Type VIII Collagen Modulates TGF-β1-induced Proliferation of Mesangial Cells

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ABSTRACT

Mesangial cells in diabetic mice and human kidneys with diabetic nephropathy exhibit increased type VIII collagen, a nonfibrillar protein that exists as a heterodimer composed of α1(VIII) and α2(VIII), encoded by Col8a1 and Col8a2, respectively. Because TGF-β1 promotes the development of diabetic glomerulosclerosis, we studied whether type VIII collagen modulates the effects of TGF-β1 in mesangial cells. We obtained primary cultures of mesangial cells from wild-type, doubly heterozygous (Col8a1+/−/Col8a2+/−), and double-knockout (Col8a1−/−/Col8a2−/−) mice. TGF-β1 bound normally to double-knockout mesangial cells. In wild-type mesangial cells, TGF-β1 inhibited proliferation, but in double-knockout cells, it stimulated proliferation, promoted cell cycle progression, and reduced apoptosis; we could reverse this effect by reconstituting α1(VIII). Furthermore, in wild-type cells, TGF-β1 mainly stimulated the Smad pathways, whereas in double-knockout cells, it activated the MAPK and PI3K/Akt pathways and induced expression of fibroblast growth factor 21 (FGF21). Inhibiting FGF21 expression by either interfering with activation of the MAPK and PI3K/Akt pathways or by FGF21 siRNA attenuated the TGF-β1-induced proliferation of double-knockout mesangial cells. In vivo, diabetic double-knockout mice had significantly higher expression of renal FGF21 mRNA and protein compared with diabetic wild-type mice. Immunohistochemistry revealed strong expression of FGF21 in both glomerular (mesangial) and tubular cells of diabetic mice. Taken together, these data suggest that type VIII collagen significantly modulates the effect of TGF-β1 on mesangial cells and may therefore play a role in the pathogenesis of diabetic nephropathy.


Diabetic nephropathy is clinically characterized by proteinuria and progressive renal insufficiency and is the leading cause of end-stage renal failure in developed countries.1 Podocyte injury, glomerular basement membrane thickening, mesangial hypertrophy, and expansion with subsequent development of glomerulosclerosis and tubulointerstitial fibrosis have long been recognized as pathologic hallmarks.2,3 Several hormones, cytokines, and growth factors are known to play an important role in the genesis and progression of diabetic nephropathy. In particular, the multifunctional cytokine TGF-β1 has been identified as stimulating the synthesis and inhibiting the degradation of extracellular matrix molecules, and promoting mesangial cell hypertrophy, one of the cardinal characteristics of diabetic glomerulopathy.4,5 It has been found that TGF-β1 mRNA and protein expressions are elevated in the glomeruli and renal cortex of humans with diabetic nephropathy and in various experimental models of diabetic nephropathy.6,7 On the
other hand, neutralization of TGF-β1 by an anti-TGF-β antibody prevented glomerular hypertrophy and attenuated enhanced extracellular matrix gene expression in experimental diabetic nephropathy. TGF-β1 initially stimulates mesangial cell proliferation but subsequently inhibits cell cycle progression by induction of cell cycle inhibitors such as p27Kip1, resulting in mesangial hypertrophy. Moreover, TGF-β1 stimulates the expression of key extracellular matrix molecules including different types of collagen, fibronectin, and laminin, and suppresses, in parallel, the degradation of these molecules. This leads to the complex accumulation of matrix, resulting in diffuse glomerular sclerosis and progressive tubulointerstitial fibrosis.

In addition to the classical collagen types I, III, IV, and VI found in diabetic nephropathy, a strong specific induction of type VIII collagen has been found in mesangial cells in diabetic mice and in kidney biopsies from patients with human diabetic nephropathy compared with normal kidneys. Type VIII collagen, a nonfibrillar collagen protein, exists as a heterodimer composed of two distinct polypeptide chains designated as α1(VIII) and α2(VIII) (genes: Col8a1 and Col8a2). Although increasing evidence indicates that type VIII collagen, expressed by a limited number of cell types, may play an important role in mesangial cell function and the pathogenesis of diabetic glomerulosclerosis, the detailed physiologic function of this matrix protein remains unclear. This study investigates the potential role of type VIII collagen in modulating the biologic effects of TGF-β1 on mesangial cells.

RESULTS

TGF-β1 Induces the Expression of Col8a1 in Mouse Mesangial Cells

Previous studies have shown that high ambient glucose induces expression of Col8a1 in mouse mesangial cells. Therefore, the effect of recombinant TGF-β1 was tested. Figure 1 shows that stimulation with 2.5 ng/ml TGF-β1 for 24 hours significantly enhanced the expression of Col8a1 mRNA in wild-type (by 78.2 ± 7.8%; Figure 1A) as well as in Col8a1+/−/Col8a2+/− heterozygous (HZ) (by 77.5 ± 16.3%; Figure 1B) mesangial cells. No specific Col8a1 mRNA or α1(VIII) protein was detected in the absence or presence of TGF-β1 in Col8a1−/−/Col8a2−/− knockout (KO) mesangial cells (Figure 1, C and D). However, α1(VII) expression could be reconstituted in KO mesangial cells using the mammalian expression vector pCEP in which the Col8a gene transcription is driven by a cytomegalovirus promoter (Figure 1, C and D).

