microRNA-21 ORCHESTRATES HIGH GLUCOSE-INDUCED SIGNALS TO TORC1 FOR RENAL CELL PATHOLOGY IN DIABETES

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Running Title: Pathologic features of diabetic nephropathy

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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 PTEN contributes to renal cell hypertrophy and matrix expansion. We identified 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 downregulation 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 the progressive glomerulosclerosis that results from accumulation of extracellular matrix components collagen, laminin and fibronectin. 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) 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 hyperhexoseemia-induced mesangial hypertrophy and fibronectin expression (13). PI 3,4,5-trisphosphate (PIP3), produced by the PI 3 kinase, activates Akt kinase (14,15). Phosphatase and tensin homolog deleted in chromosome ten (PTEN) dephosphorylates PIP3, 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 downregulates 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 microRNAs 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 (RISC), which contains the argonaute 2 and 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 (MREs) present in the mRNA 3’UTR as well as in coding sequence pose imperfect complementarity (18,19).

Mice null for the enzyme Dicer, which is necessary for pre-miR 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 and 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 coworkers 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 OVE26 type 1 diabetic mouse concomitant with reduced expression of PTEN, and
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 downregulates PTEN. Our results show that miR-21 promotes activation of TORC1 necessary for cellular hypertrophy. Finally, we demonstrate that high glucose-induced miR-21 enhanced 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.

MATERIALS AND METHODS

Materials: D-Glucose, D-Mannitol, anti β-actin antibody, anti fibronectin antibody, phenylmethylsulfonylfluoride, Na3VO4, NP-40 and protease inhibitor cocktail were obtained from Sigma, St. Louis, MO. Phospho-Akt (Ser-473), Akt, phospho-S6 kinase (Thr-389), S6 kinase, phospho-GSK3β (Ser-9) antibodies were purchased from Cell Signaling, Boston, MA. PTEN and GSK3β antibodies, and siRNA pool for Glut1 were obtained from Santa Cruz, Delaware, CA. Fugene-HD transfection reagent was purchased from Roche Molecular Biology, Indianapolis, IN. TRIZol reagent for RNA isolation was purchased from Invitrogen, Carlsbad, CA. Plasmid isolation kit and Qproteome Cell Compartment kit were obtained from Qiagen, Valencia, CA. GeneScreen Plus hybridization transfer membranes were purchased from NEN, Boston, MA. T4 polyneucleotide kinase was purchased from New England Biolabs, Ipswich, MA. ProbeQuant G-50 micro columns were purchased from GE Healthcare, UK. Ly294002 was obtained from Calbiochem, San Diego, CA. MK-2206 was obtained from Selleck Chemicals, Houston, TX. RT² real-time SYBR green/ROX PCR master mix and GAPDH RT-PCR primers for rat and mouse were obtained from SuperArray Biosciences, Frederick, MD. The primers for detection of mature miR-21, U6 (for normalization), mirVana qRT-PCR miRNA detection kit and the anti-miR-21 were obtained from Ambion, Austin, TX. 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, MIT, Boston. Fibronectin promoter-driven luciferase reporter (Fibro-Luc) has been described previously (36).

Animal Protocol: OVE26 mice and the control FBV mice were purchased from 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 three 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 hours. Then the cells were incubated in DMEM with 25 mM glucose for 24 hours. 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% confluency prior to serum starvation and incubation with DMEM plus 25 mM glucose for 24 hours.

**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 instruction.

**Immunoblotting:** Renal cells after incubation with high glucose and renal cortices from control and diabetic OVE26 mice were lysed in RIPA buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na3VO4, 1 mM PMSF, 0.1% protease inhibitor cocktail and 1% NP-40) at 4 °C for half an hour as described previously (6,41,42). The cell debris were pelleted at 10,000 × g for 20 min at 4 °C. Supernatant was collected and protein was estimated using BioRad reagent. For immunoblotting, equal amounts of cell lysates were separated by SDS polyacrylamide gel electrophoresis and transferred to PVDF membrane. Proteins present in the membrane were immunoblotted with indicated antibodies as described previously (6,42).

