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ANIMAL MODELS

Excess Podocyte Semaphorin-3A Leads to Glomerular Disease Involving PlexinA$_1$—Nephrin Interaction

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Semaphorin-3A (Sema3a), a guidance protein secreted by podocytes, is essential for normal kidney patterning and glomerular filtration barrier development. Here, we report that podocyte-specific Sema3a gain-of-function in adult mice leads to proteinuric glomerular disease involving the three layers of the glomerular filtration barrier. Reversibility of the glomerular phenotype upon removal of the transgene induction provided proof-of-principle of the cause-and-effect relationship between podocyte Sema3a excess and glomerular disease. Mechanistically, excess Sema3a induces dysregulation of nephrin, matrix metalloproteinase 9, and αvβ3 integrin *in vivo*. Sema3a cell-autonomously disrupts podocyte shape. We identified a novel direct interaction between the Sema3a signaling receptor plexinA$_1$ and nephrin, linking extracellular Sema3a signals to the slit-diaphragm signaling complex. We conclude that Sema3a functions as an extracellular negative regulator of the structure and function of the glomerular filtration barrier in the adult kidney. Our findings demonstrate a crosstalk between Sema3a and nephrin signaling pathways that is functionally relevant both *in vivo* and *in vitro*. *(Am J Pathol 2013, 183: 1156—1168; http://dx.doi.org/10.1016/j.ajpath.2013.06.022)*

The glomerular filter is a size-selective barrier composed of three layers: fenestrated endothelium, glomerular basement membrane (GBM), and podocyte foot processes.[1] Disruption of any of these components of the glomerular filtration barrier causes loss of permselectivity, proteinuria, and glomerular disease.[1] Podocyte foot processes are linked by slit diaphragms, which are modified adherens junctions composed of extracellular domains of nephrin molecules associated to a multiprotein complex.[2,3] Gene mutations in slit-diaphragm proteins and their actin-associated proteins cause familial nephrotic syndrome.[4—7] The GBM is a complex of type IV collagen (α3, α4, and α5) and laminin 521 (α5β2γ1) chains, perlecan, syndecan, entactin, and agrin. Imbalance of collagen and laminin chain expression results in abnormalities of GBM ultrastructure and proteinuria.[8,9] Loss of glomerular endothelial fenestration due to inhibition of vascular endothelial growth factor (VEGF-A) signaling or to excess soluble Flt-1 causes proteinuria and preeclampsia.[10,11]

Semaphorin-3A (Sema3a) is a secreted guidance protein involved in axon pathfinding and in cardiovascular, lung, and kidney patterning.[12,13] In the normal kidney, Sema3a is expressed in podocytes and collecting ducts.[7] Loss-of-function studies during kidney development showed that Sema3a inhibits endothelial cell migration into glomeruli and limits ureteric bud branching.[14,15] *Sema3a* gain-of-function during development resulted in glomerular hypoplasia, delayed podocyte differentiation, and absent slit diaphragms.[15] Exposure of cultured podocytes to recombinant Sema3a induced down-regulation of podocin and decreased the interactions among nephrin, podocin, and CD2AP.[16] Systemic administration of Sema3a to adult mice induced transient, reversible foot-process effacement and proteinuria similar to that induced by protamine sulfate.[17,18] We observed increased podocyte Sema3a protein and mRNA expression in mice with diabetic nephropathy.[13,19] Taken together, our previous studies suggested that excess Sema3a might disrupt the glomerular filtration barrier in the mature kidney, particularly in the setting of diabetes.

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The goal of the present study was to define whether excess podocyte Sema3a per se causes glomerular disease in adult mice, and to examine the mechanism involved. Here, we report that induction of podocyte-specific *Sema3a* overexpression in adult mice causes a proteinuric glomerular disease involving the three layers of the glomerular filtration barrier. Mechanistically, we show that excess Sema3a induces dysregulation of nephrin, MMP-9, and αvβ3 integrin *in vivo*, and we identify a novel interaction between the Sema3a signaling receptor plexinA_1 and nephrin that links Sema3a signals to the slit-diaphragm signaling complex. Collectively, these findings establish that Sema3a functions as an extracellular negative regulator of the integrity and function of the glomerular filtration barrier.

Materials and Methods

Animal protocols and procedures were approved by the institutional animal care and use committees at Albert Einstein College of Medicine and Yale University School of Medicine. Mice were housed in a pathogen-free environment. Mice were anesthetized with ketamine−xylazine (0.1 mg/kg body weight i.p.), and kidneys were removed before euthanasia. Blood and urine were obtained by cardiac and bladder puncture, respectively.

Sema3a-Overexpressing Mice

Generation of doxycycline-regulated *podocin-rtTA:tet-O-Sema3a* mice (hereafter referred to as *Sema3a*$^+$) was performed as described previously.[15] Six-week-old male *Sema3a*$^+$ mice were fed doxycycline chow (625 mg/kg chow; Harlan-Teklad, Madison, WI) for 1 month ($n = 16$). Controls were age-matched uninduced *Sema3a*$^+$ mice ($n = 18$) fed standard chow and single-transgenic (*podocin-rtTA* or *tet-O-Sema3a*) mice fed doxycycline chow for 1 month ($n = 4$). For reversibility experiments, *Sema3a*$^+$ mice were induced with doxycycline for 1 month and then were fed standard chow for 2 weeks ($n = 10$).

Noninvasive Blood Pressure Monitoring

Systolic and diastolic blood pressure were noninvasively measured on nonanesthetized mice ($n = 4$ per experimental group) previously acclimated to the procedure, by determining the tail blood volume with a volume pressure recording sensor and an occlusion tail-cuff (CODA system; Kent Scientific, Torrington, CT). Mice were acclimated before blood pressure readings were obtained (at least 15 readings per mouse).

Albuminuria and Creatinine Clearance

Equal volumes of urine were resolved by SDS-PAGE and were stained with Coomassie Blue or immunoblotted with anti−bovine serum albumin antibody (Upstate 07-248; 1:1000; EMD Millipore, Billerica, MA). Creatinine was measured in plasma and in 24-hour urine samples by high-performance liquid chromatography.[19]

Histology and Immunohistochemistry

Kidneys were fixed in 10% formalin and were paraffin embedded or processed for cryosectioning. PAS staining was performed and examined by light microscopy. Fluorescent immunostaining was performed for total laminin (Sigma-Aldrich, St. Louis, MO) and collagen IV (SouthernBiotech, Birmingham, AL) in formalin-fixed deparaffinized sections, and for nephrin (Fitzgerald Industries International, Acton, MA), podocin (Sigma-Aldrich), Sema3a (R&D Systems, Minneapolis, MN), αvβ3 integrin (EMD Millipore), and Wow-1 fragment antigen-binding region (Fab)[20] in acetone-fixed cryosections, as described previously.[11,15,19,21] Appropriate Cy2 and Cy3 fluorescent-tagged donkey secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were used to visualize immunoreactive signals, and confocal images were acquired (FluoView 300; Olympus, Tokyo, Japan). Quantitation of immunofluorescence signal was performed using ImageJ software version 1.47 (NIH, Bethesda, MD), as described by Yu et al[22] with minor modifications. In brief, the integrated density of immunofluorescence-positive signals was measured and normalized for the glomerular area in 5 to 10 glomeruli per mouse ($n = 4$ to 6 mice per experimental group).

