MicroRNA-21 in Glomerular Injury


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ABSTRACT

TGF-β1 is a pleotropic growth factor that mediates glomerulosclerosis and podocyte apoptosis, hallmarks of glomerular diseases. The expression of microRNA-21 (miR-21) is regulated by TGF-β1, and miR-21 inhibits apoptosis in cancer cells. TGF-β1-transgenic mice exhibit accelerated podocyte loss and glomerulosclerosis. We determined that miR-21 expression increases rapidly in cultured murine podocytes after exposure to TGF-β1, and is higher in kidneys of TGF-β1-transgenic mice than wild-type mice. miR-21–deficient TGF-β1-transgenic mice showed increased proteinuria and glomerular extracellular matrix deposition and fewer podocytes per glomerular tuft compared with miR-21 wild-type TGF-β1-transgenic littermates. Similarly, miR-21 expression was increased in streptozotocin-induced diabetic mice, and loss of miR-21 in these mice was associated with increased albuminuria, podocyte depletion, and mesangial expansion. In cultured podocytes, inhibition of miR-21 was accompanied by increases in the rate of cell death, TGF-β1/Smad3-signaling activity, and expression of known proapoptotic miR-21 target genes p53, Pdcd4, Smad7, Tgfbr2, and Timp3. In American-Indian patients with diabetic nephropathy (n=48), albumin-to-creatinine ratio was positively associated with miR-21 expression in glomerular fractions (r=0.6; P<0.001) but not tubulointerstitial fractions (P=0.80). These findings suggest that miR-21 ameliorates TGF-β1 and hyperglycemia-induced glomerular injury through repression of proapoptotic signals, thereby inhibiting podocyte loss. This finding is in contrast to observations in murine models of tubulointerstitial kidney injury but consistent with findings in cancer models. The aggravation of glomerular disease in miR-21–deficient mice and the positive association with albumin-to-creatinine ratio in patients with diabetic nephropathy support miR-21 as a feedback inhibitor of TGF-β signaling and functions.


TGF-β family growth factors activate large networks of signaling cascades. Their signaling activity is tightly regulated by feedback and feed-forward loops on multiple levels.1,2 MicroRNAs (miRs), small noncoding RNAs that repress gene expression post-transcriptionally, are integral components of feedback mechanisms, thereby modulating the activity of signaling pathways, including stress responses in many physiologic and pathologic processes.3 In glomerular injury, TGF-β1 regulates podocyte dysfunction and apoptosis, mesangial cell activation, and extracellular matrix (ECM) deposition.1 miR-192 and miR-200 are induced by TGF-β1 and regulate TGF-β1 signaling activity on kidney injury.4–7 miR-21 has been broadly studied in cancer biology because of its antiapoptotic effects8–10 and because its expression is regulated by TGF-β1.

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Several miR-21 target genes induce podocyte apoptosis, including Smad7 and p53. However, in models of tubulointerstitial injury, miR-21 mediates damage to the kidney through repression of several targets, including Smad7, thereby increasing TGF-β signaling activity.

Podocyte depletion predicts progressive loss of renal function in patients with diabetic nephropathy (DN). Mechanisms of podocyte apoptosis have been studied in TGF-β1–transgenic (Tgfb1-TG) mice, which are characterized by increased levels of circulating TGF-β1 and progressive glomerulosclerosis. Furthermore, mice with hyperglycemia after streptozotocin (STZ)-induced β-cell loss exhibit podocyte apoptosis. These findings prompted us to hypothesize that miR-21 antagonizes podocyte apoptosis and glomerular injury. We used miR-21–deficient Tgfb1-TG as well as miR-21–deficient mice with STZ-induced diabetes to test our hypothesis. In addition, we examined the relationship of glomerular miR-21 expression in human DN with proteinuria, an indicator of podocyte damage.

