EGF Receptor Deletion in Podocytes Attenuates Diabetic Nephropathy

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ABSTRACT

The generation of reactive oxygen species (ROS), particularly superoxide, by damaged or dysfunctional mitochondria has been postulated to be an initiating event in the development of diabetes complications. The glomerulus is a primary site of diabetic injury, and podocyte injury is a classic hallmark of diabetic glomerular lesions. In streptozotocin-induced type 1 diabetes, podocyte-specific EGF receptor (EGFR) knockout mice (EGFR<sub>podKO</sub>) and their wild-type (WT) littermates had similar levels of hyperglycemia and polyuria, but EGFR<sub>podKO</sub> mice had significantly less albuminuria and less podocyte loss compared with WT diabetic mice. Furthermore, EGFR<sub>podKO</sub> diabetic mice had less TGF-β1 expression, Smad2/3 phosphorylation, and glomerular fibronectin deposition. Immunoblotting of isolated glomerular lysates revealed that the upregulation of cleaved caspase 3 and downregulation of Bcl2 in WT diabetic mice were attenuated in EGFR<sub>podKO</sub> diabetic mice. Administration of the SOD mimetic mito-tempol or the NADPH oxidase inhibitor apocynin attenuated the upregulation of p-c-Src, p-EGFR, p-ERK1/2, p-Smad2/3, and TGF-β1 expression and prevented the alteration of cleaved caspase 3 and Bcl2 expression in glomeruli of WT diabetic mice. High-glucose treatment of cultured mouse podocytes induced similar alterations in the production of ROS; phosphorylation of c-Src, EGFR, and Smad2/3; and expression of TGF-β1, cleaved caspase 3, and Bcl2. These alterations were inhibited by treatment with mito-tempol or apocynin or by inhibiting EGFR expression or activity. Thus, results of our studies utilizing mice with podocyte-specific EGFR deletion demonstrate that EGFR activation has a major role in activating pathways that mediate podocyte injury and loss in diabetic nephropathy.


The incidence of diabetic nephropathy continues to increase worldwide in association with the epidemic rise in diabetes. In the United States, diabetic kidney disease is now the major cause of end stage kidney disease and increases the rate of other complications, such as cardiovascular disease. Twenty to forty percent of all patients with diabetes will develop some form of kidney disease during their life.

Podocytes are highly specialized cells characterized by formation of foot processes that are interconnected by the slit diaphragm, which is a critical component of the glomerular filtration barrier. In both experimental animals and patients with type 2 diabetes, a reduction in the number of podocytes per glomerulus is associated with broadening of podocyte foot processes and is thought to contribute to the progression of diabetic nephropathy. Moreover, it is now widely recognized that proteinuria, specifically microalbuminuria, is one of the earliest clinically identifiable markers of diabetes-induced renal damage, and appearance of proteinuria indicates a compromised glomerular filtration barrier. Recent studies...

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suggested that reactive oxygen species (ROS) production, mediated primarily by NADPH oxidases of the Nox family, mediates podocyte injury, including podocyte apoptosis and detachment from the glomerular basement membrane.6,7

The EGF receptor (EGFR) is a member of the ErbB family of receptor tyrosine kinases that consist of an extracellular ligand-binding domain, a single membrane-spanning region, a homologous cytoplasmic protein tyrosine kinase domain, and a C-terminal tail with multiple phosphorylation sites. Ligand binding to EGFR leads to activation of the intrinsic kinase domain and subsequent phosphorylation on specific tyrosine residues within the cytoplasmic tail, including Y1068 and Y1173. In addition, EGFR may be activated by nonlbind-associatr intracellular Src family kinases, indicated by phosphorylation at Y845.

