THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 22, pp. 20109–20116, June 3, 2011

Activation of Bone Morphogenetic Protein 4 Signaling Leads to Glomerulosclerosis That Mimics Diabetic Nephropathy*

Received for publication, August 30, 2010, and in revised form, March 22, 2011 Published, JBC Papers in Press, April 6, 2011, DOI 10.1074/jbc.M110.179382

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Diabetic nephropathy (DN) is the most common cause of chronic kidney disease. We have previously reported that Smad1 transcriptionally regulates the expression of extracellular matrix (ECM) proteins in DN. However, little is known about the regulatory mechanisms that induce and activate Smad1. Here, bone morphogenetic protein 4 (Bmp4) was found to up-regulate the expression of Smad1 in mesangial cells and subsequently to phosphorylate Smad1 downstream of the advanced glycation end product-receptor for advanced glycation end product signaling pathway. Moreover, Bmp4 utilized Alk3 and affected the activation of Smad1 and Col4 expressions in mesangial cells. In the diabetic mouse, Bmp4 was remarkably activated in the glomeruli, and the mesangial area was expanded. To elucidate the direct function of Bmp4 action in the kidneys, we generated transgenic mice inducible for the expression of Bmp4. Tamoxifen treatment dramatically induced the expression of Bmp4, especially in the glomeruli of the mice. Notably, in the nondiabetic condition, the mice exhibited not only an expansion of the mesangial area and thickening of the basement membrane but also remarkable albuminuria, which are consistent with the distinct glomerular injuries in DN. ECM protein overexpression and activation of Smad1 in the glomeruli were also observed in the mice. The mesangial expansion in the mice was significantly correlated with albuminuria. Furthermore, the heterozygous *Bmp4* knock-out mice inhibited the glomerular injuries compared with wild type mice in diabetic conditions. Here, we show that BMP4 may act as an upstream regulatory molecule for the process of ECM accumulation in DN and thereby reveals a new aspect of the molecular mechanisms involved in DN.

Diabetic nephropathy (DN)[4] is the most common cause of chronic kidney disease and end-stage renal disease in the world. DN is characterized by mesangial matrix expansion caused by an excessive deposition of extracellular matrix (ECM) proteins in the mesangial area, which ultimately progresses to glomerulosclerosis associated with renal dysfunction (1, 2). Advanced glycation end products (AGE) produced as the result of hyperglycemia are known to stimulate the production of ECM proteins, resulting in glomerulosclerosis (3–5). One of the major components of ECM is α1/α2 type IV collagen (Col4), which is overproduced in diabetic glomerulosclerosis. We have previously demonstrated that Smad1 directly binds to the promoter of Col4 and transcriptionally up-regulates Col4 expression (6). In addition, glomerular expression of Smad1 is significantly increased in diabetic rats along with mesangial expansion (7, 8). In previous reports, it has been shown that Smad1 is absent in the renal glomeruli of normal adult human and mice (6). For now, we still lack mechanistic insight into how Smad1 expression is induced and activated in DN. It is generally known that Smad1 is directly phosphorylated by TGF-β type I receptors as well as bone morphogenetic proteins (BMPs) through type I and II BMP receptors (9). Although BMPs are well known to be required for the normal development of various tissues and organs, including the kidneys (10), the role of BMPs in adults or in diseases is unclear. Moreover, the underlying molecular mechanisms of the BMP signaling pathway involved in the pathogenesis of DN remain largely unknown.

Previous studies have reported that TGF-β and its downstream signaling are critical factors in mediating mesangial cell hypertrophy and fibronectin synthesis in diabetes and that angiotensin II plays an important role in the development of mesangial matrix expansion (11, 12). Angiotensin II type 1 receptor blocker (AT1 antagonist) or an angiotensin-converting enzyme inhibitor has been proved to slow slightly the progression of DN (13, 14). However, medical research has still not found a way to halt the progression of DN. From these facts, we hypothesized that there might be other signaling pathways contributing to the progression of DN. Therefore, in this study we tried to dem-

* This work was supported by Grants-in-aid for Scientific Research of the Japan Society for the Promotion of Science 21591033, 19590973, and 22390169, Japan Science and Technology Agency Grant AS2115050G, the Mitsui Sumitomo Welfare Foundation, and The Kidney Foundation of Japan Grant JKFB09-41.

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[4] The abbreviations used are: DN, diabetic nephropathy; MCM, MerCreMer; MC, mesangial cell; ECM, extracellular matrix; AGE, advanced glycation end product; RAGE, receptor for advanced glycation end product; BMP, bone morphogenetic protein; STZ, streptozotocin; tgm, transgenic mice; GBM, glomerular basement membrane.

onstrate that the BMP signaling pathway is a regulatory molecular mechanism directly involved in the pathological features in DN by using both *in vitro* and *in vivo*. We generated inducible *Bmp4* transgenic mice (tgm) by using the tamoxifen-regulated Cre-loxP system and provided an ideal model to investigate the direct role of Bmp4 in glomerular injury *in vivo*.

EXPERIMENTAL PROCEDURES

Experimental Animals—All animal experiments were performed in accordance with Institutional Animal Care and Use Committee of Chugai Pharmaceutical Co. Ltd. and Institutional guidelines, and the ethical Review Board of Tokushima University granted permission for the procedures used in this study.