Mesangial Cell Proliferation Is Induced by TGF-β1 in Col8a1+/−/Col8a2−/− KO Mesangial Cells

We have previously shown that TGF-β1 inhibits proliferation of mesangial cells and induces cell hypertrophy. Therefore,
we further investigated whether endogenous type VIII collagen expression may modulate the influence of TGF-β1 on the proliferation. Wild-type, Col8a1+/−/Col8a2+/− and Col8a1−/−/Col8a2−/− KO mesangial cells were stimulated with 2.5 ng/ml TGF-β1 for 24 hours. Cell proliferation was assessed by direct cell counting as well as DNA synthesis rate. Under these conditions, TGF-β1 significantly increased the total cell number of Col8a1+/−/Col8a2−/− KO (by 37.5 ± 7.3%), whereas no increase was seen in Col8a1+/−/Col8a2+/− HZ and wild-type mesangial cells (Figure 2A). To confirm these findings with an alternative approach, we investigated the DNA synthesis rate under similar conditions. Cells were labeled with bromodeoxyuridine (BrdU), and the incorporation into DNA was assessed. Induction of DNA synthesis by TGF-β1 was apparent in Col8a1+/−/Col8a2−/− KO, whereas the cytokine inhibited DNA synthesis in Col8a1+/−/Col8a2+/− HZ as well as wild-type mesangial cells (Figure 2B). Forced reconstitution of Col8a1 expression using a cytomegalovirus-promoter plasmid, but not the empty control vector, in Col8a1+/−/Col8a2−/− KO cells abrogated the TGF-β1-induced proliferation (Figure 2C).

Col8a1 Expression Influences the Effect of TGF-β1 on Cell Cycle and Apoptosis

It is well known that in many cell types, TGF-β1 can affect the cell cycle and induce apoptosis.11,19 To address this, we measured cell cycle and apoptosis by fluorescence-activated cell sorter (FACS) analyses in mesangial cells exposed to 2.5 ng/ml TGF-β1 (Figure 3, A through G). Cell cycle analysis revealed that TGF-β1 increased the number of Col8a1+/−/Col8a2−/− KO cells in the S phase (by 81.4 ± 17.0%), whereas in wild-type as well as in Col8a1+/−/Col8a2+/− HZ mesangial cells, the DNA synthesis was significantly inhibited by TGF-β1 (Figure 3, A through C and G). Moreover, TGF-β1 raised the number of Col8a1+/−/Col8a2+/− KO cells in the G2/M phase (by 38.5 ± 7.2%) compared with wild-type cells (Figure 3, A through C and G), indicating completion of the cell cycle. In contrast, TGF-β1-mediated cell cycle arrest in wild-type cells was attenuated in Col8a1+/−/Col8a2−/− KO mesangial cells as demonstrated by a reduction of the number of cells in the G1 phase (by 18.9 ± 5.0%) as well as in the sub-G1 phase (by 24.6 ± 9.8%) by treatment of TGF-β1 (Figure 3, A through E). Because cells in the sub-G1 phase may be apoptotic, we assessed annexin V-fluorescein binding and propidium-iodide staining with FACS analysis (Figure 3H). TGF-β1 significantly reduced the rate of apoptotic (annexin-positive but propidium iodide-negative) Col8a1+/−/Col8a2−/− KO mesangial cells rate (by 26 ± 6%) in contrast to wild-type mesangial cells (Figure 3H).

The Receptor Affinity for TGF-β1 Is Not Different

To exclude the possibility that the different effects of TGF-β1 may be caused by changes in the expression of receptors for TGF-β1, saturation binding studies with 125I-TGF-β1 were performed. Binding occurred in a concentration-dependent manner with apparent dissociation constants of 2 ± 1 nM for wild-type and 1.2 ± 0.1 nM for TGF-β1-induced proliferation in HZ cells. The basal proliferation rate in HZ cells was significantly attenuated by TGF-β1 stimulation (Figure 2A), whereas no stimulation was observed in wild-type cells (Figure 2B). Moreover, we further investigated whether endogenous type VIII collagen expression may modulate the influence of TGF-β1 on the proliferation. Wild-type, Col8a1+/−/Col8a2−/− and Col8a1−/−/Col8a2−/− KO mesangial cells were stimulated with 2.5 ng/ml TGF-β1 for 24 hours. Cell proliferation was assessed by direct cell counting as well as DNA synthesis rate. Under these conditions, TGF-β1 significantly increased the total cell number of Col8a1+/−/Col8a2−/− KO (by 37.5 ± 7.3%), whereas no increase was seen in Col8a1+/−/Col8a2+/− HZ and wild-type mesangial cells (Figure 2A). To confirm these findings with an alternative approach, we investigated the DNA synthesis rate under similar conditions. Cells were labeled with bromodeoxyuridine (BrdU), and the incorporation into DNA was assessed. Induction of DNA synthesis by TGF-β1 was apparent in Col8a1+/−/Col8a2−/− KO, whereas the cytokine inhibited DNA synthesis in Col8a1+/−/Col8a2+/− HZ as well as wild-type mesangial cells (Figure 2B). Forced reconstitution of Col8a1 expression using a cytomegalovirus-promoter plasmid, but not the empty control vector, in Col8a1+/−/Col8a2−/− KO cells abrogated the TGF-β1-induced proliferation (Figure 2C).