**Secondary structure prediction:** 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’UTR. The RNA hybrid program ([http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/](http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/)) was used to predict the secondary structure for the duplex formation between miR-21 recognition element in the mRNA of PTEN 3’ UTR and mature miR-21 (43).

**Real time quantitative RT-PCR (qRT-PCR):** Total RNA was extracted from cells using TRIZol reagent as described previously (44). cDNA was synthesized from 1 µg of purified RNA by 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: 94°C for 10 minutes, followed by 40 cycles at 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 30 seconds. The primers used for detection of pre-mature miR-21 are as follows: Rat; forward primer: 5’-TGTACCACCTTGTGGGGTAG-3’ and reverse primer: 5’-GATACCCAAATGTGCACAG-3’; Mouse; forward primer: 5’-TGTACCACCTTGTCGGATAG-3’; reverse primer: 5’-GATACCCAAATGTGCACAG-3’; Human; Forward primer: 5’-TGTCGGGTAGCTTATCAGAC-3’; Reverse primer: 5’-TTCCAGACGCCCATCGACTG-3’. For detection of mature miRNAs, mirVana qRT-PCR primer sets for hsa-mir-21 (Ambion) were used according to 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 GFP (green fluorescence protein) mRNA was used as surrogate to detect miR-21 Sponge RNA sequence (45).
One µg RNA was used to amplify the GFP mRNA. PCR cycling conditions were: 94°C for 10 minutes, followed by 40 cycles at 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds. 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 was 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 minutes and dried at 75°C for an hour. The probes were prepared by 5’ end labeling of oligos by T4 polynucleotide kinase. 50 pmols of oligos were end-labeled using radiolabelled γ-32P-ATP (150 µCi/µl) and purified with ProbeQuant G-50 micro column following manufacturer’s protocol. The blots were hybridized overnight at 45°C in hybridization buffer containing 5X SSC, 7% SDS, 2X Denhardt’s solution and sheared salmon sperm DNA (40 µg/ml). Blots were thoroughly washed after hybridization first with 2X SSC containing 0.05% SDS followed by 0.5X SSC containing 0.05% SDS at 50°C for 15 minutes for each wash. The blots were exposed to KODAK BioMax MR photographic film at -70°C. The probe sequences are as follows: miR-21, 5´-TCAACATCAGTCTGATAAGCTA-3´; U6 5´-AAAATATGGGAACGCTTCACGGATTTG CG-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 hours.

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 mean of luciferase activity per microgram protein as arbitrary units ± SE of triplicate measurements as described previously (6,42,44,47,48).

Protein synthesis assay: Mesangial cells were starved in serum-free medium as described above and treated with 25 mM glucose for 24 hours. Protein synthesis was measured using the 35S-labeled methionine incorporation essentially as described (6,42).

Measurement of cellular hypertrophy: After incubation with 25 mM glucose, cells were trypsinized and counted using a hemocytometer. Cells were pelleted by centrifuging at 4000xg for 5 minutes at 4°C. The pellets were washed with PBS, lysed with RIPA 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 ANOVA followed by Student–Newman–Keuls analysis as described previously (6,9,42). Where necessary the data were analyzed by paired t-test. Means ± SE of indicated experiments is shown. p value less than 0.05 was considered as significant.

