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Genetic inactivation of *Semaphorin 3C* protects mice from Acute Kidney Injury.

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Running Headline: SEMA3C deleterious function in AKI

Abstract

To guide the development of therapeutic interventions for acute kidney injury, elucidating the deleterious pathways of this global health problem is highly warranted. Emerging evidence has indicated a pivotal role of endothelial dysfunction in the etiology of this disease. We found that the class III semaphorin SEMA3C was ectopically upregulated with full length protein excreted into the blood and truncated protein secreted into the urine upon kidney injury, and hypothesized a role for SEMA3C in acute kidney injury. Sema3c was genetically abrogated during acute kidney injury and subsequent kidney morphological and functional defects in two well-characterized models of acute kidney injury; warm ischemia/reperfusion and folic acid injection were analyzed. Employing a beta actin-dependent, inducible knockout of Sema3c, we demonstrate that in acute kidney injury SEMA3C promotes interstitial edema, leucocyte infiltration and tubular injury. Additionally, intravital microscopy combined with Evans Blue dye extravasation and primary culture of magnetically-sorted peritubular endothelial cells identified a novel role for SEMA3C in promoting vascular permeability. Thus, our study points to microvascular permeability as an important driver of injury in acute kidney injury, and to SEMA3C as a novel permeability factor and potential target for therapeutic intervention.

Keywords: Genetically altered and transgenic models, Vascular permeability, Neuropilin 1, VEGF, Acute Kidney Injury, Class 3 semaphorin

Translational Statement

AKI is a common clinical condition associated with high risks of mortality and subsequent progression to CKD. Emerging evidence has proved the pivotal role of endothelial dysfunction in the aetiology of AKI. Our studies on a ligand-specific mouse suggest that SEMA3C, a protein involved in axon pathfinding and cardiovascular development, mediates damaging functions in AKI. We identified an unrecognised role for SEMA3C in vascular permeability and studied how this could translate into tissue oedema, neutrophil infiltration and altered blood flow. Our work, via deepening the understanding of AKI pathophysiology, could guide the development of therapeutic interventions for this intractable disease.

Introduction

Acute Kidney Injury (AKI) represents a severe health burden impacting about 13.3 million patients annually for a global death toll of 1.7 million¹. Even if followed by renal function recovery, AKI is a major risk factor for the subsequent development of chronic kidney disease (CKD) and end-stage renal disease, a process known as AKI to CKD transition². Accumulating evidence has revealed that endothelial dysfunction plays a key role in the initiation, extension and maintenance of AKI³. Dysregulation of pro-angiogenic factors and unsolved capillary hyperpermeability contribute to microvascular hypoperfusion, oedema, hypoxia and inflammation in AKI^{4,5}. These processes not only aggravate the initial injury but have long-term effects on renal function and repair phase, predisposing to renal fibrosis and CKD.

SEMA3C is an 85 kDa glycoprotein that belongs to the large family of semaphorins initially identified for their involvement in axon pathfinding⁶. Semaphorins exist primarily as membrane-bound proteins with the exception of class III semaphorins which are secreted as soluble molecules⁷. We and others have shown that embryos deficient for *Sema3c* presented with severe kidney and cardiovascular abnormalities^{8,9}. SEMA3C also controls the development of novel and aberrant vasculature, including cancer angiogenesis¹⁰ and vascularisation within the degenerate human intervertebral disc¹¹. SEMA3C acts via receptor complexes which include ligand-binding subunit Neuropilin 1 (NRP1) or Neuropilin 2 (NRP2), and a coreceptor consisting of one of four class plexin proteins¹².

In this study, we investigated the critical pathophysiological role played by SEMA3C in two well-characterised models of AKI, the ischemia/reperfusion (IR) and the Folic Acid (FA) nephrotoxicity. We used a genetic approach to abrogate *Sema3c* during AKI initiation and progression, and analysed subsequent renal structural and functional defects in *Sema3c-ko* compared to wildtype littermates. *Sema3c-ko* proved renoprotective effects in AKI, resulting in decreased kidney tissue damage and leukocyte infiltration. Our innovative work provides concrete evidence that ectopic upregulation of SEMA3C in the context of AKI plays a pivotal role in the

etiology of the disease. It also identifies SEMA3C as a key mediator of kidney microvascular permeability, which could promote AKI.

Methods and Materials

Only novel methodologies are described below. Other standard methods with minor modifications were followed and are detailed in Supplemental Materials.

