

SOX9 Protein Induces a Chondrogenic Phenotype of Mesangial Cells and Contributes to Advanced Diabetic Nephropathy^{*,§}

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Diabetic nephropathy (DN) is the most important chronic kidney disease. We previously reported that Smad1 transcriptionally regulates the expression of extracellular matrix in DN. Phenotypic change in mesangial cells (MCs) is a key pathologic event in the progression of DN. The aim of this study is to investigate a novel mechanism underlying chondrogenic phenotypic change in MCs that results in the development of DN. MCs showed chondrogenic potential in a micromass culture, and BMP4 induced the expression of chondrocyte markers (SRY-related HMG Box 9 (SOX9) and type II collagen (COL2)). Advanced glycation end products induced the expression of chondrocyte marker proteins downstream from the BMP4-Smad1 signaling pathway in MCs. In addition, hypoxia also induced the expression of BMP4, hypoxia-inducible factor-1 α (HIF-1 α), and chondrocyte markers. Overexpression of SOX9 caused ectopic expression of proteoglycans and COL2 in MCs. Furthermore, forced expression of Smad1 induced chondrocyte markers as well. Dorsomorphin inhibited these inductions. Glomerular expressions of HIF-1 α , BMP4, and chondrocyte markers were observed in diabetic nephropathy mice. These positive stainings were observed in mesangial sclerotic lesions. SOX9 was partially colocalized with HIF-1 α and BMP4 in diabetic glomeruli. BMP4 knock-in transgenic mice showed not only similar pathological lesions to DN, but also the induction of chondrocyte markers in the sclerotic lesions. Here we demonstrate that HIF-1 α and BMP4 induce SOX9 expression and subsequent chondrogenic phenotype change in DN. The results suggested that the transdifferentiation of MCs into chondrocyte-like cells in chronic hypoxic stress may result in irreversible structural change in DN.

Diabetic nephropathy (DN)² is the most common and important chronic kidney disease and is the leading cause of

end-stage kidney disease (ESKD) worldwide. The characteristic glomerular changes resulting from DN are thickening of the glomerular basement membrane (GBM) and expansion of the mesangial area caused by increased mesangial extracellular matrix (ECM). Mesangial cells (MCs) are the major source of ECM synthesis in diabetic glomeruli (1–3). Hyperglycemia, advanced glycation end products (AGEs), and transforming growth factor- β (TGF- β) are implicated in the progression of DN by stimulating ECM production in MCs (4–6). In addition, the phenotypic modulation and dedifferentiation of MCs, which are derived from mesenchymal cells, are key pathological events (7, 8). MCs acquired phenotypes not only of activated smooth muscle cells but also of fibroblasts, and subsequently secrete excess ECM proteins.

We previously reported that Smad1 is a direct transcriptional regulator of type IV collagen (COL4), a major component of increased mesangial ECM in DN, as well as other ECM proteins such as type I collagen (COL1), osteopontin, and α -smooth muscle actin (α -SMA) (4, 9). The most critical feature of glomerulosclerosis is mesangial expansion, which is strongly correlated with decreased glomerular filtration rate (GFR) (10). Most importantly, we found that the glomerular expression levels of Smad1 are clearly correlated with the severities of mesangial matrix expansion in rodent DN (9, 11). Therefore, we have concluded that Smad1 plays a central role in the development of DN. However, we still lack mechanistic insight into how Smad1 is involved in phenotypic alteration of MCs and in the irreversible progression of DN. It is generally acknowledged that Smad1 transduces TGF- β signals and also transduces BMP signals through its receptors (12, 13). Although impairment of vascular wall integrity caused by mesangial matrix expansion leads to irreversible glomerulosclerosis, the molecular mechanisms underlying this irreversible change in the phenotypes of MCs in advanced DN are still unknown. We also demonstrated previously that a transcription factor, SRY-related HMG Box 9 (SOX9), is involved in the modulation of transcriptional enhancement of COL4 (14), but we still do not know how this influences the phenotypic alteration of MCs.

It is well recognized that SOX9 is a cartilage-specific transcription factor; that is, the expression of SOX9 is required for

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² The abbreviations used are: DN, diabetic nephropathy; BMP4, bone morphogenetic protein 4; SOX9, SRY-related HMG Box 9; pSOX9, phosphorylated SOX9; COL2, type II collagen; AGEs, advanced glycation end-products; HIF-1 α , hypoxia-inducible factor-1 α ; ESKD, end-stage kidney disease; MCs, mesangial cells; iNOS, inducible nitric-oxide synthase; Tgm, transgenic mice.

the commitment and differentiation of mesenchymal cells toward the chondrogenic lineage in all developing skeletal elements (15). Moreover, SOX9 is responsible for the deposition of ECM, which is composed predominantly of type II collagen (COL2) in chondrocytes (16). Precise regulation of both induction and silencing of SOX9 is required for the normal development of chondrocytes. Conversely, inappropriate or reactivated expression of SOX9 was reported to impair cell phenotype and promote ECM deposition in vascular diseases and fibrosis (17–20). Among the many signaling molecules involved in chondrogenesis, the biological significance of bone morphogenetic proteins (BMPs) has been well established by utilizing pluripotent C3H10T1/2 cells as a model for the initial events of mesenchymal chondrogenesis (21).

We hypothesized that BMP4 and SOX9 would be closely linked to the phenotypic alteration of MCs and to the irreversible progression of diabetic glomerulosclerosis. However, the regulatory mechanism in the interaction between BMP4-Smad1 signaling and SOX9 expression in MCs during the process of diabetic glomerular injuries has not been fully elucidated.

