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Induction of Podocyte VEGF₁₆₄ Overexpression at Different Stages of Development Causes Congenital Nephrosis or Steroid-Resistant Nephrotic Syndrome

Delma Veron,* Kimberly Reidy,[†] Arnaud Marlier,[‡]
Claudia Bertuccio,* Guillermo Villegas,[†]
Juan Jimenez,[§] Michael Kashgarian,[¶]
and Alda Tufro*

From the Departments of Pediatrics,* Internal Medicine,[‡] and Pathology,[¶] Yale University School of Medicine, New Haven, Connecticut; and the Department of Pediatrics,[†] and Analytical Imaging Facility,[§] Albert Einstein College of Medicine, Bronx, New York

The tight regulation of vascular endothelial growth factor-A (VEGF-A) signaling is required for both the development and maintenance of the glomerular filtration barrier, but the pathogenic role of excessive amounts of VEGF-A detected in multiple renal diseases remains poorly defined. We generated inducible transgenic mice that overexpress podocyte VEGF₁₆₄ at any chosen stage of development. In this study, we report the phenotypes that result from podocyte VEGF₁₆₄ excess during organogenesis and after birth. On doxycycline induction, *podocin-rtTA:tet-O-VEGF₁₆₄* mice express twofold higher kidney VEGF₁₆₄ levels than single transgenic mice, localized to podocytes. Podocyte VEGF₁₆₄ overexpression during organogenesis resulted in albuminuria at birth and was associated with glomerulomegaly, uniform podocyte effacement, very few and wide foot processes joined by occluding junctions, almost complete absence of slit diaphragms, and swollen endothelial cells with few fenestrae as revealed by transmission electron microscopy. Podocyte VEGF₁₆₄ overexpression after birth caused massive albuminuria in 70% of 2-week-old mice, glomerulomegaly, and minimal changes on light microscopy. Transmission electron microscopy showed podocyte effacement and fusion and morphologically normal endothelial cells. Podocyte VEGF₁₆₄ overexpression induced nephrin down-regulation without podocyte loss. VEGF₁₆₄-induced abnormalities were reversible on removal of doxycycline and were unresponsive to methylprednisolone. Collectively, the data suggest

that moderate podocyte VEGF₁₆₄ overexpression during organogenesis results in congenital nephrotic syndrome, whereas VEGF₁₆₄ overexpression after birth induces a steroid-resistant minimal change like-disease in mice. (Am J Pathol 2010; 177:2225–2233; DOI: 10.2353/ajpath.2010.091146)

Vascular endothelial growth factor-A (VEGF-A) is an endothelial growth factor and a vascular permeability factor required for endothelial cell differentiation and angiogenesis during embryonic development.^{1,2} VEGF-A signals induce endothelial cell proliferation and migration and enhance vascular leakage.³ In the kidney VEGF-A is expressed and secreted by podocytes, and a tight regulation of VEGF-A signaling is necessary for glomerular vascularization, development, and maintenance of the glomerular filtration barrier.^{4,5} Deletion of all VEGF isoforms in podocytes caused hydrops fetalis due to avascular glomeruli and haploinsufficiency-induced endotheliosis, whereas massive VEGF₁₆₄ overexpression in podocytes resulted in collapsing glomerulopathy in newborn mice.⁴ VEGF-A is moderately increased in multiple glomerular diseases including nephrotic syndrome and diabetic nephropathy, but its pathogenic role remains controversial.^{6,7} We recently determined that overexpression of VEGF₁₆₄ in podocytes of adult

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Address reprint requests to Alda Tufro, M.D., Ph.D., Department of Pediatrics, Yale University School of Medicine, 333 Cedar St., P.O. Box 208064, New Haven, CT 06520-8064. E-mail: alda.tufro@yale.edu.

mice causes a reversible glomerulopathy characterized by glomerulomegaly, mesangial expansion, glomerular basement membrane thickening, podocyte effacement associated with nephrin, and matrix metalloproteinase 9 down-regulation.⁸ VEGF is increased in congenital nephrotic syndrome of the Finnish type (CNSF).⁹ Nephrin mutations cause CNSF, characterized by massive albuminuria at birth due to severe glomerular filtration barrier abnormalities.¹⁰ Kidneys in CNSF fail to develop slit-diaphragms and podocyte foot processes are flattened and fused,^{10,11} whereas associated mesangial expansion, endothelial blebs, and proliferation lead to capillary obliteration.⁹ Mutations in *podocin*, *neph1*, *FAT1*, *WT1*, and *Nck1-2* genes also cause congenital nephrotic syndrome in mice,¹²⁻¹⁶ suggesting that some integral components of the slit-diaphragm and nephrin signaling partners are crucial for the development and function of the glomerular filtration barrier, whereas others become critical for its maintenance.

