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Molecules and Cells

Transcription Factor 7-Like 2 (TCF7L2) Regulates Activin Receptor-Like Kinase 1 (ALK1)/Smad1 Pathway for Development of Diabetic Nephropathy

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Smad1 has previously been shown to play a key role in the development of diabetic nephropathy (DN), by increasing synthesis of extracellular matrix. However, the regulatory mechanism of Smad1 in DN is still unclear. This study aims to elucidate molecular interactions between activin receptor-like kinase 1 (ALK1)/Smad1 signaling pathway and transcription factor 7-like 2 (TCF7L2) in the progression of DN *in vitro* and *in vivo.* The expressions of TCF7L2 and ALK1 were induced by advanced glycation end products (AGEs) in parallel with Smad1, phosphorylated Smad1 (pSmad1), and alpha-smooth muscle actin (α-SMA) through TGF-β1 in cultured mesangial cells. Constitutively active ALK1 increased pSmad1 and α-SMA expressions. The binding of TCF7L2 to ALK1 promoter was confirmed by chromatin immunoprecipitation assay. Furthermore, TCF7L2 induced promoter activity of ALK1. AGEs and TGF-β1 induced a marked increase in TCF7L2 expression in parallel with ALK1. Overexpression of TCF7L2 increased the expressions of ALK1 and Smad1. Inversely, TCF7L2 knockdown by siRNA suppressed α-SMA expression as well as ALK1 and Smad1. The iNOS transgenic mice (iNOS-Tgm), which developed diabetic glomerulosclerosis resembling human diabetic nephropathy, exhibited markedly increased expressions of ALK1, TCF7L2, Smad1, pSmad1, and α-SMA in glomeruli in association with mesangial matrix expansion. These results provide a new evidence that the TCF7L2/ALK1/Smad1 pathway plays a key role in the development of DN.

INTRODUCTION

Diabetic nephropathy (DN) is the leading cause of end-stage renal disease and a major contributing cause of morbidity and mortality in Japan as well as the United States. The typical pathological changes in DN are thickening of the glomerular basement membrane and mesangial matrix expansion, the latter of which progresses to diabetic nodular lesion and causes diabetic glomerulosclerosis. These specific lesions may be a target for investigating the pathogenesis of DN (Bojestig et al., 1994; Kanwar et al., 2008; Schrijvers et al., 2004).

We have demonstrated that Smad1 directly regulates the transcriptional activity of key molecules, such as type I and type IV collagens, in diabetic glomerulosclerosis. Moreover, we found that the level of urinary excretion of Smad1 predicted the development of mesangial matrix expansion in rat DN. Therefore, we concluded that Smad1 played a central role in the development of DN (Abe et al., 2004; Mima et al., 2008). However, the regulatory mechanism of Smad1 in DN is still unclear.

Besides endothelial cells, activin receptor-linked kinase1 (ALK1) is the upstream molecule for Smad1 in several cell types, including chondrocytes, hepatocytes, and neural cells (Finnson et al., 2008; Goumans et al., 2002; Konig et al., 2005; Wiercinska et al., 2006). Previously, we have also shown that advanced glycation end products (AGEs) induce the expression of ALK1 in cultured mesangial cells. In adult humans with DN, the expression of ALK1 and Smad1 are reemerged in the glomeruli (Abe et al., 2004; Matsubara et al., 2006). These facts suggest that ALK1 as well as Smad1 are involved in the development of diabetic glomerulosclerosis, however, the correlation between ALK1 and Smad1 is unclear.

There have been several reports on the important role of TGF-β in the development of DN (Nakamura et al., 1993; Park et al., 1997; Ziyadeh, 2004). ALK1 expression is assumed to regulate TGF-β expression since ALK1 is one of the type I receptors for TGF-β family protein and activates the Smad1/5/8 signaling pathway (Lux et al., 1999); however, the mechanism of the TGF-β-mediated regulation of ALK1 in DN is also unclear.

Importantly, the analyses of ALK1-deficient mice have shown that ALK1 is associated with vascular maturation and stabilization (Oh et al., 2000; Urness et al., 2000). Furthermore, ALK1 was shown to be responsible for both primary pulmonary hypertension (PPH) and hereditary hemorrhagic telangiectasia (HHT2) in human (Trembath et al., 2001). These facts suggest that ALK1 is also closely linked to the pathogenesis of vascular disorders. Therefore, ALK1 may be related to the common microvascular complications of diabetes mellitus, including DN; however, the molecular mechanism underlying this influence

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remains unknown.

We found the binding site for transcriptional factor (TCF) on the promoter region of ALK1. Among the TCF family molecules, transcriptional factor 7-like 2 (TCF7L2) is identified as one of the candidate genes responsible for type 2 diabetes (Florez et al., 2006; Grant et al., 2006). Recently, TCF7L2 polymorphism has been shown to be related to renal dysfunction in human DN (Kottgen et al., 2008; Sale et al., 2007). However, the precise role of TCF7L2 in the development of DN is unexplained.

Phenotypic change in the mesangial cells is an important pathological feature for the development of DN. The phenotypic change is characterized by the increased expression of alpha-smooth muscle actin (α-SMA) in many glomerular diseases (Essawy et al., 1997; Sanai et al., 2000). While there are several possible regulatory mechanisms of the expression of α-SMA (Hautmann et al., 1997; Kanematsu et al., 2007; Tang et al., 2008), few reports have discussed the mechanisms involved in DN. We have also reported that phosphorylated Smad1 (pSmad1) transcriptionally upregulates the expression of α-SMA (Matsubara et al., 2006). In this study, we investigated whether the TGF-β/TCF7L2/ALK1/Smad1 signal transduction pathway contributed to the phenotypic change as well as mesangial matrix expansion in DN.

MATERIALS AND METHODS

Cell culture

A glomerular mesangial cell line was established from glomeruli isolated from normal, 4-week-old mice (C57BL/6JxSJL/J) and was identified according to the method described previously (Davies, 1994). The mesangial cells were maintained in B medium (a 3:1 mixture of minimal essential medium/F12 modified with trace elements) (supplemented with 1 mM glutamine, penicillin at 100 units/ml, streptomycin at 100 mg/ml (Invitrogen, USA), and 10% fetal calf serum (FCS) (Davies, 1994). The cells were plated on 60 or 100-mm dishes (Nalge Nunc International, Denmark) and maintained in B medium/10% FCS.

Antibodies

Anti-ALK1 (kindly provided by Chugai Pharmaceutical Co., Ltd), anti-Smad1, anti-pSmad2/3, Histone H3 (Santa Cruz Biotechnology, Germany), anti-pSmad1, anti-TCF7L2 (Cell Signaling, USA), anti-α-SMA (Abcam, Cambridge, U.K.), anti-Flag, anti-β-actin (Sigma, USA), and TGF-β1-neutralizing antibody (R&D Systems, USA) were used for western blot analysis. Anti-TCF7L2 antibody (Cell Signaling) was used for chromatin immunoprecipitation (ChIP) assay. Anti-pSmad1,5,8, anti-TCF7L2, anti-Smad1, anti-ALK1 (Santa Cruz Biotechnology), and anti-α-SMA (Abcam), antibodies were used for Immunofluorescent staining.

A selective inhibitor of TGF-β/ activin type I receptor kinase assay

SB431542 (Sigma) was a selective inhibitor of TGF-β/ALK4, 5, 7 (Inman et al., 2002) and dissolved in dimethyl sulfoxide (DMSO). After 24 h starvation with Opti-MEM (Invitrogen), SB431542 (10 μM) was added to cells 1 h prior to treatment with TGF-β1 (10 ng/ml) for 24 h (Kou et al., 2007). On the other hand, 3×10^5 mesangial cells were seeded into 60-mm dishes coated with AGE-BSA or BSA at 1 μg/cm^2 either in the presence of SB431542 (10 μM) or DMSO. These cells were harvested for western blot analysis after 48 h incuabtion.