EGFR is widely expressed in the mammalian kidney, including the glomeruli, proximal tubules, and cortical and medullary collecting ducts.8–10 There is increasing evidence that EGFR is an important mediator of cell fate decisions, such as proliferation, cell lineage determination and differentiation, migration and even cell death.11,12 Aberrant EGFR receptor signaling pathway activations is associated with tumorigenesis,12,13 and we and other investigators have demonstrated that the EGFR activation is a pivotal mediator for renal fibrosis and may interact with TGF-β signaling.14–17

In this study, we investigated the role of podocyte EGFR in the development of diabetic nephropathy by using podocyte-specific EGFR deletion mice and determined that hyperglycemia-induced ROS production activates the Src kinase and thereby induces EGFR activation-dependent phosphorylation of ERK, activation of the TGF-β–Smad2/3 signaling pathway, downregulation of Bcl2, and upregulation of cleaved caspase 3 and leads to a reduction of the podocyte number per glomerulus and development of albuminuria, the hallmarks of early diabetic nephropathy.

RESULTS

Podocyte EGFR Deletion Attenuated Albuminuria and Podocyte Loss Induced by Hyperglycemia

Activation of EGFR in mammalian kidney cells including podocytes is an important step in the regulation of cell survival, migration, proliferation, and differentiation.10,11,18,19 Recent studies by us and others indicate that administration of EGFR tyrosine kinase inhibitors can slow progression of diabetic nephropathy in experimental animals.17,20 To study the potential role of EGFR expressed in diabetic podocytes, we developed a podocyte-specific EGFR knockout mouse (EGFR–/–) by crossing EGFRflg/flg mice21 with Podocin.Cre mice22 (Figure 1A). Effective cleavage of the EGFR floxed gene was verified by PCR analysis of isolated glomerular genomic DNA, with excision of exon 3 of the EGFR encoding gene (Figure 1B). Effective deletion of EGFR protein expression in podocytes was confirmed by immunoblotting analysis of isolated glomerular lysates (Figure 1C). Both EGFR–/– and wild-type (WT) mice were made diabetic by the streptozotocin injection 1 week after unilateral nephrectomy. The two groups of mice developed comparable levels of hyperglycemia within 6 days, which persisted through the course of the study.

Podocytes are essential for the integrity of the glomerular filtration barrier, and proteinuria is an indication of a compromised glomerular filtration barrier. The diabetic EGFR–/– mice developed significantly less albuminuria compared with the WT mice (urinary albumin/creatinine ratio WT versus EGFR–/–: 170.80±12.93 versus 80.8±6.14 µg/mg; P<0.001; n=5–7 mice/group) (Figure 2A). Electron microscopy revealed more severe segmental podocyte foot process effacement in the WT mice (Figure 2B).

Podocyte detachment from the glomerular basement membrane is proposed to be an early feature of diabetic kidney injury and may predict the progression of diabetic nephropathy, but the precise mechanisms of podocyte loss under diabetic conditions have not yet been completely elucidated. We observed significantly more podocytes loss in WT diabetic mice compared with the EGFR–/– diabetic mice (podocyte number/glomerulus: WT versus EGFR–/–: 9.71±0.56 versus 12.71±0.42, P<0.01, n=5–7 mice/group) (Figure 2, C and D). Further analysis of expression of a differentiated podocyte cell marker protein, synaptopodin,23 in isolated glomerular lysates indicated much less synaptopodin expression in WT mouse glomeruli (Figure 2, E and F). To determine the mechanism of podocyte loss, we isolated glomeruli and analyzed the expression levels of Bcl2, an antiapoptotic protein,24 and cleaved caspase3, a proapoptotic protein.25 We found that the expression of Bcl2 was downregulated and the expression of cleaved caspase3 was upregulated in WT diabetic glomeruli, and these alterations were significantly less in EGFR–/– mice (Figure 2, E and F).