Childhood idiopathic nephrotic syndrome (INS), defined by massive albuminuria and hypoalbuminemia, is most often steroid-responsive and is associated with minimal change disease (MCD).¹⁷ MCD is characterized by normal glomerular histology on light microscopic examination, podocyte foot process effacement and fusion, absence of immune deposits, and cellular infiltrates on electron microscopic examination. These abnormalities revert to normal ultrastructure on steroid treatment in the majority of affected children.¹⁸ INS is thought to be due to a circulating factor secreted by T cells, so far not identified.^{18,19} VEGF was tested as a circulating factor candidate because of its vascular permeability-enhancing properties. Initial studies revealed similar VEGF circulating levels during relapse and remission of steroid-sensitive nephrotic syndrome in children, and rats given parenteral VEGF failed to develop proteinuria, but a role of endogenous podocyte VEGF in the development of proteinuria was not excluded.²⁰ Follow-up reports showed increased plasma and urine VEGF levels during INS relapses, which were responsive to glucocorticosteroids.^{21,22} Glucocorticosteroids are negative regulators of VEGF transcription and secretion *in vitro*.²³ Thus, the role of excess endogenous VEGF as a determinant of proteinuria, and the response to steroids in patients with INS and animal models remains unclear. Recent reports indicate that dexamethasone induces transcriptional activation of nephrin and enhances nephrin expression *in vitro*, suggesting that this mechanism could be involved in the steroid response observed in minimal change disease.²⁴

To test the hypothesis that enhanced podocyte VEGF causes proteinuria in developing mice by disrupting nephrin signaling we developed an inducible mouse model of moderate podocyte VEGF₁₆₄ overexpression. Here we examined the phenotypes resulting from podocyte VEGF₁₆₄ excess at two developmental stages: during organogenesis and in the newborn mouse. We report that moderate podocyte VEGF₁₆₄ overexpression during organogenesis results in congenital nephrotic syndrome, whereas after birth VEGF₁₆₄ overexpression induces a steroid-resistant minimal

change-like disease in mice associated with nephrin down-regulation.

Materials and Methods

Inducible, Podocyte-Specific VEGF₁₆₄ Overexpression in Mice

All experiments were performed in accordance with protocols approved by the Albert Einstein College of Medicine Committee for Animal Use and Experimentation and Yale Institutional Animal Care and Use Committee. We generated tetracycline-regulated inducible transgenic mice (*podocin-rtTA: tet-O-VEGF₁₆₄*) that overexpress VEGF₁₆₄ in podocytes on induction with doxycycline⁸ by breeding *podocin-rtTA* mice²⁴ with *tet-O-VEGF₁₆₄* mice.²⁵ Transgenic mice were genotyped by PCR using reported primers.^{8,25,26} Developing mice were induced with doxycycline during embryonic and early postnatal life. To induce podocyte VEGF₁₆₄ overexpression during organogenesis, *podocin-rtTA: tet-O-VEGF₁₆₄* pregnant mice were given doxycycline in the chow from embryonic day 12 (to ensure therapeutic levels by embryonic day 14, the onset of podocin expression) until the pups were born (total $n = 55$, *podocin-rtTA:tet-O-VEGF₁₆₄* [TOPO] $n = 26$, single transgenic $n = 29$). To induce podocyte VEGF₁₆₄ overexpression after birth, doxycycline was started immediately postpartum and continued for 2 weeks (TOPO, $n = 38$). Controls were induced single transgenic littermates (*podocin-rtTA* or *tet-O-VEGF₁₆₄*, $n = 11$) and age-matched noninduced *podocin-rtTA:tet-O-VEGF₁₆₄* mice ($n = 8$).

Reversibility of VEGF-Induced Phenotype and Response to Steroids

To examine the reversibility of VEGF₁₆₄-induced changes, additional *podocin-rtTA:tet-O-VEGF₁₆₄* pups were induced with doxycycline from birth until 2 weeks ($n = 37$) and then were switched to a regular diet for 2 weeks (+/- dox, $n = 22$). Additional controls were *podocin-rtTA: tet-O-VEGF₁₆₄* pups not receiving doxycycline ($n = 8$) and their single transgenic littermates. To test whether VEGF₁₆₄-induced changes are abrogated by steroids, *podocin-rtTA:tet-O-VEGF₁₆₄* pups were induced with doxycycline from birth until 4 weeks of age ($n = 9$) and were given methylprednisolone (5 mg/kg/day i.p., $n = 5$) or vehicle ($n = 4$) from age 2 to 4 weeks. Additional controls were *podocin-rtTA:tet-O-VEGF₁₆₄* pups given methylprednisolone at an identical dose from age 2 to 4 weeks but not receiving doxycycline ($n = 5$).

