Vegfa Protects the Glomerular Microvasculature in Diabetes

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Vascular endothelial growth factor A (VEGFA) expression is increased in glomeruli in the context of diabetes. Here, we tested the hypothesis that this upregulation of VEGFA protects the glomerular microvasculature in diabetes and that therefore inhibition of VEGFA will accelerate nephropathy. To determine the role of glomerular Vegfa in the development and progression of diabetic nephropathy, we used an inducible Cre-loxP gene-targeting system that enabled genetic deletion of *Vegfa* selectively from glomerular podocytes of wild-type or diabetic mice. Type 1 diabetes was induced in mice using streptozotocin (STZ). We then assessed the extent of glomerular dysfunction by measuring proteinuria, glomerular pathology, and glomerular cell apoptosis. *Vegfa* expression increased in podocytes in the STZ model of diabetes. After 7 weeks of diabetes, diabetic mice lacking Vegfa in podocytes exhibited significantly greater proteinuria with profound glomerular scarring and increased apoptosis compared with control mice with diabetes or *Vegfa* deletion without diabetes. Reduced local production of glomerular Vegfa in a mouse model of type 1 diabetes promotes endothelial injury accelerating the progression of glomerular injury. These results suggest that upregulation of VEGFA in diabetic kidneys protects the microvasculature from injury and that reduction of VEGFA in diabetes may be harmful. ***Diabetes*** **61:2958–2966, 2012**

Diabetes is the leading cause of end-stage kidney failure in North America. A primary feature of diabetic nephropathy is dysfunction and injury of the microvasculature. To date, there has been significant scientific effort focused on understanding how hyperglycemia and other metabolic consequences of diabetes promote microvascular injury and end-organ damage. On the other hand, endogenous factors that slow or prevent development of complications are also likely to play a major role in shaping the manifestations of diabetic nephropathy. Vascular endothelial growth factor A (VEGFA) plays an important role in regulating glomerular structure and function and may also influence the outcome of diabetic kidney disease.

VEGFA is a secreted glycoprotein of the platelet-derived growth factor superfamily required for glomerular endothelial cell migration, differentiation, and survival (1). Glomerular visceral epithelial cells, known as podocytes, are a major source of VEGFA in the kidney (2). Both up- and downregulation of podocyte Vegfa levels during kidney development lead to glomerular disease in mice, while a reduction of glomerular VEGFA both in patients treated with VEGFA inhibitors and in adult transgenic mice with the deletion of *Vegfa* causes renal thrombotic microangiopathy (TMA) (1,3–5). Thus, proper regulation of VEGFA expression is critical for the healthy glomerulus.

In early stages of diabetes, Vegfa expression is increased in glomeruli. In rodents, both insulin deficiency and resistance are associated with increased production of renal Vegfa (6,7). This increase in VEGFA has been documented in renal biopsies and plasma from patients with type 1 or 2 diabetes (8,9), leading to the hypothesis that the increased level of VEGFA in diabetes is detrimental to glomerular function. In keeping with this model, overexpression of Vegfa in podocytes of transgenic mice is associated with features of diabetic nephropathy such as a thickened glomerular basement membrane and proteinuria (5,10,11). Studies of Vegfa inhibition in rodent models of diabetic nephropathy have generated mixed results, with some studies demonstrating protection from progression and others failing to show benefit (12–15). One drawback to inhibitor studies is potential lack of target specificity along with inability to determine the extent of inhibition in specific tissues.

Accordingly, we took a genetic approach that allows us to extinguish Vegfa signaling in the glomerulus with a precision and degree not possible with small molecule or other pharmacologic agents. To determine the role of local Vegfa production in podocytes in the development and progression of diabetic nephropathy, we used the streptozotocin (STZ) model of type 1 diabetes in mice. Using this approach, we show that loss of Vegfa from podocytes has adverse consequences for the glomerular structure and function in diabetic mice, resulting in global sclerosis and death within a few weeks. Our results suggest that upregulation of VEGFA is not necessarily detrimental for the diabetic glomerulus and may serve a protective function.

RESEARCH DESIGN AND METHODS

The generated transgenic mice carried the independent transgenes *Nphs2-rtTA*, *tetO-Cre*, and *Vegfa*$^{floxx/floxx}$ on a mixed background (3). All experiments were approved by the Animal Care Committee of Mount Sinai Hospital (Toronto, Canada) and were conducted in accordance with guidelines established by the Canadian Council on Animal Care. Diabetes was induced at 2.5 weeks of age by one daily intraperitoneal injection of STZ (50 mg/kg in fresh 0.1 mol/L citrate buffer, pH 4.5) for five consecutive days (http://www.diacomp.org). At 3.5 weeks of age, the *Vegfa* gene was deleted in a time-specific manner from podocytes by the administration of 2 mg/mL doxycycline in the drinking water for 1 week. In each litter, mice were randomly separated into four groups: diabetic mice and doxycycline (DM+VEGFKO; n = 46), diabetic and no doxycycline (DM; n = 33), sham buffer injection and doxycycline (VEGFKO; n = 40), or only sham buffer injections (wild type [WT]; n = 30) (summarized in

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Table 1). Mice were weighed, urine was collected, and blood glucose was monitored (Contour, Bayer) on a weekly basis. At the end point, HbA_{1c} level was measured in the tail vein blood using the Bayer Analyzer. Kidneys were harvested at various time points. The early time point was 3–4 weeks after STZ injection to confirm excision of the *Vegfa* gene and document pathology. The late time point was 7–8 weeks after STZ injection or earlier if demise was imminent.

As another control, a group of nondiabetic VEGFKO mice was given a 5-fold or 10-fold higher dose of doxycycline to ensure that differences between diabetic and nondiabetic VEGFKO groups were not due to increased doxycycline ingestion as a result of polydipsia. In theory, this might lead to improved excision of the floxed *Vegfa* gene.

Urinalysis. Spot urine was collected and examined for glucosuria and albuminuria using a urine dipstick (Chemstrip 5 L; Roche Diagnostics, Indianapolis, IN). Protein concentration was measured using the Bio-Rad Protein assay (Bio-Rad Laboratories) according to the manufacturer's instructions. Protein concentrations were normalized using the urine creatinine levels as measured by the Jaffe method (16).