RESULTS

TGF-β1 Induces miR-21 Expression through Smad Signaling

Smads regulate the processing of miR-21 from its precursor to the mature form. We tested whether miR-21 expression is regulated by TGF-β1/Smad signaling in podocytes. Mature miR-21 levels increased after exposure to TGF-β1 in wild-type (WT) podocytes but not in Smad2/3-deficient podocytes (Figure 1A). Similarly, the expression of the miR-21 precursor was strongly induced by TGF-β1 in WT podocytes but absent in Smad2/3-deficient podocytes (Figure 1B). These findings suggest that TGF-β1 regulates miR-21 expression through Smad signaling in podocytes.

To examine whether miR-21 expression is regulated by TGF-β1 in vivo, we examined mature miR-21 expression levels in kidneys of Tgfb1-TG mice. We found the expression of miR-21 to be significantly increased in kidneys of Tgfb1-TG compared with WT mice and detected higher levels of miR-21 in kidneys of TGF-β1–transgenic (Tgfb1-TG) mice, which are characterized by increased levels of circulating TGF-β1 and progressive glomerulosclerosis. These findings are consistent with previous reports of regulation of miR-21.

Lack of miR-21 Is Associated with Increased Apoptosis and ECM Deposition in Tgfb1-TG Mice

Loss of podocytes causes glomerulosclerosis. Glomerular disease in Tgfb1-TG mice is characterized by podocyte apoptosis and increased ECM deposition. Examination of cleaved caspase-3 expression, a marker of apoptosis, detected significantly more cleaved caspase-3–positive cells in glomeruli of Tgfb1-TG mice compared with WT mice (Figure 4C). To examine whether loss of miR-21 is associated with loss of podocytes, we determined the number of podocytes per glomerular tuft. Unchallenged miR-21-KO mice did not exhibit differences in WT1 mRNA or protein expression in isolated glomeruli by quantitative RT-PCR (qRT-PCR) and Western blotting, respectively, or number of WT1-positive cells per glomerular tuft compared with miR-21-WT mice (data not shown). Furthermore, TG/miR-21-KO and TG/miR-21-WT littermates have equal number of WT1-positive cells per glomerular tuft at 2 weeks of age (Figure 4A). At 4 weeks of age, when glomerulosclerosis is prominent, the number of 4’,6-diamidino-2-phenylindole– and

TRANSFECTED WITH CONTROL OLIGONUCLEOTIDES QUANTIFIED BY ANNEXIN V AND PROPIDIOUM IODIDE STAINING AS WELL AS MITOCHONDRIAL MEMBRANE POTENTIAL MEASUREMENTS (Figure 2, A and B). In addition, transfection of podocyte with miR-21 mimic decreased TGF-β1–induced apoptosis compared with control oligonucleotides (Figure 2B). The findings imply that miR-21 inhibits TGF-β1–induced podocyte apoptosis.

Tgfb1-TG/miR-21-Knockout Mice Develop Increased Proteinuria and ECM Deposition

To investigate the function of miR-21 in TGF-β–mediated glomerular injury, we crossed Tgfb1-TG mice with previously described miR-21-knockout (KO) mice to generate Tgfb1-TG/miR-21-WT (TG/miR-21-WT) and miR-21-KO (TG/miR-21-KO) littermates. miR-21–deficient mice do not have proteinuria or structural abnormalities in the kidney (Supplemental Figure 1). Kidney function and structure were examined in TG/miR-21-WT and TG/miR-21-KO littermates at 4 weeks of age. Qualitative and quantitative analyses of urine protein showed that TG/miR-21-WT mice had a normal urine protein-to-creatinine ratio, whereas over 50% of TG/miR-21-KO mice developed increased proteinuria (Figure 3, A and B). Total TGF-β1 protein concentration in plasma (Supplemental Figure 2, left panel) and glomerular Tgfb1 mRNA levels (Supplemental Figure 2, right panel) were not different between TG/miR-21-WT and TG/miR-21-KO mice. TG/miR-21-KO mice exhibited increased periodic acid–Schiff and picrosirius red staining in glomeruli but not in the tubulointerstitial area (Figure 3, C and D). Collagen III protein and collagen 1a1 (Col1a1), Col4a1, and Col6a1 mRNA expression were also increased in glomeruli of TG/miR-21-KO mice (Figure 3, C and E). The pattern of ECM deposition in glomeruli of TG/miR-21-KO was nodular (Figure 3C). These findings are consistent with accelerated TGF-β1–induced glomerulosclerosis in the absence of miR-21.
Loss of miR-21 Is Associated with Increased Proteinuria and Mesangial Expansion as Well as Decreased Podocyte Density in Diabetic miR-21-KO Mice