Figure 1. Development of podocyte-specific EGFR deletion mice. (A) Schematic for the generation of EGFR–/– mice by crossing Podocin.Cre mice with EGFRflg/flg mice. (B) EGFR deletion of exon 3 and Cre expression are verified by PCR using glomerular genomic DNA as the template. (C) EGFR attenuation in podocytes is confirmed by immunoblotting of isolated glomerular lysates. Data are representative of three separate experiments (n=5–7 mice/group).
Figure 2. Proteinuria and podocyte loss are attenuated in mice with selective deletion of EGFR in podocytes. (A) EGFR<sup>podKO</sup> mice aged 9–10 weeks and their control littermates are subjected to five consecutive STZ injections. Mouse urine is collected for 24 hours and creatinine and albumin are measured at 32 weeks after STZ injection (n=5–7 per group). (B) Representative micrographs of electron microscopy from different groups of mouse kidney (n=3–5 per group). (C) Representative kidney sections are stained with the antibody against WT1. (D) Podocyte numbers are counted randomly in 15 glomeruli/section (n=5–7 per group). (E) Immunoblotting of isolated glomeruli lysates from EGFR<sup>podKO</sup> mice and their control littermates 32 weeks after STZ injection are analyzed with the indicated antibodies (n=5–7 mice/group). (F) Densitometry of the data in E. Data are presented as the mean±SEM. *P<0.05; **P<0.001. Veh, vehicle; STZ, streptozocin.
EGFR Deletion in Podocytes Attenuated the Increased TGF-β Signaling in Diabetic Kidney

Activation of the TGF-β signaling pathway plays a major role in the progression of diabetic nephropathy through induction of extracellular matrix accumulation by enhancing synthesis of collagen, fibronectin, and laminin, as well as by inhibiting matrix metalloproteinase-mediated extracellular matrix degradation.26 Our previous study found that in response to chronic angiotensin II infusion in renal proximal tubule epithelial cells, TGF-β–Smad2/3 signaling pathway was activated through a c-Src kinase-mediated EGFR-ERK signaling pathway activation.15 To determine whether the TGF-β signaling pathway was activated in diabetic podocytes through an EGFR-dependent mechanism, we examined and found increased phosphorylation levels of EGFR at three different sites (Y845, Y1068 and Y1173) as well as increased phosphorylation of c-Src, ERK1/2 as well as Smad2/3. The expression levels of TGF-β and fibronectin were upregulated in the glomeruli isolated from WT diabetic mice. There was marked inhibition of phosphorylation of EGFR at all three tyrosine residues, ERK and Smad2/3 phosphorylation, and TGF-β and fibronectin expression in EGFRpodKO diabetic mice. However, c-Src phosphorylation was unchanged (Figure 3, A and B). Immunoﬂuorescence of the mouse kidney sections with antibodies against fibronectin conﬁrmed that upregulated ﬁbronectin in glomeruli was markedly blunted in EGFRpodKO diabetic mice (Figure 3C).

ROS-Mediated Podocyte c-Src and EGFR/ERK Phosphorylation

Elevated levels of ROS due to either increased production or decreased antioxidant function in the kidney are implicated in the initiation and progression of diabetic nephropathy.1 Our recent studies demonstrated

Figure 3. Deletion of EGFR in podocytes attenuates ERK activation, TGFβ-Smad2/3 signaling activation, and fibronectin expression, but not Src phosphorylation, in diabetic kidney glomeruli. (A) Immunoblotting of isolated glomeruli lysates from EGFRpodKO mice and their control littermates 32 weeks after STZ injection are analyzed with indicated antibodies. (B) Density of the data in A. (C) Immunoreactivity in diabetic EGFRpodKO mice and littermate controls with speciﬁc antibodies against fibronectin (green), podocyte marker synaptopodin (red), and DAPI (blue) (n=5–7 mice/group). Veh, vehicle; STZ, streptozocin.
that NADPH oxidase–derived ROS production–mediated c-Src kinase activation was an upstream mediator of EGFR activation in renal proximal tubular epithelial cells in response to chronic angiotensin II treatment. A recent study also indicated that inhibition of c-Src kinase prevented progression of diabetic nephropathy. To examine whether NADPH oxidase–dependent ROS production induced c-Src kinase–dependent activation of the EGFR-ERK signaling pathway and TGF-β-Smad2/3 signaling pathway in podocytes, WT diabetic mice were administered a mitochondria-targeted ROS scavenger, mito-tempol (10 mg/kg per day intraperitoneally), or a cell-permeable NADPH-oxidase inhibitor, apocynin (5mg/kg per day intraperitoneally), for 3 weeks with the first dose being given 2 days after initiation of diabetes. Both mito-tempol and apocynin treatments inhibited phosphorylation of c-Src, EGFR (Y845), ERK1/2, and Smad2/3; prevented upregulation of TGF-β expression; and prevented the alterations of synaptopodin, Bcl2, and cleaved caspase3 expression in diabetic mouse glomeruli. However, upregulated EGFR phosphorylation at both tyrosine 1068 (Y1068) and tyrosine 1173 was not affected by mito-tempol or apocynin administration (Figure 4).