Fixation for histology. Tissue was fixed in 10% formalin/PBS, embedded in paraffin, and sectioned 4 μm thick. These sections were stained with periodic acid–Schiff and photographed with a DC200 Leica camera mounted on a Leica DMLB microscope (Leica Microsystems).

In situ hybridization. Kidneys were washed in RNAse-free PBS and then submersed overnight in 4% paraformaldehyde/PBS. The kidneys were immersed in 30% sucrose for 24 h and embedded in Tissue-Tek O.C.T. (Sakura Finetek USA) and snap frozen. The frozen tissues were then sectioned 7 μm thick and stored at −20°C. Sections were stained with digoxigenin-labeled probes. *Wt1* and *Nphs1* mRNA probes were used in situ to identify glomerular podocytes, and the *Vegfa* mRNA probe was used to detect expression of all isoforms of *Vegfa* as previously described (17).

Glomerular morphology. Sections were scored by a renal pathologist in a blinded manner using ~30 glomerular cross-sections from each mouse. The following rubric was used: 0, normal glomerulus; +1, mesangial matrix expansion of the glomerulus; +2, severe mesangial matrix expansion; +3, severe mesangial matrix expansion and/or segmental glomerulosclerosis; and +4, global glomerulosclerosis (>50% of the glomerulus) (18,19). The mean glomerular score for each mouse was determined and averaged for each treatment group. Slides were scanned with a Pannoramic scanner (3Dhistech, Budapest, Hungary) and measured with the Pannoramic viewer software.

Immunofluorescence. Frozen paraformaldehyde-fixed sections were stained for immunofluorescence. The sections were first blocked with 3% BSA (Roche Diagnostics, Mannheim, Germany) for 1 h at room temperature and then incubated with polyclonal rabbit anti-Podocin primary antibody (1:500; Sigma) and with rat anti-mouse Pecam monoclonal antibody (1:500; BD Bioscience) overnight at 4°C. The next day, the samples were incubated with the secondary antibodies at room temperature with Alexa Fluor 488 goat anti-rabbit antibody (1:500; Invitrogen) and Cy3 goat anti-rat antibody (1:500; Invitrogen). The sections were imaged using a spinning-disk confocal microscope (Axiovert 200M; Carl Zeiss). Paraffin-embedded sections were stained for immunofluorescence for Podocin as described above; fluorescent DyLight 594–conjugated *Lycopersicon esculentum* (tomato) lectin (1:100; Vector Laboratories), and DAPI were used to visualize capillaries and nuclei, respectively (20). The tomato lectin–stained samples were imaged on a fluorescence microscope (BX61 Upright Fluorescence Microscope; Olympus).

Immunohistochemistry. Cleaved caspase-3 was used to detect apoptotic cells. Paraffin-embedded kidneys were cut into 3-μm sections. Cleaved caspase-3 was determined by rabbit polyclonal antibody ASP 175 (1:200; Cell Signaling) followed by peroxidase-labeled swine anti-rabbit IgG antibody (Dako) as previously described (21). The number of caspase-3–positive cells was determined by positive cells per 50 glomeruli at a magnification of ×400.

Reflection contrast microscopy. For stereologic measurements, kidneys were harvested from three mice from each of the four treatment groups at 7–8 weeks post-STZ (late time point). Kidney tissue was cut into 1-mm^2 blocks; fixed in 2% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.3; and embedded in Quetol-Spurr resin (Canemco). Sections (0.1 μm thick) were stained with toluidine blue. High-resolution images were obtained using reflection contrast microscopy (22). Glomerular area and perimeters of capillary profiles were measured with ImageJ (http://rsb.info.nih.gov) in nine randomly selected glomeruli from each treatment group.

Human glomerular endothelial cell culture. Primary human glomerular endothelial cells (passage 7–9, ACBRI; Cell Systems) were cultured in EGM-2 medium (Clonetech, Lonza) with 10% FBS. Cells were characterized by their expression of the endothelial tyrosine kinase receptor TEK by quantitative PCR and Western blotting (23). RNA was analyzed by real-time PCR for *TEK* using the sense primer 5-TGGAGTCAGCTTGCTCCTTT-3 and the antisense 5-ACCTCCAGTGGATCTTGGTG-3. TEK protein from cell lysates was detected with rabbit anti-Tie2 antibody (C20; Santa Cruz). Experiments in the following conditions were used for 48 h in 12-well plates in serum-free media with or without VEGFA: control, 30 mmol/L mannitol (osmotic control), 15 mmol/L D-glucose, or 30 mmol/L D-glucose. Cells were incubated with 50 μmol/L *cis*-diamminedichloroplatinum (II) (Sigma) as a positive control for cleaved caspase-3 activity (24). Cells were washed and fixed with 10% formalin for 15 min before blocking and staining with DAPI and rabbit polyclonal antibody against cleaved caspase-3 (1:500; Cell Signaling). Apoptotic cells (cleaved caspase-3 positive) versus the total number of cells (DAPI positive) were counted in nine different views in each well and used to assess the degree of apoptosis under each of the different experimental conditions.

Statistical analysis. Results are expressed as means ± SD unless otherwise stated. Group comparisons were performed using two-tailed Student *t* test or one-way ANOVA followed by post hoc Tukey or Dunnet test. Kaplan-Meier curves with log-rank analysis were used to assess the progression of proteinuria. $P \leq 0.05$ was considered statistically significant.

TABLE 1
Summary of mice involved in experiment

Treatment group	Age of STZ (weeks)	Age of Dox (weeks)	Weight at dissection (g)
WT			
Early (n = 5)			23.5 ± 3.6
Late (n = 25)			24.0 ± 3.3
DM			
Early (n = 5)	2.5		20.8 ± 2.4
Late (n = 28)	2.5		19.6 ± 5.6*
VEGFKO			
Early (n = 11)		3.5	24.6 ± 4.3
Late (n = 29)		3.5	23.5 ± 3.1
DM+VEGFKO			
Early (n = 9)	2.5	3.5	16.6 ± 6.2*
Late (n = 37)	2.5	3.5	18.1 ± 4.8†

Data are means ± SD. Mice from the early group were dissected at 3–4 weeks after STZ and from the late group were dissected at 7–8 weeks after STZ. Dox, doxycycline. *$P < 0.01$ and †$P < 0.001$ vs. nondiabetic mice.