Proteinuria and VEGF-A Analysis

Mice were anesthetized with ketamine, kidneys were harvested, blood and urine samples were obtained by heart and bladder puncture, and mice were euthanized by exsanguination. Urinary creatinine was measured by a colorimetric assay (Oxford Biomedical Research, Oxford,

MI). Albuminuria was assessed by Western analysis: urine samples, normalized for creatinine excretion, were resolved by SDS-polyacrylamide gel electrophoresis, immunoblotted with anti-bovine serum albumin antibody (Upstate Biotechnology, Lake Placid, NY). In addition, albuminuria was measured by enzyme-linked immunosorbent assay (Albuwell, Exocell, Philadelphia, PA) following the manufacturer's protocol and expressed as the albumin/creatinine ratio ($\mu\text{g}/\text{mg}$); urine creatinine was measured by high-performance liquid chromatography-tandem mass spectrometry.²⁷ Serum albumin was measured by the albumin-bromocresol green reaction (Roche COBAS Mira Plus automated chemistry analyzer).²⁸ VEGF-A was measured in whole kidney lysates by enzyme-linked immunosorbent assay (mVEGF, R&D Systems, Minneapolis, MN), following the manufacturer's protocol.

Laser Capture Microdissection and Real-Time PCR

Glomerular mRNA VEGF₁₆₄ expression was assessed by quantitative PCR after laser capture microdissection.^{29,30} In brief, glomeruli were laser-cut from 8.0- μm OCT frozen kidney sections using a Leica AS LMD system, and total RNA was purified using an RNeasy Plus Micro kit (Qiagen, Hilden, Germany). cDNAs were synthesized with hexamer random primers and SuperScript III (Invitrogen, Grand Island, NY). Quantitative PCR was performed using the SsoFast EvaGreen reaction mixture and a CFX96 real-time system (Bio-Rad, Hercules, CA). Primer pairs used for VEGF and hypoxanthine phosphoribosyltransferase (HPRT) amplification were reported previously.²⁹ The gene expression level was determined by the comparative cycle threshold method (ΔCt).⁸ Controls run in the absence of template were negative.

Histology, Transmission Electron Microscopy, and Morphometric Analysis

Kidneys were processed for light and electron microscopy or frozen in isopentane and mounted in OCT (Sakura, Finetek, Japan). PAS staining was performed to evaluate histological changes. Glomerular diameters were measured in 46 ± 4 glomeruli/section ($\times 400$ magnification) from six mice per experimental group. Glomerular volume was determined as $Gv = \beta/k \cdot (\pi \cdot r^2)^{3/2}$, where $\beta = 1.38$ is the shape coefficient for spheres, $k = 1.1$ is the size distribution coefficient, and $(\pi \cdot r^2)$ is the glomerular area.³¹ For transmission electron microscopy (TEM) $\sim 2\text{-mm}$ kidney cortex cubes were fixed with 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, postfixed in 1% osmium tetroxide followed by 2% uranyl acetate, dehydrated in ethanol, and embedded in LX112 (Ladd Research Industries, Burlington, VT). Thin sections (80 nm) were cut (Reichert Ultracut E) and stained with 0.5% uranyl acetate and 0.5% lead citrate using a Leica EM Grid Stainer and examined using a JEOL 1200EX microscope. We quan-

tified the fenestrations in glomerular capillary cross-sections in TEM samples using ImageJ (NIH, Bethesda, MD). Fenestrae were counted in endothelial cells alongside 15 to 35 μm of glomerular basement membrane (GBM) per animal from three mice per experimental group.

Immunohistochemistry

Cryosections were fixed in cold acetone, permeabilized with 0.3% Triton X-100, washed, blocked, and incubated with anti-nephrin (20R-NP002, Fitzgerald, North Acton, MA). Paraffin-embedded sections were deparaffinized, hydrated, blocked, and incubated with anti-WT1 (M3561, Dako, Carpinteria, CA) + anti-nephrin and anti-Tyr¹¹⁷⁵ VEGFR2 (19A10, Cell Signaling Technology, Danvers, MA). Sections were washed and incubated with Cy2- or Cy3-labeled anti-guinea pig, anti-goat, or anti-rabbit secondary antibodies, as appropriate (Jackson ImmunoResearch, West Grove, PA), washed with PBS, and mounted using Fluoromount-G, (Southern Biotechnology, Birmingham, AL). Paraffin sections were deparaffinized, incubated with epitope retrieval solution in 10 mmol/L citrate buffer, blocked and incubated with anti-VEGF antibody (M7273, Dako), detected with the Dako visualization reagent, and counterstained with hematoxylin. All sections were examined by confocal microscopy (FV300, Olympus, Center Valley, PA) or light microscopy (Eclipse50i, Nikon, Melville, NY); images were taken at $\times 400$ magnification. Podocyte counts were performed in dual immunofluorescent staining for WT1 and nephrin, using merge images: WT1⁺/nephrin⁺ cells were counted in 10 to 15 glomeruli/kidney from 2-week-old VEGF₁₆₄-overexpressing mice ($n = 4$) and from age-matched controls ($n = 4$).

