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 pharmacological 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.
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 AS175 (1:200; Cell Signalling) 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 blocks; fixed in 2% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.3; and embedded in Quetol-300 Spurr resin (Caneco). 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 (Clonetics, 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 (5′-GGCTCCAGTGGATCTTGGTG-3′) and actin (5′-TCCTCCAGTGGACCTGGTG-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 n-glucose, or 30 mmol/L v-glucose. Cells were incubated with 50 μmol/L cis-diaminedichlorohalogenate (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 Signalling). 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. Groups were compared using 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 ≤ 0.05 was considered statistically significant.

**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. 1A). One week after STZ injection, mice were given doxycycline for 7 days to eliminate Vegfa expression from podocytes (Fig. 1B). STZ caused hyperglycemia (blood glucose >15 mmol/L) within 2 weeks from the start of first injection (Fig. 1C), and there was no significant difference in blood glucose or weight between DM and DM+VEGFKO mice or between WT and VEGFKO alone. Likewise, HbA1c levels were similar between the two groups (Fig. 1D). 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. 2A) 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. 2B). Thus, as shown previously (25) for other diabetic models, early stages of diabetes are
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 non-diabetic 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. 3A 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. 3A). 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. 3B). 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. 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-rTFA, tetO-Cre, and Vegfa$^{flax/flax}$. 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: HbA1c 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, $n = 4$). *$P < 0.05$, **$P < 0.01$. **
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. 4C 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. 4D). 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. 4F). While DM mice

![Image](https://example.com/image1)

**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.)**

![Image](https://example.com/image2)

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

Although glomeruli from VEGFKO mice without diabetes looked histologically normal at 7–8 weeks post-STZ injection (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. 5A). 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. 5B). 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 Vegf 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. **P < 0.01. Ctrl, control; Gluc, glucose; OsmCtrl, osmotic control. (A high-quality digital representation of this figure is available in the online issue.)

diabetes.diabetesjournals.org  DIABETES, VOL. 61, NOVEMBER 2012  2963
a significant increase in the number of cleaved caspase-3–positive cells within the glomeruli of DM+VEGFKO mice compared with other groups (Fig. 5C). 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. 5A 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 caspase-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. 5D).

**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). Neangiogenesis 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 neangiogenesis 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 Vegfa knockout 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 mice; 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, diabetic 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 hyperglycemia. 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 VEGF does not occur in isolation; other angiogenic pathways are affected. In particular, we have recently shown that the levels of angio-poietin 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 VEGF, 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 VEGF 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.

ACKNOWLEDGMENTS

This work was funded by Canadian Institutes of Health Research grants MOP 77756 and 02931, National Institutes of Health Grant 1 U01 DK076136-01, and Terry Fox Grant TF0106002 (to S.E.Q.). S.E.Q. holds the Gabor-Zellerman Chair in Renal Research, University Health Network, University of Toronto. G.A.S. was funded by a graduate studentship from the Banting and Best Diabetes Centre at the University of Toronto and by an Ontario Student Opportunity Trust Fund from the Samuel Lunenfeld Research Institute.

No potential conflicts of interest relevant to this article were reported.

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.

The authors thank K. Harpal (Samuel Lunenfeld Research Institute) for the histologic stains.

REFERENCES

1. Eremina V, Cui S, Gerber H, et al. Vascular endothelial growth factor a signaling in the podocyte-endothelial compartment is required for mesangial cell migration and survival. J Am Soc Nephrol 2006;17:724–735
2. Robert B, Zhao X, Abrahamson DR. Coexpression of neuropilin-1, Flk1, and VEGF(164) in developing and mature mouse kidney glomeruli. Am J Physiol Renal Physiol 2000;279:F275–F282
3. Eremina V, Jefferson JA, Kowalewska J, et al. VEGF inhibition and renal thrombotic microangiopathy. N Engl J Med 2008;358:1129–1136
4. Eremina V, Sood M, Haigh J, et al. Glomerular-specific alterations of VEGF-A expression lead to distinct congenital and acquired renal diseases. J Clin Invest 2003;111:707–716
5. Sisson K, Eremina V, Baelde H, et al. Glomerular structure and function require paracrine, not autocrine, VEGF-VEGFR-2 signaling. J Am Soc Nephrol 2010;21:1691–1701
6. Cooper ME, Vranes D, Youssef S, et al. Increased renal expression of vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 in experimental diabetes. Diabetes 1999;48:2293–2299
7. Flyvbjerg A, Dagnaes-Hansen F, De Vriese AS, Schrijvers BF, Tilton RG, Rasch R. Acceleration of long-term renal changes in obese type 2 diabetic mice by a neutralizing vascular endothelial growth factor antibody. Diabetes 2002;51:3090–3094
8. Hohenstein B, Hausknacht B, Boehmer K, Riss S, Brekenk RA, Hugo CP. Local VEGF activity but not VEGF expression is tightly regulated during hyperglycemia. Kidney Int 2006;69:1654–1661
9. Hovind P, Tarnow L, Oestergaard PB, Parving HH. Vascular endothelial growth factor (VEGF) and VEGF(164) in developing and mature mouse kidney glomeruli. Am J Physiol Renal Physiol 2000;279:F275–F282
10. Veron D, Reidy KJ, Bertuccio C, et al. Overexpression of VEGF-A in podocytes of adult mice causes glomerular disease. Kidney Int 2010;77:989–999
11. Veron D, Bertuccio CA, Marlier A, et al. Podocyte vascular endothelial growth factor (Vegfa) overexpression causes severe nodular glomerulosclerosis in a mouse model of type 1 diabetes. Diabetologia 2011;54:1227–1241
12. Sung SH, Ziyadeh FN, Wang A, Pyagay PE, Kanwar YS, Chen S. Blockade of vascular endothelial growth factor signaling ameliorates diabetic albuminuria in mice. J Am Soc Nephrol 2006;17:3083–3104

