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

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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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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 VEGF<sub>164</sub> 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 VEGF<sub>164</sub> 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: L arginine+O₂ → citrulline+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

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NO bioavailability is associated with high VEGF-A levels.\textsuperscript{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.\textsuperscript{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.\textsuperscript{26,27} Consistent with these findings, db/db mice treated with l-arginine and sepiapterin had improved albuminuria and glomerular basement membrane (GBM) thickness, associated with reversed eNOS dimerization and phosphorylation, suggesting that improving eNOS activity delays the progression of diabetic nephropathy.\textsuperscript{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.\textsuperscript{29–32} S-Nitrosylation is a reversible, covalent addition of NO to thiol groups on specific cysteine from proteins, forming nitroso-protein (SNO).\textsuperscript{30–32} Nitrosylation induces redox-based conformational changes in target proteins that modulate signaling and function.\textsuperscript{32} Altered protein S-nitrosylation has been demonstrated in pulmonary, hematologic, neurologic, and cardiovascular diseases, as well as in cancer, preeclampsia, and diabetes.\textsuperscript{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 VEGF\textsuperscript{164} in eNOS KO mice and evaluated whether S-nitrosylation is mechanistically involved in the ensuing glomerular phenotype. Our findings indicate that podocyte VEGF\textsuperscript{164} 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 VEGF\textsuperscript{164} gain of function decreases glomerular laminin S-nitrosylation in eNOS null mice, linking this post-translational modification to nodular glomerulosclerosis.

**RESULTS**

**Podocyte VEGF\textsuperscript{164} Gain of Function Induces Proteinuria and Renal Failure in eNOS KO Mice**

Podocyte VEGF\textsuperscript{164} gain of function in eNOS KO mice (iVEGF: eNOS\textsuperscript{−/−}) resulted in massive proteinuria, assessed by SDS-PAGE/Comassie stain (Figure 1A) and quantified by ELISA. Podocyte VEGF\textsuperscript{164} 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 VEGF\textsuperscript{164} 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 VEGF\textsuperscript{164} 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.\textsuperscript{5} Surprisingly, systolic BP was normal in eNOS null mice with or without podocyte VEGF\textsuperscript{164} gain of function for 3 months (99.5±2.8 mmHg versus 97.1±3 mmHg; P=NS). Collectively, these results show that podocyte VEGF\textsuperscript{164} 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 VEGF\textsuperscript{164} 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 VEGF\textsuperscript{164} gain of function in eNOS KO mice altered VEGF excretion in a manner consistent with changes in GFR (Figure 1C, D, 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 VEGF\textsuperscript{164} 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 VEGF\textsuperscript{164} 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 VEGF\textsuperscript{164} Gain of Function Induces Nodular Glomerulosclerosis in eNOS KO mice**

Induction of podocyte VEGF\textsuperscript{164} 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 VEGF\textsuperscript{164} gain of function, glomerular lesions progressed to nodular glomerulosclerosis associated to protein casts, vascular hyalinosis, endothelial injury, mesangiolyis, 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 VEGF\textsuperscript{164} 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 filtration barrier (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 (FPE), 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.

Figure 1. Podocyte VEGF<sub>164</sub> gain of function in eNOS KO mice induces proteinuria and renal failure. (A) SDS PAGE/Coomasie stain shows severe albuminuria in eNOS<sup>−/−</sup> mice with VEGF<sub>164</sub> gain of function (iVEGF:eNOS<sup>−/−</sup>+dox) compared with control mice (iVEGF:eNOS<sup>−/−</sup>−dox). BSA standard (1 μg). (B) ELISA confirms massive albuminuria in iVEGF:eNOS<sup>−/−</sup>+dox mice (left panel), approximately 23-fold compared with control iVEGF:eNOS<sup>−/−</sup>−dox mice and iVEGF mice; (right panel) iVEGF +dox develop mild albuminuria, approximately 22-fold compared with iVEGF control mice. Note that the scale bar is 15 times smaller. (C) Creatinine clearance decreases about 55% in iVEGF:eNOS<sup>−/−</sup>+dox mice compared with control mice (iVEGF:eNOS<sup>−/−</sup>−dox); whereas in mice with intact eNOS, VEGF<sub>164</sub> gain of function (iVEGF

