Podocyte-Specific VEGF-A Gain of Function Induces Nodular Glomerulosclerosis in eNOS Null Mice

Delma Veron,* Pardeep K. Aggarwal,* Heino Velazquez,† Michael Kashgarian,‡ Gilbert Moeckel,‡ and Alda Tufro*

*Department of Pediatrics, †Department of Internal Medicine, and ‡Department of Pathology, Yale University School of Medicine, New Haven, Connecticut

ABSTRACT

VEGF-A and nitric oxide are essential for glomerular filtration barrier homeostasis and are dysregulated in diabetic nephropathy. Here, we examined the effect of excess podocyte VEGF-A on the renal phenotype of endothelial nitric oxide synthase (eNOS) knockout mice. Podocyte-specific VEGF164 gain of function in eNOS−/− mice resulted in nodular glomerulosclerosis, mesangiolysis, microaneurysms, and arteriolar hyalinosis associated with massive proteinuria and renal failure in the absence of diabetic milieu or hypertension. In contrast, podocyte-specific VEGF164 gain of function in wild-type mice resulted in less pronounced albuminuria and increased creatinine clearance. Transmission electron microscopy revealed glomerular basement membrane thickening and podocyte effacement in eNOS−/− mice with podocyte-specific VEGF164 gain of function. Furthermore, glomerular nodules overexpressed collagen IV and laminin extensively. Biotin-switch and proximity ligation assays demonstrated that podocyte-specific VEGF164 gain of function decreased glomerular S-nitrosylation of laminin in eNOS−/− mice. In addition, treatment with VEGF-A decreased S-nitrosylated laminin in cultured podocytes. Collectively, these data indicate that excess glomerular VEGF-A and eNOS deficiency is necessary and sufficient to induce Kimmelstiel-Wilson–like nodular glomerulosclerosis in mice through a process that involves deposition of laminin and collagen IV and de-nitrosylation of laminin.


Vascular glomerular endothelial factor-A (VEGF-A) is essential for the development and maintenance of normal glomerular structure and function.1 Podocytes are the most important source of glomerular VEGF-A.1–4 Glomerular VEGF-A plays a critical role in the pathogenesis of diabetic nephropathy.5–7 Transgenic mice with podocyte VEGF164 gain of function develop a glomerular phenotype indistinguishable from early diabetic nephropathy, in the context of normal blood glucose and normal systemic VEGF-A.5 In the setting of type 1 diabetes, plasma VEGF-A increases but nodular glomerulosclerosis develops only in mice with podocyte VEGF164 gain of function, demonstrating that local rather than systemic VEGF excess is critical for the progression of diabetic glomerulopathy to advanced disease.6

Nitric oxide (NO) is a product of arginine oxidation: \( \text{L arginine} + \text{O}_2 \rightarrow \text{citrulline} + \text{NO} \), catalyzed by NO synthase (NOS). The major source of endogenous NO, NOS isoforms (neuronal NOS, inducible NOS, and endothelial NOS),8–10 are expressed in the kidney.11–14 VEGF-A activates endothelial NOS (eNOS), inducing NO generation, which stimulates soluble guanylate cyclase, thereby causing vasodilatation. VEGF-A activates eNOS via phosphatidylinositol-3-kinase/Akt.15 Signals downstream from VEGF-A and NO stimulate endothelial cell proliferation and migration in human endothelium, regulate endothelial integrity, and contribute to angiogenesis.16–21 In diabetes, low
NO bioavailability is associated with high VEGF-A levels.7,8,22–25 Nakagawa et al. called this process “uncoupling of VEGF to NO,” connecting mechanistically the advanced nephropathy with the relationship between VEGF and NO in the kidney.26 Experimental diabetes induced in eNOS knockout (KO) mice resulted in severe diabetic nephropathy: nodular glomerulosclerosis, decreased GFR, and hypertension, associated with increased VEGF mRNA renal expression.26,27 Consistent with these findings, db/db mice treated with L-arginine and sepiapterin had improved albuminuria and glomerular basement membrane (GBM) thickness, suggesting that improving eNOS activity delays the progression of diabetic nephropathy.28 However, the mechanisms whereby excess VEGF-A and eNOS insufficiency lead to advanced diabetic nephropathy remain unclear.

