MicroRNA-21 Orchestrates High Glucose-induced Signals to TOR Complex 1, Resulting in Renal Cell Pathology in Diabetes

Nirmalya Dey†, Falguni Das‡, Meenalakshmi M. Mariappan†, Chandi Charan Mandal§, Nandini Ghosh-Choudhury∗, Balakuntalam S. Kasinath†‡, and Goutam Ghosh Choudhury∗†¶

From *Veterans Affairs Research and ¶Geriatric Research, Education, and Clinical Center, South Texas Veterans Health Care System, San Antonio, Texas and Departments of †Medicine and §Pathology, University of Texas Health Science Center, San Antonio, Texas 78229

Hyperglycemia induces a wide array of signaling pathways in the kidney that lead to hypertrophy and matrix expansion, eventually culminating in progressive kidney failure. High glucose-induced reduction of the tumor suppressor protein phosphatase and tensin homolog deleted in chromosome 10 (PTEN) contributes to renal cell hypertrophy and matrix expansion. We identified microRNA-21 (miR-21) as the molecular link between high glucose and PTEN suppression. Renal cortices from OVE26 type 1 diabetic mice showed significantly elevated levels of miR-21 associated with reduced PTEN and increased fibronectin content. In renal mesangial cells, high glucose increased the expression of miR-21, which targeted the 3′-UTR of PTEN mRNA to inhibit PTEN protein expression. Overexpression of miR-21 mimicked the action of high glucose, which included a reduction in PTEN expression and a concomitant increase in Akt phosphorylation. In contrast, expression of miR-21 Sponge, to inhibit endogenous miR-21, prevented down-regulation of PTEN and phosphorylation of Akt induced by high glucose. Interestingly, high glucose-stimulated miR-21 inactivated PRAS40, a negative regulator of TORC1. Finally, miR-21 enhanced high glucose-induced TORC1 activity, resulting in renal cell hypertrophy and fibronectin expression. Thus, our results identify a previously unrecognized function of miR-21 that is the reciprocal regulation of PTEN levels and Akt/TORC1 activity that mediate critical pathologic features of diabetic kidney disease.

The hallmarks of diabetic nephropathy consist of altered glomerular hemodynamics and certain structural alterations. The latter include renal hypertrophy, thickening of basement membranes, podocyte dysfunction, and progressive glomerulosclerosis that results from accumulation of extracellular matrix components collagen, laminin, and fibronectin. The elevated glomerular filtration rate associated with early stages of diabetes is followed by microalbuminuria, frank proteinuria, and finally fibrosis (1–3). Hyperglycemia-induced production of cytokines and hormones such as angiotensin II and transforming growth factor-β (TGFβ) contribute to glomerular as well as tubular hypertrophy and accumulation of matrix proteins (4, 5).

High glucose activates multiple signal transduction pathways including the phosphatidylinositol (PI)4 3-kinase/Akt cascade. We and others have shown that PI 3-kinase and Akt kinase significantly contribute to hypertrophy and increased matrix protein expression in kidney tissues in models of diabetic nephropathy and in cultured mesangial and proximal tubular epithelial cells (6–12). In fact, Akt1-null mice display reduced hyperhexosemia-induced mesangial hypertrophy and fibronectin expression (13). PI 3,4,5-trisphosphate, produced by the PI 3-kinase, activates Akt kinase (14, 15). Phosphatase and tensin homolog deleted in chromosome 10 (PTEN) dephosphorylates PI 3,4,5-trisphosphate, resulting in inactivation of Akt kinase (15). Therefore, the levels of PTEN may regulate the activation status of Akt and hence the downstream signaling pathways that affect hypertrophy and accumulation of matrix proteins. In fact, we have shown that the levels of PTEN protein are significantly reduced in a rat model of diabetic nephropathy and in cultured mesangial cells incubated with high glucose (9). This observation has recently been confirmed by other investigators (16). The mechanism by which high glucose down-regulates PTEN is not fully determined.

Post-transcriptional regulation of gene expression by microRNAs (miRNAs) has become increasingly important for diverse biologic processes involved in the pathogenesis of many diseases (17). The primary microRNA transcripts are processed by the Drosha microprocessor complex in the nucleus followed by further processing by Dicer in the cytosol (18). To suppress gene expression, the miRNAs are incorporated into the RNA-dependent silencing complex, which contains Argonaute 2 and

4 The abbreviations used are: PI, phosphatidylinositol; miR-21, microRNA-21; PTEN, phosphatase and tensin homolog deleted in chromosome 10; miRNA, microRNA; PTE, proximal tubular epithelial; qRT-PCR, quantitative RT-PCR; mTOR, mammalian target of rapamycin; TORC1, TOR complex 1.
Dicer along with other proteins. Two major mechanisms of negative regulation of gene expression by miRNAs have been reported. Perfect Watson-Crick base pairing results in deadenylation and subsequent cleavage of target mRNA. mRNA translational repression is predominantly favored because many microRNA recognition elements present in the mRNA 3’-UTR as well as in the coding sequence pose imperfect complementarity (18, 19).

Mice null for the enzyme Dicer, which is necessary for premiR maturation, display embryonic lethality, indicating the importance of miRNAs in development (20). Ablation of Dicer in the podocytes of mouse kidney resulted in glomerular foot process effacement and basement membrane injury, resulting in proteinuria (21–23). The pathology in the Dicer-null mouse was ascribed to changes in kidney-specific miR-30 family members. Changes in expression of multiple miRNAs have been reported in several kidney-related pathologies (16, 24–31). For example, high glucose has been shown to suppress the expression of miR-93 in podocytes, in renal microvascular endothelial cells, and in diabetic glomeruli (32). In contrast, both miR-216a and miR-217 are increased in type 2 diabetic mouse glomeruli and in cultured mesangial cells in the presence of high glucose and TGFβ (16, 33). In a mouse model of acute renal injury and in vitro cisplatin-treated proximal tubular epithelial cells, augmented expression of miR-34a is observed (28). miR-192 is highly expressed in the renal tissues of patients with hypertensive nephrosclerosis and IgA nephropathy (34, 35). Natarajan and co-workers have shown increased abundance of miR-192 in the renal glomeruli of type 1 and type 2 diabetic mice (26). Using mesangial cells in culture, these authors identified the Zeb2 transcriptional repressor as the target of miR-192, which increased the expression of type I collagen α2 that contributes to glomerulosclerosis (26). In the present report, we show significantly elevated expression of miR-21 in the renal cortex of the OVE26 type 1 diabetic mouse concomitant with reduced expression of PTEN and an increase in fibronectin abundance. We demonstrate in cultured glomerular mesangial and proximal tubular epithelial (PTE) cells that high concentrations of glucose increase the expression of miR-21 that down-regulates PTEN. Our results show that miR-21 promotes activation of TORC1 necessary for cellular hypertrophy. Finally, we demonstrate that high glucose-induced miR-21 enhances the expression of fibronectin in mesangial and PTE cells. These results provide a mechanism involving miR-21 for some of the pathological changes found in diabetic nephropathy.