TEM

Kidney cortex was fixed and processed for transmission electron microscopy (TEM), and samples were examined on a JEOL 1200EX microscope as described previously.[15] High-resolution digitized images (2000 dpi) were used to measure foot process width using NIH ImageJ software, as described previously.[21] Approximately 100 to 150 foot processes per kidney were measured, adjacent to ≥50 μm GBM per kidney ($n = 3$ or 4 mice per experimental group).

qPCR

Total RNA was isolated from whole-kidney tissue using TRIzol reagent (Life Technologies-Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. One microgram of isolated RNA from each animal was used to generate cDNA, using a QuantiTect kit (Qiagen, Valencia, CA). PCR reactions used pooled cDNA ($n = 4$ or 5 mice per experimental group), and amplification was performed using an Applied Biosystems SYBR Green master mix (Life Technologies) with a Mastercycler ep realplex[2] system (Eppendorf, Hauppauge, NY) . PCR primers were designed with Primer Express software version 2.0 (Life Technologies), as described previously,[15] with the following additional primers (forward and reverse): Lmx1b 5′-CATCCTTTGAGGTCTCCTCCAA-3′ and 5′-GCCAGCTTCTTCATCTTTGCTC-3′; collagen IV α2 5′-CCCATCTGACATCACACTTGTTG-3′

and 5′-TGAGATTACGCCGGGTATCC-3′; α2 laminin 5′-GACCTCTCTGGCCGCATTC-3′ and 5′-CCTCAGAGTT-ACTGATGCTGATCTG-3′; and β2 laminin 5′-GGGTTCC-AATGGTCACCATC-3′ and 5′-TGAAATGAAACTCAGC-TTCCAGG-3′. Reactions were performed in duplicate, and each experiment was repeated three times. Gene expression relative to the housekeeping gene GAPDH or ubiquitin was determined with the $2^{-\Delta\Delta Ct}$ method, as described previously.[15]

Western Blot Analysis, Immunoprecipitation, GST, and Overlay Assays

Kidneys were lysed in modified radioimmunoprecipitation assay buffer, and pooled samples of whole-kidney lysates were generated using equal amounts of protein from each mouse ($n = 4$ to 5 mice per experimental group).[15] Proteins were resolved on 8% to 15% SDS-PAGE gels, and immunoblotting was performed using standard technique with the following primary antibodies: WT1 (sc-192; Santa Cruz Biotechnology, Santa Cruz, CA), nephrin (20R-NP002; Fitzgerald Industries International), podocin (P0372; Sigma-Aldrich), CD2AP (sc-9137; Santa Cruz Biotechnology), neuropilin-1[23]; Sema3a (sc-28867; Santa Cruz Biotechnology), β1 and αvβ3 integrins (44-870G and MAB 1976Z; EMD Millipore), hMMP-9 (Chemicon AB-19016; EMD-Millipore), tubulin (T6074; Sigma-Aldrich), and actin (A2066; Sigma-Aldrich).

Whole kidney and immortalized podocytes were lysed in immunoprecipitation buffer, precleared, and incubated with purified 1 μg FLAG-tagged nephrin overnight at 4°C, and immunoprecipitated with anti-FLAG antibody (F7425; Sigma-Aldrich), as described previously.[21] Immunoprecipitates were analyzed by Western blotting using anti-nephrin (Fitzgerald Industries International), anti-plexinA_1 (Cell Signaling Technology, Danvers, MA), and anti-FLAG (Sigma-Aldrich) antibodies. HEK cells were transiently transfected with plexinA_1—Myc and nephrin—FLAG using Lipofectamine 2000 reagent (Life Technologies), according to the manufacturer's protocol; cells were lysed 24 hours later in immunoprecipitation buffer. Lysates were precleared and immunoprecipitated with nephrin[24] and plexinA_1 antibodies, as described previously.[11,25] Immunoprecipitates were analyzed by Western blotting using anti-nephrin (Fitzgerald Industries International) and anti-plexinA_1. Lysates from HEK cells transfected with plexinA_1—Myc and nephrin—FLAG were used as positive controls.

Glutathione S-transferase (GST) binding assays were performed as described previously,[25] with minor modifications.

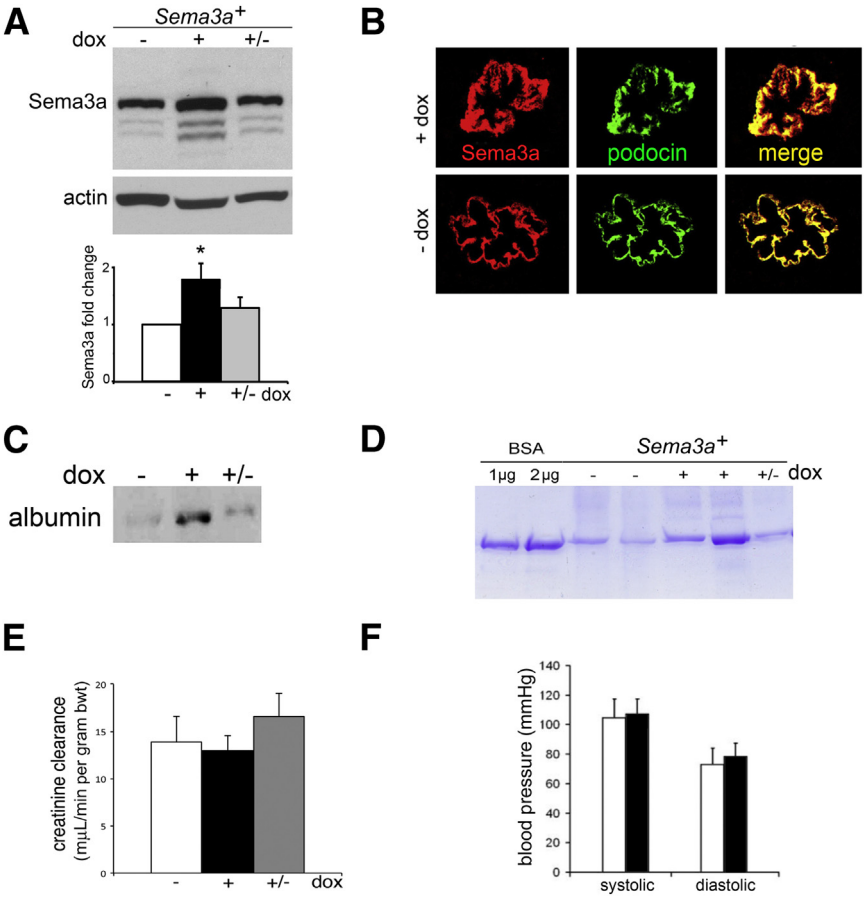

Figure 1 Podocyte *Sema3a* gain-of-function induces proteinuria. **A**: Western blotting indicates that *Sema3a*$^+$ mice overexpress kidney Sema3a approximately twofold after doxycycline-induction for 1 month. Smaller molecular weight bands represent cleaved Sema3a. Quantitation is based on at least three independent experiments. **B**: Dual immunofluorescence reveals Sema3a (red) and podocin (green) expression in developing mouse glomeruli; colocalization (yellow) indicates Sema3a expression in podocytes. **C** and **D**: Western blotting (**C**) and Coomassie Blue gel (**D**) indicate that excess Sema3a induces reversible proteinuria, based on albumin detected in urine samples corrected for creatinine. **E** and **F**: Excess Sema3a does not alter creatinine clearance (**E**) or blood pressure (**F**) within 1 month. Data are expressed as means ± SEM. White bars, control (−dox); black bars, Sema3a excess (+dox); gray bars, reversibility (+/−dox). *$P <$ 0.05. bwt, body weight. Original magnification, ×400 (**E**).