At the cellular level, binding of NO to soluble guanylate cyclase leads to increased cyclic guanosine monophosphate (cGMP) production and activation of protein kinase G, phosphodiesterases, and cGMP-gated ion channels. However, extensive evidence demonstrates that NO exerts multiple biologic functions through cGMP–independent S-nitrosylation of proteins.29–32 S-Nitrosylation is a reversible, covalent addition of NO to thiol groups on specific cysteine from proteins, forming nitroso-protein (SNO).30–32 Nitrosylation induces redox-based conformational changes in target proteins that modulate signaling and function.32 Altered protein S-nitrosylation has been demonstrated in pulmonary, hematologic, neurologic, and cardiovascular diseases, as well as in cancer, preeclampsia, and diabetes.31,33

We hypothesized that deficient S-nitrosylation of specific proteins mediates the glomerular phenotype resulting from eNOS deletion and excess VEGF-A in vivo. Here we examined the effects of increased podocyte VEGF164 in eNOS KO mice and evaluated whether S-nitrosylation is mechanistically involved in the ensuing glomerular phenotype. Our findings indicate that podocyte VEGF164 gain of function in eNOS null mice is sufficient to induce nodular glomerulosclerosis, massive proteinuria, and renal failure in the absence of diabetic milieu. Podocyte VEGF164 gain of function decreases glomerular laminin S-nitrosylation in eNOS null mice, linking this post-translational modification to nodular glomerulosclerosis.

RESULTS

Podocyte VEGF164 Gain of Function Induces Proteinuria and Renal Failure in eNOS KO Mice

Podocyte VEGF164 gain of function in eNOS KO mice (iVEGF: eNOS+/-/-) resulted in massive proteinuria, assessed by SDS-PAGE/Coomassie stain (Figure 1A) and quantified by ELISA. Podocyte VEGF164 gain of function exacerbated the albuminuria observed in eNOS KO mice (Figure 1B, left panel), whereas it induced less pronounced albuminuria in eNOS wild-type mice (Figure 1B, right panel; notice scale difference), indicating that excess VEGF-A and lack of eNOS synergistically induce proteinuria. Podocyte VEGF164 gain of function in eNOS KO mice induced renal failure: creatinine clearance decreased to 45% of control values (7.2±2.1 versus 3.3±0.7 μl/min per g body weight) (Figure 1C, left panel). Conversely, podocyte VEGF164 gain of function in eNOS wild-type mice induced hyperfiltration: creatinine clearance increased >350% (6.6±1.5 versus 23.4±3.7 μl/min per g body weight) (Figure 1C, right panel), as we previously described.5 Surprisingly, systolic BP was normal in eNOS null mice with or without podocyte VEGF164 gain of function for 3 months (99.5±2.8 mmHg versus 97.1±3 mmHg; P=NS). Collectively, these results show that podocyte VEGF164 gain of function in eNOS KO mice severely disrupts renal function, inducing massive proteinuria and kidney failure.

Plasma VEGF-A was approximately 2-fold higher in eNOS KO mice (Figure 1D, left panel) than in eNOS wild-type mice (Figure 1D, right panel), and podocyte VEGF164 gain-of-function did not alter plasma VEGF in either group of mice. VEGF-A urine excretion was lower in eNOS KO mice than in eNOS wild-type mice (Figure 1E). Notably, podocyte VEGF164 gain of function in eNOS KO mice altered VEGF excretion in a manner consistent with changes in GFR (Figure 1, C and E), as indicated by similar VEGF-A/creatinine (35±6 versus 25±15 pg/μg; P=NS). Plasma NO was several-fold higher in eNOS-deficient than in wild-type mice (Figure 1F), and podocyte VEGF164 gain of function increased circulating NO only in the latter (Figure 1F, right panel). NO excretion was similar in eNOS-deficient and wild-type mice. In addition, podocyte VEGF164 gain of function did not induce significant increase in urine NO excretion in eNOS-deficient mice but increased NO excretion several fold in eNOS wild-type mice (Figure 1G). Urine NO positively correlated with urine VEGF in mice with intact eNOS, suggesting that urine NO excretion depends on VEGF excretion (Figure 1H). However, the correlation between urine VEGF and urine NO was lost in eNOS null mice (Figure 1I), suggesting that VEGF-A and NO direct correlation is eNOS dependent.