**EXPERIMENTAL PROCEDURES**

**Materials**—D-Glucose, D-mannitol, anti-β-actin antibody, anti-fibronectin antibody, phenylmethylsulfonyl fluoride, Na3VO4, Nonidet P-40, and protease inhibitor mixture were obtained from Sigma. Phospho-Akt (Ser-473), Akt, phospho-S6 kinase (Thr-389), S6 kinase, and phospho-GSK3β (Ser-9) antibodies were purchased from Cell Signaling Technology, Boston, MA. PTEN and GSK3β antibodies and the siRNA pool for Glut1 were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. FuGENE HD transfection reagent was purchased from Roche Applied Science. TRIZol reagent for RNA isolation was purchased from Invitrogen. The plasmid isolation kit and Qproteome Cell Compartment kit were obtained from Qiagen, Valencia, CA. GeneScreen Plus hybridization transfer membranes were purchased from PerkinElmer Life Sciences. T4 polynucleotide kinase was purchased from New England BioLabs, Ipswich, MA. ProbeQuant G-50 microcolumns were purchased from GE Healthcare. Ly294002 was obtained from Calbiochem. MK-2206 was obtained from Selleck Chemicals, Houston, TX. RT2 real time SYBR Green/ROX PCR Mastermix and GAPDH RT-PCR primers for rat and mouse were obtained from SuperArray Biosciences, Frederick, MD. The primers for detection of mature miR-21 and U6 (for normalization), the mirVana quantitative RT-PCR (qRT-PCR) miRNA detection kit, and the anti-miR-21 were obtained from Ambion, Austin, TX. The Luciferase Reporter Assay System kit was purchased from Promega, Madison, WI. pCMV-miR-21 plasmid was kindly provided by Dr. A. Hata, Tufts University School of Medicine, Boston, MA. PTEN 3’-UTR-Luc reporter plasmid was a kind gift from Dr. T. Patel, Ohio University. Scrambled RNA expression plasmid was a kind gift from Dr. D. M. Sabatini, Whitehead Institute for Biomedical Research. miR-21 Sponge plasmid vector was provided by Dr. P. A. Sharp, Massachusetts Institute of Technology, Boston, MA. Fibronectin promoter-driven luciferase reporter (Fibro-Luc) has been described previously (36).

**Animal Protocol**—OVE26 mice and the control FVB mice were purchased from The Jackson Laboratories, Bar Harbor, ME. The animal protocol was approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio. The animals had free access to food and water. OVE26 mice develop significant renal hypertrophy and albuminuria at 2 months of age due to severe hyperglycemia (37, 38). At 3 months of age, the animals were euthanized, and both kidneys were removed. Cortical sections from each mouse were pooled and frozen as described previously (39).

**Cell Culture**—Rat and human kidney glomerular mesangial cells were grown as described previously (40, 41). Briefly, rat mesangial cells were propagated in DMEM with low glucose containing 17% fetal bovine serum in the presence of penicillin/streptomycin. At confluence, the cells were washed, and serum-free medium was added for 24 h. Then the cells were incubated in DMEM with 25 mM glucose for 24 h. For osmotic control, the cells were incubated with 5 mM glucose plus 20 mM mannitol. The human mesangial cells were grown in DMEM with 10% fetal bovine serum. The cells were treated with 25 mM glucose as described for the rat mesangial cells. Mouse proximal tubular epithelial cells were grown as described previously (11). Briefly, these cells were propagated in DMEM containing 7% fetal bovine serum in the presence of penicillin/streptomycin. The cells were grown to near 90% confluence prior to serum starvation and incubation with DMEM plus 25 mM glucose for 24 h.

**Preparation of Membrane Fractions**—Mesangial cells were incubated with high glucose for the indicated duration. Cells were washed with ice-cold PBS, and membrane fractions were prepared using the Qproteome Cell Compartment kit according to the manufacturer’s instructions.
The validated target in the 3'-UTR of human PTEN mRNA for miR-21 was used to search for its target sequence in the rat and mouse PTEN 3'-UTRs. The RNAhybrid program was used to predict the secondary structure for the duplex formation between the miR-21 recognition element in the mRNA of PTEN 3'-UTR and mature miR-21 (43).

Real Time qRT-PCR—Total RNA was extracted from cells using TRIzol reagent as described previously (44). cDNA was synthesized from 1 µg of purified RNA using the mirVana qRT-PCR miRNA detection kit according to the manufacturer's instructions. qRT-PCR was performed using a real time PCR machine (7900HT, Applied Biosystems). Each sample was analyzed in duplicate. PCR cycling conditions were as follows: 94 °C for 10 min followed by 40 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. The primers used for detection of premature miR-21 are as follows: rat: forward primer, 5'-TGTACCACCTTGTGGGTAG-3'; reverse primer, 5'-GATACTAAATGTCAGACAG-3'; mouse: forward primer, 5'-TGTAACCACCTTGTGGGTAG-3'; reverse primer, 5'-GATACTAAATGTCAGACAG-3'; human: forward primer, 5'-GATGCGGTAGCTTATCCGACAG-3'; reverse primer, 5'-TTTGACAGCCCACTGCGACTG-3'. For detection of mature miRNAs, mirVana qRT-PCR primer sets for hsa-mir-21 (Ambion) were used according to the manufacturer's protocol. mirVana qRT-PCR primer sets for U6 (Ambion) were used for normalization. Data analyses were done by the comparative Ct method as described previously (44).