To generate inducible *Bmp4* transgenic mouse lines, we used the tamoxifen-regulated Cre-loxP system. This system consists of two transgenes. The first transgene is the inducible *Bmp4* expression cassette, *pMacII*-floxed *GFP pA-Bmp4*, by using expression vector pMacII, consisting of cytomegalovirus enhancer, mouse β-actin promoter, and mouse β-globin genomic DNA (Fig. 4*A, panel a*). The second transgene is a construct for expression of a fusion protein of mutated murine estrogen receptor and Cre recombinase (MerCreMer (MCM)) with the pCAGGS vector (Fig. 4*A, panel b*) (15). *MCM* cDNA was a kind gift from Prof. M. Reth (16). Each of these two transgenes was microinjected to the pronuclei of C57BL/6J mouse fertilized eggs to make transgenic mouse lines. Two strains of inducible *Bmp4* transgenic mouse lines were established as double transgenic mice, C57BL/6J-Tg(Actb-GFP-Bmp4)1Csk-Tg (CAG-MerCreMer)67Csk and C57BL/6J-Tg(Actb-GFP-Bmp4)5Csk-Tg(CAG-MerCreMer)67Csk. In this system, Cre is expressed and remains in the cytoplasm of expressing cells, where it is in an inactive form bound to heat shock protein 90 (Hsp90). After injection of tamoxifen, Cre is released from the Hsp90 and translocates to the nucleus, where it becomes active and mediates recombination of DNA-carrying loxP target sequences. MerCreMer contains two mutated estrogen receptor-binding domains and confers tight dependence on tamoxifen binding for translocation to the nucleus and recombinase activity (17, 18). To induce *Bmp4* gene expression, 8-week-old transgenic mice were fed a diet (CE-2, CLEA, Japan) containing 0.02% tamoxifen citrate (Sigma).

Eight-week-old male C57BL/6 mice weighing 22–24 g were rendered diabetic by the intraperitoneal injection of 50 mg per kg body weight streptozotocin (STZ) in citrate buffer, pH 4.5, for 5 consecutive days. The diabetic state was confirmed 5 days after final injection by measurement of blood glucose level. All mice that were given STZ had a blood glucose concentration exceeding 400 mg/dl and were considered diabetic.

Bmp4 knock-out mice (C57BL/6 $Bmp4^{+/-}$) were gifts of B. Hogan and M. Saitou. Generation and characterization of this mutant mouse were described elsewhere (19). $Bmp4^{+/-}$ mice were maintained on a C57BL/6 background. Diabetes was induced in 8-week-old male mice weighing 22–24 g by the intraperitoneal injection of 100 mg per kg of body weight STZ in citrate buffer, pH 4.5, for 2 days. Mice receiving an injection of citrate buffer were used as controls. The levels of blood glucose were determined 1 week after final injection of STZ. The mice that were given STZ with blood glucose levels exceeding 400 mg/dl were considered diabetic. The mice were classified into four groups as follows: (*a*) nondiabetic C57BL/6 ($n = 6$); (*b*) nondiabetic $Bmp4^{+/-}$ ($n = 6$); (*c*) diabetic C57BL/6 ($n = 8$); and (*d*) diabetic $Bmp4^{+/-}$ ($n = 7$). These mice were killed 20 weeks after final injection of STZ and citrate buffer.

Cell Culture Experiment—A glomerular mesangial cell line was established from glomeruli isolated from normal 4-week-old mice (C57BL/6) and identified according to a method described previously (20). Phenotypically stable cells, passage 14–24, were plated in 100-mm plastic dishes (Nunc) and maintained in B medium (a 3:1 mixture of minimal essential medium/F-12 modified with trace elements) (Nissui) supplemented with 1 mM glutamine, penicillin at 100 units/ml, streptomycin at 100 mg/ml (Invitrogen), and 20% fetal calf serum (FCS) (Invitrogen). The cultured cells fulfilled the criteria generally accepted for glomerular mesangial cells (20).

AGE-BSA was prepared by incubating BSA (fraction V) in phosphate-buffered saline (10 mM, pH 7.4) with 50 mM D-glyceraldehyde for 7 days at 37 °C with protease inhibitors and antibiotics as described previously (3, 21). Unmodified BSA was incubated under the same conditions without D-glyceraldehyde as a control. Preparations were tested for endotoxin, but endotoxin was not detected. Protein concentrations were measured by the Bradford method. All AGE protein-specific fluorescence intensities were measured at a protein concentration of 1 mg/ml. AGE-BSA contained 59.4 AGE units/mg of protein, and unmodified BSA contained 2.3 AGE units/mg of protein, respectively. The glomerular mesangial culture was examined with AGE exposure as described previously (4). These cells were seeded on 100-mm plastic dishes and maintained in B medium/10% FCS. After a 48-h incubation, cells were starved for 24 h in Opti-MEM (Invitrogen) and then incubated with Bmp4 (R&D Systems) at 37 °C for the indicated times. These cells were incubated with carboxymethyl lysine (Nippi) at 10 μg/ml for 24 h followed by serum starvation.

For the receptor for AGE (RAGE) and *Bmp4* knockdown experiments, the cells were analyzed 48 h post-transfection with siRNA for *RAGE* or scrambled siRNA (Invitrogen) and siRNA for *Bmp4* or scrambled siRNA (TAKARA). *Alk3* knockdown experiments were performed by transfecting the cells with shRNA for *Alk3* or control (Takara), followed by serum starvation and stimulation with Bmp4 after 24 h.

Western Blotting—Western blots were performed with anti-Bmp4 (Santa Cruz Biotechnology), anti-Smad1 (Sp125, a mouse monoclonal antibody specific for Smad1, was generated in this study), anti-phospho-Smad1/5/8 (Cell Signaling), anti-Col4 (Southern Biotech), anti-RAGE (ABR), anti-Alk3 (Abgent), anti-β-actin (Sigma), and anti-α-tubulin (Sigma) antibodies.

Histological Examination—Tissue for light microscopy was fixed in methyl Carnoy's solution and embedded in paraffin. Sections were stained with periodic acid Schiff and periodic acid methenamine silver-stained. Morphometry was evaluated in periodic acid methenamine-stained tissues by image processor for analytical pathology as described previously (22). Kidney sections were treated with anti-Bmp4, anti-phospho-Smad1 (Upstate), anti-Smad1, anti-Col4, and Col1 (Abcam) antibod-