Preparation of AGEs

AGE-bovine serum albumin (BSA) and unmodified BSA were prepared according to the method described previously (Doi et al., 1992; Yamagishi et al., 2002). AGE-BSA and unmodified BSA were purified through 0.22-μm sterile Millipore filters (Millex-GX, Millipore) to remove contaminants. Endotoxin content in all samples was measured by *limulus* amoebocyte lysate assay (Wako) and was not detected. AGE content was estimated by fluorescence intensity at a protein concentration of 1 mg/ml. AGE-BSA contained 89.9 AGE units/mg of protein, and unmodified BSA contained 3.71 AGE units/mg of protein, respectively. Protein concentrations were determined by the method of Bradford using BSA as the standard.

Blockage of TGF-β1

The 3×10^5 mesangial cells were seeded into 60-mm dishes coated with AGE-BSA or BSA at 1 μg/cm^2 either in the presence of 10 μg/ml TGF-β1-neutralizing antibody (R&D Systems) or a control normal chicken IgY. These cells were harvested for western blot analysis after 48 h incuabtion.

RNA isolation and quantitive real-time PCR

Total RNA was isolated from mesangial cells using the TRIsol reagent (Invitrogen). RNA was converted to cDNA using Superscript III (Invitrogen) according to manufacturer's instructions. cDNA was quantified and diluted to 25 ng/μl. Relative mRNA expression levels of ALK1 were measured using TaqMan Universal PCR Master Mix (Applied Biosystems, USA), and relative mRNA expression levels of TCF7L2, TGF-β1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured using the SYBR Green PCR Master Mix (Applied Biosystems) on ABI Prism 7700 Sequence Detection System (Applied Biosystems). The ALK1 TaqMan primer/MGB probe sets were obtained from Applied Biosystems assays on demand (ID Mm0043432_m1). Primers specific for TCF7L2 are 5′-CAAG AGGCAAGATGGAGGGC-3′ (forward) and 5′-GTGATGAGAG GCGTGAGTGG-3′ (reverse), TGF-β1 are 5′-ATACCAACTATT GCTTCAGCTCCAC-3′ (forward) and 5′-CACGTAGTAGACGA TGGGCAGT-3′ (reverse), GAPDH are 5′-AAAATGGTGAAG GTCGGTGTG-3′ (forward) and 5′-AATGAAGGGGTCGTTGA TGG-3′ (reverse). Each reaction consisted of 5 ng cDNA. The cycling parameters were 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 60 s at 60°C. GAPDH, a common housekeeping gene, was used as an internal control for an equal amount of starting material. All samples were plated in triplicate. Gene expression was quantified by the comparative cycle threshold (C_T) method, normalizing C_T-values to the housekeeping gene GAPDH and calculating relative expression values (Fink et al., 1998).

Small-interfering RNA

Mesangial cells were seeded and grown until 60-80% confluent on 60-mm dishes. Small-interfering RNA (siRNA) for ALK1 (siALK1) or Smad1 (siSmad1) or TCF7L2 (siTCF7L2) was combined with DharmaFECT transfection reagent (Thermo Fisher Scientific, USA). Control siRNA (Thermo Fisher Scientific) was used as a control for comparison of effects of these siRNAs. The cells were transfected according to the recommended protocol with 200 nM of siALK1or 28 nM of siSmad1 or 100 nM of siTCF7L2 for 48 h.

Vector constructions

To construct the expression vector p3xFlag-TCF7L2, mouse cDNA encoding full-length TCF7L2 protein was amplified by PCR with Pfu DNA polymerase (Promega, USA) and subcloned into the p3xFLAG-CMV-7.1 (Sigma). To construct the expression vector wild-type ALK1 (wt-ALK1), mouse cDNA

encoding full-length ALK1 protein subcloned into the pCI-neo mamalilan expression vector (Promega). The expression vector constitutively active form of ALK1 (caALK1) (Q201D) was generated by site-directed point mutagenesis. To examine whether a transcription-factor-binding motif (TBM) (AACAAAG) (Shimokawa et al., 2003) was responsible for the promoter activity of ALK1, a 90 bp-DNA fragment (-1098 bp to -1019 bp) of the ALK1 gene promoter which included aTBM (-1061 bp to -1055 bp) was obtained by PCR amplification using genomic mouse DNA as a template and two primers: 5′-GGGGTACCTGAA GATGGTCATGTATCCAA-3′ (foward) and 5′-CCCAAGCTTCT ATGGATTACAGTATCTGC-3′ (reverse). The amplified a 90 bp-fragment was digested with *Kpn*I and *Hin*dIII (TaKaRa, Japan) and inserted into the upstream of the *firefly* luciferase reporter gene of pGL4.10-basic (Promega) to form pGL4.10-wTBM. The mutation vector (pGL4.10-mTBM) was introduced 2-base mutation (AACAAAG to gcCAAAG) at the TBM site (Korinek et al., 1997). *Renilla* luciferase control vector (pGL4.74 vector; Promega) was used as an internal control.

Transient transfection experiments treatment

Mesangial cells were seeded and grown until 60-80% confluent on 100-mm dishes. These cells were transfected with caALK1, p3xFlag-TCF7L2, wt-ALK1 and pCI-neo empty vector using FuGENE6 transfection reagent (Roche diagnostics, USA) and were taken for analysis 48 h after transfection.

ChIP assay

ChIP assays were performed essentially as described previously by Luo et al. (1998). Mesangial cells were seeded and grown until 60-80% confluent on 100-mm dishes. These cells were treated with TGF-β1 (10 ng/ml). ChIP assay was performed using ChIP assay kit (Upstate biothchnology, USA) according to manufacturer's protocol with anti-TCF7L2 antibody (Cell Signaling), or normal control rabbit IgG at 4°C. Input DNA (4%) was used as a template in the PCR reaction. PCR amplification was performed using Taq DNA polymerase (Promega) with primers to amplify the region containing TBM (AACAAAG) (Shimokawa et al., 2003) in ALK1 gene promoter. The 5′-primer was 5′-TCAAGACAGAGAGCCGCCG-3′, and the 3′ primer was 5′-ATTTCAGGGTCAGGACAGAC-3′.

Dual-luciferase reporter assay

The activities of *firefly* luciferase in pGL4.10 and of *Renilla* luciferase in pGL4.74 were determined following the dual-luciferase reporter assay protocol recommended by Promega. 3×10^4 CHO-K1 cells in 10% fetal bovine serum/Dulbecco's modified Eagle's medium were seeded into 24-well dishes. Twenty four hours later, these cells were transfected with 100 ng of pGL4.10-wTBM or pGL4.10-mTBM reporter constructs along with either 300 ng of p3xFlag-TCF7L2 or vector alone (mock). And 10 ng of pGL4.74 vector was co-transfected as an internal control. Transfection was performed with FuGENE6 transfection reagent according to the manufacturer's instructions. After 48 h, these cells were rinsed twice with PBS buffer and cell lysates were prepared by manually scraping the cells from culture plates in the presence of 1× passive lysis buffer. 20 μl of cell lysate was transferred into a luminometer tube containing 100 μl of LARII, and the *firefly* luciferase activity (M1) was measured first. The *Renilla* luciferase activity (M2) was then measured after adding 100 μl of Stop & Glo Reagent. The results were calculated and expressed as the ratio of M1/M2. The experiments were carried out more than three times with triple replicates. The data are presented as mean ± SD.

Western blotting

These cells were harvested in sample buffer and resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane (GE Healthcare UK Ltd., England), subjected to western blot analysis using primary antibodies and detected using an enhanced chemiluminescence detection system (Invitrogen).

Experimental animals

The nitric-oxide synthase cDNA fused with insulin promoter transgenic mice (iNOS-Tgm) was maintained on CD-1 mouse background (Takamura et al., 1998). Male littermates were screened for the transgenes by the PCR amplification and used for analysis. The primers used for the detection of iNOSTg were as follows: forward primer, 5′-GTGGGCTATGGGTTTGT GGAAGGAGA-3′, and reverse primer 5′-CGATGTCACATGCA GCTTGT-3′ (Yamamoto et al., 2001).