RESULTS

Expression of miR-21 in the kidneys of OVE26 type 1 diabetic mice: We have previously shown that high glucose inhibits the PTEN protein levels in mesangial cells
This downregulation 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 three 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 significant increase in fibronectin expression in diabetic renal tissues (Figs. 1A, 1B). 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 (Figs. 1C, 1D). Reduction in PTEN expression was also associated with the predicted increase in phosphorylation of Akt (Figs. 1E, 1F). 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 downregulation, 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 target 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) (Supplementary Fig. S1A). Furthermore, the minimum free energies predicted for the binding of seed sequence of miR-21 to PTEN 3'UTR of these species are less than -6 kcal/mole, which supports the critical energy requirement for optimal repression of target protein expression (Supplementary Fig. S1B) (52). In support of the notion that 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 to control mice (Figs. 1H and 1I). 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 downregulate 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) (Supplementary Fig. S2). In mesangial cells 25 mM glucose (HG) 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. Glut 12 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 minutes (Fig. 3A). To determine the involvement of Glut1 in miR-21 expression, we used an siRNA pool to downregulate the Glut1 protein (Fig. 3B). Downregulation 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 (Supplementary Fig. S3A) significantly reduced the reporter activity of PTEN 3’UTR-Luc similar 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 it did not reach significance when compared to 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 (Supplementary 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). Since 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 similar to 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 (Supplementary 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 to 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 plus high glucose were not sufficient to 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 the surrogate for the expression of ‘Sponge’ sequences as demonstrated by the Ebert et al (45). Instead of miR-21 Sponge, when endogenous miR-21 was downregulated by transfecting anti-miR-21 (Supplementary Fig. S5A), the PTEN 3’UTR-Luc activity was significantly increased (Supplementary Fig. S5B). Anti-miR-21 in the presence of high glucose reversed the downregulation of luciferase activity obtained with high glucose alone (Supplementary 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 to that in 5 mM glucose (Fig. 5B), but this was not significant (Fig. 5B, bottom panel). In contrast, miR-21 Sponge in the presence of high glucose reversed the downregulation of PTEN induced by high glucose alone (Fig. 5B). Consequently, miR-21 sponge inhibited high glucose-stimulated phosphorylation of Akt and its substrate GSK3β (Figs. 5C and 5D). Use of anti-miR-21 to downregulate endogenous miR-21 (Supplementary Fig. S6A) in the presence of high glucose reversed the inhibition of PTEN induced by high glucose alone (Supplementary Fig. S6B), similar 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 (Supplementary Figs. S6C and S6D).

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 (Supplementary 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 as observed with high glucose or miR-21 alone (Fig. 6A). However, a nonsignificant increase in phosphorylation of PRAS40 was observed with miR-21 plus high glucose as compared to that obtained with high glucose or miR-21 alone (Fig. 6A). 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 (Supplementary Fig. S8A), similar attenuation of phosphorylation of PRAS40 by high glucose was observed (Supplementary 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). We, therefore, 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 (Supplementary Fig. S9) increased the S6 kinase phosphorylation similar to 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 to 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 downregulated endogenous miR-21 expression (Supplementary Fig. S10A) and inhibited the phosphorylation of S6 kinase in response to high glucose (Supplementary 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 Supplementary Fig. S11A). However, this was completely blocked by Ly294002 (Fig. 6E and Supplementary Fig. S11A). Similarly, the Akt inhibitor MK-2206 (64), significantly attenuated miR-21-mediated phosphorylation of S6 kinase (Fig. 6F and Supplementary Fig. S11B). Ly294002 and MK-2206 did not affect the expression pattern of miR-21 (Supplementary Figs S11A and S11B). 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 (Supplementary Fig. S12A) significantly increased the protein synthesis similar to high glucose alone (Fig. 7A). Both miR-21 and high glucose together produced an analogous increase in protein synthesis with a slight additive effect (Fig. 7A), indicating 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 (Supplementary Fig. S12B) induced hypertrophy of mesangial cells as observed with high glucose alone (Fig. 7B). 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 (Figs. 7C and 7D; Supplementary Figs. S13A and S13B). Similarly, expression of anti-miR-21 to block endogenous miR-21 expression (Supplementary Figs. S14A and S14C) markedly prevented both protein synthesis and hypertrophy in mesangial cells by high glucose (Supplementary Figs. S14B and S14D). 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. 7E, 7F and Supplementary Figs. S15A and S15B). Similarly, Akt inhibitor MK-2206 also blocked miR-21-mediated protein synthesis and hypertrophy of mesangial cells (Fig. 7G, 7H and Supplementary Figs. S15C and S15D). 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). Since 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 (Supplementary 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 downregulate endogenous miR-21 expression (Supplementary Figs. S16B and S16C).