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Figure 2. TGF-β1-mediated effects on proliferation are modified by the presence of α1(V) collagen. (A) Total cell number is significantly stimulated by 2.5 ng/ml TGF-β1 for 24 hours only in Col8a1+/−/Col8a2+/− KO mesangial cells but not in wild-type or Col8a1+/−/Col8a2+/− HZ cells (n = 10 independent experiments with triplicates, ***<0.001 versus controls). (B) DNA synthesis determined as bromodeoxyuridine incorporation. Whereas 2.5 ng/ml TGF-β1 induced a significant decrease in DNA synthesis in wild-type and Col8a1+/−/Col8a2+/− HZ cells, it clearly stimulated DNA synthesis in Col8a1+/−/Col8a2−/− KO mesangial cells (n = 10 independent experiments done four times each, **<0.01 versus controls of the same genotype). (C) Effect of a Col8a1 reconstitution on cell number. Col8a1+/−/Col8a2−/− KO mesangial cells were transfected either with control plasmid pCPE or Col8a1/pCPE (each 0.8 and 1.6 μg/ml) for 12 hours and stimulated with 2.5 ng/ml TGF-β1 for a further 24 hours. Reconstitution of Col8a1 expression reduced the TGF-β1-induced proliferation to basal levels, whereas the empty control vector had no effect (n = 9, ***<0.001 versus controls without TGF-β1, #P<0.001 versus cells transfected with control vector [1.6 μg/ml] treated with TGF-β1).
TGF-β1 Signaling Pathways Differ Depending on Type VIII Collagen Expression

Because TGF-β1 can activate diverse signaling pathways including Smad and mitogen-activated protein (MAP) kinase pathways,20,21 we tested whether these secondary messengers are differentially regulated. Wild-type, Col8a1<sup>+/−</sup>/Col8a2<sup>+/−</sup> HZ, and Col8a1<sup>+/−</sup>/Col8a2<sup>−/−</sup> KO mesangial cells were exposed to 2.5 ng/ml TGF-β1 for different time periods, and cell extracts were analyzed by Western blotting with antibodies against Smad3, Erk 1,2, and Akt (Figure 4). In contrast to wild-type cells, TGF-β1 activated in Col8a1<sup>+/−</sup>/Col8a2<sup>−/−</sup> KO cells activated MAP kinase and Akt pathways rather than Smad3 (Figure 4). Furthermore, phosphorylated and total Erk 1,2 and PI3K/Akt was increased in Col8a1<sup>+/−</sup>/Col8a2<sup>−/−</sup> KO mesangial cells compared with the wild-type even in the absence of TGF-β1.

Difference in TGF-β1-induced Gene Expression in Wild-type and Col8a1<sup>+/−</sup>/Col8a2<sup>−/−</sup> KO Mesangial Cells

We next assessed potential differences in the TGF-β1-induced transcriptome in wild-type and Col8a1<sup>+/−</sup>/Col8a2<sup>−/−</sup> KO mesangial cells using microarray analysis. Out of the more than 39,000 transcripts on the mouse Affymetrix array, 945 showed a reliable signal. The number of genes induced or repressed at least two-fold was 110. Gene array analysis was performed according the MIAME Guidelines,22,23 and data have been submitted the microarray informative database of the European Bioinformatics Institute (accession number E-MEXP-2658). Further information how the analysis of gene arrays was performed and selected results are shown in the supplemental materials. Thirty-three known
genes were upregulated, and 77 were down-regulated (data not shown). We found that genes involved in, for example, extracellular matrix formation, cell cycle, apoptosis, survival, MAP kinase signaling, and DNA replication showed altered expression levels. Several genes (collagen type V [2.9-fold], laminin [3.4-fold], TGF-β1 [7.3-fold], TGF-β receptor type II [3.5-fold], fibroblast growth factor 2 [3.0-fold], fibroblast growth factor (FGF21) [10.7-fold], and inhibitor of apoptosis protein [2.1-fold]) were highly induced by TGF-β1 in Col8a1−/−/Col8a2−/− KO compared with TGF-β1-challenged wild-type mesangial cells (Figure 5A). Real-time PCR analyses of NF-κB and PI3K, both well known as important regulators of cell survival,24 confirmed the finding that TGF-β1 is able to induce components of cell survival pathways in the absence of type VIII collagen expression (increase of p110α expression by 40.9 ± 12.7%) (Figure 5B).

**TGF-β1-induced FGF21 Expression Is Increased in the Absence of Type VIII Collagen**

Affymetrix analysis indicated that the TGF-β1-mediated FGF21 gene expression is much higher in Col8a1−/−/Col8a2−/− KO compared with TGF-β1-treated wild-type mesangial cells. Because it has been recently reported that FGF21 is a novel metabolic factor regulating glucose uptake,25 we further focused on this cytokine. As shown in Figure 6A, FGF21 mRNA expression was inducible by TGF-β1 in all three mesangial cells, but induction of FGF21 mRNA was significantly higher (approximately three-fold) in Col8a1−/−/Col8a2−/− KO mesangial cells than in wild-type cells. This finding was confirmed by Western blot analysis (Figure 6B). Time-course analysis of FGF21 expression in Col8a1−/−/Col8a2−/− KO mesangial cells showed that TGF-β1 stimulated expression of FGF21 protein in a time-dependent manner (Figure 6C). Next, we investigated whether the TGF-β1-