Podocyte VEGF164 Gain of Function Induces Nodular Glomerulosclerosis in eNOS KO mice

Induction of podocyte VEGF164 gain of function in eNOS KO mice for 1 month resulted in mesangial sclerosis, while non-induced controls with identical genotype showed normal glomerular histology (Figure 2, 1–3). After 3 months of podocyte VEGF164 gain of function, glomerular lesions progressed to nodular glomerulosclerosis associated to protein casts, vascular hyalinosis, endothelial injury, mesangiolysis, and microaneurysms (Figure 2, 4–15). Quantitation of glomerular periodic acid-Schiff (PAS)–positive nodules revealed nodules in approximately 6% of glomeruli from eNOS KO mice with podocyte VEGF164 gain of function, whereas no nodules were observed in glomeruli from control eNOS KO mice (0%) (n=146±11 glomeruli/kidney; P<0.05) (Figure 2, Table 1).
A semi-quantitative pathologic score, including nodules, mesangial sclerosis, mesangiolysis, and interstitial fibrosis, confirmed the observation that podocyte VEGF<sub>164</sub> gain of function in eNOS KO mice significantly increases glomerular abnormalities (Figure 2, Table 1). Nonrandom association of eNOS KO+VEGF<sub>164</sub> gain of function with glomerular nodules and mesangiolysis was further confirmed by Fischer exact tests ($P=0.001$ and $P=0.01$, respectively).

Ultrastructural analysis showed that eNOS KO have normal glomerular ultrastructure (Figure 3A). By contrast, podocyte VEGF<sub>164</sub> gain of function in eNOS KO mice results in podocyte effacement and GBM thickening (Figure 3B). A semi-quantitative score including foot process effacement, mesangiolysis, endothelial injury and GBM thickening showed increased ultrastructural damage in eNOS KO mice with podocyte VEGF<sub>164</sub> gain of function (Table 2). eNOS wild-type mice showed normal glomerular histology and ultrastructure (Supplemental Figure 1).

Podocyte VEGF<sub>164</sub> gain of function in eNOS wild-type mice induced glomerulomegaly, mesangial expansion, GBM thickening with absence of lamina rara interna.
and externa, and partial FPE, changes indistinguishable from early diabetic nephropathy, as previously described.\(^5\) Together, these findings indicate that podocyte VEGF\(_{164}\) gain of function in eNOS KO mice results in a glomerular phenotype indistinguishable from advanced diabetic nephropathy in the context of normal blood glucose (Table 3).

**Laminin and Collagen IV Upregulation in Glomerular Nodules**

We observed that podocyte VEGF\(_{164}\) gain of function in eNOS KO mice induced a significant increase in immunoreactive laminin and collagen IV colocalized to the glomerular nodules (Figure 4A). Eosinophilic PAS-stained nodules observed in consecutive kidney sections confirmed the presence of excess laminin and collagen IV in the nodules (Figure 4A, middle and right panels). By contrast, both collagen IV and laminin were limited to the GBM in glomeruli without nodules (Figure 4, left panels and right bottom panels). Quantitation of immunofluorescence signals revealed significantly increased laminin and collagen IV in glomeruli from eNOS-deficient mice with podocyte VEGF\(_{164}\) gain of function (Figure 4B), while quantitation of whole kidney lysate laminin by Western analysis showed similar laminin expression level in both groups of eNOS KO mice (Figure 4C), suggesting that the excess laminin is limited to glomerular nodules.

We previously reported that podocyte VEGF\(_{164}\) gain of function during 12 weeks in diabetic mice induces nodular glomerulosclerosis and advanced glomerulopathy.\(^6\) Here, we immunostained kidney sections from those mice and detected both laminin and collagen IV colocalized mainly in diabetic nodules (Figure 5A), a pattern similar to that observed in eNOS KO mice with podocyte VEGF\(_{164}\) gain of function (Figure 5B). Collectively, these findings suggest that in mice with insufficient eNOS function, induced by diabetes mellitus or by eNOS deletion, increased glomerular VEGF-A causes laminin and collagen IV accumulation in glomerular nodules.