To extend our findings of increased proteinuria, podocyte loss, and glomerular ECM deposition in TG/miR-21-KO mice, we investigated the effect of loss of miR-21 in mice with hyperglycemia after STZ injection, a murine model of DN that exhibits increased podocyte apoptosis and mesangial expansion\(^20\) and significantly increased miR-21 expression in the kidney (Supplemental Figure 3). Blood glucose levels were significantly elevated after 2 weeks reaching 600 mg/dl at 20 weeks after STZ exposure independent of genotype (Figure 5A). Elevated albuminuria developed appropriately 4 weeks after STZ exposure independent of genotype (Figure 5B). Eight weeks after STZ exposure, miR-21-KO had significantly higher albuminuria than miR-21-WT littermates with hyperglycemia that persisted, with miR-21-heterozygous exhibiting levels of albuminuria between KO and WT (Figure 5B). Furthermore, diabetic miR-21-KO mice had significantly higher serum creatinine levels compared with diabetic WT littermates (Figure 5C), suggesting that loss of miR-21 was associated with decreased kidney function under diabetic stress conditions.

Histologic analysis of glomeruli revealed that diabetic miR-21-KO mice showed significantly increased mesangial index 20 weeks after STZ using quantitative image analysis of periodic acid–Schiff staining compared with diabetic miR-21-WT mice (Figure 5D). At the same time, determination of podocyte number indicated decreased podocyte density in diabetic miR-21-KO compared with diabetic miR-21-WT littermates (Figure 5E), suggesting that loss of miR-21 was associated with decreased kidney function under diabetic stress conditions.

Glomerular miR-21 Expression Is Positively Associated with Albumin-to-Creatinine Ratio in Patients with DN

To determine the relevance of these findings for patients with DN, we examined miR-21 expression in microdissected glomeruli and tubulointerstitial fractions of 48 kidney biopsies of American-Indian patients with DN.\(^27\) The general characteristics of the cohort are listed in Supplemental Table 1. Patients exhibited a broad range of albumin-to-creatinine ratios (ACRs), whereas the mean

WT1-positive nuclei per glomerular tuft was decreased in TG/miR-21-KO compared with the WT mice (Figure 4B). Thus, the decreased podocyte number per glomerular tuft at 4 weeks of age is caused by loss of podocytes rather than developmental podocyte deficiency. In addition, Col4a1 mRNA as well as fibronectin mRNA and protein expression were increased in glomeruli but not in tubulointerstitial area of TG/miR-21-KO mice (Figure 3E and F), suggesting accelerated fibrotic response in glomeruli of TG/miR-21-KO mice.

