FOG2 Protein Down-regulation by Transforming Growth Factor-β1-induced MicroRNA-200b/c Leads to Akt Kinase Activation and Glomerular Mesangial Hypertrophy Related to Diabetic Nephropathy*

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Jung Tak Park, Mitsuo Kato, Hang Yuan, Nancy Castro, Linda Lanting, Mei Wang, and Rama Natarajan

From the Division of Molecular Diabetes Research, Department of Diabetes, Beckman Research Institute of City of Hope, Duarte, California 91010

**Background:** The mechanism of TGF-β1-induced Akt kinase activation in diabetic nephropathy (DN) is not fully elucidated.

**Results:** FOG2 down-regulation by TGF-β1-induced miR-200b/c activates Akt, which leads to glomerular mesangial hypertrophy.

**Conclusion:** FOG2 and miR-200b/c are novel modulators of TGF-β1-induced Akt activation in glomerular mesangial cells.

**Significance:** These results reveal new mediators of TGF-β1 actions related to the pathogenesis of DN.

Diabetic nephropathy (DN) is a major complication of diabetes. It is characterized by glomerular mesangial expansion caused by mesangial cell hypertrophy, followed by extracellular matrix protein accumulation and subsequent glomerulosclerosis (1–4). Cellular hypertrophy, a maladaptive increase in total cell volume and protein content without a concomitant increase in total cell number, is one of the early abnormalities found in DN (5). In addition, chronic fibrotic changes have been found to be preceded by hypertrophic growth (4, 6). Therefore, understanding the mechanisms associated with pathogenesis of mesangial hypertrophy is important for the development of approaches to treat DN.

The phosphatidylinositol 3-kinase (PI3K)-Akt pathway plays a key role in cellular hypertrophy. The PI3K enzyme, a well known upstream mediator of Akt kinase activation, is composed of a catalytic subunit, p110, and a regulatory subunit, p85α. When activated, the catalytic subunit of PI3K recruits Akt kinase to the membrane and activates it by phosphorylation (7). Activated Akt phosphorylates several downstream proteins that play central roles in hypertrophy, cell growth, cell survival, and protein synthesis.

Transforming growth factor-β1 (TGF-β) is a major promoter of diabetic renal manifestations. Enhanced expression of TGF-β in renal cells can lead to glomerular mesangial hypertrophy and fibrosis under diabetic conditions (2, 8–11). High glucose conditions increase the transcription of TGF-β1 (12, 13). Moreover, many of the effects of high glucose in renal cells or the progression of DN were found to be attenuated by neutralizing TGF-β1 (10, 14). Therefore, TGF-β is a critical medi-
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ator of the effects of high glucose in models of DN. Recent studies have found that PI3K-Akt activation by TGF-β plays a key role in its downstream actions (15–18). Interestingly, recent studies have shown that down-regulation of phosphatase and tensin homolog (PTEN) is a key mechanism by which TGF-β activates PI3K-Akt and that this occurs via microRNAs (miRNAs) such as miR-216a/miR-217 that target PTEN (16, 19).

Growing evidence suggests that miRNAs play critical roles in the progression of DN. miRNAs are a group of endogenously produced, short noncoding RNAs that bind to the 3′ UTRs of target mRNAs through base pairing and inhibit mRNA translation or promote mRNA degradation (20, 21). Several miRNAs expressed in the kidney were found to be mis-regulated in the renal cortex and glomeruli of animal models of diabetes as well as in mouse and human mesangial cells treated with TGF-β or high glucose, and they could modulate the expression of extra-cellular matrix genes, suggesting a functional role in DN pathophysiology (3, 19, 22–34).

Recently, a novel protein FOG2 was found to bind with the p85α, regulatory subunit of PI3K, thereby inhibiting PI3K activation (35). In addition, miR-200 was reported to decrease FOG2 expression by targeting the 3′ UTR of the FOG2 mRNA, thereby altering PI3K activity and regulating the insulin signaling pathway and metabolism (35). However, the role of FOG2 in TGF-β signaling and cellular hypertrophy has not been examined.

In this study, we show that TGF-β up-regulates miR-200b/c in mouse mesangial cells (MMC) and subsequently down-regulates its target FOG2, which leads to PI3K-Akt activation and glomerular mesangial hypertrophy. Furthermore, we observed that a FOG2 siRNA could directly increase Akt activity and cellular hypertrophy, whereas inhibitors of miR-200b/c could attenuate TGF-β-induced mesangial cellular hypertrophy. These results reveal a novel new role for FOG2 regulated by miR-200b/c in TGF-β-induced PI3K-Akt activation in the pathogenesis of DN.