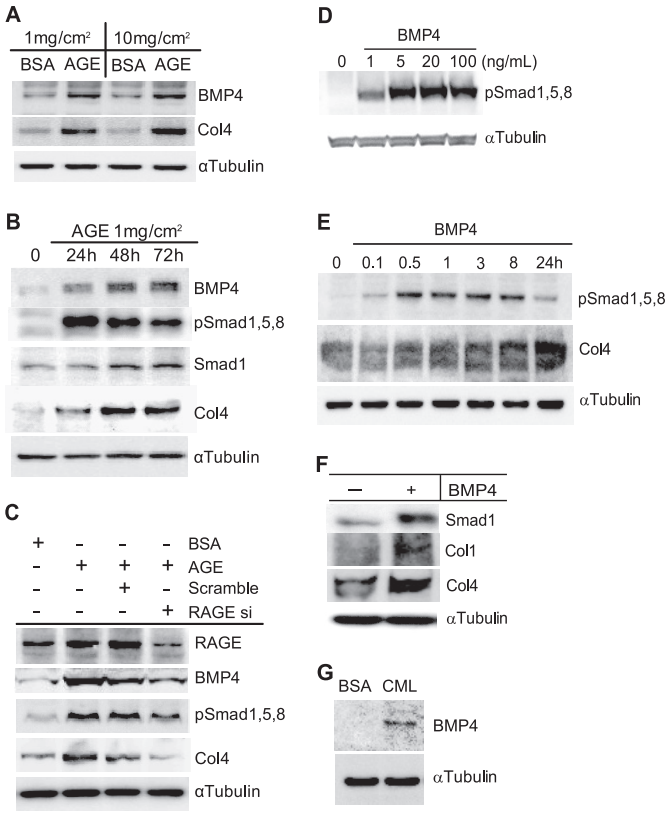


FIGURE 1. **AGE and *Bmp4* showed increased ECM proteins in mouse mesangial cells.** *A,* expression of Bmp4 and Col4 detected by immunoblot after treatment with AGE (1 or 10 μg/cm^2) and BSA (1 or 10 μg/cm^2) for 48 h. Equivalent protein loading was confirmed by α-tubulin. Specific proteins were detected by Bmp4 and Col4. *B,* expression of Bmp4, pSmad1/5/8, Smad1, and Col4 after stimulation by AGE 1 μg/cm^2 for the indicated times by immunoblot. *C,* expression of RAGE, Bmp4, and Col4 after treatment with AGE detected by immunoblot with RAGE siRNA (*siRAGE*) or scrambled siRNA (*scramble*) for 48 h. *D,* phosphorylation of Smad1/5/8 after stimulation by Bmp4 (1–100 ng/ml) for 30 min detected by immunoblot. *E,* phosphorylation of Smad1/5/8 and expressions of Col4 after stimulation by Bmp4 (20 ng/ml) for 0.1–24 h detected by immunoblot. *F,* expression of Smad1 and Col1 after stimulation by Bmp4 (20 ng/ml) for 24 h detected by immunoblot. *G,* expression of Bmp4 detected by immunoblot after treatment with CML (10 μg/ml). The data are representative of three independent experiments.

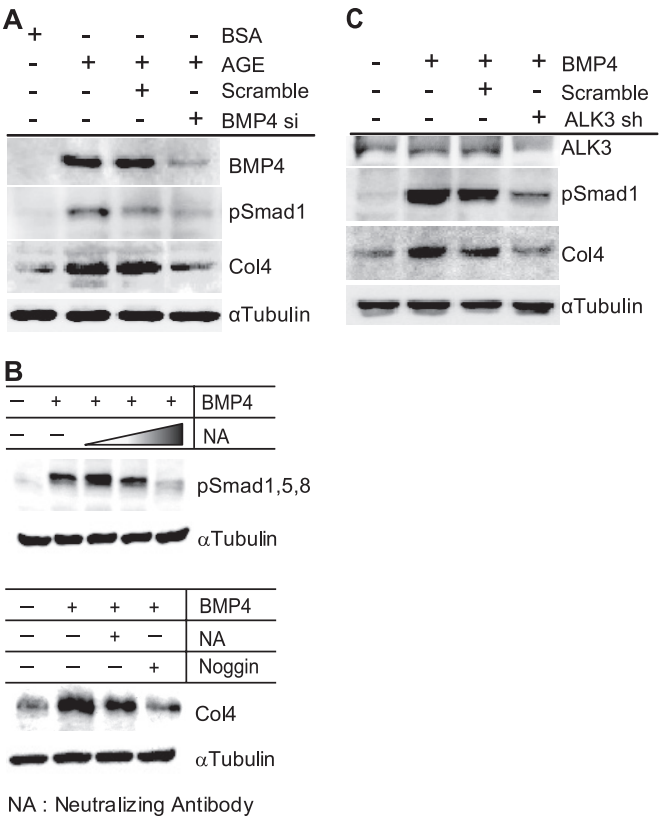


FIGURE 2. **Modulation of Bmp4 signaling.** *A,* expression of Bmp4 and Col4 after treatment with AGE, detected by immunoblot with *Bmp4* siRNA (*siBmp4*) or scrambled siRNA (scramble) for 48 h. *B,* phosphorylation of Smad1/5/8 and expressions of Col4 after stimulation by Bmp4 (20 ng/ml) detected by immunoblot with and without Bmp4-neutralizing antibody or Noggin. *C,* phosphorylation of Smad1/5/8 and expressions of Col4 after stimulation by Bmp4 (20 ng/ml) detected by immunoblot with shRNA for *Alk3* (*shALK3*) or control vector. The data are representative of three independent experiments.

ies. The immunoreactivity of Bmp4 was quantified by Bmp4-positive areas in the glomeruli using image processor for analytical pathology. Tissue for electron microscopy was fixed in 2.5% glutaraldehyde. The mean GBM thickness was calculated with Image J (National Institutes of Health).

RESULTS

Bmp4 Up-regulates Smad1-Col4 Expression through Activation of the AGE-RAGE Signaling Pathway—We previously demonstrated that Smad1 is induced by AGE stimulation in mouse mesangial cells (MCs). Therefore, we first determined the expression of Bmp4 in mouse MCs treated with AGE. Expression of Bmp4 and Col4 was increased by AGE stimulation (1 and 10 μg/cm^2) (Fig. 1*A*). Longer exposure to AGE (48 and 72 h) remarkably up-regulated the expression of Bmp4 as well as those of Smad1 and Col4 (Fig. 1*B*). Knockdown of the RAGE, which is thought to be the main receptor for AGE, attenuated the inductions of Bmp4 and Col4 by AGE (Fig. 1*C*) (23–25). Phosphorylation of Smad1 (pSmad1) was strongly induced by Bmp4, and induction of Col4 by Bmp4 was observed in a time-dependent manner in mouse MCs (Fig. 1, *D* and *E*). Moreover, Bmp4 induced the expression of Smad1 and type 1 collagen (Col1) as well as Col4 in mouse MCs (Fig. 1*F*). Another ligand carboxymethyl lysine for RAGE also induced the expression of Bmp4 in MCs (Fig. 1*G*). These results suggest that Bmp4 is involved in the regulation of Smad1 activation and subsequent production of ECM proteins downstream of the RAGE signaling pathway in MCs.

