphorylated-Smad1 was more evident in diabetic Smad1-Tg mice than in their littermates (Fig. 2H) and was largely localized in mesangial and/or endocapillary cells (Fig. 2I).
Diabetic changes in the regulatory factors of Smad1 phosphorylation

Our data demonstrated that Smad1 is phosphorylated under diabetic conditions. It is generally accepted that BMPs/BMPRs are potent stimulators of Smad1. Among the BMPs, BMP2, 4, and 7 are expressed at various sites in different embryonic stages of renal development (27-29). We observed that Bmp7 was abundantly expressed both in non-diabetic and diabetic glomeruli. However, there was no difference in its expression between the non-diabetic and diabetic groups of mice (Fig. 3A). Notably, the glomerular expression of Bmp4 was increased by approximately 2-fold after STZ treatment both in WT and Smad1-Tg mice (Fig. 3B), although Bmp2 was not detected by RT-qPCR using the same aliquot (data not shown). These data suggest that BMP4 is involved in the progression of diabetic nephropathy. BMPs induce Smad1 phosphorylation by forming heterotetrameric complexes with two major types of membrane-bound serine/threonine kinase receptors, the type I ALK receptors and the type II receptors (30). In vitro binding assays suggest that ALK3/6 are type I receptors for BMP4 (31, 32). Therefore, we examined the glomerular expression of BMPRII, Alk3, and Alk6 in these mice. The glomerular expression of BmprII and Alk3 but not Alk6 was increased after STZ treatment. Next, we studied the localization of BMP4 and ALK3 by immunohistochemistry. In non-diabetic mice, BMP4 was barely detected in the glomeruli (data not shown). In contrast, 36 weeks after diabetes induction, BMP4 was extensively expressed in the podocytes and partly in the mesangium (Fig. 3B). Double
immunostaining for ALK3 and desmin (Fig 3C left panel), PDGF receptor β (Fig 3C middle panel), or nephrin (Fig 3C right panel) revealed that increased ALK3 in diabetic glomeruli was mainly localized in the mesangium. These data suggest that BMP4/ALK3/Smad1 signaling contributes to the progression of diabetic nephropathy.

Neutralizing BMP4 ameliorates the exacerbation of glomerular injuries in diabetic mice

To further delineate the role of BMP4 in the development of diabetic nephropathy, we administered a neutralizing antibody against BMP4 to both diabetic Smad1-Tg mice and their diabetic littermates. First, we evaluated the specificity of the neutralizing activity of the antibody using an assay measuring Smad1 phosphorylation in mesangial cells induced by BMPs and TGF-β. The addition of the antibody completely inhibited the phosphorylation of Smad1/5/8 induced by BMP4 but not by BMP2, BMP7, or TGF-β (Fig. 4A), indicating its specificity. Next, we administered the neutralizing antibody or control IgG to each group of mice (Table 2). The administration of the neutralizing antibody attenuated the nuclear translocation of phosphorylated Smad1 (Fig. 4B), ameliorated the glomerular accumulation of type IV collagen (Fig. 4D), and inhibited mesangial matrix expansion (Fig. 4C and 4F) both in diabetic Smad1-Tg mice and littermates. Furthermore, RNA quantification by RT-qPCR from isolated glomeruli by laser-manipulated microdissection revealed that treatment with the
BMP4-neutralizing antibody also improved glomerular expression of αSMA both in diabetic Smad1-Tg mice and littermates (Fig. 4E). However, the BMP4-neutralizing antibody did not have any effect on albuminuria (Fig. 4G).