Previously we have shown 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 (Supplementary Fig. S17A) also augmented the luciferase activity similar 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 (Supplementary Fig. S17B) significantly inhibited the transcription of fibronectin (Fig. 8D). Similarly, transfection of anti-miR-21 to downregulate endogenous miR-21 (Supplementary Fig. S18A) prevented the high glucose-induced fibronectin transcription (Supplementary 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 PI 3 kinase and Akt inhibitors Ly294002 and MK-2206, which attenuated miR-21-induced expression of fibronectin (Figs. 8E, 8F and Supplementary Figs. S19A and S19B). 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. Figs. 9A and 9B show that 25 mM glucose significantly increases the expression of pre-miR-21 and miR-21. To examine the role of miR-21 on PTEN 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. Similar 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 (Supplementary 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 showed inhibition of PTEN protein levels (Fig. 9D). Expression of miR-21 Sponge (Supplementary Fig. S20B) significantly prevented the downregulation of PTEN by high glucose (Fig. 9D).
However, miR-21 Sponge plus high glucose did not completely reverse PTEN protein levels to that 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 upregulate 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 (Figs. 9E and 9F).

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 (Supplementary 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 (Figs. 9I, 9J and Supplementary Fig. S20D and S20E). Finally, we examined the involvement of miR-21 in fibronectin expression in PTE cells. Expression of miR-21 Sponge (Supplementary 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 Supplementary 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 (Figs. 10A, 10B and Supplementary Figs. S21A and S21B). Both PI 3 kinase and Akt inhibitors, Ly294002 and MK-2206, respectively inhibited the phosphorylation of S6 kinase (Fig. 10A and 10B), resulting in attenuation of miR-21-induced protein synthesis (Fig. 10C, 10E and Supplementary Figs. S21C and 21E) and hypertrophy of PTE cells (Figs. 10D and 10F and Supplementary Figs. S21D and S21F). Furthermore, Ly294002 and MK-2206 blocked miR-21-induced expression of fibronectin in PTE cells (Figs. 10G, 10H and Supplementary Figs. S21G and S21H). 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 identified that high glucose-sensitive miR-21 expression enhanced phosphorylation of Akt, resulting in inactivation of PRAS40 to increase TORC1 activity necessary for hypertrophy of renal cells. Finally, we demonstrate the involvement of miR-21 in 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. 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 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 downregulation of PTEN (65,66). Interestingly, these mice also display proteinuria associated with glomerular hypertrophy 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 hypertrophy and matrix expansion. In mesangial and PTE cells, high glucose augmented miR-21, which targets 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 high glucose-stimulated downregulation 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 previously reported (67). In contrast, Tatsuguchi et al showed a negative regulatory effect of miR-21 on cardiomyocyte size (68). More recently, using four different approaches, Olson and coworkers conclusively demonstrated that miR-21 is not necessary for induction of cardiac hypertrophy (69). Contrary to these observations, our results conclusively demonstrate a positive regulatory role of miR-21 in the induction of hypertrophy of mesangial and PTE cells via targeting PTEN.

We and others have established a significant role of mTOR, especially TORC1 for pathologic renal hypertrophy including that induced by diabetes (6,42,57-60,70). TORC1 is comprised of five proteins, mTOR, raptor, mLST8/GβL, PRAS40 and deptor (62,71). Although mLST8/GβL is dispensable, raptor is absolutely necessary for TORC1 activity and contains a docking site for its substrates including S6 kinase (63,72). PRAS40 directly binds raptor through its KSLP and TOS motifs with very high affinity and acts as an endogenous inhibitor of TORC1 activity (62,73). We and others have shown that phosphorylation of PRAS40 inactivates its inhibitory function to unmask the substrate binding site of raptor. Consequently, S6 kinase can bind raptor and be phosphorylated by the mTOR subunit of the TORC1 (6,62,74). In the present study, we demonstrate a role of miR-21 in the phosphorylation of PRAS40 and TORC1 activation in response to high glucose.