Bmp4-Alk3 Signal Transduction Pathway Is Important for the Expression of Col4—We next investigated the effects of the inhibition of Bmp4 signaling in the stimulation of AGE. The increased expression of Col4 caused by AGE was significantly inhibited by the knockdown of *Bmp4* (Fig. 2*A*). Similar results were obtained from an inhibition assay using a neutralizing antibody for Bmp4 or Noggin (Fig. 2*B*). Although the neutralizing antibody for Bmp4 clearly inhibited the phosphorylation of Smad1 (Fig. 2*B*), the inhibitory effect for Col4 was partial. Two BMP type I receptors, activin-like kinase 3 and 6 (Alk3 and Alk6), can bind Bmp4 *in vitro* and are also expressed during kidney and urinary tract development (26). We then performed RT-PCR analyses for mouse MCs in the presence or absence of AGE. Expression of *Alk3*, but not *Alk6*, was detected in MCs in both conditions of AGE (data not shown). Therefore, we carried out knockdown of *Alk3* and confirmed the inhibitory effect of phosphorylation of Smad1 (Fig. 2*C*). Expression of Col4 was

A

	Control	Diabetes
n	6	8
Body weight (g)	31.2 ± 7.2	25.8 ± 6.2
Blood glucose (mg/dl)	168 ± 32	558 ± 76*

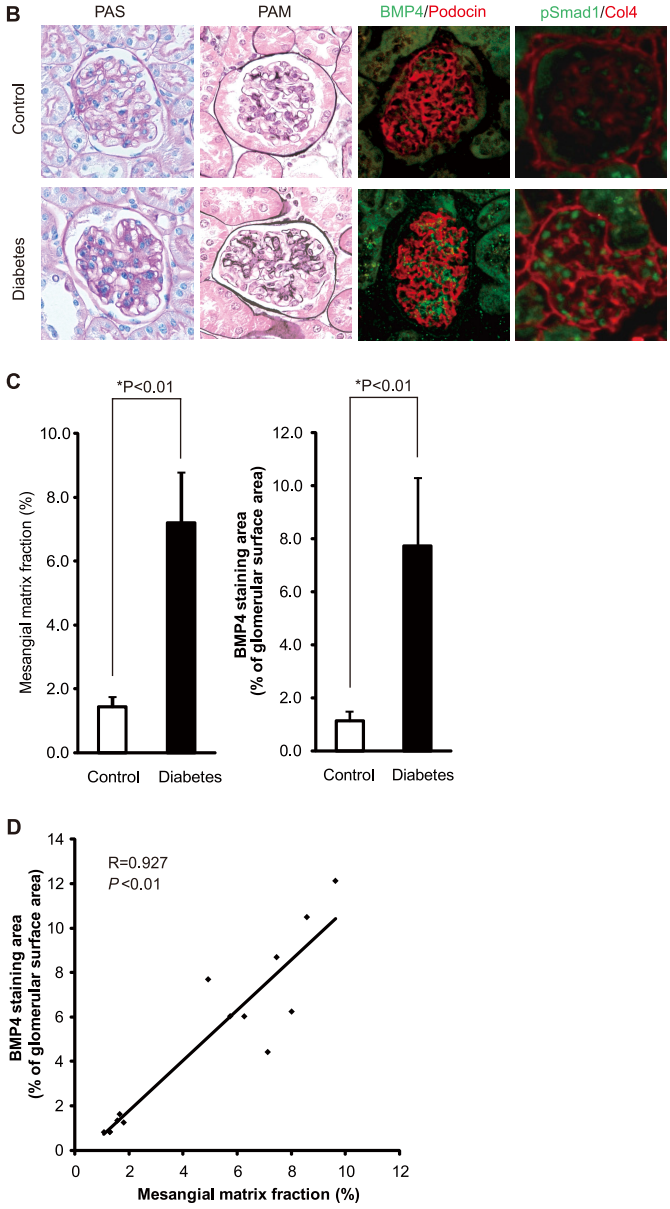


FIGURE 3. **Morphological changes in experimental models of diabetic nephropathy.** *A,* biochemical data in *Control* (nondiabetic mouse) and *Diabetes* (STZ injection mouse) ($n = 6$ for Control; $n = 8$ for Diabetes; results expressed as mean ± S.D. *, $p < 0.01$, *t* test). *B,* representative light microscopy and immunohistochemistry for Control (*upper panels*) and Diabetes (*lower panels*) after 20 weeks of STZ or citrate buffer injection. *PAM*, periodic acid methenamine. *C,* mesangial sclerotic fraction was determined as percentage of mesangial matrix area per total glomerular surface area. The expression of Bmp4 was determined as percentage of Bmp4-positive staining area per total glomerular surface area ($n = 6$ for control; $n = 8$ for Diabetes; $p < 0.01$, *t* test). *D,* correlation between glomerular Bmp4-positive area and mesangial matrix fraction ($R = 0.927$; *, $p \leq 0.01$, *t* test).

also suppressed by *Alk3* silencing in mouse MCs (Fig. 2*C*). These findings implicate that Bmp4 and its receptor Alk3 affect the activation of Smad1 and Col4 expression in mouse MCs.