Figure 1. miR-21 expression levels are induced by TGF-β1 in podocytes and Tgfb1-TG mice. (A) Levels of mature miR-21 and miR-21 precursor increased in WT mouse podocytes up to 2.5-fold after exposure to TGF-β1, which was completely abolished in Smad2/3-deficient podocytes (n=3 independent experiments; measured by qRT-PCR). *P<0.05 compared with WT at the same time point. (B) miR-21 precursor levels increased 4-fold in the same cell samples (measured by qRT-PCR). *P<0.05 compared with WT at the same time point. (C) In kidneys of Tgfb1-TG mice, miR-21 levels were significantly higher in kidneys of Tgfb1-TG mice with severe phenotype (TG/severe; n=8) and mild phenotype (TG/mild; n=6) compared with WT (n=5) inferred from histology score.\(^23\)**P<0.001 compared with WT. (D) Similarly, TGF-β1 mRNA levels were significantly higher in TG/severe (n=3) compared with WT (n=3) in kidneys of Tgfb1-TG mice. *P<0.05.
GFR measured by iothalamate clearance was above 90 ml/min per 1.73 m² at the time of biopsy in all patients. miR-21 expression in glomeruli measured by RNA sequencing showed highly significant correlation with ACR ($r=0.6$, $P<0.001$). Whereas miR-21 levels of patients with normoalbuminuria (ACR $<30$ mg/mg) or microalbuminuria ($30 \leq$ ACR $<300$ mg/mg) were not different, patients with macroalbuminuria (ACR $\geq 300$ mg/mg) exhibited significantly increased miR-21 levels compared with patients with normoalbuminuria (Figure 5F). In contrast, tubulointerstitial miR-21 levels were not associated with albuminuria ($r=0.05$, $P=0.80$), miR-21 expression did not show a significant correlation with GFR in either tissue compartment. Quantification of glomerular miR-21 expression using qRT-PCR showed high correlation with read number obtained by RNA sequencing ($r=0.6$, $P<0.001$) and confirmed the positive association with ACR ($r=0.7$) and significantly higher levels in patients with macroalbuminuria versus nonalbuminuria ($P=0.01$).

**Inhibition or Loss of miR-21 Results in Increased Expression of Multiple Proapoptotic Pathways**

To identify the underlying mechanism for inhibition of podocyte apoptosis by miR-21, we searched the published literature for genes known to promote apoptosis that are regulated by miR-21. TGF-β receptor 2 (Tgfbr2) and Smad7 are members of the TGF-β–signaling cascade mediating TGF-β–induced apoptosis, and they are repressed by miR-21 through binding to their respective 3′ untranslated regions (3′UTRs) (Figure 6A) as is programmed cell death 4 (Pdcd4) (Figure 7). p53 (Trp53) is indirectly suppressed by miR-21. TGF-β1 mediates podocyte apoptosis through Smad3, and murine podocytes transfected with miR-21 inhibitor showed increased Smad3 phosphorylation 4 and 24 hours after exposure to TGF-β1 (Figure 6B). Meanwhile, Pdcd4 protein expression was decreased by TGF-β1, and inhibition of miR-21 further increased Pdcd4 protein expression (Figure 6C). Although inhibition of miR-21 in unchallenged podocytes had no significant effect on Smad7, which mediates TGF-β–induced podocyte apoptosis, inhibition of miR-21 led to additional increase of Smad7 mRNA expression 24 hours after TGF-β1 (Figure 6D). In vivo, expression of Tgfbr2, Tgfb1, Smad7, and Trp53 mRNAs was increased in glomeruli of TG/miR-21-KO compared with TG/miR-21-WT littermates (Figure 6E). These results suggest that the antiapoptotic capacity of miR-21 is mediated by inhibition of multiple proapoptotic signals.

**Inhibition or Loss of miR-21 Alters Expression of Multiple Regulators of ECM Deposition**

ECM deposition is enhanced by increased collagen production and/or decreased breakdown of extracellular collagen by metalloproteinases. Tissue inhibitors of metalloproteinase (TIMPs) diminish the degradation capacity of extracellular metalloproteinases. In glomeruli of TG/miR-21-KO mice, Col4a1 as well as Timp3 mRNA were increased (Figures 3E and 6E). Both genes are predicted targets of miR-21 (Figure 6A), and Timp3 has been experimentally confirmed as an miR-21 target in glioma cells. Furthermore, Timp3 mRNA levels were increased in cultured murine podocytes transfected with...
miR-21-inhibitor±TGF-β1 (Figure 6D). Other predicted targets of miR-21 implicated in TGF-β signaling, such as Ras homolog gene family member B, remained unchanged (Figure 6E), and no difference in TGF-β1 mRNA expression was detected in kidney cortex between diabetic miR-21 KO and WT mice (data not shown).

