Semaphorin3a regulates endothelial cell number and podocyte differentiation during glomerular development

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Semaphorin3a (Sema3a), a chemorepellent guidance protein, plays crucial roles in neural, cardiac and peripheral vascular patterning. Sema3a is expressed in the developing nephron, mature podocytes and collecting tubules. Sema3a acts as a negative regulator of ureteric bud branching, but its function in glomerular development has not been examined. Here we tested the hypothesis that Sema3a regulates glomerular vascular development using loss- and gain-of-function mouse models. Sema3a deletion resulted in defects in renal vascular patterning, excess endothelial cells within glomerular capillaries, effaced podocytes with extremely wide foot processes and albuminuria. Podocyte Sema3a overexpression during organogenesis resulted in glomerular hypoplasia, characterized by glomerular endothelial cell apoptosis, delayed and abnormal podocyte foot process development, a complete absence of slit diaphragms and congenital proteinuria. Nephrin, WT1 and VEGFR2 were downregulated in Sema3a-overexpressing kidneys. We conclude that Sema3a is an essential negative regulator of endothelial cell survival in developing glomeruli and plays a crucial role in podocyte differentiation in vivo. Hence, a tight regulation of Sema3a dosage is required for the establishment of a normal glomerular filtration barrier.

KEY WORDS: Semaphorin, Glomerular development, Podocyte differentiation, Endothelial cell migration, Mouse

INTRODUCTION

Glomerulogenesis requires spatially directed cell migration, cell differentiation and modulation of cell-cell and cell-matrix interactions to generate the tri-layered structure of the mature glomerular filter. The signaling mechanisms that guide these processes are not fully understood (Quaggin and Kreidberg, 2008). Vascular endothelial growth factor (VEGFA) induces vasculogenesis and stimulates endothelial cell proliferation and migration into the vascular cleft of S-shaped bodies to form glomerular capillaries (Eremina et al., 2003; Gerber et al., 1999; Kitamoto et al., 1997; Tufro, 2000; Tufro et al., 1999). As capillaries form, adjacent epithelial cells envelop the capillary loop and differentiate into podocytes, forming foot processes and slit diaphragms (SDs) that replace the immature cell-cell interactions [occluding junctions (OJ)]. Gene deletion studies have identified several proteins (including nephrin, podocalyxin, LMX1β, POD1, podocin, kreisler and GLEPP1) that are required to establish podocyte foot processes and slit diaphragms, although the mechanisms involved are unclear. Recruitment of mesangial cells into the developing vascular tuft is dependent upon endothelial cell secretion of platelet-derived growth factor B (Lindahl et al., 1998). Together, podocytes and endothelial cells synthesize the glomerular basement membrane (GBM) and assemble the tri-layered glomerular filtration barrier (Abrahamson et al., 1998; Sariola, 1984). Major GBM components, such as laminin α5, form a structural framework for glomerular development, and both laminin α5 and its receptor α3 integrin are required for glomerulogenesis (Kreidberg et al., 1996; Miner and Li, 2000).

Sema3a gene dosage results in perinatal lethality (Behar et al., 1996). Sema3a signaling is mediated by a complex of the binding receptor neuropilin 1 and the signaling receptors plexinA1 or A3 (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997; Tamagnone et al., 1999). Both Sema3a gene deletion results in perinatal lethality (Behar et al., 1996). Sema3a signaling functions, including axon pathfinding, cardiac and peripheral vascular patterning and branching morphogenesis. Sema3a gene deletion resulted in defects in renal vascular patterning, excess endothelial cells within glomerular capillaries, effaced podocytes with extremely wide foot processes and albuminuria. Podocyte Sema3a overexpression during organogenesis resulted in glomerular hypoplasia, characterized by glomerular endothelial cell apoptosis, delayed and abnormal podocyte foot process development, a complete absence of slit diaphragms and congenital proteinuria. Nephrin, WT1 and VEGFR2 were downregulated in Sema3a-overexpressing kidneys. We conclude that Sema3a is an essential negative regulator of endothelial cell survival in developing glomeruli and plays a crucial role in podocyte differentiation in vivo. Hence, a tight regulation of Sema3a dosage is required for the establishment of a normal glomerular filtration barrier.

We hypothesized that Sema3a plays a role in the formation of the glomerular filtration barrier. We examined the effect of Sema3a gene dosage on glomerular filter development using loss- and gain-of-function models. Here we show that Sema3a gene deletion causes defective renal vascular patterning, excess endothelial cells and poor glomerular capillary lumen development, whereas podocyte Sema3a overexpression during kidney organogenesis leads to glomerular hypoplasia, glomerular endothelial apoptosis and abnormal podocyte differentiation with a complete absence of slit diaphragms. Thus, a tightly regulated Sema3a dosage is required for the development of a normal glomerular filtration barrier.

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MATERIALS AND METHODS

All experiments were performed in accordance with approved protocols and AECOM Institute of Animal Studies regulations. Mice were housed in a pathogen-free environment.

**Sema3a-null mutant mice**

*Sema3a*−/− mice (Behar et al., 1996) were bred and their progeny (n = 31) were examined at birth. *Sema3a*−/− mice were bred with *Flk1-lacZ*−/− mice (Shalaby et al., 1995). Progeny from *Flk1-lacZ*−/−: *Sema3a*−/− mice were examined at birth (n = 29). Mice were genotyped using appropriate PCR primers (Behar et al., 1996; Shigehara et al., 2003) for *tet-O-Sema3a*, the primers 5′-TGTCAGTCGATGCGTC-3′ and 5′-CACTGGACCTTGTCATC-3′ were used. *Flk1-lacZ*−/− mice were identified by lacZ staining (Lobe et al., 1999).