Surprisingly, fibronectin expression was similar in eNOS KO mice with or without podocyte VEGF\(_{164}\) gain of function, as assessed by immunohistochemistry (IHC) and immunoblotting (Supplemental Figure 2), suggesting that fibronectin is not involved in glomerular nodule development. To assess the basis for the PFE, slit-diaphragm proteins were examined by immunoblot and IHC. Podocyte VEGF\(_{164}\) gain of function in eNOS KO mice decreased nephrin expression, while podocin and Wilms’ tumor 1 remained unchanged (Figure 6, A and B), suggesting that increased VEGF-A signaling and decreased NO induce nephrin downregulation without podocyte loss, as we previously described.\(^5\) Collectively, these findings indicate that nephrin is not involved in glomerular nodule development. To assess the basis for the PFE, slit-diaphragm proteins were examined by immunoblot and IHC. Podocyte VEGF\(_{164}\) gain of function in eNOS KO mice decreased nephrin expression, while podocin and Wilms’ tumor 1 remained unchanged (Figure 6, A and B), suggesting that increased VEGF-A signaling and decreased NO induce nephrin downregulation without podocyte loss, as we previously described.\(^5\) Collectively, these findings indicate that nephrin is not involved in glomerular nodule development. To assess the basis for the PFE, slit-diaphragm proteins were examined by immunoblot and IHC. Podocyte VEGF\(_{164}\) gain of function in eNOS KO mice decreased nephrin expression, while podocin and Wilms’ tumor 1 remained unchanged (Figure 6, A and B), suggesting that increased VEGF-A signaling and decreased NO induce nephrin downregulation without podocyte loss, as we previously described.\(^5\)

**Laminin Denitrosylation Is Involved in Nodular Glomerulosclerosis**

Oxidative addition of an NO molecule to the thiol group of cysteine residues is a physiologically important posttranslational protein modification, implicated in several metabolic and pathophysiologic events.\(^33\) We tested whether podocyte VEGF\(_{164}\) gain of function disrupts protein S-nitrosylation in eNOS KO mice. IHC using a nitrosocysteine antibody revealed that VEGF-A excess induces significant decrease in SNO of glomerular proteins (Figure 7A). Dual immunostaining showed that SNO signals merge with laminin, particularly in glomeruli from eNOS KO mice (Figure 7A). Furthermore,
Table 1. Semiquantitative pathologic score from iVEGF:eNOS<sup>−/−</sup>

<table>
<thead>
<tr>
<th>Variable</th>
<th>−dox (Control)</th>
<th>+dox 1 Month</th>
<th>+dox 3 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesangial nodules</td>
<td>0±0</td>
<td>0±0</td>
<td>5.7±2.2&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mesangiolysis (%)</td>
<td>0±0</td>
<td>8.5±1.3</td>
<td>28.2±9.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mesangial sclerosis (%)</td>
<td>6.8±1</td>
<td>13.3±1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.8±9.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Interstitial fibrosis (%)</td>
<td>2±0</td>
<td>13.4±3.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.8±3.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Controls were age-matched with +dox×1 month (n=5) and age-matched with +dox×3 months (n=4). The number of glomeruli containing nodules, mesangial sclerosis, or mesangiolysis was counted in PAS-stained kidney sections and expressed as percentage of total glomeruli; interstitial fibrosis represents the area of injured tissue as percentage of total kidney section area. Data are expressed as mean±SEM.

<sup>a</sup>P<0.05 compared with control.

<sup>b</sup>P<0.05 comparing 1 month versus 3 months of doxycycline induction.

Figure 3. Podocyte VEGF<sub>164</sub> gain of function induces GBM thickening, podocyte effacement in eNOS KO mice. Transmission electron microscopy reveals the following: (A) control eNOS KO mice (iVEGF:eNOS<sup>−/−</sup>−dox) glomeruli showing normal ultrastructure and (B) glomeruli from eNOS KO mice with VEGF<sub>164</sub> gain of function (iVEGF:eNOS<sup>−/−</sup>+dox) showing GBM thickening, absence of lamina rara interna and externa, and FPE. Images were taken at 5000× magnification.