End Point RT-PCR to Detect miR-21 Sponge—Expression of green fluorescent protein (GFP) mRNA was used as a surrogate to detect the miR-21 Sponge RNA sequence (45).
was used to amplify the GFP mRNA. PCR cycling conditions were as follows: 94 °C for 10 min followed by 40 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. The primers used for detection of GFP mRNA are as follows: forward primer, 5'-ACGGCAAGCTGACCCTGAAG-3'; reverse primer, 5'-GGGTGCTCAGGTAGTGGTTG-3'.

Northern Blotting—25 μg of total RNA were separated on a 15% denaturing polyacrylamide gel and then electroblotted onto GeneScreen Plus hybridization transfer membrane as described previously (46). Following transfer, the membrane was UV-cross-linked for 2 min and dried at 75 °C for an hour. The probes were prepared by 5'-end labeling of oligos by T4 polynucleotide kinase. 50 pmol of oligos were end-labeled using radiolabeled [γ-32P]ATP (150 Ci/μl) and purified with a ProbeQuant G-50 microcolumn according to the manufacturer's protocol. The blots were hybridized overnight at 45 °C in

FIGURE 2. High glucose increases miR-21 to target 3'-UTR of PTEN mRNA in mesangial cells. A, rat mesangial cells were transfected with PTEN 3'-UTR-Luc followed by incubation with 25 mM glucose (HG) and 5 mM glucose plus 20 mM mannitol (NG) for 24 h. The cell lysates were assayed for luciferase activity as described under “Experimental Procedures.” The mean ± S.E. of six measurements is shown, *, p = 0.0004 versus 5 mM glucose plus 20 mM mannitol. B-D, rat mesangial cells were incubated with 25 mM glucose (HG) for 24 h. Total RNAs were used to detect pre-miR-21 and mature miR-21 by real time qRT-PCR (B and D) as described under “Experimental Procedures.” n = 9 for both panels. *, p = 0.0001 versus 5 mM glucose plus 20 mM mannitol in B; *, p = 0.02 versus 5 mM glucose plus 20 mM mannitol in D. In C, the total RNA was used in Northern analysis to detect mature miR-21 as described under “Experimental Procedures.” The bottom panel shows quantification of the miR-21 band. n = 9 for both panels. *, p = 0.0481 versus 5 mM glucose plus 20 mM mannitol.

FIGURE 3. High glucose-stimulated Glut1 translocation to membrane of mesangial cells contributes to expression of miR-21. A, mesangial cells were incubated with 25 mM glucose (HG) for the indicated periods of time. As a control, 5 mM glucose plus 20 mM mannitol (NG) was used. Membrane fractions were prepared as described under “Experimental Procedures.” The membrane extracts were immunoblotted with Glut1 and actin antibodies. The histogram at the bottom shows quantification of the Glut1 protein. B, Glut1 siRNA (siGLUT1) reduces Glut1 protein expression. Mesangial cells were transfected with an siRNA pool against Glut1. The cell lysates were immunoblotted with Glut1 and actin antibodies. C, mesangial cells were transfected with an siRNA pool targeting Glut1 followed by incubation with 25 mM glucose for 24 h. Total RNA was used in real time qRT-PCR for detection of mature miR-21. The bottom panel shows down-regulation of Glut1 protein. In A, n = 4. *, p < 0.01 versus 5 mM glucose plus 20 mM mannitol. In C, n = 4. *, p < 0.01 versus 5 mM glucose plus 20 mM mannitol; **, p < 0.05 versus 25 mM glucose.
hybridization buffer containing 5× SSC, 7% SDS, 2× Denhardt’s solution, and sheared salmon sperm DNA (40 μg/ml). Blots were thoroughly washed after hybridization first with 2× SSC containing 0.05% SDS followed by 0.5× SSC containing 0.05% SDS at 50 °C for 15 min for each wash. The blots were exposed to Eastman Kodak Co. BioMax MR photographic film at 70 °C. The probe sequences are as follows: miR-21, 5′-TCAACATCAGTCTGATAAGCTA-3′; U6, 5′-AAAA-TATGGAACGCTTCACGAATTTGCG-3′.

Transient Transfection—Mesangial and PTE cells were transfected with the indicated plasmids using FuGENE HD as described previously (6, 42, 47). A plasmid expressing scrambled RNA was used as control. The same protocol was used to transfect miR-21 inhibitor (anti-miR) and control scrambled RNA. Transfected cells were starved as described above and treated with 25 mM glucose for 24 h.

Luciferase Assay—The cells were transfected with the reporter plasmid along with the indicated vector or miRNA inhibitor. Luciferase activity was determined in the cell lysate using a luciferase assay kit as described previously (44, 47). The data are presented as the mean of luciferase activity/microgram of protein as arbitrary units ± S.E. of triplicate measurements as described previously (6, 42, 44, 47, 48).

Measurement of Cellular Hypertrophy—After incubation with 25 mM glucose, cells were trypsinized and counted using a hemocytometer. Cells were pelleted by centrifugation at 4000 × g for 5 min at 4 °C. The pellets were washed with PBS and lysed with radioimmune precipitation assay buffer as described above, and the total protein content was determined. Hypertrophy was expressed as a measure of cellular protein content per cell as described previously (6, 9, 42).

Statistics—The significance of the data was determined by analysis of variance followed by Student-Newman-Keuls analysis as described previously (6, 9, 42). Where necessary, the data were analyzed by paired t test. The means ± S.E. of indicated experiments are shown. A p value less than 0.05 was considered significant.
RESULTS

Expression of miR-21 in Kidneys of OVE26 Type 1 Diabetic Mice—We have shown previously that high glucose inhibits the PTEN protein levels in mesangial cells (9). This down-regulation of PTEN was associated with increases in mesangial cell hypertrophy and matrix protein fibronectin expression (9). To investigate this phenomenon in vivo, we used the type 1 diabetic OVE26 mouse. This pancreatic β-cell-specific calmodulin transgenic mouse develops hyperglycemia within 3 days of birth and displays pathologic features of diabetic nephropathy including renal hypertrophy, increased mesangial volume, and matrix expansion (37, 38). Expression of fibronectin was examined in the renal cortices prepared from 3-month-old diabetic OVE26 mice. The results show a significant increase in fibronectin expression in diabetic renal tissues (Fig. 1, A and B).