Mouse strains

We used the *Sema3c*^{flox/flox} (MGI:107557)⁸, *Sema3c*^{+/-} and *CAGGCre-ERTM* (MGI:2182767)¹³ alleles for breeding appropriate animals for the experimental study. Generation of the *Sema3c*^{+/-} allele was obtained by breeding *Sema3c*^{flox/flox} with germline beta-actin-CRE (MGI: 2176050). *Sema3c*^{-/-} embryos presented with the well-characterised renal and cardiac defects (Supplemental Figure 5). *Sema3c* conditional mutants (FA-*Sema3c* ko : *CAGGCre-ERTM*;*Sema3c*^{flox/-}) and wt littermate controls (FA-CTR: *Sema3c*^{flox/+} or *Sema3c*^{flox/flox}) were treated with Tamoxifen (1 mg/40 g body weight) for 5 consecutive days and then subjected to a single intraperitoneal dose of FA (FA-treated animals) or vehicle (Sham animals). Successful *Sema3c* deletion was demonstrated by recombination PCR and by the reduction of SEMA3C induction upon FA treatment (Supplemental Figure 2). Mice were maintained on a mixed genetic background consisting of C57BL/6J and CD1. Genotyping primers are *Sema3c* Flox Forw 5'- GAATCTGGCAAAGGACGATG-3', *Sema3c* Flox Rev 5'- GACCACTGGGCTTGAGAGAG-3', *Sema3c* Null 5'-AAGGCGCATAACGATAACCAC-3', CRE Forw 5'-TGGAAAATGCTTCTGTCCGTTTGC-3' and CRE Rev 5'-AACGAACCTGGTCGAAATCAGTG-3'. The exact number of mice per strain and/or samples (n) is listed in Figure Legends.

Intravital microscopy

Intravital scanning was performed using an inverted Olympus IX83 microscope equipped with a 37°C heated chamber. Briefly, the left kidney of anaesthetised wild type mice was exposed by flank incision and gently pulled to allow insertion of a slit plate at the level of the renal artery and vein. This system allows the physical separation of the organ from the rest of the body. At that time, a total volume of 200µl composed of FITC-Albumin (100µg/g body weight; 50mg/ml stock solution, Sigma) mixed with either 0.9%NaCl (control, CTR), or 5µg recombinant mouse VEGF-A (VEGF-164, R&D Systems), or 5µg recombinant mouse SEMA3C (R&D Systems), was intravenously injected in the eye sinus. The animal was then flipped over to expose the kidney onto the microscope slide and image acquisition (1 image every 15 seconds for 30 minutes) could start

instantaneously. Tiling acquisition was performed in widefield microscopy with IX83 and DP73 camera (Olympus).

In vivo permeability Miles assay

8-week old mice of the designated genotype were anaesthetised and subjected to left renal artery clamping for 30 minutes at 37°C followed by 24 hours of reperfusion. At that time, retro-orbital injection (10µl/g of body weight) of Evans Blue dye (5mg/ml dissolved in 0.9% NaCl) was performed. 35 minutes post injection; anaesthetised animals were thoroughly perfused with 0.9%NaCl via the left ventricle to remove all blood from the vasculature. Perfused kidneys were extracted, weighed and incubated in 100% Formamide overnight at 56°C. Extracted dye concentration was measured using a spectrophotometer at 600nm and the value obtained was reported as $OD_{600}/10^{-3}$ Kg of tissue. Intradermic injection (50µl) of recombinant mouse SEMA3C (2µg), mouse SEMA3A (2µg) and mouse VEGF-A (0.5µg) (R&D Systems) was performed on anaesthetised wildtype mice kept on a 37°C heated waterbed at all time. The appearance of a blue spot was monitored, photographed and quantified 30 mins post injection as described above.

Results

In AKI SEMA3C is expressed de novo in the adult kidney

In the embryonic kidney, *Sema3c* expression is detected in both early nephronic epithelium and ureteric bud-derived structures at E17.5 (black arrowhead and asterisk respectively, Figure 1A)⁹, restricted to the papilla at birth (red arrowhead), and no longer detected in the adult. In the diseased kidney following surgically-induced (IR) and chemically-induced (FA) acute kidney injury, SEMA3C is ectopically upregulated compared to control kidneys and overexpression was detected as early as 3 hrs post FA (Figure 1B-C and Supplemental Figure 1A) and 2.5hrs post IR (Figure 1D). SEMA3C was predominantly found in cortical tubules (white arrowheads, Figure 1C-D), in scattered tubular parts of the corticomedullary region and in glomerular parietal epithelial cells (yellow arrowheads, Figure 1C). Increased serum urea, as an indicator of abnormal kidney function, confirms the efficiency of the IR and FA procedures (Figure 1E-F). Quantification of tissue expression was performed in both IR- and FA-collected samples at different time points post injury, using RT-PCR and a previously validated set of primers⁸ (Supplemental Figure 1C-D).

Next, we performed SEMA3C immunohistochemistry in kidney biopsies from patients with AKI. Tubular immunostaining was evident in all four biopsies tested: two from transplanted kidneys (ATN1, ATN2), one from vancomycin-induced AKI (ATN3), and one from AL-amyloidosis derived from lymphoma (ATN4). We concluded that SEMA3C upregulation was a conserved response to acute kidney injury both in mice and humans.

