Semaphorin3a disrupts podocyte foot processes causing acute proteinuria

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Semaphorin3a was discovered as a secreted guidance protein that acts as a chemorepellent to migrating axons and endothelial cells. In the adult mouse kidney, it is expressed in podocytes and collecting tubules. Here, we show that exogenous semaphorin3a caused acute nephrotic range proteinuria associated with podocyte foot process effacement and fusion, endothelial cell damage, decreased vascular endothelial growth factor-A receptor expression, and downregulation of the slit-diaphragm proteins podocin, nephrin, and CD2-associated protein. When vascular endothelial growth factor 165 was administered at the same time as Semaphorin3a, no proteinuria or renal ultrastructural abnormalities occurred, suggesting that semaphorin3a effects may be mediated, in part, by downregulation of vascular endothelial growth factor receptor 2 signaling. Our findings indicate that a balance of semaphorin3a to vascular endothelial growth factor-A may be important for glomerular filtration barrier homeostasis.

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Class 3 semaphorins are a family of secreted guidance proteins.[1,2] Semaphorin3a (*Sema3a*) plays an important role in neural and cardiovascular patterning.[3] *Sema3a* generates chemorepellent cues by inducing growth cone collapse and inhibiting endothelial and neural crest cell migration.[4–7] Semaphorin3a (SEMA3A) binds to neuropilins 1 and 2, also known as co-receptors for the vascular endothelial growth factor-A (VEGF-A) isoform $VEGF_{165}$, and signals through plexins A_{1-3} and D_1.[4,8,9] SEMA3A signaling decreases integrin function and interferes with the actin cytoskeleton.[7,10,11] Semaphorins and their receptors have been detected in endothelial cells, neurons, podocytes, and tubular epithelial cells *in vivo* and *in vitro*.[10,12–16]

Deletion of the *Sema3a* gene in mice causes perinatal lethality associated with atrial dilatation, septum defects, and vascular patterning abnormalities.[10,17] Plexin D_1 disruption in mice and zebrafish induces cardiovascular malformations consistent with *Sema3a*, *Sema3c*, and $VEGF_{165}$ null phenotypes, suggesting that class 3 semaphorins and VEGF-A have coordinated functions in cardiovascular patterning.[3,12,13]

SEMA3A expression in the kidney is developmentally regulated. Initially detected in all epithelial cells, *Sema3a* mRNA transcripts localize to podocytes and collecting tubules in the mature organ.[15] In cultured podocytes, SEMA3A signaling regulates the expression and interactions of slit-diaphragm proteins and decreases podocyte survival.[16] However, the function of SEMA3A *in vivo* remains unknown.

We hypothesized that SEMA3A counterbalances VEGF-A functions in the podocyte and that together they regulate slit-diaphragm homeostasis. Hence, excess or lack of either protein could cause glomerular filtration barrier permeability abnormalities and proteinuria. Excess and neutralization of VEGF-A have been shown to result in proteinuria.[18–23] The potential role of SEMA3A in the pathogenesis of proteinuria has not been explored. Here, we asked whether excess circulating SEMA3A causes proteinuria and renal damage. We report that remarkably, systemic SEMA3A injection into healthy mice causes acute and transient massive proteinuria, podocyte foot process effacement, endothelial cell damage, and downregulation of slit-diaphragm proteins as well as downregulation of the signaling VEGF-A receptor 2 (VEGFR2). These abnormalities are abrogated by co-administration of SEMA3A and VEGF-A.

RESULTS

To examine the effect of systemic SEMA3A on glomerular filtration barrier function, we infused recombinant SEMA3A intraperitoneally in adult healthy mice. SEMA3A administration induced nephrotic range proteinuria within 4 h, whereas injection of denatured SEMA3A, vehicle, or an irrelevant protein did not do so (Figure 1a). SEMA3A-induced proteinuria resolved by 24 h (Figure 1a). At the end of the experiment (4–48 h), kidneys were harvested, processed, and examined by light and electron microscopy. No histological abnormalities were detected by light microscopy in SEMA3A-treated or control kidneys (data not shown). Kidneys from mice injected with vehicle or denatured SEMA3A (controls) were indistinguishable and showed normal podocyte, glomerular basement membrane, and endothelial cell ultrastructure (Figure 1b and c). Transmission electron microscopy revealed extensive fusion and effacement of podocyte foot processes in all kidneys examined 4 h after SEMA3A infusion (Figure 1d–g). In the fused foot processes, slit diaphragms were replaced by occludens junction-like structures (Figure 1f). Patchy similar lesions were observed in most of the mice (4/5) examined at