**Generation of podocyte-specific Sema3a-overexpressing mice**

*Sema3a* cDNA (NM_017310) was cloned into the *MuLt1* and *Nhe1* sites of pBII-EGFP (Clontech) carrying the Tet-responsive promoter (Tet-On System). Tet-O-*Sema3a* mice were generated by pronuclear injection of DNA into FVB embryos (Transgenic Facility, AECOM) and were bred with *podocin-rtTA* mice (Shigehara et al., 2003) to yield bi-transgenic *podocin-rtTA: tet-O-Sema3a* mice. Isolated glomeruli were used to confirm *Sema3a* transgene induction. Glomeruli were isolated from adult *podocin-rtTA: tet-O-Sema3a* mice and single transgenics induced with doxycycline for 7 days, and un-induced *podocin-rtTA: tet-O-Sema3a* mice (n = 4 per group), using a reported protocol (Takemoto et al., 2006). *Sema3a* mRNA was measured by qPCR as described below.

Pregnant bi-transgenic dams were fed doxycycline (625 mg/kg feed, Harlan-Teklad, Madison, WI, USA) from embryonic day 12 (E12) until birth (NB1) or 2 weeks of age. Doxycycline dosing was started at E12 to allow for therapeutic doxycycline levels to be reached by the onset of podocin expression in the developing embryos at E14. *Podocin-rtTA: tet-O-Sema3a* progeny were examined at both NB1 and 2 weeks of age and were compared with induced single-transgenic littermates and with age-matched un-induced *podocin-rtTA: tet-O-Sema3a* as controls.

**Cells**

Mouse glomerular endothelial cells (MGEC) were isolated from *Flk1-lacZ*−/− mice as previously described (Tufto-McReddie et al., 1997; Tufto, 2000). Endothelial cells were cloned and characterized by immunocytochemistry, β-Gal and Fluorescent Lectin stainings. Primary antibodies were used anti-Flk1 (sc-315, Santa Cruz), anti-CD31 (Pharmingen), anti-α smooth muscle actin clone 1A4 (#A2547, Sigma) and anti-WT1 (sc-192, Santa Cruz).

**Migration assays**

MGEC/Kidney co-culture

Cell Tracker-labeled MGEC were co-cultured with embryonic kidneys (E12) and exposed for 24 hours to either media plus 250 ng/ml rat recombinant *Sema3a* or media plus vehicle. MGEC migration towards the explants was examined 12 and 24 hours later by phase- and confocal microscopy (Olympus Fluoview FV300).

**Quantitative migration assay**

5 × 10⁴ MGEC were plated on 8 μm pore filters (Nunc, 137443) in complete MGEC media and allowed to attach for 1 hour, starved for 1 hour in serum-free media. Serum-free media, with or without recombinant SEMA3A (500 ng/ml), was added in the lower chamber, and cells were incubated at 37°C for 6 hours. At the end of the experiment, cells on the upper side of the insert were mechanically removed, and the cells on the lower side of the insert were fixed in 10% buffered formalin for 25 minutes, stained with 0.1% Crystal Violet for 30 minutes, then counted. Experiments were performed in duplicate and repeated four times.

**Immunoblotting**

Kidneys were lysed in a modified radio immuno precipitation assay (RIPA) buffer (Karihaloo et al., 2005). Pooled samples of whole kidney lysates were generated using equal micrograms of protein from each mouse. Proteins were resolved by 8-15% SDS-PAGE and immunoblotting was performed using standard western blotting technique with the following primary antibodies: anti-WT1 (Santa Cruz, sc192, 1:200); anti-nephrin (Fitzgerald, 1:500); anti-podocin (gift from Peter Mundel, 1:500); anti-CD2AP (Santa Cruz, sc-9137, 1:200); anti-PAX2 (Zymed, 71-6000, 1:250); anti-VEGF (Santa Cruz, sc507, 1:200); anti-VEGFR2 (Santa Cruz, sc-315, 1:500); anti-neurlipin 1 (gift from Alex Kolodkin, 1:1000) and anti-actin (Sigma, A2066, 1:1000). To assess albuminuria, equal volumes of urine were resolved on SDS-PAGE and immunoblotted with anti-BSA antibody (Upstate, 07-248, 1:1000).

**Immunohistochemistry (IHC)**

Freshly harvested kidneys were either fixed in 10% formalin for 24 hours and embedded in paraffin or incubated in 18% sucrose for 10 minutes, embedded in optimal cutting temperature (OCT) compound and frozen in isopentine mixed with dry ice. Cryosections were fixed in −20°C acetone (or 4% paraformaldehyde for PAX2/synaptopodin IHC), permeabilized with 0.3% Triton-X, and blocked in 2.5% donkey serum, 1% BSA, 0.1% gelatin, 0.1% Triton-X, 0.05% Tween and 0.05% sodium azide. For the WT1/anti-activated caspase 3 dual-immunostaining, sections from paraffin-embedded, formalin-fixed kidneys were rehydrated, microwaved in citrate buffer (pH 6.0) and blocked as above. For F4/80 IHC, antigen retrieval was with proteinase K, with detection by Vectastain ABC kit and DAB (Vector Labs). The following primary antibodies and dilutions were used: anti-claved caspase 3 (Cell Signaling, 1:300), anti-CD31 (BD Pharmingen, 1:50), anti-F4/80 (E Bioscience, 1:100), anti-nephrin (Fitzgerald, 1:500), anti-PAX2 (Zymed, 1:25), anti-PCNA (Santa Cruz, 1:50), anti-podocin (1:50) and anti-NFAT-synaptopodin (gifts from P. Mundel, 1:200) and anti-WT1 (Dako, 1:50). Secondary antibodies were fluorescent-tagged donkey Cy2 and Cy3 (Jackson ImmunoResearch Laboratories, Pennsylvania, USA). Confocal images were obtained (Olympus Fluoview 300/ Leica AOBBS). For negative controls, primary antibodies were omitted.