De novo excretion and secretion of SEMA3C upon acute kidney injury

We next tested serum and urine samples of FA-treated mice for the presence of SEMA3C by Western Blot at the indicated time (Figure 2B). SEMA3C is cleaved, like other class III semaphorins, by furin-like endoproteases. Furin protein levels were reported to be low in blood¹⁴,¹⁵, while Furin proteolytic activity was increased in the urine post IR¹⁶. Cleaved SEMA3C was

detected in the urine of both FA- and IR-injured animals, but not in sham controls (Figure 2B, top). We also found that the 85 kDa full-length SEMA3C was induced in the serum of FA- and IR-injured mice (Figure 2B, bottom); a 109 kDa SEMA3C recombinant protein was used as a positive control.

SEMA3C receptors NRP1 and NRP2 are expressed in the renal vasculature during AKI

In addition to their well-documented expression in glomerular endothelial and mesangial cells^{17, 18}, we found that NRP1 and NRP2 expression colocalised with CD31-positive endothelial cells in peritubular capillaries following FA injection (yellow arrowhead, Figure 2C) and IR (Supplemental Figure 2B). NRP1 and NRP2 glomerular expression was also conserved post FA injury (Supplemental Figure 2A). We also observed some NRP1-positive, CD31-negative cells which were previously reported to be NG2-positive pericytes (red asterisk, Figure 2C)¹⁸. The same expression pattern was observed for both NRP1 and NRP2 in IR-treated animals (Supplemental Figure 2B) and is in agreement with previous finding at the single-cell level post IR¹⁹. We found no expression for either NRP1 or NRP2 in kidney tubules, suggesting that SEMA3C is not involved in an autocrine loop of regulation. No double-positive NRP1-CD45 cells were observed in FA-treated kidneys (Supplemental Figure 2C), indicating that NRP1 does not seem to be expressed in leukocytes during kidney injury. We concluded that secreted SEMA3C from damaged tubules could potentially act in a paracrine fashion on the kidney vasculature.

*Genetic downregulation of *Sema3c* by conditional mutagenesis abrogates AKI*

We next investigated the role of SEMA3C in the pathogenesis of AKI by deleting *Sema3c* in FA- and IR-injured mice. *Sema3c* conditional mutants (FA-*Sema3c-ko* or IR-*Sema3c-ko* : *CAGGCre-ERTM;Sema3c^{flox/-}*) and wt littermate controls (FA-WT or IR-WT: *Sema3c^{flox/+}* or *Sema3c^{flox/flox}*) were treated with Tamoxifen for 5 consecutive days (day 1 to 5) and subjected to either a single

intraperitoneal injection of FA (FA-treated animals) or vehicle (Sham animals) on day 8 or IR injury on day 8 (cf supplemental material and methods). Successful *Sema3c* deletion was demonstrated by recombination PCR, real-time PCR and by the reduction of SEMA3C induction upon FA treatment (Supplemental Figure 3). The analysis of Periodic Acid-Schiff (PAS)-stained kidney sections in FA-WT or IR-WT mice (*Sema3c^{fllox/+}* or *Sema3c^{fllox/fllox}*) showed characteristic features of ATN (Figure 3A, C), including loss of brush border (black arrowhead), flattening of the renal tubular cells (red arrowheads) due to tubular dilatation (red asterisk) and intratubular cast formation (black arrows) compared to Sham mice. These pathological changes and corresponding injury scores were significantly attenuated in FA-*Sema3c* ko and IR- *Sema3c* ko (*CAGGCre-ERTM;Sema3c^{fllox/-}*) as revealed by a reduced number of dilated tubules and by brush border maintenance in some tubular regions (Figure 3A, C). A significant diminution of serum urea levels is also observed in FA-*Sema3c* ko compared to FA-WT mice and in IR-*Sema3c* ko compared to IR-WT, meaning that downregulation of *Sema3c* also ameliorated kidney function in AKI (Figure 3A, C). Of note, no changes in kidney function or injury were observed in vehicle-injected *Sema3c* ko (data not shown). We also found no significant SEMA3C ectopic upregulation in other organs such as the lungs, liver and heart, in neither the FA- nor the IR-induced AKI models (Supplemental Figure 4A). Therefore, it is rather unlikely that in a 48hours timeframe, our analysis based on kidney injury reflects any other pathophysiological changes caused by *Sema3c* deletion in other organs. Because the degree of AKI correlates with neutrophil infiltration in mice and rats²⁰, we investigated neutrophil extravasation in our model and found a considerable reduction in renal neutrophil infiltration in FA-*Sema3c-ko* compared to FA-WT mice (Figure 3B). Neutrophil extravasation contributes to tubular injury, and a significant drop in the apoptotic marker cleaved caspase 3 was detected in FA-*Sema3c-ko* compared to FA-WT animals 48 hrs post injury (Supplemental Figure 4B). We also tested another inflammatory cell macrophage that represents the major leukocyte infiltrating into the kidney during IR, and found that F4/80-positive macrophages were less abundant in IR-*Sema3c-ko* compared to IR-WT animals 48 hrs post injury