+dox) increases creatinine clearance approximately 3-fold compared with controls (iVEGF−dox). (D) Plasma VEGF-A increased approximately 2-fold in all eNOS<sup>−/−</sup> mice compared with mice with intact eNOS (iVEGF); podocyte VEGF<sub>164</sub> gain of function did not alter plasma VEGF-A levels in either group. (E) Urine VEGF is lower in eNOS<sup>−/−</sup> mice than in mice with intact eNOS and decreases further with VEGF<sub>164</sub> gain of function, whereas it increases in mice with intact eNOS. (F) Plasma NO is about 9-fold higher in eNOS<sup>−/−</sup> than in mice with intact eNOS; VEGF<sub>164</sub> gain of function increases plasma NO approximately 3-fold only in mice with intact eNOS. (G) NO urine excretion in iVEGF:eNOS<sup>−/−</sup> mice is normal; podocyte VEGF<sub>164</sub> gain of function increases NO excretion only in mice with intact eNOS. (H) NO urine excretion in eNOS<sup>−/−</sup> mice is positively correlated with urine VEGF. (I) Correlation between urine NO and urine VEGF is abrogated in iVEGF:eNOS<sup>−/−</sup> mice. *P<0.05 versus respective control, #P<0.05 compared with iVEGF control.
and externa, and partial FPE, changes indistinguishable from early diabetic nephropathy, as previously described. Together, these findings indicate that podocyte VEGF164 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 VEGF164 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 VEGF164 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 VEGF164 gain of function during 12 weeks in diabetic mice induces nodular glomerulosclerosis and advanced glomerulopathy. 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 VEGF164 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 VEGF164 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 FPE, slit-diaphragm proteins were examined by immunoblot and IHC. Podocyte VEGF164 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 in eNOS wild-type mice. Podocyte VEGF164 gain of function in eNOS wild-type mice showed laminin and collagen IV expression similar to control mice with identical genotype (Supplemental Figure 3), whereas nephrin expression decreased in VEGF164 overexpressing mice.

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. We tested whether podocyte VEGF164 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\textsuperscript{−/−}.

<table>
<thead>
<tr>
<th>Variable</th>
<th>−dox (Control)</th>
<th>+dox 1 Month (n=5)</th>
<th>+dox 3 Months (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesangial nodules (%)</td>
<td>0±0</td>
<td>0±0</td>
<td>5.7±2.2\textsuperscript{a,b}</td>
</tr>
<tr>
<td>Mesangiolysis (%)</td>
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<td>8.5±1.3</td>
<td>28.2±9\textsuperscript{a,b}</td>
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<tr>
<td>Mesangial sclerosis (%)</td>
<td>6.8±1</td>
<td>13.3±1.2\textsuperscript{a}</td>
<td>39.8±9.4\textsuperscript{a,b}</td>
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<tr>
<td>Interstitial fibrosis (%)</td>
<td>2±0</td>
<td>13.4±3.4\textsuperscript{a}</td>
<td>14.8±3.9\textsuperscript{a}</td>
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Controls were age-matched with +dox x 1 month (n=5) and age-matched with +dox x 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.

\textsuperscript{a}P<0.05 compared with control.

\textsuperscript{b}P<0.05 comparing 1 month versus 3 months of doxycycline induction.

DISCUSSION

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\textsubscript{164} gain of function develop massive proteinuria, renal failure, and nodular glomerulosclerosis, a phenotype indistinguishable from advanced diabetic nephropathy, in the absence of diabetic milieu.

eNOS KO mice are susceptible to developing renal failure when renal mass is reduced experimentally.\textsuperscript{21} Similarly, type 1 and type 2 diabetes decrease GFR in eNOS KO mice.\textsuperscript{26,27,35} We previously reported that induction of VEGF\textsubscript{164} gain of function in diabetic mice causes less hyperfiltration than in nondiabetic mice.\textsuperscript{6} Here we find that podocyte VEGF\textsubscript{164} gain of function in eNOS KO mice causes renal failure, suggesting that eNOS function is required to maintain GFR when glomerular VEGF-A is upregulated. Moreover, we show that VEGF\textsubscript{164} gain of function and eNOS deletion effects on proteinuria are synergistic, >10-fold above either individual genotype. Proteinuria in eNOS-deficient mice with VEGF\textsubscript{164} gain of function is associated with nephrin downregulation without change in podocin and Wilms’ tumor 1 expression, suggesting no significant podocyte loss, as previously reported in diabetic and nondiabetic mice with VEGF\textsubscript{164} gain of function.\textsuperscript{5,6} VEGF-A function as a podocyte survival factor\textsuperscript{3,36} and nephrin–
VEGF2–β-integrin signaling through direct protein-protein interactions probably underlie both the absence of podocyte 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.6