Since Akt activation is required for TORC1 activity, any modulation of miRNA expression that impacts expression of proteins influencing PI 3 kinase/Akt signaling will have a significant effect on cell function. Thus PTEN, which influences this signaling pathway and which undergoes downregulation in response to increased expression of miR-21 by high glucose in mesangial and PTE cells will impact TORC1 activity. Activation of S6 kinase by TORC1-mediated phosphorylation has recently been shown to be necessary for cell hypertrophy (75). We have also shown recently that high glucose-induced mesangial cell hypertrophy also requires S6 kinase (6). Our results demonstrate for the first time that high glucose-stimulated activation of TORC1 to phosphorylate and activate S6 kinase depends upon the increased miR-21. Furthermore, we show that the high glucose-induced increase in miR-21 contributes to hypertrophy of mesangial and PTE cells. We found increased expression of miR-21 in the renal tissues of type 1 diabetic mice at the age of three months at which time their kidneys are known to be hypertrophied (37,38). This
upregulation of miR-21 was also associated with inhibition of PTEN abundance and increased Akt phosphorylation. These results provide evidence for a role of miR-21 in renal cell hypertrophy in vivo in diabetes.

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 ubiquitously expressed miR-17-5p have been reported to directly target the 3’ UTR of fibronectin mRNA (76,77). Whether miR-17-5p is downregulated by high glucose to increase fibronectin expression needs to be investigated. More recently, 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 upregulation 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 SOD1 and SOD2 mRNA, which are known to play important roles in the pathology of diabetic renal diseases and fibronectin expression (78-81).

We have previously reported that PI 3 kinase/Akt signal transduction regulates the expression of fibronectin by a transcriptional mechanism (36). Furthermore, we showed 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 upregulation of fibronectin by high glucose may be a direct effect of PTEN downregulation and resulting Akt activation.

Many miRNAs have been valued as disease markers. Here we show that miR-21 acts as a central moderator 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.

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REFERENCES


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LEGENDS TO THE FIGURES

Figure 1. Expression of fibronectin is associated with reduced PTEN abundance and increased miR-21 in OVE26 mice renal cortex. (A, C, E) Renal cortical lysates from 3-month old control FVB and OVE26 type 1 diabetic mice were immunoblotted with fibronectin, PTEN, phospho-Akt, Akt and actin antibodies as indicated. Panels B, D and F are the quantification of the results shown in panel A, C and E respectively. C, control and D, OVE26 diabetic mouse. n = 3 in each group. *p = 0.0286 vs control in panel B; *p = 0.0497 vs control in panel D; *p = 0.019 vs control in panel F. (G) Sequence complementarity between miR-21 and its target sites in the 3’ UTR of human (hsa), mouse (mmu) and rat (rno) PTEN mRNAs. (H and I) Total RNA from renal cortices of control and OVE 26 mice was used in real time qRT-PCR to detect the pre-miR-21 (panel H) and mature miR-21 (panel I) levels as described in the Materials and Methods. n = 4 mice in each group. *p = 0.002 vs control in panel H; *p = 0.003 vs control in panel I.

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 hours. The cell lysates were assayed for luciferase activity as described in the Materials and Methods. Mean ± SE of six measurements is shown. *p = 0.0004 vs NG. (B – D). Rat mesangial cells were incubated with 25 mM glucose (HG) for 24 hours. Total RNAs were used to detect pre-miR-21 and mature miR-21 by real time qRT-PCR (panels B and D) as described in the Materials and Methods. n = 9 for both panels. *p = 0.0001 vs NG in panel B; *p = 0.02 vs NG in panel D. In panel C, the total RNA was used in Northern analysis to detect mature miR-21 as described in the Materials and Methods. Bottom panel shows quantification of the miR-21 band. n = 9. *p = 0.0016 vs NG. (E) Human mesangial cells were incubated with 25 mM glucose (HG) or 5 mM glucose plus 20 mM mannitol (NG) for 24 hours. Total RNA was used to detect mature miR-21 as described in the Materials and Methods. n = 3. *p = 0.0481 vs NG.

Figure 3. High glucose-stimulated Glut1 translocation to the membrane of mesangial cells contributes to the expression of miR-21. (A) Mesangial cells were incubated with 25 mM glucose (HG) for indicated periods of time. As control, 5 mM glucose plus 20 mM mannitol was used (NG). Membrane fractions were prepared as described in the Materials and Methods. The membrane extracts were immunoblotted with Glut1 and actin antibodies. The histogram at the bottom shows quantification of the Glut1 protein. (B) Glut1 siRNA reduces Glut1 protein expression. Mesangial cells were transfected with siRNA pool against Glut1. The cell lysates were immunoblotted with Glut1 and actin antibodies. (C) Mesangial cells were transfected with siRNA pool targeting Glut1 followed by incubation with 25 mM glucose for 24 hours. Total RNA was used in real time qRT-PCR for detection of mature miR-21. Bottom panel shows downregulation of Glut1 protein. In panel A, n = 4. *p < 0.01 vs NG. In panel C, n = 4. *p < 0.01 vs NG; **p < 0.05 vs HG.