Western Analysis

Kidneys from VEGF₁₆₄-overexpressing mice receiving doxycycline from birth until 2 or 4 weeks of age (+ dox, $n = 5$ and $n = 9$, respectively) or not receiving doxycycline (– dox, $n = 10$ age-matched) and receiving doxycycline + methylprednisolone (+ dox + pred, $n = 5$) or methylprednisolone alone (+ pred, $n = 5$) were lysed in radioimmunoprecipitation assay buffer containing protease inhibitors (Complete Mini EDTA-Free, Roche, Indianapolis, IN), 1 mmol/L sodium orthovanadate, and 1 mmol/L NaF. Protein concentration was determined with a BCA Protein Assay Kit (Pierce, Rockford, IL). Equal amounts of protein lysate from individual kidneys were combined into separate pools (+ dox; – dox; + dox + pred; + dox + vehicle; and + pred), and 200 μg of protein/lane was resolved in 8 to 10% SDS-polyacrylamide gel electrophoresis. Primary antibodies used were anti-nephrin, anti-podocin (P0372, Sigma-Aldrich, St. Louis, MO), and anti-WT1 (sc192, Santa Cruz Biotechnology, Santa Cruz, CA). Anti-actin (A2066, Sigma-Aldrich) was used as a control for protein loading. Secondary antibodies were anti-rabbit, anti-mouse horseradish peroxidase-IgG (Amersham, Piscataway NJ), or anti-guinea pig horseradish peroxidase-IgG (Fitzgerald), as appropriate. Signals were visualized by enhanced chemolumi-

Figure 1. Podocyte VEGF₁₆₄ overexpression during organogenesis. **A–C:** VEGF-A immunohistochemistry showing increased immunoreactive VEGF-A (brown) in glomeruli from *pod-rtTA:tet-O-VEGF*₁₆₄ mice on doxycycline (**A**, + dox) versus single transgenic littermates (**B**, + dox) and uninduced *pod-rtTA:tet-O-VEGF*₁₆₄ mice (**C**, - dox). **D:** VEGF measured by enzyme-linked immunosorbent assay in whole kidney lysate, showing a twofold increase in mice overexpressing podocyte VEGF₁₆₄ (TOPO) versus controls. **P* < 0.05 compared with control. **E** and **F:** Glomerular laser capture. Representative glomeruli are shown before and after capture; original magnification, ×400. **G:** VEGF mRNA expression in isolated glomeruli from VEGF₁₆₄-overexpressing mice (+ dox) was approximately twofold higher than that in controls (- dox); VEGF mRNA ΔCt values were normalized to hypoxanthine phosphoribosyltransferase mRNA. **P* < 0.05, + dox versus - dox.

nescence (Amersham) and quantified by densitometric analysis using ImageJ software.

Statistical Analysis

All values are expressed as means ± SEM. To determine statistical significance, we used an unpaired Student's *t*-test and one-way analysis of variance for comparisons between two groups or multiple groups, respectively. *P* < 0.05 was deemed statistically significant.

Results

Podocyte VEGF₁₆₄ Overexpression during Kidney Organogenesis Causes Congenital Nephrotic Syndrome

VEGF₁₆₄ overexpression in podocytes during organogenesis resulted in normal litter size (9 ± 0.9 pups/litter, *n* = 5) and body weight (1.5 ± 0.05 versus 1.6 ± 0.07 g, NS). *Podocin-rtTA/Tet-O-VEGF*₁₆₄ mice (*n* = 23) were indistinguishable from their single transgenic littermates (*n* = 21) at birth. Podocyte VEGF₁₆₄ overexpression in *podocin-rtTA/Tet-O-VEGF*₁₆₄ newborn mice resulted in an increase in immunoreactive glomerular VEGF-A (Figure 1A) compared with that in their single transgenic littermates (Figure 1B) and with mice of identical genotype not receiving doxycycline (Figure 1C). In VEGF₁₆₄-overexpressing mice, whole kidney VEGF-A was increased twofold (Figure 1D). Likewise, VEGF mRNA expression in glomeruli isolated by laser capture was significantly increased in VEGF₁₆₄-overexpressing mice (Figure 1, E–G).