In brief, GST−nephrin cytoplasmic domain (GST-CD-nephrin) and GST−control fusion proteins expressed in *Escherichia coli* BL21 (Stratagene, La Jolla, CA) were purified using batch purification on glutathione−Sepharose 4B beads (GE Healthcare, Little Chalfont, UK). PlexinA_1−FLAG was purified from HEK cells transiently transfected with pFlag−CMV−plexinA_1 construct[26] using anti-FLAG M2 affinity gel and was eluted by competition with 3× FLAG peptide (F4799; Sigma-Aldrich). One microgram of purified plexinA_1−FLAG was incubated overnight with 0.25 μg GST fusion proteins in PBS and 0.05% Triton-X, washed, eluted by boiling in Laemmli buffer, resolved, immunoblotted with anti-FLAG (Sigma-Aldrich) and anti-GST (sc-33613; Santa Cruz Biotechnology) antibodies, and detected by enhanced chemiluminescence.

Blot overlay assays were performed as described previously.[25] Two to ten micrograms of GST-tagged fusion proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, probed with 0.2 μg/mL purified plexinA_1−FLAG, immunoblotted with anti-FLAG antibody, and detected by enhanced chemiluminescence.

Generation of the *Sema3a*$^+$ Podocyte Cell Line

The *Sema3a*$^+$ mice (*podocin-rtTA:tet-O-Sema3a*[15]) were bred with *H-2Kb-tsA58* mice[27] (Immortomouse; Jackson Laboratory, Bar Harbor, ME) to generate a conditionally immortalized podocyte cell line overexpressing Sema3a in a doxycycline-regulated manner. Glomeruli were isolated from triple-transgenic mice using magnetic beads under sterile conditions,[22,28,29] plated on collagen I−coated dishes, and cultured in RPMI 1640 medium (Life Technologies) under permissive conditions (33°C), as described previously.[25] Podocyte primary cultures were propagated and subjected to dilution cloning. Clones were selected on the basis of podocyte-specific protein expression, morphology, and doxycycline-regulated *Sema3a* expression and then were

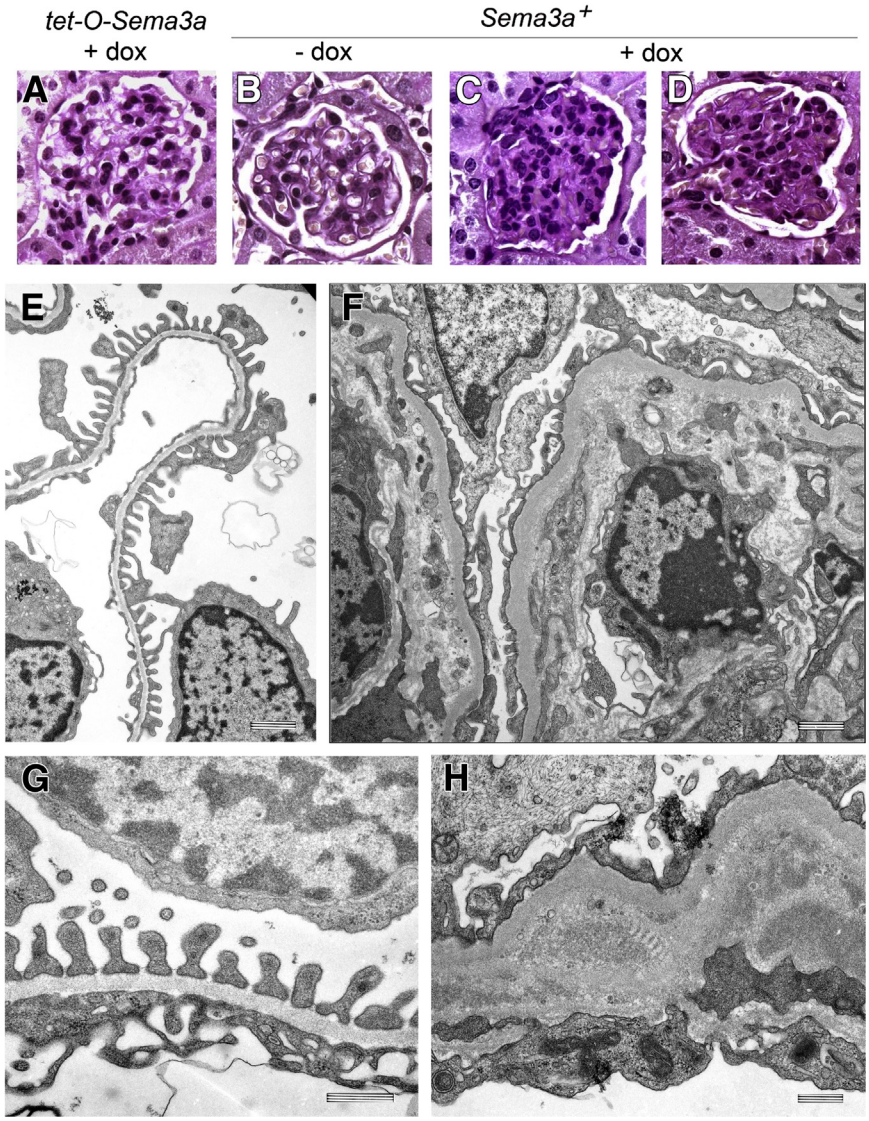

Figure 2 Podocyte Sema3a gain-of-function induces mesangial expansion foot process effacement and endothelial injury. **A** and **B**: PAS staining reveals normal glomeruli in kidneys of single-transgenic mice with doxycycline (+dox) and in uninduced *Sema3a*$^+$ mice (−dox). **C** and **D**: Two representative Sema3a-overexpressing glomeruli (+dox) exhibit mesangial expansion. **E**−**H**: TEM reveals normal glomerular filtration barrier ultrastructure in uninduced *Sema3a*$^+$ kidneys (**E** and **G**). Sema3a overexpression induces foot process effacement, endothelial cell swelling, and marked GBM expansion (**F** and **H**). Original magnification, ×400 (**A**−**D**). Scale bars: 1 μm (**E** and **F**) and 500 nm (**G** and **H**).