RESULTS

Generation of a genetic model to study the effect of Vegfa reduction in glomeruli of diabetic mice. To define the role of increased Vegfa expression in glomeruli of diabetic mice, we used an inducible podocyte-specific *Vegfa* knockout mouse (Fig. 1*A*). One week after STZ injection, mice were given doxycycline for 7 days to eliminate Vegfa expression from podocytes (Fig. 1*B*). STZ caused hyperglycemia (blood glucose >15 mmol/L) within 2 weeks from the start of first injection (Fig. 1*C*), and there was no significant difference in blood glucose or weight between DM and DM+VEGFKO mice or between WT and VEGFKO alone. Likewise, HbA_{1c} levels were similar between the two groups (Fig. 1*D*). Only diabetic mice were positive for glucosuria by dipstick (not shown). By in situ analysis, we confirmed that *Vegfa* mRNA expression was markedly reduced in both VEGFKO and VEGFKO+DM mice (Fig. 2*A*) and *Vegfa* levels were increased in glomeruli of diabetic mice compared with nondiabetic control mice consistent with previous expression studies (2,12,25). The approximate degree of *Vegfa* excision after Cre activation was 70% of podocytes—slightly lower than previously reported (3) and is likely due to the shorter doxycycline induction period used in the current study (1 vs. 2 weeks in the study by Eremina et al. [3]). By contrast, there was no change in expression of the podocyte-specific markers Wilms tumor 1 (*Wt1*) or Nephrin (*Nphs1*) at the early time point (Fig. 2*B*). Thus, as shown previously (25) for other diabetic models, early stages of diabetes are

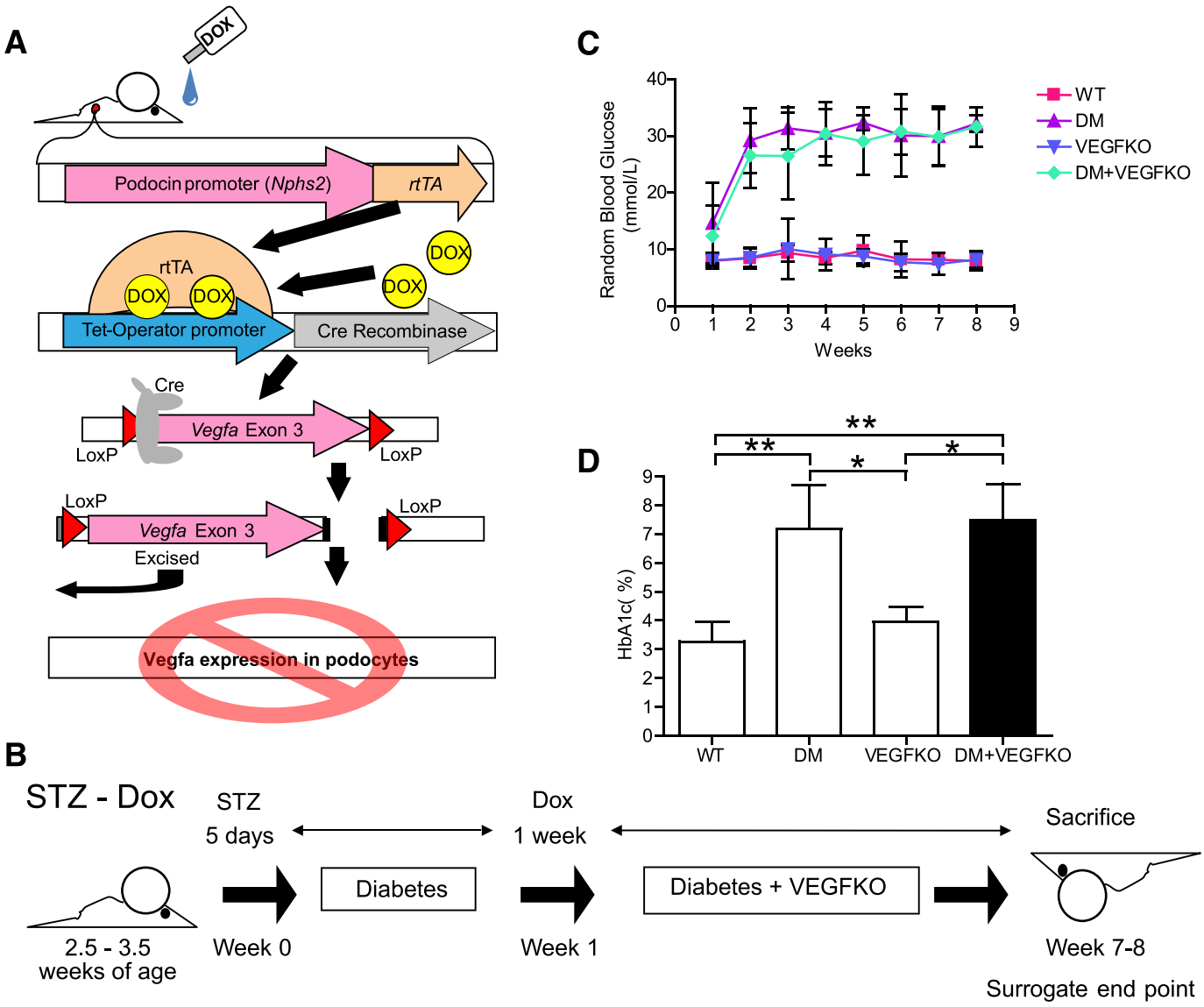

FIG. 1. Generation of the genetic mouse model. *A*: To delete *Vegfa* from podocytes at specified time points, a transgenic line was created carrying four independent transgenes: *Nphs2-rtTA*, *tetO-Cre*, and *Vegfa*$^{flox/flox}$. *B*: Mice were divided into four groups: WT (no STZ, no doxycycline [Dox]), DM (injected with STZ at week 0), VEGFKO (induced with doxycycline at week 1 of treatment), and DM+VEGFKO (injected with STZ at week 0 and induced with doxycycline at week 1). *C*: Random blood glucose levels were higher in diabetic groups ($P < 0.001$). There was no difference between DM (n = 8–27) and DM+VEGFKO (n = 9–38) or between WT (n = 9–24) and VEGFKO (n = 7–27). *D*: HbA_{1c} measurements at time of dissection were not different between diabetic groups (DM, n = 8, vs. DM+VEGFKO, n = 4) or between nondiabetic groups (WT, n = 6, vs. VEGFKO, n = 4). *$P < 0.05$, **$P < 0.01$.

associated with enhanced expression of *Vegfa* mRNA in the glomerulus in the STZ model.