EXPERIMENTAL PROCEDURES

Animals—All animal studies were conducted according to protocol approved by the Institutional Animal Care and Use Committee at the Beckman Research Institute of City of Hope. Type 2 diabetic db/db mice and genetic control nondiabetic db/+ mice (10–12 weeks old, eight per group), were obtained from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 mice (The Jackson Laboratory) were injected with 50 mg/kg of streptozotocin (STZ) intraperitoneally on 5 consecutive days. Mice injected with diluent served as controls. Diabetes was confirmed by tail vein blood glucose levels (fasting glucose >300 mg/dl). Each group was composed of eight mice. All mice were sacrificed at 16 weeks post-induction of diabetes. Glomeruli were isolated from freshly harvested kidneys by a sieving technique. Renal capsules were removed, and the cortical tissue of each kidney was separated by dissection. The cortical tissue was then carefully strained through a stainless sieve with a pore size of 150 μm by applying gentle pressure. Enriched glomerular tissue below the sieve was collected and transferred to another sieve with a pore size of 75 μm. After several washes with cold PBS, the glomerular tissue remaining on top of the sieve was collected. The pooled glomeruli were centrifuged, and the pellet was collected for RNA extraction. Each glomeruli sample was composed of tissue pooled from two mice.

Cell Culture Experiments—MMC were obtained and cultured as described previously in RPMI 1640 medium supplemented with 10% FBS (22). Passages 5–7 were used for experiments. Recombinant human TGF-β1 was from R&D Systems (Minneapolis, MN). LY-294002 was from Calbiochem, and MK-2206 was purchased from Selleck Chemicals (Houston, TX). LY-294002 and MK-2206 were dissolved in dimethyl sulfoxide (DMSO) and used at a final concentration of 20 and 1 μM, respectively, as described previously (36, 37).

Immunohistochemistry—Formalin-fixed, paraffin-embedded sections of mouse kidneys were mounted onto positively charged slides, deparaffinized, washed with water, blocked with Dako protein block (Dako, Carpinteria, CA), and incubated with FOG2 antibody (1:25) for 30 min. Slides were washed with Dako wash, treated with hydrogen peroxide for 5 min, washed with PBS, incubated with anti-rabbit secondary antibody conjugated with a peroxidase polymer (Dako, Carpinteria, CA), and washed and incubated with 3,3′-diaminobenzidine for 8 min. Slides were counterstained with hematoxylin and mounted. Images were taken at ×40 magnification using an Olympus BX51 microscope with In Studio (Pixera Corp., Santa Clara, CA) software to collect images. ImagePro software (Media Cybernetics Inc., Rockville, MD) was used to quantify staining.

Real Time Quantitative PCR—RNA was extracted using miNeasy columns (Qiagen, Inc. Valencia, CA). miRNA expression analysis was performed with the qScript miRNA cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD) and PerfeCTa SYBR Green Supermix (Quanta Biosciences). GeneAmp RNA PCR kit (Applied Biosystems, Carlsbad, CA) and POWER SYBR Green mix (Applied Biosystems) were used for mRNA quantification. Extracted mature miRNAs were first polyadenylated with poly(A) polymerase followed by reverse transcription into cDNA using oligo(dT) primer with universal tag. miRNAs were amplified using specific mature miRNA sequences as forward primers and the universal primer provided in the kit as reverse primer. Real time quantitative PCRs were performed on the 7500 real time PCR system (Applied Biosystems, Foster City, CA). PCR primer sequences were as follows: FOG2, 5′-GAGCTTGG-AAGACGTGGAGT-3′ and 5′-CCAGGCTGTCCCTGTT-TGTC-3′; and TGF-β1, 5′-CAACGCCATCTATGAGA-3′ and 5′-AAGCCCTGTATTCCGTCTCC-3′.

Western Blot Analysis—Immunoblotting was performed as described previously (22). Cells were lysed in Laemmlı’s sample buffer. Lysates were fractionated on 10% SDS-polyacrylamide gels (Bio-Rad) and transferred to nitrocellulose membrane. Membranes were immunoblotted with appropriate antibodies. Antibody against FOG2 was from Santa Cruz Biotechnology (1:500). Antibodies against phospho-Akt, Akt, and β-actin were from Cell Signaling (Beverly, MA). Blots were scanned using GS-800 densitometer and quantified with Quantity One software (Bio-Rad).

miRNA Oligonucleotides—Oligonucleotides representing the miR mimics, negative control for mimics (NC-M), the miR-
200b/c inhibitors, and the negative control for the inhibitors (NC-I) were all obtained from Thermo Fisher Scientific Inc. (Waltham, MA).