SEMA3C does not sustain peritubular capillary loss during AKI

Because SEMA3C exerts potent inhibiting effects in cellular models of angiogenesis²¹, we first hypothesised that by acting on NRP1- and NRP2-positive peritubular ECs, secreted SEMA3C could affect peritubular capillary density in AKI. We used a similar approach and magnetically isolated peritubular ECs from wt kidneys as previously described (Figure 4A)²². When treated with increasing amounts of SEMA3C, purified ECs surprisingly increased their proliferative capacity as shown by a significant rise in ki67 positivity (Figure 4C, top), and underwent capillary-network formation on Matrigel in a dose-dependent manner (Figure 4C, bottom). *In vivo*, EC proliferation was only detected 48 hours post injury as previously described²³. Indeed, some elongated cells double labelled for ki67 and CD34, indicative of EC origin, could be detected 48 hours post IR and FA-injuries (yellow arrows, Figure 4D).

We next investigated the effect of *Sema3c* downregulation on peritubular vasculature *in vivo* using protein extracted from *Sema3c-ko* and wt littermates. *Sema3c-ko* had no effect on the expression of VEGFR2, adherens junction VE-cadherin, or plasmalemma vesicle-associated protein PLVAP on capillary endothelia (Figure 5). Solely, the expression of the tight junction (TJ) epithelial adhesion molecule Occludin was downregulated in FA-treated wt kidneys and upregulated in *Sema3c-ko* protein extracts (Figure 5C-C'). In regard to the large amount of epithelial cell death observed in AKI, Occludin change in expression is more likely to reflect the amount of tubular injury, which is less pronounced in *Sema3c-ko* compared to wt littermates (Figure 3), than a modulation in TJ associated permeability.

In AKI, Sema3c-ko reduces capillary leakage, interstitial oedema and microvascular congestion

Increased vascular permeability leading to tissue oedema was observed as early as 6 hours post IR in the mouse²⁴. 24 hours post unilateral IR, the L/R kidney ratio had significantly increased (Figure 6A). Likewise, kidney to body weight ratio following 24 hrs of FA treatment had dramatically risen compared to control sham kidneys (Figure 6A). These results suggest that in addition to tubular dilatation, a substantial infiltration of fluid in the renal parenchyma could have occurred. Remarkably, the kidney to body weight ratio was significantly lower in FA-treated *Sema3c-ko* compared to FA-WT controls (Figure 6A), suggesting that downregulation of *Sema3c* might be able to reduce tissue oedema in the FA-induced AKI nephropathy. A non-significant drop in L/R kidney ratio was also observed in IR-*Sema3c-ko* compared to IR-WT controls (Figure 6A). However, when we used a more sensitive method that consists in measuring Evans Blue dye (EB) extravasation in the left ischemic kidney following unilateral IR (Figure 6B), we found that disrupting SEMA3C signalling was also able to reduce vascular permeability in IR (Figure 6B). Results showed that vascular permeability has (i) increased 24hours post reperfusion in the ischemic kidney and (ii) significantly dropped in IR-*Sema3c-ko*. Interstitial oedema participates in the diminished microvascular flow and alters oxygen diffusion observed in AKI pathologies. Microangiography performed with high molecular weight dextran (FITC-Dextran (2000 kDa)) on sham animals revealed that normal vascular blood flow allows FITC-Dextran to be dispatched throughout the kidney without significant extravasation from the vessels and highlight peritubular capillaries and glomeruli of control sham mice (Figure 6C-D). However, only very scattered vascular regions of the kidneys were positive for FITC-Dextran in FA-CTR 10mins post injection, implying severe microvascular leakage and hypoperfusion (Figure 6C). Conversely, significantly more kidney vascular regions were stained for FITC-Dextran in FA-*Sema3c-ko* (Figure 6C). Likewise, *Sema3c* downregulation in IR-*Sema3c ko* was able to attenuate the microvascular congestion observed in IR-WT mice (Figure 6D). Our results suggest that downregulation of *Sema3c* is able to induce a diminution in interstitial oedema and an improvement of renal blood flow.