Bmp4 Is Induced in the Glomeruli of Diabetic Mice—We next investigated whether the Bmp4-Smad1 signaling pathway acts *in vivo* by using experimental models of DN. STZ mice exhibited glomerulosclerosis (Fig. 3*B*). A positive staining of Bmp4 was observed in expanded mesangial areas, which was merged with Col4 but not Podocin (Fig. 3*B*). The pSmad1 expression was also positive with a nucleic pattern in the glomeruli, indicating activation of the Smad1 signaling pathway in diabetic nephropathy mice (Fig. 3*B*), although neither were detected in control mice. The increase in glomerulosclerotic areas detected by silver staining (periodic acid methenamine) was remarkable in STZ mice (7.2 ± 1.5%) compared with wild type mice (1.4 ± 0.3%) (Fig. 3*C*). Immunohistochemical positivity of Bmp4 levels was observed in accordance with the levels of glomerulosclerosis (correlation factor $r = 0.927$, $p < 0.01$) (Fig. 3*D*). Taken together, Bmp4 may contribute to Smad1 activation and subsequent overproduction of ECM proteins in these experimental models of DN. Hence, we propose the hypothesis that Bmp4 plays a critical role in the development of renal changes that are characteristic of DN.

Generation of Inducible Bmp4 Transgenic Mice by the Tamoxifen-regulated Cre-loxP System—To examine whether Bmp4 is responsible for the formation of characteristic renal changes and albuminuria in the development of DN, we generated transgenic mice with inducible expression of *Bmp4* using the tamoxifen-regulated Cre/LoxP system in a nondiabetic condition (Fig. 4*A*). It is well known that Bmp4 is a multifunctional signaling molecule required for normal embryonic development. Because no specific promoter-directed expression in MCs has been developed, we decided to use the CMV enhancer and β-actin promoter, which has been reported to be activated only in glomeruli but not in tubules, arteries, and interstitium (27). We confirmed that the CMV/actin promoter drives the transgene expression predominantly in the whole glomeruli by monitoring GFP expression (Fig. 4*C*). To discuss the direct interaction of Bmp4 induction and pathophysiological changes in this inducible transgenic mouse system, we set several negative controls to exclude the possible nonspecific factors as follows: environmental and feeding conditions, insertion of the transgenes in the genome, and pharmacological actions of tamoxifen and nuclear translocation of MCM. The *Bmp4* transgenic mice did not have any abnormalities before induction with tamoxifen as we show in the *upper row* of Fig. 5*A*, which indicates that neither environmental and feeding conditions nor insertion of the transgenes in the genome has any significant effect on their health. Additionally, the *MCM* transgenic mice did not have any obvious symptoms after they were fed with tamoxifen-containing diet (data not shown), even though translocation of activated MCM into nuclei takes place. These results indicate that both pharmacological actions of tamoxifen and nuclear translocation of the MCM did not have significant effects. Therefore, we concluded that pathophysiological changes in the *Bmp4* transgenic mice described below are a direct consequence of induced Bmp4 overexpression.

Induced Overexpression of Bmp4 in the Glomeruli of Adult Mice Leads to Glomerulosclerosis and Advanced Albuminuria—Tamoxifen-inducible *Bmp4* transgenic mice revealed extensive expansion of the mesangial matrix, compared with noninducible glomeruli. To quantify the extent of mesangial expansion, we used an image processor for analytical pathology system,

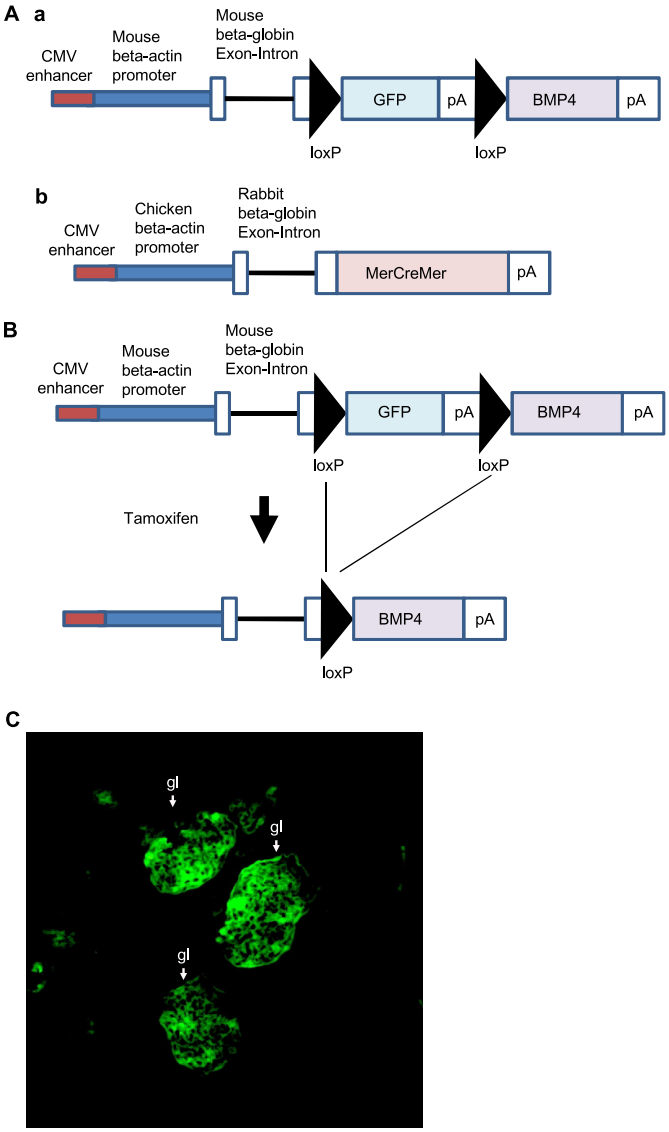


FIGURE 4. **Experimental overview of the generation of tamoxifen-inducible *Bmp4* transgenic mice.** *A, panel a,* transgene for inducible murine *Bmp4* (*pMac II*-floxed *GFP-Bmp4*) was constructed using an expression vector for mammalian cells, pMac II. pMacII is composed of a CMV enhancer and mouse β-actin promoter as transcription regulatory elements, followed by the genomic DNA of mouse β-globin for appropriate splicing of the transgene and addition of the poly(A) signal. *Renilla reniformis* GFP cDNA with mouse β-globin poly(A) signal flanked by directly repeated *loxP* sequences (*loxP-GFP-pA-loxP*) was inserted into the cloning site of pMac II. Finally, a full length of murine *Bmp4* cDNA with Kozak sequence (GCCACC) was inserted downstream from the *loxP-GFP-pA-loxP*. *Panel b,* tamoxifen-inducible cre recombinase, MCM, is a site-specific recombinase cre fused with two ligand-binding domains of a mutated murine estrogen receptor. The *MCM* expression vector was constructed by inserting the *MCM* cDNA into the pCAGGS. *B,* for induction of *Bmp4* gene expression, 8-week old double transgenic mice were fed a diet (CE-2, CLEA Japan) containing 0.02% tamoxifen citrate (Sigma). *C,* GFP expression area displayed predominance at the glomeruli (*gl*).