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**Figure 4.** TGF-β1 induced signal transduction is different in wildtype and Col8a1−/−/Col8a2−/− KO mesangial cells. (A) Signal transduction pathways. Wild-type, Col8a1−/−/Col8a2−/− HZ cells, and Col8a1−/−/Col8a2−/− KO mesangial cells were stimulated for 5 minutes or 3 hours with 2.5 ng/ml with (+) or without (−) TGF-β1, and phosphorylation of Smad3, Erk1,2, and Akt was analyzed by Western blots using phospho-specific antibodies. (B through D) Statistical analysis after densitometry of the blots. Whereas TGF-β1 leads to phosphorylation of Smad3 and only relatively little phosphorylation of Erk1,2 and Akt in wild-type cells, MAP kinase activation and Akt phosphorylation was significantly stronger in Col8a1−/−/Col8a2−/− KO mesangial cells after challenge with TGF-β1 (C and D, *P < 0.05 versus no TGF-β1, **P < 0.01 versus no TGF-β1, n = 3 independent experiments). In contrast Smad3 phosphorylation was attenuated. Col8a1−/−/Col8a2−/− cells compared with the wild-type (B, *P < 0.05 versus no TGF-β1, **P < 0.01 versus no TGF-β1, n = 3 independent experiments) HZ mesangial cells revealed an intermediate response. The blots are representative for three independent experiments with qualitatively similar results.
induced FGF21 expression is dependent on Erk 1,2 and PI3K/Akt pathways. Interference with the Erk 1,2 (with PD98059) as well as PI3K/Akt (with LY294002) pathways attenuated the TGF-β1-mediated upregulation of FGF21 mRNA in Col8a1−/−/Col8a2−/− KO mesangial cells (Figure 6D). Moreover, TGF-β1-induced FGF21 protein was reversed to basal levels by PD and LY (Figure 6E). This Western blot also clearly demonstrates that the inhibitors prevented phosphorylation of Erk 1,2 and Akt (Figure 6E). To test the cell specificity of the TGF-β1-induced FGF21 mRNA expression, podocytes as well as a mouse mesangial cell line (MMC) were studied. As shown in Figure 6F, TGF-β1 significantly stimulated FGF21 mRNA expression in mesangial cells with the highest induction in Col8a1−/−/Col8a2−/− KO mesangial cells but not in podocytes.

Inhibition of FGF21 Expression Attenuates the TGF-β1-mediated Proliferation in Col8a1−/−/Col8a2−/− KO Mesangial Cells

Inhibition of Erk 1,2 or PI3K/Akt pathways attenuated the TGF-β1-stimulated proliferation of Col8a1−/−/Col8a2−/− KO mesangial cells (Figure 7A). To further investigate the role of FGF21 in the TGF-β1-induced proliferation of Col8a1−/−/Col8a2−/− KO mesangial cells, FGF21 expression was inhibited by siRNA. 5 and 10 nM FGF21 siRNA, but not control siRNA, inhibited TGF-β1-mediated FGF21 protein expression in Col8a1−/−/Col8a2−/− KO mesangial cells (Figure 7B). Inhibition of FGF21 protein expression using specific siRNA inhibited the TGF-β1-stimulated proliferation of Col8a1−/−/Col8a2−/− KO mesangial cells (Figure 7C).

Enhanced Renal FGF21 Expression in Diabetic Col8a1−/−/Col8a2−/− KO Mice

To address the in vivo situation, we investigated the levels of FGF21 in serum and kidney homogenate in the streptozotocin-induced diabetic wild-type, Col8a1+/−/Col8a2+− HZ, and Col8a1−/−/Col8a2−/− KO mice. Because it has been recently demonstrated that serum FGF21 levels and FGF21 mRNA expression in different tissues are increased in obese and diabetic mice, we used db/db diabetic mice as a control. Serum FGF21 concentrations significantly increased only in db/db mice, a model of type 2 diabetes, compared with nondiabetic db littermates, whereas no significant difference was observed in streptozotocin-induced diabetic mice compared with nondiabetic controls (Figure 8A). However, renal FGF21 mRNA (Figure 8B) and protein (Figure 8C) expression was significantly higher in diabetic Col8a1−/−/Col8a2−/− KO mice compared with diabetic wild-type animals.

DISCUSSION

Mesangial cells play an important role in the development of diabetic glomerulosclerosis. In vitro and in vivo studies have demonstrated that mesangial cells exposed to high glucose or the diabetic environment initially undergo a very limited proliferation and become subsequently arrested in the G1 phase of the cell cycle. This G1 phase arrest is associated with the development of cellular hypertrophy and production of extracellular matrix proteins leading to mesangial expansion and sclerosis. TGF-β1 is a key factor induced by high glucose, mediating G1 phase arrest by induction of cell cycle inhibitors such as p27Kip1,11,12