**Role of BMP4 in mesangial cell αSMA expression**

In this study, we have demonstrated that overexpression and subsequent phosphorylation of Smad1 results in an increase in the glomerular expression of αSMA, whereas the inhibition of Smad1 phosphorylation using an anti-BMP4 antibody leads to the improvement of glomerular expression of αSMA. To further elucidate the relationship between Smad1 phosphorylation and αSMA, we generated Smad1 mutants: a constitutively active mutant in which two serine residues at the carboxyl termini were substituted with aspartic acid (Smad1-DVD) (26) and a dominant negative mutant in which the carboxyl termini were lacking (Smad1-ΔC) (Fig. 5A). The expression of Smad1-DVD increased the transcriptional activity of 3GC2-Lux, a Smad1-dependent reporter, whereas the expression of Smad1-ΔC did not (Fig. 5B). In mesangial cells, Smad1-DVD was localized in the nuclei, whereas Smad1-ΔC was localized mainly in the cytoplasm (Fig. 5C). Therefore, we asked whether the transcriptional activity of αSMA is modulated by the constitutive activation of Smad1 in mesangial cells. As expected, the expression of Smad1-DVD but not Smad1-ΔC increased the transcriptional activity of
αSMA in mesangial cells (Fig. 5D). Finally, we examined whether dorsomorphin, a small molecule inhibitor of BMP signaling (33), can affect the expression of αSMA by inhibiting the phosphorylation of Smad1 in mesangial cells. As shown in Fig. 5E and 5F, BMP4 induced the phosphorylation of Smad1 along with increased αSMA expression, which was blocked by dorsomorphin. These data indicate that BMP4 mediates αSMA expression via the phosphorylation of Smad1 in mesangial cells.

**Heterozygous SMAD1 knockout mice exhibit attenuated mesangial sclerosis in diabetes**

We further investigated whether the reduction of Smad1 expression improved the diabetic glomerular changes using heterozygous SMAD1 knockout mice, which were kindly provided from Dr. Anita B Roberts (34). After the induction of diabetes, body weights, blood pressure, and HbA1c in diabetic SMAD1 heterozygous KO mice did not differ from their diabetic WT mice (Supplement table 4). The glomerular expression of Smad1 was reduced by approximately 30% both in diabetic and non-diabetic SMAD1 heterozygous KO mice (Figure 6A). We observed a partial attenuation of albuminuria (Figure 6B) and mesangial matrix expansion (Figure 6C and 6D) along with the improvement of the glomerular expression of Col4a1/a2 and Col1a1/a2 (Figure 6E) in diabetic SMAD1 heterozygous KO mice compared with diabetic wild type mice. These data suggest that Smad1 is a critical determinant for the development of diabetic
nephropathy.

Discussion

In this study, we clearly demonstrate that Smad1 plays a critical role in the development of diabetic nephropathy. Smad1 is the only molecule demonstrated *in vivo* to be a direct regulator of Col4a1/a2. Therefore, it seems reasonable to consider Smad1 or its modifier as therapeutic targets for diabetic nephropathy. In this regard, BMP4 could be a potential candidate. We recently reported that heterozygous BMP4 knockout mice exhibit attenuated diabetic nephropathy (18). However, because these mice display renal abnormalities (35), this attenuation might be partly due to the effect of the congenital kidney anomalies. Therefore, in this study, we used a more physiological method for modulating BMP4 action using the BMP4-blocking antibody and clearly demonstrated that the inhibition of BMP4 ameliorated diabetic glomerular injuries.

It is noteworthy in this study that BMP4 could aggravate diabetic nephropathy, although BMP7 is known to protect against diabetic nephropathy. BMP4 could have several distinct roles from BMP7 in its structure and function. First BMP4 has only 58% homology in peptide sequence to BMP7. Second, the functional targets differ between BMP4 and BMP7 during kidney development. For example, BMP4 promotes glomerulogenesis, but BMP7 does not. On the other hand, BMP7 but not BMP4 contributes to mesenchymal survival. (36). Finally, the downstream target of BMP4 is
Smad1 in mesangial cells, as demonstrated in this study, whereas the downstream target of BMP7 in mesangial cells is Smad5 (37). Thus, it is suggested that each BMP has its own function and signaling targets both in the development and disease progression of the kidney.

It remains to be determined whether BMP4/Smad1 signaling is specific for diabetic nephropathy. We previously reported increased glomerular expression of BMP4/Smad1 in another diabetic model mice (38) and also identified PDGF-B as another activator of Smad1 in mesangial cells via Src in murine experimental glomerulonephritis (39). Smad1 also operates during chronic stages of fibrosis in scleroderma (40). Thus, we speculate that the Smad1 signaling is a common pathway among various glomerular injuries and organ fibrosis. To determine the specificity of BMP4 in diabetic nephropathy, the glomerular expression of BMP4 should be tested in other disease models in the future experiments.