To further confirm the role of ROS in activation of the EGFR-ERK pathway and TGF-β-Smad2/3 signaling pathway in podocytes, we utilized a conditionally immortalized mouse podocyte line. The cells were maintained at 33° in culture medium with IFNγ and then differentiated at 37°C in culture medium without IFNγ for 10 days, as previously described (Figure 5A). When the differentiated cultured podocytes were exposed to high glucose (25 mM) after 24 hours of quiescence, ROS production increased significantly within 4 hours, and treatment of the cells with either apocynin or mito-tempol markedly blunted the increased ROS (Figure 5B).

High-glucose treatment of the differentiated podocytes for 24 or 48 hours markedly increased phosphorylation of c-Src at Y416 and EGFR at Y1068, Y1173, and Y845, and also increased TGF-β, phosphor-Smad2/3, and cleaved caspase3 expression and decreased Bcl2 expression (Figure 6, A and B). Pretreatment of the differentiated podocytes with the EGFR tyrosine kinase inhibitor, AG1478, not only blocked EGFR to ERK and TGF-β-Smad2/3 signals but also prevented altered expression of cleaved caspase3 and Bcl2 (Figure 6, C and D). Apocynin or mito-tempol pretreatment of the differentiated podocytes markedly inhibited c-Src and EGFR tyrosine 845 (Y845) phosphorylation and activation of TGF-β-Smad2/3 signaling, but not EGFR phosphorylation at tyrosine 1068 (Y1068) or tyrosine 1173 (Y1173). In addition, both elevation of cleaved caspase3 and reduction of Bcl2 expression in response to high-glucose treatment were prevented by either apocynin or mito-tempol treatment (Figure 7, A and B). To confirm that c-Src kinase phosphorylation is upstream of EGFR phosphorylation and its downstream signaling, we

Figure 4. Inhibition of ROS production blunts the signaling pathway alterations in WT diabetic mouse glomeruli. WT 129svj mice aged 9–10 weeks are subjected to five consecutive STZ injections. (A and C) Apocynin (A) or mito-tempol (C) is administered by intraperitoneal injections starting 2 days after STZ injection for 3 weeks. Immunoblotting of isolated glomeruli lysates from control, diabetic mice with or without treatment is performed by using indicated antibodies. (B and D) Densitometry of the data in A and C, respectively (n=5–7 mice/group). Veh, vehicle; STZ, streptozocin.
determined that the c-Src kinase inhibitor, PP2, inhibited EGFR tyrosine 845 (Y845) phosphorylation-mediated TGF-β–Smad2/3 signaling pathway activation in the differentiated podocytes in response to high-glucose treatment, but not EGFR phosphorylation at tyrosine 1068 (Y1068) and tyrosine 1173 (Y1173). (Figure 7, C and D).

**Knocking Down EGFR-Inhibited TGF-β–Smad2/3 Signaling and Prevented Alterations of Bcl2 and Cleaved Caspase3 Expression in Podocytes in Response to High-Glucose Treatment**

To further investigate whether EGFR activation was essential for activation of the TGF-β–Smad2/3 signaling pathway in podocytes in response to high-glucose treatment, we knocked down EGFR protein expression by transfection of specific mouse small interfering RNA (siRNA) sequences in the cultured immortal mouse podocytes and found that downregulation of EGFR expression markedly attenuated TGF-β–Smad2/3 activation and alteration of cleaved caspase3 and Bcl2 expression in response to high-glucose exposure (Figure 8, A and B). For further investigation of cell morphologic changes in response to high-glucose treatment, the differentiated podocytes were stained with fluorescence-labeled phalloidin, which binds polymerized f-actin, after mannitol or glucose treatment. There was a striking reduction in actin stress fiber formation when the cells were exposed to high glucose. Knockdown of EGFR expression by siRNA limited the reduction in actin stress fiber formation in response to high-glucose treatment (Figure 8C).