13. Schrijvers BF, Flyvbjerg A, Tilton RG, De Vriese AS, Lameire NH, De Vriese AS. A neutralizing VEGF antibody prevents glomerular hypertrophy in a model of obese type 2 diabetes, the Zucker diabetic fatty rat. Nephrol Dial Transplant 2006;21:324–329

14. Ku CH, White KE, Dei Cas A, et al. Inducible overexpression of a flt-1 in podocytes ameliorates glomerulopathy in diabetic mice. Diabetes 2008;57:2824–2833

15. Schrijvers BF, De Vriese AS, Tilton RG, et al. Inhibition of vascular endothelial growth factor (VEGF) does not affect early renal changes in a rat model of lean type 2 diabetes. Horm Metab Res 2005;37:21–25

16. Husdan H, Rapoport A. Estimation of creatinine by the Jaffe reaction. A comparison of three methods. Clin Chem 1968;14:222–238

17. Piscione TD, Wu MY, Quaggin SE. Expression of Hairy/Enhancer of Split genes, Hes1 and Hes5, during murine nephron morphogenesis. Gene Expr Patterns 2004;4:707–711

18. Raji L, Azar S, Keane W. Mesangial immune injury, hypertension, and progressive glomerular damage in Dahl rats. Kidney Int 1984;36:137–143

19. Tervaet TW, Mooyaart AL, Amann K, et al.; Renal Pathology Society. Pathologic classification of diabetic nephropathy. J Am Soc Nephrol 2010;21:596–593

20. Inai T, Mancuso M, Hashizume H, et al. Inhibition of vascular endothelial growth factor (VEGF) signaling in cancer causes loss of endothelial fenestrations, regression of tumor vessels, and appearance of basement membrane ghosts. Am J Pathol 2004;165:35–52

21. Pinkse GG, Bouwman WP, Jiawan-Lalai R, Terpstra OT, Bruijn JA, de Heer E. Integrin signaling via RGD peptides and anti-beta1 antibodies confers resistance to apoptosis in islets of Langerhans. Diabetes 2006;55:312–317

22. Prins FA, Bruijn JA, De Heer E. Applications in renal immunopathology of reflection contrast microscopy, a novel superior light microscopical technique. Kidney Int 1996;49:261–266

23. Dumont DJ, Gradwohl GJ, Fong GH, Auerbach R, Brettman ML. The endothelial-specific receptor tyrosine kinase, tek, is a member of a new subfamily of receptors. Oncogene 1998;2:1293–1301

24. Dumun B, Hie Z, Somerset H, Oh DJ, Faubel S, Edelstein CL. Caspasases and calpain are independent mediators of cispalatin-induced endothelial cell necrosis. Am J Physiol Renal Physiol 2006;291:F578–F587

25. Jeansson M, Gawlik A, Anderson G, et al. Animal Models of Diabetic Complications Consortium. Mouse models of diabetic nephropathy. J Am Soc Nephrol 2009;20:2503–2512

26. Jeansson M, Gawlik A, Anderson G, et al. Angiopoietin-1 is essential in mouse vasculature during development and in response to injury. J Clin Invest 2011;121:2278–2289

27. Brosius FC 3rd, Alpers CE, Bottinger EP, et al.; Animal Models of Diabetic Complications Consortium. Mouse models of diabetic nephropathy. J Am Soc Nephrol 2009;20:2503–2512