Glomerular nodules observed in eNOS KO mice with podocyte VEGF164 gain of function are characterized by a laminated structure, 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.39 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.40 In the present study, all anesthetized eNOS KO were normotensive, while glomerular nodules developed only in those with VEGF-A 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.41,42 Diabetic eNOS KO mice26,35 and diabetic VEGF164 gain-of-function mice6 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.5,6 Consistent with this, increasing eNOS activation with l-arginine or a BH4 analogue in diabetic mice improves proteinuria and GBM thickness.28 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.43,44 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.45 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.46,47 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 nitrosylated 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.\textsuperscript{48-49} VEGF-A induces \(\beta\)-catenin S-nitrosylation in an eNOS-dependent manner in endothelial cells, and \(\beta\)-catenin S-nitrosylation is inhibited in eNOS null mice.\textsuperscript{50} 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.\textsuperscript{32,33,50}

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 \(\alpha\beta\gamma\) heterotrimers that undergo a maturation process involving protein folding, post-translation modifications; and polymerization among \(\alpha\), \(\beta\), and \(\gamma\) subunits.\textsuperscript{51,52} Defects in protein coding sequence or protein maturation can lead to disease. A mutation of \(\beta_2\) laminin (R246Q) impairs LM521 secretion.\textsuperscript{53} 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-\(\beta\).\textsuperscript{54-56} 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,\textsuperscript{57} suggesting that laminin trimer defects are probably not involved in glomerular nodule formation. Laminin polymerization is \(\text{Ca}^{2+}\)-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

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**Figure 5.** Podocyte VEGF164 gain of function in diabetic mice and in eNOS KO nondiabetic mice induces similar glomerular laminin and collagen IV expression patterns. IHC shows (top) remarkable laminin (green) and collagen IV (red) accumulation in glomerular nodules from (A) diabetic mice with intact eNOS (DM-IVEGF) and (B) from eNOS KO nondiabetic mice (iVEGF:eNOS–/+) when podocyte VEGF164 gain of function is induced; notice that laminin and collagen IV colocalize [merge=yellow] and have similar expression patterns. IHC also shows (bottom) glomeruli without nodules with less abundant laminin and collagen IV expression in both groups of mice (A and B).

**Figure 6.** Podocyte VEGF164 gain of function in eNOS KO mice downregulates nephrin. (A) IHC shows that VEGF164 gain of function decreases immunoreactive nephrin in eNOS KO glomeruli (iVEGF:eNOS–/+dox). Scale bar=50 μm. (B) Representative immunoblots show that nephrin expression decreases in kidney lysate from eNOS–/− mice with VEGF164 gain of function (+dox) versus control mice (−dox), while podocin and Wilms’ tumor 1 (WT1) remain unchanged. Quantitations were performed in four or more immunoblots per protein. *P<0.05 versus control.
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 podocytes37 using total laminin rabbit polyclonal antibody (Sigma-Aldrich) and S-nitrosocysteine mouse monoclonal antibody (AG Scientific),55,56 using detection reagents orange according to the Duolink II fluorescence protocol (Olink Bioscience, Uppsala, Sweden),60,61 as detailed in the Supplemental Methods.

Biotin Switch Assay
We assessed laminin S-nitrosylation by biotin switch assay34 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.

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DISCLOSURES
None.