Mice overexpressing VEGF₁₆₄ during kidney organogenesis had albuminuria at birth, whereas single transgenic littermates and uninduced mice did not (Figure 2A). Light microscopy examination of PAS-stained kidney sections of newborn mice overexpressing podocyte VEGF₁₆₄ showed normal histology, except for glomerular enlargement and protein-containing tubules (Figure 2B, + dox), which were not present in uninduced controls (Figure 2B, - dox). More mature glomeruli were ob-

served in VEGF₁₆₄-overexpressing mice than in their single transgenic littermates, underscoring the local effects of VEGF on glomerular development. Morphometric analysis demonstrated glomerulomegaly, with ~60% larger glomerular volume in kidneys overexpressing podocyte VEGF₁₆₄ than in single transgenic littermate controls (Fig-

Figure 2. Podocyte VEGF₁₆₄ overexpression during organogenesis causes congenital nephrotic syndrome. VEGF₁₆₄-overexpressing mice showed (**A**) albuminuria at birth; (**B**) protein cast and glomerulomegaly (+ dox), quantified in **C**. **D** and **F:** TEM showed effaced podocytes (epf), fused by occluding junctions (thick arrows), and endothelial cells with few fenestrae protruding into capillary lumina (double-headed thin arrows). Uninduced (- dox) *pod-rtTA:tet-O-VEGF*₁₆₄ mice appeared normal on light microscopy (**B**, - dox) and ultrastructure **E** and **G**: Slim foot processes (fp) joined by slit-diaphragms (small arrows) and fenestrated endothelial cells (arrowheads). Scale bars: 50 μm (**B**); 1 μm (**D** and **E**); 500 nm (**F** and **G**). P, podocyte; CL, capillary lumen; EC, endothelial cell.

ure 2C). TEM revealed uniform podocyte effacement, with few wide foot processes (Figure 2, D and F) in capillary loop and maturing stage glomeruli from newborn kidneys overexpressing VEGF₁₆₄ ($n = 6$). Slit-diaphragms were almost completely absent, replaced by occluding junctions (Figure 2, D and F, thick arrows). The GBM was largely unaffected. Endothelial cells looked bigger than in control littermates, and their cytoplasm was found to protrude into the lumen, occupying a vast portion of most capillary lumina. Endothelial cells had few fenestrae, and diaphragms were not observed (Figure 2, D and F, thin double-headed arrows). In contrast, uninduced *podocin-rtTA/Tet-O-VEGF*₁₆₄ pups had normal ultrastructure, ie, well developed podocyte foot processes linked by slit-diaphragms, thin tri-layered GBM, and fenestrated endothelium (including diaphragms occasionally) in the glomerular capillary loops ($n = 5$, Figure 2, E and G), as did single transgenic littermate kidneys ($n = 5$, not shown). Quantification showed 0.07 ± 0.01 fenestrae/ μm of GBM in glomerular endothelial cells from kidneys overexpressing podocyte VEGF₁₆₄, and 0.55 ± 0.018 fenestrae/ μm of GBM in uninduced control mice, $P = 0.00059$. Together, these findings are suggestive of congenital nephrosis induced by podocyte VEGF₁₆₄ overexpression.

Podocyte VEGF₁₆₄ Overexpression after Birth Causes MCD

Induction of podocyte VEGF₁₆₄ overexpression after birth caused massive albuminuria in 70% of *podocin-rtTA/Tet-O-VEGF*₁₆₄ mice by 2 weeks of age, whereas single transgenic littermates and uninduced age-matched control mice did not develop albuminuria (Figure 3A). Urine albumin/creatinine ratios were 18-fold higher in VEGF₁₆₄-overexpressing mice than in uninduced controls (273 ± 37.2 versus 15 ± 4.8 $\mu\text{g}/\text{mg}$, $P < 0.001$). Plasma albumin was lower in VEGF₁₆₄-overexpressing mice than in control littermates (2.6 ± 0.07 versus 3.3 ± 0.09 g/dl, $P < 0.05$). Glomerular light microscopy was normal in all mice at 2 weeks of age, with the exception of glomerular enlargement in VEGF₁₆₄-overexpressing mice (Figure 3, B and C). TEM revealed podocyte effacement and fusion, with slit-diaphragms replaced by occluding junctions (Figure 3E, thick arrows, Supplemental Figure 1, A and B, see <http://ajp.amjpathol.org>) in kidneys overexpressing VEGF₁₆₄ since birth ($n = 11$), albeit less widespread than that in newborns induced *in utero*. Notably, endothelial cells appeared normal or focally swollen and had fenestrations (Figure 3E, arrowheads), the GBM appeared normal, and no podocyte detachment was observed. Single transgenic littermates ($n = 4$) (Figure 3D) and uninduced *podocin-rtTA/Tet-O-VEGF*₁₆₄ kidneys ($n = 4$) (not shown) had normal ultrastructure. Quantitation of glomerular endothelial cell fenestration in glomerular endothelial cells from kidneys overexpressing podocyte VEGF₁₆₄ showed no significant difference with controls (0.64 ± 0.15 versus 0.99 ± 0.25 fenestrae/ μm of GBM, $P = 0.1464$), suggesting that a moderate excess of VEGF-A does not disrupt endothelial cell fenestration postnatally.