**MMC Transfection**—Cells (1 × 10⁶/transfection) were transfected with siRNA or miRNA oligonucleotides using an Amaxa Nucleofector (Lonza, Basel, Switzerland) according to the manufacturer’s protocols as described previously (19). MMC were trypsinized and resuspended in Basic Nucleofection Solution at 1 × 10⁶/ml. Subsequently, 100 µl of cell suspension (1 × 10⁶ cells) was mixed with miRNA mimic, hairpin inhibitor oligonucleotides, or ON-TARGET plus siRNA or negative controls (Thermo Fisher Scientific Inc., Waltham, MA) as indicated. Transfected cells were harvested for RNA and protein isolation at indicated times.

**Measurement of Cellular Hypertrophy**—Hypertrophy was assessed by measurement of cellular protein/cell counts as described previously (19). MMC were trypsinized and counted using a Coulter Counter with 100-µm aperture (Beckman Coulter, Brea, CA). Cells were lysed, and total protein content was measured using protein assays from Bio-Rad.

**Statistical Analysis**—Statistical analysis was performed using PRISM software (Graph-Pad, San Diego, CA) for data analysis with Student t tests or analysis of variance. p < 0.05 was considered statistically significant. All data were expressed as means ± S.E.

**RESULTS**

**FOG2 Expression Is Down-regulated in Glomeruli of Diabetic Mice**—To evaluate whether glomerular FOG2 is related to the progression of DN, we first examined the expression of FOG2 in glomeruli of type 2 and type 1 diabetic mice. The immunohistochemical staining of FOG2 was significantly decreased in glomeruli from type 2 diabetic db/db mice compared with nondiabetic control mice (db/+ ) (Fig. 1A–C). FOG2 mRNA levels were also significantly lower in the renal glomeruli of the diabetic db/db mice when compared with control db/+ mice (Fig. 1D). Conversely, TGF-β1 gene expression levels were increased in the glomeruli from db/db mice (Fig. 1E). A similar decrease in FOG2 staining was observed in glomeruli from streptozotocin-induced type 1 diabetic mice (STZ) compared with nondiabetic control mice (db/+ ) (Fig. 1A–C). FOG2 mRNA levels were lower in STZ glomeruli (Fig. 1F), whereas TGF-β1 expression levels were significantly higher (Fig. 1F).

**FOG2 Expression Is Down-regulated in MMC Treated with High Glucose and with TGF-β**—To further clarify whether the glomerular expression of FOG2 is regulated by diabetic conditions in vitro, MMC were cultured with either normal glucose (NG, 5.5 mM) or high glucose (HG, 25 mM) for 72 h. The expression of FOG2 mRNA and protein was significantly down-regulated by HG relative to NG treatment (Fig. 2A–C). The down-regulation of FOG2 in HG-treated MMC was associated with an increase in Akt phosphorylation (Fig. 2B and D). These changes were accompanied by a significant increase of TGF-β1 expression in HG-treated MMC (Fig. 2E). Because TGF-β is increased in renal cells under diabetic conditions, is up-regulated by HG, and plays a key role in the pathogenesis of DN, we next examined the effects of TGF-β. TGF-β treatment in MMC resulted in a significant decrease in FOG2 mRNA (from 6 to 0.01 and ** indicate p < 0.01 and p < 0.05, respectively.

**FIGURE 1. Decreased levels of FOG2 in glomeruli from mouse models of type 2 (db/db) and type 1 diabetes (streptozotocin-induced).** A and B, representative immunohistochemistry staining of FOG2 in glomeruli from genetic control db/+ mouse (scale bar, 20 μm) (A) and from diabetic db/db mouse (B). C, percentage of FOG2-positive area. Significant decrease of FOG2 staining is observed in glomeruli from db/db mice compared with those from genetic control db/+ mice. To quantify the expression of FOG2, the positive stained areas/glomerular areas (%) were measured using ImageJ (National Institutes of Health). For each animal, 10 glomeruli were evaluated. Mean ± S.E. D, FOG2 mRNA expression in glomeruli from db/db mice compared with those from genetic control db/+ mice. E, TGF-β1 mRNA expression in glomeruli. Significant increase in TGF-β1 mRNA in glomerul from db/db mice compared with those from genetic control db/+ mice. Glomeruli were isolated by a sieving technique from eight mice in each group. Results were normalized with internal control 18S. Mean ± S.E. ** and * indicate p < 0.01 and p < 0.05, respectively.
24 h; Fig. 3A) and protein levels (from 1 to 24 h; Fig. 3, B and C). Furthermore, in parallel, there was a significant increase in Akt phosphorylation in the TGF-β-treated MMC (Fig. 3, B and D).