Figure 7. VEGF164 gain of function decreases laminin nitrosylation in eNOS KO mice and in cultured podocytes. (A) IHC shows that immunoreactive laminin (green) and nitroso-cysteine (red) colocalize in glomeruli from eNOS KO (iVEGF:eNOS−/−−dox) mice and that VEGF164 gain of function decreases nitroso-cysteine in eNOS KO mice (iVEGF:eNOS−/−+dox). (B) PLA shows laminin and nitroso-cysteine interaction, indicating the presence of SNO-laminin in eNOS KO glomeruli (iVEGF:eNOS−/−−dox); VEGF164 gain of function decreases this interaction (iVEGF:eNOS−/−+dox). Hoechst33342 (blue) labels nuclei. All scale bars=50 μm. (C) Biotin switch assay (BST) shows (top) laminin immunoblot following pulldown of S-nitrosylated proteins depicting SNO-laminin (neg, negative control). (Bottom) Input shows total laminin. (Right) SNO-laminin quantitation by densitometric analysis from three independent experiments shows that VEGF164 gain of function decreases laminin nitrosylation in eNOS KO kidneys (iVEGF:eNOS−/−+dox). *P<0.05 versus iVEGF:eNOS−/−−dox and iVEGF kidneys. (D) Dual IHC shows colocalization of nitroso-Cys (red) and laminin (green) in podocytes exposed to 100 μM L-NAME with or without 50 ng/ml VEGF165 for 48 and 24 hours, respectively. (E) PLA demonstrates laminin S-nitrosylation in L-NAME+VEGF165, and examined by dual immunocytochemistry or PLA as described in the Supplemental Methods.
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This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2013070752/-/DCSupplemental.
SUPPLEMENTAL INFORMATION

Podocyte VEGF-A gain-of-function induces nodular glomerulosclerosis in eNOS null mice. Veron et al.

Material and methods

Generation of inducible podocyte VEGF_{164} overexpression in eNOS KO mice: eNOS knockout mice (eNOS KO, C57BL/6j-Nos3tm1Unc, Jackson Laboratory, Bar Harbor, ME) were crossbred with podocin-rtTA:tet-O-VEGF_{164} mice that overexpress VEGF_{164} in podocytes upon induction with doxycycline, and backcrossed >10 generations to ensure a stable C57BL/6j genetic background. Podocin-rtTA: tet-O-VEGF_{164}: eNOS^{−/−} mice (iVEGF:eNOS^{−/−}) mice were viable, fertile with normal litter size. General parameters from iVEGF:eNOS^{−/−} mice are shown in Table 3. Mice were genotyped by PCR as previously described. We studied male 112 ± 5 days old mice fed standard diet (iVEGF:eNOS^{−/−} control, n=22) or doxycycline containing diet (0.625 mg/g chow; Harlan-Teklad, Madison, WI, USA) for 1 and 3 months (iVEGF:eNOS^{−/−} +dox, n=25). Additional controls were podocin-rtTA: tet-O-VEGF_{164} mice fed standard diet (iVEGF control n=5), or doxycycline-diet for 2 months (iVEGF +dox n=5). In addition, we performed IHC in kidney tissue from iVEGF diabetic mice previously reported. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the Yale University School of Medicine.

Functional parameters: Glomerular filtration rate was estimated by creatinine clearance. Plasma and 24 h urine creatinine were measured by HPLC. Albuminuria was assessed by western blot, dipstick (Albustix, Bayer) and ELISA (Bethyl) and was
expressed in µg/day. Denatured urine samples (15µl) and albumin standard were resolved in 8% SDS–PAGE and stained with Coomassie blue. Immunoblots were performed with anti-bovine serum albumin antibody (Millipore 07-248).

Blood pressure was measured under anesthesia (1.5–2% isoflurane/O₂), through a PE-50 catheter placed in the carotid artery. The catheter was connected to a pressure transducer, and analyzed using PowerLab/8SP system (Chart; AD Instruments, Colorado Springs, CO, USA), as we previously described.⁶

Plasma and urine VEGF-A was quantified by ELISA (mVEGF-A; R&D, Minneapolis, MN, USA), following the manufacturer’s protocol. Plasma and urine total NO was measured using a colorimetric nitrate reductase-based NO detection kit (Enzo, Life Sciences INT’L, INC, Plymouth Meeting, PA, USA). Random blood glucose was measured by glucose oxidase biosensor blood glucose meter (One-Touch-Ultra-2; LifeScan, Milpitas, CA, USA).