**Quantitative real-time PCR (qPCR)**

RNA was isolated from whole kidney tissue using Trizol Reagent (Invitrogen) as per the manufacturer’s instructions or from isolated glomeruli using RNeasy Minikit (Qiagen). 1 μg of isolated RNA from each animal was used to generate cDNA (Qiagen Quantitect kit). PCR reactions used individually isolated glomerular cDNA or pooled cDNA [Sema3a−/− and Sema3a+/−, n = 7 mice per group; podocin-rtTA: tet-O-Sema3a+ newborns (−dox (doxycycline) and +dox, n = 6 mice per group), and amplification was performed using Applied Biosystems SYBR-Green Mastermix with an Eppendorf Realplex Mastercycler. PCR primers were designed with Primer Express software and were as follows [the melting temperature (Tm) of each primer pair is shown in parentheses]:

- **Sema3a** (60°C), 5′-GATGTTGCTCATGCCCTAC-3′ (forward) and 5′-TGTTGCTGCAAGTCAGAGCAG-3′ (reverse);
- **Podocin** (58°C), 5′-TGCTCAACTCTCCCTGAG-3′ (forward) and 5′-ACCACCTGCAGAAATTCGG-3′ (reverse);
- **Nephrin** (59°C), 5′-GACATGGGGTCGTCGTC-3′ (forward) and 5′-GGATGTCCTTGGATGGAAGG-3′ (reverse);
- **GAPDH** (58°C), 5′-GGATGTCGCTTGATGGA-3′ (forward) and 5′-GATGTTGGCTCCCTATGAAAC-3′ (reverse);
- **VWF** (57°C), 5′-AGGTTGCTCTCTTTGCCCTCA-3′ (forward) and 5′-AGGGCCCACACTAAAAGT-3′ (reverse);
- **Sema3f** (60°C), 5′-AGGGCCCAGAATGCTGCGG-3′ (forward) and 5′-CATCAAGGTGATCTTCTGATGG-3′ (reverse) and 5′-ACTCAGGAGTCTTCTGGACTC-3′ (reverse);
- **Sema3g** (60°C), 5′-ACTTCACCACCTGGGGAGT-3′ (forward) and 5′-GCCCAAAGCAGACCCGG-3′ (reverse);
- **Gapdh** (58-60°C), 5′-GATGTTGGCTCCTGGTCTTC-3′ (forward) and 5′-GCCGGCTGCTCCACCTCTCT-3′ (reverse);
- **Actin** (60°C), 5′-GCCCATGTTTACCAAGCCAGG-3′ (forward) and 5′-GTCCTTTTATACGTGTTGAGAA-3′ (reverse).

Reactions were run in duplicate and each experiment was repeated three times. Gene expression relative to housekeeping genes *Gapdh* or ubiquitin was determined with the 2−ΔΔCt method (Schmittgen and Livak, 2008).
Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL)

Apoptotic nuclei were identified using In Situ Cell Death Detection Kits (Chemicon) following the manufacturer’s protocol. Detection was performed by either immunoperoxidase-DAB, counterstained with Periodic Acid Schiff (PAS), or fluorescein-conjugated anti-digoxigenin, followed by application of rhodamine-labeled Griffonia simplicifolia Lectin I (Vector Labs; 1:150) to label endothelial cells (Laitinen, 1987). Apoptotic cells were counted in each glomerulus of the kidney sections (n=4-5 mice per group, mean=25 glomeruli per mouse).

Transmission electron microscopy (TEM)

For TEM, the kidney cortex was fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, post-fixed with 1% osmium tetroxide followed by 1% Uranyl Acetate, dehydrated and embedded in LX112 resin (LADD Research Industries, Burlington, VT, USA). Ultrathin (80 nm) sections were cut on a Reichert Ultracut UCT, stained with Uranyl Acetate followed by Lead Citrate and viewed on a JEOL 1200EX transmission electron microscope at 80 kv.

Histologic and TEM morphometric analysis

Kidney sections were stained with PAS solution and glomerular volume was determined by measuring the diameters of all glomeruli within three sections. Glomerular volume was calculated as G=β/k–(r–r2)3/2, where β=1.38 is the shape coefficient for spheres, k=1.1 is the size distribution coefficient and (r–r2) is the glomerular area (Hirose et al., 1982). To determine glomerular endothelial cell number in Sema3a mutant mice, endothelial cell nuclei were identified in 1-3 capillary loops per mouse on 5000× TEM images of Sema3a+/+ and Sema3a–/– mice. Image J software (NIH) was used to determine the number of cell-cell interactions per μm of GBM (pore density) and foot process width, and to quantify WT1 and CD31 images (n=3 kidneys per group, 15-40 glomeruli per section). The total GBM length examined per group was: Sema3a+/+, 58,064 nm; Sema3a–/–, 117,842 nm; newborn podocin-rtTA: tet-O-Sema3a–/– mice (+dox), 156,312 nm; newborn podocin-rtTA: tet-O-Sema3a–/– mice (+dox), 95,477 nm; newborn Sema3a–/– mice (–dox), 117,842 nm; newborn Sema3a–/– mice (–dox), 156,312 nm; newborn Sema3a–/– mice (+dox), 167,516 nm.

Statistical analysis

Student’s unpaired t-test was used to compare groups. P<0.05 was deemed statistically significant.