SEMA3C is a vascular permeability factor

To determine whether SEMA3C could directly increase blood vessel permeability, we used two distinct procedures. Intradermal injections of both VEGF-A and SEMA3A, which are key inducers of vascular permeability²⁵, caused increased extravasation of EB dye into the tissue compared to PBS (Figure 7). Similarly, SEMA3C was able to induce a significant increased vascular leakage of the EB dye, establishing a novel function for SEMA3C as a vascular permeability factor (Figure 7). To test whether SEMA3C induced vascular permeability in kidneys, we next used intravital microscopy and retro-orbital injection of VEGF-A or SEMA3C combined with tracer FITC-Albumin. In this experiment, we monitored the presence of green tubular casts, which represents the rapid passage of the usually unfiltered FITC-Albumin, indicating elevated glomerular endothelial permeability. As expected, we did not find any casts in mice receiving FITC-Albumin alone over 30mins post injection (Figure 8 and Supplemental Movie 1). However, we observed a remarkable effect on glomerular vascular permeability when we injected 5 μ g of VEGF-A (Figure 8A and Supplemental Movie 2). The first cast was observed as early as 12 mins post injection and was followed by a quick dynamic of cast formation and excretion, indicative of the powerful permeability effect of VEGF-A on kidney vasculature (Supplemental Movie 2). When mice were injected with 5 μ g of SEMA3C, the first cast was observed at around 23 mins (Figure 8A, Supplemental Movie 3) and 4 out of 8 treated mice presented with casts at 30 mins post injection. At 35mins, 7 out of 8 injected mice had accumulated FITC-Albumin in their kidney tubules (Figure 8B). A similar dynamic of cast formation was observed when VEGF-A concentration was lowered to 2 μ g (Supplemental movie 4). We also examined peritubular capillary branches, which correspond to the reported sites of EB dye extravasation in pathological conditions²⁶. In both VEGF-A and SEMA3C injected mice, we observed the accumulation of FITC-Albumin dots at the

capillary intersections and, to a lesser extent, along the longitudinal axis of the microvessels in VEGF-A (red arrowhead Figure 8A). Overall, our data show that SEMA3C is a vascular permeability factor of the peripheral and internal vasculature.

Discussion

By taking advantage of a repertoire of *in vivo* and *ex vivo* techniques, we have characterised the function of the class III semaphorin SEMA3C in the context of AKI. First, we show that in AKI SEMA3C is expressed *de novo* in renal tubules, the most likely source for increased full length (secreted) SEMA3C in the blood, as well as of truncated (excreted) SEMA3C in the urine. Second, we found that *Sema3c* conditional deletion ameliorated tissue injury and renal function in AKI. Third, we showed that SEMA3C is a vascular permeability factor. We demonstrated that mice lacking *Sema3c* presented with reduced interstitial oedema and leukocyte infiltration and improved renal blood flow. Our combined analysis, via revealing a novel deleterious tubulovascular cross-talk, improves the mechanistic understanding of AKI.

In this study, we demonstrated that the ectopic upregulation of SEMA3C promotes AKI. Since upregulation of SEMA3C protein precedes that of its mRNA, we hypothesise that SEMA3C is, at least partly, regulated at the post-transcriptional level, as it is the case for SEMA3A in AKI¹⁶. At the transcriptional level, a possible regulator could be the zinc finger transcription factor GATA6 which drives SEMA3C expression in physiological conditions²⁷ and which is actively upregulated upon IR injury²⁸.

Considering the central role played by semaphorins and neuropilins in angiogenesis, it was valueable to test whether SEMA3C could interfere with normal EC functions in AKI. We provide *in vitro* evidence for a pro-angiogenic effect of SEMA3C on peritubular capillary endothelia. However, at least during the first 48 hrs after induction of AKI, we found no evidence for angiogenesis *in vivo*.

We next tested whether SEMA3C could promote renal microvascular hyperpermeability, as previously reported for SEMA3A and SEMA3C receptor NRP1^{33, 34}. We first showed that SEMA3C was able to induce vascular permeability of dermal and kidney ECs *in vivo*. In this context, we found that SEMA3C's capacity to induce increased permeability was on a par with SEMA3A at the dose tested. This consideration is important as SEMA3A role in permeability was reported to be the key detrimental event involved in ischemia-induced brain tissue damage²⁵, and in the loss of endothelial barrier integrity in glioblastoma³⁵. We further showed that downregulation of SEMA3C was able to (i) reduce EB dye vascular leakage through peritubular ECs in unilateral IR-induced nephropathy and (ii) ameliorate microvascular blood flow in FA-and IR-induced nephropathy. Our results relate to previous studies showing that, renal microvascular hyperpermeability contributes to an overall decline in GFR through interstitial oedema, increased tubular pressure and tubular obstruction³⁶. In addition, efforts to attenuate vascular leakage through inhibiting $\alpha\beta5$ integrin showed significant protective effects in IR³⁷.