Together, these results suggest that FOG2 expression is decreased in mesangial cells under diabetic conditions in vivo and in vitro, and this is associated with an increase in Akt phosphorylation. In addition, the findings imply that these changes are associated with TGF-β expression, suggesting a link between FOG2 inhibition and Akt phosphorylation in glomerular mesangial cells under diabetic conditions.

**Knockdown of FOG2 Activates Akt in Mouse Mesangial Cells—**

Next, we tested the functional relevance of FOG2 down-regulation in MMC. Recently, FOG2 and its fly analog U-shaped (USH) were found to suppress PI3K activity and thereby repress insulin receptor signaling in human cell lines and Drosophila melanogaster to control growth (35). Therefore, we investigated whether FOG2 also affects the phosphorylation of Akt, a downstream target of PI3K, in MMC. FOG2 gene silencing by transfection with specific siRNAs (siFOG2) resulted in a significant decrease in FOG2 mRNA (Fig. 4A) as well as FOG2 protein levels (Fig. 4, B and C) relative to nontargeting control siRNA (siNTC). Furthermore, siFOG2 transfection also significantly increased the phosphorylation of Akt relative to siNTC transfection in MMC (Fig. 4, B and D), suggesting that the down-regulated FOG2 in TGF-β-treated cells may have a functional relationship with Akt activation in MMC.

Previous studies have shown that glomerular mesangial hypertrophy, a major feature of DN, is a downstream consequence of Akt activation (16, 19). We next tested whether the increase in Akt phosphorylation ensued by FOG2 knockdown also results in increased cellular hypertrophy in MMC. TGF-β significantly increased cellular protein content, verifying that MMC hypertrophy is induced by TGF-β (Fig. 4E). Transfection of siFOG2 similarly induced hypertrophy in serum-depleted
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First, miR-200b/c expression levels were evaluated in HG-treated MMC. miR-200b and miR-200c levels were significantly increased in MMC after 72 h of HG treatment (Fig. 5A). A similar increase in miR-200b and miR-200c was also found in MMC treated with TGF-β (24 h) (Fig. 5B). miR-200b/c levels were further evaluated in glomeruli of db/db and STZ mice. miR-200b and miR-200c levels were significantly increased in glomeruli of db/db mice (Fig. 5C) as well as STZ mice (Fig. 5D) compared with corresponding control mice as reported previously (27, 28), verifying that miR-200b and miR-200c are up-regulated in glomerular mesangial cells and renal glomeruli under diabetic conditions.

**FOG2 Is Down-regulated by miR-200b/c in MMC**—Next, we examined whether miR-200b/c could down-regulate FOG2 in MMC. Transfection of MMC with oligonucleotide mimics of miR-200b (200b-M) or miR-200c (200c-M) significantly increased the levels of miR-200b and miR-200c, respectively, verifying the effectiveness of the mimics (Fig. 6A). Furthermore, results showed that 200b-M and 200c-M transfection significantly down-regulated both FOG2 mRNA (Fig. 6B) and protein levels (Fig. 6C and D) relative to those transfected with negative control mimic (NC-M) oligonucleotides, suggesting that FOG2 abundance can be decreased by the up-regulated miR-200b/c in glomerular mesangial cells under diabetic conditions.

**Akt Is Activated by miR-200b/c in MMC**—Because our results showed that FOG2 can suppress the phosphorylation of Akt, we next evaluated whether the 200b-M and 200c-M induced down-regulation of FOG2 could also up-regulate Akt phosphorylation in MMC. Results showed that Akt phosphorylation was indeed significantly increased in MMC transfected with 200b-M and 200c-M compared with NC-M-transfected cells (Fig. 6, C and D), which occurred in parallel, to the down-regulation of FOG2 (Fig. 6, C and D).