DM+VEGFKO mice develop marked proteinuria. As a clinically relevant indicator of glomerular injury, protein-to-creatinine ratio was measured in diabetic and nondiabetic mice weekly. We found that diabetic VEGFKO mice began to manifest significantly higher protein-to-creatinine ratios beginning at 3 weeks after administration of STZ. Significant proteinuria was defined as ≥20 mg/mg; Fig. 3*A* shows the fraction of mice reaching this end point over the study period. Strikingly, a much greater portion of DM+VEGFKO mice reached this end point much earlier in the study (Fig. 3*A*). Levels of protein excretion remained higher in the DM+VEGFKO group throughout the period of study, reaching levels that were approximately fivefold higher than in controls by the end of the study period ($P < 0.01$) (Fig. 3*B*). It is well established that the degree of proteinuria varies among mouse strains after STZ induction of diabetes (26), and we observed some variability in levels of protein excretion in these mice with mixed genetic backgrounds. Nonetheless, the robust increase in proteinuria in the diabetic VEGFKO was consistently and statistically significantly higher than in controls.

Previous studies have shown that loss of Vegfa alone from podocytes results in TMA (3). To compare the impact of DM alone versus VEGFKO alone, we followed a cohort of DM (n = 7) and VEGFKO (n = 7) mice up to 10 weeks after STZ or sham injection, respectively. VEGFKO mice given different doses of doxycycline developed proteinuria by 8 weeks (17.7 ± 3.95 mg/mg) that progressed until 11 weeks (29.8 ± 9.70 mg/mg) when the study was stopped. In contrast, the cohort of DM mice did not develop

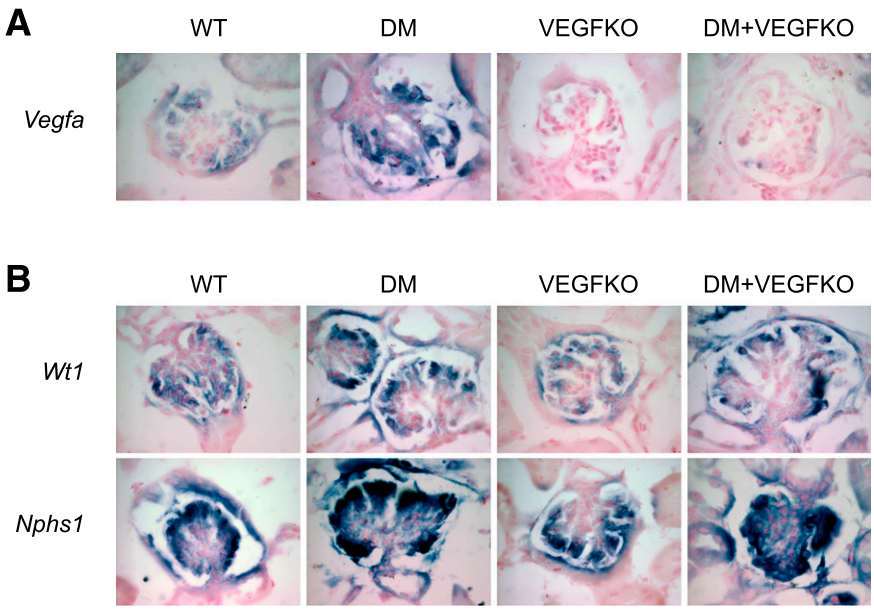

FIG. 2. In situ mRNA expression of *Vegfa*, *Wt1*, and *Nphs1* in the kidney. *A*: *Vegfa* expression was increased in glomeruli of DM compared with WT mice but reduced in the VEGFKO and DM+VEGFKO groups (n = 4). *B*: *Wt1* and *Nphs1* expression appeared similar in all groups (n = 4). Staining was performed at the late time point. (A high-quality digital representation of this figure is available in the online issue.)

significant proteinuria, compared with controls, 10 weeks after STZ (1.87 ± 0.66 mg/mg). No DM+VEGFKO mice were followed to this late time point, as they had succumbed to renal disease prior to the 10-week time point. Notably, no phenotype was ever observed in mice treated with doxycycline alone.

Accelerated glomerular injury occurs in diabetic mice lacking Vegfa in podocytes. Kidneys were harvested from each of the four treatment groups at 3–4 weeks after STZ injection (early time point) and at 7–8 weeks after STZ injection (late time point) (Table 1). The kidney tissue was examined for structural change, glomerular injury, and features of diabetic nephropathy (Fig. 4). At the early time point, the glomerular area was similar but the glomerular score was significantly worse in the DM+VEGFKO group only (Fig. 4*C* and *E*). At the later time point, kidneys from DM mice with diabetes alone showed mild mesangial matrix expansion and hypertrophy of tubules but the glomerular area was not significantly increased compared with controls (Fig. 4*D*). By contrast, at the late time point kidneys from DM+VEGFKO mice showed evidence of dramatic kidney injury including significant glomerular matrix expansion and sclerosis, with enlarged tubules containing protein deposits (Fig. 4*F*). While DM mice

