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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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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.

Glomerular hypertrophy is a hallmark of diabetic nephropathy. Akt kinase activated by transforming growth factor-β1 (TGF-β) plays an important role in glomerular mesangial hypertrophy. However, the mechanisms of Akt activation by TGF-β are not fully understood. Recently, miR-200 and its target FOG2 were reported to regulate the activity of phosphatidylinositol 3-kinase (the upstream activator of Akt) in insulin signaling. Here, we show that TGF-β activates Akt in glomerular mesangial cells by inducing miR-200b and miR-200c, both of which target FOG2, an inhibitor of phosphatidylinositol 3-kinase activation. FOG2 expression was reduced in the glomeruli of diabetic mice as well as TGF-β-treated mouse mesangial cells (MMC). FOG2 knockdown by siRNAs in MMC activated Akt and increased the protein content/cell ratio suggesting hypertrophy. A significant increase of miR-200b/c levels was detected in diabetic mouse glomeruli and TGF-β-treated MMC. Transfection of MMC with miR-200b/c mimics significantly decreased the expression of FOG2. Conversely, miR-200b/c inhibitors attenuated TGF-β-induced decrease in FOG2 expression. Furthermore, miR-200b/c mimics increased the protein content/cell ratio, whereas miR-200b/c inhibitors abrogated the TGF-β-induced increase in protein content/cell. In addition, down-regulation of FOG2 by miR-200b/c could activate not only Akt but also ERK, which was also through PI3K activation. These data suggest a new mechanism for TGF-β-induced Akt activation through FOG2 down-regulation by miR-200b/c, which can lead to glomerular mesangial hypertrophy in the progression of diabetic nephropathy.

Diabetic nephropathy (DN)[2] 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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[2] The abbreviations used are: DN, diabetic nephropathy; MMC, mouse mesangial cell; PTEN, Phosphatase and tensin homolog; miRNA, MicroRNA; CON, nondiabetic control mice; STZ, streptozotocin-induced diabetic mice; LY, LY-294002; MK, MK-2206; DMSO, dimethyl sulfoxide; NG, normal glucose; HG, high glucose; siFOG2, FOG2 siRNA; siNTC, nontarget control siRNA; 200b-M, miR-200b mimic; 200c-M, miR-200c mimic; NC-M, negative control mimic; 200b-I, miR-200b inhibitor; 200c-I, miR-200c inhibitor; NC-I, negative control inhibitor.

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 extracellular 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 miRNeasy 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′-GAGCTGCGAAGACGTGGAGT-3′ and 5′-CCAGGCTGTCCTGGTTTGTC-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 Laemmli'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 Fischer Scientific Inc. (Waltham, MA).

MMC Transfection—Cells (1×10^6/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 1×10^7/ml. Subsequently, 100 μl of cell suspension (1×10^6 cells) was mixed with miRNA mimic, hairpin inhibitor oligonucleotides, or ON-TARGET plus siRNA or negative controls (Thermo Fischer 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. 1, *A–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. 1*D*). Conversely, *TGF-β1* gene expression levels were increased in the glomeruli from *db/db* mice (Fig. 1*E*). A similar decrease in FOG2 staining was observed in glomeruli from streptozotocin-induced type 1 diabetic mice (STZ) compared with nondiabetic control mice (CON) (Fig. 1, *F–H*). *FOG2* mRNA levels were lower in STZ glomeruli (Fig. 1*I*), whereas *TGF-β1* expression levels were significantly higher (Fig. 1*J*).

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. 2, *A–C*). The down-regulation of FOG2 in HG-treated MMC was associated with an increase in Akt phosphorylation (Fig. 2, *B* and *D*). These changes were accompanied by a significant increase of *TGF-β1* expression in HG-treated MMC (Fig. 2*E*). 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