Though hypoxia has been considered a pivotal contributor to tubular atrophy and interstitial fibrosis in the progression of kidney disease (22), recent studies revealed that hypoxia or ischemic stress also affects MCs in various kidney diseases (23–25). Therefore, in addition to hyperglycemia, local hypoxia or ischemic stress and hypoxia-regulated gene expression also could be important factors at the advanced phase of diabetic glomerulosclerosis. A key mediator in cellular responses to hypoxic conditions is hypoxia-inducible factor-1 (HIF-1), a heterodimeric transcription factor consisting of a constitutively expressed β subunit and an O_2 -regulated α subunit. A recent report revealed that high glucose also induces HIF-1 α expression in MCs (26). Therefore, HIF-1 α also could be an important exacerbating factor in the development of glomerulosclerosis. The chondrocyte is a unique cell located at a permanent state of physiological hypoxia, and hypoxia and HIF-1 α support cartilaginous matrix accumulation (27, 28) and are permissive factors in chondrocyte differentiation (29, 30). Furthermore, chondrocyte-related main matrix proteins, such as COL2a1 and aggrecan, are not direct targets of HIF but are up-regulated by hypoxia through cartilage-specific transcription factor SOX9 (31).

Based on these findings, we hypothesized that MCs acquire a chondrocyte-like phenotype that SOX9 mediates during glomerulosclerosis progression. We describe here a novel mechanism of chondrogenic phenotype change of MCs in the developing DN.

EXPERIMENTAL PROCEDURES

Cell Culture Experiment—C3H10T1/2 cells were obtained from the American Type Culture Collection. For routine passage, C3H10T1/2 cells were plated as monolayer cultures and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) (Invitrogen). A glomerular MC line was established from glomeruli isolated from normal 4-week-old mice (C57BL/6JxSJL/J) and was identified according to a previously described method (32). The MCs were maintained in B medium (a 3:1 mixture of minimal essen-

tial medium/F12 modified with trace elements) supplemented with 1 mM glutamine, penicillin at 100 units/ml, streptomycin at 100 mg/ml (Invitrogen), and 10% fetal calf serum (FCS). The cells were plated on 100-mm dishes (Nalge Nunc International, Roskilde, Denmark) and maintained in B medium/10% FCS. Before each assay, the cells were serum starved for 24 h in the medium containing 0.2% FCS. Stimulation with BMP4 (100 ng/ml) (Sigma) was carried out in serum-free culture medium for 24 h. For treatment with dorsomorphin (5 μ M), a small-molecule BMP inhibitor (33), dissolved in dimethyl sulfoxide (DMSO) was added to cells 1 h prior to treatment with BMP4 for 24 h. For the hypoxic culture, the cells were cultured at 37 °C with 5% CO_2 and 5% O_2 in a Hypoxia Incubator Chamber (Stemcell Technologies, Vancouver BC, Canada) (34). AGE exposure was performed as previously described (35).

Preparation of AGEs—AGE-BSA was prepared by incubating BSA in phosphate-buffered saline (10 mM, pH 7.4) with 50 mM glucose 6-phosphate for 8 weeks at 37 °C as described previously (36). Unmodified BSA was incubated under the same conditions without glucose 6-phosphate as a control. 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 and control BSA contained 61.3 and 8.31 units of AGE per milligram of protein, respectively.

Chondrogenic Differentiation—To assess the extent of chondrogenesis, micromass cultures of C3H10T1/2 cells or MCs were performed as previously described (37). Cells were trypsinized and resuspended in 10% FCS at a concentration of 10^7 cells/ml. A 10- μ l drop of cell suspension was placed in the center of a Labtek Permanox chamber slide (Nunc, Naperville, IL). The cells were allowed to adhere for 2–3 h at 37 °C and 5% CO_2 , and then 1 ml of F12 medium with or without 100 ng/ml recombinant human BMP4 (Sigma) was added to the culture. The medium was changed every 3 days. Alcian blue staining (pH 1.0) or immunocytochemistry was performed at day 9.

Alcian Blue Staining and Immunocytochemistry—Culture cells were fixed in 4% PFA, washed with water, and stained with Alcian blue 8-GX (Sigma) in 0.1 N HCl for 2 h and then washed twice in 0.1 N HCl to remove nonspecific staining. Immunocytochemistry was performed with anti-SOX9 (Abcam, Cambridge, UK), anti-COL2 antibody (EMD Chemicals, Gibbstown, NJ) after being fixed in 4% PFA followed by incubation with chondroitinase ABC (Seikagaku Biobusiness, Tokyo, Japan). For nuclear staining, DAPI was applied using Cellstain-DAPI solution (Dojindo Molecular Technologies, Kumamoto, Japan). Appropriate fluorescent secondary antibodies were used for visualization, and imaging was done using a fluorescent microscope.

Plasmid Constructs—To construct the expression vector p3xFlag-SOX9, mouse cDNA encoding full-length SOX9 protein was amplified by PCR with Pfu DNA polymerase (Promega, Madison, WI) and subcloned into the p3xFLAG-CMV-7.1 (Sigma). To construct the expression vector pCMV-Myc-Smad1, mouse cDNA encoding full-length Smad1 protein was subcloned into the pCMV-Myc Mammalian Expression Vector (Clontech, Mountain View, CA). The accuracy of the constructs was confirmed by DNA sequencing.

Transient Transfection of MCs—MCs were seeded and grown until 60–80% confluent on 100-mm dishes. The cells were transfected with 0, 1, and 5 μ g of expression vector encoding full-length SOX9 or Smad1 by using Fugene6 Transfection Reagent (Roche, Penzberg, Germany) according to the manufacturer's instructions. The cells were harvested in lysis buffer after 24 h incubation, and Western blotting was performed as reported below or trypsinized and resuspended for the micro-mass culture method.

