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 [5], 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.[5,54] 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.[6] 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.[6] 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/O_2), 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. [5,6] 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. *S*ections 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 $NaVO_4$ + 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.[5] 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.[5]

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).[55,56] 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 $\pm$ 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:

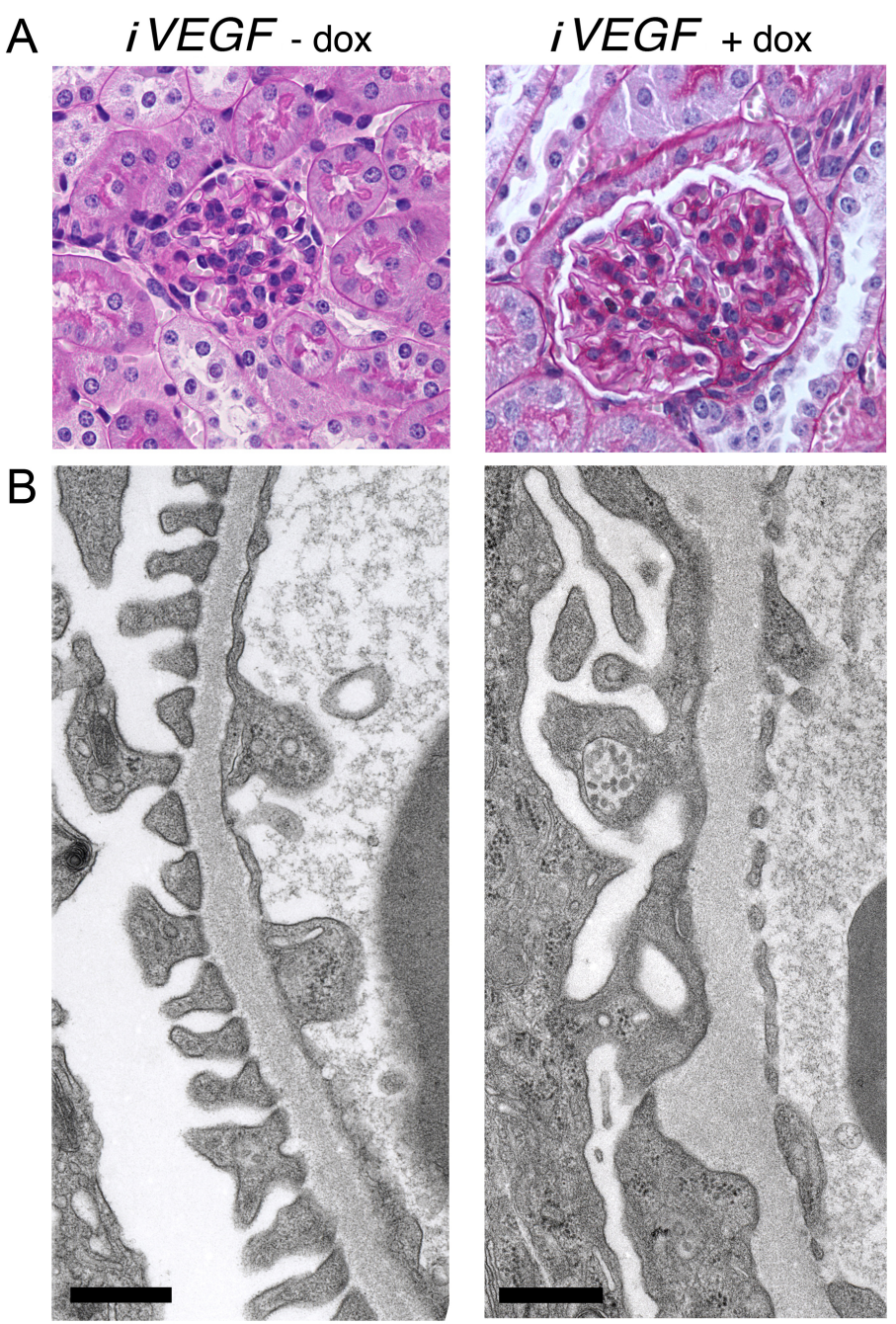
Supplementary Figure 1: **Podocyte *$VEGF_{164}$* gain-of-function in mice with intact *eNOS* induces glomerulomegaly, mesangial expansion, GBM thickening, and podocyte effacement.** (A) PAS stain shows normal glomeruli in control (*iVEGF* – dox) mice. Glomeruli from *iVEGF* +dox kidneys show mesangial 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 immnunoblot shows similar expression level in *iVEGF:eNOS*$^{-/-}$ - dox and in *iVEGF:eNOS*$^{-/-}$ + dox.