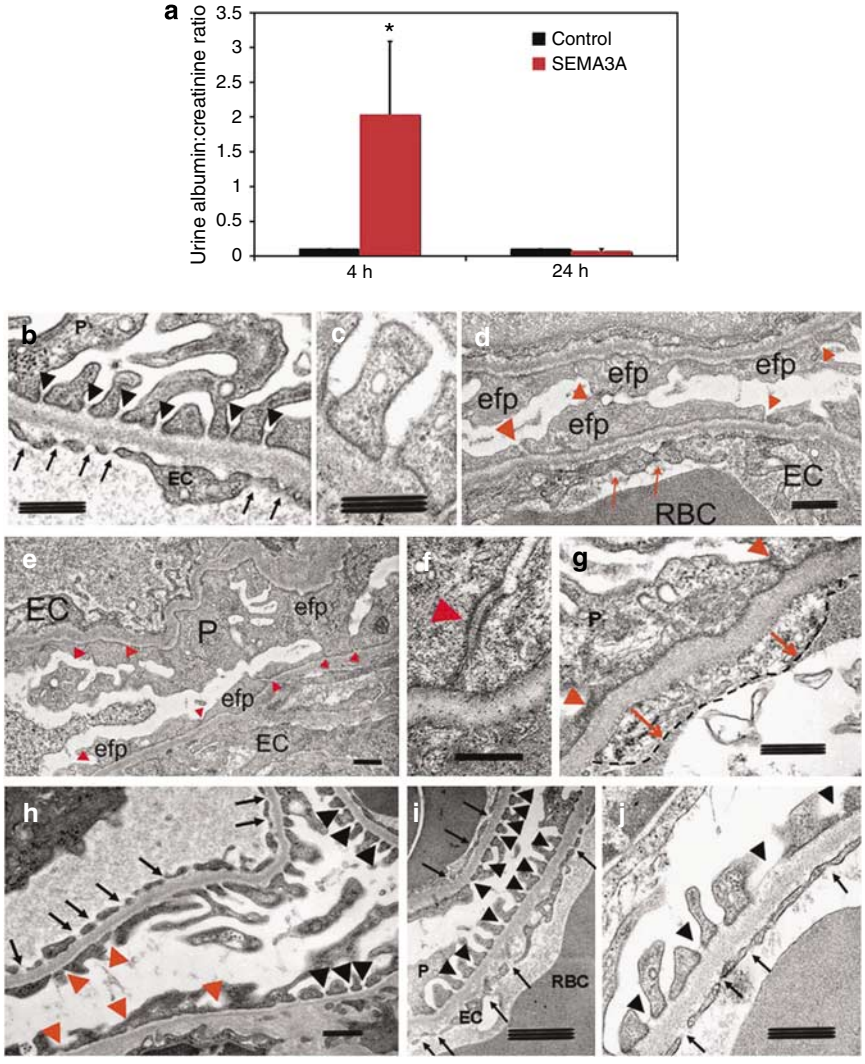

Figure 1 | SEMA3A induced massive transient proteinuria via podocyte foot process effacement and fusion and endothelial cell damage. (**a**) Quantification of proteinuria 4 and 24 h after SEMA3A, vehicle, or denatured sema 3A injection (control); data are expressed as mean + s.e.m. Albumin:creatinine ratio in the urine. (**b**–**j**) Transmission electron microscopy images: (**b**) control kidney 4 h after vehicle injection showing normal foot processes separated by slit diaphragms (black triangles), normal GBM, and an endothelial cell with normal fenestrations (black arrows); (**c**) control kidney showing two foot processes linked by a slit diaphragm at higher magnification. (**d**–**g**) SEMA3A-treated kidneys 4 h after SEMA3A injection: (**d**) effacement and fusion of foot processes (red triangles), endothelial cell swelling, and lack of fenestrations (single-ended red arrows); (**e**) extensive foot process effacement and fusion (red triangles); (**f**) an occluding junction between adjacent podocytes instead of the slit diaphgram; and (**g**) endothelial cell detachment (red arrows and black dotted line), vacuoles and damage, and podocyte foot process fusion (red triangles); (**h**) SEMA3A-treated kidney 24 h after injection showing patchy podocyte lesions (red and black triangles) and normal endothelial fenestrations (black arrows); (**i** and **j**) SEMA3A-treated kidney 48 h after injection shows normal podocyte and endothelial cell ultrastructure; (**j**) intact slit diaphragms (black triangles) and fenestrae (black arrows) at higher magnification. Note that the glomerular basement membrane (GBM) remains intact throughout. Bar = 200 nm.