Western Blotting—Proteins from whole-cell extract or mouse total kidney were separated by 10% SDS-PAGE, transferred onto a nitro-cellulose membrane, and subjected to Western blotting using anti-BMP4 (Santa Cruz Biotechnology), anti-HIF-1 α (Novus Biologicals, Littleton, CO), anti-Smad1 (Santa Cruz Biotechnology), anti-phospho-Smad1/5/8, anti-phospho-Smad2, anti-phospho-Smad3 (Cell Signaling Technology, Beverly, MA), anti-type-II collagen (Millipore, Billerica, MA), anti-SOX9 (Millipore), anti-pSOX9 (Abcam), anti- β -actin (Sigma), anti-Flag M2 (Sigma), and anti-Myc (MBL International, Woburn, MA) antibodies. Proteins were visualized using HRP-conjugated secondary antibodies (Dako, Glostrup, Denmark) and ECL detection reagents (GE Healthcare, Milwaukee, WI).

Histological Examination—Light microscopy: Tissue for light microscopy was fixed in methyl Carnoy's solution and embedded in paraffin. Sections (2 μ m thick) were stained with periodic acid-Schiff's reagent (PAS) and periodic acid-Schiff methenamine (PASM).

Immunohistochemistry—Kidney sections were processed for immunohistochemistry following standard procedures. To examine the COL4 expression in kidney, ethyl Carnoy's solution-fixed and paraffin-embedded tissue blocks were used. Kidney sections were rehydrated and treated with 0.3% hydrogen peroxide in methanol for 30 min. To eliminate nonspecific staining, sections were incubated with the appropriate preimmune serum for 60 min at room temperature and then incubated with AvidinD and Biotin blocking solutions (Vector Laboratories, Burlingame, CA) for 15 min each. Sections were incubated with anti-COL4 antibody and then incubated with the appropriate biotinylated secondary antibodies followed by incubation with the avidin-biotin peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories). Peroxidase conjugates were subsequently localized using diaminobenzidine tetrahydrochloride. To examine the expression of BMP4, HIF-1 α , SOX9, pSOX9, and COL2, an immunofluorescence study was performed using frozen kidney sections. The sections (4- μ m thick) were fixed in 4% PFA, blocked with 10% donkey serum, and incubated with anti-BMP4, anti-HIF-1 α (Santa Cruz Biotechnology, R&D Systems Minneapolis, MN), anti-SOX9 (Millipore, Santa Cruz Biotechnology), anti-pSOX9 (Abcam) and anti-COL2 (Santa Cruz Biotechnology) antibodies followed by incubation with fluorescent secondary antibody. Staining with DAPI was performed to identify the nuclei of cells.

Animals—All animal experiments were performed in accordance with the Institutional Animal Care and Use Committee of Chugai Pharmaceutical Co. Ltd. and its institutional guidelines. The Review Board of Tokushima University granted ethical permission to undertake this study. The animals were

housed under specific pathogen-free conditions at the animal facility of the University of Tokushima.

As a model for advanced DN, transgenic mouse expressing inducible nitric-oxide synthase (iNOS) under control of insulin promoter (iNOS Tgm) was examined in this study. The model showed iNOS expression at the only β cells in pancreas and nitric oxide produced by cytokine-induced iNOS can cause the degeneration of β cells. The destruction of β cells results in a markedly reduced pancreatic islet mass and in the development of type 1 diabetes mellitus. The iNOS Tgm was maintained on CD-1 mouse background (38). No expression of iNOS protein, whose synthesis is directed only in pancreatic islets, was detected in the kidney. Male littermates were screened for the transgenes by PCR amplification and used for analysis. The primers used for the detection of iNOS Tgm were as follows: forward primer, 5'-GTGGGCTATGGGTTTGTGGAAGG-AGA-3' and reverse primer, 5'-CGATGTCACATGCAGCT-TGT-3' (39). The iNOS Tgm having blood glucose levels of more than 400 mg/dl were used as diabetic compared with 100–150 mg/dl in control mice. At 48 weeks of age, the mice were killed and analyzed.

To generate tamoxifen-inducible BMP4 transgenic mouse lines, the mutated murine estrogen receptor-Cre (MerCreMer) transgene and the loxP-GFP-loxP-BMP4 transgene under the control of CMV enhanced- β actin promoter of mouse and chicken were used, respectively. For the induction of BMP4 gene expression, 8-week-old double transgenic mice were fed a diet (CE-2, CLEA Japan, Tokyo, Japan) containing 0.02% tamoxifen citrate (Sigma).

RESULTS

Chondrogenic Differentiation of MCs in Response to BMP4 under High Density Micromass Culture Conditions—We first examined the effects of BMP4 in chondrogenic differentiation by using C3H10T1/2 stem cells. These cells were originally isolated from C3H mouse embryos; they behave like mesenchymal stem cells (24) and obtain a chondrocyte phenotype by BMP stimulation in micromass culture systems (40). The formation of chondrogenic nodules in C3H10T1/2 cells treated with BMP4 was confirmed under conditions favorable for chondrogenesis (Fig. 1A), indicating that BMP4 acts as an inducer of chondrogenesis. Next, to determine whether or not MCs have chondrogenic potential, we performed a micromass culture method for MCs in the presence or absence of BMP4. The effect of BMP4 on MCs was observed as Alcian blue-positive multicellular nodules similar to those in C3H10T1/2 cells (Fig. 1B). COL2, a major chondrocyte-specific component of the cartilage ECM, was induced in BMP4-stimulated MCs (Fig. 1C). Moreover, we found that the expression of SOX9 and that of COL2 were significantly induced by BMP4 treatment in the monolayer culture (Fig. 1D). From these findings, it is clear that MCs have chondrogenic potential in response to BMP4 *in vitro*.