Next, we determined the expression of PTEN. The level of PTEN in the renal cortex of OVE26 mice was significantly lower than that in the control mice (Fig. 1, C and D). Reduction in PTEN expression was also associated with the predicted increase in phosphorylation of Akt (Fig. 1, E and F). These results suggest a possible role of PTEN in the pathology of diabetic kidney disease in OVE26 mice.

To investigate the mechanism of PTEN protein down-regulation, we investigated miR-21, which has been experimentally validated to target the 3′-UTR of human PTEN mRNA (49, 50). Recent studies demonstrate that nearly 25% of the miRNA tar-
Pathologic Features of Diabetic Nephropathy
get sites in the 3′-UTR are conserved in humans and mice (51). Analysis of human, mouse, and rat PTEN 3′-UTR showed the presence of the highly conserved miR-21 recognition element (Fig. 1G). The predicted minimum free energies (ΔG) for binding of miR-21 and PTEN 3′-UTR for human, mouse, and rat are comparable (−15.2, −13.7, and −14.4 kcal/mol, respectively) (supplemental Fig. S1A). Furthermore, the minimum free energies predicted for the binding of the seed sequence of miR-21 to PTEN 3′-UTR of these species are less than −6 kcal/mol, which supports the critical energy requirement for optimal repression of target protein expression (supplemental Fig. S1B) (52).

Supports the critical energy requirement for optimal repression of PTEN 3′-UTR (Fig. 4). In support of the notion that the 3′-UTR of PTEN mRNA is a direct target for miR-21, we found increased expression of pre-miR-21 and mature miR-21 in the kidney cortex of diabetic mice compared with control mice (Fig. 1, H and I). These results indicate that miR-21 may contribute to the reduction in PTEN that may regulate the pathologic features of diabetic nephropathy.

**High Glucose Increases miR-21 to Down-regulate PTEN Expression in Mesangial Cells**—To investigate systematically the role of high glucose in regulation of PTEN, we examined the effect of 25 mM glucose on the reporter activity of a plasmid in which the PTEN 3′-UTR is fused to luciferase cDNA (PTEN 3′-UTR-Luc) (supplemental Fig. S2). In mesangial cells, 25 mM glucose significantly inhibited the reporter activity driven by the PTEN 3′-UTR (Fig. 2A). 25 mM glucose significantly enhanced the expression of pre-miR-21 as determined by real time qRT-PCR (Fig. 2B). Northern analysis of total RNA with anti-miR-21 probe showed a marked increase in mature miR-21 expression in response to 25 mM glucose (Fig. 2C). Induced expression of mature miR-21 by high glucose was also confirmed by real time qRT-PCR in rat (Fig. 2D) and human (Fig. 2E) mesangial cells. Different isoforms of glucose transporters contribute to the transport of glucose into kidney cells. Glut1 is predominantly present in the collecting ducts and distal tubules and in cultured distal tubular epithelial cells (53, 54). Glut1 is expressed in mesangial and proximal tubular epithelial cells in culture and in diabetic renal cortex (53, 55). Involvement of Glut1 in glucose transport in mesangial cells has been established (55, 56). Incubation of mesangial cells with 25 mM glucose significantly increased the membrane localization of Glut1 within 15 min (Fig. 3A). To determine the involvement of Glut1 in miR-21 expression, we used an siRNA pool to down-regulate the Glut1 protein (Fig. 3B). Down-regulation of Glut1 in mesangial cells significantly inhibited the high glucose-stimulated expression of mature miR-21 (Fig. 3C). Together these data suggest that Glut1-mediated transport of glucose contributes to the expression of miR-21.

We next tested the effect of miR-21 on PTEN expression. Plasmid-derived expression of miR-21 (supplemental Fig. S3A) significantly reduced the reporter activity of PTEN 3′-UTR-Luc similarly to that induced by 25 mM glucose (Fig. 4A). High glucose treatment along with expression of exogenous miR-21 further inhibited PTEN 3′-UTR-Luc activity (Fig. 4A), indicating that the effects are additive; however, this result did not reach significance when compared with the effect induced by miR-21 alone. These results suggest the existence of some degree of miR-21-independent effect of high glucose. Vector-derived expression of miR-21 (supplemental Fig. S3B) significantly attenuated the expression of PTEN protein, analogous to the effect obtained by high glucose (Fig. 4B) and high glucose plus miR-21 expression (Fig. 4B). This reduction in protein levels by miR-21 was associated with a significant increase in Akt phosphorylation, similar to the effect seen with high glucose alone and high glucose in the presence of miR-21 expression (Fig. 4C). Because increased phosphorylation of Akt results in activation of its kinase activity (15), we examined Akt function by phosphorylation of one of its substrates, GSK3β. In mesangial cells, expression of miR-21 alone enhanced the phosphorylation of GSK3β similarly to that seen with high glucose treatment (Fig. 4D). These results suggest that miR-21 regulates the expression of PTEN in response to high glucose in mesangial cells.

To confirm the involvement of miR-21 in PTEN expression, we used a plasmid vector expressing seven copies of the bulged miR-21 binding site placed in the 3′-UTR of GFP mRNA (supplemental Fig. S4). Expression of this construct acts as a “Sponge” that reduces miR-21 (45). Mesangial cells were transfected with miR-21 Sponge or vector alone along with PTEN 3′-UTR-Luc reporter plasmid. As expected, 25 mM glucose reduced the luciferase reporter activity (Fig. 5A). Expression of miR-21 Sponge alone significantly increased the luciferase activity as compared with that in the presence of 5 mM glucose (Fig. 5A). miR-21 Sponge in the presence of high glucose significantly prevented the inhibition of luciferase activity induced by high glucose alone (Fig. 5A). However, the effect of miR-21 Sponge plus high glucose did not restore PTEN 3′-UTR-Luc activity to the levels obtained with miR-21 Sponge alone. These results indicate that miR-21 Sponge significantly reduces the increased endogenous miR-21 induced by high glucose; therefore, miR-21 Sponge and high glucose were not sufficient to