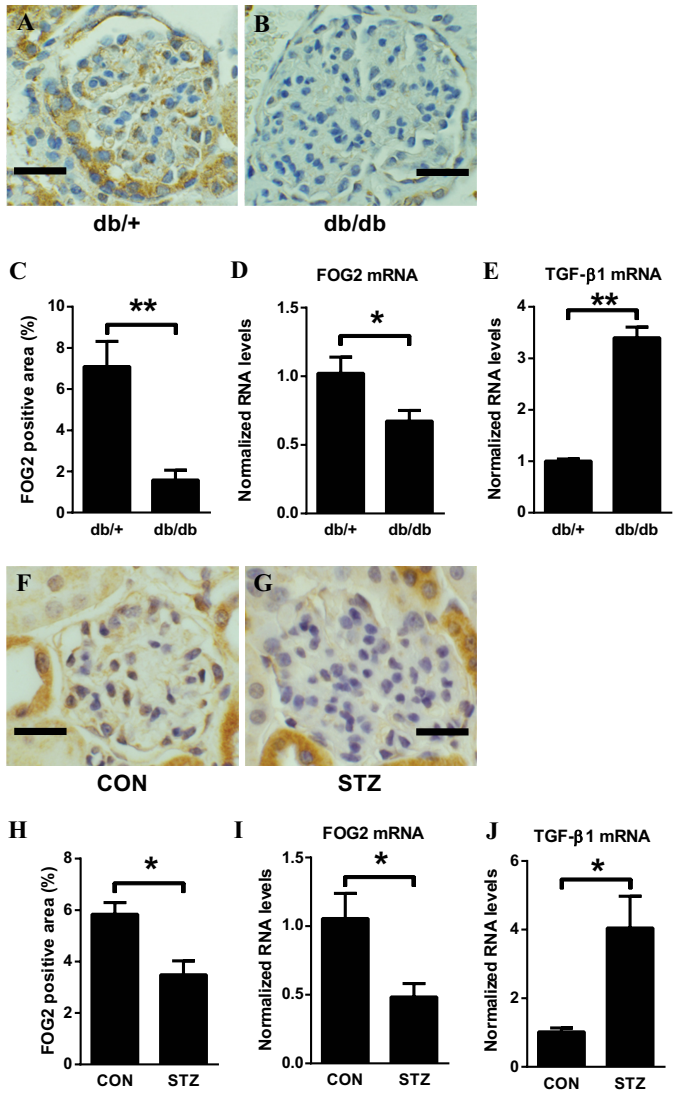

FIGURE 1. **Decreased levels of FOG2 in glomeruli from mouse models of type 2 (*db/db*) and type 1 diabetes (streptozotocin-induced).** *A* and *B*, representative immunohistochemistry staining of FOG2 in glomeruli from genetic control *db/+* mouse (*scale bar*, 20 μm) (*A*) and from diabetic *db/db* 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. Significant decrease of *FOG2* mRNA 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 glomeruli 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 18 S. Mean ± S.E. *F* and *G*, representative immunohistochemistry staining of FOG2 in glomeruli from control (*CON*) mouse (*scale bar*, 20 μm) (*F*) and from STZ-induced diabetic mouse (*G*). *H*, percentage of FOG2-positive area. Significant decrease of FOG2 staining is observed in glomeruli from STZ compared with those from CON mice. For each animal, 10 glomeruli were evaluated. Mean ± S.E. *I*, *FOG2* mRNA expression in glomeruli. Significant decrease of *FOG2* mRNA in glomeruli from STZ mice compared with those from CON mice. *J*, *TGF-β1* mRNA expression in glomeruli. Significant increase in *TGF-β1* mRNA in glomeruli from STZ mice compared with those from CON mice. Glomeruli were isolated by a sieving technique from eight mice in each group. Results were normalized with internal control 18 S. Mean ± S.E. ** and * indicate $p < 0.01$ and $p < 0.05$, respectively.

24 h; Fig. 3*A*) 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*).

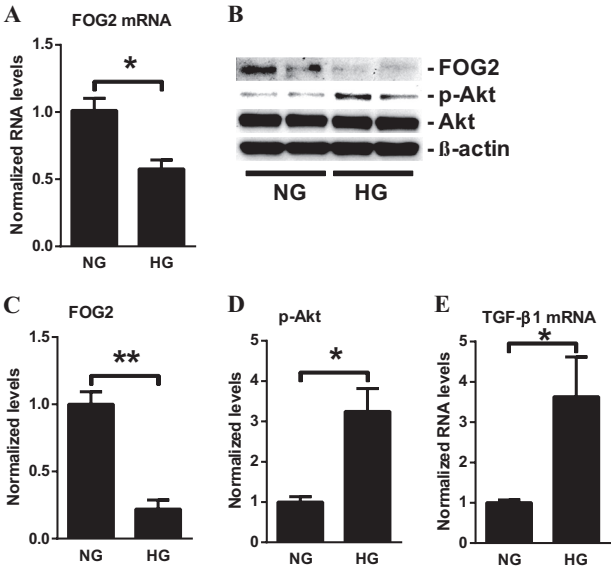

FIGURE 2. **High glucose decreases the expression of FOG2 in MMC.** *A, FOG2* mRNA levels in MMC treated with NG (5.5 mM) or HG (25 mM). Significant decrease was detected at 72 h after HG treatment compared with NG. Results were normalized with internal control 18 S. Mean ± S.E. ($n = 4$). *B*, immunoblotting analysis of MMC after treatment with HG (25 mM for 72 h). *C,* HG treatment significantly decreased protein levels of FOG2 (normalized to β-actin). Mean ± S.E. ($n = 4$). *D,* phosphorylation of Akt (*p-Akt*) (normalized to total Akt) significantly increased with HG treatment. Mean ± S.E. ($n = 4$). *E*, *TGF-β1* mRNA levels in MMC with HG treatment. Significant increase in *TGF-β1* mRNA in MMC treated with HG for 72 h compared with NG. Results were normalized with internal control 18 S. Mean ± S.E. ($n = 4$). ** and * indicate $p < 0.01$ and $p < 0.05$, respectively *versus* NG.