Diabetic nephropathy is a devastating complication leading to renal failure that affects up to 40% of diabetic patients worldwide. Recent studies demonstrated that VEGF-A and eNOS play a critical role in the pathogenesis of advanced diabetic nephropathy, although the molecular mechanisms involved are not fully understood. Here, we present evidence that excess glomerular VEGF-A and eNOS deficiency are necessary and sufficient to induce Kimmelstiel-Wilson–like nodular glomerulosclerosis in mice. Indeed, eNOS KO mice with podocyte VEGF<sub>164</sub> gain of function develop massive proteinuria, renal failure, and nodular glomerulosclerosis, a phenotype indistinguishable from advanced diabetic nephropathy, in the absence of diabetic milieu.

Collectively, these findings demonstrate laminin S-nitrosylation in vivo and in cultured podocytes and show that excess VEGF-A downregulates laminin S-nitrosylation in the absence of eNOS or NOS activity, suggesting that laminin de-nitrosylation may contribute to glomerular nodule development.

DISCUSSION
Table 2. Transmission electron microscopy semiquantitative pathologic score from iVEGF:eNOS−/−

<table>
<thead>
<tr>
<th>Variable</th>
<th>iVEGF:eNOS−/− –dox (n=4)</th>
<th>iVEGF:eNOS−/− +dox (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPE (%)</td>
<td>25±6.5</td>
<td>53.3±11a</td>
</tr>
<tr>
<td>Mesangiolysis (%)</td>
<td>1.2±1.2</td>
<td>7.5±4.8</td>
</tr>
<tr>
<td>Endothelial Injury (%)</td>
<td>11.2±6.6</td>
<td>15.8±11.4</td>
</tr>
<tr>
<td>GBM thickening (%)</td>
<td>13.8±8.9</td>
<td>40.5±8.6</td>
</tr>
</tbody>
</table>

All values represent the percentage area of glomerular capillary tuft involving the indicated abnormalities in mice that received doxycycline for 3 months and their age-matched controls. Values are provided as mean±SEM.

*P<0.05 compared with control.

Table 3. General parameters from iVEGF:eNOS−/− mice

<table>
<thead>
<tr>
<th>Variable</th>
<th>–dox (Control) (n=10)</th>
<th>+dox 1 Month (n=14)</th>
<th>–dox (Control) (n=12)</th>
<th>+dox 3 Months (n=11)</th>
</tr>
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<tr>
<td>Age (d)</td>
<td>74±7</td>
<td>83±5</td>
<td>140±9</td>
<td>146±4</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>21±1</td>
<td>24±2a</td>
<td>23±1</td>
<td>25±1a</td>
</tr>
<tr>
<td>Kidney weight (mg)</td>
<td>146±8</td>
<td>163±11a</td>
<td>161±5</td>
<td>159±10</td>
</tr>
<tr>
<td>Urine volume (ml/d)</td>
<td>0.5±0.1</td>
<td>0.6±0.2</td>
<td>0.7±0.09</td>
<td>0.4±0.04</td>
</tr>
<tr>
<td>Blood glucose (mg/dl)</td>
<td>221±18</td>
<td>203±17</td>
<td>189±16</td>
<td>188±13</td>
</tr>
<tr>
<td>Proteinuria dipstick (+)</td>
<td>1.8±0.6</td>
<td>3±0.4a</td>
<td>1.5±0.3</td>
<td>3.2±0.2a</td>
</tr>
</tbody>
</table>

Values are provided as mean±SEM.

*P<0.05 compared with control.

VEGFR2–β-integrin signaling through direct protein-protein interactions probably underlie both the absence of podocytopenia and the FPE observed in this study. Systemic VEGF-A level and urine VEGF-A excretion are not useful markers of advanced nephropathy in the context of eNOS insufficiency because high systemic VEGF-A overrides glomerular VEGF-A, as shown here in eNOS KO mice and previously in diabetic mice.

Glomerular nodules observed in eNOS KO mice with podocyte VEGF164 gain of function are characterized by a lamina structured, accumulation of acellular PAS-positive material surrounded by cellular nuclei and accumulation of collagen IV and laminin, a pattern similar to that observed in diabetic mice. Mesangiolysis has been linked to nodular formation in a landmark study of renal biopsy specimens from diabetic patients. In the present work, significant mesangiolysis coexisted with nodular glomerulosclerosis, suggesting that even in the absence of diabetic milieu VEGF-A excess leads to nodule generation in a mouse model susceptible to endothelial injury. An additional mechanism thought to contribute to nodule formation in eNOS KO mice is hypertension. In the present study, all anesthetized eNOS KO were normotensive, while glomerular nodules developed only in those with VEGF gain of function. Hence, the data demonstrate that in eNOS KO mice podocyte VEGF164 gain of function leads to Kimmelstiel-Wilson–like nodule formation associated with mesangiolysis in the absence of diabetic milieu or hypertension.