We have recently found in biopsies from patients with diabetic nephropathy that type VIII collagen mRNA and protein expression (Fig. 9) is upregulated compared with healthy control kidneys. Furthermore, type VIII collagen expression in human diabetic nephropathy was not correlated with renal function and proteinuria, suggesting that the diabetic environment itself may be primarily responsible for the induction of...
In fact, high glucose-containing medium induced Col8a1 mRNA expression independent of its osmolarity in a MMC, but not in murine tubular and glomerular endothelial cells. Col8a2 was not induced by high glucose in mesangial cells. Further studies in STZ-induced diabetic Col8a1−/−/Col8a2−/− KO mice revealed less mesangial matrix deposition and albuminuria compared with diabetic wild-type animals. Because it has been previously shown that type VIII collagen may stimulate migration of smooth vascular muscle cells but may inhibit proliferation of endothelial cells, this study addresses whether type VIII collagen modulates the influence of TGF-β1, one of the major cytokines in diabetic nephropathy, on cell cycle regulation in mesangial cells. We used mesangial cells isolated from genetically modified mice deficient in both Col8 genes to test whether there is a relationship between the biologic effects of exogenous TGF-β1 and the expression of type VIII collagen. We provide evidence that TGF-β1 induced proliferation of mesangial cells in the absence of endogenous type VIII collagen expression. TGF-β1-induced signal transduction changed from utilization of the Smad system to activation of Erk 1,2 and PI3K/Akt pathways in the absence of type VIII collagen. A greater renal expression of FGF21 was also seen in diabetic Col8a1−/−/Col8a2−/− KO mice compared with diabetic wild-type animals. FGF21 is a recently discovered novel endocrine and paracrine metabolic regulator. When given to diabetic animals, FGF21 stimulates glucose uptake into cells and lowers plasma glucose as well as triglycerides. On the other hand, FGF21 mRNA expression itself is induced in the liver by glucose. When given to diabetic animals, FGF21 stimulates glucose uptake into cells and lowers plasma glucose as well as triglycerides. On the other hand, FGF21 mRNA expression itself is induced in the liver by glucose. Renal effects of FGF21 have not been previously described. FGF21 signals through cell-surface receptors composed of the classic FGF receptors complexed with β-Klotho but can also act through all four FGR isotypes. Wente et al. have previously demonstrated that FGF21 stimulates insulin gene transcription and insulin biosynthesis via activation of Erk 1,2 signaling in the pancreatic β-cell, promotes β-cell survival, and inhibits β-cell apoptosis via activation of PI3K/Akt signaling, which resulted in a strong reduction in circulating glucose levels and an increased number of islets and β-cells in diabetic mice after long-term administration of FGF21. Recent studies in STZ-induced diabetic mice have shown that FGF21 mediates the TGF-β1-induced Smad activation to the noncanonical pathway and these cells have been found to also express FGF receptors. Thus, our findings that FGF21 mediates the TGF-β1-induced proliferation in Col8a1−/−/Col8a2−/− KO mesangial cells are in accordance with these earlier findings. Because it has been previously shown that FGF21 expression is induced by the PI3K/Akt pathway and Erk 1,2 pathways, the shift from TGF-β1-induced Smad activation to the noncanonical pathway may play a role in mesangial cell proliferation.

Figure 6. (A through E) FGF21 expression induced by TGF-beta is strongest in Col8a1−/−/Col8a2−/− KO mesangial cells. (A) Although TGF-β1 for 24 hours increased FGF21 mRNA expression in all three cell types, the induction was much stronger in Col8a1−/−/Col8a2−/− KO mesangial cells. Quantitative RT-real-time PCR normalized to 18 S rRNA (n = 12, *P < 0.01 versus controls, **P < 0.001 versus controls of the same phenotype). (B) Western blot analysis for FGF21 protein expression. Stimulation of wild-type mesangial cells for 24 hours with TGF-β1 resulted in a detectable band, whereas Col8a1−/−/Col8a2−/− HZ and Col8a1−/−/Col8a2−/− KO mesangial cells clearly expressed FGF21 protein after TGF-β1 treatment. This blot is representative of three independent experiments with qualitatively similar results. (C) Time-course analysis of FGF21 protein expression in Col8a1−/−/Col8a2−/− KO mesangial cells. The cells were stimulated with 2.5 ng/ml TGF-β1 (+) or control medium without TGF-β1 (−) for the indicated length of time, and FGF21 protein expression was analyzed by Western blot. TGF-β1-mediated FGF21 protein expression occurred after 3 hours, peaked at 6 to 9 hours, and diminished after 24 hours. This blot is representative of two separate experiments.
whole kidney FGF21 mRNA expression as well as the enhanced serum concentration of FGF21 in diabetic animal is likely due to stimulated tubular expression as detected in the immunohistochemistry. Nevertheless, the discrete increase in mesangial cell staining for FGF21 in diabetic Col8a1/−/Col8a2/− KO mice but not in the diabetic wild-type is presumably sufficient to stimulate local mesangial proliferation.

How exactly type VIII collagen modulates TGF-β1-associated signal transduction pathways is currently unclear, but a few suggestions can be made. Older studies have shown that...
collagen type V is required for the growth and inhibitory actions of TGF-β1 nontransformed epithelial cells. The NC1 domain of the α1 chain of type VIII collagen inhibits the proliferation of bovine aortic endothelial cells, but the mechanisms behind these anti-proliferative effects were not further investigated. Several studies have also demonstrated that various collagens can exert anti-proliferative effects on mesangial cells. For example, collagen type I suppresses Erk 1,2 activation and is antiproliferative for mesangial cells. Discoidin domain receptors (DDR1 and 2) are receptor tyrosine kinases that function as receptors for various collagens. DDR1 is expressed on mesangial cells, and type VIII collagen binds to and activates this receptor. Interestingly, Curat and Vogel report that DDR1-knockout mesangial cells exhibited an increased proliferation and activation of Erk 1,2. Thus, the lack of type VIII collagen synthesis after TGF-β1 stimulation in Col8a1−/−/Col8a2−/− KO mesangial cells may lead to a reduced DDR1 activation with concomitant stimulation of the Erk 1,2 pathway. This, together with the induced FGF21 synthesis and activation of other pathways (e.g. NF-κB), ultimately leads to TGF-β1-induced proliferation and not hypertrophy in Col8a1−/−/Col8a2−/− KO mesangial cells. Further studies that are beyond the scope of this report are necessary to define the exact role of DDR1 in TGF-β1-induced proliferation of Col8a1−/−/Col8a2−/− KO mesangial cells.