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. 4*A*) 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. 4*E*). Transfection of siFOG2 similarly induced hypertrophy in serum-depleted

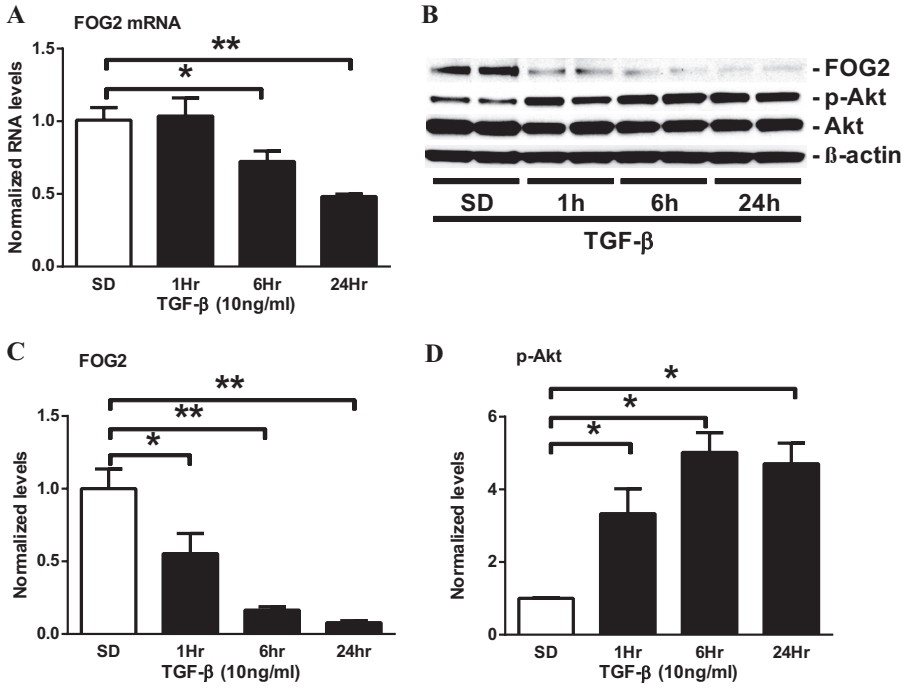

FIGURE 3. **TGF-β decreases the expression of FOG2 in MMC.** *A, FOG2* mRNA levels in MMC treated with TGF-β. Significant decrease was detected at 6 and 24 h after TGF-β treatment. Results were normalized with internal control 18 S. Mean ± S.E. ($n = 4$). *B–D,* immunoblotting analysis of MMC after treatment with TGF-β (10 ng/ml for 1 h to 24 h). *C,* TGF-β treatment significantly decreased protein levels of FOG2 at 1–24 h (normalized to β-actin). Mean ± S.E. ($n = 4$). *D,* phosphorylation of Akt (*p-Akt*) (normalized to total Akt) significantly increased with TGF-β treatment. Mean ± S.E. ($n = 4$). ** and * indicate $p < 0.01$ and $p < 0.05$, respectively *versus* S.D.