**miR-200b/c Inhibitors Can Attenuate the Down-regulation of FOG2 by TGF-β in MMC**—To verify that FOG2 is regulated by miR-200b/c in glomerular mesangial cells under diabetic conditions, we further investigated whether TGF-β-induced down-regulation of FOG2 can be reversed by oligonucleotide inhibitors of miR-200b/c. Transfection with hairpin inhibitor oligonucleotides of miR-200b (200b-I) and miR-200c (200c-I) resulted in a significant decrease in miR-200b and miR-200c levels, respectively (Fig. 7A). Treatment of MMC with TGF-β and NC-I significantly down-regulated both FOG2 mRNA (Fig. 7B) and protein levels (Fig. 7, C and D) relative to MMC with NC-I alone. However, the FOG2 mRNA (Fig. 7B) and protein levels (Fig. 7, C and D) decreased by TGF-β treatment were both significantly restored in cells transfected with a mixture of 200b-I and 200c-I. These results further verify that TGF-β-induced miR-200b/c under diabetic conditions can down-regulate the expression of FOG2 in glomerular mesangial cells.

**miR-200b/c Inhibitors Attenuate Akt Activation in TGF-β-treated MMC**—Because the inhibition of miR-200b/c attenuated the down-regulation of FOG2 in TGF-β-treated MMC, and FOG2 down-regulates Akt phosphorylation, we further tested whether inhibition of miR-200b/c could also reverse the

glomerular mesangial cells (Fig. 4E), confirming that the down-regulation of FOG2 can enhance cellular hypertrophy in MMC. Together these results suggest that the down-regulation of FOG2 by TGF-β under diabetic conditions can augment Akt kinase activation and subsequently result in glomerular mesangial cell hypertrophy.

**miR-200b/c Are Up-regulated in MMC Treated with HG and TGF-β, as Well as in Glomeruli of Diabetic Mice**—miR-200b and miR-200c were recently shown to target FOG2 in human cell lines. Similarly, in D. melanogaster the corresponding fly analog miR-8 could target U-shaped (USH) (35). In addition, the 3′UTR of FOG2 is highly conserved in humans and mice. Therefore, we examined whether FOG2 could also be down-regulated by miR-200b/c under diabetic conditions in MMC.

First, miR-200b/c expression levels were evaluated in HG-treated MMC. miR-200b and miR-200c levels were significantly increased in MMC after 72 h of HG treatment (Fig. 5A). A similar increase in miR-200b and miR-200c was also found in MMC treated with TGF-β (24 h) (Fig. 5B). miR-200b/c levels were further evaluated in glomeruli of db/db and STZ mice. miR-200b and miR-200c levels were significantly increased in glomeruli of db/db mice (Fig. 5C) as well as STZ mice (Fig. 5D) compared with corresponding control mice as reported previously (27, 28), verifying that miR-200b and miR-200c are up-regulated in glomerular mesangial cells and renal glomeruli under diabetic conditions.

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**Akt Is Activated by miR-200b/c in MMC**—Because our results showed that FOG2 can suppress the phosphorylation of Akt, we next evaluated whether the 200b-M and 200c-M induced down-regulation of FOG2 could also up-regulate Akt phosphorylation in MMC. Results showed that Akt phosphorylation was indeed significantly increased in MMC transfected with 200b-M and 200c-M compared with NC-M-transfected cells (Fig. 6, C and D), which occurred in parallel, to the down-regulation of FOG2 (Fig. 6, C and D).

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**miR-200b/c Inhibitors Attenuate Akt Activation in TGF-β-treated MMC**—Because the inhibition of miR-200b/c attenuated the down-regulation of FOG2 in TGF-β-treated MMC, and FOG2 down-regulates Akt phosphorylation, we further tested whether inhibition of miR-200b/c could also reverse the

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**FIGURE 4.** Down-regulation of FOG2 induces Akt phosphorylation in MMC. A, transfection with siFOG2 significantly decreased mRNA levels of FOG2 in MMC relative to nontargeting control siRNA (siNTC). Results were normalized with internal control 18 S. Mean ± S.E. (n = 4). B–D, immunoblotting analysis of MMC after transfection with siFOG2 (5 and 10 nM for 24 h). C, FOG2 protein levels significantly decreased after transfection with siFOG2 (normalized to β-actin). Mean ± S.E. (n = 4). D, transfection with siFOG2 significantly increased phosphorylation of Akt (normalized to total Akt). Mean ± S.E. (n = 4). E, cellular protein levels were calculated as ratio of total protein amount/total cell number. Protein content (hypertrophy) was increased by TGF-β in serum-depleted cells. FOG2 down-regulation by siFOG2 transfection induced hypertrophy in MMC to a similar extent as TGF-β. Mean ± S.E. (n = 4). ** and * indicate p < 0.01 and p < 0.05, respectively.
TGF-β-induced increase in Akt phosphorylation in MMC. Treatment of MMC with TGF-β significantly up-regulated Akt phosphorylation relative to untreated MMC as expected (Fig. 7, C and E). However, in 200b-I- and 200c-I-transfected MMC, TGF-β-induced Akt phosphorylation was significantly attenuated compared with that seen in NC-I-transfected MMC (Fig. 7, C and E).