Figure 4. Expression of miR-21 mimics the effect of high glucose on PTEN expression and on downstream signaling. (A) miR-21 inhibits PTEN 3’UTR reporter activity. Rat mesangial cells were cotransfected with the PTEN 3’ UTR-Luc reporter plasmid and pCMV-miR-21 (miR-21) or scrambled RNA expression vector (Scr) followed by incubation with 25 mM glucose (HG) and 5 mM glucose plus 20 mM mannitol (NG) for 24 hours. The cell lysates were assayed
for luciferase activity as described in the Materials and Methods. Mean ± SE of 6 measurements is shown. *p < 0.001 vs NG. (B – D) miR-21 inhibits the expression of PTEN resulting in phosphorylation of Akt and GSK3β similar to high glucose. Mesangial cells were transfected with CMV-miR-21 expression vector followed by incubation with high glucose as described in the panel A. The cell lysates were immunoblotted with PTEN, phospho-Akt, Akt, phospho-GSK3β, GSK3β and actin antibodies as indicated in different panels. Histogram at the bottom part of each panel shows quantification of the protein bands. n = 6; *p < 0.001 vs NG in panels B, C and D. #p < 0.01 vs NG in panel B.

**Figure 5.** Expression of miR-21 Sponge inhibits the effect of high glucose on expression of PTEN and on downstream signaling. (A) miR-21 Sponge prevents high glucose-induced downregulation of PTEN 3’ UTR reporter activity. Rat mesangial cells were cotransfected with the PTEN 3’ UTR-Luc reporter plasmid and miR-21 Sponge expression vector followed by incubation with high glucose as described in the legend of Fig. 4A. The cell lysates were assayed for luciferase activity as described in the Materials and Methods. Mean ± SE of 6 measurements is shown. *p < 0.05 vs NG; #p < 0.001 vs NG; **p < 0.01 vs HG. Bottom panel shows expression of GFP mRNA in one of the samples performed in parallel. (B – D) Expression of miR-21 Sponge prevents downregulation of PTEN induced by high glucose, resulting in phosphorylation of Akt and GSK3β. Rat mesangial cells were transfected with miR-21 Sponge followed by incubation with high glucose as described in the legend of Fig. 4A. The cell lysates were immunoblotted with PTEN, phospho-Akt, Akt, phospho-GSK3β, GSK3β and actin antibodies as indicated in different panels. Expression of GFP mRNA is shown in the lysates prepared in parallel. Histogram at the bottom of each panel shows quantification of the protein bands. n = 6. *p < 0.01 vs NG and **p < 0.01 vs HG in panel B. *p < 0.001 vs NG; **p < 0.001 vs HG in panels C and D.

**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 RNA followed by incubation with high glucose (HG) as described in the legend of Fig. 4. The cell lysates were immunoblotted with phospho-PRAS40, PRAS40, phospho-S6 kinase, S6 kinase and actin antibodies as indicated. Histograms show quantification of the protein bands. n = 4; *p < 0.01 vs NG; #p < 0.001 vs NG in panel A. *p < 0.001 vs NG; #p < 0.01 vs NG (n = 6) in panel 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, 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. Bottom histograms show quantification of the protein bands. n = 4; *p < 0.001 vs NG; **p < 0.001 vs HG in panel B. *p < 0.05 vs NG; **p < 0.05 vs HG (n = 6) in panel D. (E and F) 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 Ly294002 (25 µM; panel E) or Akt inhibitor MK-2206 (1 µM; panel 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 panel E, n = 4. *p < 0.001 vs control; **p < 0.001 vs miR-21-transfected. In panel F, n = 4. *p < 0.001 vs control; **p < 0.001 vs miR-21 transfected.