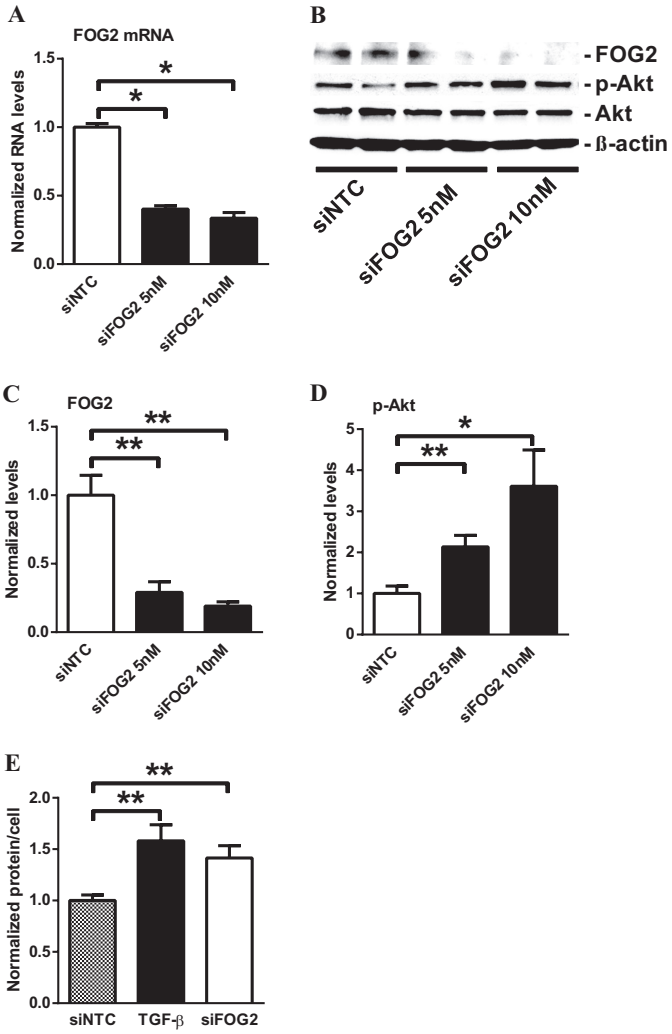

FIGURE 4. **Down-regulation of FOG2 induces Akt phosphorylation in MMC.** *A,* transfection with siFOG2 significantly decreased mRNA levels of *FOG2* in MMC relative to nontargeting control siRNA (*siNTC*). Results were normalized with internal control 18 S. Mean ± S.E. ($n = 4$). *B–D,* immunoblotting 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.

glomerular mesangial cells (Fig. 4*E*), 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. 5*A*). A similar increase in miR-200b and miR-200c was also found in MMC treated with TGF-β (24 h) (Fig. 5*B*). 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. 5*C*) as well as STZ mice (Fig. 5*D*) 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. 6*A*). Furthermore, results showed that 200b-M and 200c-M transfection significantly down-regulated both FOG2 mRNA (Fig. 6*B*) and protein levels (Fig. 6, *C* 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 *E*), 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. 7*A*). Treatment of MMC with TGF-β and NC-I significantly down-regulated both FOG2 mRNA (Fig. 7*B*) and protein levels (Fig. 7, *C* and *D*) relative to MMC with NC-I alone. However, the FOG2 mRNA (Fig. 7*B*) 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

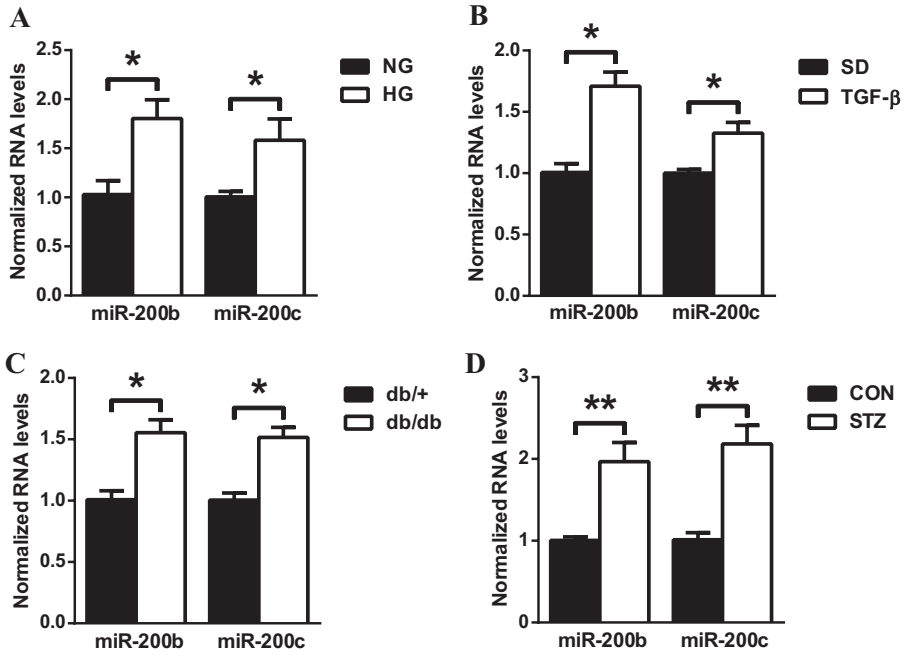

FIGURE 5. **Increased levels of miR-200b/c in TGF-β or HG-treated MMC and glomeruli from *db/db* or STZ-induced diabetic mice.** *A,* expression levels of miR-200b/c in MMC treated with NG (5.5 mM) or HG (25 mM) for 72 h. miR-200b and miR-200c levels were increased in HG-treated MMC relative to NG-treated MMC. Mean ± S.E. ($n = 4$). *B,* expression levels of miR-200b/c in MMC treated with or without TGF-β (10 ng/ml for 24 h). miR-200b and miR-200c levels were significantly increased by TGF-β. Mean ± S.E. ($n = 4$). *C,* miR-200b and miR-200c levels were increased in diabetic (*db/db*) mice glomeruli relative to respective control nondiabetic (*db/+*) mice. Glomeruli were isolated by a sieving technique from eight mice in each group. *D,* miR-200b and miR-200c levels were increased in STZ-induced diabetic mice compared with nondiabetic CON mice. Glomeruli were isolated by a sieving technique from eight mice in each group. Mean ± S.E. Results were normalized with internal control U6. ** 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. 10*A*). 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. 10*B*). 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).