24 h (Figure 1h). Glomerular endothelial cell swelling, lack of fenestrations, and damage, as indicated by vacuoles and partial detachment, were observed 4 h after sema 3A infusion (Figure 1d and g). The glomerular basement membrane did not show significant changes at any time point examined. Podocyte and endothelial cell lesions resolved completely at 48 h (Figure 1i and j).

To begin to elucidate the molecular basis of sema 3A-induced abnormalities, we examined the expression level and distribution of the slit-diaphragm proteins podocin, CD2AP, and nephrin in control and SEMA3A-treated mice by western blot analysis and immunocytochemistry. To enrich the kidney lysates for podocyte proteins, we used isolated glomeruli. SEMA3A induced a significant decrease in CD2AP, podocin, and in the nephrin protein level 4 h post-injection (Figure 2a–c). These changes were transient and resolved by 24–48 h, as did the proteinuria and the ultrastructural abnormalities (Figure 1). SEMA3A did not alter the localization of podocin, nephrin, and CD2AP to podocytes, and it did not change the localization of synaptopodin, a prototypical marker of differentiated podocytes (Figure 2d).

Next, we asked whether systemic SEMA3A regulates renal/glomerular VEGF-A and VEGFR2 expression. We determined that SEMA3A does not alter VEGF-A protein level (Figure 3a), but rather induces a significant decrease in VEGFR2 kidney protein level in SEMA3A-treated mice as compared to control mice (Figure 3b), suggesting that SEMA3A effects may be mediated, in part, by downregulation of VEGFR2 signaling.