**FOG2 Down-regulation Activates ERK as Well as Akt in a PI3K-dependent Manner in MMC**—MMC transfected with siFOG2 and 200b-M were treated with a PI3K inhibitor, LY-294002, and Akt-specific inhibitor, MK-2206 (36, 38), to confirm that increased Akt phosphorylation is via PI3K signaling mediated by miR-200b/c-induced FOG2 down-regulation. FOG2 protein levels were significantly decreased in siFOG2-transfected MMC compared with siNTC-transfected MMC (Fig. 8, A and B). Akt was activated in siFOG2-transfected MMC. This increase in Akt phosphorylation was significantly reduced in MMC treated with LY-294002 and MK-2206 (Fig. 8, A and C). The activation of ERK was also evaluated in MMC transfected with siFOG2 and 200b-M treated with LY-294002 and MK-2206 to investigate whether Akt was the only downstream pathway affected by FOG2 and miR-200b/c. ERK was significantly activated in siFOG2-transfected MMC as evidenced by increased phospho-ERK levels. This activation was significantly attenuated by LY-294002 treatment. However, ERK activation was not significantly affected by MK-2206 (Fig. 8, A and D).

Similarly, in 200b-M transfected MMC, FOG2 protein levels were significantly decreased (Fig. 9, A and B). This was accompanied by an increase in Akt phosphorylation. Activated Akt in 200b-M-transfected MMC was attenuated in LY-294002- and MK-2206-treated MMC (Fig. 9, A and C), showing that Akt activation associated with FOG2 down-regulation induced by miR-200b/c is PI3K-dependent. In addition, ERK phosphorylation was significantly increased in 200b-M-transfected MMC, and this was significantly attenuated by LY-294002 treatment. However, increased ERK phosphorylation induced by 200b-M was not blocked by MK-2206, suggesting that ERK is not downstream of Akt in MMC (Fig. 9, A and D). These results suggest that, in addition to Akt, ERK is also activated through miR-200b/c-induced FOG2 down-regulation. Moreover, the results also suggest that FOG2-regulated activation of both Akt and ERK is through a common upstream effector, PI3K.

**miR-200b/c Enhance Hypertrophy in MMC**—Because miR-200b/c could down-regulate FOG2 and subsequently enhance Akt phosphorylation, we further examined whether miR-200b/c can also directly increase glomerular mesangial cell hypertrophy, a downstream consequence of Akt or ERK phosphorylation. Transfection of MMC with 200b-M or 200c-M significantly increased cellular protein content compared with control NC-M-transfected MMC (Fig. 10A). In addition, transfection with a mixture of 200b-I and 200c-I significantly attenuated the TGF-β-induced increase in cellular protein content relative to that seen in NC-I-transfected MMC (Fig. 10B). These results suggest that, in response to TGF-β or diabetic conditions, up-regulated miR-200b/c, which targets FOG2, can lead to a down-regulation of FOG2 and subsequent increase in Akt (and ERK) phosphorylation to promote hypertrophy in MMC (Fig. 11).
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In this study, we showed for the first time that diabetic conditions can down-regulate FOG2 and that the down-regulation of FOG2 plays a role in TGF-β-induced Akt activation in MC. In addition, FOG2 down-regulation by miR-200b and miR-200c mimics. Transfection with 200b-M or 200c-M significantly decreased FOG2 mRNA levels. Results were normalized with internal control 18 S. Mean ± S.E. (n = 4). C–E, immunoblotting analysis of MMC after transfection with 200b-M and 200c-M. D, FOG2 protein levels normalized to β-actin were significantly decreased after transfection with 200b-M or 200c-M. Mean ± S.E. (n = 4). E, transfection with 200b-M or 200c-M significantly increased phosphorylation of Akt (p-Akt) normalized to total Akt. Mean ± S.E. (n = 4). ** and * indicate p < 0.01 and p < 0.05, respectively.