Figure 3. Podocyte VEGF₁₆₄ overexpression after birth causes MCD. **A:** Western blot showing massive albuminuria in VEGF₁₆₄-overexpressing mice two weeks after induction, whereas single transgenic littermates and uninduced mice had minimal proteinuria or none. **B:** PAS stain from single transgenic kidney showed normal histology. **C:** PAS stain from VEGF₁₆₄-overexpressing kidney showed enlarged glomeruli. **D:** TEM showing normal ultrastructure in single transgenic mouse kidney: foot processes (fp), slit-diaphragms (small arrows), and endothelial cell (EC) fenestrae (arrowheads). **E:** TEM from VEGF₁₆₄-overexpressing kidney showing effaced foot processes fused by large occluding junctions (thick arrows), occasional slit-diaphragms (small arrow), and fenestrated ECs (arrowheads). **F–I:** the VEGF₁₆₄ overexpression phenotype is reversible two weeks after removal of doxycycline (+/- dox). **F:** VEGF immunohistochemistry. **G:** TEM shows reversibility of VEGF-induced podocyte effacement: normal slit-diaphragms (small arrows) and EC fenestrae (arrowheads). **H:** Albuminuria, assessed by Western blot. Scale bars: 50 μm (B, C, and F); 200 nm (D and E); 1 μm (G). P, podocyte; RBC, red blood cell, EC, endothelial cell. * $P < 0.05$, + dox compared with - dox and +/- dox.

Next, we tested the reversibility of VEGF₁₆₄-induced phenotype by removing doxycycline induction from weeks 2 to 4 of life and confirmed that VEGF-A podocyte expression had returned to baseline (Figure 3F). Notably, functional and ultrastructural changes induced by podocyte VEGF₁₆₄ overexpression in newborn mice were reversible: 2 weeks after doxycycline removal no abnormalities were observed by TEM (Figure 3G), and the hypoalbuminemia (2.6 ± 0.07 versus 3.3 ± 0.15 g/dl, + dox versus +/- dox, $P < 0.05$) and albuminuria were resolved (Figure 3, H and I). Collectively these findings are consistent with a minimal change-like disease induced by podocyte VEGF₁₆₄ overexpression in developing mice.

To gain insight into the molecular basis of VEGF₁₆₄-induced podocyte effacement and fusion we examined

Figure 4. Podocyte VEGF₁₆₄ overexpression induces nephrin down-regulation without podocyte loss, and increased VEGFR2 phosphorylation. **A:** Nephrin immunofluorescence decreased on doxycycline induction (+ dox), and appeared close to normal two weeks after doxycycline removal (+/- dox). **B:** Western blot showing baseline nephrin expression in single transgenic kidneys on doxycycline and uninduced *pod-rtTA:tet-O-VEGF*₁₆₄ kidneys, down-regulation on transgene induction (+ dox), and reversibility of nephrin down-regulation (+/-). **C:** Expression levels were normalized for actin and expressed as fold mean $\pm$ SEM changes from uninduced mice (- dox). **D** and **E:** Western blot showing no changes in podocin expression. **F** and **G:** Western blot showing no changes in WT1 expression. **H:** Dual immunofluorescence for nephrin (red) and WT1 (green) showing similar podocyte numbers in control (- dox) and VEGF₁₆₄-overexpressing glomeruli (+ dox). **I:** Dual immunofluorescence for nephrin and Tyr¹¹⁷⁵-VEGFR2 (P-VEGFR2) showing increased P-VEGFR2 immunostaining in VEGF₁₆₄-overexpressing mice. Scale bar: 20 μ m. * P < 0.05 compared with - dox; ** P < 0.05 compared with + dox.