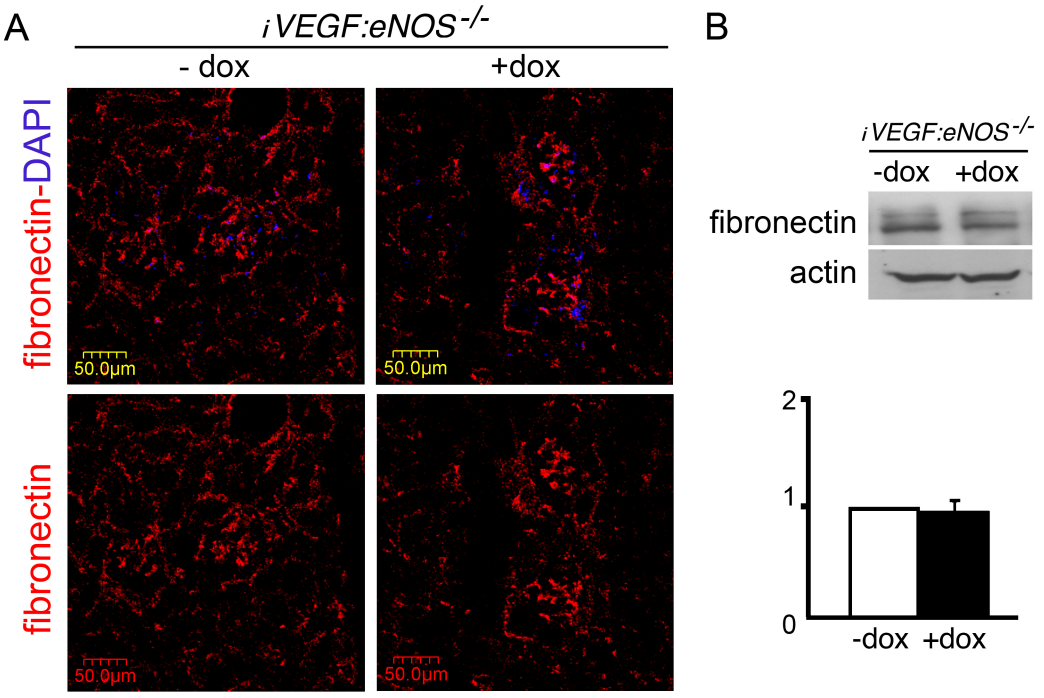
Supplementary Figure 3: Podocyte *$VEGF_{164}$* gain-of-function in *iVEGF* mice does not alter glomerular laminin and collagen IV expression. Immunoreactive laminin (green) and collagen IV (red), show similar expression in glomeruli from *iVEGF*- dox and *iVEGF* + dox mice. (400x magnification).

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.

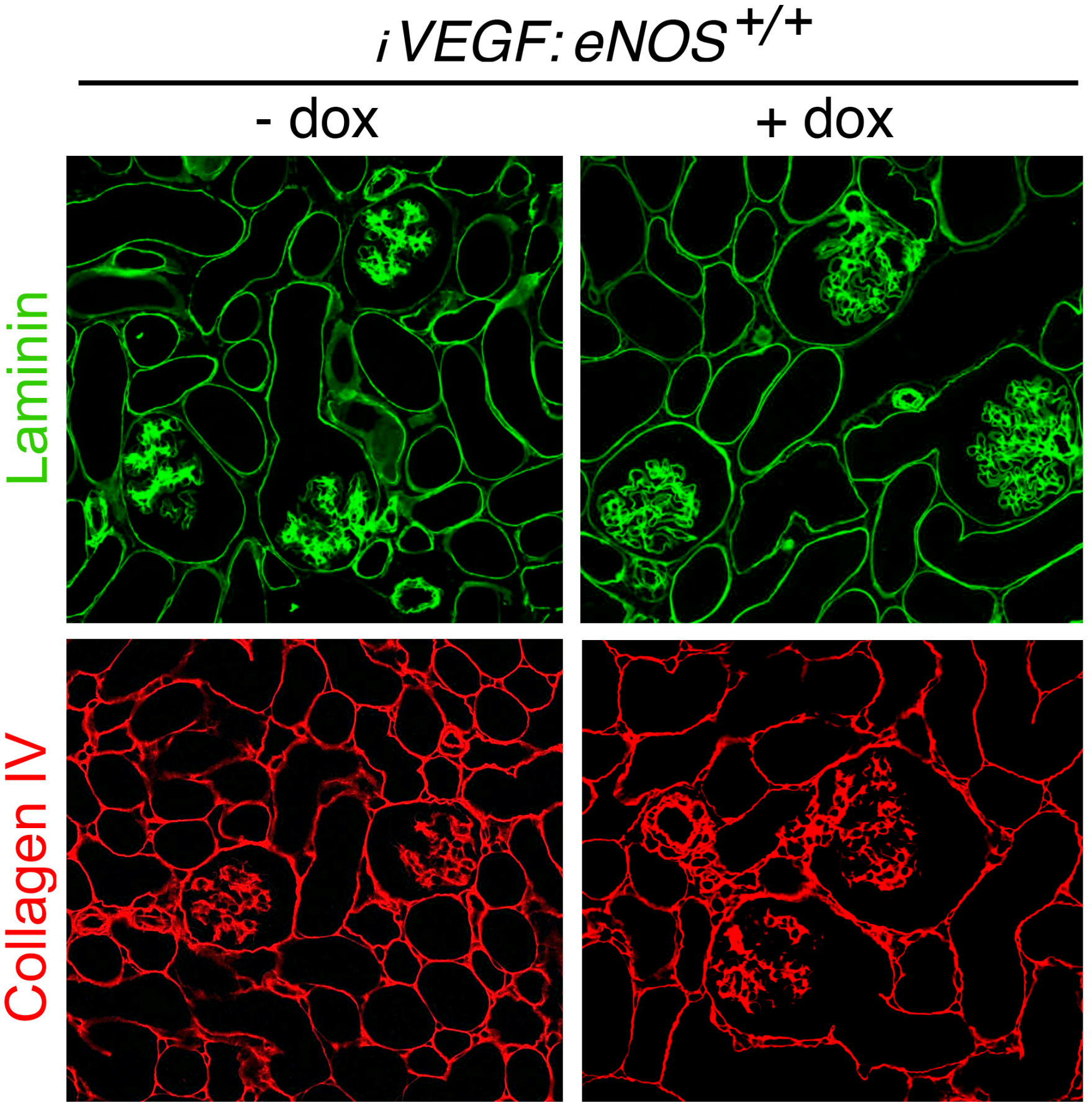
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure 4