**FIGURE 6. miR-21 regulates phosphorylation of PRAS40 and S6 kinase.** A and C, expression of miR-21 mimics the effect of high glucose on PRAS40 and S6 kinase phosphorylation. Rat mesangial cells were transfected with pCMV-miR-21 or vector expressing scrambled (Scr) RNA followed by incubation with high glucose (HG) as described in the legend of Fig. 4. The cell lysates were immunoblotted with phospho-PRAS40 (p-PRAS40), PRAS40, phospho-S6 kinase (p-S6), S6 kinase (S6K), and actin antibodies as indicated. Histograms show quantification of the protein bands. n = 4, *p < 0.01 versus 5 mM glucose plus 20 mM mannitol (HG); #p < 0.001 versus 5 mM glucose plus 20 mM mannitol in A, *p < 0.001 versus 5 mM glucose plus 20 mM mannitol; #p < 0.001 versus 5 mM glucose plus 20 mM mannitol (n = 6) in C. B and D, miR-21 Sponge inhibits high glucose-induced phosphorylation of PRAS40 and S6 kinase. Mesangial cells were transfected with miR-21 Sponge or vector plasmid followed by incubation with high glucose (HG) as described in the legend of Fig. 4. The cell lysates were immunoblotted with phospho-PRAS40, phospho-S6 kinase, S6 kinase, and actin antibodies as indicated. Panels indicated by GFP and GAPDH show their mRNAs in samples prepared in parallel. The bottom histograms show quantification of the protein bands. n = 4, *p < 0.001 versus 5 mM glucose plus 20 mM mannitol; **p < 0.001 versus high glucose in B. *, p < 0.05 versus 5 mM glucose plus 20 mM mannitol; **, p < 0.05 versus high glucose (n = 6) in D. E and F, the PI 3-kinase/Akt axis regulates miR-21-mediated S6 kinase phosphorylation. Rat mesangial cells were transfected with CMV-miR-21 expression vector followed by incubation with PI 3-kinase inhibitor Ly294022 (25 μM, E) or Akt inhibitor MK-2206 (1 μM, F). The cell lysates were immunoblotted with phospho-S6 kinase, S6 kinase, and actin antibodies as indicated. Histograms in the bottom panels show quantification of the protein bands. In E, n = 4, *p < 0.001 versus control; **p < 0.001 versus miR-21-transfected. In F, n = 4, *p < 0.001 versus control; **p < 0.001 versus miR-21-transfected.
increase the PTEN 3'-UTR-Luc activity to the level of miR-21 Sponge alone. Detection of GFP mRNA (Fig. 5A, bottom panel) acts as a surrogate for the expression of Sponge sequences as demonstrated by the Ebert et al. (45). When endogenous miR-21 was down-regulated by transfecting anti-miR-21 instead of miR-21 Sponge (supplemental Fig. S5A), the PTEN
3′-UTR-Luc activity was significantly increased (supplemental Fig. S5B). Anti-miR-21 in the presence of high glucose reversed the down-regulation of luciferase activity obtained with high glucose alone (supplemental Fig. S5B).

Next, we examined the effect of miR-21 Sponge on the expression of PTEN protein in response to high glucose. Expression of miR-21 Sponge alone slightly increased the PTEN protein levels as compared with that in 5 mM glucose (Fig. 5B). However, this was not significant (Fig. 5B, bottom panel). In contrast, miR-21 Sponge in the presence of high glucose reversed the down-regulation of PTEN induced by high glucose alone (Fig. 5B). Consequently, miR-21 Sponge inhibited high glucose-stimulated phosphorylation of Akt and its substrate GSK3β (Fig. 5, C and D). The use of anti-miR-21 to down-regulate endogenous miR-21 (supplemental Fig. S6A) in the presence of high glucose reversed the inhibition of PTEN induced by high glucose alone (supplemental Fig. S6B) similarly to the results obtained with miR-21 Sponge (Fig. 5B). Also, anti-miR-21 significantly inhibited high glucose-stimulated phosphorylation of Akt, resulting in attenuation of GSK3β phosphorylation (supplemental Fig. S6, C and D).

miR-21 Regulates High Glucose-induced TORC1 Activation—We and others have shown a significant role of mTOR, especially TORC1, in diabetes-induced kidney injury (57–60). PRAS40, a component of TORC1, inhibits the activity of this complex (6, 61). The mechanism of activation of TORC1 involves the phosphorylation of PRAS40 at Thr-246, thus inhibiting its suppression of TORC1 (6, 62). Therefore, we tested the involvement of miR-21 in the phosphorylation of PRAS40 in mesangial cells. 25 mM glucose increased the phosphorylation of PRAS40 (Fig. 6A). Expression of miR-21 (supplemental Fig. S7) resulted in a similar increase in PRAS40 phosphorylation (Fig. 6A). miR-21 expression in the presence of high glucose had the same effect on PRAS40 phosphorylation (Fig. 6A). However, a nonsignificant increase in phosphorylation of PRAS40 was observed with high glucose or miR-21 alone (Fig. 6A). Moreover, these data suggest that high glucose may have some miR-21-independent effect on PRAS40 phosphorylation. In contrast, expression of miR-21 Sponge significantly inhibited the high glucose-induced phosphorylation of PRAS40 (Fig. 6B). miR-21 Sponge did not completely inhibit the effect of high glucose, further suggesting a miR-21-independent mechanism. Also, when anti-miR-21 was used to inhibit endogenous miR-21 expression (supplemental Fig. S8A), attenuation of phosphorylation of PRAS40 by high glucose was observed (supplemental Fig. S8B).

S6 kinase is a direct substrate of TORC1. Phosphorylation of S6 kinase at Thr-389 is considered a marker for TORC1 activation (6, 63). Therefore, we examined the effect of miR-21 on S6 kinase phosphorylation. As expected, incubation of mesangial cells with high glucose increased phosphorylation of S6 kinase (Fig. 6C). Expression of miR-21 (supplemental Fig. S9) increased the S6 kinase phosphorylation similarly to that seen with high glucose treatment (Fig. 6C). The slight increase in phosphorylation of S6 kinase observed with miR-21 in the presence of high glucose as compared with miR-21 or high glucose alone was not significant (Fig. 6C and the quantification at the bottom). On the other hand, expression of miR-21 Sponge significantly inhibited the high glucose-stimulated phosphorylation of S6 kinase (Fig. 6D). Similarly, expression of anti-miR-21 down-regulated endogenous miR-21 expression (supplemental Fig. S10A) and inhibited the phosphorylation of S6 kinase in response to high glucose (supplemental Fig. S10B). These results indicate that augmented expression of miR-21 contributes to high glucose-induced activation of TORC1.