Diabetic and Hypoxic Conditions Induced Chondrocyte-related Proteins in MCs—Given the evidence that MCs have chondrogenic potential under BMP4 treatment, we examined whether or not diabetic and hypoxic conditions can induce the expression of chondrocytic markers in MCs. Because AGEs have been known as the major factors that contribute to the

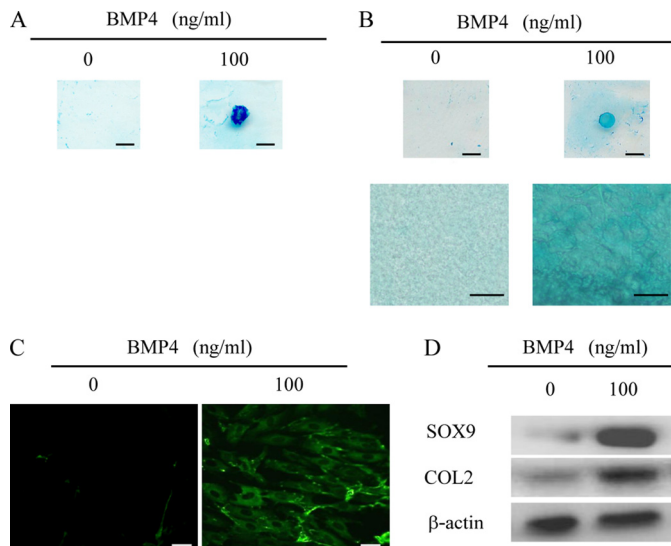


FIGURE 1. Chondrogenic potential of MCs. A, C3H10T1/2 pluripotent stem cells were cultured under the micromass culture method. Cells were stained with Alcian blue to determine chondrogenesis at day 9 (scale bar, 1 mm). B and C, MCs were cultured under the micromass culture method. B, on day 9, cells were stained with Alcian blue. The upper panels are macroscopic findings (scale bar, 1 mm), and the lower panels are microscopic findings (original magnification $\times 400$, scale bar, 30 μm). C, on day 9, cells were stained with anti-COL2 antibody (original magnification $\times 400$, scale bar, 30 μm). D, Western blot analysis of SOX9 and COL2 protein expression in cultured mouse MCs treated with BMP4 for 24 h prior to harvest. β -Actin is the internal control. A representative result is shown from three independent experiments.

pathogenesis of diabetes complications (40), treatment with AGEs was examined in MCs. AGE induced chondrocytic markers (SOX9 and COL2) along with the up-regulation of BMP4 in MCs (Fig. 2A). In MCs treated with AGE, Smad2 and Smad3, as well as Smad1, were phosphorylated compared with BSA under exposure to AGE. The treatment with SB431542, a selective inhibitor of TGF- β signaling, and dorsomorphin, a selective small molecule inhibitor of BMP signaling, inhibited the phosphorylation of Smad2/3 and Smad1, respectively (Fig. 2B). The increases in SOX9 and COL2 were not inhibited by SB431542 but were inhibited by dorsomorphin (Fig. 2C). Next, we investigated whether or not hypoxic stress, which emerges in the advanced stage of DN, affects the chondrocytic change in the MC phenotype. The dramatically increased expression levels of BMP4, HIF-1 α , and chondrocytic markers were observed and compared with the control (Fig. 2D). These data indicate that diabetic and hypoxic conditions significantly affected the activation of the BMP4-Smad1 signaling pathway and the induction of chondrocyte-related proteins in MCs.

Overexpression of SOX9 Induces Chondrocytic Markers in MCs—To examine whether or not SOX9 has an important role in the induction of chondrogenic phenotype changes in MCs, we carried out an overexpression experiment using a SOX9 expression plasmid. Transient overexpression of SOX9 induced COL2 protein in a dose-dependent manner on monolayer cultures of MCs (Fig. 3A). Moreover, the synthesis of proteoglycans was confirmed by Alcian blue staining in a micromass culture of MCs with forced expression of SOX9 (Fig. 3B). Double immunofluorescence microscopic analysis revealed that SOX9 was induced mainly in the nucleus and subsequently COL2 was expressed in the cytoplasm of MCs transiently trans-