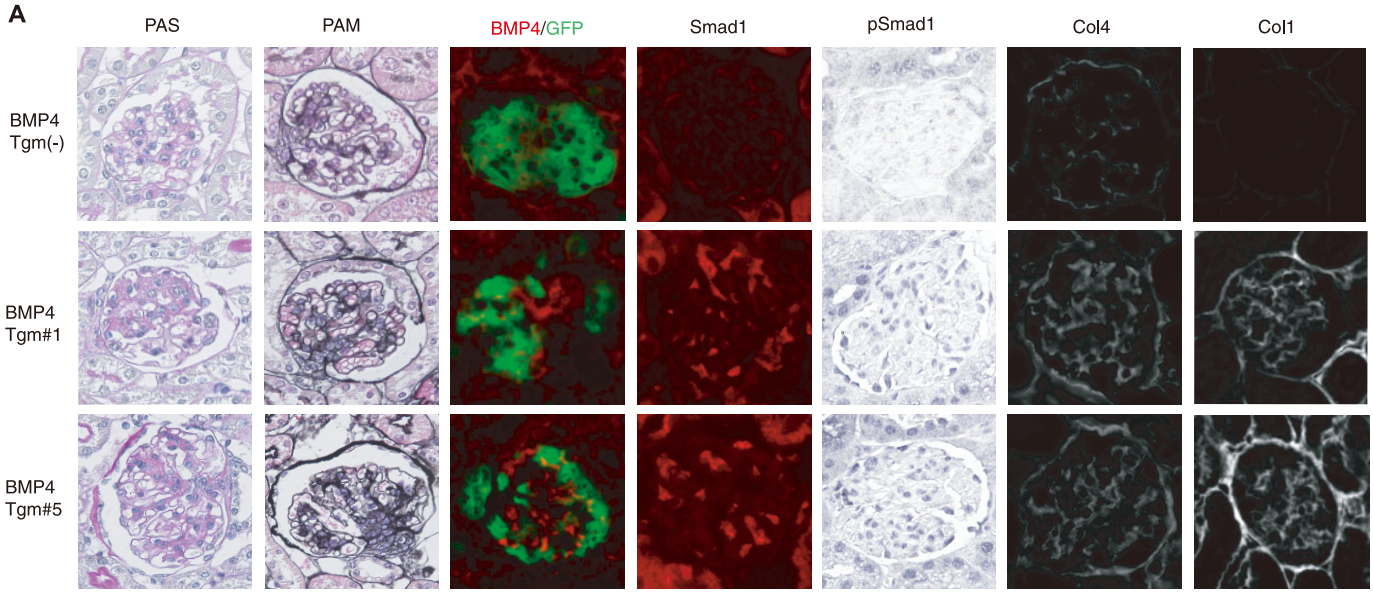
which showed an increase in the mesangial matrix fraction compared with noninducible mice (Fig. 5, *A* and *B*). These transgenic mice also showed significant induction of glomerular expressions of Smad1, pSmad1, Col4, and Col1 compared with noninducible mice (Fig. 5*A*). Furthermore, the *Bmp4* tgm exhibited marked thickening of the GBM as well as mesangial expansion in electron microscopic analyses (Fig. 5, *C* and *D*), both of which are characteristic of human DN. Albuminuria was dramatically increased in inducible *Bmp4* tgm compared with noninducible mice. Moreover, the degree of expansion of mesangial areas in inducible *Bmp4* tgm was significantly correlated with the degree of albuminuria (Fig. 5, *E* and *F*). These changes were similarly observed in another line of *Bmp4* tgm. Collectively, inducible *Bmp4* tgm were able to mimic diabetic changes in glomeruli by exhibiting pathological features remarkably resembling human DN in a nondiabetic condition.

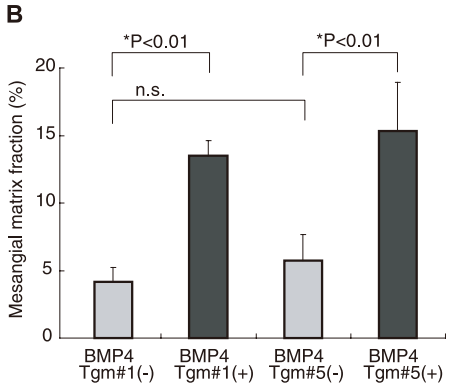
Heterozygous Bmp4 Knock-out Mice Exhibited Attenuation of Glomerulosclerosis in Diabetes—Although forced expression of Bmp4 in glomeruli caused renal changes similar to DN, there still remains the possibility that *Bmp4* tgm showed renal changes of different renal diseases. Therefore, we investigated whether the reduction of Bmp4 expression improved the diabetic glomerular changes by using heterozygous *Bmp4* knock-out mice. Blood glucose was increased, and body weight was decreased in both diabetic mouse groups compared with the control mouse group (Fig. 6*A*). We observed the attenuation of mesangial matrix expansion in diabetic $Bmp4^{+/-}$ mice compared with diabetic wild type mice (Fig. 6*B*). Furthermore, the pSmad1 expression was decreased in $Bmp4^{+/-}$ STZ mice compared with C57BL/6 STZ mice (Fig. 6*B*). We also found that glomerular expression of Col4 was reduced in diabetic $Bmp4^{+/-}$ mice, compared with diabetic wild type mice (6.3 ± 2.4% in $Bmp4^{+/-}$ STZ mice *versus* 9.7 ± 0.7% in C57BL/6 STZ mice, $p < 0.05$) (Fig. 6*C*). These data suggest that BMP4 is a critical determinant for the development of DN.