**DISCUSSION**

This study provided evidence for the first time that podocyte-specific deletion of EGFR in mice attenuated albuminuria and podocyte loss in the diabetic kidney. In an attempt to determine the underlying mechanism, we found that EGFR deletion in podocytes inhibited TGF-β upregulation and activation of Smad2/3 in glomeruli of diabetic mice. Administration of a mitochondrial-targeted antioxidant (mito-tempol) or a cell-permeable NADPH oxidase inhibitor (apocynin) to the WT diabetic mice inhibited c-Src phosphorylation at Y416 (which is known to activate Src kinase), inhibited EGFR at Y845 (a c-Src kinase-mediated phosphorylation site), inhibited the TGF-β–Smad signaling pathway, and prevented proapoptotic mediators both in glomeruli from diabetic mice and in cultured podocytes exposed to hyperglycemia. Therefore, our results suggest that ROS-mediated activation of EGFR by c-Src kinase is a key mechanism of podocyte dysfunction and loss during the development of diabetic nephropathy.

In isolated diabetic mouse glomeruli from WT diabetic mice, inhibition of NADPH oxidase by apocynin or mito-tempol reduced EGFR phosphorylation at tyrosine 845 (Y845), but did not affect EGFR phosphorylation at tyrosine 1068 (Y1068) and at tyrosine 1173 (Y1173), which are well known autophosphorylation sites mediated by EGFR ligands. In vitro, mito-tempol or apocynin treatment of the differentiated podocytes not only blocked ROS production, but also inhibited c-Src kinase activation and phosphorylation of EGFR at tyrosine 845 (Y845) without affecting EGFR phosphorylation at tyrosine 1068 (Y1068) and at tyrosine 1173 (Y1173).

There is increasing evidence that continuous and aberrant EGFR activation is related to renal fibrogenesis. In this regard, we recently reported that in a model of accelerated diabetic nephropathy (endothelial nitric oxide synthase–streptozotocin mice), administration of the EGFR tyrosine kinase inhibitor, erlotinib, markedly reduced albuminuria and glomerulosclerosis. Similarly, studies in diabetic rats reported that a different EGFR tyrosine kinase inhibitor, PKI 166, attenuated early glomerular enlargement and preserved podocytes. Deletion of podocyte EGFR did not reduce c-Src kinase activation in diabetic glomeruli. By contrast, inhibition of ROS production in vivo or in vitro inhibited not only c-Src activation but also phosphorylation of EGFR at Y845, without decreasing EGFR phosphorylation at Y1068 and Y1173, suggesting that EGFR may be activated by both ligand-mediated and ligand-independent pathways in diabetes. In this regard, we recently reported increased expression of the EGFR ligand, HB-EGF, in diabetes that was mediated by...
endothelial dysfunction, and endothelial-specific HB-EGF deletion reduced progression of diabetic nephropathy.34

Previous studies by us and others indicated that activation of EGFR contributes to recovery from AKI35–37 by accelerating tissue regeneration but is detrimental in response to chronic kidney insults by increasing profibrotic pathways. We suggest that mild or moderate AKI induces acute and self-limited EGFR activation to promote cell repair and proliferation, whereas more severe AKI or a continuous chronic kidney injury will induce continued EGFR activation, which in turn will activate TGF-β and other signaling pathways mediating progressive renal fibrosis.

Increased ROS production has been posited to be a mediator of the injury associated with diabetes in various target organ systems, including the kidney.38,39 There are a number of potential sources for increased ROS production in the diabetic kidney, including NADPH oxidase, advanced glycation end products, defects in the polyol pathway, uncoupled nitric oxide synthase, and alterations in the mitochondrial respiratory chain via oxidative phosphorylation.40 It was proposed that overproduction of superoxide by mitochondria provides a unifying explanation for many of the pathophysiologic mechanisms underlying diabetic complications,41 although recent studies questioned this hypothesis.42 However, it was noteworthy that in the current studies, hyperglycemia-induced ROS production in cultured podocytes was inhibited both by an NADPH oxidase inhibitor, apocynin, and a mitochondria-targeted SOD, mito-tempol.