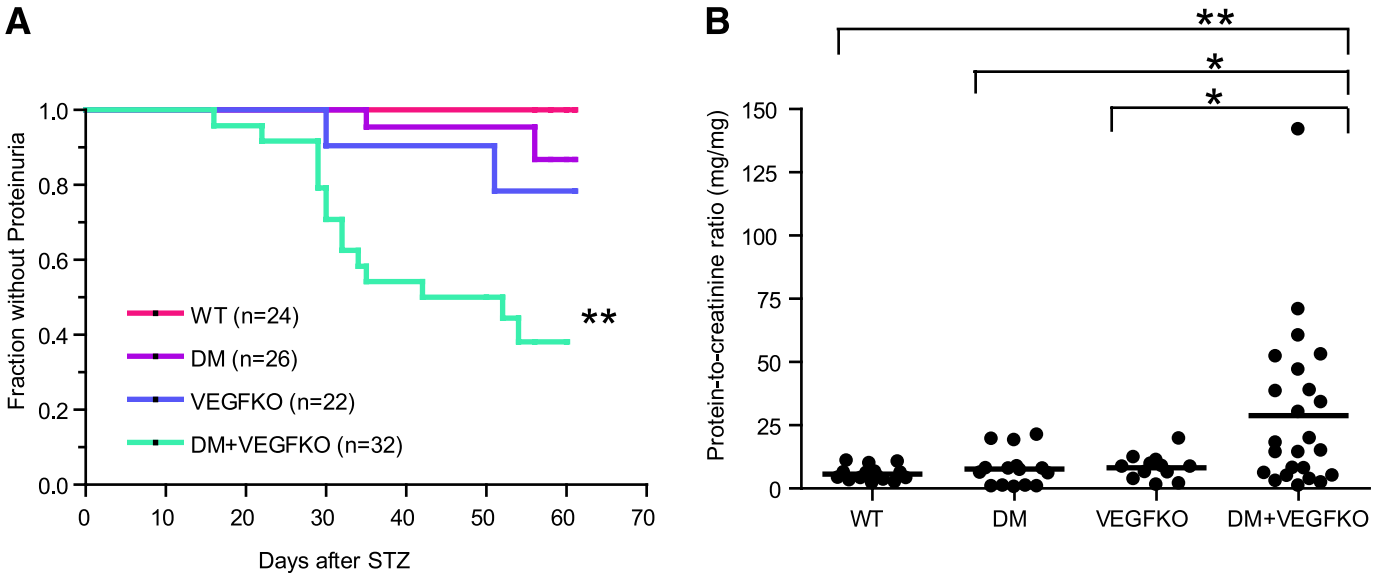

FIG. 3. Observed proteinuria in DM+VEGFKO mice. *A*: A Kaplan-Meier curve shows a number of mice remaining without significant proteinuria (defined as protein-to-creatinine ratio >20 mg/mg or 4+ on dipstick). Significant proteinuria began earlier and was observed in a much larger proportion of DM+VEGFKO mice by the end of study (60%) compared with all other groups. *B*: Protein-to-creatinine ratio of mice dissected at 7–8 weeks post-STZ showed a significantly higher degree of proteinuria in DM+VEGFKO mice compared with all other groups. *P < 0.05, **P < 0.01.