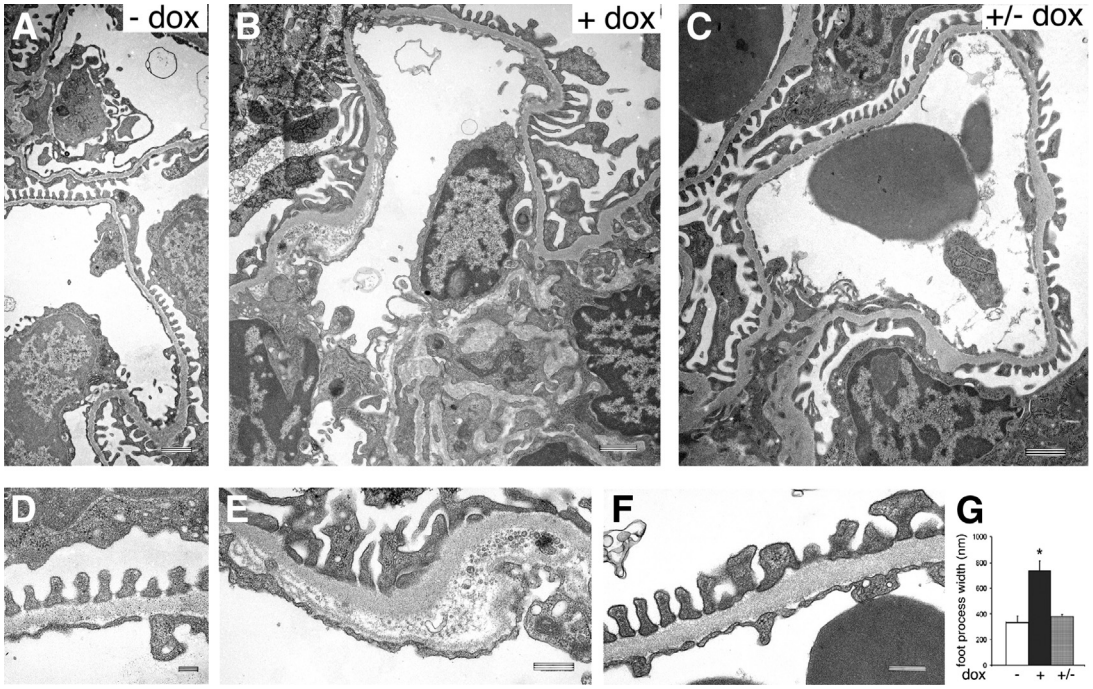

Figure 3 Sema3a-induced glomerular ultrastructural abnormalities are reversible. **A** and **D**: TEM reveals normal glomerular filtration barrier ultrastructure in uninduced *Sema3a*$^{+}$ kidneys. **B** and **E**: *Sema3a* podocyte overexpression induces foot process effacement, mesangial matrix expansion, and GBM lamination. **C** and **F**: At 2 weeks after doxycycline removal (+/−dox), the Sema3a-induced foot process effacement, GBM, and endothelial cell abnormalities resolve. **G**: Morphometry confirms reversibility of foot process effacement. Data are expressed as means ± SEM. *$P < 0.05$. Scale bars: 1 μm (**A**–**C**); 200 nm (**D**); 500 nm (**E** and **F**).

propagated and induced to differentiate at 37°C for at least 7 days before experiments.

Differentiated *Sema3a*$^{+}$ podocytes plated on collagen I–coated glass slide chambers were kept in standard medium or were exposed to 50 to 500 ng/mL recombinant mouse Sema3a[16] for 1 to 12 hours, fixed in 4% paraformaldehyde, and stained with rhodamine phalloidin. Images were acquired using a Zeiss Axiovert microscope (Carl Zeiss Microscopy, Jena, Germany) equipped with an ApoTome imaging system. Podocyte area (μm^2) was measured using Zeiss AxioVision software version 4.8 freehand area selection on images acquired at ×600 magnification ($n = 43 \pm 8$ cells per experimental condition in four independent experiments). Data are expressed as means ± SEM.

Statistical Analysis

Student's unpaired *t*-test or analysis of variance followed by Bonferroni correction was used to compare groups, as appropriate. $P < 0.05$ was deemed statistically significant.

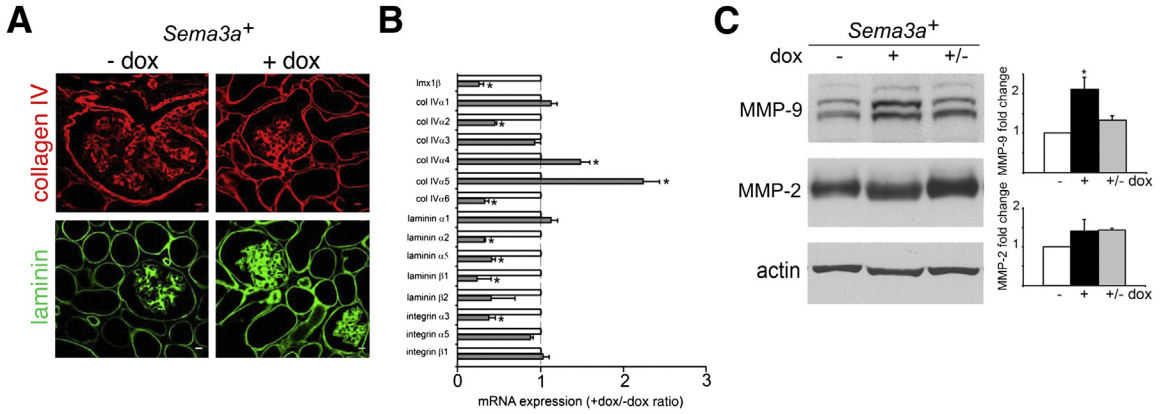

Figure 4 *Sema3a* gain-of-function disrupts GBM composition. **A**: IHC reveals similar distribution of collagen IV and laminin. **B**: qPCR indicates Sema3a-induced dysregulation of individual collagen IV and laminin chain mRNA, normalized for control mRNA expression levels. **C**: Western blotting indicates Sema3a-induced reversible MMP-9 up-regulation; MMP-2 remains unchanged. Quantitation of fold change is based on at least four independent experiments. Data are expressed as means ± SEM. *$P < 0.05$. col, collagen. Scale bar = 400 μm (**A**).