In this study, albuminuria did not reflect mesangial expansion in BMP4 antibody-treated diabetic mice and diabetic Smad1-Tg mice, although diabetic Smad1 heterozygous KO mice exhibited slightly improved albuminuria. The mechanisms responsible for these results remain unknown. Previous reports indicated that albuminuria was not correlated with the severity of mesangial expansion in incipient diabetic nephropathy both in humans (41) and a rodent model (15). Treatment with an anti-TGF-β antibody does not attenuate albuminuria in db/db mice despite its beneficial effects on
glomerular matrix expansion (42). These data suggest distinct mechanisms may underlie albuminuria and mesangial matrix expansion in diabetic nephropathy.

In conclusion, this study of Smad1-overexpressing mice revealed that both the induction and phosphorylation of Smad1 play critical roles for the development of diabetic glomerulopathy \textit{in vivo}. BMP4 might be responsible for the phosphorylation of Smad1 and represents a novel therapeutic target for diabetic nephropathy.
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Duality of Interest

T.D received Collaborative research from Chugai Pharmaceutical Co., Ltd.

Author contributions

N.F, H. Arai, and T.D conceived and designed the experiments. T.M, M.A, H. Abe, U.O, C.G, N.F performed the experiments. U.O and K.J established the transgenic mice. K.M established the neutralizing antibody. T.M, A.M, T.T, M.A, K.T, S.K, K.N, N.I, N.F, and T. Kita analyzed the data. T.M, H. Abe, H. Arai, and T.D wrote the paper. T.M and H. Abe are the guarantors of this work, had full access to all the data, and take full responsibility for the integrity of data and the accuracy of data analysis.

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Prior Presentation

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References


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Table 1

Characteristics of non-diabetic (Control) and streptozotocin induced diabetic (STZ) wild type (WT) and Smad1transgenic (Tg) mice

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*p<0.05 versus control. BW, body weight; SBP, systolic blood pressure; rKW, right kidney weight; CCr, creatinine clearance
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*p<0.05 versus control

IgG, mice treated with control IgG antibody; αBMP4IgG, mice treated with BMP4 neutralizing antibody
Figure Legends

Figure 1: Smad1-overexpressing mice exhibit severe glomerular injuries after the induction of diabetes

A: Schematic structure of the transgene. The transgene construct carried a chicken β-actin promoter with a CMV enhancer, rabbit β-globin intron, 1.4-kb human Smad1 cDNA, and rabbit β-globin polyA.

B: Southern blot analysis of genomic DNA extracted from the mouse tail and digested with EcoRI. The 0.9-kb and 1.4-kb transgenes (open arrowheads) were detected in line 59 using a cDNA probe (thick black line in A). A closed arrowhead indicates the genomic Smad1.

C: Northern blot analysis of Smad1 mRNA in whole kidney of wild type and Smad1-transgenic mice (Smad1-Tg). The Smad1 mRNA from the transgene is approximately 1.4 kb (open arrowhead), which is smaller than the endogenous transcript (3.3 kb, closed arrowhead). Transgene-specific signals were detected in the kidney of Smad1-Tg #59 and Smad1-Tg #60 mice. Smad1-Tg #32 was the non-expression line.

D: Western blot analysis of Smad1 protein from various tissues of Smad1-Tg mice. The upper two panels were blotted using an anti-Smad1 antibody that detects both human and mouse Smad1. The lower two panels were blotted using an anti-human
Smad1-specific antibody. The transgene was expressed mainly in muscle, heart, and testis. The vertical dividing lines between lane 2 and 3, and lane 6 and 7 indicate where irrelevant lanes were deleted from the final image.

E: Representative western blot analysis and relative bar graph quantification of proteins isolated from the glomeruli in WT (n=4) and Smad1-Tg mice (n=4). Glomeruli isolation was performed using Dynabeads as described in “Research design and methods”. β-actin was used as an internal control.

F: Immunohistochemistry of total Smad1 in the glomerulus. Smad1 was clearly detected in the glomerulus of Smad1-Tg mice and was mainly localized in the mesangial area (middle panel). The right panel is the kidney section of Smad1-Tg mice stained with control IgG. Sections were counter-stained with hematoxylin solution.