Is TGF-β1-mediated mesangial proliferation in the absence of type VIII collagen better than hypertrophy? Regression of diabetic mesangial matrix expansion and glomerulosclerosis have occurred in humans and in experimental models of diabetic nephropathy. It is reasonable to assume that this process requires some limited proliferation of mesangial cells to restore an intact environment besides proteolytic clearance of the deposited extracellular matrix proteins. Diabetic p27Kip1 knockout mice have a reduced glomerular matrix expansion and less albuminuria despite
an increase in the mesangial cell number compared with diabetic wild-type animals. Bone marrow-derived cells can differentiate into mesangial cells after renal injury. Bone-marrow infusion can ameliorate progressive glomerulosclerosis in different experimental models. Although de novo formation of endothelial cells is important in this process, there is experimental evidence that the repair also involves mesangial cells. Glomerular mesangial cells provide structural support, and a repair process after diabetic nephropathy likely requires a well-balanced recruitment of glomerular endothelial and mesangial cells. Alternatively, one may see the increase in type VIII collagen expression during diabetic nephropathy as an intrinsic renal attempt to limit progression of disease.

In summary, we found that TGF-β1 induces a proliferation of mesangial cells in the absence of concomitant type VIII collagen expression. This effect is in strong contrast to wild-type mesangial cells in which TGF-β1 is anti-prolifer-
FGF21 expression was only found in diabetic animals. A weak increase in mesangial FGF21 was also observed in nondiabetic animals with qualitatively similar results (magnification, ×400).

**CONCISE METHODS**

**Animal Experiments**
All of the animal experiments were approved by the Thüringer Landesamt für Lebensmittelsicherheit und Verbraucherschutz and done in accordance with the German Animal Protection Law. The generation and phenotype of Col8a1+/−/Col8a2+/− KO mice has been previously described in detail.13 Col8a1+/−/Col8a2+/− KO and Col8a1+/−/Col8a2+/− HZ mice were crossed back for at least 20 generations into the C57Bl/6 background,13 and wild-type mice were maintained in a pathogen-free facility. All of the animals had free access to water and were on the standard rodent chow. Ten- to twelve-week-old mice were randomly divided into groups treated with streptozotocin (STZ; Sigma, St. Louis, MO) or left untreated. STZ was dissolved in sterile 10 mM sodium citrate (pH 5.5) and injected intraperitoneally (50 mg/kg body wt) for five consecutive days. To render the animals hyperglycemic without becoming ketoacidotic, a subcutaneous insulin implant (LinShin, Toronto, Canada) was administered.15 Venous blood glucose concentrations were measured with Free Style Lite (Abbott Diabetes Care, Wiesbaden, Germany). The mice were sacrificed after 40 days. In addition, diabetic db/db mice and control db/mice (Jackson Laboratory, Bar Harbor, ME) were used at the age of 4 months. At the end of the experiment, venous blood was collected in tubes on ice containing aprotonin (0.6 TIU/ml of blood; Phoenix Pharmaceuticals, Burlingame, CA) to inhibit the activity of proteinases. FGF21 protein concentrations were determined in serum samples using a mouse FGF21 RIA kit (Phoenix Pharmaceuticals, Burlingame, CA). In addition, the kidneys were harvested and mechanically homogenized with the homogenizer SpeedMill P12 (Analytik Jena Bio Solutions, Jena, Germany), and the total RNA and protein were isolated as described below.

**Isolation and Culture of Mesangial Cells**
Mesangial cells were obtained from the isolated glomeruli of Col8a1+/−/Col8a2+/− wild-type, Col8a1+/−/Col8a2+/− HZ, and Col8a1+/−/Col8a2+/− KO mice according to previously described protocols of sieving and centrifugation for the preparation of primary mesangial cell cultures.15,25 Our studies used subcultures of the fifteenth to twentieth passages of mesangial cells grown in RPMI 1640 medium (Promo Cell, Heidelberg, Germany), containing 5 mM glucose and 10% heat-inactivated FCS at 37°C in 5% CO₂. Routine identification of the mesangial cells was performed by indirect immunofluorescence microscopy using antibodies against α-smooth-muscle actin (Progen Biotechnik, Heidelberg, Germany), synaptopodin (Sigma), E-cadherin (Abcam, Cambridge, UK), and PECAM-1 (Santa Cruz Biotechnology, Santa Cruz, CA). Used cells stained positive for α-smooth-muscle actin but were negative for synaptopodin, E-cadherin, and PECAM-1. The cells were seeded in appropriate tissue culture plates and cultured for 24 hours. The cells were serum-starved (0.1% FCS) overnight and exposed as described for the indicated lengths of time.

**Proliferation Assays**
A colorimetric immunoassay kit for BrdU incorporation (Roche Diagnostics, Mannheim, Germany) was used for quantification of DNA synthesis.58 1 × 10⁴ cells were seeded in 96-well plates; after incubation in RPMI supplemented with 0.1% FCS for 24 hours, the cells were treated with 2.5 ng/ml human recombinant TGF-β1 (Peprotech, Hamburg, Germany) for 24 hours. Mesangial cells were then labeled with BrdU for 4 hours at 37°C, and the incorporation was determined with the assay kit following the manufacturer’s instructions. The ab-

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**Figure 9.** Increase in FGF21 mesangial expression only in diabetic Col8a1+/−/Col8a2+/− KO mice. (A) through (H) Immunohistology for FGF21-protein expression. (A) Negative control with normal rabbit serum. (B) Nondiabetic Col8a1+/−/Col8a2+/− wild-type mice. (C) Diabetic Col8a1+/−/Col8a2+/− wild-type animals. (D) Nondiabetic Col8a1+/−/Col8a2+/− heterozygous mice. (E) Diabetic Col8a1+/−/Col8a2+/− heterozygous mice. (F) Nondiabetic Col8a1+/−/Col8a2+/− KO mice. (G) Diabetic Col8a1+/−/Col8a2+/− KO mice. (H) Nondiabetic db/db mice. (I) diabetic db/db mice (at 4 weeks). Although all diabetic animals revealed an increase in tubular staining for FGF21, a weak increase in mesangial FGF21 expression was only found in diabetic Col8a1+/−/Col8a2+/− KO mice (G). Interestingly, diabetic db/db mice showed an increase in FGF21 expression in visceral cells of Bowman’s capsule. No specific staining was found with nonimmune serum (A). This staining is representative of five sections obtained from each kidney from five individual animals with qualitatively similar results (magnification, ×400).
sorbance of the samples was measured at 450 nm using a microplate reader (Tecan, Crailsheim, Germany).