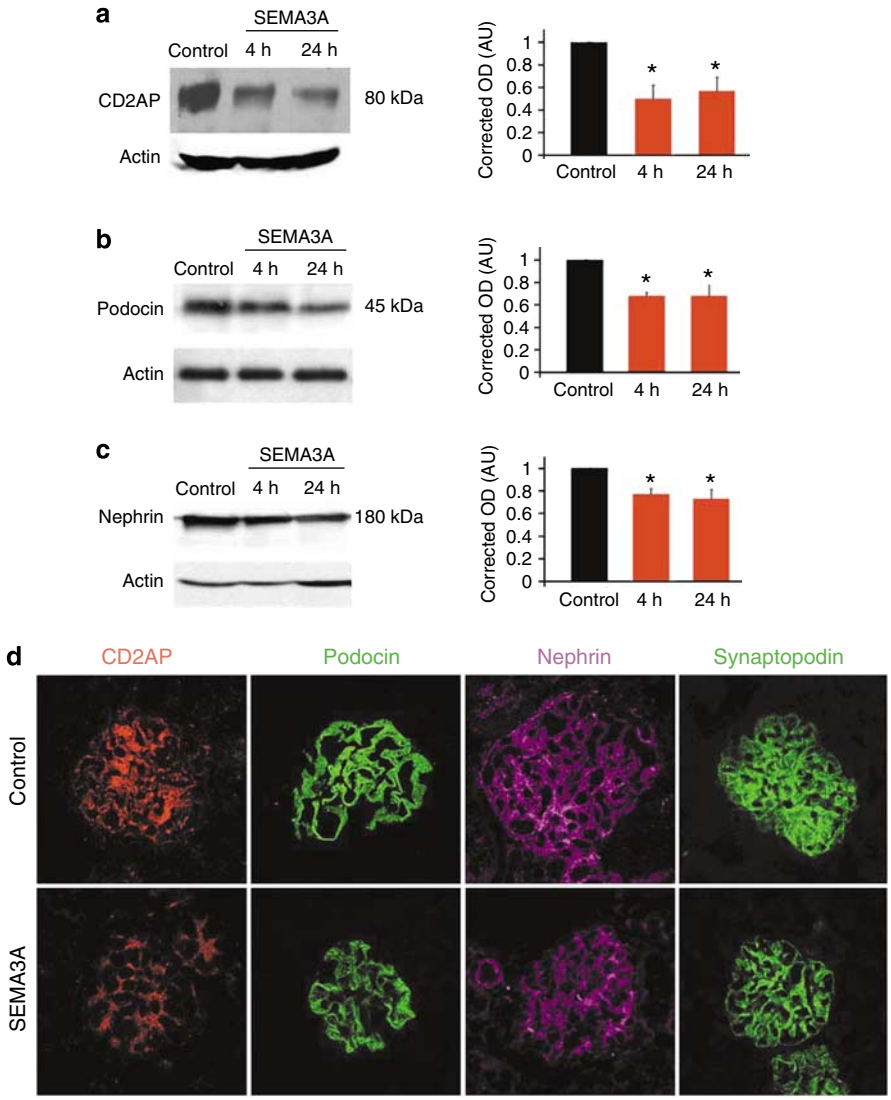

Figure 2 | SEMA3A induces downregulation of CD2AP, podocin, and nephrin. Proteins from ⩾4 mouse kidneys were pooled before SDS-PAGE in all conditions. (**a**) *Left*: western blot showing decreased CD2AP level after SEMA3A injection and *right*: quantification of densitometric changes as compared to control, after correction for actin (loading control). Data are expressed as mean + s.e.m., $n = 5$, $^*P < 0.05$. (**b**) Western blot showing decreased podocin level after SEMA3A injection, quantification as described in (**a**), $n = 5$. (**c**) Western blot showing decreased nephrin expression level after SEMA3A injection, quantification as described in (**a**), $n = 4$; actin expression levels were used to normalize for protein loading. (**d**) Fluorescence immunohistochemistry showing localization of CD2AP, podocin, nephrin, and synaptopodin to podocytes in control and SEMA3A-treated kidney sections, original magnification × 600.

To gain insight into SEMA3A/VEGF-A interactions, we used an immortalized mouse podocyte cell line[24] expressing both ligands and their receptors.[16,25] We determined that SEMA3A induces a decrease in VEGFR2 mRNA (Figure 3c) and VEGFR2 protein level, whereas addition of $VEGF_{165}$ in equimolar concentration prevents these changes (Figure 3d), supporting a role for SEMA3A/VEGF-A balance in podocyte signaling.

Next, we investigated whether co-administration of equimolar doses of SEMA3A and $VEGF_{165}$ can prevent proteinuria or glomerular filtration barrier damage induced by SEMA3A *in vivo*. We determined that SEMA3A + $VEGF_{165}$-treated mice did not develop acute proteinuria (Figure 4a). Transmission electron microscopy showed a normal glomerular ultrastructure in SEMA3A + $VEGF_{165}$-treated mice (Figure 4b). Accordingly, nephrin, podocin, and VEGFR2 expression levels were normal in SEMA3A + $VEGF_{165}$-treated mice (Figure 4c and d). Collectively, these findings suggest that $VEGF_{165}$ competition for neuropilin 1 binding prevents SEMA3A signaling and abrogates SEMA3A-induced podocyte and endothelial cell damage.

In summary, our results indicate that SEMA3A causes acute proteinuria, associated with reversible podocyte and endothelial cell damage, which are prevented by $VEGF_{165}$, suggesting that SEMA3A/VEGF-A balance is involved in glomerular filtration barrier homeostasis.