RESULTS

Sema3a loss-of-function results in renal vascular defects

To define the effect of Sema3a deletion on glomerular development we compared kidneys from newborn Sema3a+/+ and Sema3a–/– mice histologically and by TEM. Light microscopy examination of Sema3a+/+ glomeruli revealed poor capillary lumen development and increased nuclei within the loops, whereas Sema3a–/– glomeruli had normal wide-open capillary loops (Fig. 1A). On examination with TEM, Sema3a–/– mice had open glomerular capillaries with fenestrated endothelium, podocyte foot processes linked by SDs and a normal GBM (Fig. 1B). By contrast, TEM revealed significant abnormalities in Sema3a+/+ glomeruli. There was no visible capillary lumen in Sema3a+/+ glomeruli, even in the most mature glomeruli, and multiple endothelial cells were visualized within the capillary loops (Fig. 1B). Fenestrae were absent in these abnormal endothelial cells. Morphometric analysis of TEM images showed that Sema3a+/+ glomeruli had twice the number of endothelial cells per capillary loop as compared with Sema3a–/– glomeruli (Fig. 1C). The GBM appeared morphologically normal in Sema3a+/+ newborn mice. However, many podocyte foot processes were extremely wide and linked by OJs rather than SDs (Fig. 1B). Quantification of these abnormalities demonstrated a broad variation in podocyte foot process width in Sema3a+/+ glomeruli, with some foot processes 30-fold wider than the average width of Sema3a–/– foot processes, whereas others were only 1.5-fold wider or normal (Fig. 1B,C). Remarkably, the morphologic abnormalities in the glomerular endothelium and foot process development of Sema3a–/– mice were associated with impaired glomerular filtration barrier function as evidenced by albuminuria on immunoblotting (Fig. 1D).

The defects in glomerular capillary formation suggested the possibility of more-widespread patterning defects. To define the vascular phenotype resulting from Sema3a gene deletion, we bred Sema3a–/– mice (Behar et al., 1996) with Flk1-lacZ–/– knock-in mice (Shalaby et al., 1995), and generated double-heterozygotes Flk1-lacZ+/–: Sema3a–/– (see Table S1 in the supplementary material).

Fig. 1. Sema3a deletion results in excess glomerular endothelial cells and podocyte foot process effacement. (A) PAS-stained wild-type glomerulus (+/+ with open capillaries; Sema3a-null (–/–) glomerulus shows multiple blue nuclei in the capillary loops and few open lumina. (B) TEM: newborn Sema3a+/+ (3A+/+) glomeruli demonstrate intact foot processes (fp), slit diaphragms (SDs), GBM and endothelial cells (EC). Sema3a+/+ (3A+/+) glomeruli show multiple EC within the capillary loop, wide foot processes (efp: effaced foot process, red arrowheads) joined by occluding-junctions (OJ), P, podocyte; Cap, capillary lumen. (C) TEM morphometric analysis: the number of EC per capillary loop is two-fold higher, the foot processes are seven-fold wider, and the SD density is five-fold lower in Sema3a–/– glomeruli versus wild-type glomeruli (n=8 mice per group, P=0.0002). *, P<0.05. (D) Western blot showing albuminuria in Sema3a–/– newborn mice. Scale bars: in A, 20 μm; in B, 1 μm for upper panel, 200 nm in lower panel.
Kidneys from the newborn progeny of Fk1-lacZ+/–: Sema3a+/– were stained for lacZ to examine renal endothelial cell patterning (Fig. 2A). Fk1-lacZ+/–: Sema3a+/– kidneys exhibited increased Fk1-positive cells throughout in a disorganized pattern, indicating an abnormal number and distribution of endothelial cells with loss of Sema3a function (Fig. 2A). To confirm the vascular phenotype, endothelial cells were immunostained with anti-CD31 antibody. Sema3a–/+ kidneys had a 50% increase in cortical CD31-positive immunofluorescence as compared with Sema3a+/+ kidneys, confirming a global excess of renal endothelial cells in Sema3a–/+ mice (Fig. 2B,C). Congruent with the findings of increased renal endothelial cells on TEM, the CD31-positive area within Sema3a–/+ glomeruli was 38% larger than the CD31-positive area within Sema3a+/+ glomeruli (Fig. 2D).

Excess glomerular endothelial cells could be the result of increased endothelial cell proliferation, decreased endothelial cell apoptosis or increased endothelial cell migration. Dual-label immunostaining with anti-CD31 and anti-PCNA antibodies was performed to identify endothelial and proliferating cells, respectively. PCNA staining in both Sema3a–/+ and Sema3a+/– newborn kidneys localized predominantly to tubules and not in glomeruli. Proliferating endothelial cells were rarely observed within glomeruli and the nephrogenic cortex and occurred in less than 1% of the total cortical area (Fig. 2B), suggesting that the majority of endothelial cells were not proliferating in newborn Sema3a+/– kidneys.