the expression of podocyte-specific proteins by immunoblotting and immunohistochemistry. Notably, podocyte VEGF₁₆₄ overexpression resulted in down-regulation of nephrin, which was abrogated by removal of doxycycline (Figure 4, A–C). Single transgenic mice receiving doxycycline had normal nephrin expression (Figure 4B), excluding the possibility that doxycycline, per se, may have caused nephrin dysregulation. In contrast, podocin and WT1 expression levels did not change in podocyte VEGF₁₆₄-overexpressing kidneys (Figure 4, D–G), suggesting that nephrin down-regulation was not due to podocyte loss. Moreover, podocyte counts performed in kidney sections with dual immunostaining for nephrin and WT1 were similar (11 ± 0.6 versus 12.8 ± 0.6 podocytes/glomerulus, NS) (Figure 4H) in VEGF₁₆₄-overexpressing glomeruli ($n = 57$) and controls ($n = 64$). VEGF receptor 2 (VEGFR2) signaling was activated in VEGF₁₆₄-overexpressing podocytes, as shown by dual P¹¹⁷⁵-VEGFR2

Figure 5. VEGF-induced MCD is steroid-resistant. **A:** Western blot showing albuminuria from induced *pod-rtTA:tet-O-VEGF*₁₆₄ (+ dox), which does not improve with methylprednisolone, whereas uninduced mice (- dox) receiving methylprednisolone do not develop proteinuria. **B:** TEM images showing extensive podocyte effacement in induced kidneys (+ dox, top panels), which did not improve with methylprednisolone (top right panel), and normal ultrastructure in uninduced kidneys (- dox, bottom panels), unchanged by methylprednisolone (bottom right panel). efp, effaced foot process; cap, capillary; P, podocyte; EC, endothelial cell. **C:** Western blots showing VEGF₁₆₄-induced decreased nephrin levels, further decreased by methylprednisolone; expression level changes are shown as fold mean $\pm$ SEM change from uninduced baseline ($n = 6$ blots). Podocin and WT1 immunoblots showed no protein level changes ($n = 4$ blots each). * P < 0.05 compared with - dox and methylprednisolone.

and nephrin immunostaining, where most immunoreactive P¹¹⁷⁵-VEGFR2 signals merge with nephrin signals (Figure 4I).

Next, we tested whether parenteral methylprednisolone would reverse nephrin down-regulation induced by VEGF₁₆₄ overexpression in developing mice. Mice overexpressing podocyte VEGF₁₆₄ since birth and uninduced control mice were given methylprednisolone or vehicle intraperitoneally daily from 2 to 4 weeks of age. Methylprednisolone did not reverse or improve the albuminuria, podocyte effacement, or nephrin down-regulation induced by podocyte VEGF₁₆₄ overexpression in developing mice (Figure 5, A–C) and did not alter podocin and WT1 expression (Figure 5C). Collectively, these results suggest that VEGF-A signaling disrupts nephrin expression without causing podocyte loss and that this is not reversible by steroid therapy.

In summary, we report that moderate podocyte VEGF₁₆₄ overexpression during organogenesis causes massive podocyte effacement and proteinuria consistent with congenital nephrosis, and during early postnatal life it causes reversible nephrotic syndrome mimicking steroid-resistant nephrotic syndrome in mice.

Discussion

Inducible podocyte VEGF₁₆₄ overexpression during mouse organogenesis impaired the normal differentiation of podocyte foot processes and slit-diaphragms, as well as endothelial cell fenestration, resulting in severely abnormal glomerular filtration barrier and proteinuria at birth, consistent with congenital nephrosis. These abnormalities are very similar to those described in nephrin- or podocin-null mice^{11,32} and to podocyte and endothelial cell abnormalities described in kidneys from infants with CNSF.^{9,33,34} This finding suggests that moderate podocyte VEGF₁₆₄ overexpression during organogenesis is sufficient to disrupt the development of mature podocyte foot processes, cell-cell interactions, and glomerular capillaries. VEGF-A is critical for fenestrae development and maintenance in mature glomerular endothelial cells.³⁵ However, excess local VEGF₁₆₄ seems to disrupt fenestration of immature endothelial cells *in vivo*, probably by lack of thinning of the cell periphery, thought to be required before fenestra formation and due to dysregulation of the actin cytoskeleton.^{36,37} These findings clearly differ from the collapsing glomerulopathy induced by massive podocyte VEGF₁₆₄ overexpression in nephrin-driven VEGF transgenic mice, underscoring the notion that a tight regulation of VEGF-A dosage is required for normal renal morphogenesis.⁴

When moderate podocyte VEGF₁₆₄ overexpression is induced in newborn mice, massive proteinuria develops, associated with effacement and fusion of previously formed podocyte foot processes, in the absence of podocyte detachment or morphological endothelial cell and GBM abnormalities by TEM and normal glomeruli by light microscopy, consistent with minimal change disease.^{33,38} Reversibility of all functional and ultrastructural abnormalities on removal of doxycycline induction confirmed both the cause-and-effect relationship between VEGF₁₆₄ overexpression and the observed phenotype and its similarity to minimal change disease.