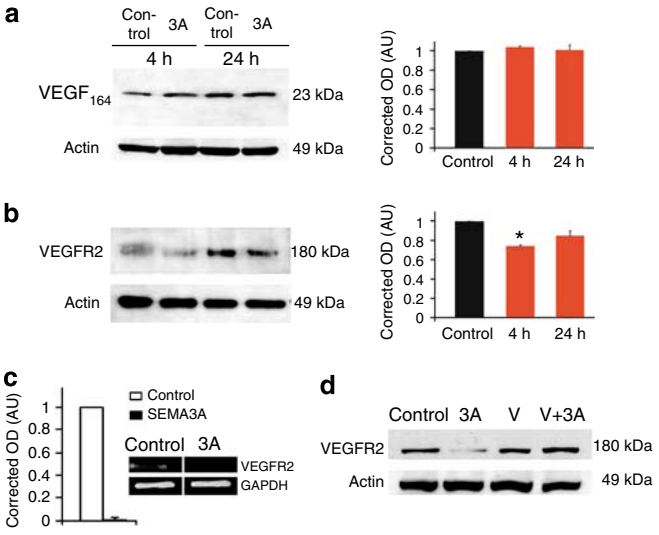

Figure 3 | SEMA3A decreases VEGFR2 expression *in vivo* and in cultured podocytes. (**a**) Representative western blot showing that $VEGF_{164}$ expression is not altered by SEMA3A, actin = loading control. (**b**) Representative western blot showing that SEMA3A downregulates VEGFR2 expression, actin = loading control; quantification in (**a** and **b**) as described in Figure 2a, $n = 4$; proteins from $\geqslant 4$ mouse kidneys were pooled before SDS-PAGE in all conditions. (**c**) RT-PCR amplification showing that SEMA3A decreases VEGFR2 mRNA, optical density corrected for GAPDH, and expressed as mean + s.e.m., $n = 4$, $^*P < 0.05$. (**d**) Western blot from podocyte whole-cell lysate showing SEMA3A-induced VEGFR2 downregulation, which is abrogated by $VEGF_{165}$, actin = loading control.

DISCUSSION

This study demonstrates that excess circulating SEMA3A causes acute proteinuria associated with podocyte foot process effacement and fusion, as well as endothelial cell damage and downregulation of slit-diaphragm proteins and VEGFR2. Both the podocyte ultrastructural abnormalities and the increased permeability of the glomerular filtration barrier are transient and reversible. To rule out nonspecific effects triggered by a heterologous protein, we injected

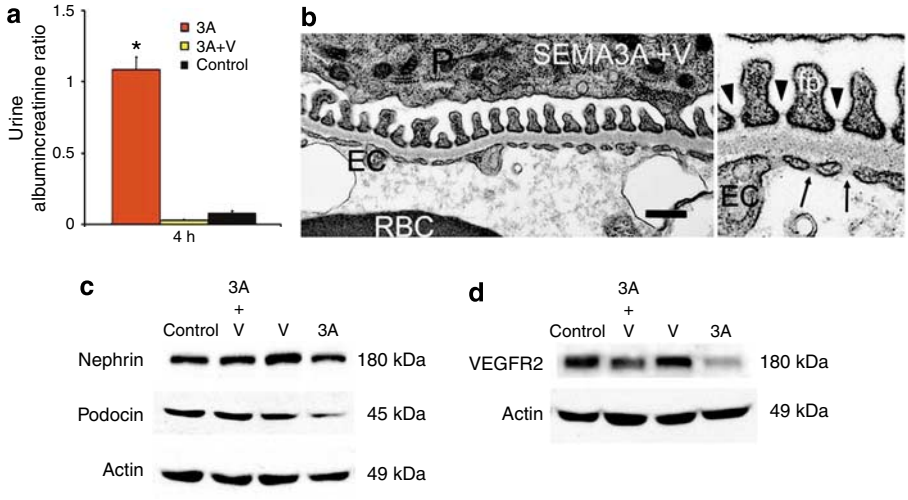

Figure 4 | $VEGF_{165}$ abrogates SEMA3A effects on glomerular permeability, ultrastructure, and gene expression *in vivo*. (**a**) Albumin:creatinine ratio in the urine 4 h after SEMA3A (3A), SEMA3A + $VEGF_{165}$ injection (3A + V), or vehicle (con), data are expressed as mean + s.e.m. (**b**) TEM of a SEMA3A + $VEGF_{165}$-treated kidney showing normal glomerular ultrastructure, inset shows intact slit diaphragms, GBM, and endothelial cell fenestrations at higher magnification, bar = 500 nm. (**c**) Western blots showing that nephrin and podocin protein levels in SEMA3A + $VEGF_{165}$-treated kidneys are not different from controls, whereas both proteins decrease in SEMA3A-treated kidneys, actin = loading control. (**d**) Western blot showing that VEGFR2 protein level in SEMA3A + $VEGF_{165}$-treated kidneys is not different from controls, whereas VEGFR2 decreases in SEMA3A-treated kidneys and increases in $VEGF_{165}$-treated kidneys, actin = loading control. Proteins from $\geqslant 3$ mouse kidneys were pooled before SDS-PAGE in all conditions.

denatured SEMA3A or collagen I and we confirmed that neither of them induced proteinuria or renal ultrastructural abnormalities, arguing that the effect of the intact SEMA3A was specific and induced by signaling.