G: Representative views of light microscopy 36 weeks after STZ treatment of the kidney from non-diabetic non-Tg (upper left), non-diabetic Smad1-Tg#59 (upper right), diabetic non-Tg (lower left), and diabetic Smad1-Tg#59 (lower right). Bar represents 100 μm (PASM staining, X 400 original magnification)

H: Mesangial matrix fraction 36 weeks after STZ treatment in each group of mice. Glomerular surface area and PASM-positive area were determined as described in Research design and methods. The mesangial sclerotic fraction is reported as the percentage of mesangial matrix area per total glomerular surface area. n=6 in
non-diabetic WT mice. n=6 in non-diabetic Smad1-Tg#59 mice. n=10 in STZ-induced diabetic WT mice. n=10 in STZ-induced diabetic Smad1-Tg#59 mice. Comparisons were made using two-way ANOVA followed by the Tukey’s HSD test. The results indicated significant main and interaction effects for genotype and for diabetes. The data are presented as the mean ± SD. # p < 0.05, versus non-diabetic mice of the same genotype. * p<0.05, versus diabetic WT mice of the same diabetic condition.

I: Urinary albumin excretion (UAE) after the induction of diabetes in Smad1-Tg#59 mice and littermates. Open triangles, open squares, closed triangles and closed triangles represent vehicle-treated non-Tg mice (WT Cont: n=6), Smad1-Tg#59 mice (Tg Cont: n=6), STZ-treated non-Tg mice (WT STZ: n=10), and Smad1-Tg#59 (Tg STZ: n=10), respectively. Urinary albumin excretion revealed a marked increase in diabetic Smad1-Tg#59 mice from 24 weeks. Data were logarithmically transformed, and comparisons were made using MANOVA followed by the Tukey’s HSD test for multiple comparisons. The data are presented as the mean ± SD. * p < 0.05, versus non-diabetic mice of the same genotype at each period compared with the pre-treatment period.

**Figure 2: Diabetic Smad1-Tg exhibit increased glomerular expression of type IV collagen, type I collagen, and αSMA as well as increased phosphorylation of Smad1.**

A: Quantification of Col4a1, Col4a2, Col1a1, Col1a2, and αSMA mRNA by RT-qPCR in isolated glomeruli 36 weeks after STZ treatment as described in Research design and methods. Ribosomal RNA 18S expression was used as a control.

B: Representative immunohistochemical views of each group of mice. Immunostaining for type IV collagen (first column), type I collagen (second column), and αSMA (third column) in non-diabetic WT mice (WT Cont: first row), non-diabetic Smad1-Tg#59 mice
(Tg Cont: second row), diabetic WT mice (WT STZ: third row), or diabetic Smad1-Tg mice (Tg STZ: fourth row) are presented. Bar: 50 µm.

C: Representative views of GBM in each group of mice are presented. Bar: 1 µm.

D: Quantification of GBM thickness in each group of mice as described in the Materials and methods.

E: Quantification of Col4α3 mRNA by RT-qPCR in isolated glomeruli 36 weeks after STZ treatment as described in the Materials and methods. Ribosomal RNA 18S expression was used as a control.

F: Quantification of the mRNA expression of Smad1 in the glomeruli 36 weeks after STZ treatment.

G: Percentage of the cells with phosphorylated nuclear Smad1 by immunohistological staining in each group of mice.

A, D, E, F, and G: n=6 in non-diabetic WT mice. n=6 in non-diabetic Smad1-Tg#59 mice. n=10 in STZ-induced diabetic WT mice. n=10 in STZ-induced diabetic Smad1 Tg#59 mice. Comparisons were made using two-way ANOVA followed by the Tukey’s HSD test. The results indicated significant main effects for genotype and for diabetes for all factors as well as significant effects for the genotype x diabetes interaction with respect to Col1a1, αSMA, pSmad1 and the thickness of GBM; no significant interaction effects were observed with respect to Col4α1/α2, Col1α2, Col4α3, and Smad1. # p < 0.05, versus non-diabetic mice of the same genotype. * p<0.05, versus WT mice in the same diabetic
condition.

H: Representative immunohistochemistry of phosphorylated Smad1 in WT and Smad1-Tg#59 mice at 36 weeks with or without STZ treatment. Bar: 50 µm.