As for the mechanism by which SEMA3C could regulate vascular permeability, previous reports strongly suggest that adherens junction (AJ) modulation might play a role in our model. On one hand, SEMA3C could inhibit AJ integrity through VE-Cadherin endocytosis in 30 mins²¹, and on the other hand, SEMA3C receptor NRP1 activation leads to the phosphorylation and subsequent internalisation of VE-cadherin to open AJs and increase vascular permeability³⁸. However, the precise mechanisms leading to increase vascular permeability will require further investigation.

In regards to the large amount of interstitial fluid accumulation and neutrophil infiltration, we cannot eliminate a possible effect on renal lymphatic vasculature. Indeed, the inactivation of SEMA3E/Plexin-D1 pathway could ameliorate cardiac function in a mouse model of acute cardiac injury by promoting reactive lymphangiogenesis to alleviate oedema³⁹. Considering that SEMA3C is also capable of interacting with Plexin-D1⁴⁰ and inhibiting lymphangiogenesis⁴¹, SEMA3C downregulation might mitigate renal tissue injury via increasing lymphangiogenesis, which could also involve NRP2⁴².

The thorough study of tissue-specific *Nrp1* and *Nrp2* mouse mutants could be informative to carefully dissect the essential contribution of NRP-driven pathways in AKI. However, the fact that NRP1 binds both VEGF-A and class III semaphorins¹² and that NRP2 can sometimes compensate for the loss of NRP1⁸ adds to the level of complexity. An interesting study reported that a specific NRP1 blocking peptide directed to class III semaphorin SEMA3A was able to alleviate IR-induced AKI⁴³. However, the vascular contribution to AKI was not investigated by the authors. Nevertheless, the combined SEMA3C-SEMA3A analyses underscore the importance of the deleterious activity of the Class III semaphorins-NRP1 axis in AKI and offer options for therapeutic strategies. In particular, specific compounds directed to the NRP1-class III semaphorin binding site could possibly reduce tissue oedema without compromising NRP1-VEGF-A-dependant vessel growth and renal microvasculature maintenance.

Finally, we found that SEMA3C was *de novo* excreted and secreted in FA-induced injury and that SEMA3C urinary levels rise significantly and well before urea levels elevate in the serum of FA-treated mice. In that respect, further studies in human patients should investigate whether SEMA3C could serve as an early marker of AKI and/or a predictive marker of AKI to CKD progression.

In summary, our data indicate that SEMA3C is produced by damaged tubular cells during AKI and suggests that it is a key detrimental mediator of renal injury. Our study is supported by recent single-cell RNAseq analysis showing *Sema3c* upregulation in novel epithelial-to-stromal cross-talks following AKI⁴⁴. Furthermore, AKI to CKD transition is associated with an increased *Sema3c* expression⁴⁵. Our analysis unravels an important signalling pathway of AKI and emphasises the role of vascular permeability as an important underlying cause of the disease. In that sense, our study provides an improved mechanistic understanding of the disease and the first evidence for the role of SEMA3C in kidney disease.

Authors Contributions

ACai, GY, SP, PF, BS, RK, MG and AC conducted the experiments and acquired data. XDYC provided human samples. AC designed the research study. SP and PF helped design the intravital permeability assay. CChatziantoniou, PJS and AC provided the funding. ACai and AC wrote the manuscript with contributions from all the authors.

Disclosure

The authors have declared that no conflict of interest exists (please refer to the disclosure statement document)

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Figure Legends

Figure 1. Ectopic upregulation of SEMA3C in two well-characterised mouse models of AKI.

(A) *Sema3c* in situ hybridisation on E17.5 newborn (NB) and adult wildtype (wt) mouse kidneys. Black arrowhead and asterisk indicate nephronic epithelium and ureteric bud-derived structures, respectively; red arrowhead indicates the renal papilla. (B) SEMA3C immunostaining on kidneys 24 hrs post IR or FA injury (n=3). (C) Serial kidney sections of mice immunostained for SEMA3C at 5 or 48 hrs post FA injury at the papilla, medulla and cortex levels (n=3). The boxed area is shown at higher magnification. White and yellow arrowheads indicate SEMA3C upregulation in cortical tubules and in glomerular parietal epithelial cells, respectively. (D) SEMA3C immunostaining of control kidneys (contralateral) and kidneys at 2.5 hrs post IR injury at the corticomedullary junction. (E, F) Serum urea levels in FA- and IR-AKI model. * $P \leq 0.05$, *** $P \leq 0.001$. Scale bar: 100 μm

Figure 2. SEMA3C ectopic upregulation in human biopsies, de novo excretion and secretion of SEMA3C upon mouse kidney injury.