To examine whether the effect of miR-21 on TORC1 activity is mediated by PI 3-kinase, we used Ly294002, a pharmacological inhibitor of this lipid kinase. As before, expression of miR-21 increased phosphorylation of S6 kinase (Fig. 6E and supplemental Fig. S11A). However, this was completely blocked by Ly294002 (Fig. 6E and supplemental Fig. S11A). Similarly, the Akt inhibitor MK-2206 (64) significantly attenuated miR-21-mediated phosphorylation of S6 kinase (Fig. 6F and supplemental Fig. S11B). Ly294002 and MK-2206 did not affect the expression pattern of miR-21 (supplemental Fig. S11 and A and B). These data conclusively demonstrate the involvement of PI 3-kinase/Akt in miR-21-induced activation of TORC1.

miR-21 Increases Mesangial Cell Hypertrophy—High glucose contributes to renal and especially mesangial cell hypertrophy (1, 3, 5). We have shown recently that activation of TORC1 kinase is necessary for high glucose-induced hypertrophy of mesangial cells (6). We tested the role of miR-21 in high glucose-stimulated mesangial cell hypertrophy. First, we used protein synthesis as a surrogate for hypertrophy of cells (6, 9, 57). As expected, incubation of mesangial cells with high glucose increased protein synthesis (Fig. 7A). Expression of miR-21 (supplemental Fig. S12A) significantly increased the protein synthesis similarly to that seen with high glucose alone (Fig. 7A). Both miR-21 and high glucose together produced an anal-
A heterogeneous increase in protein synthesis with a slight additive effect (Fig. 7A), indicating that a miR-21-independent effect of high glucose may be active. Mesangial cell hypertrophy was also determined by the ratio of total protein content to cell number. miR-21 expression (supplemental Fig. S12B) induced hypertrophy of mesangial cells as observed with high glucose alone (Fig.

**FIGURE 8. miR-21 regulates high glucose-stimulated fibronectin expression in mesangial cells.** A and B, rat mesangial cells were transfected with pCMV-miR-21 or scrambled (Scr) RNA-expressing vector (A) and miR-21 Sponge or vector plasmid (B) followed by incubation with high glucose for 24 h as described in the legend of Fig. 4. The cell lysates were immunoblotted with fibronectin and actin antibodies as indicated. In B, the parts indicated by GFP and GAPDH show their mRNA expression in samples run in parallel. Bottom histograms show the mean ± S.E. of six independent experiments. In A, *, p < 0.001 versus 5 mM glucose plus 20 mM mannitol (NG). In B, *, p < 0.001 versus 5 mM glucose plus 20 mM mannitol; **, p < 0.001 versus high glucose (HG). C and D, mesangial cells were cotransfected with Fibro-Luc reporter plus pCMV-miR-21 or scrambled RNA expression vector (C) and Fibro-Luc plus miR-21 Sponge or vector plasmid (D). Transfected cells were treated with high glucose as described in the legend of Fig. 4A. The cell lysates were used for luciferase activity as described under “Experimental Procedures.” The mean ± S.E. of six measurements is shown. In C, *, p < 0.05 versus 5 mM glucose plus 20 mM mannitol. In D, *, p < 0.001 versus 5 mM glucose plus 20 mM mannitol; **, p < 0.001 versus high glucose. The PI 3-kinase/Akt axis regulates miR-21-mediated fibronectin expression in mesangial cells. E and F, rat mesangial cells were transfected with CMV-miR-21 expression vector followed by incubation with PI 3-kinase inhibitor Ly294002 (25 μM; E) and Akt inhibitor MK-2206 (1 μM; F), respectively, for 24 h. The cell lysates were immunoblotted with fibronectin and actin antibodies as indicated. The histograms at the bottom show quantification of the protein bands. In E, n = 4, *, p < 0.01 versus control; **, p < 0.001 versus miR-21-transfected. In F, n = 4, *, p < 0.01 versus control; **, p < 0.05 versus miR-21-transfected.
Expression of miR-21 in the presence of high glucose did not further increase the mesangial cell hypertrophy (Fig. 7B). To confirm the role of miR-21 in hypertrophy, we again used the miR-21 Sponge. Expression of miR-21 Sponge significantly inhibited the high glucose-induced protein synthesis and hypertrophy in mesangial cells (Fig. 7, C and D, and supplemental Fig. S13, A and B). Similarly, expression of anti-miR-21 to block endogenous miR-21 expression (supplemental Fig. S14, A and C) markedly prevented
Pathologic Features of Diabetic Nephropathy

both protein synthesis and hypertrophy in mesangial cells by high glucose (supplemental Fig. S14, B and D). These results suggest that miR-21 regulates hypertrophy of mesangial cells in response to high glucose.

Next, we determined the requirement of PI 3-kinase in mesangial cell hypertrophy. Use of LY294002 to inhibit PI 3-kinase showed significant attenuation of miR-21-induced protein synthesis and hypertrophy (Fig. 7, E and F, and supplemental Fig. S15, A and B). Similarly, Akt inhibitor MK-2206 also blocked miR-21-mediated protein synthesis and hypertrophy of mesangial cells (Fig. 7, G and H, and supplemental Fig. S15, C and D). We conclude that PI 3-kinase/Akt signaling regulates the effect of miR-21 in mesangial cell hypertrophy.

miR-21 Regulates High Glucose-induced Fibronectin Expression—One of the cardinal manifestations of diabetic nephropathy is glomerulosclerosis, which results from increased expression of matrix proteins by the mesangial cells (5). We have shown previously that Akt kinase regulates the expression of one such matrix protein, fibronectin (9, 36). Because we found regulation of this kinase by miR-21, we investigated its involvement in expression of fibronectin by high glucose. Incubation of mesangial cells with 25 mM glucose enhanced the expression of fibronectin as expected (Fig. 8A). Expression of miR-21 (supplemental Fig. S16A) similarly increased fibronectin expression in these cells (Fig. 8A). In contrast to this observation, expression of miR-21 Sponge showed marked inhibition of high glucose-induced fibronectin expression (Fig. 8B). Identical results were obtained when we used anti-miR-21 to down-regulate endogenous miR-21 expression (supplemental Fig. S16, B and C).