SEMA3A is known to control endothelial cell migration and axon pathfinding by inducing rearrangements of the actin cytoskeleton and decreasing integrin activity.[7,10] We have previously shown that the *Sema3a* gene is expressed in podocytes throughout life,[15] and that cultured podocytes express *Sema3a* and its receptors neuropilin 1, 2, and plexins A1–3 and D1.[16] Podocyte exposure to recombinant SEMA3A induced apoptosis, downregulation of podocin expression, and decreased interactions between podocin/CD2AP and podocin/nephrin *in vitro*.[16] These studies suggested that sema 3A regulates the function of the slit-diaphragm signaling complex and thus could alter the permeability of the glomerular filtration barrier *in vivo*.

The results reported here show that a single injection of recombinant SEMA3A causes transient massive proteinuria, foot process effacement and fusion, and endothelial damage. These changes were observed 4 h after SEMA3A infusion and resolved within 48 h. Interestingly, the slit diaphragms were replaced by ladder-like structures similar to occluding junctions, such as those described during podocyte differentiation at the capillary loop stage of glomerulogenesis,[26,27] suggesting that foot process fusion may be a dynamic de-differentiation process.[26] Similar rapid changes have been described following protamine sulfate infusion, where foot process effacement involved nephrin phosphorylation and was reversed by heparin.[28,29] Replacement of slit diaphragms by occluding junctions have also been observed in aminonucleoside nephrosis, in congenital nephrotic syndrome of the Finnish type, and in minimal change disease, consistently associated with transient or permanent proteinuria.[27,30,31]

To begin to elucidate the molecular basis of the proteinuria and the ultrastructural changes induced by *in vivo* SEMA3A infusion, we examined the expression of slit-diaphragm proteins. We found that SEMA3A acutely and transiently decreased podocin, CD2AP, and nephrin protein levels, consistent with disregulating the slit-diaphragm signaling complex. The mechanism(s) of SEMA3A-induced podocin, CD2AP, and nephrin downregulation are unknown and need to be explored. Although the mRNA and protein half-life for these molecules has not been determined, given the observed changes in 4 h, it is likely that the reduction in protein levels involves protein degradation rather than transcriptional or translational regulation. We cannot exclude the possibility of SEMA3A-induced changes in post-translational modifications, such as nephrin phosphorylation, that in turn may alter its signaling and protein interactions.[29,32] The recovery of podocin, CD2AP, and nephrin protein levels lagged behind the resolution of the proteinuria and the improvement of the ultrastructural changes, suggesting that there are additional, unidentified molecules involved in these processes. The localization of slit-diaphragm proteins to podocytes was not altered by SEMA3A. Podocin localizes to the podocyte foot process cell membrane and physically associates with CD2AP and nephrin.[33,34] Disruption of the podocin gene results in massive proteinuria, mesangial sclerosis, and renal failure in mice.[35] Downregulation of podocin has been reported in the puromycin aminonucleoside nephrotic syndrome model and in LMX1B-mutant mice.[36,37] Podocin mutations in humans cause autosomal-recessive familial focal glomerulosclerosis.[38] CD2AP is an adapter protein that interacts with podocin and nephrin and anchors them to the actin cytoskeleton.[34,39] CD2AP mutation results in massive proteinuria and foot process effacement, progressive glomerulosclerosis, and renal failure in mice.[40] In humans, CD2AP haploinsufficiency is associated with proteinuric disease.[41] Nephrin is an integral component of the slit diaphragm that functions as an adhesion, a scaffold and a signaling molecule.[33] Nephrin mutations cause congenital nephrotic syndrome of the Finnish type in humans and massive proteinuria, lack of slit diaphragms, and foot process fusion in mice.[42,43] In view of the known functions of these proteins,[44] the functional and ultrastructural changes induced by SEMA3A strongly suggest that downregulation of podocin, CD2AP, and nephrin played a mechanistic role in the glomerular filtration barrier permeability changes and ultrastructural abnormalities observed.