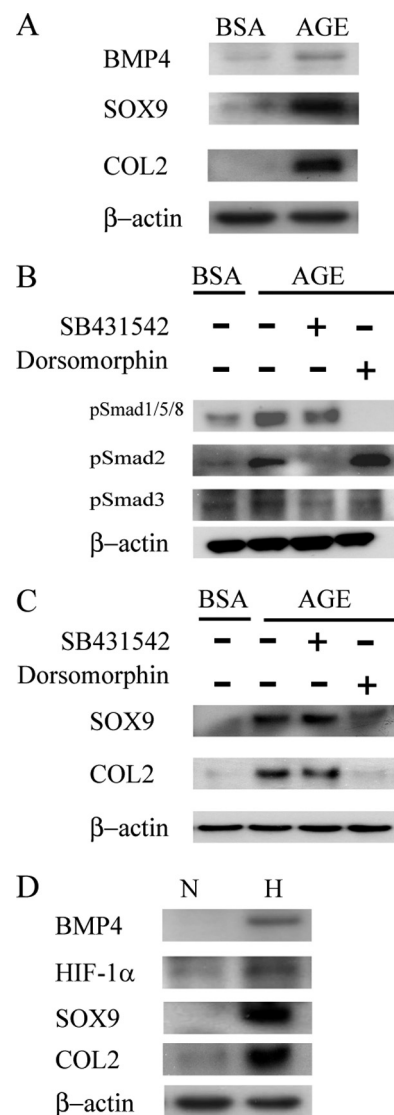


FIGURE 2. Induction of chondrocyte-related proteins in response to AGEs and hypoxia in mouse MCs. A, cultured MCs were treated with AGEs (5 $\mu\text{g}/\text{cm}^2$) and BSA (5 $\mu\text{g}/\text{cm}^2$) for 48 h. Whole-cell extracts subjected to SDS/PAGE and BMP4, SOX9, and COL2 were detected by Western blotting. B, Western blot analysis of pSmad1/5/8, pSmad2, and pSmad3 protein expression in cultured mouse MCs treated with AGEs (5 $\mu\text{g}/\text{cm}^2$) and BSA (5 $\mu\text{g}/\text{cm}^2$) for 6 h prior to harvest. The cells exposed to AGEs were treated with SB431542 or dorsomorphin. C, Western blot analysis of BMP4, SOX9, and COL2 protein expression in cultured mouse MCs treated with AGEs (5 $\mu\text{g}/\text{cm}^2$) and BSA (5 $\mu\text{g}/\text{cm}^2$) for 48 h prior to harvest. The cells exposed to AGEs were treated with SB431542 or dorsomorphin. D, cultured MCs were treated with normoxia (21% O_2) or hypoxia (5% O_2) for 24 h. Whole-cell extracts were subjected to SDS/PAGE; HIF-1 α , BMP4, SOX9, and COL2 were detected by immunoblotting. β -actin is the internal control. N, normoxia; H, hypoxia. A representative result is shown from three independent experiments.

fected with FLAG-SOX9 (Fig. 3C). These findings indicate that MCs are capable of acquiring the chondrocyte phenotype through the induction of SOX9.

Smad Signaling Mediates Phenotypic Change in MCs—To investigate the interaction between BMP4-Smad1 signaling and SOX9 expression in MCs, we focused on Smad1, because we previously reported that Smad1 is induced and regulates the expression of other phenotypic markers, such as α -SMA, under diabetic conditions (4). The Smads are intermediates in the BMP signaling pathways of various cell types (41, 42). MCs were

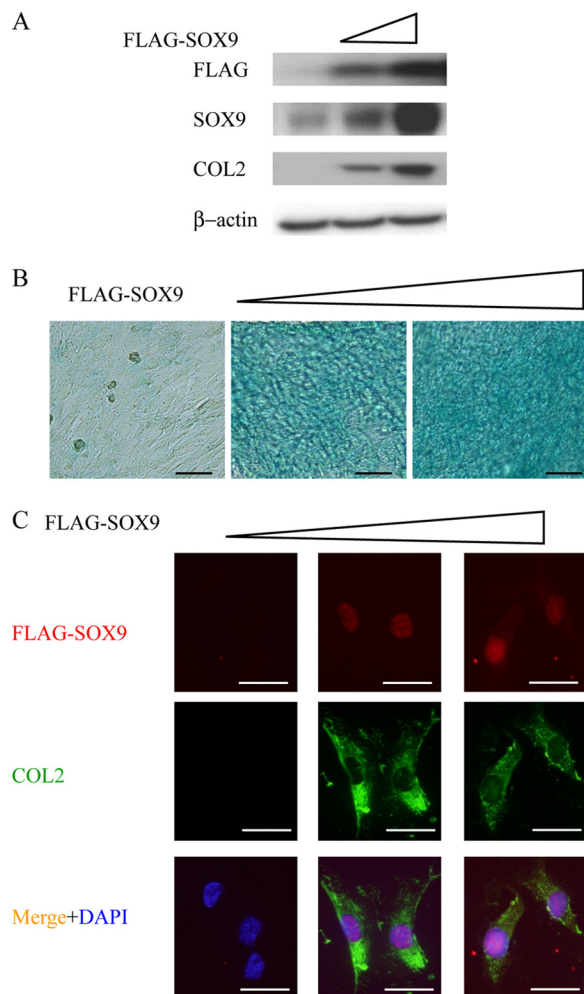


FIGURE 3. Overexpression of SOX9 induces chondrocytic phenotypic change in the absence of BMP4. The cells were transfected with full-length SOX9 expression vector (0, 1, and 5 μ g plasmid DNA/well) in a dose-dependent manner. *A*, after 24 h cell lysates were prepared, and equal levels of extract protein were separated by SDS/PAGE and immunoblotted with the antibodies indicated. *B*, after 24 h, the cells were trypsinized and cultured under micromass conditions, and on day 3 were stained with Alcian blue (original magnification $\times 400$, scale bar; 30 μ m). *C*, after 24 h, the cells were trypsinized and cultured under micromass conditions, and on day 3 were stained with the antibodies indicated (original magnification $\times 400$, scale bar; 30 μ m). A representative result is shown from three independent experiments.

either treated with Smad1 expression plasmids to overexpress Smad1 or with 100 ng/ml BMP4 pretreatment with dorsomorphin. Overexpression of Smad1 induced the expression of SOX9 and COL2 in MCs (Fig. 4*A*). Pretreatment with dorsomorphin suppressed the phosphorylation of Smad1/5/8 and diminished the increased expression of SOX9 and COL2 in the presence of BMP4 (Fig. 4*B* and *C*). These results provide evidence that Smad1 signaling mediates phenotypic change into chondrocyte-like cells from MCs.

Glomerular Expression of HIF-1 α and of Chondrocyte-related Marker in Mice with Diabetic Nephropathy—To further confirm the chondrogenic phenotypic change in MCs in diabetes *in vivo*, we examined an experimental advanced diabetic nephropathy model mouse that carries human cDNA for iNOS under the control of the insulin promoter (iNOS Tgm). These mice expressed NOS2 constitutively in pancreatic β cells and devel-