Histology

Light Microscopy: After removal of kidney tissue, tissue blocks for light microscopy examination were fixed in ethyl Carnoy's solution and embedded in paraffin. Sections (1 μm) were stained with periodic acid-Schiff's (PAS) reagent. Immunofluorescent staining: Cryopreserved kidney tissues were cut in 4-μm-thick sections and fixed in 4% paraformaldehyde with picric acid for 15 min at 4°C. To eliminate nonspecific staining, sections were incubated with 3% bovine serum albumin diluted with 1× PBS for 30 min at room temperature, followed by incubation with primary antibodies, such as anti-pSmad1,5,8, anti-TCF7L2, anti-Smad1, anti-ALK1 (Santa Cruz Biotechnology), anti-ALK1, and anti-α-SMA (Abcam) antibodies overnight at 4°C.

Statistics

The data are expressed as the means ± SD. Comparison among more than two groups was performed by one-way analysis of variance followed by the post hoc analysis (Bonferroni/Dunn test) to evaluate statistical significance. All analyses were performed using StatView (SAS Institute, USA). Statistical significance was defined as $P < 0.01$.

RESULTS

AGEs induced the expression of ALK1 in a TGF-β1-dependent manner

TGF-β1 mRNA levels increased approximately 2.2-fold 24 h after AGEs treatment in cultured mesangial cells (Fig. 1A). AGEs stimulation significantly increased the expression of ALK1 in cultured mesangial cells. This increase in expression of ALK1 was completely inhibited by the TGF-β1 neutralizing antibody (NA) (Fig. 1B). Similarly, the increased expression of Smad1 and α-SMA by AGEs were attenuated by a TGF-β1 NA in parallel with that of ALK1 (Fig. 1B), indicating that TGF-β1 induced the expression of ALK1, Smad1, and α-SMA under experimental conditions that mimic the diabetic state.

TGF-β1 induced ALK1 and Smad1 expression in an ALK5/Smad2/3-independent manner

TGF-β1 (10 ng/ml) induced the expression levels of ALK1 mRNA significantly in time-dependent manner (Fig. 1C). Further, TGF-β1 induced its expression in dose-dependent manners for 24 h (Fig. 1D). TGF-β1 also phosphorylated Smad2/3 in mouse mesangial cells, and this phosphorylation was blocked by SB431542, which is a selective inhibitor of TGF-β/ALK4, 5, and 7. However, the increased expressions of ALK1

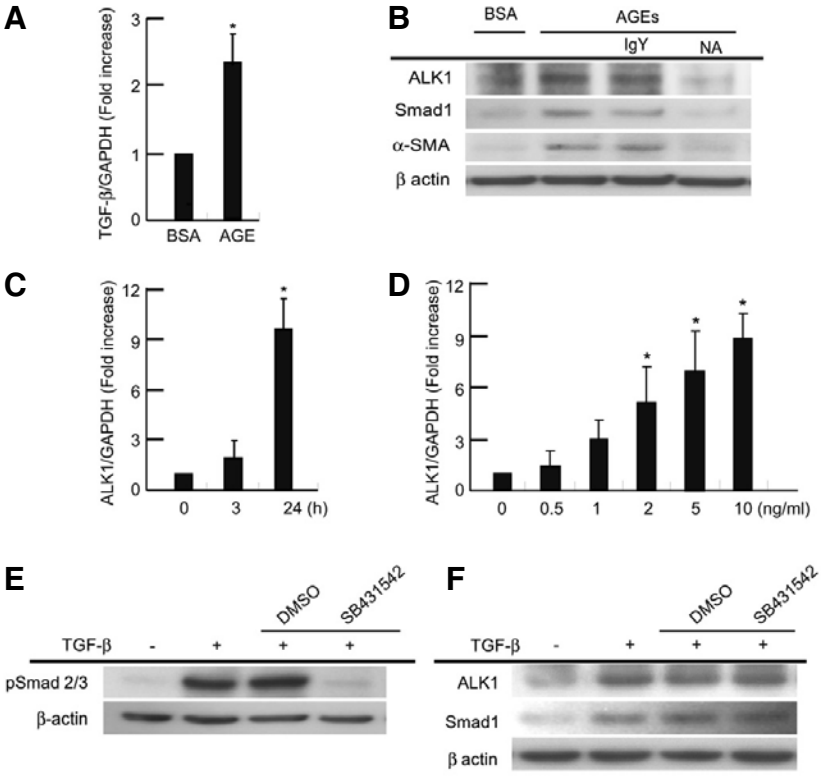

Fig. 1. AGEs induced the expression of ALK1, Smad1, and α-SMA through a TGF-β1-dependent pathway in mouse mesangial cells, and this induction occurred independently of the ALK5 pathway. (A) Quantitative real-time PCR analysis of TGF-β1 mRNA expression in total RNA lysates from mesangial cells treated with BSA or AGEs for 24 h. GAPDH was used as an internal control. A representative exposure is shown from 3 independent experiments. The data are presented as mean ± SD. The asterisks indicate statistically significant differences in comparison with the control (*; $P < 0.01$; Student's *t*-test). (B) The cultured mouse mesangial cells were treated with BSA or AGEs for 48 h prior to harvesting for Western blot analysis of ALK1, Smad1, and α-SMA. The cells exposed to AGEs were treated with neutralizing antibody (NA) for TGF-β1 or control chicken IgY. The data from 1 of 3 independent experiments are shown. (C, D) Quantitative real-time PCR analysis of ALK1 mRNA expression in total RNA lysates from mesangial cells treated with TGF-β1 in a time- (C) and dose-dependent (D) manner. GAPDH was used as an internal control. A representative exposure is shown from 3 independent experiments. The data are presented as mean ± SD. The asterisks indicate statistically significant differences in comparison with the control (*; $P < 0.01$; Bonferroni/Dunn test). (E) Western blot analysis of pSmad2/3 protein expression in cultured mouse mesangial cells treated with TGF-β1 for 1 h prior to harvest. (F) Western blot analysis of ALK1 and Smad1 protein expression in cultured mouse mesangial cells treated with TGF-β1 for 24 h prior to harvest. β-actin is the internal control. All Western blot analysis data from 1 of 3 independent experiments are shown.

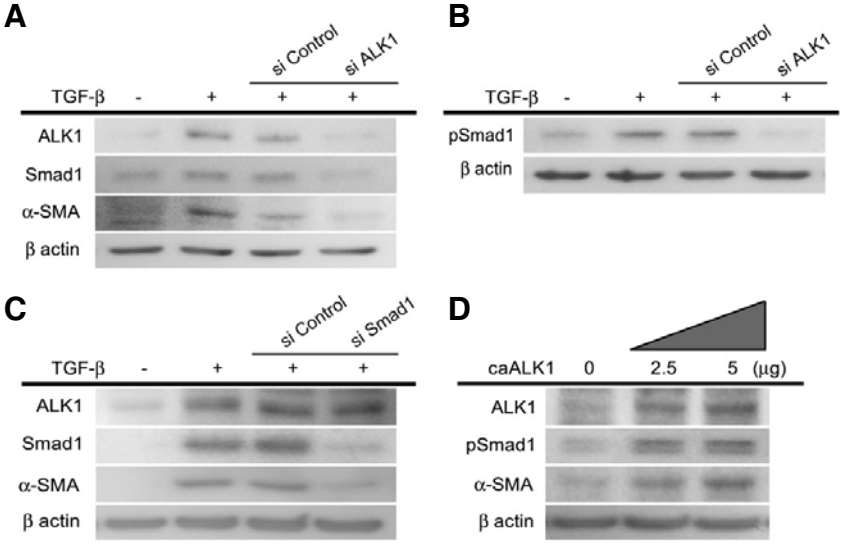

Fig. 2. Effects of introduction of siRNA for ALK1 or Smad1, or introduction of the constitutively active form of ALK1 into mouse mesangial cells, or TGF-β1 on ALK1-introduced mesangial cells. (A) Cultured mouse mesangial cells were transfected with siRNA for ALK1 (siALK1) or siControl. After 48 h, the cells were cultured in the presence of TGF-β1 (10 ng/ml) for 24 h. ALK1, Smad1, and α-SMA protein expressions were detected by Western blot analysis. (B) Cultured mouse mesangial cells were transfected with siALK1 or siControl. After 48 h, the cells were cultured in the presence of TGF-β1 (10 ng/ml) for 1 h prior to harvesting for Western blot analysis of pSmad1 protein expression. (C) Cultured mesangial cells were transfected with siRNA for Smad1 (siSmad1) or siControl. After 48 h, the cells were cultured in the presence of TGF-β1 (10 ng/ml) for 24 h. ALK1, Smad1, and α-SMA protein expressions were confirmed by Western blot analysis. (D) The cells were transfected with a constitutively active form of ALK1 (caALK1) expression vector (0, 2.5, and 5 μg plasmid DNA/well) in a dose-depen-dent manner. After 48 h, cell lysates were prepared, and ALK1, pSmad1, and α-SMA were visualized by western blot analysis. β-actin was used as an internal control.

and Smad1 by TGF-β1 (10 ng/ml) were not affected by SB431542 (Figs. 1E and 1F). These results suggest that the ALK1/Smad1 signaling pathway is activated in an ALK5-independent manner in the developmental process of DN.