28. UK Prospective Diabetes Study (UKPDS) Group. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). Lancet 1998;352:877–883

29. Möllsten A, Marklund SL, Wessman M, et al. A functional polymorphism in the manganese superoxide dismutase gene and diabetic nephropathy. Diabetes 2007;56:265–269

30. The Diabetes Control and Complications (DCCT) Research Group. Effect of intensive therapy on the development and progression of diabetic nephropathy in the Diabetes Control and Complications Trial. Kidney Int 1995;47:1703–1729

31. Mathews MK, Merges C, McLeod DS, Lutty GA. Vascular endothelial growth factor and vascular permeability changes in human diabetic retinopathy. Invest Ophthalmol Vis Sci 1997;38:2729–2741

32. Waisbourd M, Goldstein M, Loevenstein A. Treatment of diabetic retinopathy with anti-VEGF drugs. Acta Ophthalmol 2011;89:203–207

33. Root HF, Mirtsy S, Dittrz J. Proliferative retinopathy in diabetes mellitus; review of eight hundred forty-seven cases. J Am Med Assoc 1959;169:905–909

34. Osterby R, Asplund J, Bangstad HJ, et al. Neovascularization at the vascular pole region in diabetic glomerulopathy. Nephrol Dial Transplant 1999;14:348–352

35. Drummond K, Mauer M; International Diabetic Nephropathy Study Group. The early natural history of nephropathy in type 1 diabetes: II. Early renal structural changes in type 1 diabetes. Diabetes 2002;51:1580–1587

36. Najafian B, Mauer M. Progression of diabetic nephropathy in type 1 diabetic patients. Diabetes Res Clin Pract 2009;83:1–8

37. Fioretto P, Mauer M. Diabetic nephropathy: diabetic nephropathy-challenges in pathologic classification. Nat Rev Nephrol 2010;6:508–510

38. Kosugi T, Nakayama T, Li Q, et al. Soluble Flt-1 gene therapy ameliorates albuminuria but accelerates tubulointerstitial injury in diabetic mice. Am J Physiol Renal Physiol 2010;298:F809–F816

39. Stokes MB, Erazo MC, D’Agati VD. Glomerular disease related to anti-VEGF therapy. Kidney Int 2008;74:1487–1491

40. Rini BI, Garcia JA, Cooney MM, et al. Toxicity of sunitinib plus bevacizumab in renal cell carcinoma. J Clin Oncol, 2010;28:e284–e285

41. Izzedine H, Brocheriou I, Deray G. Thrombotic microangiopathy and anti-VEGF agents. Nephrol Dial Transplant 2007;22:1451–1452

42. Prangé C, Le.faucheuc C, Medioni J, Jacquot C, Hill GS, Nochy D. Renal thrombotic microangiopathy caused by anti-VEGF-antibody treatment for metastatic renal-cell carcinoma. Lancet Oncol 2007;8:177–178

43. Q1 Z, Fujita H, Jin J, et al. Characterization of susceptibility of inbred mouse strains to diabetic nephropathy. Diabetes 2005;54:2628–2637

44. Tesch GH, Nikolic-Paterson DJ. Recent insights into experimental mouse models of diabetic nephropathy. Nephron Exp Nephrol 2006;104:e57–e62

45. Wolf G, Chen S, Ziyadeh FN. From the periphery of the glomerular capillary wall toward the center of disease: podocyte injury comes of age in diabetic nephropathy. Diabetes 2005;54:1626–1634

46. Baelde HJ, Eikmans M, Lappin DW, et al. Reduction of VEGF-A and CTGF expression in diabetic nephropathy is associated with podocyte loss. Kidney Int 2008;73:1358–1365

47. Isermann B, Vinnikov IA, Madhusudhan T, et al. Activated protein C protects against diabetic nephropathy by inhibiting endothelial and podocyte apoptosis. Nat Med 2007;13:1349–1358

48. Lip PL, Sutton and J. Cane GJ, et al. Plasma vascular endothelial growth factor, angiopeitin-2, and soluble angiopeitin receptor tie-2 in diabetic retinopathy; effects of laser photocoagulation and angiotensin receptor blockade. Br J Ophthalmol 2004;88:1543–1546

49. Bollée G, Patey N, Cazajous G, et al. Thrombotic microangiopathy secondary to VEGF pathway inhibition by sunitinib. Nephrol Dial Transplant 2009;24:682–685

50. McKnight AJ, Maxwell AP, Patterson CC, Brady HR, Savage DA. Association of VEGF-1493C–>T polymorphism with diabetic nephropathy in type 1 diabetes mellitus. J Diabetes Complications 2007;21:242–245