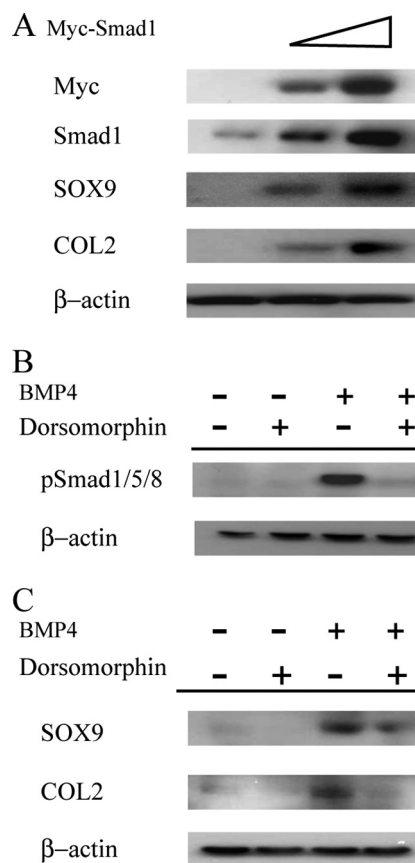


FIGURE 4. Activation of the BMP4-Smad1 signal pathway causes chondrogenic phenotypic change of MCs. The cells were transfected with full-length Smad1 expression vector (0, 1, and 5 μ g plasmid DNA/well) in a dose-dependent manner. *A*, after 24 h, cell lysates were prepared, and equal levels of extract protein were separated by SDS/PAGE and immunoblotted with the antibodies indicated. *B* and *C*, cells were treated with a specific BMP signaling inhibitor, dorsomorphin (10 μ M) 1 h before treatment with BMP4 (100 ng/ml). *B*, after 0.5 h cell lysates were prepared, and equal levels of extract protein were separated by SDS/PAGE and immunoblotted with the antibodies indicated. *C*, after 24 h cell lysates were prepared, and equal levels of extract protein were separated by SDS/PAGE and immunoblotted with the antibodies indicated. A representative result is shown from three independent experiments.

oped severe type 1 DM. These mice have been reported to develop severe mesangial matrix expansion and glomerulosclerosis (39) compared with STZ mice and resembled advanced stage of human DN. A marked increase in COL4 expression was observed in iNOS Tgm along with the severe mesangial expansion and glomerulosclerosis (Fig. 5, *A–F*). These histopathological findings importantly resembled those of the irreversible progression of human DN. Furthermore, the iNOS Tgm showed remarkable up-regulation of the expression of BMP4, HIF-1 α , SOX9, pSOX9, and COL2 in sclerotic lesions of diabetic glomeruli (Fig. 5, *L–P*). In contrast, the expression of these proteins was nearly absent in normal glomeruli (Fig. 5, *G–K*). In addition, Western blot analyses showed induction of these proteins, corresponding to the histopathological examinations, compared with control mice (Fig. 5*Q*). These data suggest that HIF-1 α and chondrocyte-related proteins were induced in advanced damaged glomeruli of DN mice.

Colocalization of HIF-1 α , BMP4, and SOX9 in Diabetic Glomeruli—To examine the colocalization of HIF-1 α and SOX9, double immunofluorescence staining was carried out.

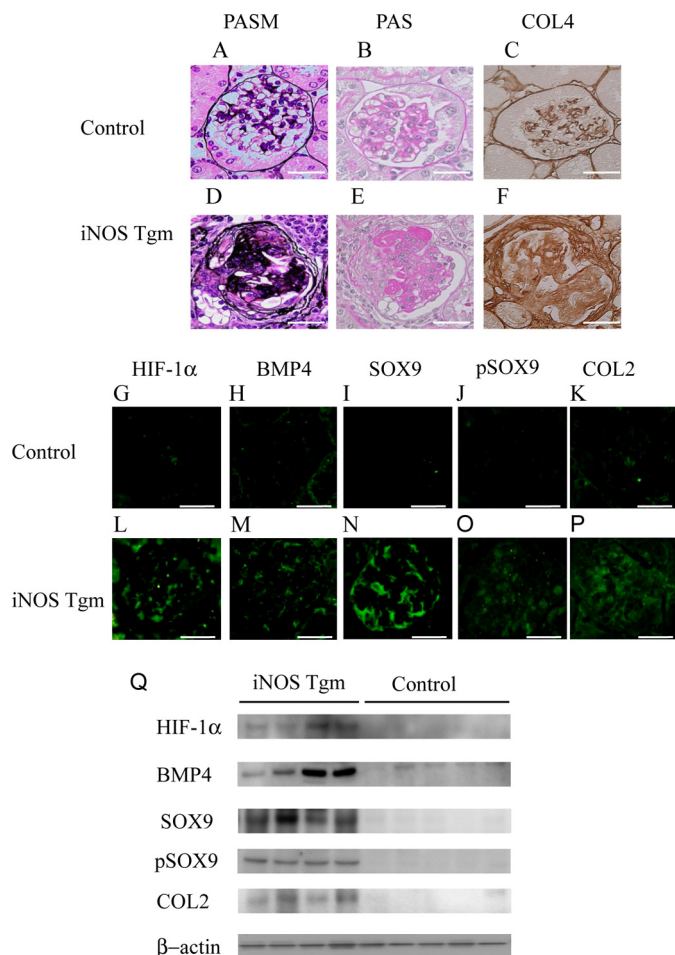


FIGURE 5. Expressions of HIF-1 α , BMP4, SOX9, pSOX9, and COL2 in mouse DN. A–F, representative photomicrographs of periodic acid-Schiff methenamine (PASM), periodic acid-Schiff staining (PAS), and immunohistochemical staining of COL4 are shown (original magnification $\times 400$, scale bar; $30\ \mu\text{m}$). G–P, representative photomicrographs of immunohistochemical staining of HIF-1 α , BMP4, SOX9, pSOX9, and COL2 are shown (original magnification $\times 400$, scale bar; $30\ \mu\text{m}$). The upper panels are control mice, and the bottom panels are iNOS Tgm at 48 weeks of age. Q, Western blots for the whole kidney lysates from each mice are shown ($n = 5$ for control; $n = 4$ for iNOS Tgm). In all, $10\ \mu\text{g}$ of each sample was analyzed with the antibodies indicated. Each lane represents representative data.