In summary, we found that in isolated glomerular lysates from streptozotocin injection induced type 1 diabetic WT or EGFRpodKO mice or cultured immortal podocytes after exposure to high-glucose medium, increased glomerular ROS production, subsequent Src kinase activation was upstream of EGFR activation in podocytes, and the activated EGFR induced TGF-β–Smad2/3 signaling and proapoptotic signaling, which led to reduction of podocyte number by apoptosis. In light of recent studies indicating that detrimental effects of TGF-β in diabetic nephropathy may be mediated by induction of mitochondrial Nox4 and increased ROS production via activation of TGF-β receptor dependent–Smad2/3 signaling,43,44 we propose that there may exist a vicious cycle, with high glucose directly activating NADPH oxidase–dependent ROS production and subsequent Src kinase–mediated EGFR signaling leading to TGF-β–Smad2/3 activation, which may in turn further activate mitochondrial NADPH oxidase to increase ROS production and Src kinase activity, thereby enhancing TGF-β–mediated podocyte injury.

**Figure 6.** Blocking EGFR phosphorylation inhibits the effects of high-glucose treatment on cultured podocytes. (A) With exposure of the differentiated podocytes to high glucose (25 mM) for either 24 or 48 hours, phosphorylation of Src at Y416; EGFR at Y845, Y1173, and Y1068; and Smad2/3 is increased. TGFβ and cleaved caspase3 expression is increased, but Bcl2 expression is inhibited. (B) Densitometry of the data in A. (C) In cultured differentiated podocytes, AG1478 treatment inhibits high-glucose–induced phosphorylation of EGFR and ERK, activation of TGFβ–Smad2/3 signaling, and expression alterations of cleaved caspase3 and Bcl2. (D) Densitometry of the data in C. Results from three separate experiments are shown. Veh, vehicle.
CONCISE METHODS

Reagents and Antibodies
Antibodies against EGFR, p-EGFR (Y1173, Y1068, and Y845), p-SRC (Y416), Bcl2, cleaved Caspase3, TGF-β1, and p-Smad2/3 were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against WT1, p-ERK1/2, total ERK, and all secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 488– or Alexa Fluor 594–conjugated secondary antibodies were from Life Technologies (Grand Island, NY). The specific EGFR tyrosine kinase inhibitor AG1478 and the inhibitor of Src tyrosine kinase PP2 were from EMD Millipore (Billerica, MA). Antibodies against fibronectin, streptozocin, 2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ymin)-2-oxoethyl) triphenylphosphonium chloride (mito-tempol), 4'-hydroxy-3'-methoxyacetophenone (apocynin), 2',7'-dichlorodihydrofluorescein diacetate, and all other reagents were from Sigma-Aldrich (St. Louis, MO).

Generation of EGFRpodKO Null Mice
The Vanderbilt University Institutional Animal Care and Use Committee approved all experiments and all experiments were conducted according to National Institutes of Health guidelines. EGFRfl/fox mice, which were generated as we previously described, were back-crossed onto a 129 Svj background for 10 generations followed by crossing with transgenic mice carrying Cre recombinase under the control of the podocin promoter (Pod-Cre), which were also back-crossed onto the 129 Svj background for 10 generations. Age-matched EGFRfl/fox littermates lacking the pod-Cre transgene were used as WT controls for Pod-Cre(+) EGFRfl/fox (EGFRpodKO) mice lacking the EGFR in podocytes. Mice were genotyped by PCR with the following primers: 5'-CTTTGGAGAACCTGCAGATC-3' and 5'-CTGCTACTGGCTCAAGTTTC-3' for verification of the EGFR gene flanked mice, 5'-ACACTAGCACTGACTGCTGG-3' and 5'-CTGCTACTGGCTCAAGTTTC-3' for verification of the EGF gene floxed mice, 5'-ACACTAGCACTGCTGGG-3' and 5'-CTGCTACTGGCTCAAGTTTC-3' for verification of the EGFR pod-Cre expression.