SEMA3A and $VEGF_{165}$ compete for binding to neuropilin 1 and have multiple opposite effects on endothelial cells, neurons, and podocytes.[7,16,45] Interestingly, both proteins colocalize in podocytes.[15,16,25,46] We propose a model where a dynamic counterbalance between SEMA3A and VEGF-A functions regulates the homeostasis of the slit-diaphragm proteins and thus the permeability of the glomerular filtration barrier. Several lines of evidence from *in vivo* and *in vitro* studies support this hypothesis. Elegant experiments showed transient proteinuria associated with ultrastructural changes in mice injected with anti-VEGF antibody or soluble flt-1 receptor.[22] Similar abnormalities have been observed in cancer patients treated with anti-VEGF antibody and in women with preeclampsia.[19,23,47] These studies proved that neutralization of circulating VEGF-A (or excess 'decoy' receptor, soluble flt-1) induced nephrin downregulation and suggested that podocyte VEGF-A signaling is necessary to maintain the normal function and structure of the glomerular filtration barrier. Here, we demonstrate that excessive circulating SEMA3A induces downregulation of podocin, CD2AP, and nephrin and results in similar functional consequences, that is, increased permeability of the glomerular filtration barrier and ensuing proteinuria. In addition, we show that SEMA3A induces downregulation of VEGFR2 without altering the VEGF-A kidney protein level *in vivo*, suggestive of another mechanism decreasing VEGF-A signals.

The *in vivo* results reported here are consistent with our previous *in vitro* data showing that VEGF-A increases podocin expression and its association with CD2AP, whereas SEMA3A has the opposite effect, that is downregulation of podocin and its interactions with CD2AP and nephrin.[16,25]

VEGF-A and SEMA3A also have opposite effects on podocyte survival: VEGF-A is a podocyte survival factor whereas SEMA3A induces podocyte apoptosis *in vitro*.[16,25,48] We also showed that VEGF-A induces VEGFR2 upregulation in cultured podocytes.[25] In this study, we confirmed and extended these *in vitro* findings, showing that SEMA3A induces downregulation of podocyte VEGFR2 mRNA and protein, and that addition of $VEGF_{165}$ abrogates these changes, supporting the notion that SEMA3A effects are mediated, at least in part, by downregulation of VEGFR2 signaling. Moreover, we demonstrated that co-administration of SEMA3A and $VEGF_{165}$ *in vivo* abrogates the glomerular ultrastructural abnormalities induced by SEMA3A, prevents the development of proteinuria, and the downregulation of slit-diaphragm proteins and VEGFR2. These results imply that $VEGF_{165}$ competes with SEMA3A for neuropilin 1 signaling and restores glomerular filtration barrier homeostasis.

In summary, we report a novel mechanism of proteinuria induced by SEMA3A in mice. The physiologic levels and the role of SEMA3A in proteinuric states are unknown. However, the finding that excessive SEMA3A causes acute proteinuria by disregulating slit-diaphragm proteins and VEGFR2 signaling may be important to uncover pathogenic mechanisms of proteinuric diseases, and may have potential therapeutic implications, such as neutralization of SEMA3A to prevent or decrease podocyte apoptosis and support slit-diaphragm protein expression.

MATERIALS AND METHODS

Recombinant SEMA3A

As previously described,[16] rat *Sema3a* cDNA (NM_017310) was cloned into the EcoRI/NotI sites of the pHis.parallel vector.[49] *Escherichia coli* BL21 DE3 Codon Plus (Stratagene, La Jolla, CA, USA) were transformed with pHis.parallel/*Sema3a* and induced to produce the recombinant protein with 1 mM IPTG (isopropyl-beta-D-thiogalactopyranoside). SEMA3A was extracted from inclusion bodies under denaturing conditions, refolded with a reducing buffer followed by an oxidation buffer, dialyzed against decreasing urea concentrations (2 M–7 mM) and concentrated using Amicon Ultra filters (Millipore, Billerica, MA, USA).

SEMA3A injection and evaluation of proteinuria

Swiss-Webster 6-week-old male mice ($n=28$) were given a single sema 3A intraperitoneal injection (0.5 μg per g body weight), SEMA3A + $VEGF_{165}$ (0.15 μg per g body weight, ≈molar dose, $n=3$) or $VEGF_{165}$ (0.15 μg per g body weight, $n=3$). Weight-matched controls received a single intraperitoneal bolus of denatured SEMA3A ($n=4$) or an irrelevant protein (rat collagen I 0.5 μg per g body weight, $n=4$) or 0.5 ml vehicle (100 mM NaCl, 50 mM Tris-Cl, 192 mM glycine, 7 mM urea, and pH 7.5), ($n=20$). Four and 24 h after the intraperitoneal injection, mice were anesthetized according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Urine was collected by bladder puncture and kidneys were harvested. Urinary albumin and creatinine concentrations were determined by enzyme-linked immunosorbent assay (Albuwell M Elisa kit; Exocell, Philadelphia, PA, USA) and colorimetric assay (creatinine colorimetric assay; Oxford Biomedical Research, Oxford, MI, USA). Urine albumin excretion was estimated as albumin:creatinine ratio.