DISCUSSION

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tion that FOG2 levels are lower in the glomeruli of both type 1 and type 2 diabetic mice. It also suggests that FOG2 might not play a significant role in renal tubular pathophysiology under diabetic conditions. Furthermore, the decreased FOG2 transcript expression in glomeruli from human subjects with DN suggests that the proposed miR-200-FOG2-Akt pathway may be significant not only in experimental animal models but also in human diabetic kidney disease as well.

**FIGURE 7.** TGF-β-induced down-regulation of FOG2 restored by inhibitors of miR-200b (200b-I) and miR-200c (200c-I) in MMC. A, expression levels of miR-200b/c in MMC transfected with miR-200b or miR-200c inhibitors. Transfection with 200b-I or 200c-I significantly decreased miR-200b or miR-200c expression levels, respectively, relative to negative control inhibitor (NC-I) oligonucleotides. Results were normalized with internal control U6. Mean ± S.E. (n = 3). B, expression of FOG2 mRNA levels in MMC treated with TGF-β after transfection with negative control inhibitor (NC-I) or 200b-I and 200c-I. Treatment of MMC with TGF-β down-regulated FOG2 mRNA levels. Transfection with miR-200b-I and 200c-I reversed this effect. Results were normalized with internal control 18 S. Mean ± S.E. (n = 5). C–E, immunoblotting analysis of MMC after TGF-β treatment and transfection with 200b-I and 200c-I. D, FOG2 protein levels (normalized to β-actin) were significantly decreased after TGF-β treatment for 24 h. Transfection with 200b-I and 200c-I significantly restored FOG2 protein levels decreased by TGF-β. Mean ± S.E. (n = 4). E, treatment with TGF-β significantly increased phosphorylation of Akt. Transfection with 200b-I and 200c-I blocked TGF-β-induced up-regulation of Akt phosphorylation (normalized to total Akt). Mean ± S.E. (n = 4). ** and * indicate p < 0.01 and p < 0.05, respectively.
It is possible that TGF-β induced in mesangial cells under diabetic conditions can up-regulate miR-200b/c via Smad3. A recent report demonstrated that Smad3 directly binds to a Smad-binding element located in the promoter region of miR-200b, functioning as a transcriptional activator in gastric cancer cells (47). In addition, our previous results have shown that miR-192 induced by TGF-β can target and down-regulate Zeb1/2, functioning as a transcriptional activator in gastric cancer cells (47). In addition, our previous results have shown that miR-200b was reported to be up-regulated not only in mesangial cells but also in endothelial cells and podocytes treated with high glucose (28). In addition, increased levels of the miR-200 family members were also observed in human kidneys from hypertensive nephrosclerosis, IgA nephropathy, and lupus nephritis patients, suggesting a key role for miR-200 in renal diseases and TGF-β actions (48–50). We recently demonstrated a pro-fibrotic role for miR-200b/c in enhancing the expression of TGF-β, collagen type I α-2, and collagen type IV α-1, by targeting Zeb1/2, repressors that bind to the promoters of these genes (27). In this study, we found that miR-200b/c can down-regulate FOG2, an inhibitor of PI3K. Our observation
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FIGURE 10. miR-200b and miR-200c regulate TGF-β-induced hypertrophy in MMC. A and B, cellular protein levels were calculated as ratios of total protein amount/total cell number. A, protein levels (hypertrophy) were increased in cells transfected with miR-200b and miR-200c mimics. Mean ± S.E. (n = 4). B, TGF-β treatment increased hypertrophy in serum-depleted cells. Transfection with miR-200b and miR-200c inhibitors attenuated TGF-β-induced hypertrophy. Mean ± S.E. (n = 4). ** and * indicate p < 0.01 and p < 0.05, respectively.

FIGURE 11. Schematic model of Akt and ERK activation and hypertrophy through miR-200b/c and FOG2 pathway in response to TGF-β in the pathogenesis of diabetic nephropathy. TGF-β induced by diabetic conditions up-regulates miR-200b/c which targets FOG2, ensuing a down-regulation of FOG2. The decrease in FOG2 increases PI3K and Akt activation, leading to MMC hypertrophy. In addition, increased PI3K by FOG2 down-regulation also activates ERK, which can play a role in fibrosis and other mechanisms related to diabetic nephropathy.