For automated cell counting, 5 × 10^4 cells were seeded in 12-well culture plates and cultured for 24 hours. The cells were serum-starved overnight and exposed to agonists for a further 24 hours. The cells were detached from the culture dishes by trypsinization and counted.

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For reconstitution experiments, full-length Col8a1 cDNA was cloned into pCEP-Pu vector or pCEP-Pu control vector were transfected into mesangial cells (80 to 90% confluence) using Lipofectamine™ 2000 reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer’s protocol.

**Cell Cycle Analysis and Apoptosis**

Cell cycle analysis and apoptosis assay were performed using flow cytometry. After 24 hours of treatment with 2.5 ng/ml TGF-β1, the cells were harvested by trypsinization and washed twice with PBS. The cells were suspended in 200 μl of PBS with 2% FCS and incubated in staining solution (10 mM 1,4-piperazinediethanesulfonic acid, 2 mM MgCl2, 0.1 M NaCl, 0.1% Triton X-100, 25 μg/ml RNase [Roche Diagnostics], 10 μg/ml propidium iodide [Sigma]) at 4°C for 30 minutes in the dark. Then each sample was analyzed using a FACScalibur flow cytometer (Becton Dickinson, Franklin Lake, NJ), and the percentages of cells within the sub-G1, G1, S, and G2/M phases of the cell cycle were determined. The total number of cells analyzed for each sample was 10,000, and raw data were processed using CellQuestPro and WinMDI software.

Annexin V binding was determined with an apoptosis assay kit (Roche Diagnostics) following the manufacturer’s instructions. Fluorescence was measured on the FACScalibur flow cytometer.

**TGF-β1 Receptor Binding Assay**

5 × 10^4 Col8a1^+/+ or Col8a2^+/+ wild-type and Col8a1^-/- or Col8a2^-/- KO mesangial cells were plated per well in a 12-well plate and grown for 24 hours. After serum deprivation for 12 hours, the cells were washed once with ice-cold PBS and preincubated with binding buffer (serum-free RPMI with 5 mM MgCl2, 10 mM 6-amino-capric acid, 1 mM phenylmethylsulfonyl fluoride, 0.05% aprotinin, and 1 mg/ml bovine serum albumin) on a shaking platform at 4°C for 30 minutes. For saturation binding assays, increasing amounts (0.5 to 25 pM) of human recombinant I^-125I-TGF-β1 (specific activity, 3287 Ci/mmol) (PerkinElmer, Waltham, MA) were added in binding buffer. Nonspecific binding was determined in the presence of 0.5 to 25 nM human recombinant TGF-β1 (Peprotech, Hamburg, Germany). After incubation on a shaking platform at 4°C for 1 hour, the cells were gently washed three times with ice-cold binding buffer and lysed with 1 ml of 5 M NaOH with 0.5% Triton X-100. The amount of radioactivity was counted in a gamma scintillation counter. Control wells without radioactivity were trypsinized, and the cell number was determined with a CASY Cell Counter (Schräfe System, Reutlingen, Germany). Nonspecific binding was subtracted, and the data were analyzed with GraphPad Prism (GraphPad Software, La Jolla, CA). The results are presented as Scatchard plots of saturation binding data, and each point represents the mean of three individual experiments.

**RNA Extraction, cDNA Synthesis, and Reverse Transcription (RT) Real-time PCR**

For RT real-time PCR analysis, total RNA was isolated from treated cells or kidney homogenates using the RNeasy kit (Qiagen, Hilden, Germany), and 1 μg was reverse-transcribed using the Reverse Transcription System (Promega, Madison, WI) following the manufacturer’s instructions. The expression levels of genes were determined by quantitative PCR using LightCycler-FastStart DNA Master SYBR Green I (Roche Diagnostics) on a Real-Plex Mastercycler (Eppendorf, Hamburg, Germany). PCRs were carried out in a total volume of 20 μl containing 2 μl of cDNA and sense and antisense primers in a concentration of 0.25 μM each. To normalize for differences in the amount of cDNA in each sample, we performed amplification of 18S ribosomal RNA as an endogenous control. The amplification program included an initial denaturation step (10 minutes, 95°C), 40 cycles of amplification (denaturation for 10 seconds at 95°C), primer annealing (temperature see Table 1) for 15 seconds, extension for 10 seconds at 72°C, an additional heating step to melt potential primer dimers for 5 seconds at 95°C), and a melting curve program (denaturation for 10 seconds at 95°C, cooling and holding for 15 seconds at 60°C, and then heating at a speed of 0.1°C/s to 95°C). Finally, a cooling step of 2 minutes at 40°C was performed. Table 1 shows the sequences of the primer pairs. The transcript levels were normalized to the mean value of samples from the unstimulated controls.