Superficial glomeruli appeared more mature in mice overexpressing podocyte VEGF₁₆₄ than in control littermates, confirming the reported effect of VEGF on glomerular vascularization.³⁹ Glomerular size is not usually assessed in MCD.³⁸ However, glomerular enlargement, equivalent to that observed in developing mice overexpressing podocyte VEGF₁₆₄, has been reported in patients with MCD, some of whom developed focal segmental glomerulosclerosis in later biopsy specimens.⁴⁰

The severe podocyte effacement and fusion observed in developing mice overexpressing VEGF₁₆₄ was associated with and probably due to down-regulation of nephrin expression, which was also reversible. Podocyte VEGF₁₆₄ overexpression did not alter the ex-

pression of other podocyte-specific proteins, such as podocin and WT1, and podocyte counts were similar to those in control mice, arguing that nephrin down-regulation was not due to podocyte loss but was caused by VEGF₁₆₄ overexpression. In line with this observation, no podocyte detachment was observed by TEM. In patients with MCD, reduced nephrin with granular distribution correlated with podocyte effacement and improved during remission.⁴¹ Dysregulation of nephrin phosphorylation has also been reported after protamine sulfate injection,⁴² a model of acute and reversible disruption of the slit-diaphragm,⁴³ adriamycin-induced nephrosis, and human nephrosis.^{44,45} Podocyte autocrine VEGF-A signals mediated by VEGFR2 alter slit-diaphragm protein expression *in vitro*.⁵ Recently, we showed that podocyte VEGF₁₆₄ overexpression in adult mice induces podocyte VEGFR2 phosphorylation.⁸ Furthermore, we identified a VEGFR2-nephrin interaction *in vivo*.⁸ In the present study we show that these mechanisms also occur in developing mice (Figure 4H and data not shown), resulting in podocyte effacement. Signals downstream from the VEGFR2-nephrin interaction mediating nephrin down-regulation remain to be determined.

It is well established that a vast majority of pediatric patients with minimal change disease are responsive to high-dose steroids.¹⁷ This response has been interpreted as a consequence of the immunosuppressive effect of glucocorticosteroids.⁴⁶ However, the cell and molecular mechanism(s) mediating the effect of glucocorticosteroids on foot process effacement are poorly understood. In cultured podocytes dexamethasone induces activation of nephrin transcription.^{24,47} Dexamethasone promotes nephrin trafficking by stimulating glycosylation and release from the endoplasmic reticulum in HEK cells transfected with human nephrin⁴⁸ and improves nephrin expression in puromycin aminonucleoside and adriamycin-induced nephrosis.^{49,50} Glucocorticosteroids are known to function as negative regulators of VEGF transcription and secretion *in vitro*.^{23,49,51} In children with steroid-sensitive nephrotic syndrome, whose serum and urine VEGF levels were elevated, glucocorticosteroid treatment reduced VEGF levels.²² Collectively, these data suggest that glucocorticosteroids should improve VEGF-induced nephrosis. However, in developing mice overexpressing podocyte VEGF₁₆₄, parenteral administration of methylprednisolone, at a dose equivalent to a therapeutic dose in humans, did not alter the VEGF-induced phenotype, as assessed by albuminuria, ultrastructure, or nephrin expression. This finding suggests that increased VEGF signaling interferes with nephrin function and signaling downstream from glucocorticosteroid-regulated transcription and trafficking, resulting in steroid-resistant MCD. The possibility that the doxycycline-regulated VEGF transgene expression overrides the physiological regulation of VEGF by glucocorticosteroids cannot be excluded. However, this regulation underscores a potential role of excessive VEGF-A in the pathogenesis of steroid-resistant INS.

In summary, moderate podocyte VEGF₁₆₄ overexpression during organogenesis results in congenital nephrosis, whereas postnatal induction in newborn mice, after

foot process differentiation, causes a minimal change-like disease not responsive to steroids, mimicking childhood steroid-resistant nephrotic syndrome. This inducible model of minimal change disease should prove useful to further characterize the molecular mechanisms mediating VEGF-induced podocyte effacement during development and to test therapeutic interventions.

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