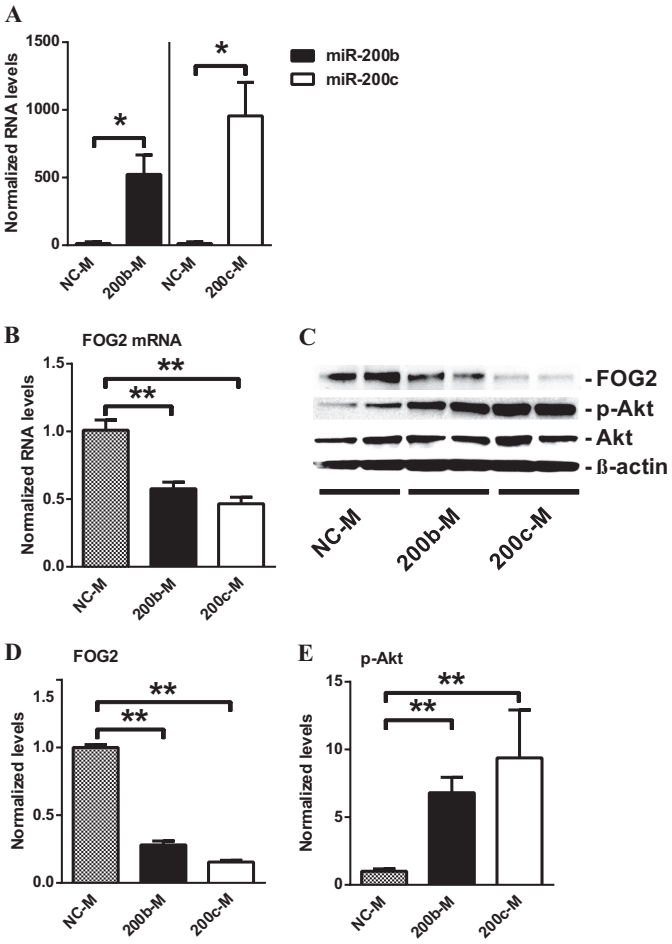

FIGURE 6. **FOG2 is down-regulated by mimics of miR-200b (*200b-M*) and miR-200c (*200c-M*) in MMC.** *A,* levels of miR-200b/c in MMC transfected with miR-200b and miR-200c mimics. Transfection with 200b-M or 200c-M significantly increased miR-200b and miR-200c levels, respectively, relative to NC-M oligonucleotides. Results were normalized with internal control U6. Mean ± S.E. ($n = 3$). *B,* expression of *FOG2* mRNA in MMC transfected with 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

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 was demonstrated to induce hypertrophy in MMC, thus identifying FOG2 as a new regulator in the pathogenesis of DN. Moreover, we have shown that, in addition to Akt, ERK, which is also one of the key players implicated in DN, could also be activated by the down-regulation of FOG2.

Our current results demonstrate an additional novel pathway by which Akt is activated by TGF-β. Accumulating evidence shows that TGF-β plays a key role in the pathogenesis of DN, which has been attributed to the activation of its downstream targets (1, 2, 10, 11). The Smad signaling pathway has been the best characterized effector of TGF-β receptor activation and signaling in DN progression (39, 40). However, the PI3K-Akt pathway has also been implicated in mediating the cellular effects of TGF-β in DN (15, 17, 18). Reports show that the PI3K-Akt signaling cascade triggered by TGF-β induces glomerular mesangial and renal tubular hypertrophy, important pathologic phenotypic changes in the course of DN progression (16, 41). Because of its well known role as a signal transducer of TGF-β in DN, the mechanisms by which TGF-β activates Akt has elicited much interest. Down-regulation of PTEN by TGF-β has been implicated in Akt activation (16), and recently, we showed that miR-216a- and miR-217-mediated down-regulation of PTEN, which de-phosphorylates phosphatidylinositol 1,4,5-trisphosphate to inhibit Akt activation, is a key mechanism of Akt activation by TGF-β in MMC (19). In addition to these previously suggested pathways, our current results demonstrate that another mechanism involving miR-200b/c targeting FOG2, which inhibits PI3K activity, can also contribute to TGF-β-induced Akt activation in mesangial cells under diabetic conditions. These results imply that TGF-β activates Akt through several indirect mechanisms mediated by key miRNAs and signaling intermediates. One report showed that, although TGF-β receptor activation induces PI3K activity, the TGF-β receptor and the regulatory subunit of PI3K do not bind directly (18) thereby suggesting several modes of Akt activation mechanisms, including our proposed indirect models.