that there is a more rapid decrease in FOG2 protein levels compared with mRNA levels after TGF-β treatment in MMC (Fig. 3, A and B) further supports the operation of this post-transcriptional regulation. The current results suggest that, in addition to accelerating renal fibrosis, miR-200b/c play a role in transducing TGF-β signals by activating the PI3K-Akt pathway, thereby promoting glomerular mesangial hypertrophy. It is also noteworthy that a single miRNA (miR-200) family can act on multiple targets (Zeb1/2 and FOG2), which work in conjunction to manifest the pathologic changes seen in the kidney under diabetic conditions.

miR-200a has also been reported to be decreased in some models of increased renal fibrosis and epithelial to mesenchymal transition (32), which could be due to differences in the models and experimental conditions used as well as cell type specificity in the actions of miR-200 family members. However, Kim et al. (51) showed that miR-200a and miR-141 are not efficiently extracted because of low GC content. Therefore, it is possible that in some instances, the levels of low GC content miRNAs like miR-200a may be underestimated even though they are not decreased (and likely even increased). miR-200b (not affected by extraction methods according to the same paper) was shown to be increased in some reports (27, 28). In this study, we also detected increased levels of miR-200b and miR-200c under our experimental conditions.

It is possible that FOG2 is also regulated by other miRNAs besides miR-200b/c. miRNAs such as miR-103 and miR-183 have potential binding sequences in the 3’UTR of FOG2 (predicted by TargetScan). Several miRNAs that are highly expressed in the kidney have been previously found to be up-regulated in the glomeruli of diabetic animal models compared with corresponding controls (3, 19, 22, 27–29, 34, 52, 53). In addition, some of these miRNAs were also shown to be increased in HG- or TGF-β-treated mesangial cells (27–29, 34). However, in these studies, miRNAs potentially targeting FOG2, other than miR-200b/c, were not found to be differentially expressed in the diabetic environment in the kidney or mesangial cells, thus supporting the notion that miR-200b/c could be the main miRNAs regulating FOG2 in renal glomeruli or MC under diabetic conditions. Nevertheless, the involvement of other miRNAs cannot be fully ruled out.

Akt has been shown to be activated in response to TGF-β signaling in several cell types (15, 19, 54). TGF-β also enhances Akt phosphorylation in multiple renal cells, including glomerular mesangial cells, podocytes, and renal tubular cells (15, 19, 37, 55, 56). However, in hematopoietic cells, TGF-β has been shown to inhibit Akt phosphorylation by inducing the phosphatase SHIP, an inhibitor of Akt activation (57). In addition, in adult hepatocytes, TGF-β failed to activate Akt (58). Therefore, although our results demonstrate that FOG2 can modulate TGF-β-Akt signaling in glomerular mesangial cells, the role of FOG2 in TGF-β signal transduction may also be cell type- as well as disease-specific.

Interestingly, ERK was also activated during miR-200b/c-induced FOG2 down-regulation. ERK activation is known to be involved in TGF-β-induced collagen expression as well as HG-induced cellular hypertrophy in mesangial cells (59–61). Blockage of ERK by specific ERK inhibitors has been shown to attenuate high glucose-induced cellular hypertrophy and fibronectin expression in mesangial cells and renal tubular cells (62, 63), suggesting ERK as one of the signaling pathways in the progression of DN along with Akt (64). However, the molecular mechanisms by which TGF-β activates ERK are not fully understood. The present findings raise the possibility that FOG2 down-regulation by miR-200b/c could be a common pathway in PI3K-dependent activation of both Akt and ERK, regulating both the early hypertrophic features as well as later glomerular fibrotic characteristics of DN. Therefore, the pathologic changes found in mesangial cells under diabetic conditions might be due to the activation of both Akt and ERK through a common effector, PI3K.

The up-regulation of FOG2 by miR-200b/c inhibitors in TGF-β-treated cells was sufficient not only to attenuate Akt
activation but also to abrogate TGF-β-induced hypertrophy. Renal hypertrophy is recognized as an early pathologic finding in the course of DN progression (5). In addition, hypertrophic growth precedes fibrotic changes in the kidney under diabetic conditions (6). Therefore, repressing renal hypertrophy by inhibiting miR-200b/c and restoring FOG2 in the early stages of DN could be novel approaches to slow down disease progression. Further in vivo investigations are necessary to evaluate this.

In summary, the current results demonstrate that post-transcriptional regulation of FOG2, a target of miR-200b/c which are induced by TGF-β in MMC, plays a role in activating the PI3K-Akt-ERK pathway under diabetic conditions (Fig. 11). This new link could be an additional novel mechanism by which TGF-β induces cellular hypertrophy and fibrosis in DN, thereby leading to renal disease progression.

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