Induction of Diabetes in EGFRpodKO and WT Mice
Ten-week-old male EGFRpodKO and their WT mice or WT 129 Svj mice were injected daily with streptozocin (prepared freshly in 0.1 mol/L citrate buffer, pH 4.5, and given at a dose of 50 mg/kg body weight intraperitoneally) or vehicle alone for 5 consecutive days to induce type 1 diabetes. One week before streptozocin injection, unilateral nephrectomy surgery was performed in those mice that were used to analyze urine creatinine and albumin. Blood glucose was measured weekly.

Figure 7. Blocking ROS production or c-Src kinase activity inhibits the effects of high-glucose treatment on cultured podocytes. (A) Pretreatment of the differentiated podocytes with either apocynin or mito-tempol inhibited phosphorylations of c-Src at Y416 and EGFR at Y845, damped TGF-β-Smad2/3 signaling activation and blunted the alterations of cleaved caspase3 and Bcl2 expression in response to high glucose treatment. (B) Densitometry of the data in A. (C) Pretreatment of the differentiated podocytes with PP2 not only inhibited c-Src phosphorylation, but also restricted phosphorylation of EGFR and activation of TGF-β-Smad2/3 signaling pathway in response to high glucose treatment. (D) Densitometry of the data in C. Representative data from three separate experiments are shown. Veh, vehicle.
measured using the OneTouch Basic Blood Glucose Monitoring System (LifeScan, Milpitas, CA) on blood samples obtained via the saphenous vein after 5 hours of food deprivation. Mice were euthanized at indicated time points.

Measurement of Urine Creatinine and Albumin

We collected 24-hour urine samples from uninephrectomized EGFR<sup>podKO</sup> and WT mice 32 weeks after either streptozocin or vehicle injection. Urinary albumin levels were detected by a murine microalbuminuria ELISA kit (AlbuwellM) and the urinary creatinine was measured by using a microplate assay kit (Creatinine Companion; Exocell, Philadelphia, PA). All measurements were performed in triplicate and the ratio of urinary albumin (in micrograms per milliliter) to creatinine (in milligrams per milliliter) was calculated and expressed in micrograms per milligram. Results are expressed as the mean±SEM.

Isolation of Glomeruli by Using Dynabeads M-450

Three weeks after induction of hyperglycemia by streptozocin injection, diabetic and control mouse glomeruli were isolated as previously described with minor modifications. Briefly, mice were anesthetized by an intraperitoneal injection of Nembutal Sodium solution (50 mg/kg) and perfused with 4×10<sup>7</sup> Dynabeads (0.25 ml of commercial Dynabeads M-450 Tosylactivated beads) diluted in 20 ml of PBS through the heart. The kidneys were removed, minced, and digested in collagenase (1 mg/ml collagenase A, 100 U/ml deoxyribonuclease I in HEPES-buffered salt solution) at 37°C for 30 minutes with gentle agitation. The collagenase-digested tissue was gently pressed through a 100-μm cell strainer using a flattened pestle, and the cell strainer was then washed with 5 ml of HEPES-buffered salt solution. The filtered cells were passed through a new cell strainer without pressing and the cell strainer was washed with 5 ml of HEPES-buffered salt solution. The cell suspension was then centrifuged at 200×g for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in 2 ml of HEPES-buffered salt solution. Finally, glomeruli containing Dynabeads were gathered by a magnetic particle concentrator and washed three times with ice-cold HEPES-buffered salt solution. The isolated glomeruli were lysed in RIPA buffer followed by immunoblotting analysis.

Cell Culture

Immortalized mouse podocyte cells, from Dr. Peter Mundel (Harvard Medical School), were cultured as previously described. Briefly, cells were maintained at 33°C in RPMI 1640 medium containing 100 U/ml IFNγ and 10% FBS and induced to differentiate by shifting them to 37°C and culturing in RPMI 1640 medium containing 10% FBS without IFNγ for 10 days. The differentiated podocytes were made quiescent in RPMI 1640 medium containing 5.5 mM of glucose and 1% FBS for 24 hours followed by treatment with 25 mM mannitol or glucose with or without metformin (300 μM) or apocynin (500 μM), AG1478 (1 μM), and PP2 (5 μM) for 24 or 48 hours.