Previous in vitro studies indicated that Sema3a negatively regulates cell survival (Bagnard et al., 2001; Guan et al., 2006; Guttmann-Raviv et al., 2007; Moretti et al., 2008). Apoptotic cells, identified by either TUNEL or immunodetection of cleaved caspase 3, were scarce in newborn glomeruli (Fig. 3A,B). Although no difference in overall apoptotic rate could be detected between Sema3a+/– and Sema3a+/+ glomeruli (Fig. 3A,B). Co-localization of TUNEL-positive cells and endothelial cells labeled by Griffonia simplicifolia Lectin (Laitinen, 1987) demonstrated a five-fold decrease in glomerular endothelial apoptotic rate in Sema3a mutants (0.05±0.02 vs 0.01±0.01 endothelial apoptotic cells per glomeruli, in Sema3a+/+ and Sema3a+/–, respectively, P<0.05). This suggests that decreased endothelial apoptosis contributes to the excess endothelial cells observed in Sema3a+/– kidneys. Next, we examined Sema3a effects on glomerular endothelial cell migration using in vitro assays (Tufro, 2000). Recombinant SEMA3A decreased glomerular endothelial cell migration across membranes and towards embryonic kidneys (Fig. 3C-E; also see Fig. S1 in the supplementary material), suggesting that Sema3a acts as a chemorepellant and impairs glomerular endothelial cell migratory response to chemotaxtactant stimuli. Collectively, in vivo and in vitro data suggest that loss of Sema3a promotes endothelial cell survival and migration, respectively, resulting in an excess of renal endothelial cells and renal vascular patterning defects.

Sema3a and its receptors are expressed in macrophages and induce apoptosis (Ji et al., 2009). Thus, we examined whether Sema3a loss-of-function altered macrophage number in the kidney using F4/80 IHC. No F4/80-positive cells were identified in glomeruli of either Sema3a–/– or Sema3a+/– kidneys (see Fig. S2 in the supplementary material). Interstitial macrophages were present, but F4/80-positive cell counts in Sema3a–/– and Sema3a+/+ kidneys were similar [10.3±0.7 vs 10.3±0.7 F4/80-positive cells per 400× field, in Sema3a–/– and Sema3a+/+, respectively, P not significant (ns)].

**Sema3a deletion alters PAX2 expression in the developing kidney**

We next examined the expression levels of VEGFA and its receptors VEGFR2 and neuropilin 1 in Sema3a+/– mice (Fig. 4). Wild-type and Sema3a+/– kidneys had similar levels of expression of both VEGFA and its receptors, suggesting that the renal vascular phenotype occurring upon Sema3a deletion was not due to upregulation of VEGFA but rather to an imbalance between VEGFA and Sema3a signaling pathways. To explore further the abnormalities underlying the glomerular phenotype observed in Sema3a mutant mice, we examined the expression of podocyte-specific proteins and transcription factors relevant to podocyte differentiation. We determined that nephrin, podocin and WT1 expression was unchanged, whereas PAX2 expression was decreased by 30% in Sema3a+/– mice (Fig. 4). Localization of WT1, synaptopodin and PAX2 by immunofluorescence was not altered in Sema3a+/– kidneys (Fig. 3B, also see Fig. S3A in the supplementary material).
Sema3a gain-of-function during nephrogenesis impairs podocyte differentiation

To define further the function of Sema3a, we examined the effects of podocyte Sema3a overexpression during kidney organogenesis in vivo using an inducible tetracycline-regulated model (Tet-On). Tet-O-Sema3a transgenic mice were generated as described in the Material and methods and were bred with podocin-rtTA mice (Shigehara et al., 2003). Uninduced podocin-rtTA: tet-O-Sema3a mice have normal life spans, are fertile and have normal size litters. Following a one-week induction with doxycycline, adult podocin-rtTA: tet-O-Sema3a mice exhibited a four-fold higher Sema3a mRNA level in isolated glomeruli as compared with controls (Fig. 5A). Controls included single transgenic mice on doxycycline and uninduced podocin-rtTA: tet-O-Sema3a mice. Podocin-rtTA: tet-O-Sema3a mice were induced from E12 until either birth or two weeks after birth, at completion of nephrogenesis (see Table S1 in the supplementary material). Controls were single transgenic littermates and age-matched uninduced podocin-rtTA: tet-O-Sema3a mice. Mice overexpressing Sema3a during kidney organogenesis exhibited smaller glomeruli at birth, with a 30% decrease in mean glomerular volume (Fig. 5B,C). On examination with TEM, glomeruli from control newborns exhibited fully differentiated podocyte foot processes linked by SDs, a fenestrated endothelium and a normal newborn GBM (Fig. 5D). In contrast, glomeruli from Sema3a-overexpressing mice had immature, cuboidal podocytes that lacked differentiated foot processes (Fig. 5D) and extended flat and wide processes (~six-fold wider than those of uninduced controls; Fig. 5E). In contrast to the variable podocyte abnormalities observed in Sema3a−/− glomeruli, the podocyte processes of glomeruli from Sema3a-overexpressing mice were uniformly flat and extremely wide. Their immaturity was further demonstrated by the complete absence of SDs, with adjacent processes being solely joined by OJs (Fig. 5E). There was a decrease in total cell-cell interaction density (pore density), as the number of OJs did not compensate for the lack of SDs (Fig. 5E). In addition, glomerular endothelial cells of newborn Sema3a-overexpressing...
mice were swollen and vacuolated, with decreased fenestrations, whereas the GBM appeared morphologically similar to that seen in control newborns (Fig. 5D).

The absence of foot processes and SDs observed in newborn Sema3a-overexpressing mice could represent Sema3a-mediated impairment of foot process and SD formation or a developmental delay. Hence, we examined Sema3a-overexpressing mice at completion of nephrogenesis. Two-week-old control glomeruli exhibited normal podocyte foot processes with SDs, GBM and fenestrated endothelium as described in the newborn (Fig. 6A). By contrast, TEM images from two-week-old Sema3a-overexpressing glomeruli revealed incompletely differentiated and abnormal podocyte foot processes. Foot processes from two-week-old Sema3a-overexpressing kidneys were flat and 50% wider than age-matched controls (Fig. 6A,B). Strikingly, these processes failed to form SDs and were joined by OJs (Fig. 6A). Glomerular endothelial cells remained swollen and had decreased fenestration, whereas the GBM was morphologically normal (Fig. 6A).