We have shown previously that expression of fibronectin in mesangial cells is regulated by a transcriptional mechanism (36). Therefore, we examined the effect of miR-21 on the transcription of fibronectin using a plasmid construct in which the fibronectin promoter drives the luciferase reporter gene (36). Incubation of mesangial cells transfected with the reporter plasmid with 25 mM glucose produced the predicted increase in luciferase activity, a measure of increased transcription of fibronectin (Fig. 8C). Expression of miR-21 (supplemental Fig. S17A) also augmented the luciferase activity similarly to the effect obtained with high glucose alone or with miR-21 plus high glucose (Fig. 8C). As opposed to this observation, expression of miR-21 Sponge (supplemental Fig. S17B) significantly inhibited the transcription of fibronectin (Fig. 8D). Similarly, transfection of anti-miR-21 to down-regulate endogenous miR-21 (supplemental Fig. S18A) prevented the high glucose-induced fibronectin transcription (supplemental Fig. S18B). These results indicate that miR-21 contributes to the high glucose-stimulated fibronectin expression in mesangial cells.

To determine the involvement of PI 3-kinase/Akt, we used both a PI 3-kinase inhibitor and an Akt inhibitor, LY294002 and MK-2206, respectively, which attenuated miR-21-induced expression of fibronectin (Fig. 8, E and F, and supplemental Fig. S19, A and B). These data conclusively demonstrate a role of PI 3-kinase/Akt in mediating the effect of miR-21.

miR-21 Regulates Hypertrophy and Fibronectin Expression in Proximal Tubular Epithelial Cells—The results described above demonstrate that miR-21 regulates the signal transduction pathways necessary for mesangial cell hypertrophy as well as fibronectin expression. However, the PTE cells predominantly contribute to renal hypertrophy and fibrosis during the progression of diabetic kidney disease (1, 3, 5). We tested the expression of miR-21 in PTE cells. Fig. 9, A and B, show that 25 mM glucose significantly increases the expression of pre-miR-21 and miR-21. To examine the role of miR-21 in PTE expression in PTE cells, we transfected PTEN 3′-UTR-Luc along with miR-21 Sponge plasmid into these cells followed by incubation with 25 mM glucose. Similarly to the effect on the PTEN 3′-UTR-driven reporter activity found in mesangial cells (Fig. 4A), high glucose significantly inhibited the luciferase activity in PTE cells (Fig. 9C). Expression of miR-21 Sponge (supplemental Fig. S20A) blocked the high glucose-induced inhibition of luciferase activity (Fig. 9C). However, miR-21 Sponge in the presence of high glucose was not sufficient to completely reverse the luciferase activity to the level obtained with miR-21 Sponge alone. These results suggest that miR-21
Sponge reduces the increased level of high glucose-induced expression of endogenous miR-21. Therefore, in the presence of high glucose, miR-21 Sponge may not be sufficient to increase the PTEN 3′-UTR-Luc activity to the level observed with miR-21 Sponge alone.

Next, we studied the involvement of miR-21 in PTEN-regulated signal transduction. Incubation of PTE cells with 25 mm glucose resulted in inhibition of PTEN protein levels (Fig. 9D). Expression of miR-21 Sponge (supplemental Fig. S20B) significantly prevented the down-regulation of PTEN by high glucose (Fig. 9D). However, miR-21 Sponge plus high glucose did not completely reverse PTEN protein levels to the level obtained with miR-21 Sponge alone (Fig. 9D). These results suggest that miR-21 Sponge reduces the increased endogenous levels of miR-21 induced by high glucose but may not be able to up-regulate PTEN protein to the level observed with miR-21 Sponge alone. Furthermore, miR-21 Sponge attenuated high glucose-stimulated phosphorylation of Akt and its substrate GSK3β (Fig. 9, E and F).

To investigate the role of miR-21 in activation of TORC1 in PTE cells, we examined the phosphorylation of PRAS40. Expression of miR-21 Sponge (supplemental Fig. S20C) significantly inhibited the high glucose-stimulated PRAS40 phosphorylation (Fig. 9G), resulting in attenuation of phosphorylation of S6 kinase (Fig. 9H). miR-21 Sponge blocked both protein synthesis and hypertrophy of PTE cells in response to high glucose (Fig. 9, I and J, and supplemental Fig. S20, D and E). Finally, we examined the involvement of miR-21 in fibronectin expression in PTE cells. Expression of miR-21 Sponge (supplemental Fig. S20F) significantly inhibited the high glucose-stimulated fibronectin protein expression (Fig. 9K). This action of miR-21 Sponge was due to its inhibitory effect on transcription of fibronectin (Fig. 9L and supplemental Fig. S20G).

Next, we studied the involvement of PI 3-kinase and Akt in mediating the effect of miR-21 in PTE cells. Expression of miR-21 in PTE cells increased phosphorylation of S6 kinase (Fig. 10, A and B, and supplemental Fig. S21, A and B). Both a PI 3-kinase inhibitor and an Akt inhibitor, Ly294002 and MK-2206, respectively, inhibited the phosphorylation of S6 kinase (Fig. 10, A and B), resulting in attenuation of miR-21-induced protein synthesis (Fig. 10, C and E, and supplemental Fig. S21, C and E) and hypertrophy of PTE cells (Fig. 10, D and F, and supplemental Fig. S21, D and F). Furthermore, Ly294002 and MK-2206 blocked miR-21-induced expression of fibronectin in PTE cells (Fig. 10, G and H, and supplemental Fig. S21, G and H). These results suggest that PI 3-kinase/Akt signal transduction regulates the effect of miR-21 in PTE cell hypertrophy and fibronectin expression.