Transmission electron microscopy

Kidneys were fixed in 2% paraformaldehyde + 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, postfixed with 1% osmium tetroxide followed by 1% uranyl acetate, dehydrated, and embedded in LX112 resin (LADD Research Industries, Williston, VT, USA). Ultrathin (80 nm) sections were cut (Reichert, Heidelberg, Germany, Ultracut UCT), stained with uranyl acetate/lead citrate, and examined by transmission electron microscopy (JEOL 1200EX).

Mouse glomerular isolation and western analysis

Mice previously treated for indicated time periods with vehicle, SEMA3A, SEMA3A + $VEGF_{165}$, or $VEGF_{165}$ were anesthetized as described above, and perfused with magnetic beads/PBS via left ventricle puncture (Dynabeads; Dynal Corp, Oslo, Norway). Kidneys were harvested and glomeruli were isolated using a magnet.[50] Glomeruli (average purity ~85%) were lysed in RIPA buffer + protease inhibitors (Roche, Indianapolis, IN, USA) + 1 mM Na orthovanadate + 1 mM NaF. Protein concentration was determined by bicinchoninic acid assay (Sigma, St Louis, MO, USA). A total of 50–100 μg protein per lane were resolved in 6–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Polyclonal primary antibodies used for immunoblotting were anti-podocin (Podo 11A; Alpha Diagnostics Inc., San Antonio, TX, USA); anti-CD2AP (no. sc-9137; Santa Cruz Technology, Santa Cruz, CA, USA); anti-nephrin (Nephr 11A; Alpha Diagnostics); anti-VEGF-A (no. sc-507; Santa Cruz Technology); anti-VEGFR2 (no. sc-315; Santa Cruz Technology). Anti-actin (no. A2066; Sigma) was used as a control for protein loading. Secondary antibodies used were anti-rabbit or anti-mouse HRP-IgG (Amersham, Piscataway, NJ, USA). Signals were visualized by enhanced chemoluminescence (Amersham).

Immunohistochemical studies

Kidneys were embedded in optical coherence tomography (Sakura, Torrance, CA, USA), snap-frozen in dry ice/isopentane and kept at −80 °C. Six micrometer cryosections were air dried, fixed in cold acetone for 5 min, permeabilized with 0.3% TritonX-100, washed, and exposed to blocking serum for 30 min. Sections were incubated with the following primary antibodies for 2 h: anti-nephrin (NPHN11-A; Alpha Diagnostic Inc.), anti-podocin (Podo 11-A; Alpha Diagnostic Inc.), anti-CD2AP (no. sc-9137, Santa Cruz), and anti-synaptopodin (clone G1D4, Biodesign International). Following appropriate washes, sections were incubated with Cy2- or Cy3-labeled secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) for 45 min, washed with PBS, and mounted (Vectashield; Vector Laboratories, Burlingame, CA, USA). All sections were examined by confocal microscopy (FV300; Olympus, Center Valley, PA, USA) and images were taken at ×60 magnification.

Cell culture

A conditionally immortalized mouse podocyte cell line was maintained in RPMI 1640 + 10% fetal bovine serum as described previously.[16,24] Differentiated podocytes were serum starved for 4 h and then exposed to serum-free media, serum-free media + recombinant sema 3A (50 ng ml^{-1}), serum-free media + $VEGF_{165}$ 20 ng ml^{-1}, or serum-free media + sema 3A 50 ng ml^{-1} + $VEGF_{165}$ 20 ng ml^{-1} for 4 h as indicated. At the end of the experiments,

podocytes were lysed and processed for western analysis as described above.

Statistical analysis

All experiments were performed $\geqslant 3$ times. Glomeruli from four mice in each experimental condition were pooled, proteins were extracted for immunoblotting. Densitometric analysis of reverse transcription-PCR and western blotting were performed, optical density was corrected for protein loading and expressed as a proportion of control signals (mean ± s.e.m.). In all experiments, control and experimental conditions were compared using *t*-test or analysis of variance, as appropriate. $P<0.05$ was considered statistically significant.

DISCLOSURE

The authors have no financial interest to disclose.

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