I: Enlarged glomerular image of the dashed square in Fig. 2H. Positive nuclear staining for phosphorylated Smad1 was localized in the mesangial area (asterisk) and endocapillary area (open arrowhead). Sections were counter-stained with hematoxylin solution. Note that glomerular parietal cells were negative for phosphorylated Smad1 (closed arrowhead). Bar: 50 µm

Figure 3: Glomerular expression of BMP4, Type II BMP receptor (BMPRII), and type I BMP receptor (ALK3) are increased in diabetic mice.

A: Quantification of BMP7, BMP4, BMPRII, and ALK3 mRNA expression by RT-qPCR in isolated glomeruli 36 weeks after STZ treatment. Ribosomal RNA 18S expression was used as control. Specific primers for each gene transcript are listed in supplemental table 2. n=6 in non-diabetic WT mice. n=6 in non-diabetic Smad1-Tg#59 mice. n=10 in STZ-induced diabetic WT mice. n=10 in STZ-induced diabetic Smad1-Tg#59 mice. Comparisons were made using two-way ANOVA followed by the Tukey’s HSD test. The results indicated significant main effects for diabetes but no significant main effects for genotype or for the genotype x diabetes interaction effects with respect to BMP4, BMPRII, and ALK3. # p < 0.05, versus non-diabetic mice of the same genotype. *
p<0.05, versus WT mice in the same diabetic condition. # p < 0.05, versus non-diabetic mice of the same genotype. ns: not significant.

B: Representative immunohistochemistry of BMP4 in diabetic Smad1-Tg mice (Tg STZ). Note that the glomerular expression of BMP4 was mainly localized in podocytes (arrowhead). Bar: 50 µm. Inserted photo in E is an enlarged image of the area around the arrowhead. Sections were counter-stained with hematoxylin solution. Bar: 25 µm

C: Double immunofluorescent staining for ALK3 with desmin (left panel), PDGF receptor β (PDGFRβ) (middle panel), or nephrin (right panel). Desmin is a marker of mesangial cells or injured podocytes, PDGFRβ is a marker of mesangial cells, and nephrin is a marker of podocytes. ALK3 (first row), desmin (second row in the right panel), PDGFRβ (second row in the middle panel), nephrin (second row in the left panel), and merged images (third row) from non-diabetic mice (Tg#59 Cont: left column) and diabetic mice (Tg#59 STZ: right column) are presented. Note that ALK3 is hardly detectable, or is presumably localized only around the vascular pole of the non-diabetic glomeruli (white arrowhead in middle and right panel). On the other hand, ALK3 is increased in diabetic glomeruli and colocalized with desmin (third row in left panel) and PDGFRβ (third row in middle panel) but not with nephrin (third row in right panel).

Figure 4: Blocking BMP4 ameliorates glomerular injuries in diabetic mice

A: Neutralizing effect of the anti-BMP4 antibody in mesangial cells. Treatment with the anti-BMP4 antibody inhibits the phosphorylation of Smad1/5/8 induced by BMP4 but not
by TGF-β, BMP7, or BMP2. Mesangial cells were preincubated with anti-BMP4 (αB) or control IgG antibody (C) for 30 min before stimulation by BMP2, BMP4, BMP7, or TGF-β. B-E: Treatment with anti-BMP4 antibody attenuated an increase in phosphorylated Smad1 (B), mesangial matrix expansion (C), and glomerular accumulation of type IV collagen (D) and improved the glomerular expression of αSMA (E) both in diabetic Smad1-Tg mice and their diabetic littermates. Morphometric analyses were performed as described. To quantify αSMA, mRNA was extracted from isolated glomeruli by laser-manipulated microdissection. C: Control IgG, αB: BMP4-neutralizing antibody. The number of mice in each group in this experiment is listed in table 2. Comparisons were made using one-way ANOVA followed by the Tukey’s HSD test. #: p < 0.05 versus non-diabetic mice, *: p < 0.05 versus diabetic littermates, **: p < 0.05 versus mice treated with control IgG, §: not examined.

F: Representative histological views after treatment with the BMP4 antibody. The neutralizing anti-BMP4 antibody was subcutaneously injected into each group of mice once every two weeks from 20 to 36 weeks after diabetes induction. Periodic acid silver methenamine staining results are presented. WT (upper row) and Smad1-Tg (lower row) mice were non-diabetic and treated with control IgG (left column); diabetic and treated with control IgG (middle column); or diabetic and treated with anti-BMP4 antibody (right column). Sections were counter-stained with hematoxylin solution. Bar 50 µm.