**Figure 7.** mir-21 regulates high glucose-induced protein synthesis and hypertrophy of mesangial cells. Rat mesangial cells were transfected with pCMV-miR-21 or vector expressing scrambled RNA (panels A and B) and miR-21 Sponge or vector (panels C and D) followed by incubation with high glucose (HG) for 24 hours as described in the legend of Fig. 4. In panels A and C, incorporation of $^{35}$S-methionine was determined as described in the Materials and Methods. In panels B and D, the cells were trypsinized, counted and protein content determined. The cell hypertrophy was expressed as a ratio of total protein per cell as described in the Materials and Methods. Mean ± SE of triplicate measurements is shown. In panel A, *p < 0.001 vs NG; #p < 0.05 vs NG. In panel B, *p < 0.01 vs NG; #p < 0.05 vs NG. In panel C, *p < 0.05 vs NG; **p < 0.05 vs HG. In panel D, *p < 0.001 vs NG; **p < 0.001 vs HG. (E – H) PI 3 kinase/Akt axis regulates miR-21-mediated protein synthesis and hypertrophy of mesangial cells. Rat mesangial cells were transfected with CMV-miR-21 expression vector followed by incubation with PI 3 kinase and Akt inhibitors Ly294002 (25 µM; panels E and F) and MK-2206 (1 µM; panels G and H), respectively for 24 hours. $^{35}$S-methionine incorporation (panels E and G) and cell hypertrophy (panels F and H) were determined as described above. Mean ± SE of triplicate measurements is shown for panels E – H. In panel E, *p < 0.01 vs control; **p < 0.01 vs miR-21-transfected. In panel F, *p < 0.001 vs control; **p < 0.001 vs miR-21-transfected. In panel G, *p < 0.01 vs control; **p < 0.01 vs miR-21-transfected. In panel H, *p < 0.001 vs control; **p < 0.001 vs miR-21-transfected.

**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 scramble RNA expressing vector (panel A) and miR-21 Sponge or vector plasmid (panel B) followed by incubation with high glucose for 24 hours as described in the legend of Fig. 4. The cell lysates were immunoblotted with fibronectin and actin antibodies as indicated. In panel B, the parts indicated by GFP and GAPDH show their mRNA expression in samples run in parallel. Bottom histograms show mean ± SE of 6 independent experiments. In panel A, *p < 0.001 vs NG. In panel B, *p < 0.001 vs NG; **p < 0.001 vs HG. (C and D) Mesangial cells were cotransfected with Fibro-Luc reporter plus pCMV-miR-21 or scrambled RNA expression vector (panel C) and Fibro-Luc plus miR-21 Sponge or vector plasmid (panel 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 in the Materials and methods. Mean ± SE of 6 measurements is shown. In panel C, *p < 0.05 vs NG. In panel D, *p < 0.001 vs NG; **p < 0.001 vs HG. PI 3 kinase/Akt axis regulates miR-21-mediated fibronectin expression in mesangial cells. Rat mesangial cells were transfected with CMV-miR-21 expression vector followed by incubation with PI 3 kinase and Akt inhibitors Ly294002 (25 µM; panel E) and MK-2206 (1 µM; panel F), respectively for 24 hours. The cell lysates were immunoblotted with fibronectin and actin antibodies as indicated. The histograms at the bottom show quantification of the protein bands. In panel E, n = 4. *p < 0.001 vs control; **p < 0.001 vs miR-21-transfected. In panel F, n = 4. *p < 0.01 vs control; **p < 0.05 vs miR-21-transfected.