(A) SEMA3C immunostaining of time-zero control (T0) and acute tubular necrosis (ATN) patient tissue biopsies. ATN1-2, kidney transplant patients diagnosed with pronounced (ATN1) and mild ATN (ATN2). ATN3, vancomycin in HIV patients ATN. ATN4, renal amyloidosis derived from lymphoma. (B) Top: representative western blot image of cleaved SEMA3C isoform excretion in mouse urine at 2.5 hrs (n=3) or at 6 hrs (n=3) after FA or Sham injections (left) or in mouse urine at 48 hrs (n=4) post IR (right). Bottom: representative western blot image of full-length SEMA3C secretion in mouse serum 48 hrs post FA injection (n=2) (left) or 48 hrs post IR (n=2) (right). #1 dil. and #2 dil. represent a 20 times dilution of sample #1 and #2, respectively. S3C rec. represents SEMA3C 109 kDa recombinant protein. (C) Kidneys were immunolabelled for NRP1 or NRP2 and endothelial marker CD31 24 hrs post FA injection. Yellow arrowheads indicate NRP1/CD31 (left) or NRP2/CD31 (right) double-positive peritubular ECs; red asterisk indicates NRP1 positive, CD31 negative pericytes. Scale bar: 100 μm .

Figure 3. Genetic downregulation of Sema3c by conditional mutagenesis abrogates AKI.

(A) PAS-stained sections of Sham, wildtype (FA-WT) and Sema3c ko (FA-S3C ko) mouse kidneys at 48 hrs post FA injection with corresponding quantification of serum urea and kidney injury score (n \geq 5). (B) Sections of Sham, FA-WT and FA-S3C ko mouse kidneys were immunolabelled with

anti-neutrophil antibody (red). Corresponding staining were quantified (% area relative to DAPI-positive cells) (n=4). (C) PAS-stained sections of Control, wildtype (IR-WT) and *Sema3c* ko (IR-S3C ko) mouse kidneys at 48 hrs post IR injury with corresponding quantification of serum urea and kidney injury score (n≥5). (D) Sections of Control, IR-WT and IR-S3C ko mouse kidneys were immunolabelled with anti-macrophage antibody F4/80 (red) Black arrowheads indicate loss of brush border; red arrowheads indicate flattening of renal tubular cells; red asterisks indicate tubular dilatation; black arrows indicate intratubular cast formation. *P<0.05, **P ≤ 0.01, ***P ≤ 0.001. Scale bar: 100 μm (A), 50 μm (C).

Figure 4. SEMA3C promotes peritubular capillary EC proliferation in vitro. (A) Schematic procedure of magnetic isolation of adult mouse peritubular ECs. (B) Left: phase-contrast image of isolated peritubular ECs, black arrows indicate magnetic beads conjugated with CD31 antibodies. Right: primary ECs stained with Endomucin and CD31. Indicated numbers mean % of positivity for each marker (performed on two sets of isolation). (C) Isolated primary ECs were cultured with 0 (CTR), 0.3 and 0.7 μg of recombinant SEMA3C for either 48 hrs and stained for Ki67 (n=5 per condition, upper) or seeded on Matrigel and cultured for 2 weeks (n≥3 per condition, lower). (D) Ki67 and CD34 double-staining of kidneys 24 hours or 48 hours post FA- and IR-induced nephropathies. Yellow arrows indicate Ki67/CD34 double-positive ECs (n=3). *P<0.05, **P ≤ 0.01. Scale bars: 20 μm (B), 50 μm (C), 100 μm (D).

Figure 5. SEMA3C does not sustain peritubular capillary loss during AKI. (A) Western blot analysis of EC marker VEGFR2 at different time points (3, 6 and 24 hours) post IR-induced kidney injury. (B) Western blot analysis of EC marker VEGFR2 at 48 hrs post FA-induced kidney injury in wildtype mouse and *Sema3c-ko*. (C) Western blot analysis of endothelial adhesion molecule VE-cadherin, EC plasmalemma vesicle-associated protein PLVAP and TJ molecule Occludin at 48 hrs post FA-induced kidney injury in wildtype mouse and *Sema3c-ko*. (A'-C') Corresponding quantification (n≥3) of (A-C). *P<0.05, **P<0.01, ***P ≤ 0.001. ns: not significant.

Figure 6. Sema3c downregulation ameliorates microvascular blood flow and decreases kidney vascular permeability leading to tissue oedema in AKI. (A) Kidney/bodyweight ratios of FA-treated animals at the indicated genotype (left) or left/right kidney ratios of IR-treated animals