The role of FOG2 in disease pathophysiology has not been clearly investigated although its physiologic functions have been demonstrated only recently. Previous studies have been mainly focused on the role of FOG2 in hematopoiesis and heart development (42, 43). In addition, FOG2 was found to be down-regulated in lung and prostate cancers, suggesting that it may possess tumor suppressor properties (44, 45). As indicated earlier, a recent study suggested that FOG2 plays a key role in insulin signaling in liver cells and adipocytes, although the physiologic relevance is not fully clear (35). Insulin receptor activation is known to transduce its signal through the PI3K-Akt pathway. FOG2 was shown to act as a negative modulator of the PI3K-Akt pathway by directly binding to p85α, the regulatory subunit of PI3K. Additionally, by targeting FOG2, the miR-200 family was implicated in the down-regulation of FOG2 to facilitate insulin signaling (35). Although these new players, miR-200 and FOG2, have broadened the understanding of physiologic Akt activation mechanisms in insulin signaling, the role of FOG2 in disease states is less understood. The results of this study reveal a novel new protective role for FOG2 against pathologic PI3K-Akt activation and hypertrophy in the glomerular mesangium in a disease state such as DN. In addition, they demonstrate that down-regulation of FOG2 can augment TGF-β-mediated signaling and Akt activation.

Interestingly, a recent transcriptome analysis of human renal glomeruli and tubular tissue from patients with diabetic kidney disease showed that *FOG2* (also known as *ZFPM2*) transcript expression was significantly decreased in renal glomeruli of patients with DN compared with glomeruli from control healthy kidneys. However, FOG2 expression levels did not differ in tubular tissue from DN patients compared with healthy controls (46). This report supports the significance of FOG2 down-regulation in diabetic glomerulopathy and our observa-

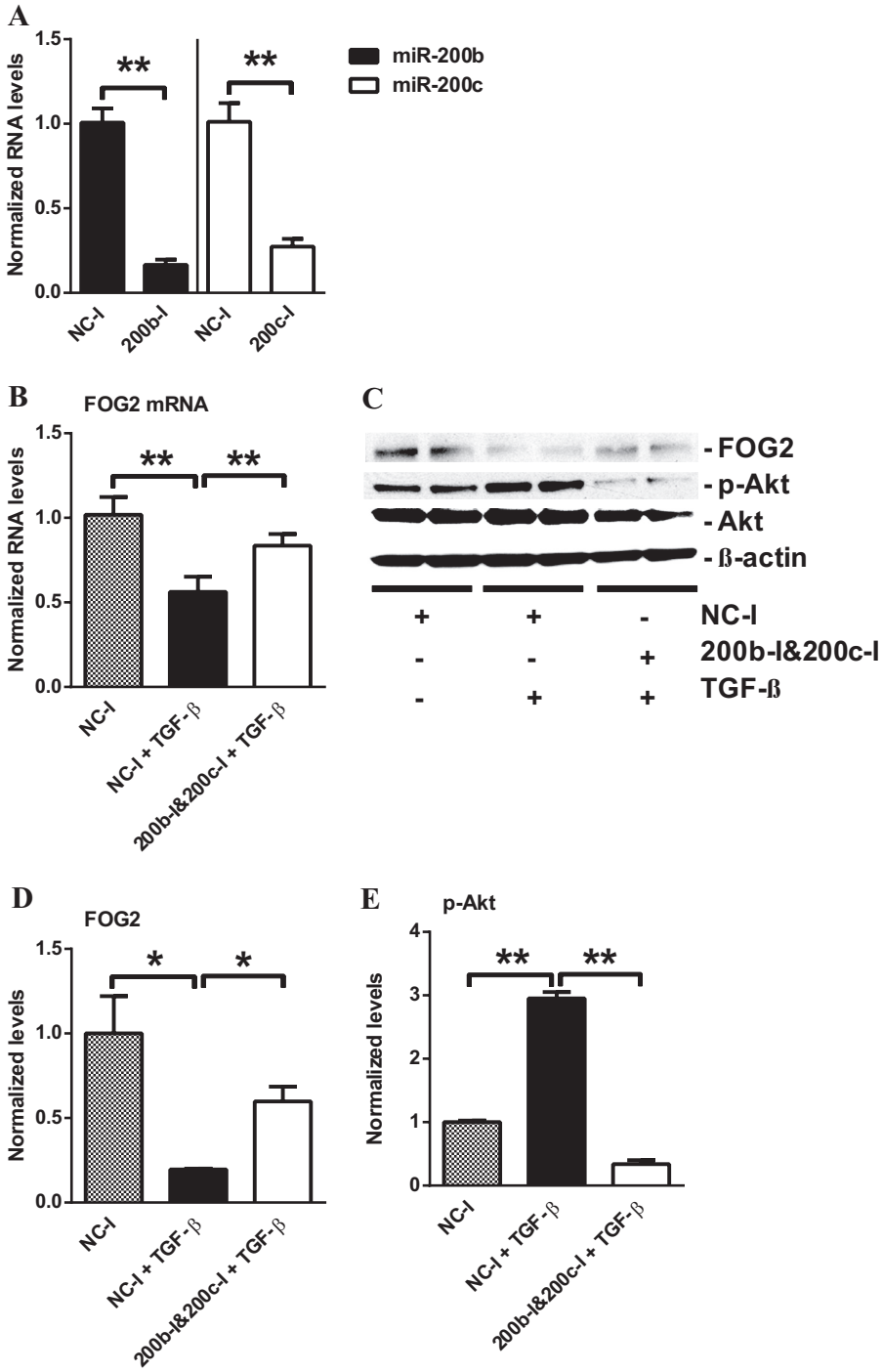

FIGURE 7. **TGF-*β*-induced down-regulation of FOG2 restored by inhibitors of miR-200b (*200b-I*) and miR-200c (*200c-I*) in MMC.** *A,* expression levels of miR-200b/c in MMC transfected with miR-200b or miR-200c inhibitors. Transfection with 200b-I or 200c-I significantly decreased miR-200b or miR-200c 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.