Coexistence of HIF-1 α and an active form of SOX9 (pSOX9) was observed in the nuclei in diabetic glomeruli (Fig. 6A). A recent study showed that HIF-1 α protein is induced in MCs in a diabetic condition (26). Taken together, these data suggest that these genes expressed in diabetic glomeruli mainly occurred in MCs. Furthermore, SOX9 expression also partially coincided with BMP4 expression in the diabetic glomeruli (Fig. 6B).

Evaluation of Chondrogenic Potential in Tamoxifen-inducible BMP4 Transgenic Mice—It is well known that BMP4 is critically involved in the normal embryonic development. We have just recently reported that BMP4 acts as an upstream regulatory molecule for the process of ECM accumulation in DN and provide a new aspect of molecular mechanisms in DN (43). We have developed transgenic mice with inducible expression of BMP4 by using the tamoxifen-regulated Cre/LoxP system. BMP4 transgenic mice (BMP4 Tgm) exhibited pathological features, an expanded mesangial area and a thickened glomerular basement membrane in glomeruli, that remarkably resembles

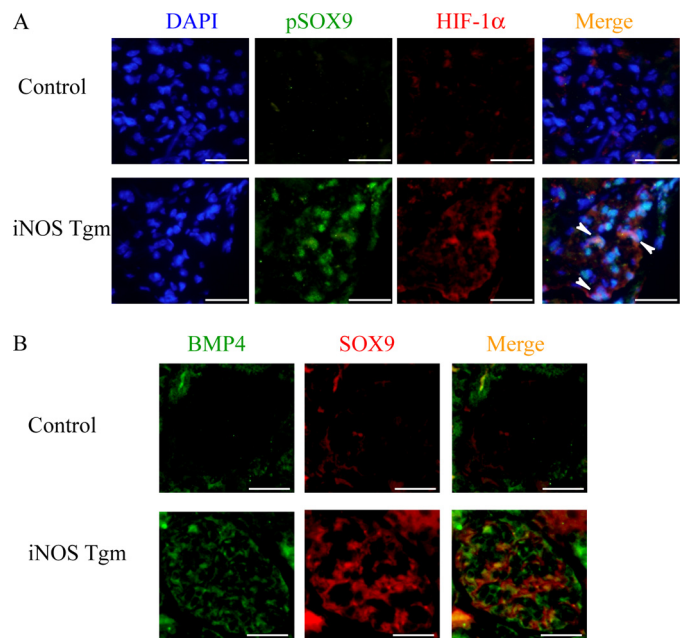


FIGURE 6. Colocalization of HIF-1 α , BMP4, and pSOX9 in mouse DN. A, triple staining of pSOX9 (green), HIF-1 α (red), and DAPI (blue) is shown. Arrowheads indicate the areas of overlap of HIF-1 α and SOX9 in the nuclei. B, double staining with BMP4 (green) and SOX9 (red) is shown. The upper panels are control mice, and the bottom panels are iNOS Tgm at 48 weeks of age. Original magnification $\times 400$, scale bar; $30\ \mu\text{m}$.

human DN. Tamoxifen-inducible BMP4 Tgm revealed extensive expansion of the mesangial matrix compared with non-transgenic mouse glomeruli (Fig. 7A and supplemental Fig. S1). After tamoxifen administration, BMP4 was induced in the areas of GFP disappearance. And these Tgm also showed significant induction of glomerular expression of SOX9 and of COL2 compared with the control mice (Fig. 7B). Furthermore, Western blot analyses showed induction of expression of each of these proteins, corresponding to the histological examinations, compared with control mice (Fig. 7C). These data suggest that chondrocyte-related proteins were induced by BMP4 *in vivo*.

DISCUSSION

MC, a mesenchymally derived cell, provides structural support to the glomerulus by producing ECM components that form the mesangial matrix (44). Mesenchymal progenitor or stem cells have multipotential differentiation capacity, allowing development along the chondrogenic, osteogenic, myogenic, and adipogenic lineages. In addition, phenotypic change in MCs is an important pathological change in glomerular injury and is the forerunner of glomerulosclerosis. Although many reports have demonstrated that the trans-differentiation of adult MCs to activated myofibroblastic cells is a characteristic change, in this study we showed that MCs have chondrogenic potential under the micromass culture method, thus demonstrating that MCs have chondrogenic potential in DN. This novel potential of trans-differentiation is mediated by the induction of SOX9, a master transcriptional factor of chondrogenesis. Recently, several strands of evidence have implicated unexpected SOX9 expression in diseases such as within the media of calcified vasculature (45), in skin keloid lesions (areas of excessive scarring following trauma) (46), and in liver fibrosis