ALK1 is an upstream regulator of Smad1

Because ALK1, Smad1, and α-SMA show coordinated expressions in mouse mesangial cells, we examined the regulatory pathway by using the siRNAs for ALK1 or Smad1 in mouse

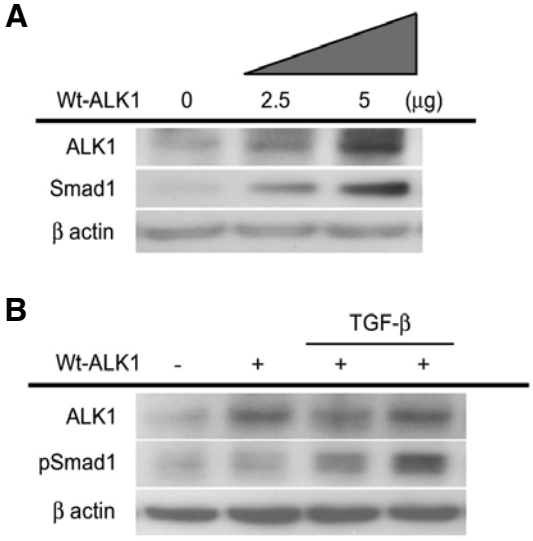

Fig. 3. Effect of TGF-β1 on ALK1-introduced mesangial cells. (A) The cells were transfected with full-length ALK1 (wt-ALK1) expression vector (0, 2.5, and 5 μg plasmid DNA/well) in a dose-dependent manner. After 48 h, cell lysates were prepared, and Smad1 was visualized by Western blot analysis. (B) Cultured mouse mesangial cells were transfected with wild-type ALK1 (Wt-ALK1) expression vector (5 μg of plasmid DNA/well). After 48 h, the cells were cultured in the presence of TGF-β1 (0.5 ng/ml) for 1 h prior to harvesting for Western blot analysis of ALK1 and pSmad1. β-actin was used as an internal control. All data from 1 of 3 independent experiments are shown.

mesangial cells. Transfection with the siRNA for ALK1 suppressed the TGF-β1-induced expressions of Smad1, α-SMA, and pSmad1 (Figs. 2A and 2B). In contrast, siRNAs for Smad1 did not show any effects of ALK1 expression (Fig. 2C). To confirm this regulatory pathway, we examined the effect of caALK1. Introduction of caALK1 induced the expression of both pSmad1 and α-SMA in a dose-dependent manner in mesangial cells (Fig. 2D). The introduction of wt-ALK1 induced Smad1 expression in a dose-dependent manner in mesangial cells (Fig. 3A). The TGF-β1 stimulated activation of pSmad1 was enhanced by the increase of ALK1 (Fig. 3B). Further analysis was carried out to examine the effect of TGF-β1 on Wt-ALK1-induced mesangial cells. These results suggest that ALK1 plays a central role in Smad1 activation in the progression of DN.

TCF7L2 contributed to ALK1 expression by binding to the ALK1 promoter

To identify the molecules that regulate the expression of ALK1, the MatInspector program, which predicts the transcription-factor-binding sites *in silico*, was used to search the regions 2000 bp upstream and 200 bp downstream from the transcription-initiation sites of the ALK1 sequence (GenBank accession number NM_009612). We found a TBM (AACAAAG) (Shimokawa et al., 2003) in the ALK1 gene promoter (Fig. 4A). In this study, we focused on TCF7L2, which is related to type 2 diabetes (Florez et al., 2006; Grant et al., 2006), because TBM had the ability to bind to TCF families containing high mobility group (HMG) DNA-binding domains (Waterman, 2004). Then, we examined the role of TCF7L2 in the gene expression of ALK1. After the transfection of TCF7L2 into the mesangial cells, TGF-β1 enhanced the translocation of TCF7L2 to the nucleus (Fig. 4B). To confirm the binding of TCF7L2 to the ALK1 promoter *in vivo*, we performed a ChIP assay. Immunoprecipitated DNA, which was purified using the specific antibody for TCF7L2, was analyzed for the ALK1 gene promoter. TGF-β1 induced a significant specific binding between TCF7L2 and TBM of ALK1

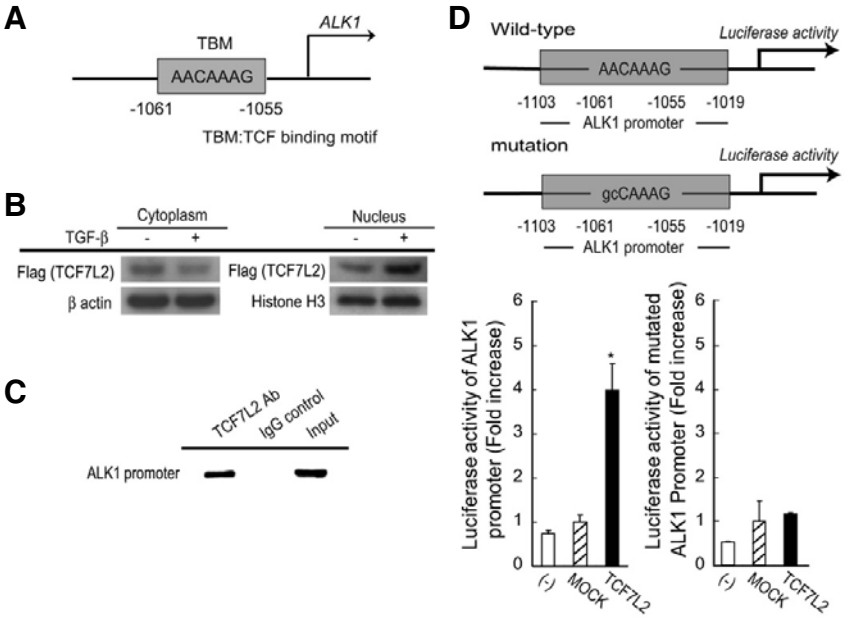

Fig. 4. TCF7L2 binds to ALK1 promoter and regulates ALK1 transcription activity. (A) The putative transcription-binding motif (TBM) located from 1055 bp to 1061 bp upstream from the transcription-initiation site of ALK1 sequence (NM_009612) is obtained using MatInspector program *in silico*. (B) Cultured mesangial cells transfected with p3xFlag-TCF7L2 were incubated in the presence or absence of TGF-β1 (10 ng/ml) for 24 h prior to harvest. These cell lysates were divided into the cytoplasmic and nuclear fractions for western blot analysis. β-actin was used as the cytoplasmic control. And Histone H3 was used as nuclear control. (C) ChIP assay was performed using anti-TCF7L2 antibody with mesangial cells treated with or without TGF-β1 (10 ng/ml) for 24 h. PCR was performed using primers for the TBM. After sonication, DNA from 4% solutions of each sample was saved as the input fraction (Input). All data from 1 of 3 independent experiments are shown. (D) The results of dual-luciferase reporter assay. After 48 h, the cells were lysed. Then, the activation of wild-type (pGL4.10-wTBM) or mutant type (pGL4.10-mTBM) reporter constructs were measured in CHO-K1 cells cotransfected with p3xFlag-TCF7L2, vector alone (mock), or in the absence of p3xFlag-TCF7L2; pGL4.74 was used as an internal control. A representative exposure is shown from 3 independent experiments. The data are presented as mean ± SD. The asterisks indicate statistically significant differences when compared with the control (*; $P < 0.01$; Bonferroni/Dunn test).