G: Urinary excretion of albumin (UAE) in each group of mice. The number of mice in
each group used in this experiment is listed in table 2. Data were logarithmically transformed, and comparisons were made using one-way ANOVA followed by Tukey’s HSD test. # p < 0.05, versus non-diabetic mice of the same genotype.

Figure 5: Constitutively active Smad1 induces the expression of αSMA in mesangial cells

A: Construction of mutant Smad1. Serine 463 and 465 of Smad1 were substituted with aspartic acid (Smad-DVD) to create constitutively active Smad1, or the C-terminus was deleted (Smad1-ΔC) to create an inactive Smad1. Both Smad1 mutants were recognized by the anti-Smad1 antibody.

B: Transcriptional activities of Smad1 mutants by luciferase assay in Cos7 cells. Mutant Smad1 or mock expression vector was transfected with 3GC2-lux into Cos7 cells. All transfections were quadruplicated and repeated at least three times. * p < 0.05 versus mock vector. # p < 0.05 versus Smad1-DVD.

C: Localization of Smad1 mutants in murine cultured mesangial cells. Mouse mesangial cells transfected with each Smad1 mutant vector were stained with anti-Smad1 antibody (green) and DAPI (purple) without stimulation. Note that Smad1-DVD was detected in the nucleus, whereas Smad1-ΔC was detected mainly in the cytoplasm.

D: Smad1-DVD increased the transcriptional activity of αSMA in mesangial cells. Each
Smad1 mutant vector was transfected with mouse αSMA-promoter luciferase construct into mesangial cells. After 24 hours, the luciferase activities of cell lysate were measured as described. Data represent the mean ± SD of triplicate determinations. * p < 0.05

E and F: BMP4 increases αSMA in mesangial cells by phosphorylation of Smad1. Mesangial cells were preincubated with dorsomorphin (1 µM) for 30 min, followed by the stimulation with BMP4 (25 ng/ml). Twenty-four hours after stimulation, cells were harvested. One representative blot is presented in E, and the mean optical densitometry of αSMA/β-actin from three independent experiments is presented in F. * p <0.05 versus control without dorsomorphin or BMP4; # p < 0.05 versus BMP4 stimulation without dorsomorphin.

**Figure 6: Heterozygous SMAD1 knockout mice exhibit attenuated mesangial sclerosis in diabetes.**

A: Quantification of the Smad1 mRNA expression in the glomeruli 36 weeks after diabetes. Comparisons were made using two-way ANOVA followed by the Tukey’s HSD test. The results indicated significant main effects for genotype and for diabetes but no significant interaction effects. # p<0.05, versus non-diabetic mice of the same genotype. * p < 0.05, diabetic WT versus KO mice.

B: Urinary albumin excretion (UAE) 36 weeks after the induction of diabetes in SMAD1 heterozygous KO mice and littermates. Comparisons were made using two-way ANOVA
followed by the Tukey’s HSD test. The results indicated significant main effects and interaction effects for genotype and diabetes. # p<0.05, versus non-diabetic mice of the same genotype. * p < 0.05, diabetic WT versus KO mice.

C: Representative light microscopy images of the kidney from non-diabetic WT (upper left), non-diabetic *SMAD1* heterozygous KO (upper right), diabetic WT (lower left), and diabetic *SMAD1* heterozygous KO (lower right) mice 36 weeks after STZ treatment. Bar: 100 µm (PASM staining, X 400 original magnification)

D: Mesangial matrix fraction 36 weeks after STZ treatment in each group of mice. Comparisons were made using two-way ANOVA followed by the Tukey’s HSD test. The results indicated significant main and interaction effects for genotype and diabetes. # p < 0.05, versus non-diabetic mice in the same genotype. * p < 0.05, diabetic WT versus KO mice

E: Quantification of Col4a1/a2 and Col1a1/a2 mRNA by RT-qPCR in isolated glomeruli of the kidney 36 weeks after STZ treatment. Comparisons were made using two-way ANOVA followed by the Tukey’s HSD test. The results indicated significant main effects for genotype and for diabetes in all factors as well as significant interaction effects with respect to Col4a1 but no significant interaction effects with respect to Col4a2 and Col1a1/a2. # p < 0.05, versus non-diabetic mice of the same genotype. * p < 0.05, diabetic WT versus KO mice.