**Microarray Experiments**

After stimulation of Col8a1^+/+ or Col8a2^+/+ wild-type and Col8a1^-/- or Col8a2^-/- KO mesangial cells with 2.5 ng/ml TGF-β1 for 24 hours, total RNA was extracted as described above. The GeneChip Mouse Genome 430 2.0 Array containing probes of over 39000 transcripts (Affymetrix, Santa Clara, CA) was used for analysis. Array hybridization was performed according to the supplier’s instructions using the GeneChip Expression 3’ Amplifikation One-Cycle Target Labelling and Control reagents from Affymetrix. The scanning of the microarray was done with the GeneChip Scanner 3000 (Affymetrix) at 1.56 μ resolution. Data analysis was performed with the MAS 5.0 (Microarray Analysis System, Affymetrix) software.

**Table 1. List of primer pairs and annealing temperature**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense Primers</th>
<th>Antisense Primers</th>
<th>T anneal</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA</td>
<td>5’-CAGCGGCGGGGAAGTACAGTGAAA-3’</td>
<td>5’-GATCTGGATATCGTGACCG-3’</td>
<td>58°C</td>
</tr>
<tr>
<td>Col8a1</td>
<td>5’-CGGAGTGGAGAAAGACCGAGGAGT-3’</td>
<td>5’-CCTGCCCAAGAAGCCCGAA-3’</td>
<td>62°C</td>
</tr>
<tr>
<td>FGF21</td>
<td>5’-ATGGAATGGATGAGATCTAGAGTTGG-3’</td>
<td>5’-CTTGGTGTGTCATCTGAGTGAGG-3’</td>
<td>62°C</td>
</tr>
<tr>
<td>NFkB</td>
<td>5’-GATCACCTGCAAGAGACCGAGAT-3’</td>
<td>5’-AGATGCTGTGAGGAGATCGTA-3’</td>
<td>58°C</td>
</tr>
<tr>
<td>p110α</td>
<td>5’-GACCCAGTAGGGCACCCTGGA-3’</td>
<td>5’-GCATCCTCCCAGCACATT-3’</td>
<td>56°C</td>
</tr>
<tr>
<td>Vinculin</td>
<td>5’-GCAGCAACGAGCTGACAGATA-3’</td>
<td>5’-TCTCTTTCTGGTGTGAGC-3’</td>
<td>62°C</td>
</tr>
</tbody>
</table>

ray Suite statistical algorithm; Affymetrix) probe level analysis using GeneChip Operating Software (GCOS 1.4), and the final data extraction was done with the DataMining Tool 3.1 (Affymetrix). Each experiment (stimulation of cells, isolation of RNA, chip hybridization, and analysis) was independently performed three times, and only differentially expressed transcripts in all sets of experiments were further analyzed. For the details of data analysis according to the MIAME Guidelines, see the online supplemental materials.

**FGF21 siRNA**

To inhibit the FGF21 mRNA expression, mesangial cells were transfected with siRNA targeting FGF21 (5′-TTGGAATAATAAGAATGCTCTGA-3′; NM_020013) or control siRNA (Qiagen) using HiPerfect Transfection Reagent (Qiagen). After incubation with the transfection complexes under normal growth conditions overnight, the medium was changed, and the cells were stimulated with 2.5 ng/ml TGF-β1 for 24 hours.

**Western Blot Analysis**

Cultured mesangial cells and kidney homogenate were lysed using Complete LysisM (Roche Diagnostics) and supplemented with the phosphate buffer-sodium orthovanadate (100 μM). Lysates were cleared by centrifugation, and 50 μg of protein/lane was separated by SDS-PAGE. Western blotting was performed as described previously. The primary antibodies used were as follows: anti-mouse collagen α1(VIII) (Abcam, Cambridge, UK), anti-phospho-Erk 1,2, anti-phospho-Akt, anti-phospho-Smad3 (Cell Signaling Technology, Danvers, MA), and anti-mouse FGF21 (R&D Systems, Minneapolis, MN). The blots were subsequently reprobed with anti-α-actinulin (Sigma), anti-Erk 2, anti-Akt (Santa Cruz Biotechnology), and anti-Smad3 (Cell Signaling Technology). Peroxidase-labeled secondary antibodies to rabbit IgG and mouse IgG (1:10,000) were purchased from KPL (Gaithersburg, MD). Western Lightning Chemiluminescence Reagent Plus (ECL; PerkinElmer LAS, Boston, MA) was used for detection of signals, and the images were captured using a Fuji LAS-3000 imaging system (Fujifilm Life Science, Düsseldorf, Germany) as described previously.

**Immunohistochemistry for FGF21 Expression**

Paraffin-embedded kidneys were sectioned at 4 μm. For the detection of FGF21, a rabbit polyclonal to FGF21 (Abcam, Cambridge, UK) with a concentration of 0.30 mg/ml was used in a 1:100 dilution (this antibody reacts with human and murine FGF21). Detection was performed with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) using an anti-phospho secondary antibody to rabbit IgG (1:10,000) were purchased from KPL (Gaithersburg, MD). Western Lightning Chemiluminescence Reagent Plus (ECL; PerkinElmer LAS, Boston, MA) was used for detection of signals, and the images were captured using a Fuji LAS-3000 imaging system (Fujifilm Life Science, Düsseldorf, Germany) as described previously.

**Statistical Analyses**

The results given in this article are presented as the means ± SEM. The results were analyzed with the Kruskal-Wallis test for multigroup comparison followed by the Mann-Whitney U-test. A P value of <0.05 was considered significant.

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**DISCLOSURES**

None.

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