The dramatic defects in podocyte differentiation and endothelial cell damage induced by podocyte Sema3a gain-of-function during organogenesis were associated with altered glomerular permeability. Albuminuria was evident on immunoblotting both in newborn (Fig. 5F) and two-week-old (Fig. 6C) Sema3a-overexpressing mice. Collectively, these
findings indicate that podocyte Sema3a plays a crucial role in podocyte differentiation and SD formation during glomerular development.

To investigate the molecular mechanism underlying the undifferentiated podocyte phenotype, we next examined expression of SD proteins. Nephrin expression decreased to half of normal levels in Sema3a-overexpressing kidneys, as determined by immunoblotting and confirmed by immunofluorescence (Fig. 7A,B). By contrast, podocin expression was unchanged, suggesting that the decrease in nephrin expression was not due to loss of podocytes (Fig. 7A,B). The expression level of the adaptor protein, CD2AP, was also not altered by Sema3a overexpression (Fig. 7A). Furthermore, Sema3a-overexpressing mice had a greater than 50% decrease in WT1 expression in comparison with controls (Fig. 7A). A decrease in the intensity of the nuclear WT1 staining in Sema3a-overexpressing glomeruli as compared with controls was also observed, although the podocyte cell count was unchanged (Fig. 8D,E).

Synaptopodin immunostaining demonstrated similar patterns of expression in Sema3a-overexpressing and control kidneys, as reported in another model of abnormal podocyte differentiation (Miner et al., 2002) (see Fig. S3 in the supplementary material). Thus, despite their morphologic immaturity as observed on TEM, Sema3a-overexpressing podocytes were capable of expressing cytoskeletal, but not SD markers characteristic of mature podocytes.

Fig. 6. Podocyte foot process development is delayed and abnormal in mice overexpressing podocyte Sema3a. (A,B) TEM: Sema3a-overexpressing (+dox) glomeruli at completion of nephrogenesis (2 weeks) have wide podocyte foot processes (arrowheads) and absent slit diaphragms; a morphometric analysis is shown in B. Note that the endothelial cells (EC) are swollen (double-headed arrow). Controls (−dox) exhibit intact podocyte foot processes (fp), normal GBM and fenestrated endothelium. Cap: capillary lumen. (C) Urine albumin immunoblot demonstrates albuminuria in 2-week-old induced Sema3a-overexpressing mice (+dox), which is absent in controls (−dox). Scale bars: 1 μm.

Fig. 7. Podocyte Sema3a overexpression downregulates nephrin, WT1 and VEGFR2. (A) Immunoblots showing nephrin, WT1 and VEGFR2 downregulation in Sema3a-overexpressing mice (+dox E12 to 2 weeks of age). Representative blots and densitometric analysis of three separate experiments are shown; data are expressed as fold change ± s.e.m. after correction for actin; +dox vs −dox were compared using pooled samples of n=13-20 mice per group; *, P<0.05. (B) Immunostaining demonstrating that nephrin expression is decreased in newborn Sema3a-overexpressing glomeruli (+dox), whereas podocin expression is unchanged. (C) Proposed model of the Sema3a effect on PAX2 and WT1 signaling. Previously described pathways are shown in blue: PAX2 is capable of either upregulation or repression of WT1 expression, depending upon the absence or presence, respectively, of the transcriptional co-repressor groucho TLE4. WT1 stimulates nephrin expression, and it downregulates PAX2 through a negative-feedback loop. The effects of Sema3a on these pathways are shown in red: Sema3a negatively regulates WT1 and nephrin and might positively regulate PAX2. Therefore, upon Sema3a deletion, PAX2 expression is downregulated, and, upon Sema3a overexpression, WT1 and nephrin expression is decreased.
The localization and expression level of the immature nephron transcription factor PAX2, known to downregulate WT1 and nephrin (Cai et al., 2003; Wagner et al., 2006), was normal in Sema3a-overexpressing kidneys (Fig 7A; also see Fig. S3 in the supplementary material). Together, these data suggest that the phenotype observed in Sema3a-overexpressing kidneys is not secondary to podocyte loss and that excess Sema3a regulates podocyte differentiation downstream of PAX2 by downregulating WT1 and nephrin (Fig. 7C).

Podocyte Sema3a overexpression during organogenesis did not alter renal VEGFA and neuropilin 1 expression levels (Fig. 7A). By contrast, Sema3a-overexpressing mice demonstrated a 75% reduction in expression of renal VEGFR2 as compared with controls (Fig. 7A), suggesting that the effects on podocyte phenotype might in part be mediated by decreased VEGF signaling.

Sema3a is a negative regulator of glomerular endothelial and epithelial cell survival
Sema3a negatively regulates cell survival in vitro (Guan et al., 2006; Guttmann-Raviv et al., 2007). Thus, we examined if Sema3a overexpression induced apoptosis in vivo by TUNEL assay. Newborn Sema3a-overexpressing glomeruli exhibited an 8-fold increase in apoptotic cells as compared with controls (Fig. 8A,B).