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.

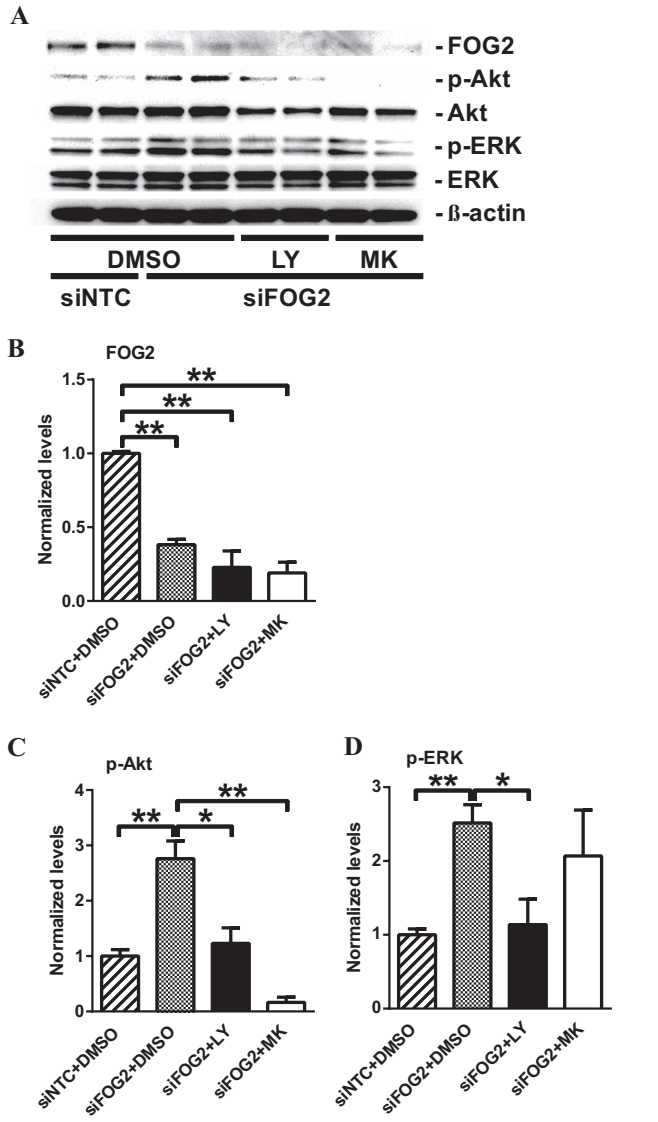

FIGURE 8. **FOG2 down-regulation phosphorylates Akt as well as ERK in a PI3K-dependent manner in MMC.** *A–D,* immunoblotting analysis of MMC after siFOG2 transfection followed by LY-294002 (*LY*) and MK-2206 (*MK*) treatment. siFOG2-transfected MMC were treated with either 20 μM LY or 1 μM MK for 24 h. *B*, FOG2 protein levels significantly decreased after siFOG2 transfection (normalized to β-actin). Mean ± S.E. ($n = 4$). *C,* transfection with siFOG2 increased phosphorylation of Akt. This increase in Akt phosphorylation was significantly attenuated by LY and MK treatment (normalized to total Akt). Mean ± S.E. ($n = 4$). *D*, siFOG2 transfection significantly increased phosphorylation of ERK. This increased ERK phosphorylation was significantly decreased by LY, but not by MK treatment (normalized to total ERK). Mean ± S.E. ($n = 4$). ** and * indicate $p < 0.01$ and $p < 0.05$, respectively.