Cross-talk and positive feedback between VEGF-A and NO pathways play an important role in the pathogenesis of diabetic nephropathy. Diabetic eNOS KO mice and diabetic VEGF164 gain-of-function mice share similar diabetic milieu and nephropathy pathogenic mechanism (i.e., increased glomerular VEGF-A and eNOS insufficiency), resulting in advanced disease phenotype (i.e., Kimmelstiel-Wilson–like nodular glomerulosclerosis and severe proteinuria). Conversely, nondiabetic mice with intact eNOS and VEGF164 gain of function develop a phenotype similar to early diabetic nephropathy. Consistent with this, increasing eNOS activation with L-arginine or a BH4 analogue in diabetic mice improves proteinuria and GBM thickness. Collectively, the data demonstrate that in the context of eNOS insufficiency, increased glomerular VEGF-A is necessary and sufficient to induce advanced nephropathy.

NOS activity and NO production are preserved and neuronal NOS is upregulated in eNOS KO mice. In keeping with this, we find that plasma NO is elevated (as is plasma VEGF-A), while iNOS expression level and NO excretion are normal in eNOS-deficient mice, suggesting that other sources of NO located in the vicinity of podocytes are stimulated by increased glomerular VEGF-A in eNOS KO mice. Conversely, eNOS KO in Murphy Roths Large/lpr mice, a genetic model of lupus nephritis, decreases NO excretion, probably because of autoimmunity, iNOS, or differences in genetic background. Triple KO mice lacking nNOS, iNOS, and eNOS have impaired renal cyclic adenosine monophosphate production and aquaporin-2 expression, resulting in nephrogenic diabetes insipidus-like phenotype, with no apparent glomerular disease. These discrepancies underscore the intriguing possibility that alternative sources of NO (nonenzymatic) may have pathogenic effects, warranting further studies.

A key finding of this study is that VEGF-A decreases S-nitrosylation in renal glomeruli. We show for the first time that laminin is nitrrosylated in vivo and that VEGF164 gain of function decreases laminin nitrosylation in eNOS KO mice, while VEGF-A excess does not alter laminin nitrosylation in the
context of sufficient eNOS. In cultured podocytes laminin is nitrosylated at baseline or under NOS inhibition, and exposure to VEGF-A downregulates laminin S-nitrosylation cell autonomously, suggesting that laminin de-nitrosylation observed in vivo is not a consequence of severe glomerular injury but rather is triggered by VEGF signaling in podocytes. VEGF-A and eNOS can modulate protein S-nitrosylation in endothelial cells. VEGF-A induces β-catenin S-nitrosylation in an eNOS-dependent manner in endothelial cells, and β-catenin S-nitrosylation is inhibited in eNOS null mice.

We demonstrate laminin S-nitrosylation in vivo using three independent methods: dual-label immunofluorescence, proximity link assay, and biotin switch assay, an accepted approach for documenting SNO. Collectively, our data suggest that VEGF-A–induced loss of laminin S-nitrosylation is involved in the development of nodular glomerulosclerosis, raising the important question of whether loss of S-nitrosylation causes laminin aggregate deposition forming glomerular nodules. Laminins are secreted as αβγ heterotrimers that undergo a maturation process involving protein folding, post-translation modifications; and polymerization among α, β, and γ subunits. Defects in protein coding sequence or protein maturation can lead to disease. A mutation of β2 laminin (R246Q) impairs LM521 secretion. Misfolded proteins can be retained in the endoplasmic reticulum, be targeted for proteasomal degradation or become resistant to degradation by proteases, and accumulate ectopically, as described for nephrin, cystic fibrosis transmembrane conductance regulator, and amyloid-β. VEGF-A–induced loss of laminin nitrosylation may disrupt folding, assembly, or polymerization of laminin isoforms; increase their secretion; or disrupt their normal interactions with collagen IV.