Figure 3. Loss of miR-21 is associated with accelerated glomerular damage in Tgfb1-TG mice. (A) In TG/miR-21-KO mice, urine protein-to-creatinine ratio was strongly increased in 8 of 19 TG/miR-21-KO but 0 TG/miR-21-WT mice (n=12) at 4 weeks of age, consistent with previous reports showing heterogeneity in this mouse model.11,18 (B) The urine of TG/miR-21-KO mice with severe proteinuria showed strongly increased amounts of albumin (Coomassie blue stain; normalized by loading 2 µg creatinine equivalents of urine for each sample). (C) Histologic examination of kidney tissue by periodic acid-Schiff (PAS) staining showed increased deposition of PAS-positive material and decreased cellularity in glomeruli of 4-week-old TG/miR-21-KO mice compared with TG/miR-21-WT littermates. Picrosirius red staining showed increased signal intensity, with a diffuse and nodular pattern in glomeruli of TG/miR-21-KO compared with TG/miR-21-WT littermates. Consistent with increased ECM deposition detected by picrosirius red staining, collagen III deposition was increased in glomeruli of TG/miR-21-KO detected by immunohistochemistry staining. Interestingly, the tubulointerstitial area appeared normal by PAS, picrosirius red, and collagen III staining independent of genotype. (D) Quantitative image analysis of picrosirius red staining intensity showed significantly higher staining intensity in glomeruli of TG/miR-21-KO (n=7) versus TG/miR-21-WT littermates (n=9), whereas no significant difference was noted in the tubulointerstitial area (P=0.08). *P<0.05. (E) Col1a1, Col4a1, Col6a1, and Fibronectin mRNA expressions determined by qRT-PCR were strongly increased in isolated glomeruli of TG/miR-21 KO mice (n=3) compared with WT littermates (n=4). *P<0.05. (F) Quantitative image analysis of fibronectin immunohistochemistry staining showed significantly higher staining intensity in glomeruli of TG/miR-21-KO (n=3) versus TG/miR-21-WT littermates (n=3), whereas no significant difference was noted in the tubulointerstitial area (P=0.40). *P<0.05.
Mining existing mRNA expression data from the same samples used for miR-21 profiling determined that **TGFBR2** and **TIMP3** expressions were inversely correlated with miR-21 levels in glomeruli of patients with DN (Figure 7).34

**DISCUSSION**

In this study, we determined that lack of miR-21 is associated with increased podocyte loss and accelerated glomerular disease in two different mouse models, that miR-21 inhibits apoptosis in cultured podocytes, and that miR-21 deficiency is associated with increased expression of proapoptotic known targets of miR-21. Furthermore, albuminuria was positively associated with miR-21 expression in glomeruli but not in the tubulointerstitial compartment in patients with DN. These findings suggest a complex role of miR-21 in kidney disease.

miR-21 is abundantly expressed and elevated in most human cancers.35 Because miR-21 inhibits apoptosis and promotes metastasis, it is considered an oncomiR and therefore, explored as
miR-21 has been implicated in both promotion and protection from tubulointerstitial injury. In mouse models of tubulointerstitial injury, miR-21 promotes fibrosis through regulation of multiple signaling pathways. Our finding that miR-21 deficiency results in increased podocyte loss is consistent with the prosurvival function of miR-21 observed in various cancer model systems. Podocytopenia is a robust predictor of disease progression in human DN and detected in various animal models of glomerular injury, including DN and Tgfβ1-TG mice. Loss of podocytes is sufficient to cause glomerulosclerosis in mice and podocyte apoptosis is induced by TGF-β. Therefore, the positive correlation of glomerular miR-21 levels with albuminuria in patients with DN suggests that increased miR-21 serves as a protective mechanism in glomerular injury.