**Histology, morphometric analysis and transmission electronic microscopy (TEM):**

Renal phenotype was characterized by light and electron microscopy. A renal pathologist (GM) examined each kidney specimen in a blinded fashion. Morphometric analysis of renal cortex including glomeruli and interstitium was performed using point-counting technique on PAS-stained sections. Points falling on glomeruli with mesangial sclerosis and interstitial fibrosis were counted and the percentage calculated by dividing points on lesion per 100 points counted. All these pathologic features were expressed as percentage of injured tissue or injured glomerular area.⁶ Mesangiolysis and glomerular nodules were quantified as percentage glomeruli/section containing mesangiolysis/nodules as was previously described.⁶,³⁹
Kidneys were fixed in 3% glutaraldehyde for transmission electron microscopy (TEM) and processed as previously described. Ultrastructural features were quantified by a renal pathologist (GM) as percentage of entire glomerular capillary tuft from 6 images/mice with a total of 4 mice in each experimental group. The following features were analyzed: foot processes effacement, mesangiolysis, endothelial injury and glomerular basal membrane thickening (Table 2).

**Immunohistochemistry:** Kidneys were embedded in OCT (Sakura), and snap frozen in dry ice-isopentane, or fixed in formalin and paraffin embedded. Deparaffinized kidney sections were incubated in sodium citrate for antigen retrieval, blocked with donkey serum, incubated with primary antibody followed by secondary antibody. Cryosections were air dried, fixed in cold acetone, permeabilized with 0.3% triton-X, washed and exposed to blocking donkey serum. Dual–immunolabeling was performed using simultaneously two primary antibodies. Sections were incubated overnight at 4°C with the following primary antibodies: anti-nephrin (Fitzgerald 20R-NP002), anti-collagen IV (Southern Biotech), anti-laminin (Sigma L9393), anti fibronectin (Sigma F3648), and anti S-nitrosocysteine (AG Scientific 1078 and Sigma N5411). Secondary antibodies were Cy2 or Cy3 labeled anti-rabbit, anti-mouse or anti-goat secondary antibodies, as appropriate (Jackson Immunoresearch). Nuclei were stained with Hoechst33342 (Molecular Probes, Invitrogen). Sections were examined by light microscopy (Nikon, Eclipse 50i) or by confocal microscopy (FV300, Olympus). Images were taken at x200 and x400 magnification.

**Immunoblotting:** Kidneys from iVEGF:eNOS−/− mice receiving standard chow (control, n=10) or doxycycline during 3 months (+dox, n=10) were lysed in RIPA buffer +
protease inhibitors (Roche) + 1mM NaVO₄ + 1mM NaF. Protein concentration was determined using bicinchoninic acid (Pierce BCA, Thermo Scientific, Rockford, IL, USA). Equal amount of protein lysate from individual kidneys were combined into separate pools, and 200µg protein/lane were resolved in 8-10% SDS PAGE and immunoblotted. Primary antibodies were nephrin (Fitzgerald Inc.), podocin (Sigma), WT1 (Santa Cruz), laminin (Sigma), fibronectin (Sigma); actin (Sigma) or tubulin (Santa Cruz) was used as protein loading controls. Signals were detected by HRP-labeled secondary antibodies and ECL standard method.

**In situ proximity ligation assay (PLA):** To detect laminin S-nitrosylation PLA was performed using total laminin rabbit polyclonal antibody (Sigma) and S-nitrosocysteine mouse monoclonal antibody (AG Scientific). Kidney frozen sections were fixed in cold acetone 10 minutes, permeabilized with 0.1% triton-X and blocked with 10% donkey serum, 0.3% hydrogen peroxide in PBS. Sections were incubated overnight simultaneously with both primary antibodies. Subsequently, we followed the Duolink® II fluorescence protocol using detection reagents orange, (Olink Bioscience, Uppsala, Sweden). We added secondary antibodies conjugated with oligonucleotides, (PLA probes: donkey anti-rabbit and anti-mouse) and the slides were incubated 1h at 37°C. After two washes with PBS, the sections were incubated with ligation solution consisting of oligonucleotides and ligase, during 30 minutes at 37°C. Ligation of oligonucleotides generates a circular DNA strand that serves as a template if the probes are in close proximity. Next, sections were incubated with the amplification solution, consisting of fluorescently labeled oligonucleotides and polymerase during 100 minutes at 37°C. The amplification reaction product attached to the antibody protein complex was visualized.
through the hybridization of fluorescently labeled oligonucleotides as a distinct fluorescent signal. Kidney sections were washed and mounted with mounting medium with DAPI. Cy3 and DAPI fluorescence signals were detected by confocal microscope (FV300, Olympus). Images were taken at x200 and x400 magnification.