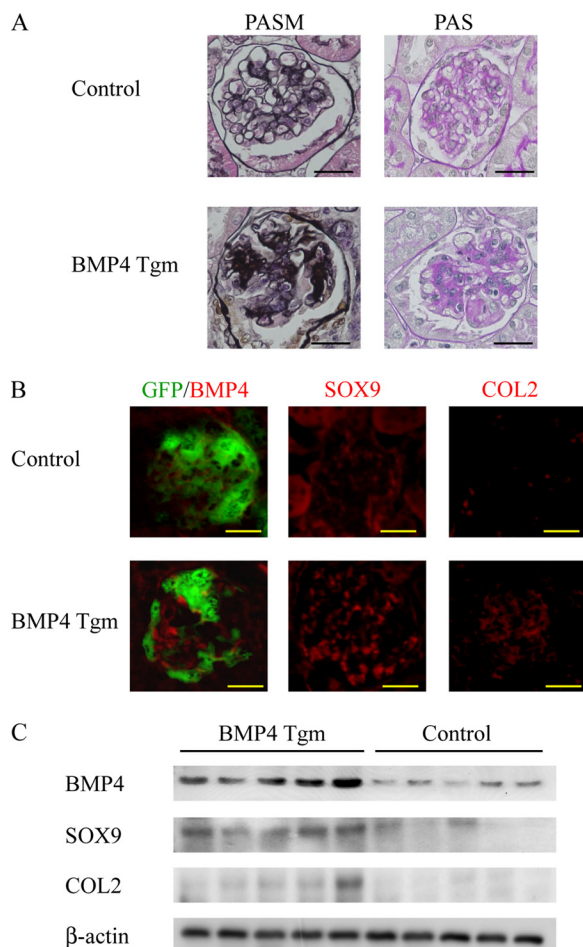


FIGURE 7. Expression of BMP4, SOX9, and COL2 in sclerotic regions of BMP4 Tgm. Representative light microscopy (A) and immunohistochemistry (B) pictures of glomeruli in BMP4 Tgm-treated (BMP4 Tgm) and not treated (Control) with tamoxifen. Original magnification $\times 400$, scale bar; 30 μ m. C, Western blots for the whole kidney lysates from all mice are shown ($n = 5$ for control; $n = 5$ for BMP4 Tgm). In all, 10 μ g of each sample was analyzed with the antibodies indicated. Each lane represents representative data.

(18), causing irreversible changes along with abnormal ECM deposition. Therefore, the acquisition of the chondrocytic phenotype in MCs may be involved in the irreversible changes of diabetic glomerulosclerosis.

In response to a variety of injuries, MCs are generally known to transdifferentiate into myofibroblasts, a specialized population of mesenchymal cells that synthesize an array of different extracellular matrix proteins (*i.e.* type I and type III collagens) that are not normally present in the mesangial matrix and markedly up-regulate the expression of smooth-muscle-like proteins (*i.e.* SMA) (4, 47, 48). We previously demonstrated that Smad1 transcriptionally regulates the expression of SMA and collagens. Moreover, we have shown that SOX9 influences the transcriptional enhancement of COL4 (14), but we still lack mechanistic insight into how SOX9 expression is regulated in DN. Here we have demonstrated that the expression of SOX9 can be induced by BMP4 treatment in MCs. AGE treatment also induced SOX9 expression as well as BMP4 in MCs, suggesting that the BMP4-SOX9 signaling pathway is activated in MCs in diabetes.

The chronic hypoxia conditions of cartilage do not compromise chondrocyte viability, as these cells appear to have devel-

oped specific mechanisms to adapt to this environment (49). HIF-1 is a fundamental mediator that is required for the regulation of chondrocyte metabolism. Although hypoxia has been considered a pivotal factor contributing to tubular atrophy and interstitial fibrosis in the progression of kidney disease (23), the role of hypoxia on the progression of irreversible changes in diabetic glomeruli has yet to be determined. In the present study, we have shown that hypoxic stress as well as hyperglycemia induces phenotypic alteration of MCs. In addition to hyperglycemia, hypoxia is thought to be an important microenvironmental factor in the progression of diabetic kidney injury. This trans-differentiation into a chondrogenic phenotype to adapt to chronic hypoxia may be a critical event during the development of glomerulosclerosis. Although HIF-1 is essential for hypoxia-induced angiogenesis in the brain, heart, lungs, or muscle (50), similar adaptive angiogenic responses do not occur in advanced diabetic glomerulosclerosis due to the tuft of glomerular capillary blood vessels located in Bowman's capsule. In general, tissue-level matrix stiffness has a definitive effect on the cell lineage specification (51), and thereby the hypoxia-induced chondrogenic phenotypic change in MCs and subsequent chondrogenic ECM production, which are characteristics of chondrocytes, can be implicated in irreversible glomerular structural changes leading to ESKD because of the up-regulated production of ECM.

The histological hallmark of the DN is mesangial sclerosis, which is characterized by an accumulation of ECM in the mesangial areas, leading to progressive obliteration of the vascular spaces and subsequent ischemic state in its advanced stage. Therefore, we analyzed iNOS Tgm, because they exhibited mesangial expansion and nodular-like lesions resembling an advanced stage of human DN, on the other hand, diabetic models including streptozotocin-induced model show minor glomerular changes. We selected the male heavy proteinuria-positive line from iNOS Tgm. They developed a generalized and diffuse hyperplasia of the mesangial matrix, GBM thickening, and heavy albuminuria. These changes are significantly similar to human DN. The responsible molecule of this established model remains to be elucidated, however, these glomerular lesions were ameliorated by blocking the AGE signaling pathway. These findings suggest iNOS Tgm is an appropriate model for investigating an advanced stage of human DN (39, 52, 53).

In the present study, iNOS Tgm developed severe glomerulosclerosis and showed ectopic induction of BMP4, SOX9, and COL2. In particular, BMP4 is a crucial regulator in the process of chondrogenesis that increases the condensation of limb mesenchyme and directly induces the expression of chondrogenic genes, including SOX9 (54, 55). Furthermore, we very recently revealed that BMP4 plays a central role in the development of DN. BMP4 Tgm showed pathological changes consistent with the features of typical diabetic mesangial matrix hyperplasia and also showed ectopic induction of SOX9 and COL2. As described above, the BMP signal pathway and SOX9 are required for the onset of chondrogenesis, suggesting that BMP4 and SOX9 are candidate regulators of phenotypic change of MC in the advanced stage of DN.

In conclusion, our study suggests that MCs have chondrogenic potential, a new mechanism of impaired differentiation

via the induction of ectopic SOX9. Chondrocytes are the only cells found in cartilage, an avascular and hypoxic mesenchymal tissue (56). Therefore, it makes sense that MCs acquire the chondrogenic phenotype mediated by ectopic SOX9 to operate a cellular-protective mechanism for chronic pathological hypoxic stress in the kidney. BMP4 and HIF-1 α , which are known to be upstream molecules of SOX9, have important influences on the phenotypic change of MC into a chondrocyte-like cell in DN. This impaired differentiation mechanism of MCs could be a key event in the development and progression of the irreversible stage of DN.

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