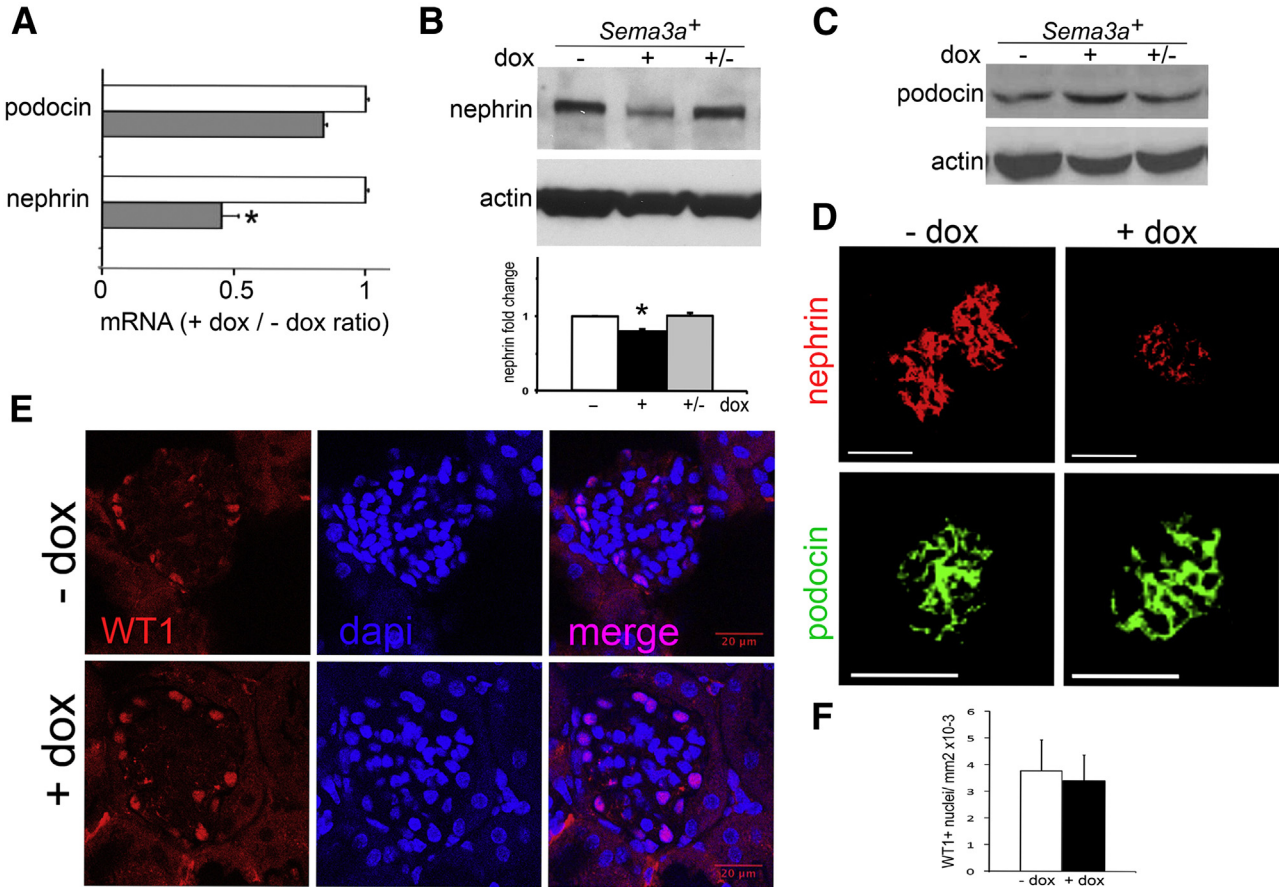

Figure 5 *Sema3a* gain-of-function down-regulates nephrin expression. **A:** Quantitative PCR indicates decreased nephrin mRNA and stable podocin mRNA in *Sema3a*-overexpressing mice. **B** and **C:** Western blotting for nephrin (**B**) and podocin (**C**) in whole-kidney lysate indicates that excess Sema3a induces reversible nephrin down-regulation but does not affect podocin expression. Quantitation is based on at least four independent experiments. **D:** Immunofluorescence reveals down-regulation of nephrin and stable expression of podocin localized to glomerular podocytes. **E:** Immunofluorescence reveals no change in podocyte number ($WT1^+$ nuclei) in *Sema3a*-overexpressing mice versus controls. DAPI labels nuclei; merged images confirm nuclear WT1 staining. **F:** Quantitation of $WT1^+$ cells per standard area. Data are expressed as means ± SEM. $n = 3$ mice per experimental group (approximately 20 glomeruli quantified per mouse). Scale bars: 100 µm (**D**); 20 µm (**E**).

Results

Podocyte *Sema3a* Gain-of-Function in Adult Mice Causes Glomerular Disease

To determine whether increased podocyte Sema3a has a pathogenic role, we used $Sema3a^+$ podocyte-specific, inducible *Sema3a* gain-of-function adult mice.[15] In these mice, the *Sema3a* transgene is expressed in podocytes in a doxycycline-regulated manner[15] (Figure 1, A and B). Uninduced adult $Sema3a^+$ mice are fertile and have normal life spans, normal renal histology, and normal glomerular ultrastructure, as assessed by TEM (Figure 2, B, E, and G). $Sema3a^+$ mice were induced with doxycycline for 1 month. Uninduced $Sema3a^+$ and single-transgenic mice receiving doxycycline served as controls.

Podocyte *Sema3a*-overexpressing mice developed proteinuria within 4 weeks (Figure 1, C and D); their creatinine clearance and blood pressure were normal (Figure 1, E and F). Light microscopy revealed mesangial matrix expansion and mild hypercellularity (Figure 2, C and D), and TEM revealed marked glomerular abnormalities in *Sema3a*-overexpressing kidneys (Figure 2, F and H). Expansion of the mesangial matrix was confirmed by TEM (Supplemental Figure S1). Glomerular endothelial cells were swollen and exhibited interdigitation (Figure 2, F and H), indicating that podocyte *Sema3a* overexpression induces endothelial cell injury. The GBM was thickened; the lamina rara interna was widened by lamination, electron-dense material, and mesangial matrix interposition (Figure 2, F and H). Significant podocyte foot process effacement was observed adjacent to the GBM abnormalities (Figure 2, F and H). Morphometric analysis of TEM images indicated increased foot process width in *Sema3a*-overexpressing mice, compared with uninduced mice of identical genotype or single-transgenic *tet-O-Sema3a* mice (737 ± 77 nm versus 370 ± 13 nm or 333 ± 50 nm, respectively) ($P < 0.05$). Thus, *Sema3a*-overexpressing mice developed a distinct glomerular disease characterized by mesangial matrix and GBM expansion, focal foot process effacement, endothelial injury, and proteinuria.

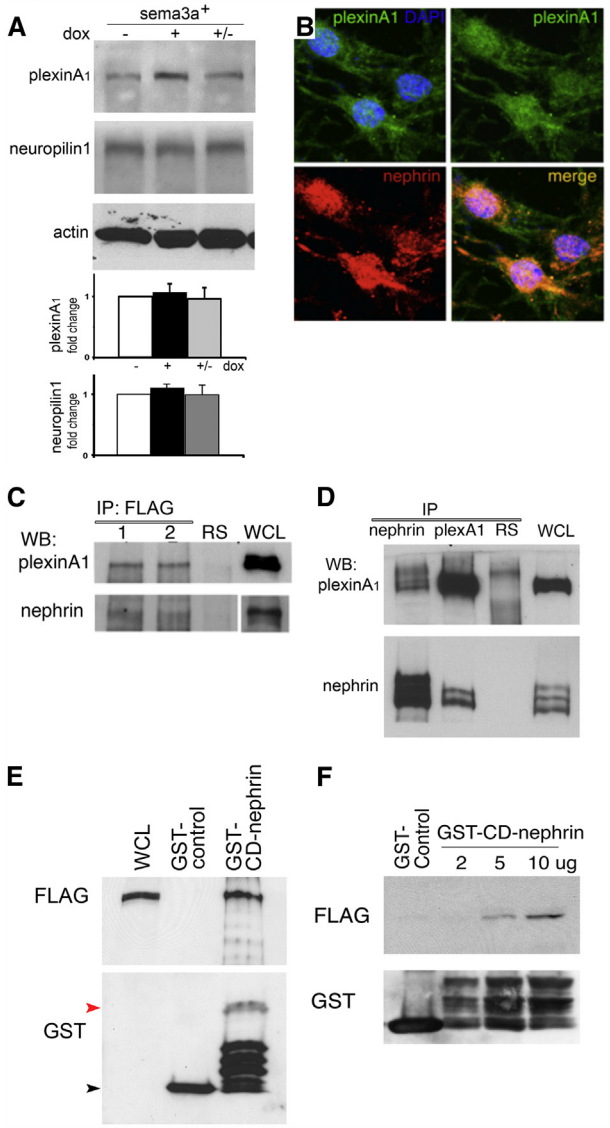

Figure 6 Sema3a signaling receptor plexinA_1 interacts with nephrin. **A:** Representative Western blots and quantitation indicate no significant changes in plexinA_1 or neuropilin 1 expression in whole kidneys after doxycycline induction. **B:** Immunofluorescence reveals colocalization of plexinA_1 and nephrin in cultured podocytes. **C:** Co-immunoprecipitation reveals association of endogenous plexinA_1 and FLAG-nephrin. Lane 1, cultured podocytes; lane 2, whole kidney. **D:** Reciprocal nephrin and plexinA_1 coimmunoprecipitation reveals nephrin—plexinA_1 interaction in transfected HEK cells. **E** and **F:** GST binding assay (**E**) indicates direct interaction of purified FLAG-plexinA_1 with nephrin cytoplasmic domain (GST-CD-nephrin, approximately 60 kDa; **red arrowhead**); the GST-control is approximately 25 kDa (**black arrowhead**). Overlay assay (**F**) indicates that plexinA_1—nephrin interaction is direct. Purified FLAG-plexinA_1 binds increasing amounts of GST-CD-nephrin blotted on cellulose membrane, as detected by FLAG immunoblotting; the GST Western blot confirms equal loading. Data are representative of at least three independent experiments. IP, immunoprecipitation; RS, rabbit serum; WB, Western blot; WCL, whole-cell lysate. Original magnification, ×400 (**B**).