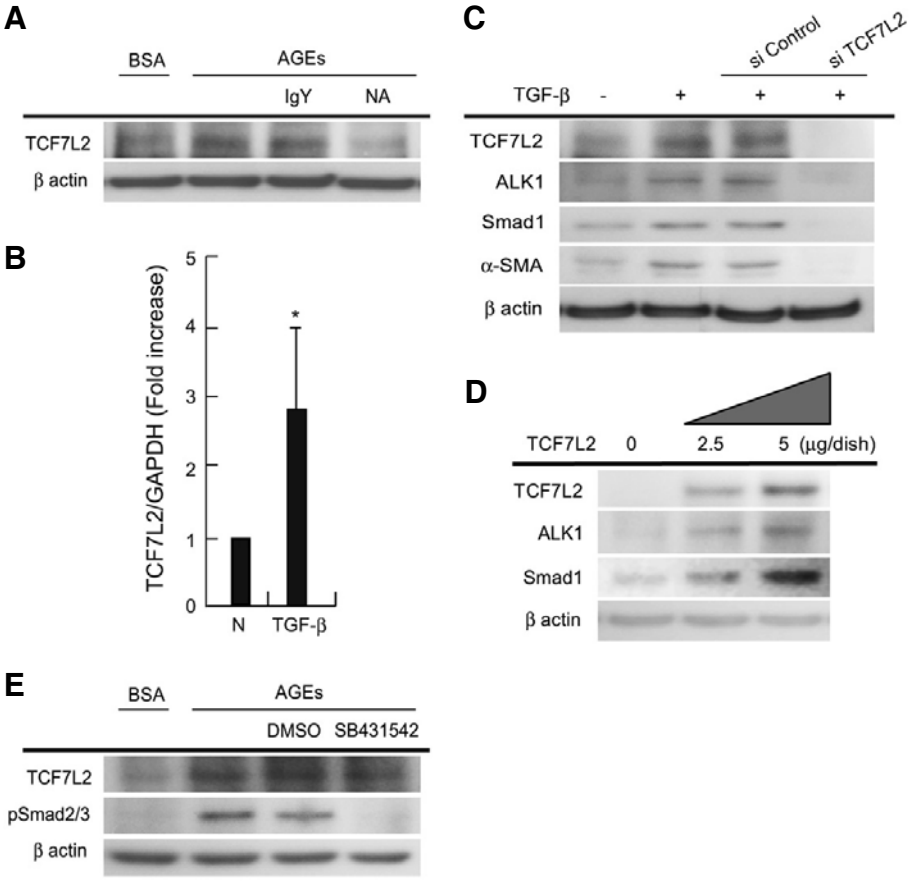

Fig. 5. Regulation of ALK1, Smad1, and α-SMA by TCF7L2. (A) Western blot analysis of ALK1, Smad1, and α-SMA protein expressions in cultured mouse mesangial cells treated with AGEs in the presence of neutralizing antibody (NA) for TGF-β1 or control chicken IgY. (B) Quantitative real-time PCR analysis of TCF7L2 mRNA expression in total RNA lysates from mesangial cells treated with TGF-β1 for 24 h. Expression of GAPDH served as an internal control. A representative exposure is shown from 3 independent experiments. The data are presented as mean ± SD. The asterisks indicate statistically significant differences in comparison with the control (*; $P < 0.01$; Student's *t*-test). (C) Cultured mouse mesangial cells were transfected with siTCF7L2 or siControl. After 48 h, the cells were cultured in the presence of TGF-β1 for 24 h prior to harvest for western blot analysis of TCF7L2, ALK1, Smad1, and α-SMA. (D) The cells were transfected with the TCF7L2 expression vector (0, 2.5, and 5 μg plasmid DNA/well) in a dose-dependent manner. After 48 h, cell lysates were prepared, and TCF7L2, ALK1, and Smad1 were visualized by western blot analysis. (E) Western blot analysis of TCF7L2 and pSmad2/3 protein expression in cultured mouse mesangial cells treated with AGEs for 48 h prior to harvest. β-actin was used as an internal control. All Western blot analysis data from 1 of 3 independent experiments are shown.

in the mesangial cells (Fig. 4C). The transcriptional activity of the ALK1 gene was examined by a reporter assay. Cotransfection of the ALK1 promoter gene and the TCF7L2 gene into CHO-K1 cells revealed approximately 4-fold increases in transcriptional activity, in comparison with the activities after transfection with the control vector or that in the absence of the TCF7L2 gene. To confirm the specificity, cotransfection of mutated TBM sites of the ALK1 promoter gene in the presence of TCF7L2 showed no increase in transcriptional activity (Fig. 4D). These results suggested that TCF7L2 could bind to TBM of *ALK1* promoter in nuclei, thereby increasing ALK1 expression. Thus, the development of DN may be determined by whether ALK1 expression is induced through TCF7L2.

Regulation of TCF7L2

AGEs increased the expression of TCF7L2 in mouse mesangial cells, which was inhibited by the addition of TGF-β1 NA (Fig. 5A) in the same way as the expression of ALK1, Smad1, and α-SMA as shown in Fig. 1A. Quantitative real-time PCR and Western blot analysis revealed that the addition of TGF-β1 (10 ng/ml) induced both mRNA and protein expressions of TCF7L2 (Figs. 5B and 5C). Introduction of siRNA against TCF7L2 into the mesangial cells blunted the TGF-β1-induced elevated expressions of TCF7L2, ALK1, Smad1, and α-SMA (Fig. 5C). Furthermore, after introducing TCF7L2 into mesangial cells, the expressions of ALK1, and Smad1 were parallel to the TCF7L2 expression (Fig. 5D). AGEs phosphorylated Smad2/3 in mouse mesangial cells, and this phosphorylation was blocked by SB431542. However, the increased expressions of TCF7L2 by AGEs was not affected by SB431542 (Fig. 5E). These findings indicate that in DN, the α-SMA expression in the mesangial cells is regulated by the AGEs/TGF-β/TCF7L2/ ALK1/Smad1 signaling pathway in an ALK5-independent manner.

Glomerular expressions of TCF7L2 and ALK1 in iNOS-Tgm

To confirm the expressions of TCF7L2, ALK1, Smad1, pSmad1, and α-SMA in the diabetic kidney *in vivo*, we investigated the glomerular expression of these factors in mice DN model (iNOS-Tgm). Periodic acid-Schiff (PAS) staining revealed that the glomerular sclerotic lesion in the iNOS-Tgm was more severe than that in the control mice (Figs. 6A and 6G). Increased glomerular expressions of TCF7L2, ALK1, Smad1, pSmad1, and α-SMA were observed in the diabetic renal glomeruli (Figs. 6H-6L). In contrast, these expressions were nearly absent in normal glomeruli (Figs. 6B-6F). These histological observations support the finding that the TCF7L2/ ALK1/Smad1/α-SMA signaling pathway is linked to the phenotypic change and extracellular matrix expansion in DN *in vivo*.