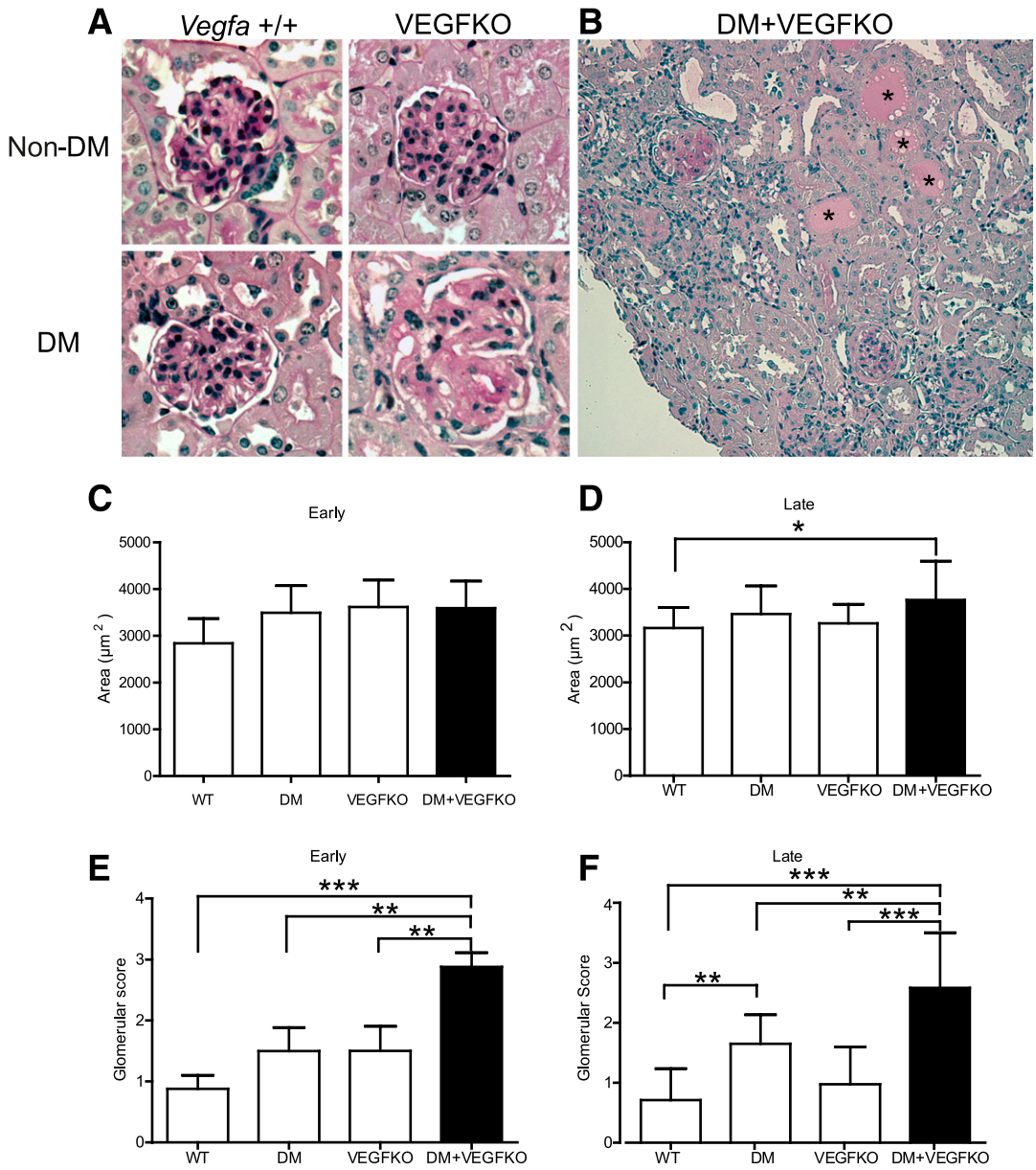

FIG. 4. DM+VEGFKO mice had severe glomerular injury and significant glomerular expansion. *A*: Light micrographs of glomeruli from each of the four treatment groups. At 7–8 weeks post–STZ injection, glomerular injury was evident in the DM+VEGFKO group only. Original magnification ×400. *B*: A lower-power view of a kidney in the DM+VEGFKO group demonstrates glomerular injury, proteinaceous casts in the tubules (*), and interstitial infiltrate. Original magnification ×200. *C* and *D*: Glomerular expansion was increased in DM+VEGFKO group at the late time point. *E* and *F*: Glomerular injury scoring showed DM+VEGFKO mice had significantly more injury at both early and late time points. In contrast, DM group alone showed less severe but significant glomerular injury compared with control group at the late time point. Early: WT (n = 2), DM (n = 3), VEGFKO (n = 4), and DM+VEGFKO (n = 3). Late: WT (n = 14), DM (n = 14), VEGFKO (n = 10), and DM+VEGFKO (n = 20). *P < 0.05, **P < 0.01, ***P < 0.001. (A high-quality digital representation of this figure is available in the online issue.)

alone had increased mesangial matrix expansion and glomerular sclerosis compared with the WT controls ($P <$ 0.01) (Fig. 4*F*), the glomerular injury was much greater in DM+VEGFKO compared with all other treatment groups ($P < 0.001$) (Fig. 4*F*).

Although glomeruli from VEGFKO mice without diabetes looked histologically normal at 7–8 weeks post-STZ (i.e., 5–6 weeks post-doxycycline), by 10 weeks post-sham (citrate) injection, histologic analysis of VEGFKO glomeruli showed features of TMA without glomerular hypertrophy (Supplementary Fig. 1). In DM mice, glomerular hypertrophy without endothelial damage was observed (not shown). Taken together, these data demonstrate that VEGFKO alone is detrimental to glomerular health but the addition of diabetes greatly accelerates injury. Conversely, 10 weeks of diabetes alone is not sufficient to cause significant glomerular injury in mice but is greatly accelerated in the absence of locally produced Vegfa.

Endothelial injury and apoptosis dominate in glomeruli of DM+VEGFKO mice. Given the well-established vascular protective role of Vegfa, we speculated that Vegfa loss might enhance microvascular injury in diabetes. Accordingly, we visualized the glomerular endothelial compartment in each of the four treatment groups using the endothelial marker Pecam. Podocin was used to identify podocytes. While enlarged glomeruli were observed in

both DM and DM+VEGFKO groups, the most striking finding was loss of endothelial cells and simplification of the capillary loop structure in the DM+VEGFKO group (Fig. 5*A*). These stains were performed at the late time point, and the results were replicated using fluorescent DyLight 594-conjugated *L. esculentum* (tomato) lectin (Supplementary Fig. 2). To quantify capillary loss, we measured capillary perimeter per glomerular area via reflection contrast microscopy and found that DM+VEGFKO mice at 7–8 weeks post-STZ had a significant reduction in capillaries per glomerular area compared with all other treatment groups (Fig. 5*B*). At the very late time points (10 weeks post–sham injection), glomeruli from VEGFKO mice also exhibited profound changes in the endothelial compartment with dropout of endothelial cells and loss of capillary loops (Supplementary Fig. 3).

To determine whether Vegfa influences glomerular cell apoptosis in diabetes, we stained kidney sections from DM and DM+VEGFKO groups for cleaved caspase-3 and quantified the number of apoptotic cells. There was