Discrepancy of miR-21 function in different injury models of the same organ has been reported in the heart, where miR-21 expression is increased as well; however, its function remains controversial, because it has been implicated in promotion and protection from tubulointerstitial injury as well as glomerular injury. In murine models of tubulointerstitial injury, miR-21 promotes fibrosis through regulation of multiple signaling pathways.

Figure 5. miR-21–deficient diabetic mice exhibit increased proteinuria, decreased kidney function, and decreased podocyte number. (A) miR-21 mutant mice developed hyperglycemia within 2 weeks of STZ treatment, and blood glucose level, were not different among different genotypes (n=7 for each genotype). (B) All STZ-treated miR-21 mutant mice developed albuminuria 4 weeks after STZ treatment, but miR-21-KO mice develop significantly increased albuminuria compared with miR-21-heterozygous (HET) and miR-21-WT mice after 8 weeks of STZ treatment. Diabetic miR-21-HET mice exhibit significantly increased albuminuria 16 weeks after STZ treatment versus WT mice (n=5–8 in each genotype). The miR-21 expression is reduced by approximately 50% in kidneys of unchallenged miR-21–HET mice (data not shown). *P<0.05 compared with miR-21-WT mice. (C) Diabetic miR-21-KO mice had higher serum creatinine level versus WT mice at 20 weeks after STZ treatment (n=7 in each genotype). *P<0.05. (D) Increased deposition of periodic acid–Schiff (PAS)–positive material was detected in glomeruli of diabetic miR-21-KO mice compared with treated miR-21-WT mice 20 weeks after STZ treatment (PAS staining). The calculated mesangial index showed significant mesangial expansion in glomeruli of diabetic miR-21-KO mice compared with diabetic miR-21-WT littermates (n=5 for each genotype). *P<0.05. (E) The number of WT1-positive nuclei (podocytes) per glomerular tuft was significantly decreased in diabetic miR-21-KO mice compared with diabetic miR-21-WT littermates 20 weeks after STZ treatment (n=5; normalized to diabetic miR-21-WT mice and presented as a percentage). *P<0.05. (F) In microdissected glomeruli of American-Indian patients with DN, miR-21 levels (log2-transformed sequence reads by RNA sequencing) were significantly higher in patients with macroalbuminuria (n=7) compared with patients with normoalbuminuria (n=19). The levels of miR-21 in patients with microalbuminuria (n=22) were between the levels in patients with normoalbuminuria and macroalbuminuria. *P<0.05.
Figure 6. miR-21 represses the expression of multiple transcripts that regulate apoptosis. (A) Predicted target sites of miR-21 in 3’UTRs of Tgfbr2, Tgfb1, Smad7, Pdcd4, Timp3, and Col4a1 (www.targetscan.org). (B) Smad3 phosphorylation was increased in cultured podocytes transfected with miR-21 inhibitor compared with podocytes transfected with control oligonucleotide and 24 hours after exposure to TGF-β1 (5 ng/ml; n=3; podocytes were transfected with oligonucleotides 20 hours before TGF-β1 treatment). Protein loading control: Glyceraldehyde 3-phosphate dehydrogenase (Gapdh). *P<0.05. (C) PDCD4 protein level was decreased in podocytes 24 hours after TGF-β1 treatment compared with buffer (n=4). PDCD4 protein expression was increased in podocytes transfected with control oligonucleotide (n=3). Exposure of podocytes transfected with miR-21 inhibitor to TGF-β1 for 24 hours resulted in increased PDCD4 protein levels compared with podocytes transfected with control oligonucleotide and treated with TGF-β1 (n=4). *P<0.05. (D) Smad7 and Timp3 mRNA were increased in miR-21 inhibitor-transfected podocytes without TGF-β1 treatment (n=3). *P<0.05. (E) TG/miR-21-WT and TG/miR-21-KO mice (n=3) exhibit higher glomerular mRNA expression of Tgfbr2, Tgfb1, Smad7, Timp3, and Col4a1 assayed by qRT-PCR. The level of Ras homolog gene family member B (RhoB), also a predicted target of miR-21, did not differ between TG/miR-21-WT and TG/miR-21-KO mice (P=0.90). *P<0.05.
contributes to myocardial disease but ameliorates heart failure and is not essential for stress-dependent cardiac remodeling. These observations suggest cell type- and context-specific functions of miR-21. Recent data indicate that the level of association of an endogenous miR with the RNA interference-induced silencing complex is regulated by the available RNA targetome and predicts miR function. In addition, accessibility of 3’UTRs and the presence or absence of other miRs can contribute to cell type-specific functions of miRs. Furthermore, identification of miR-21 functions is affected by mode of inhibition or genetic deletion.