### DISCUSSION

Using renal samples from OVE26 type 1 diabetic mice, we provide evidence for an inverse relationship between the expression of miR-21 and PTEN, which is associated with increased fibronectin abundance. We show a causal effect of miR-21 on reduced PTEN expression in response to high glucose in renal cells. We found that high glucose-sensitive miR-21 expression enhanced phosphorylation of Akt, resulting in the inactivation of PRAS40 and thereby increasing TORC1 activity necessary for hypertrophy of renal cells. Finally, we demonstrate the involvement of miR-21 in the high glucose-induced increase in fibronectin expression.

In a recent report, miRNA-mediated post-transcriptional inhibition of PTEN to regulate renal function is convincingly documented (16). miR-17-5p and miR-19 coded by the polycistronic miR-17–92 cluster target the PTEN 3′-UTR directly to reduce its protein expression. Transgenic mice expressing the miR-17–92 cluster have a lymphoproliferative disease that is mainly due to direct down-regulation of PTEN (65, 66). Interestingly, these mice also display proteinuria associated with glomerular hypertension and mesangial expansion, characteristic pathologic features of diabetic nephropathy (66). In conjunction with these results, we show significantly increased expression of a single hairpin structure-derived miR-21 in the kidneys of OVE26 type 1 diabetic mice, which manifest renal hypertension and matrix expansion. In mesangial and PTE cells, high glucose augmented miR-21, which targets the PTEN 3′-UTR to reduce expression of PTEN. Furthermore, our results conclusively demonstrate the involvement of miR-21 in activation of Akt kinase. These results are in line with our previous observation indicating that high glucose-stimulated down-regulation of PTEN is associated with augmented activation of Akt in response to high glucose (9).

We and others have shown a direct relationship between inhibition of PTEN and high glucose-induced mesangial hypertrophy and matrix protein expression (9, 16). In fact, activation of Akt induced mesangial cell hypertrophy and fibronectin expression (6, 9). Therefore, our results conclusively demonstrate that high glucose-stimulated expression of miR-21, which increases Akt activation, may play a role in renal hypertrophy. Indeed, a positive role of miR-21 in cardiomyocyte hypertrophy has been reported previously (67). In contrast, Tatsuguchi et al. (68) showed a negative regulatory effect of miR-21 on cardiomyocyte size. More recently, using four diff-

**FIGURE 10.** PI 3-kinase/Akt axis regulates phosphorylation of Akt, protein synthesis, hypertrophy, and fibronectin expression in renal PTE cells. A and B, PTE cells were transfected with CMV-miR-21 followed by incubation with Ly294002 (25 μM; A) or MK-2206 (1 μM; B). The cell lysates were immunoblotted with phospho-S6 kinase (p-S6K), S6 kinase (S6K), and actin antibodies as indicated. The histograms at the bottom show quantification of the protein bands. In A, n = 4, *p < 0.001 versus control; **p < 0.001 versus miR-21-transfected. In B, n = 4, *p < 0.001 versus control; **p < 0.001 versus miR-21-transfected. C–F, PTE cells were transfected with CMV-miR-21 followed by incubation with Ly294002 (25 μM; C and D) or MK-2206 (1 μM; E and F). [35S]Methionine incorporation (C and E) and cell hypertrophy (D and F) were determined as described under “Experimental Procedures.” In C–F, the mean ± S.E. of triplicate measurements is shown. In C, *p < 0.01 versus control; **p < 0.001 versus miR-21-transfected. In D, *p < 0.01 versus control; **p < 0.001 versus miR-21-transfected. In E, *p < 0.01 versus control; **p < 0.001 versus miR-21-transfected. In F, *p < 0.001 versus control; **p < 0.001 versus miR-21-transfected.
Pathologic Features of Diabetic Nephropathy

One of the pathologic features of diabetic nephropathy is enhanced abundance of matrix proteins including fibronectin, which contributes to fibrosis of the kidney and loss of renal function (5). Two miRNAs, the heart-specific miR-1 and the ubiquitously expressed miR-17-5p have been reported to directly target the 3'-UTR of fibronectin mRNA (76, 77). Whether miR-17-5p is down-regulated by high glucose to increase fibronectin expression needs to be investigated. More recently, the level of miR-377 has been shown to be increased in mesangial cells in the presence of high glucose and also in type 1 diabetic mouse kidney (78). This up-regulation of miR-377 was associated with increased fibronectin expression, suggesting a possible role for the miRNA (78). In fact, miR-377 was found to target the 3'-UTRs of the mRNAs of SOD1 and SOD2, which are known to play important roles in the pathology of diabetic renal diseases and fibronectin expression (78–81).

We have reported previously that PI 3-kinase/Akt signal transduction regulates the expression of fibronectin by a transcriptional mechanism (36). Furthermore, we showed the involvement of PTEN in expression of fibronectin in mesangial cells (9). Now, we demonstrate that miR-21 regulates the fibronectin protein abundance by a transcriptional mechanism in response to high glucose in mesangial and PTE cells. Additionally, we demonstrate an inverse correlation between miR-21 expression and PTEN abundance in the type 1 diabetic kidney, which shows increased expression of fibronectin. Our results support the notion that the miR-21-mediated up-regulation of fibronectin by high glucose may be a direct effect of PTEN down-regulation and the resulting Akt activation.

Many miRNAs are valued as disease markers. Here we show that miR-21 acts as a central modulator of signal transduction pathways involving PTEN, Akt, and TORC1 that contribute to pathologies of diabetic nephropathy. Targeting miR-21 may be beneficial for the patients with diabetic renal dysfunction.

Acknowledgment—We thank Dr. Anthony J. Valente for critically reading and editing the manuscript.

REFERENCES
Pathologic Features of Diabetic Nephropathy


MicroRNA-21 Orchestrates High Glucose-induced Signals to TOR Complex 1, Resulting in Renal Cell Pathology in Diabetes
Nirmalya Dey, Falguni Das, Meenalakshmi M. Mariappan, Chandi Charan Mandal, Nandini Ghosh-Choudhury, Balakuntalam S. Kasinath and Goutam Ghosh Choudhury
doi: 10.1074/jbc.M110.208066 originally published online May 25, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M110.208066

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2011/05/25/M110.208066.DC1

This article cites 81 references, 39 of which can be accessed free at
http://www.jbc.org/content/286/29/25586.full.html#ref-list-1