A, B, D, and E: n=5 in non-diabetic WT mice. n=4 in non-diabetic *SMAD1* heterozygous
KO mice. n=6 in diabetic WT mice. n=5 in diabetic SMAD1 heterozygous KO mice. The data are presented as the mean ± SD.
Fig 1

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B

Fsl

WT

Tg#59

C

WT

Tg#52(8D)

Tg#32(E1)

Tg#59(F1)

Tg#60(8D)

D

Smad1(total)

Smad1(human)

E

Smad1(total)

Smad1(human)

β-actin

G

WT

Tg#59

H

Mesangial matrix fraction (%)

WT

Tg #59

Cont

STZ

I

UAE (μg/day)

Pre 8 16 24 32

Duration after diabetes (wks)

J

# *
Fig. 2

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WT Cont  Tg#59

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Fig 3

A

BMP7

WT Tg #59

Cont STZ

ns

ns

ns

BMP4

WT Tg #59

Cont STZ

ns

# #

BMPRII

WT Tg #59

Cont STZ

ns #

ns

ALK3

WT Tg #59

Cont STZ

ns #

#

C

Tg#59 Cont Tg#59 STZ

ALK3

desmin

PDGFRβ

merge

Tg#59 Cont Tg#59 STZ

ALK3

desmin

PDGFRβ

merge

Tg#59 Cont Tg#59 STZ

ALK3

desmin

PDGFRβ

merge

BMP4 (Tg#59 STZ)

△△
Fig. 6

A

B

C

D

E

169x117mm (300 x 300 DPI)
Supplemental table 1

Characteristics of each group of mice

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*p<0.05 versus control
### Supplemental table 2

**Primers for real-time RT-PCR**

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<td>AGCGAATTGTGCCCACTCTCACA TAACTGGGAAATCGGCTGGTC</td>
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<tr>
<td>ALK3</td>
<td>TGAGGACATGCGTGGAGGGT GCTGAAAGACATGCTGGCG</td>
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<tr>
<td>Smad1</td>
<td>ATGGTTTCACAGATCCGCTCA TCCCAATATGTCGCCTGGTG</td>
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</table>
**Supplemental table 3**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Amplification</th>
<th>Fixation</th>
<th>Section</th>
<th>Ag retrieval</th>
<th>Dilution</th>
<th>Company (Cat. No)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smad1</td>
<td>SA-HRP</td>
<td>FA</td>
<td>Paraffin</td>
<td>10mM citrate pH6.0</td>
<td>1:15</td>
<td>Abnova (1D3)</td>
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<td>α-SMA</td>
<td>SA-HRP</td>
<td>FA</td>
<td>Paraffin</td>
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<td>Neo Markers (MS-113B)</td>
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<td>Type IV Collagen</td>
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<td>Paraffin</td>
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<td>Carbiochem</td>
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<td>pSmad1/5/8</td>
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<td>FA</td>
<td>Paraffin</td>
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<td>1:400</td>
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<td>BMP4</td>
<td>SA-HRP</td>
<td>FA</td>
<td>Paraffin</td>
<td>10mM citrate pH6.0</td>
<td>1:50</td>
<td>Abgent</td>
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<td>BMPR1a (ALK3)</td>
<td>SA-HRP</td>
<td>FA</td>
<td>Paraffin</td>
<td>10mM citrate pH6.0</td>
<td>1:20</td>
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<tr>
<td>Desmin</td>
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<td>FA</td>
<td>Paraffin</td>
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<td>Nephrin</td>
<td>SA-HRP</td>
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<td>Paraffin</td>
<td>10mM citrate pH6.0</td>
<td>1:100</td>
<td>eBioscience</td>
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<td>PDGFRI</td>
<td>SA-HRP</td>
<td>FA</td>
<td>Paraffin</td>
<td>10mM citrate pH6.0</td>
<td>1:100</td>
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</tr>
</tbody>
</table>