Immunoblotting

Immunoblotting procedures were performed as previously described with minor modifications. Briefly, for in vitro experiments, differentiated podocytes were cultured for 24 or 48 hours in quiescent
medium containing 25 mM of mannitol or glucose with or without mito-tempol, apocynin, or AG1478 followed by harvesting in RIPA buffer. For in vivo experiments, isolated EGFR<sup>podKO</sup> and/or WT mouse glomeruli were lysed in RIPA buffer and equal amounts of protein lysate were loaded directly onto 7.5%–15% SDS-PAGE gels, transferred onto Immobilon-P transfer membranes (EMD Millipore, Bedford, MA), and probed with the indicated primary antibody. The primary antibodies were detected with peroxidase-labeled goat anti-rabbit IgG or goat anti-mouse IgG and were exposed on film by using enhanced chemiluminescence (Amersham Biosciences, Ltd., Buckinghamshire, UK).

Transfection of EGFR siRNA
Mouse EGFR ON-TARGETplus SMARTpool siRNA (L-040411-00-0005) or negative control siRNA (Thermo Fisher Scientific, Lafayette, CO) were transfected into differentiated mouse podocytes by the Lipofectamine method (Invitrogen, Carlsbad, CA) as previously described.<sup>14</sup> Three days after transfection, the cells were made quiescent in RPMI 1640 medium containing 5.5 mM of glucose and 1% FBS for 24 hours followed by treatment with 25 mM of mannitol or glucose for 24 hours. Cells were harvested in RIPA buffer and the lysates were analyzed by immunoblotting or stained by phalloidin-FITC.

Measurement of Intracellular ROS Generation
Differentiated podocytes were cultured in 24-well plates and made quiescent in RPMI 1640 medium (containing 5.5 mM of glucose) containing 1% FBS for 24 hours, followed by washing once with HEPES-buffered salt solution (pH=7.4) (0.5 ml/well) containing 25 mM HEPES, 120 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl<sub>2</sub>, 25 mM NaHCO<sub>3</sub>, and 5.5 mM glucose. The cells were then left untreated or treated with mito-tempol (300 μM) or apocynin (500 μM) for 30 minutes before the addition of 25 mM glucose or 25 mM mannitol and 2',7'-dichlorodihydrofluorescein diacetate (100 μM) for 4 hours. The fluorescence intensity was measured using a fluorescence multwell plate reader with excitation and emission wavelengths of 485 nm and 530 nm, respectively.

Immunofluorescence Analyses
For mouse kidney tissues, immunofluorescence was performed on paraffin-embedded tissues fixed by 4% paraformaldehyde, using standard techniques as previously described.<sup>15</sup> Five-micrometer kidney sections were deparaffinized, rehydrated, subjected to antigen retrieval, and then incubated with rabbit primary antibodies (WT1, p-Smad2/3, and fibronectin) and mouse primary antibodies (synaptopodin) in 5% goat serum in PBS for 1 hour followed by incubation with Alexa Fluor 594– or Alexa Fluor 488–conjugated secondary antibodies for 1 hour. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). For cultured differentiated podocytes, the cells were fixed with 4% paraformaldehyde/PBS, permeabilized with 0.1% Triton X-100/PBS, and washed three times with PBS. After incubation with mouse anti-synaptopodin antibody (1:500) for 1 hour at room temperature. Cells were washed three times with PBS and then incubated with Alexa Fluor 488–conjugated donkey anti-mouse antibody for 1 hour. Nuclei were counterstained with DAPI. Cells were treated with 25 mM of mannitol or glucose for 24 hours after 24 hours of quiescence as described above. The differentiated podocytes were incubated with phalloidin/FITC (50 μg/ml) at room temperature for 40 minutes. The cells were washed once and counterstained with DAPI followed by three times of PBS washing before being covered with coverslips. Images were captured by using an Axioplan2 fluorescence microscope and an AxioCam HRc digital camera (Carl Zeiss).

Statistical Analyses
Data are presented as the mean±SEM for at least three separate experiments (each in triplicate). An unpaired t test was used for statistical analyses. ANOVA and Bonferroni t tests were used for multiple-group comparisons. A P value <0.05 compared with the control was considered statistically significant.

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DISCLOSURES
None.

REFERENCES