Sema3a-Induced Glomerular Disease Is Partially Reversible

To determine whether the effects of podocyte *Sema3a* gain-of-function are reversible, *Sema3a*$^+$ mice were induced for 1 month with doxycycline and examined 2 weeks after doxycycline removal (+/−dox). Sema3a-induced albuminuria resolved 2 weeks after doxycycline removal in approximately half of the mice (6 of 11 mice) (Figure 1, C and D). Notably, TEM revealed that the Sema3a-induced foot process effacement and the GBM and endothelial damage (Figure 3, B and E) also resolved upon removal of transgene induction (Figure 3, C and F), although some mesangial matrix expansion and focal GBM changes remained (Supplemental Figure S1). TEM morphometric analysis confirmed that foot process width returned to baseline (from 737 ± 77 to 380 ± 15 nm; $P < 0.05$) (Figure 3G). Although Sema3a-induced abnormalities were only partially reversible, these findings provide a proof-of-principle of the cause-and-effect relationship between podocyte Sema3a excess and glomerular disease.

Podocyte *Sema3a* Gain-of-Function Disrupts GBM Composition

To further define the GBM phenotype induced by excess Sema3a, we examined mRNA and protein expression of GBM components in induced *Sema3a*$^+$ and uninduced (control) kidneys. Expression of total collagen IV and laminin, as assessed by immunohistochemistry, was similar in induced *Sema3a*$^+$ and control kidneys (Figure 4A). Collagen IV and laminin chain immunostaining revealed no change in localization of individual chains (data not shown); however, α2 laminin expression was decreased in *Sema3a*-overexpressing kidneys, compared with controls (data not shown). qPCR demonstrated a significant increase in α4 and α5 collagen IV mRNA, whereas laminin α2, α5, β1, and β2 mRNAs were decreased to less than half their control levels (Figure 4B), suggesting that the total laminin observed with IHC may include abnormal laminin chains. Expression level of the LIM homeobox transcription factor 1-beta, a major regulator of the GBM encoded by *Lmx1b*, decreased significantly in Sema3a-overexpressing kidneys, as determined by qPCR (Figure 4B). Next, we examined the glomerular metalloproteinases MMP-2 and MMP-9 by immunoblotting. MMP-9 was up-regulated in *Sema3a*-overexpressing kidneys and returned to baseline on removal of the transgene induction, whereas expression of MMP-2 did not change (Figure 4C). Taken together, these findings suggest that the Sema3a-induced GBM phenotype in adult kidney results from both increased MMP-9 expression and subtle changes in collagen and laminin chain composition.

Sema3a Gain-of-Function Decreases Nephrin Expression

To define the molecular basis of podocyte effacement in *Sema3a*$^+$ adult mice, we examined the expression of slit-diaphragm proteins. Nephrin expression decreased to approximately 50% of control levels, as demonstrated by qPCR,