SA-HRP: Streptavidin-horseradish Peroxidase (Perkin Elmer), TSA: Tyramide Signal

Amplification (Perkin Elmer)
**Supplemental table 4**

**Characteristics of non-diabetic (Control) and streptozotocin induced diabetic (STZ) wild type (WT) and Smad1 hetero KO (Smad1 KO) mice**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>STZ</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td>Smad1 KO</td>
</tr>
<tr>
<td>Number</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>BW (g)</td>
<td>44.8±8.3</td>
<td>43.1±3.8</td>
</tr>
<tr>
<td>HbA1c(NGSP) (%)</td>
<td>4.4±0.4</td>
<td>4.2±0.2</td>
</tr>
<tr>
<td>HbA1c(IFCC) (mmol/mol)</td>
<td>25.0±4.4</td>
<td>22.2±2.2</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>120±1.6</td>
<td>123±3.7</td>
</tr>
<tr>
<td>rKW/BW (mg/g)</td>
<td>4.5±0.7</td>
<td>4.4±0.5</td>
</tr>
</tbody>
</table>

*p<0.05 versus control. BW, body weight; SBP, systolic blood pressure; rKW, right kidney weight
Supplement figure legends

Supplemental figure 1

A: Establishment of primary cultured mesangial cells from Smad1-Tg mice

Left: Western blotting of Smad1 protein in isolated glomeruli and primary cultured mesangial cells from wild type and Smad1-Tg mice. Mesangial cells were established from glomeruli isolated from a 4-week-old wild type or Smad1-Tg mice (C57BL/6J) as described. The cultured cells fulfilled the criteria generally accepted for glomerular mesangial cells. Note that Smad1 protein from transgene was also expressed in mesangial cells from Smad1-Tg mice. Human Smad1 specific antibody was used.

B: E-cadherin expression of primary cultured cells from Smad1-Tg#59

Cells and tissues were homogenized in RIPA buffer and subjected to immunoblotting. Fifteen µg of lysate was loaded in each lane. Cell lysate from A549, human alveolar adenocarcinoma cell line was used as positive control of Smad1 and E-cadherin. Anti E-cadherin (1:1000) antibody was from BD Bioscience (San Jose, CA). Anti-Smad1 antibody (#9512, 1:500) was from Cell signaling (Danvers, MA). Anti-αSMA (1:1000) antibody was from SIGMA (St. Louis, MO).
Supplemental figure 2

Generation of Inducible Smad1 overexpressing mice

A: Generation of inducible Smad1 overexpressing mice. Ubiquitously expressed MCM protein leads to the excision of the “floxed” GFP and polyA, resulting in the expression of Smad1 after the treatment of tamoxifen.

GFP (green) was seen in glomeruli of inducible Tg mice (B, E, and H). The same section was immunostained (Red) for von willbrand factor (C), podocin (F), and desmin (I), which are the markers for endothelial cells, podocytes, and mesangial cells respectively. Overlay of GFP and each staining was seen (D, G, and J). Note that GFP positive area were not in endothelial cells, but mainly in podocytes. Moreover, part of mesangium was also positive for GFP (J, K). Bar 50 µm.

L: Western blot analyses of Smad1 protein extracted from various tissues before and after tamoxifen treatment. Eighty µg of protein obtained from each sample was loaded. Smad1 expression was markedly induced after tamoxifen in many tissues including psoas muscle, heart, liver, and stomach. Note that transgene was slightly induced in kidney and ovary.

M: Inducible Smad1-Tg#5 mice also showed mesangial matrix expansion after induction of diabetes.

Morphometric analysis of mesangial matrix expansion in wild type mice, MerCreMer-Tg mice, and double transgenic mice with or without diabetes is shown.
All the groups of mice were treated with tamoxifen. Mesangial matrix fraction was measured at 36 weeks after STZ treatment in each group of mice. Glomerular surface area and PASM-positive area were determined as described in Research design and methods. The mesangial sclerotic fraction was determined as percentage of mesangial matrix area per total glomerular surface area. The number of each group of mice was listed in supplemental table 1. Comparisons were made by the Tukey’s HSD test. # p < 0.05, versus non-diabetic mice in the same genotype. * p<0.05, versus diabetic WT mice in the same diabetic condition.

**N: Inducible Smad1-Tg#5 mice did not show more albuminuria after induction of diabetes.**

Urinary albumin excretion 36 weeks after diabetes in each group of mice was shown. The number of each group of mice was listed in supplemental table 1. Comparisons were made by the Tukey’s HSD test. # p < 0.05, versus non-diabetic mice in the same genotype.