DISCUSSION

DN is characterized by typical pathological changes, and mesangial matrix expansion, resulting in renal dysfunction (Kanwar et al., 2008; Schrijvers et al., 2004). We have previously reported that Smad1 signal transduction plays a central role in the development of mesangial matrix expansion in DN (Abe et al.,

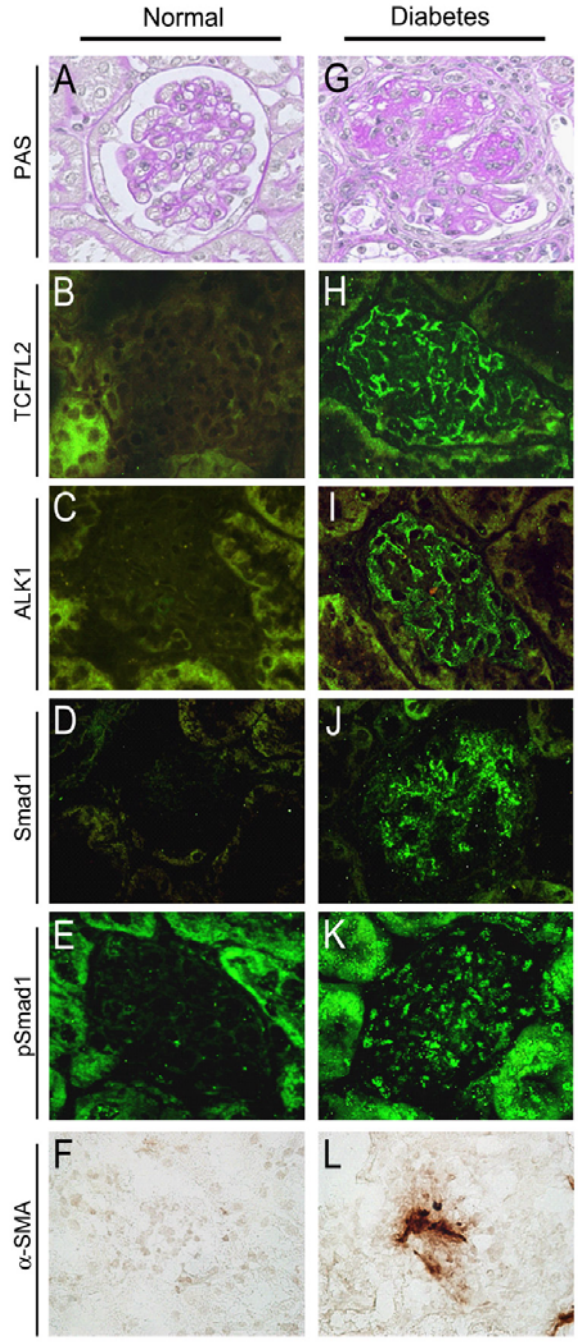

Fig. 6. Expressions of TCF7L2, ALK1, Smad1, pSmad1, and α-SMA in mouse DN. (A, G) The kidney paraffin sections derived from control mice (A) and iNOS-Tgm (G) were stained by periodic acid-Schiff staining (PAS) reagent. (B-F and H-L) The cryopreserved kidney sections derived from control mice (B-F) and iNOS-Tgm (H-L) were used for immunofluorescent staining: TCF7L2 (B, H), ALK1 (C, I), Smad1 (D, J), pSmad1 (E, K), and α-SMA (F, L). All data from 1 of 3 independent experiments are shown.

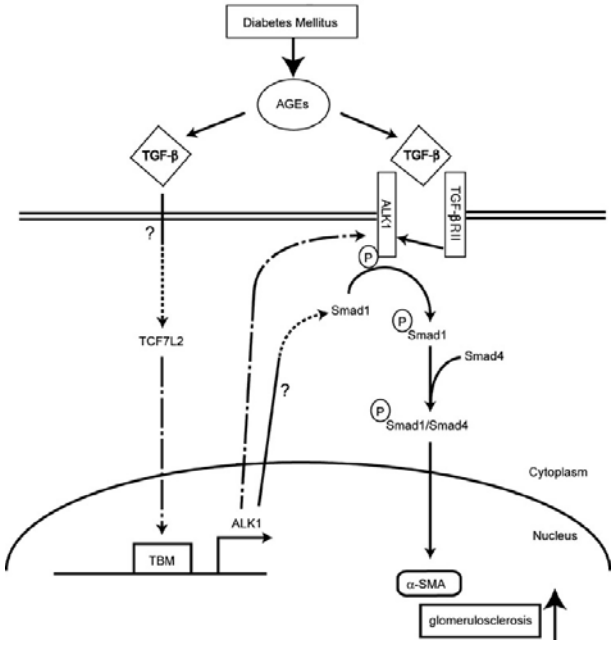

Fig. 7. The proposed model for the role of TCF7L2/ALK1/Smad1 signal transduction pathway in diabetic condition. The figure illustrates TCF7L2 binding to ALK1 promoter and the role of ALK1 and does not depict other possible mechanisms.

2004; Matsubara et al., 2006; Mima et al., 2008). In this study, we have further demonstrated a novel upstream mechanism for Smad1 signaling in DN. The fact that Smad1 is induced and activated by ALK1 suggests that TGF-β/ALK1 is directly involved in the development of diabetic glomerulosclerosis; therefore, ALK1 has the potential to become a novel therapeutic target of DN. While we had reported that urinary Smad1 was useful as a superior predictor for the development of DN (Matsubara et al., 2006), it was difficult to establish suitable tests, because Smad1 was a transcriptional factor. However, ALK1 may be easily detected, and it reflects the direct change in diabetic glomeruli because ALK1 is a transmembrane protein and its expression restarted in the kidney under diabetic conditions, whereas, previous studies have reported that the TGF-β/ALK5/Smad2/3 signaling pathway is not associated with the diabetic microangiopathy characterized by endothelial cell proliferation (Wang and Hirschberg, 2009), suggesting that this pathway has relatively low significance as a predictor of early DN. The TGF-β/ALK1/Smad1/5/8 signaling pathway is considered to be a highly specific marker and a novel therapeutic target for DN, including early-phase DN. However, further investigation is required to understand the regulatory mechanism for ALK1 expression.

This study has clearly proved that TCF7L2 acts as a key transcription factor for ALK1 in the development of DN. TCF7L2 is an HMG box-containing transcription factor, and then the variant of TCF7L2 is strongly associated with the pathophysiology of type 2 diabetes, DN, and chronic kidney disease (CKD) (Florez et al., 2006; Grant et al., 2006; Kottgen et al., 2008; Sale et al., 2007). The regulatory mechanisms of TCF7L2 have only been reported in the case of proinsulin processing by TCF7L2 in the pancreatic beta cells (Liu and Habener, 2008; Shu et al., 2008). This study indicates a new role of TCF7L2 in the development of diabetic complications. TGF-β induced the translocation of TCF7L2 from the cytoplasm to the nucleus in mouse mesangial cells, resulting in an increase in the transcriptional activation of ALK1. Furthermore, TGF-β directly induced the expression of TCF7L2 in mesangial cells *in vitro*, and then,

the expression of TCF7L2 dramatically increased in mouse diabetic glomerulosclerosis *in vivo*. Other studies have reported similar phenomena in which TCF7L2 expression was observed in Zucker diabetic fatty rat islets and human pancreatic islets of type 2 diabetes (Lyssenko et al., 2007; Parton et al., 2006). This study is the first report that TCF7L2 expression is increased in mouse mesangial cells treated with TGF-β and in glomeruli of diabetic mice. Therefore, the regulation of TCF7L2 by TGF-β may play a new key role in the development of DN. TBM in the ALK1 promoter may connect with various TCFs, including TCF7, TCF7L1, TCF7L2, or their variants. In this study we have focused on wild-type TCF7L2 because the protein conformation of variant TCF7L2 was not known, and the function of both TCF7 and TCF7L1 was unclear (Elbein et al., 2009; Sale et al., 2007; Weedon et al., 2005).

Phenotypic changes in mesangial cells are an important pathological change in glomerular injury, which is usually associated with glomerulosclerosis. Although several mechanisms have been proposed for the occurrence of phenotypic changes in the mesangial cells, no conclusive evidence has been obtained. This study shows that ALK1/Smad1 signaling is a key determinant for increased expression of α-SMA as well as mesangial matrix expansion. TGF-β and ALK1 achieve a synergistic effect on Smad1 activation and α-SMA expression; in addition, blocking of ALK1 can completely inhibit these effects. These findings show that TGF-β is an upstream regulator for these pathological features, whereas ALK1 is a critical determinant for the development of phenotypic change.

About 40% of type 2 diabetic patients develop DN in the long-term course of their disease. DN has an important impact on morbidity/mortality in diabetic patients. In contrast, it is generally acknowledged that there are long-standing diabetic patients without nephropathy (Bojestig et al., 1994; Schrijvers et al., 2004). The reasons for these clinical differences have remained unknown. However, recent reports showed that these differences may be attributed to the allelic odds ratio of TCF7L2 (OR = 1.45-2.41) (Florez et al., 2006; Grant et al., 2006). We think that the development of diabetic glomerulosclerosis may be regulated by the expression levels of TCF7L2 and the binding affinity of TCF7L2 to ALK1. Elucidation of this molecular mechanism is important.