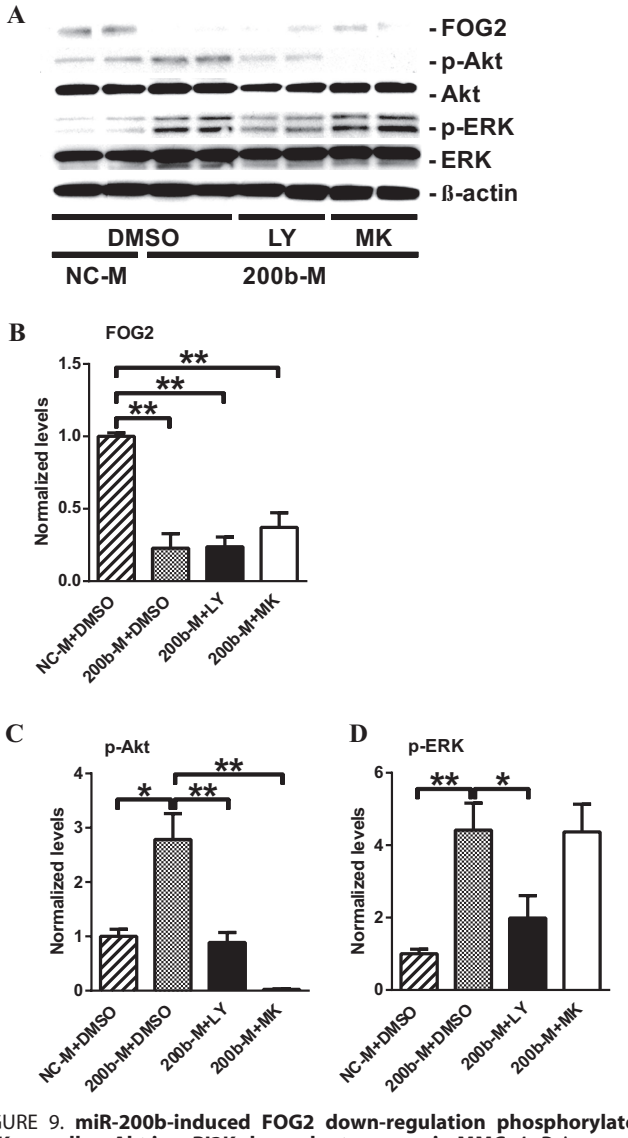

FIGURE 9. **miR-200b-induced FOG2 down-regulation phosphorylates ERK as well as Akt in a PI3K-dependent manner in MMC.** *A–D,* immunoblotting analysis of MMC after miR-200b mimic (*200b-M*) transfection followed by LY-294002 (*LY*) and MK-2206 (*MK*) treatment. 200b-M transfected MMC were treated with either 20 μM LY or 1 μM MK for 24 h. *B*, FOG2 protein levels significantly decreased after 200b-M transfection (normalized to β-actin). Mean ± S.E. ($n = 4$). *C,* transfection with 200b-M increased phosphorylation of Akt. This increase in Akt phosphorylation was significantly attenuated by LY and MK treatment (normalized to total Akt). Mean ± S.E. ($n = 4$). *D*, 200b-M transfection significantly increased phosphorylation of ERK. This increased ERK phosphorylation was significantly decreased by LY, but not by MK treatment (normalized to total ERK). 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 (E-box repressors) and thereby enhance the expression of miR-200b/c probably through E-boxes in their promoters (27). Moreover, miR-200 family also targets Zeb1/2, further enhancing the expression of miR-200 family itself through a positive feedback loop under diabetic conditions (27). 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

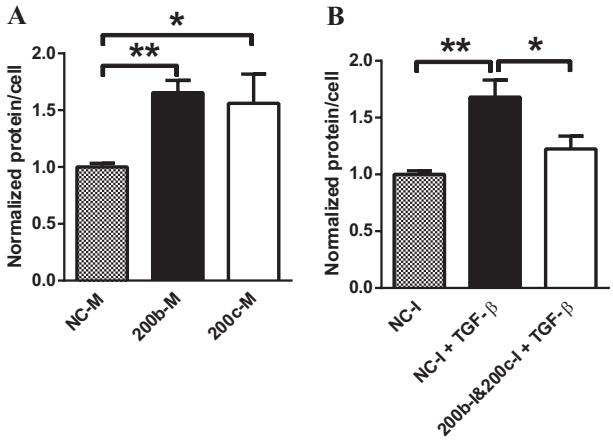

FIGURE 10. **miR-200b and miR-200c regulate TGF-β-induced hypertrophy in MMC.** *A* and *B*, cellular protein levels were calculated as ratios of total protein amount/total cell number. *A*, protein levels (hypertrophy) were increased in cells transfected with miR-200b and miR-200c mimics. Mean ± S.E. ($n = 4$). *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.

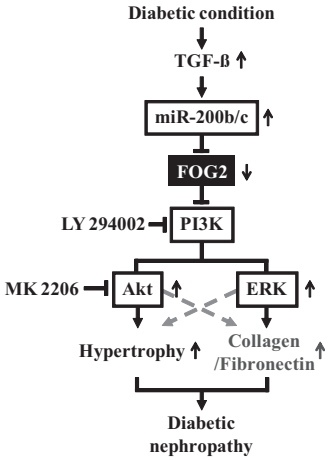

FIGURE 11. **Schematic model of Akt and ERK activation and hypertrophy through miR-200b/c and FOG2 pathway in response to TGF-β in the pathogenesis of diabetic nephropathy.** TGF-β induced by diabetic conditions up-regulates miR-200b/c which targets FOG2, ensuing a down-regulation of FOG2. The decrease 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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