**Figure 9.** miR-21 regulates high glucose-induced downregulation of PTEN and downstream signal transduction to induce renal PTE cell protein synthesis, hypertrophy and fibronectin
expression. (A and B). High glucose stimulates miR-21 expression. PTE cells were incubated with 25 mM glucose (HG) or 5 mM glucose plus 20 mM mannitol (NG) for 24 hours. Total RNAs were used to detect pre-miR-21 (panel A) and mature miR-21 (panel B) expression using real time qRT-PCR as described in the Materials and Methods. n = 6. *p = 0.0111 vs NG in panel A. *p = 0.0008 vs NG in panel B. (C) High glucose inhibits PTEN 3’ UTR-Luc activity via miR-21 in PTE cells. Cells were cotransfected with PTEN 3’ UTR-Luc and miR-21 Sponge or vector plasmid followed by incubation with 25 mM glucose or 5 mM glucose plus 20 mM mannitol for 24 hours. The cell lysates were assayed for luciferase activity as described in the Materials and Methods. Mean ± SE of triplicate measurements is shown. *p < 0.05 vs NG; #p < 0.01 vs NG; **p < 0.05 vs HG. (D – H) miR-21 regulates PTEN downregulation and downstream TORC1 signaling in PTE cells. Cells were transfected with miR-21 Sponge or vector plasmid followed by incubation with glucose as described above in panel C. The cell lysates were immunoblotted with PTEN, phospho-Akt, Akt, phospho-GSK3β, GSK3β, phospho-PRAS40, PRAS40, phospho-S6 kinase, S6 kinase and actin antibodies as indicated. Histograms at the bottom of each panel show quantification of protein bands. In panel D, *p < 0.01 vs NG; **p < 0.05 vs HG (n = 4). In panel E, *p < 0.001 vs NG; **p < 0.001 vs HG (n = 4). In panel F, *p < 0.01 vs NG; **p < 0.01 vs HG (n = 4). In panel G, *p < 0.01 vs NG; **p < 0.01 vs HG (n = 4). In panel H, *p < 0.001 vs NG; **p < 0.001 vs HG (n = 4). (I and J) miR-21 regulates PTE cell protein synthesis and hypertrophy. PTE cells were transfected with miR-21 Sponge or vector plasmid followed by incubation with high glucose as described in the Fig. 4. Protein synthesis and hypertrophy were determined as described in the Materials and Methods. Mean ± SE of triplicate measurements is shown. In panel I, *p < 0.05 vs NG; **p < 0.05 vs HG. In panel J, *p < 0.001 vs NG; **p < 0.001 vs HG. (K and L) mir-21 regulates high glucose-stimulated fibronectin expression in PTE cells. (K) Cells were transfected with miR-21 Sponge or vector plasmid followed by incubation with high glucose as described in the legend of Fig. 4. The cell lysates were immunoblotted with fibronectin and actin antibodies respectively as indicated. The bottom histogram shows quantification of 4 independent experiments. *p < 0.001 vs NG; **p < 0.001 vs HG. (L) Cells were transfected with Fibro-Luc reporter plus miR-21 Sponge or vector plasmids followed by incubation with high glucose as described in the legends of Fig. 8C and 8D. The cell lysates were used for luciferase activity as described in the Materials and Methods. Mean ± SE of triplicate measurements is shown. *p < 0.001 vs NG; **p < 0.001 vs HG.

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; panel A) or MK-2206 (1 µM; panel B). The cell lysates were immunoblotted with phospho-S6 kinase, S6 kinase and actin antibodies as indicated. The histograms at the bottom show quantification of the protein bands. In panel A, n = 4. *p < 0.001 vs control; **p < 0.001 vs miR-21-transfected. In panel B, n = 4. P < 0.001 vs control; **p < 0.001 vs miR-21-transfected. (C – F) PTE cells were transfected with CMV-miR-21 followed by incubation with Ly294002 (25 µM; panels C and D) or MK-2206 (1 µM; panels E and F). 35S-methionine incorporation (panels C and E) and cell hypertrophy (panels D and F) were determined as described in the Materials and Methods. In panels C – F, mean ± SE of triplicate measurements is shown. In panel C, *p < 0.01 vs control; **p < 0.001 vs miR-21-transfected. In panel D, *p < 0.001 vs control; **p < 0.001 vs miR-21 transfected. In panel E, *p < 0.01 vs control; **p < 0.01 vs miR-21-transfected. In panel F, *p < 0.001 vs control; **p < 0.001 vs miR-21-transfected. (G and H) PTE cells were transfected with CMV-miR-21
followed by incubation with Ly294002 (25 μM; panel G) or MK-2206 (1 μM; panel 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 panels G and H, n = 4; *p < 0.001 vs control; **p < 0.001 vs miR-21 transfected.
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microRNA-21 orchestrates high glucose-induced signals to TORC1 for renal cell pathology in diabetes
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