Multiple lines of evidence suggest that miR-21 regulates TGF-β signaling. We had previously shown that inhibition of miR-21 stimulates hematopoiesis and improves disease manifestations through inhibition of TGF-β signaling in a murine model of myelodysplastic syndrome. Here, we show that miR-21 is induced by TGF-β/Smad signaling and inhibits Smad3 phosphorylation, suggesting a negative feedback loop in which miR-21 contributes to termination of TGF-β/Smad signaling. Our finding that miR-21 expression is increased in patients with DN and macroalbuminuria suggests persistent activation of TGF-β/Smad signaling and/or regulation of miR-21 expression by other signaling mechanisms, including the Jak/Stat pathway.

To elucidate the underlying mechanisms of miR-21-regulated podocyte apoptosis, we focused on genes that have been previously shown to harbor an miR-21 binding site in their 3’UTRs and regulate podocyte apoptosis. Tgfbr2 regulates Smad activation by TGF-b1 and is repressed by miR-21 through binding to its 3’UTR. TGF-b1 induces podocyte apoptosis by Smad signaling; thus, increased expression of Tgfbr2 after loss of miR-21 likely results in increased Smad3 phosphorylation and promotes podocyte loss. Smad7 is an inhibitor of Smad2/3 phosphorylation, induces podocyte apoptosis, and inhibits fibrosis in tubulointerstitial injury. Recently, Smad7 mRNA was found to be a sequence-dependent target of miR-21 in renal and lung epithelial cells and human embryonic kidney-293 cells (Supplemental Figure 5) and be present in RNA interference-induced silencing complex in podocytes. Therefore, repression of Smad7 by miR-21 may explain the antiapoptotic function in podocytes as well as the antifibrotic capabilities of miR-21 in tubulointerstitial kidney injury. P53 has been implicated as a mediator of TGF-β-induced podocyte apoptosis, and our finding of increased p53 expression in the miR-21–deficient context is consistent with observations in cancer models showing repression of multiple Tp53-binding proteins by miR-21, leading to decreased p53 protein levels. In addition, Pdcdd mediates TGF-β–induced apoptosis in human hepatocellular carcinoma cells and is a sequence-dependent target of miR-21. In addition, Tgibi promotes apoptosis in cancer cells and is a predicted target of miR-21. The roles of Pdcdd and Tgibi in podocyte apoptosis have not been determined. The findings that plasma and intrarenal TGF-β1 levels were not different between genotypes (Supplemental Figure 2) are consistent with repression of TGF-β target genes by miR-21 rather than increased TGF-β1 expression per se.

In addition, we detected increased ECM deposition in glomeruli of miR-21–deficient Tgfb1-TG mice. It has been proposed that miR-21 regulates genes related to ECM, particularly Col4a1 and Timp3. Loss of Timp3 exacerbates DN. In glomeruli and other tissues, Timp3 and miR-21 expressions are inversely correlated, and Timp3 has been confirmed that miR-21 directly targets the Timp3 3’UTR. Col1a1 was found to be predictive of progressive disease in Tgfb1-TG mice, but its function in glomerular disease is not known. These results suggest that miR-21 also regulates ECM deposition and further support the hypothesis that repression of multiple transcripts by miR-21 leads to the observed phenotype.