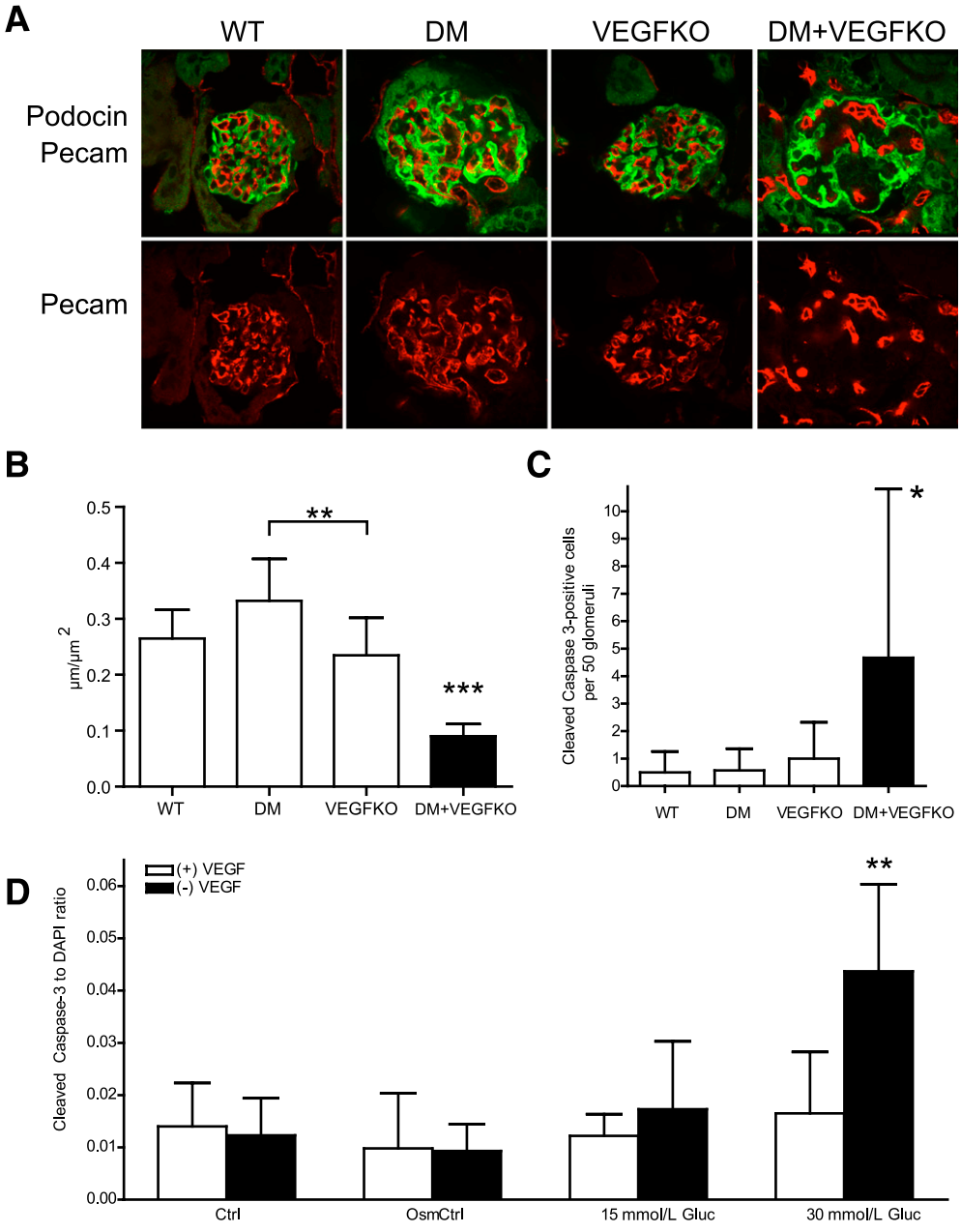

FIG. 5. The reduction of VEGFA results in glomerular cell apoptosis under high-glucose conditions in vivo and in vitro. *A*: Immunofluorescence staining demonstrates simplification and dropout of glomerular capillaries in glomeruli from DM+VEGFKO mice. Pecam staining of the endothelial cells confirms vascular defects in a representative glomerulus from the DM+VEGFKO group; Podocin (green) and Pecam (red). The bottom row shows endothelial cell staining alone to highlight differences (late time point, n = 2–3). *B*: Reflection contrast microscopy shows that the mean capillary profile perimeter per glomerular area for the DM+VEGFKO mice is reduced compared with all other groups (late time point, n = 9). **P < 0.01. ***P < 0.001. *C*: An increased number of cleaved caspase-3–positive cells were observed in glomeruli from DM+VEGFKO mice (Dunnet test). Late: WT (n = 8), DM (n = 7), VEGFKO (n = 9), and DM+VEGFKO (n = 12). *P < 0.05. *D*: Bar graph shows an increase in cleaved caspase-3–positive human endothelial cells with exposure to high glucose in the absence of VEGFA (n = 6). **P < 0.01. Ctrl, control; Gluc, glucose; OsmCtrl, osmotic control. (A high-quality digital representation of this figure is available in the online issue.)

a significant increase in the number of cleaved caspase-3–positive cells within the glomeruli of DM+VEGFKO mice compared with other groups (Fig. 5*C*). Although it was not possible to determine with confidence the cell type(s) undergoing apoptosis in the glomeruli of DM+VEGFKO, we speculated that endothelial cells were a likely target given the loss of endothelial markers shown in Fig. 5*A* and the well-established prosurvival effects of Vegfa on endothelial cells.

To explore the combined effect of diabetes and VEGFA loss on glomerular endothelial cells, we determined the rate of apoptosis in human glomerular endothelial cells exposed to normal or high glucose in the presence or absence of VEGFA. The basal percentage of cells expressing cleaved casapse-3 was 1.4 and 1.2% in normoglycemic conditions with or without Vegfa and rose to 1.6% under high-glucose (30 mmol/L) conditions. Removal of Vegfa from high-glucose media enhanced the rate of apoptosis, which rose significantly to 4.4% (Fig. 5*D*).

DISCUSSION

Diabetic nephropathy is the leading cause of end-stage kidney failure in North America and is increasing at alarming rates throughout the Western and developing worlds. The pathogenesis of diabetic nephropathy is complex, with genetic, environmental, and metabolic components (27–29). While 30–40% of patients with type 1 diabetes develop diabetic nephropathy after 20 years of disease, the other 60–70% do not, despite similar levels of hyperglycemia (30). These data suggest that endogenous protective factors exert major modifying effects in this disease. In contrast to the many studies devoted to disturbances and pathways initiated by poor glycemic control, less work has been devoted to understanding the role of endogenous factors that may protect tissues in diabetes. These pathways may represent new therapeutic targets and biomarkers that are critical pieces of the puzzle.

Here, we explored the role of one such factor—VEGFA—in the development and progression of diabetic nephropathy. VEGFA has received much attention in the complications of diabetes, as both circulating and local tissue levels of VEGFA are increased in diabetes (8,9). Indeed, we confirm in this study that renal Vegfa expression is enhanced in mice with STZ-induced DM. VEGFA is a potent angiogenic factor that signals through its tyrosine kinase receptor, VEGFR2, to promote new vessel sprouting, endothelial migration, proliferation, differentiation, and survival (1). Neoangiogenesis resulting in the formation of leaky, immature blood vessels is a common feature of both diabetic retinopathy and also diabetic nephropathy, suggesting that the elevated VEGFA levels are pathogenic (31). Accordingly, VEGFA has been considered a logical therapeutic target for treating devastating complications of diabetes. Indeed, ongoing clinical studies are exploring the effect of VEGFA inhibition in diabetic retinopathy (32).

The clinical significance of neoangiogenesis in diabetes was first recognized in the retina, where it results in vitreal hemorrhage and fibrosis (33). While vascular proliferation is clearly problematic for the diabetic eye, it has also been observed in the kidney (34). Renal biopsies taken in the first decade of diabetes clearly show abnormal blood vessels with increased diameters, increased vessel length, and proliferating new blood vessels at the vascular pole and in Bowman capsule, which surrounds the urinary space (35). This is associated with changes in vascular permeability that result in micro- followed by macroalbuminuria. Full-blown diabetic nephropathy develops over years and is characterized by nodular glomerulosclerosis, thickening of the glomerular basement membrane, Kimmelstiel-Wilson nodules, and fibrin cap lesions (35,36). During the early angiogenic phase of diabetic nephropathy, VEGFA levels are elevated in renal podocytes similar to the increased VEGFA levels found in cells of the retina. The similarity in pathologic findings in microvascular structures and elevated VEGFA expression in local tissue, as well as the fact that almost all patients with type 1 diabetes and diabetic nephropathy also have diabetic retinopathy, strongly argues for a common pathway (37). Nonetheless, it does not necessarily follow that increased VEGFA expression in the glomerulus is detrimental or, conversely, that elimination of VEGFA will be beneficial. We considered the possibility that elevated glomerular VEGFA levels may represent a compensatory response to limit endothelial injury and dysfunction, and we provide evidence supporting such a model.