Immortalized mouse podocytes were exposed to 100µM L-NAME (L-N^G-Nitroarginine methyl ester) for 24-48 hours or control medium, then 50ng/ml VEGF_{165} or control medium were added for 24 hours. Podocytes were fixed in 4% paraformaldehyde, and IHC and PLA were performed as described above.

**Biotin switch assay (BST):** We assessed laminin S-nitrosylation by BST^{35} (S-nitrosylated protein detection kit, Cayman Chemical Co.) as per manufacturer’s instructions. Briefly, equal amount of kidney lysate (1500 µg) was re-suspended in blocking buffer, acetone precipitated and labeled with labeling reagent in the presence of reducing agent. Proteins were precipitated using acetone and the protein pellets obtained were re-suspended in HENS/10 + 1% SDS buffer. Equal volumes were used to pull down biotinylated proteins using streptavidin-agarose beads (Fluka). Beads were washed 5 times and bound proteins were eluted in 2X sample buffer and laminin presence in the eluates was detected using standard immunoblot technique.

**Statistical analysis**

Data were expressed as mean ± SEM. To determine statistical significance we used unpaired Student t-test and one-way ANOVA to compare two or multiple experimental groups, respectively. To evaluate the association between two numerical variables we used Pearson correlation and between categorical variables we used Fisher’s exact test. P<0.05 was deemed statistically significant.
Supplemental Figure legends:

Supplementary Figure 1: Podocyte $VEGF_{164}$ gain-of-function in mice with intact eNOS induces glomerulomegaly, mesangial expansion, GBM thickening, and podocyte effacement. (A) PAS stain shows normal glomeruli in control ($iVEGF$ – dox) mice. Glomeruli from $iVEGF$ +dox kidneys show mesangial expansion, and glomerulomegaly (400x magnification). (B) TEM: glomerulus from control ($iVEGF$ – dox) shows normal glomerular filtration barrier ultrastructure; glomerulus from $iVEGF$ + dox kidneys shows GBM thickening, absence of lamina rara interna and lamina rara externa, and podocyte effacement. Scale bars=500nm.

Supplementary Figure 2: Podocyte $VEGF_{164}$ gain-of-function does not alter fibronectin expression in glomeruli from eNOS KO mice. (A) IHC: immunoreactive fibronectin expression pattern is similar in $iVEGF$:eNOS$^{−/−}$ - dox and $iVEGF$:eNOS$^{−/−}$ +dox mice. DAPI (blue) labels nuclei. Scale bar=50µm. (B) Quantitation of total laminin by immunoblot shows similar expression level in $iVEGF$:eNOS$^{−/−}$ - dox and in $iVEGF$:eNOS$^{−/−}$ + dox.

Supplementary Figure 3: Podocyte $VEGF_{164}$ gain-of-function in $iVEGF$ mice does not alter glomerular laminin and collagen IV expression. Immunoreactive laminin (green) and collagen IV (red), show similar expression in glomeruli from $iVEGF$- dox and $iVEGF$ + dox mice. (400x magnification).
Supplementary Figure 4: Podocyte $VEGF_{164}$ gain-of-function in $iVEGF;eNOS^{-/-}$ mice does not alter $iNOS$ expression in the kidney. Whole kidney lysate immunoblots from $iVEGF:eNOS^{-/-}$ mice that received standard (-dox) or doxycycline containing chow (+dox) showed similar iNOS expression (n=3). Tubulin immunoblots were used as control for protein loading.
Supplemental Figure 1

A  $i\text{VEGF} - \text{dox}$

B  $i\text{VEGF} + \text{dox}$
Supplemental Figure 2

(A)  

\[ i \text{VEGF:eNOS}^{-/-} \]

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50.0 μm 50.0 μm

(B)  

\[ i \text{VEGF:eNOS}^{-/-} \]

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Bar graph:

- dox + dox

0 1 2
Supplemental Figure 3

\[ iVEGF: eNOS^{+/+} \]

- dox    + dox

Laminin

Collagen IV
Supplemental Figure 4

iVEGF:eNOS-/-

iNOS

tubulin

dox - +