The complexity of miR-21 function may also be caused by the complex function of TGF-β. TGF-β activates multiple signaling cascades, exhibits cell type- and context-specific functions, and is integrated in a complex regulatory network with feed-forward and feedback loops. Furthermore, maintaining a tight homeostasis of TGF-β signaling is essential for proper function of cells and organ system underscored by the fact that overly abundant TGF-β1 leads to organ fibrosis, whereas TGF-β1 deficiency leads to excessive inflammatory responses. Multiple miRs have been found to be involved in feedback regulation of TGF-β signaling in DN similar to miR-21, including miR-192 and miR-200a, and additional miRs are likely to contribute.

Few studies have explored miR expression in patients with DN. Krupa et al. have identified miRs that differentially expressed in kidney tissues of 22 patients with progressive and nonprogressive DN and detected miR-21 to be increased in patients with progressive disease. These data likely reflect tubulointerstitial changes, because pooled formalin-fixed material from whole-kidney biopsies was used, which constitutes mainly tubulointerstitium. Furthermore, patients had significant differences.
lower eGFR, and miR-16 was used as the reference miR. Additionally, urinary miR-21 levels were higher in adolescent Hong Kong Chinese with albuminuria than without albuminuria. Our findings suggest a functional role of miR-21 in glomerular injury and podocyte loss but require additional studies in patients and additional exploration of compartment-specific functions of miR-21 and its potential role as a marker for progressive glomerular disease.

In summary, we can show that glomerular miR-21 expression is positively associated with ACR in patients with DN and that loss of miR-21 is associated with accelerated glomerular damage and podocyte apoptosis in a murine model of DN and Tgfb1-TG mice. These findings suggest a protective role of miR-21 in glomerular injury and further underscore the context-dependent functions of miR-21.

CONCISE METHODS

Mice Model and Kidney Function Examination
miR-21-KO mice were generated from disruption of the miR-21 sequence as described. Procedures were in accordance with the policies of the University of Michigan Institutional Animal Care and Use Committee. More detail is in Supplemental Material.

Cell Culture and miR Transfection
Conditionally immortalized murine podocytes were cultured as described and considered differentiated 14 days after transfer to 37°C. Smad2/3 double null podocytes were generated as described. More detail is in Supplemental Material.

Human Subjects and miR Expression Profiling
Kidney biopsy samples were collected from southwestern American Indians enrolled in a randomized, placebo-controlled clinical trial to test the renoprotective efficacy of Losartan in early type 2 diabetic kidney disease (ClinicalTrials.gov no. NCT00340678). Glomeruli and tubulointerstitial fractions were isolated from kidney biopsies by microdissection, and RNA was isolated using silica-membrane columns. The small RNA fraction was used for RNA sequencing using barcoded deep sequencing of a cDNA library prepared from multiplexed RNA as described. In addition, glomerular miR expression was validated using the TaqMan qRT-PCR array from the same RNA pool as described. More information is in Supplemental Material.

qRT-PCR
For expression analysis of miR-21 and mRNAs, TaqMan primers (Applied Biosystems) and the 7900HT Fast Real-Time PCR System (Applied Biosystems) were used according to the manufacturer’s protocols as described. For quantification of pri-miR-21, primers previously described were used. More detail is in Supplemental Material.

Apoptosis Assay and Immunoblot Assay
Cell apoptosis was quantified in vivo by detection of cleaved caspase-3 by immunohistochemistry (antibody: Asp175; Cell Signaling Technology). In vitro apoptosis and cell survival were determined by flow cytometry and mitochondrial membrane potential, respectively, as described. More detail is in Supplemental Material.

Statistical Analyses
$t$ Tests were used to compare picrosirius red intensity, cell numbers, mRNA, and protein levels between miR-21-WT and miR-21-KO mice. More information is in Supplemental Material.

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DISCLOSURES
None.

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