Microalbuminuria is a sign of renal dysfunction and is currently considered to be an independent predictor of cardiovascular events and mortality in diabetic patients (Gerstein et al., 2001). Although CKD is recognized as an independent cardiovascular disease (CVD) risk state (Go et al., 2004), the differences in the molecular mechanisms between CKD and CVD are unclear. Previous reports suggested that ALK1 was closely associated with the expression of α-SMA and the development of atherosclerosis (Yao et al., 2007), which was responsible for CVD (Keelan et al., 2001). Furthermore, recent reports describe TCF7L2 SNP as being associated with a higher risk of metabolic syndrome (MetS) (Sjogren et al., 2008; Warodomwichit et al., 2009), which is a cluster of factors contributing to increased risk of CVD and type 2 diabetes (Isomaa et al., 2001), however, the unifying mechanisms have not been identified. Our findings may provide new evidence showing that TCF7L2 is a key molecule in the relationship between CKD and CVD.

In conclusion, AGEs induced TCF7L2 expression through TGF-β in mouse mesangial cells. The TCF7L2 translocation to the nucleus increased and TCF7L2 bound to the ALK1 promoter, thereby resulting in increased ALK1 expression. The role of ALK1 in the increase of Smad1 is uncertain; however, increased ALK1 enhances the effect of TGF-β and further promotes the phosphorylation of Smad1 (Fig. 7). These interactions cause the phenotypic changes in mesangial cells, and these changes are characterized by the increased expression of α-SMA, thereby resulting in the development of glomerulosclerosis. These results suggest that the AGEs/TGF-β/TCF7L2/ALK1/Smad1 signaling pathway plays a key role in the development of diabetic nephropathy.

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REFERENCES

Abe, H., Matsubara, T., Iehara, N., Nagai, K., Takahashi, T., Arai, H., Kita, T., and Doi, T. (2004). Type IV collagen is transcriptionally regulated by Smad1 under advanced glycation end product (AGE) stimulation. J. Biol. Chem. *279*, 14201-14206.

Bojestig, M., Arnqvist, H.J., Hermansson, G., Karlberg, B.E., and Ludvigsson, J. (1994). Declining incidence of nephropathy in insulin-dependent diabetes mellitus. N. Engl. J. Med. *330*, 15-18.

Davies, M. (1994). The mesangial cell: a tissue culture view. Kidney Int. *45*, 320-327.

Doi, T., Vlassara, H., Kirstein, M., Yamada, Y., Striker, G.E., and Striker, L.J. (1992). Receptor-specific increase in extracellular matrix production in mouse mesangial cells by advanced glycosylation end products is mediated via platelet-derived growth factor. Proc. Natl. Acad. Sci. USA *89*, 2873-2877.

Elbein, S.C., Das, S.K., Hallman, D.M., Hanis, C.L., and Hasstedt, S.J. (2009). Genome-wide linkage and admixture mapping of type 2 diabetes in African American families from the American Diabetes Association GENNID (Genetics of NIDDM) Study Cohort. Diabetes *58*, 268-274.

Essawy, M., Soylemezoglu, O., Muchaneta-Kubara, E.C., Shortland, J., Brown, C.B., and el Nahas, A.M. (1997). Myofibroblasts and the progression of diabetic nephropathy. Nephrol. Dial. Transplant *12*, 43-50.

Fink, L., Seeger, W., Ermert, L., Hanze, J., Stahl, U., Grimminger, F., Kummer, W., and Bohle, R.M. (1998). Real-time quantitative RT-PCR after laser-assisted cell picking. Nat. Med. *4*, 1329-1333.

Finnson, K.W., Parker, W.L., ten Dijke, P., Thorikay, M., and Philip, A. (2008). ALK1 opposes ALK5/Smad3 signaling and expression of extracellular matrix components in human chondrocytes. J. Bone Miner. Res. *23*, 896-906.

Florez, J.C., Jablonski, K.A., Bayley, N., Pollin, T.I., de Bakker, P.I., Shuldiner, A.R., Knowler, W.C., Nathan, D.M., and Altshuler, D. (2006). TCF7L2 polymorphisms and progression to diabetes in the Diabetes Prevention Program. N. Engl. J. Med. *355*, 241-250.

Gerstein, H.C., Mann, J.F., Yi, Q., Zinman, B., Dinneen, S.F., Hoogwerf, B., Halle, J.P., Young, J., Rashkow, A., Joyce, C., et al. (2001). Albuminuria and risk of cardiovascular events, death, and heart failure in diabetic and nondiabetic individuals. JAMA *286*, 421-426.

Go, A.S., Chertow, G.M., Fan, D., McCulloch, C.E., and Hsu, C.Y. (2004). Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. N. Engl. J. Med. *351*, 1296-1305.

Goumans, M.J., Valdimarsdottir, G., Itoh, S., Rosendahl, A., Sideras, P., and ten Dijke, P. (2002). Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. EMBO J. *21*, 1743-1753.

Grant, S.F., Thorleifsson, G., Reynisdottir, I., Benediktsson, R., Manolescu, A., Sainz, J., Helgason, A., Stefansson, H., Emilsson, V., Helgadottir, A., et al. (2006). Variant of transcription factor 7-like 2 (TCF7L2) gene confers risk of type 2 diabetes. Nat. Genet. *38*, 320-323.

Hautmann, M.B., Madsen, C.S., and Owens, G.K. (1997). A transforming growth factor beta (TGFbeta) control element drives

TGFbeta-induced stimulation of smooth muscle alpha-actin gene expression in concert with two CArG elements. J. Biol. Chem. *272*, 10948-10956.

Inman, G.J., Nicolas, F.J., Callahan, J.F., Harling, J.D., Gaster, L.M., Reith, A.D., Laping, N.J., and Hill, C.S. (2002). SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. Mol. Pharmacol. *62*, 65-74.

Isomaa, B., Almgren, P., Tuomi, T., Forsen, B., Lahti, K., Nissen, M., Taskinen, M.R., and Groop, L. (2001). Cardiovascular morbidity and mortality associated with the metabolic syndrome. Diabetes Care *24*, 683-689.

Kanematsu, A., Ramachandran, A., and Adam, R.M. (2007). GATA-6 mediates human bladder smooth muscle differentiation: involvement of a novel enhancer element in regulating alpha-smooth muscle actin gene expression. Am. J. Physiol. Cell Physiol. *293*, C1093-1102.

Kanwar, Y.S., Wada, J., Sun, L., Xie, P., Wallner, E.I., Chen, S., Chugh, S., and Danesh, F.R. (2008). Diabetic nephropathy: mechanisms of renal disease progression. Exp. Biol. Med. *233*, 4-11.

Keelan, P.C., Bielak, L.F., Ashai, K., Jamjoum, L.S., Denktas, A.E., Rumberger, J.A., Sheedy, I.P., Peyser, P.A., and Schwartz, R.S. (2001). Long-term prognostic value of coronary calcification detected by electron-beam computed tomography in patients undergoing coronary angiography. Circulation *104*, 412-417.

Konig, H.G., Kogel, D., Rami, A., and Prehn, J.H. (2005). TGF-{beta}1 activates two distinct type I receptors in neurons: implications for neuronal NF-{kappa}B signaling. J. Cell Biol. *168*, 1077-1086.

Korinek, V., Barker, N., Morin, P.J., van Wichen, D., de Weger, R., Kinzler, K.W., Vogelstein, B., and Clevers, H. (1997). Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. Science *275*, 1784-1787.

Kottgen, A., Hwang, S.J., Rampersaud, E., Coresh, J., North, K.E., Pankow, J.S., Meigs, J.B., Florez, J.C., Parsa, A., Levy, D., et al. (2008). TCF7L2 variants associate with CKD progression and renal function in population-based cohorts. J. Am. Soc. Nephrol. *19*, 1989-1999.

Kou, I., Nakajima, M., and Ikegawa, S. (2007). Expression and regulation of the osteoarthritis-associated protein asporin. J. Biol. Chem. *282*, 32193-32199.

Liu, Z., and Habener, J.F. (2008). Glucagon-like peptide-1 activation of TCF7L2-dependent Wnt signaling enhances pancreatic beta cell proliferation. J. Biol. Chem. *283*, 8723-8735.