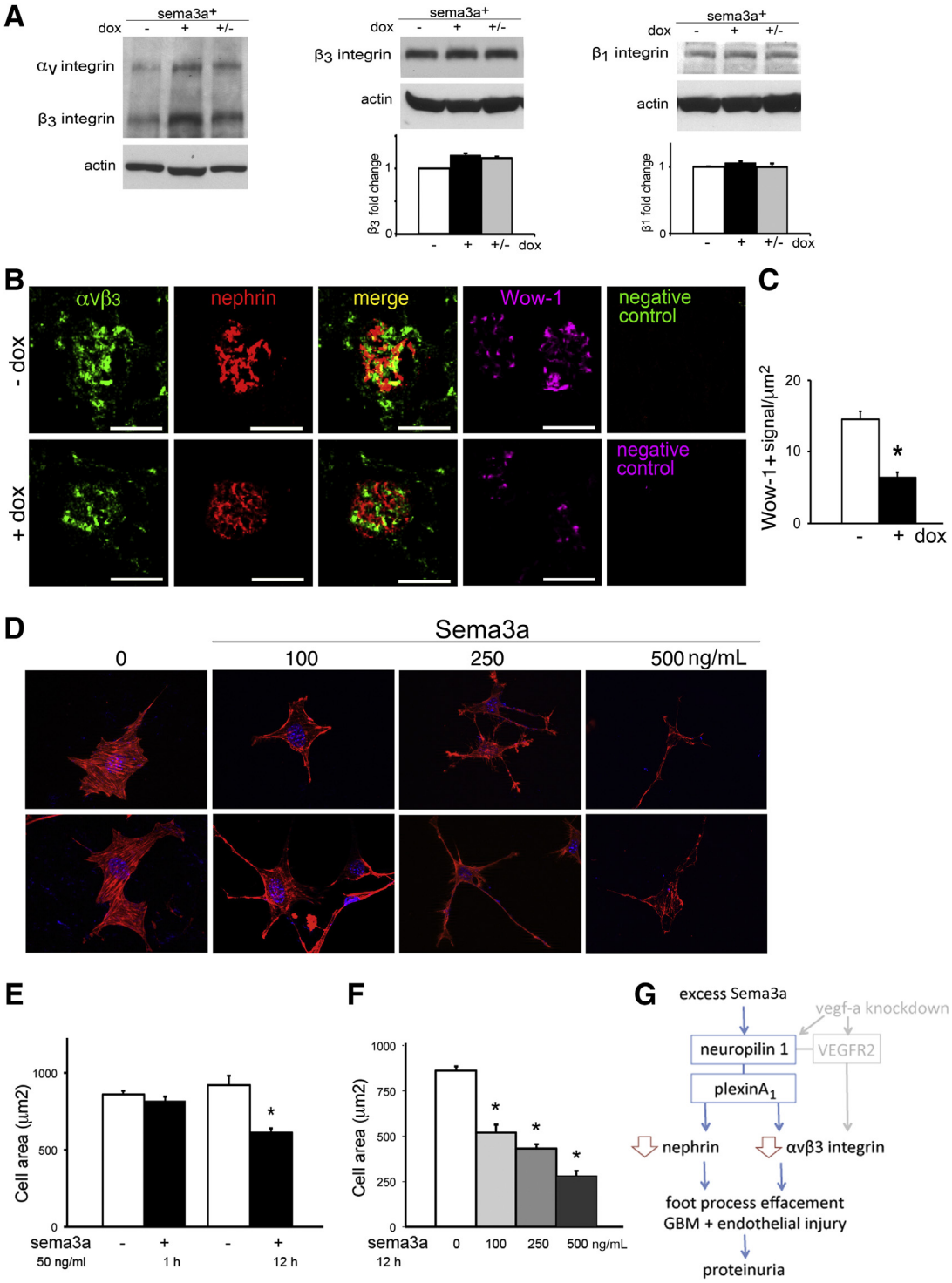

Figure 7 Sema3a decreases αvβ3 integrin glomerular activity and induces podocyte contraction and shape change. **A**: Western blotting and quantitation indicate that αvβ3, β3, and β1 integrin whole-kidney expression levels were not changed by *Sema3a* overexpression. **B**: Immunofluorescence reveals decrease of both glomerular total αvβ3 integrin and Wow-1 (active αvβ3 integrin) in *Sema3a*-overexpressing kidneys. The αvβ3 integrin and nephrin partially colocalize; negative controls demonstrate no signal in the absence of the primary antibodies (total αvβ3 integrin, nephrin, and active αvβ3 integrin). **C**: Quantitation of active αvβ3-positive signals (Wow-1), normalized for glomerular area (integrated density/μm^2) indicates an approximately 50% decrease in αvβ3 integrin activity. **D**: Sema3a induced cell contraction and shape change in a dose-dependent manner; two representative images from each condition are shown (rhodamine phalloidin staining). Note decreasing F-actin staining at higher Sema3a concentration. **E**: Differentiated *Sema3a* podocytes exposed to vehicle or 50 ng/mL recombinant mouse Sema3a for 1 hour or 12 hours exhibited significant change in cell size. **F**: Quantitation after 12 hours of Sema3a exposure indicates dose-dependent decrease in cell area. **G**: Model for Sema3a-induced glomerular disease mechanism. Data are expressed as means ± SEM, representative of four independent experiments (approximately 50 cells per experiment). *$P < 0.05$. Scale bar = 100 μm (**B**). Original magnification, ×600 (**D**).

immunoblotting, and immunohistochemistry, whereas podocin expression level was not altered by Sema3a excess (Figure 5, A–C), suggesting that the decreased nephrin expression was not a result of podocyte loss. Similar podocyte numbers in control and *Sema3a*-overexpressing glomeruli was confirmed by counts of $WT1^+$ nuclei (Figure 5, D and E). On removal of doxycycline induction, nephrin expression returned to control levels (Figure 5B), demonstrating a cause-and-effect relationship between Sema3a excess and nephrin down-regulation. The concomitant reversibility of podocyte foot process effacement and proteinuria independently confirmed the lack of podocyte loss and the Sema3a-induced pathogenic mechanism (ie, nephrin loss disrupts slit-diaphragm integrity, leading to increased glomerular filtration barrier permeability).

The Sema3a Signaling Receptor PlexinA$_1$ Interacts with Nephrin

We examined the molecular mechanisms involved in the crosstalk between Sema3a and nephrin signaling. Neuropilin-1 and plexinA$_1$, Sema3a binding, and signaling receptors[12,30,31] were detected in kidney lysates and cultured podocytes by immunoblotting and immunofluorescence (Figure 6, A and B, and Supplemental Figure S2). Purified nephrin–FLAG was found to associate with endogenous plexinA$_1$ *in vivo* and in cultured mouse podocytes, as determined by co-immunoprecipitation (Figure 6C). Furthermore, plexinA$_1$ co-immunoprecipitated with nephrin in HEK cells transiently transfected with the corresponding constructs (Figure 6D). Overlay and GST binding assays were performed to further evaluate plexinA$_1$–nephrin interaction. These experiments showed that purified plexinA$_1$–FLAG physically associates with the cytoplasmic domain of nephrin (GST-CD-nephrin) (Figure 6, E and F). Taken together, these findings demonstrate a direct plexinA$_1$–nephrin interaction.

Sema3a Decreases Glomerular αvβ3 Integrin Activity and Modulates Podocyte Shape

Sema3a signaling inhibits integrin activity and thereby modulates cell adhesion and motility.[12,32] We therefore compared αvβ3 and β1 integrin expression and activity in *Sema3a*-overexpressing and control kidneys. Although quantitation of αvβ3, β1, or β3 integrin expression in whole-kidney lysates was similar (Figure 7A), *Sema3a* gain-of-function decreased glomerular αvβ3 integrin expression and activity, as assessed by immunolabeling with αvβ3 integrin and Wow-1 antibodies, which recognize total and activated αvβ3 integrin, respectively (Figure 7B). Quantitation of Wow-1 signals by morphometric analysis confirmed the observation that *Sema3a* gain-of-function results in an approximately 50% decrease in glomerular active αvβ3 integrin (Figure 7C), suggesting that low αvβ3 integrin activity contributes to the observed glomerular phenotype.

To determine whether Sema3a influences podocyte shape cell-autonomously, we exposed cultured mouse podocytes to recombinant Sema3a[16] and examined cell shape, size, and actin cytoskeleton by rhodamine phalloidin staining (Figure 7, D–F). We observed that podocyte shape changes develop within 1 to 12 hours of Sema3a exposure. Morphometric analysis of time- and dose-response experiments demonstrated that Sema3a induces significant podocyte contraction and F-actin collapse, resulting in decreased size and dramatic shape changes (Figure 7, D–F).

Discussion

While our previous studies showed that excess Sema3a disrupts slit-diaphragm development, the present findings demonstrate that a tight regulation of podocyte Sema3a is important for the maintenance of glomerular filtration barrier structure and function in the adult kidney. In the present study, we report that podocyte *Sema3a* gain-of-function in adult mice causes glomerular disease, and *Sema3a* overexpression induces reversible dysregulation of nephrin, MMP-9, and αvβ3 integrin, resulting in foot process effacement, a striking GBM phenotype, mesangial expansion, and endothelial cell injury.

An important finding of the present study is that the glomerular disease caused by excess podocyte Sema3a in adult mice is partially reversible. The evidence is that Sema3a-induced foot process effacement and endothelial injury are reversible 2 weeks after removal of the transgene induction. Moreover, we demonstrated that down-regulation of nephrin induced by *Sema3a* gain-of-function is also reversible, and the latter results in recovery of glomerular permselectivity. Proteinuria resolved in more than half of the mice; persistent proteinuria in the remainder was likely due to the observed persistent excess extracellular matrix and focal GBM abnormalities. Taken together, these findings provide proof-of-principle of the cause-and-effect relationship between podocyte Sema3a excess and glomerular disease. Consistent with these data, we had previously found that mice overexpressing *Sema3a* during glomerular development lacked slit diaphragms at birth and that their nephrin and WT-1 expression was decreased.[15] We had also found that cultured podocytes exposed to recombinant Sema3a had decreased association of nephrin–podocin–CD2AP and down-regulated podocin, indicating that Sema3a dysregulates slit-diaphragm proteins[16] (albeit not precisely in the same manner as it does *in vivo*, likely because of cell culture conditions).

Another key finding of the present study is that in podocytes the Sema3a signaling receptor plexinA$_1$ interacts with nephrin *in vivo* and *in vitro*, as shown by co-immunoprecipitation of native plexinA$_1$ and purified nephrin and confirmed using transfected cells and purified proteins. Overlay and GST binding assays demonstrated that plexinA$_1$–nephrin interaction is direct. Taken together, these findings

indicate that plexinA_1 is a novel component of the nephrin signaling complex. PlexinA_1—nephrin interaction links Sema3a extracellular signals to the nephrin signaling pathway and provides a mechanism for Sema3a-induced foot process effacement. The plexinA_1 extracellular domain is known to be autoinhibitory, and the inhibition is released on Sema3a binding to neuropilin-1.[24,33] Downstream plexinA_1 signaling is complex and involves microtubule and actin cytoskeleton regulation via integrins, CRMPs, Rho-GTPases, and protein-methionine sulfoxide oxidase MICAL1.[12,33–35] Further studies would be required for detailed characterization of the downstream intracellular signaling pathways mediating the Sema3a effects on podocytes described here. Nonetheless, consistent with previous reports documenting Sema3a-induced growth-cone collapse in neurons,[12] as well as F-actin collapse and inhibition of motility and migration in endothelial cells,[15,36] our present findings indicate that Sema3a causes podocyte F-actin collapse and podocyte shape change, in a time- and concentration-dependent manner. Sema3a-induced cell shape changes may be mediated in part by αvβ3 integrin inactivation, and may also involve microtubules and myosin.[37,38]