The vast majority (95%) of TUNEL-positive nuclei in Sema3a-overexpressing glomeruli (+dox) versus virtually none in controls (–dox), *P<0.05. (C,D) Identification of apoptotic cell type is shown by TUNEL assay and EC labeling (C) or by activated-caspase-3 immunofluorescence and podocyte labeling (D); nuclei are labeled with DAPI (blue). (C) In Sema3a-overexpressing glomeruli (+dox), apoptotic nuclei (green) co-localize with Griffonia simplicifolia Lectin I-labeled endothelium (red), indicating endothelial cell apoptosis (yellow in merged images; insets show apoptotic nuclei at a higher magnification). Two representative glomeruli are shown. (D) WT1 (red) and activated caspase 3 (green) immunostaining. Control glomeruli (–dox) show WT1-positive podocytes and no apoptosis (negative for activated caspase 3). Two representative glomeruli from Sema3a-overexpressing mice (+dox) are shown. In most glomeruli, apoptotic nuclei are WT1-negative (top inset), indicating that the apoptotic cells are not podocytes. Only 10% of apoptotic cells were deemed to be podocytes (WT1-positive and caspase-3-positive, shown in yellow, bottom inset). (E) Podocyte number quantification (WT1-positive nuclei per mm2 glomerular area) showing similar podocyte numbers in controls (white bar) and Sema3a-overexpressing mice (gray bar). n=40-100 glomeruli per group; P=ns. Scale bars: in C, 5 μm; in D, 10 μm.
A small number of podocytes in Sema3a-overexpressing mice were also undergoing apoptosis, but this did not result in net podocyte loss (Fig. 8E).

**Compensatory changes in class 3 semaphorin gene expression**

Redundant functions among class 3 semaphorins have been reported in multiple cell types (Guttmann-Raviv et al., 2007). Thus, we asked whether expression levels of other class 3 semaphorins are altered in developing Sema3a–/– kidneys. Real-time PCR showed a two-fold increase in Sema3e mRNA expression in Sema3a–/– as compared with Sema3a+/+ kidneys, whereas expression of Sema3b, 3c, 3d and 3g was unchanged (see Fig. S4 in the supplementary material). Sema3a was downregulated to minimal levels in Sema3a–/– kidneys, as expected (see Fig. S4 in the supplementary material). By contrast, podocyte Sema3a overexpression resulted in a 2.8-fold upregulation of Sema3b expression, and no change in total kidney Sema3a, 3c, 3d, 3e or 3g was identified as compared with controls (see Fig. S4 in the supplementary material).

**DISCUSSION**

Sema3a loss- and gain-of-function studies in mice demonstrated that a tightly regulated Sema3a gene dosage is necessary for normal glomerular development and glomerular filtration barrier function.

**Sema3a acts as a negative regulator of endothelial cell survival and migration during kidney organogenesis**

Sema3a deletion increased renal endothelial cell number and altered renal vascular patterning. The glomerular endothelial cell increase resulted from reduced apoptosis. Sema3a inhibited endothelial cell migration in migration assays, consistent with previous reports (Guttmann-Raviv et al., 2007; Miao et al., 1999; Serini et al., 2003). Glomerular capillary development involves both vasculogenesis and angiogenesis, and podocyte VEGFA provides chemo-attractive signals that direct glomerular endothelial cell migration (Abrahamson et al., 1998; Eremina et al., 2003; Tufro-McReddie et al., 1997; Tufro, 2000; Tufro et al., 1999). Sema3a deletion did not alter kidney VEGFA, neuropilin 1 or VEGFR2, suggesting that Sema3a does not regulate their expression level. Sema3a is known to compete with VEGFA for binding to neuropilin 1 and probably acts as a gatekeeper for endothelial cells (Bagnard et al., 2001; Miao et al., 1999). Hence, in the Sema3a-null kidneys, unopposed VEGFA signaling might have contributed to the abnormalities of vascular patterning.

Neuropilin-1-deficient mice phenocopy Sema3a–/– mutants and exhibit lethal defects in neural and cardiovascular patterning; conversely, overexpression of neuropilin 1 results in embryonic lethality owing to an excess of capillary formation (Kawasaki et al., 1999; Kitsukawa et al., 1995). However, recently the role of Sema3a in vascular development has been called into question by reports of no obvious large vessel defects in mice expressing a mutant neuropilin 1 incapable of binding to Sema3a, and a recent study that failed to replicate vascular defects described in Sema3a–/– mice (Gu et al., 2003; Vieira et al., 2007). Notably, these studies did not examine the kidney vasculature.

Predictably, newborn mice overexpressing podocyte Sema3a did not have abnormal capillary development, given that the gain-of-function occurred after E14.5 when most of the vascular patterning had already been established. Endothelial cell apoptosis was induced by podocyte Sema3a gain-of-function, as revealed by TEM and TUNEL analysis, indicating that Sema3a participates in the crosstalk between these cell types by a non-cell-autonomous mechanism.

Increased glomerular endothelial cell apoptosis was reported in mice overexpressing podocyte angiopoietin 2, associated with VEGFA downregulation (Davis et al., 2007). Taken together, our data suggest that Sema3a negatively regulates endothelial cell survival during glomerular development and that a tight regulation of Sema3a dosage is necessary for normal glomerular vascularization.

**Sema3a regulates podocyte differentiation**

Remarkably, Sema3a deletion results in abnormal podocyte morphology despite normal expression levels of SD proteins. This suggests that the podocyte phenotype in Sema3a–/– mice might be secondary to aberrant crosstalk between the podocytes and endothelial cells, as has been proposed in other models of endothelial injury (Eremina et al., 2003). However, given that podocyte development occurs in metanephric cultures in the absence of glomerular capillary formation, and that Sema3a receptors are present in podocytes (Avner et al., 1988; Guan et al., 2006; Harper et al., 2001), the abnormal podocyte structure might also represent a Sema3a cell-autonomous requirement for normal podocyte differentiation. Interestingly, transcription factor Pax2 was downregulated in Sema3a–/– newborn mice. Deletion of the Pax2 gene results in complete renal agenesis and prevents mesenchymal-epithelial transition (Torres et al., 1995), and, although the degree of downregulation was mild and was not sufficient to impair nephron induction, it suggests that, during kidney development, Sema3a signaling might impact a fundamental pathway required for epithelial differentiation.