All LM521 isoforms are increased in advanced human diabetic nephropathy, suggesting that laminin trimer defects are probably not involved in glomerular nodule formation. Laminin polymerization is Ca++ dependent and is modulated by heparin, and VEGF-A may directly modulate these steps, in addition to increasing collagen IV secretion. Future studies will uncover these molecular mechanisms, as well as identify the laminin isoforms and the specific Cys undergoing S-nitrosylation. The inherent reversibility of S-nitrosylation implies that this potential pathogenic mechanism may be a target for therapeutic intervention in DN. Because laminin is an important component of all basement membranes and VEGF-A has pleiotropic effects, our findings may be relevant for multiple organs and diseases.
In summary, excess glomerular VEGF-A and eNOS deficiency are necessary and sufficient to induce Kimmelstiel-Wilson–like nodular glomerulosclerosis in mice, a glomerular phenotype indistinguishable from advanced diabetic nephropathy, in the absence of diabetic milieu. The development of glomerular nodules involves laminin and collagen IV deposition and decreased laminin S-nitrosylation.

**CONCISE METHODS**

**Generation of Inducible Podocyte VEGF164 Overexpression in eNOS KO Mice**

eNOS KO mice58 (eNOS KO, C57BL/6j-Nos3tm1Unc; The Jackson Laboratory, Bar Harbor, ME) were crossbred with podocin-rtTA:tet-O-VEGF164 mice,5 backcrossed to stable C57BL/6j genetic background, and fed standard or doxycycline-containing chow for 1 or 3 months. All experimental protocols were approved by the Institutional Animal Care and Use Committee at Yale University School of Medicine.

**Functional Parameters**

GFR was estimated by creatinine clearance, albuminuria was assessed by Coomassie blue staining and ELISA, plasma and urine VEGF-A was quantified by ELISA, random blood glucose was measured by glucose oxidase biosensor, and BP was measured under anesthesia, as previously described.6

**Histology, Morphometric Analysis, and Transmission Electron Microscopy**

Renal phenotype was characterized by light and electron microscopy. A renal pathologist (G.M.) examined each kidney specimen in a blinded fashion. Morphometric analysis was performed using point-counting technique on PAS-stained sections; pathologic features were expressed as percentage of injured tissue or injured glomerular area.6 Mesangiolysis and glomerular nodules were quantified as percentage glomeruli per section containing mesangiolysis or nodules, as previously described.6 Transmission electron microscopy was performed as previously described.5,6 Ultrastructural features were quantified by a renal pathologist (G.M.) as percentage of entire glomerular capillary tuft from six images per mouse in four mice per experimental group. The following features were analyzed: FPE, mesangiolysis, endothelial injury, and GBM thickening (Table 2). IHC and immunoblotting were performed as previously described.5,6 Quantitation of immunofluorescent signals was performed in 5–10 glomeruli per mouse, n=5/experimental group using ImageJ software (National Institutes of Health, Bethesda, MD), as previously described.59

**Podocyte Assay**

Immortalized mouse iVEGF podocytes were cultured in control medium as previously described37 or exposed to VEGF165, L-NAME, or...
L-NAME+VEGF165, and examined by dual immunocytochemistry or PLA as described in the Supplemental Methods.

**In Situ PLA**

PLA was performed in kidney frozen sections and immortalized mouse podocytes using total laminin rabbit polyclonal antibody (Sigma-Aldrich) and S-nitrosocysteine mouse monoclonal antibody (AG Scientific), using detection reagents orange according to the Duolink II protocol (Olink Bioscience, Uppsala, Sweden), as detailed in the Supplemental Methods.

**Biotin Switch Assay**

We assessed laminin S-nitrosylation by biotin switch assay in whole kidney lysates using a S-nitrosylated protein detection kit (Cayman Chemical, Co.), as per manufacturer’s instructions with minor modifications.

**Statistical Analyses**

Data are expressed as mean±SEM. Statistical significance (P<0.05) was determined by unpaired t test and one-way ANOVA to compare two or multiple experimental groups, respectively. Association between two variables was evaluated by Pearson correlation or Fischer exact test.

**ACKNOWLEDGMENTS**

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**DISCLOSURES**

None.
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