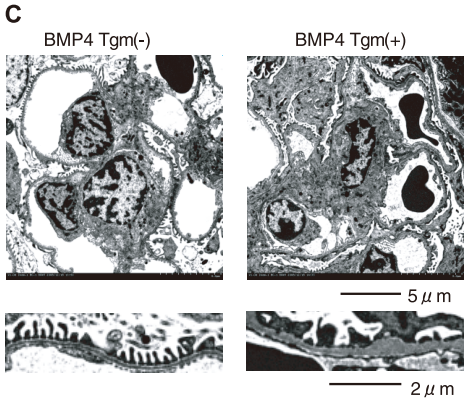
DISCUSSION

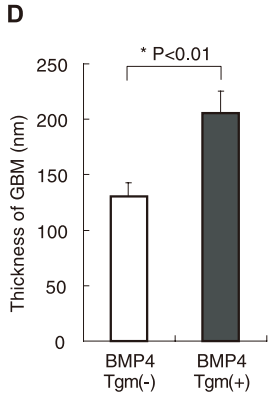
DN is the leading cause of end-stage renal disease and a major contributing cause of morbidity and mortality in patients with diabetes throughout the world. Typical features include thickening of the GBM and mesangial matrix hyperplasia (so-called glomerulosclerosis) (28). Only the latter pathological finding is related to the decrease in the glomerular filtration rate (29), which is the general clinical event for renal damage in type 1 and 2 diabetes mellitus. There are several factors associated with the development of DN, including AGE, protein kinase C, oxidative stress, and an increase in glomerular hemodynamics. Among them, we have recently reported that Smad1 is induced and phosphorylated in diabetic kidneys, but the factors driving the induction as well as phosphorylation of Smad1 in diabetes mellitus have remained elusive. Here, we provide the first direct evidence that the BMP4 signaling pathway may play a crucial role in the development of diabetic glomerulosclerosis.

Prolonged exposure to hyperglycemia is now recognized as the principal causal factor of diabetic complications (30, 31). Its deleterious effects are attributable to the formation of sugar-derived protein adducts and cross-links known as AGE. Exposure of cultured mesangial cells to AGE results in a receptor-mediated up-regulation of mRNA and protein secretion of Col4 (4, 32). We have previously shown that AGE/RAGE system is critical for progression of glomerulosclerosis, as shown in the study AGE breaker and *RAGE* knock-out mice (25, 33–35). However, there is little information regarding the downstream









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	BMP4 Tgm(-)	BMP4 Tgm(+)
n	6	7
Albuminuria (mg/g•CRE)	12.9 ± 3.5	242.1 ± 295.0

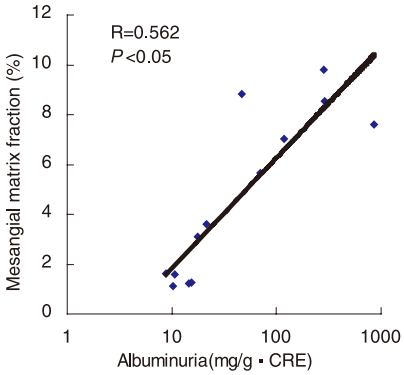

FIGURE 5. **Morphological changes in *Bmp4* tgm glomeruli.** *A,* representative light microscopy and immunohistochemistry pictures of glomeruli. The *upper row* is an uninduced mouse, and the *middle* and *lower rows* are two representative *Bmp4* tgm at 5 days after the induction. *B,* mesangial sclerotic area ($n = 6$ for control; $n = 7$ for *Bmp4* tgm; $p < 0.01$, *t* test, *n.s.*, not significant). *C,* electron micrographs of glomeruli in uninduced (*left panels*) and induced (*right panels*) *Bmp4* tgm. *Upper panels* show low magnification (*bar,* 5 μm), although lower panels show high magnification (*bar* = 2 μm). *D,* thickness of the GBM measuring 50 sections at three different sites. Areas of wrinkled GBM were excluded. The mean thickness of the GBM was 130 ± 12 nm in noninduced mice and 206 ± 20 nm in induced mice ($p < 0.01$, *t* test). *E,* albuminuria in each group ($n = 6$ for control; $n = 7$ for *Bmp4* tgm; results expressed as means ± S.D.). *F,* correlation between albuminuria and mesangial matrix fraction ($R = 0.562$, $p \leq 0.05$, *t* test).