Gain-of-function studies provided strong evidence for a role of Sema3a in podocyte differentiation. The complete absence of SDs and the congenital proteinuria observed in mice overexpressing Sema3a in podocytes are reminiscent of mice deficient in nephrin and the clinical correlate congenital nephrotic syndrome (Kestila et al., 1998; Putaala et al., 2001). Moreover, the undifferentiated podocyte morphology in mice overexpressing Sema3a is associated with downregulation of WT1, nephrin and VEGFR2, indicating that Sema3a signaling impacts major podocyte signaling pathways and structural proteins (Done et al., 2008). Dominant-negative and loss-of-function mutations in WT1 have been identified in infantile nephrotic syndrome associated with Denys-Drash and Frasier syndromes (Little et al., 1995). WT1 knockout mice exhibited downregulation of nephrin and diffuse mesangial sclerosis, similar to patients with Denys-Drash, indicating that the transcription factor WT1 is a regulator of nephrin expression and is required for proper podocyte differentiation (Guo et al., 2002). Together, the data suggest that the undifferentiated podocyte phenotype observed in Sema3a-overexpressing mice is a consequence of the downregulation of WT1 and nephrin. Downregulation of WT1 and nephrin, in association with glomerular hypoplasia and proteinuria, was also noted in mice with inappropriate Pax2 expression postnatally (Wagner et al., 2006); however, no change in Pax2 expression was found in mice overexpressing Sema3a. Pax2 signaling is complex: Pax2 can activate WT1 expression (Dehbi et al., 1996; McConnell et al., 1997), but, in the presence of the transcriptional co-repressor groucho TLE4, the opposite occurs, and WT1 expression is repressed (Cai et al., 2003; Wagner et al., 2006). WT1, in turn, is a positive regulator of nephrin expression (Guo et al., 2004; Wagner et al., 2004) and a negative regulator of Pax2 through a feedback loop (Ryan et al., 1995). Collectively, downregulation of WT1 and nephrin by Sema3a overexpression, and downregulation of Pax2 in Sema3a–/– mice, suggest that Sema3a might function as a modifier of Pax2 and WT1 signaling during podocyte differentiation (Fig. 7C). Thus, Sema3a may act as
a negative regulator of podocyte differentiation pathways by inducing downregulation of WT1 and nephrin. The differences in gene expression seen in Sem3a deletion and overexpression models are remarkable in light of the paradoxical similarity of the morphologic podocyte changes. Epithelial cells are known to occasionally exhibit similar phenotypes upon gene knock-in and knockdown (Rogers et al., 2003); therefore, podocyte effacement might represent a final common pathway that occurs with disruption of podocyte differentiation.

The podocyte Sem3a overexpression phenotype strikingly resembles the Lmxb gene deletion phenotype. Lmxb is mutated in nail-patella syndrome, and gene deletion in mice results in cuboidal podocytes that lack foot processes and SDs, as well as glomerular hypoplasia with impaired glomerular capillary development (Miner et al., 2002; Rohr et al., 2002). Distinct differences between the two mouse models are the downregulation of WT1 and nephrin and lack of GBM phenotype in the newborn mice overexpressing podocyte Sem3a, whereas in Lmxb knockouts, expression of these two podocyte proteins was not affected, podocin and VEGFA were downregulated and the GBM was abnormally expanded (Rohr et al., 2002). Dysregulation of Sem3a in newborn mice did not induce morphologic abnormalities in the GBM; however, defective GBM function and podocyte effacement might have contributed to the abnormal glomerular permeability observed in both models. The Sem3a-induced downregulation of VEGFR2 reported here could result in decreased VEGFA signaling, as was previously shown in cultured podocytes and in mice following systemic Sem3a administration (Guan et al., 2006; Tapia et al., 2008). Podocyte Sem3a overexpression induced mild podocyte apoptosis that did not alter podocyte number. This effect is consistent with, but of a lesser degree than, results previously described in vitro, and was likely attributable to a dose effect (Guan et al., 2006).

**Semaphorin compensation**

We identified Sem3a upregulation in Sem3a+/- mice. SEMA3E uniquely signals through plexin D1 (PLXND1) independently of neuropilins, and mutations in Sem3a are associated with CHARGE syndrome (Gu et al., 2005). Sem3a and Plxnd1 gene deletion studies indicate that the ligand-receptor pair functions to restrict vessel growth and branching. Given the variability of vascular phenotypes of different strains of Sem3a+/- mice (Behar et al., 1996; Serini et al., 2003; Vieira et al., 2007), upregulation of Sem3a could provide an interesting mechanism by which compensation might occur in some Sem3a+/- mice.

In Sem3a-overexpressing mice, Sem3b was upregulated. SEMA3B binds to neuropilin 2 preferentially, antagonizes Sem3a-neuropilin-1 function on neurons, has been identified as an inhibitor of the AKT pathway and a tumor suppressor gene with loss of heterozygosity in lung, kidney and ovarian cancers (Castro-Rivera et al., 2004). The upregulation of Sem3b in response to Sem3a overexpression suggests a possible role for Sem3b in podocytes.

Semaphorins have been proposed as modulators of epithelial morphogenesis (Hinck, 2004), but no prior studies examined their role in glomerular development. The findings reported here provide in vivo evidence that Sem3a signaling is required for the regulation of renal vascular development and plays a crucial role in podocyte differentiation. Further study is warranted to determine the molecular mechanisms by which Sem3a interacts with Pax2, nephrin, Wt1 and Vegfr2 genes.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/22/3979/DC1

**References**