Luo, R.X., Postigo, A.A., and Dean, D.C. (1998). Rb interacts with histone deacetylase to repress transcription. Cell *92*, 463-473.

Lux, A., Attisano, L., and Marchuk, D.A. (1999). Assignment of transforming growth factor beta1 and beta3 and a third new ligand to the type I receptor ALK-1. J. Biol. Chem. *274*, 9984-9992.

Lyssenko, V., Lupi, R., Marchetti, P., Del Guerra, S., Orho-Melander, M., Almgren, P., Sjogren, M., Ling, C., Eriksson, K.F., Lethagen, A.L., et al. (2007). Mechanisms by which common variants in the TCF7L2 gene increase risk of type 2 diabetes. J. Clin. Invest. *117*, 2155-2163.

Matsubara, T., Abe, H., Arai, H., Nagai, K., Mima, A., Kanamori, H., Sumi, E., Takahashi, T., Matsuura, M., Iehara, N., et al. (2006). Expression of Smad1 is directly associated with mesangial matrix expansion in rat diabetic nephropathy. Lab. Invest. *86*, 357-368.

Mima, A., Arai, H., Matsubara, T., Abe, H., Nagai, K., Tamura, Y., Torikoshi, K., Araki, M., Kanamori, H., Takahashi, T., et al. (2008). Urinary Smad1 is a novel marker to predict later onset of mesangial matrix expansion in diabetic nephropathy. Diabetes *57*, 1712-1722.

Nakamura, T., Fukui, M., Ebihara, I., Osada, S., Nagaoka, I., Tomino, Y., and Koide, H. (1993). mRNA expression of growth factors in glomeruli from diabetic rats. Diabetes *42*, 450-456.

Oh, S.P., Seki, T., Goss, K.A., Imamura, T., Yi, Y., Donahoe, P.K., Li, L., Miyazono, K., ten Dijke, P., Kim, S., et al. (2000). Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. Proc. Natl. Acad. Sci. USA *97*, 2626-2631.

Park, I.S., Kiyomoto, H., Abboud, S.L., and Abboud, H.E. (1997). Expression of transforming growth factor-beta and type IV collagen in early streptozotocin-induced diabetes. Diabetes *46*, 473-480.

Parton, L.E., McMillen, P.J., Shen, Y., Docherty, E., Sharpe, E., Diraison, F., Briscoe, C.P., and Rutter, G.A. (2006). Limited role for SREBP-1c in defective glucose-induced insulin secretion from Zucker diabetic fatty rat islets: a functional and gene profiling analysis. Am. J. Physiol. Endocrinol. Metab. *291*, E982-994.

Sale, M.M., Smith, S.G., Mychaleckyj, J.C., Keene, K.L., Langefeld, C.D., Leak, T.S., Hicks, P.J., Bowden, D.W., Rich, S.S., and Freedman, B.I. (2007). Variants of the transcription factor 7-like 2 (TCF7L2) gene are associated with type 2 diabetes in an African-American population enriched for nephropathy. Diabetes *56*, 2638-2642.

Sanai, T., Sobka, T., Johnson, T., el-Essawy, M., Muchaneta-Kubara, E.C., Ben Gharbia, O., el Oldroyd, S., and Nahas, A.M. (2000). Expression of cytoskeletal proteins during the course of experimental diabetic nephropathy. Diabetologia *43*, 91-100.

Schrijvers, B.F., De Vriese, A.S., and Flyvbjerg, A. (2004). From hyperglycemia to diabetic kidney disease: the role of metabolic, hemodynamic, intracellular factors and growth factors/cytokines. Endocr. Rev. *25*, 971-1010.

Shimokawa, T., Furukawa, Y., Sakai, M., Li, M., Miwa, N., Lin, Y.M., and Nakamura, Y. (2003). Involvement of the FGF18 gene in colorectal carcinogenesis, as a novel downstream target of the beta-catenin/T-cell factor complex. Cancer Res. *63*, 6116-6120.

Shu, L., Sauter, N.S., Schulthess, F.T., Matveyenko, A.V., Oberholzer, J., and Maedler, K. (2008). Transcription factor 7-like 2 regulates beta-cell survival and function in human pancreatic islets. Diabetes *57*, 645-653.

Sjogren, M., Lyssenko, V., Jonsson, A., Berglund, G., Nilsson, P., Groop, L., and Orho-Melander, M. (2008). The search for putative unifying genetic factors for components of the metabolic syndrome. Diabetologia *51*, 2242-2251.

Takamura, T., Kato, I., Kimura, N., Nakazawa, T., Yonekura, H., Takasawa, S., and Okamoto, H. (1998). Transgenic mice overexpressing type 2 nitric-oxide synthase in pancreatic beta cells develop insulin-dependent diabetes without insulitis. J. Biol. Chem. *273*, 2493-2496.

Tang, Y., Urs, S., and Liaw, L. (2008). Hairy-related transcription factors inhibit Notch-induced smooth muscle alpha-actin expression by interfering with Notch intracellular domain/CBF-1 complex interaction with the CBF-1-binding site. Circ. Res. *102*, 661-668.

Trembath, R.C., Thomson, J.R., Machado, R.D., Morgan, N.V., Atkinson, C., Winship, I., Simonneau, G., Galie, N., Loyd, J.E., Humbert, M., et al. (2001). Clinical and molecular genetic features of pulmonary hypertension in patients with hereditary hemorrhagic telangiectasia. N. Engl. J. Med. *345*, 325-334.

Urness, L.D., Sorensen, L.K., and Li, D.Y. (2000). Arteriovenous malformations in mice lacking activin receptor-like kinase-1. Nat. Genet. *26*, 328-331.

Wang, S., and Hirschberg, R. (2009). Diabetes-relevant regulation of cultured blood outgrowth endothelial cells. Microvasc. Res. *78*, 174-179.

Warodomwichit, D., Arnett, D.K., Kabagambe, E.K., Tsai, M.Y., Hixson, J.E., Straka, R.J., Province, M., An, P., Lai, C.Q., Borecki, I., et al. (2009). Polyunsaturated fatty acids modulate the effect of TCF7L2 gene variants on postprandial lipemia. J. Nutr. *139*, 439-446.

Waterman, M.L. (2004). Lymphoid enhancer factor/T cell factor expression in colorectal cancer. Cancer Metastasis Rev. *23*, 41-52.

Weedon, M.N., Owen, K.R., Shields, B., Hitman, G., Walker, M., McCarthy, M.I., Hattersley, A.T., and Frayling, T.M. (2005). A large-scale association analysis of common variation of the HNF1alpha gene with type 2 diabetes in the U.K. Caucasian population. Diabetes *54*, 2487-2491.

Wiercinska, E., Wickert, L., Denecke, B., Said, H.M., Hamzavi, J., Gressner, A.M., Thorikay, M., ten Dijke, P., Mertens, P.R., Breitkopf, K., et al. (2006). Id1 is a critical mediator in TGF-beta-induced transdifferentiation of rat hepatic stellate cells. Hepatology *43*, 1032-1041.

Yamagishi, S., Inagaki, Y., Okamoto, T., Amano, S., Koga, K., Takeuchi, M., and Makita, Z. (2002). Advanced glycation end product-induced apoptosis and overexpression of vascular endothelial growth factor and monocyte chemoattractant protein-1 in human-cultured mesangial cells. J. Biol. Chem. *277*, 20309-20315.

Yamamoto, Y., Kato, I., Doi, T., Yonekura, H., Ohashi, S., Takeuchi, M., Watanabe, T., Yamagishi, S., Sakurai, S., Takasawa, S., et al. (2001). Development and prevention of advanced diabetic nephropathy in RAGE-overexpressing mice. J. Clin. Invest. *108*, 261-268.

Yao, Y., Zebboudj, A.F., Torres, A., Shao, E., and Bostrom, K. (2007). Activin-like kinase receptor 1 (ALK1) in atherosclerotic lesions and vascular mesenchymal cells. Cardiovasc. Res. *74*, 279-289.

Ziyadeh, F.N. (2004). Mediators of diabetic renal disease: the case for tgf-Beta as the major mediator. J. Am. Soc. Nephrol. *15*, S55-57.