The outcomes of Vegfa inhibition in experimental animal models of diabetes have been conflicting, with some papers showing amelioration and others showing no improvement (7,12,14,15) or even more aggressive disease (38). A major difficulty in interpreting these studies is that it is difficult to ascertain the degree, specificity, and location of *Vegfa* inhibition achieved in each case. Furthermore, across these studies, different classes of inhibitors were used, from small-molecule inhibitors of the Vegfa receptors (tyrosine kinase inhibitors) to specific antibodies against Vegfa or Vegfa receptors to competing Vegfa aptamers. More recently, in clinical trials, it has become apparent that VEGFA inhibition has significant renal toxicity in nondiabetic patients, raising additional safety concerns (3,39,40).

In the current study, we took a genetic approach to extinguish Vegfa production in a cell- and time-specific manner in diabetic mice. The inducible Cre-loxP system permits precision in timing and location of Vegfa inhibition, allowing us to determine the role of glomerular Vegfa in progression of diabetic nephropathy in a mouse model. While deletion of *Vegfa* postnatally at 3.5 weeks results in glomerular injury in nondiabetic mice by 3 months of age, there are no overt glomerular defects in nondiabetic VEGFKO mice within the first 7–8 weeks after excision. However, overt glomerular injury and TMA developed in VEGFKO mice, starting from 8 weeks and progressing rapidly—findings similar to those of previous reports (3). We also confirmed that higher doses of doxycycline did not result in better excision and/or enhanced glomerular injury in nondiabetic VEGFKO; this control was included because diabetic mice have polydipsia and could receive a higher dose of doxycycline than nondiabetic littermates. Although this artifact is possible in theory, two protocol details made it unlikely: induction with doxycycline was given for only 1 week in the diabetic mice, and it was given at a time point when their glucose levels were only mildly elevated. Regardless, our data show no difference.

In a previous study, we showed that *Vegfa* knockout in a healthy glomerulus is harmful (3); however, here we show that *Vegfa* knockout in a diabetic glomerulus is disastrous. Why is this the case? Vegfa produced by podocytes is required to signal in a paracrine fashion to adjacent glomerular endothelial cells to maintain a healthy fenestrated glomerular vasculature via Vegfr2 activation

and subsequent intracellular signaling. Loss of glomerular Vegfa or Vegfr2 results in endothelial swelling followed by endothelial cell loss and TMA that occurs 8–12 weeks after excision (3,41,42). In this study, we hypothesized that the loss of podocyte-derived Vegfa would accelerate the progression of diabetic kidney disease, recognizing that differences would need to occur before 8 weeks of *Vegfa* deletion.

In the STZ model of type 1 diabetes in mice, glomerular injury is relatively mild and not apparent until late in the course—usually after 20 weeks of hyperglycemia (43,44). By contrast, loss of glomerular Vegfa in this diabetic model results in aggressive glomerular injury, scarring, apoptosis, and proteinuria. A number of studies have reported enhanced podocyte loss in diabetes (45–47). Here, we show that the endothelial compartment is a primary target and that glomerular endothelial injury also contributes to diabetic glomerular injury. Indeed, the dramatic reduction in endothelial markers and simplification of capillary loops suggest that the endothelial compartment was most severely affected. This is also true in mice lacking Vegfa in their podocytes in the absence of diabetes; however, the defects are greatly accelerated in the presence of hyperglcyemia. Cell culture studies confirm that endothelial cells exposed to a "double hit"—high glucose and removal of VEGFA—undergo accelerated apoptosis. Additional mechanisms of cell death such as anoikis and autophagy may also play a role in vivo.

In diabetes, dysregulation of VEGFA does not occur in isolation; other angiogenic pathways are affected. In particular, we have recently shown that the levels of angiopoietin 2 (Angpt2) produced by glomerular endothelial cells increase in diabetic mice, and elevated circulating levels have been found in patients (25,48). ANGPT2 functions to antagonize the endothelial tyrosine kinase receptor TEK. In the absence of VEGFA, ANGPT2 promotes vessel regression and endothelial cell apoptosis. Thus, it is important to consider the interaction of different pathways, and it is unlikely that inhibition of a single angiogenic factor will improve glomerular structure and function; in fact, as in this case, it has potential to do harm.

Our genetic model provides a robust knockdown of Vegfa production in the podocyte; it remains possible that a more moderate reduction in Vegfa will not be harmful. Additional insights regarding local regulation of VEGFA in the glomerulus may provide strategies to more precisely titrate levels. However, given the need for correct and tight regulation of the VEGFA-VEGFR2 signaling pathway in the healthy glomerulus, this will be a difficult goal to accomplish in practice. The incidence of significant renal toxicity in patients receiving VEGFA inhibitors as treatment for various solid tumors underscores this point (3,41,42,49).

Taken together, the results from our study clearly demonstrate that Vegfa is required for glomerular health and that this requirement is more critical in the setting of hyperglycemia (diabetes). Further, our study emphasizes the importance of endogenous factors to protect and/or damage the diabetic vasculature. Thus, while normalization of glucose levels and metabolic disturbances is important, additional insights and leverage in treatment can be gained from understanding the role of endogenous factors. In support of this possibility, recent genetic studies have identified an association between a polymorphism in the *VEGFA* gene and risk of diabetic nephropathy in an Irish cohort (50). Additional studies are needed to validate these findings and to determine the functional consequence of genetic variants in the progression of diabetic nephropathy—our study suggests future avenues to explore. Finally, thrombotic injury is also enhanced in the setting of diabetes, raising a note of caution for clinicians using anti-VEGFA agents in various clinical settings.

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G.A.S. performed the animal and cell culture studies, participated in the design of experiments, and wrote the first draft of the manuscript. M.J. provided cell culture expertise and reviewed and edited the manuscript. Y.M. provided expertise in immunohistochemistry data. V.E. provided help for in situ analysis and reviewed and edited the manuscript. H.J.B. performed the glomerular scoring and caspase-3 staining in tissues. S.E.Q. designed the experimental study, supervised all aspects of the project, and edited the manuscript. S.E.Q. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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