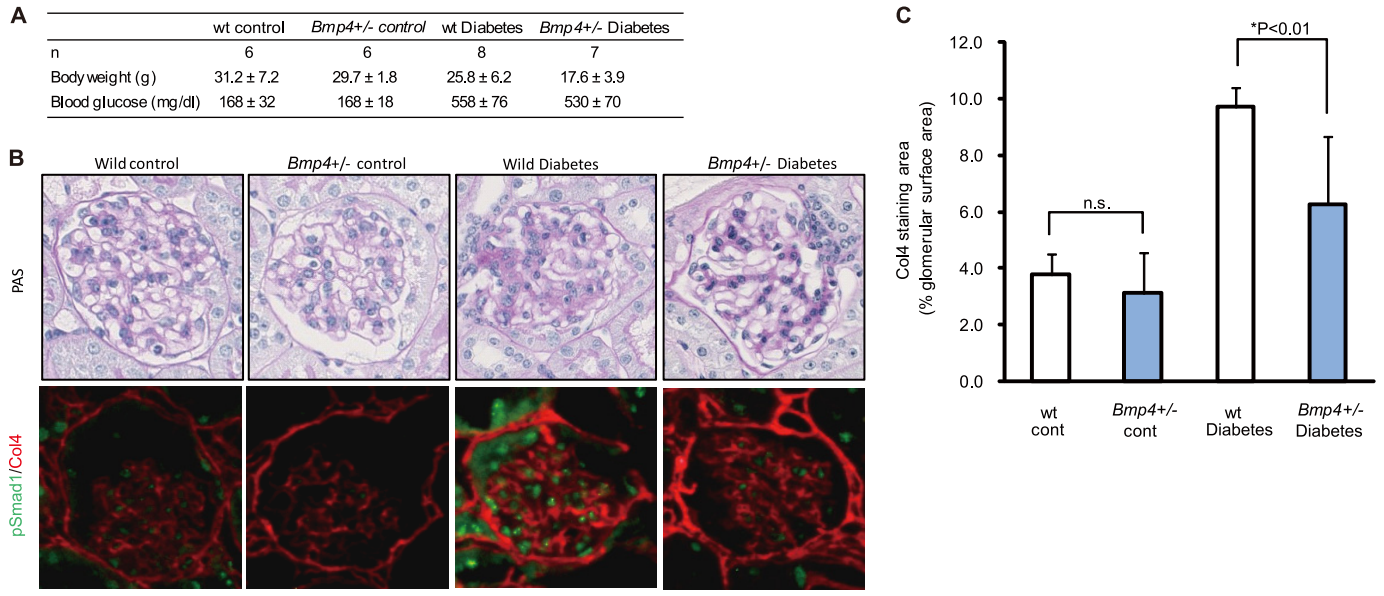

FIGURE 6. **Morphological changes in diabetic *Bmp4*$^{+/-}$ mice.** *A*, biochemical data in each group. *Bmp4*$^{+/+}$ control = C57BL/6 nondiabetic mouse; *Bmp4*$^{+/-}$ control = *Bmp4*$^{+/-}$ nondiabetic mouse; *Bmp4*$^{+/+}$ *Diabetes* = C57BL/6 diabetic mouse; *Bmp4*$^{+/-}$ *Diabetes* = Bmp4$^{+/-}$ diabetic mouse; results expressed as mean ± S.D. *B*, representative light microscopy (*upper panels*) and immunohistochemistry of pSmad1 and Col4 (*lower panels*) for *Bmp4*$^{+/+}$ control, *Bmp4*$^{+/-}$ control, *Bmp4*$^{+/+}$ *Diabetes*, and *Bmp4*$^{+/-}$ *Diabetes* at after 20 weeks of STZ or citrate buffer injection. *C*, expansion of mesangial fraction was determined as percentage of Col4-positive area per total glomerular surface area ($p < 0.01$, *t* test).

signal transduction of the AGE-RAGE axis in DN. Previous studies have shown that TGF-β increases the levels of ECM protein in DN (36–38). It is generally known that Smad3 functions as a key intracellular signal transducer for profibrotic TGF-β responses in various cells. Although the role of the Smad3 pathway in the pathogenesis of DN has only been demonstrated for interstitial fibrosis in a model of obstructive nephropathy (39), the interruption of Smad3 signaling did not improve albuminuria in STZ-diabetic *Smad3* knock-out mice (40). Similarly, albuminuria failed to improve in diabetic db/db mice treated with an anti-TGF-β antibody (41). These results suggest the existence of another signaling pathway involved in the development of DN. Here, we first confirmed that *RAGE* knockdown leads to the decreased expression of Bmp4 and the subsequent decrease of ECM production. In addition, we demonstrated that Bmp4 not only phosphorylated but also directly induced Smad1 in mesangial cells. Because it has been shown that Smad1 is absent in the normal glomeruli of adult mice and humans (6, 42), Bmp4 may be involved in the initiation of glomerulosclerosis. Expression of Bmp4 levels were also correlated with the severity of glomerulosclerosis in STZ mice. Therefore, there is a possibility that the extent of induction of BMP4 via AGE-RAGE signal correlates with pathological changes in glomeruli in DN.

The BMP family of proteins belongs to the TGF-β superfamily; these proteins exert their effects through association with two different types of serine/threonine kinase receptors, type I and type II receptors (43–45). The type IA receptor (BMPR-IA/ALK3) and BMPR-IB/ALK6 share a high similarity and specifically bind several BMP family members. First, we confirmed that Smad1 was phosphorylated through Alk3, but not Alk6, and induced the expression of Col4. We then investigated the possibility that Bmp2 and Bmp4, which originate from a single ancestral gene highly similar to the fly *dpp* gene (46, 47) and share greater than 90% amino acid homology in the ligand domain, may influence the Smad1-induced Col4 expression. Notably, the inducible *Bmp4* transgenic mice exhibited remarkable glomerulosclerosis and albuminuria, and Smad1 activation and elevated expression of Col1 and Col4 were observed in the glomeruli. These findings in the kidney are all consistent with the features of diabetic glomerulosclerosis. Therefore, forced expression of Bmp4 in glomeruli was able to mimic DN in a nondiabetic condition, strongly suggesting that Bmp4 plays a central role for the development of DN. Moreover, from the point of view of a therapeutic approach to DN, we induced diabetes on heterozygous *Bmp4* knock-out mice and analyzed them. Diabetic heterozygous *Bmp4* knock-out mice exhibited the reduction of glomerular changes compared with diabetic wild type mice, suggesting that the expression levels of Bmp4 correlates with the degree of expansion of mesangial areas in diabetes. In addition, a recent report demonstrates that Col4 itself contributes to acceleration of ECM expansion through the direct regulatory effect of Col4 on Bmp4 signaling (48). Accordingly, the activation of the BMP4 signaling pathway and overproduction of COL4 may cooperatively contribute to the progression of diabetic glomerulosclerosis. Taken together, these results provide direct and specific evidence for the causal role of BMP4 in the pathogenesis of DN *in vivo*. Because the BMPs are a large family of proteins that share common structural features, highly specific inhibitors or neutralizing antibodies for BMP4 would offer us novel therapeutic tools to suppress the progression of diabetic nephropathy.

In summary, we have clearly shown that the activation of BMP4 signal transduction through Smad1 may play an essential role for the initiation and progression of diabetic changes in the kidney. Preferential inhibition of the BMP4 signaling pathway

may provide a novel therapeutic approach to DN without undesirable adverse effects, because, for instance, BMP2 might have a protective effect against renal damage (49).

Acknowledgments—We thank Akiko Doi for critical reading of the manuscript; Toshio Mori for technical support for pathological examinations; Yumiko Oikawa for transgenic vector construction; Yousuke Kawase, Toshio Hani, and Hiromi Tateishi for manipulation of mouse embryos; Takanori Tachibe for breeding the transgenic mice; Atsuhiko Kato for histopathological examination; and Satomi Uchida and Yumiko Nakajima for excellent technical assistance. We are grateful to Drs. B. Hogan and M. Saitou for the knock-out mice for Bmp4.

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