Sema3a gain-of-function induces a laminated GBM phenotype similar to that of Alport or Pierson syndrome and that of integrin knockout and *Vegf-a* knockdown mice, likely through its effects on podocytes and endothelial cells, which together secrete the GBM components.[39] The mechanisms that regulate GBM assembly are not fully understood, and changes in GBM composition are involved in the pathogenesis of a variety of human proteinuric kidney diseases. These include classic GBM diseases such as Alport syndrome, diabetic nephropathy, and lupus nephritis.[9,40] Interestingly, our research group and others have reported *Sema3a* dysregulation in diabetic nephropathy and in lupus nephritis, respectively.[19,41]

A proper balance of collagen chain synthesis appears to be required for generation of the triple helix of collagen IV and laminin chains; indeed, permeability defects in the Alport mouse model are exacerbated by inappropriate laminin chain synthesis in response to α3 collagen IV deficit.[8] Our present findings suggest that excess Sema3a dysregulates MMP-9, as well as various extracellular matrix components (α2, α5, and β1 laminins) and integrin receptors (α3 and αvβ3) in the glomerulus. Laminin α5, a major component of the adult glomerular basement membrane laminin 521 (α5β2γ1), is required for mesangial cell adhesion and glomerular development and is down-regulated in diabetic nephropathy.[42,43] Mutations of β2 laminin cause Pierson syndrome, a rare form of nephrotic syndrome.[44,45] Abnormal glomerular deposition of α2 laminin has been reported in Alport syndrome.[46] Down-regulation of the *Lmx1b* protein suggests that Sema3a might regulate the molecular composition of the GBM, although similar to podocyte-specific *Lmx1b*-knockout mice and to patients with nail—patella syndrome, we did not identify the decreased expression of podocin, CD2AP, or α3 collagen IV that has been described in *Lmx1b*-null mice.[47–50]

Integrin α3 facilitates podocyte attachment to the GBM and is required during development to organize glomerular capillary loops and GBM assembly.[51,52] Deletion of podocyte β1 integrin induces podocyte effacement and laminated GBM, demonstrating a role in podocyte attachment; glomerular endothelium remains normal.[53] Deletion of the tetraspanin *CD151* (which regulates α3 integrin—mediated adhesion) induces GBM abnormalities similar to those observed in Alport mice and similar to our present findings, suggesting that defects in podocyte adhesion can induce abnormalities in GBM structure.[54] Sema3a inhibits endothelial cell adhesion to the extracellular matrix and cell motility via inhibition of integrin activity *in vitro*.[32,35] In the kidney, αvβ3 integrin is expressed by the glomerular endothelium and podocytes, the target cells for Sema3a paracrine[13,36] and autocrine[7,15,16] effects. Podocyte *Sema3a* gain-of-function down-regulates glomerular αvβ3 integrin expression and activity *in vivo*. However, Sema3a-induced changes in podocyte shape, cytoskeleton, and αvβ3 integrin did not result in podocyte loss *in vivo*.

Remarkably, podocyte *Vegfa* knockdown induces a glomerular phenotype similar to that of *Sema3a* gain-of-function, mediated at least in part by down-regulation of glomerular αvβ3 integrin expression and activity.[11] Vegf-a and Sema3a compete to bind neuropilin-1, which functions as a coreceptor for Vegf-a and a binding receptor for Sema3a.[12,23,36] Of note, Vegf-a and neuropilin-1 expression are not altered in *Sema3a* gain-of-function mice, and *Sema3a* expression is not dysregulated in mice with podocyte *Vegfa* knockdown (data not shown). Taken together, these findings suggest that down-regulation of αvβ3 integrin expression and activity plays a key role in disrupting the integrity and function of the glomerular filtration barrier. Notably, this occurs downstream of both Sema3a excess (the present study) and *Vegfa* knockdown,[11] suggesting that these two proteins secreted by podocytes mediate opposite signals (in the same or independent pathways) that merge to down-regulate integrin activity *in vivo* (Figure 7G), as described in cultured cells,[32,36] resulting in glomerular phenotypes similar to integrin deletion models.

We have previously shown that *Sema3a* expression in the kidney is developmentally regulated and that *Sema3a* loss or gain-of-function disrupts normal glomerular filtration barrier development.[7,15] The transcriptional and translational regulation of *Sema3a* in health and disease are poorly understood. Similarly, the regulation of Sema3a secretion (if any) is unknown; most studies of semaphorins have focused on the downstream signaling, developmental, and pathological effects.[12,13,56,58] Our research group previously reported up-regulation of Sema3a protein expression in type 1 diabetic mice,[19] and we have also observed increased Sema3a in renal biopsies from patients with advanced diabetic nephropathy (D. Veron and A. Tufro, unpublished data), raising the possibility that excess

Sema3a may contribute to the pathogenesis of proteinuric kidney disease, including diabetic nephropathy. Quantitation of Sema3a mRNA expression in glomeruli from db/db mice reported by our group and others showed discrepant results (up-regulation and down-regulation, respectively),[13,55] whereas neuropilin-1 mRNA decreased in diabetic mice and humans,[55] providing further indication that the complexity of glomerular Sema3a biology warrants further study.

Accumulating evidence suggests that Sema3a negatively regulates immune responses via Sema3a, neuropilin-1 and plexinA_4 expressed in T cells, regulatory B cells, and activated monocytes.[41,56] Sema3a dysregulation was recently reported in human systemic lupus erythematosus; serum Sema3a was mildly decreased, whereas Sema3a was increased in renal tubules.[56,57] Further studies are needed to elucidate whether dysregulation of Sema3a signaling in T cells or B cells is pathogenic in systemic lupus erythematosus and whether it is mechanistically involved in lupus nephritis. However, given the role of Sema3a as negative regulator of immune response and angiogenesis, as well as its osteoprotective effect, it has been suggested that Sema3a could be used therapeutically in immune diseases, cancer, and osteoporosis.[56,58,59] Off-target effects of Sema3a should therefore be carefully examined.

Taken together, our present findings demonstrate that excess podocyte Sema3a causes glomerular disease in adult mice. Thus, tight Sema3a regulation is critical for the maintenance of the glomerular filtration barrier structure and function. Our findings suggest that, while Sema3a-induced down-regulation of nephrin leads to podocyte effacement, up-regulation of MMP-9 and decreased $\alpha v\beta 3$ integrin activity in the glomerulus result in disruption of the integrity of the GBM and endothelial injury. Direct Sema3a effects on podocyte and endothelial cell actin cytoskeleton are likely to contribute to podocyte effacement and endotheliosis *in vivo*. PlexinA_1—nephrin interaction links Sema3a signals to the nephrin signaling pathway, providing a mechanism for Sema3a-induced foot process effacement. Insight into precisely how plexinA_1—nephrin interaction modulates nephrin turnover and podocyte cytoskeleton will require additional studies, including identification of the responsible actin-binding protein. Elucidation of the Sema3a signaling pathway downstream from plexinA_1 in podocytes may reveal novel molecular and cellular mechanisms involved in the pathogenesis of proteinuric renal disease and diabetic nephropathy, and may identify novel therapeutic targets as well.

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Supplemental Data

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