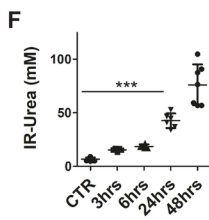
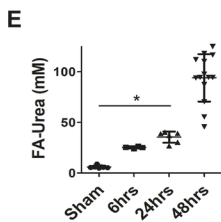
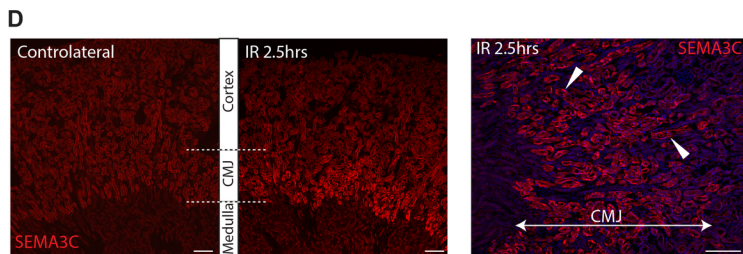
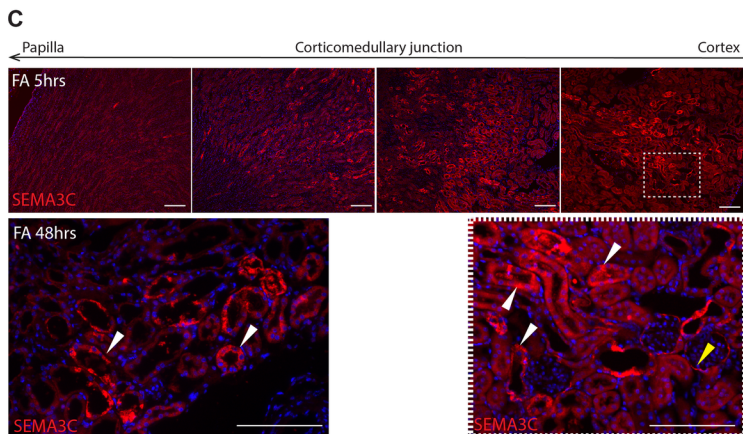
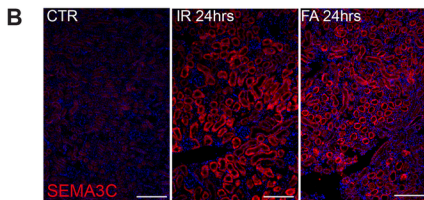
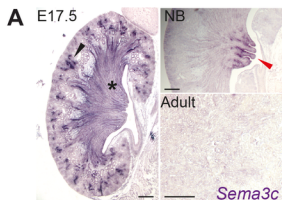
at the indicated genotypes (right) were quantified (n>3). (B) Schematic procedure of a modified Mile's assay performed 24 hours following unilateral IR in mice (left). EB dye extravasation from peritubular capillaries of the left ischemic kidney is photographed and compared to the contralateral kidney in both controls and *Sema3c-ko* (middle). Quantification of EB dye extravasation is presented as OD600 per 10⁻³ Kg of tissue weight for contralateral kidneys, IR-treated left kidneys and IR-treated left kidneys from *Sema3c-ko* (right). (n≥5). (C-D) 2000 kDa FITC-Dextran was intravenously injected into either Shams, FA-treated wildtype and FA-treated *Sema3c-ko* (C) or Controls, IR-WT and IR- *Sema3c-ko* (D) to evaluate microvascular blood flow 10mins post FA injection or IR surgery. FITC-Dextran positive areas were quantified. *P≤ 0.05, ***P≤ 0.001. Ctrlateral: contralateral. (n=3). Scale bars: 10mm (A), 100 μm (B,C)

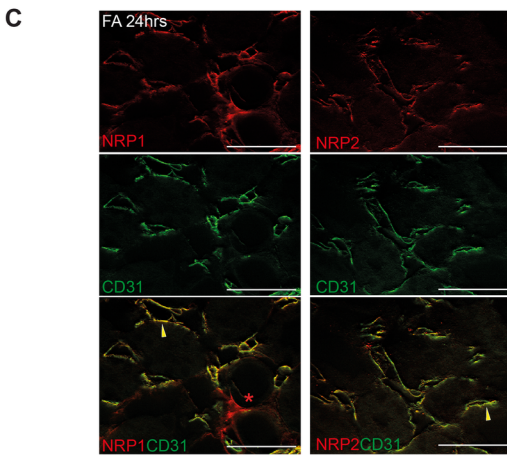
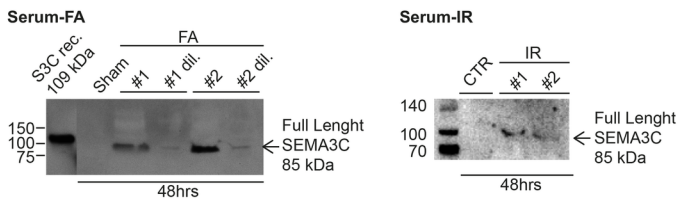
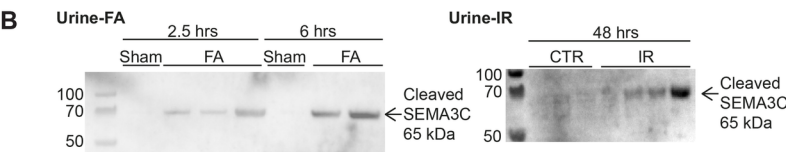
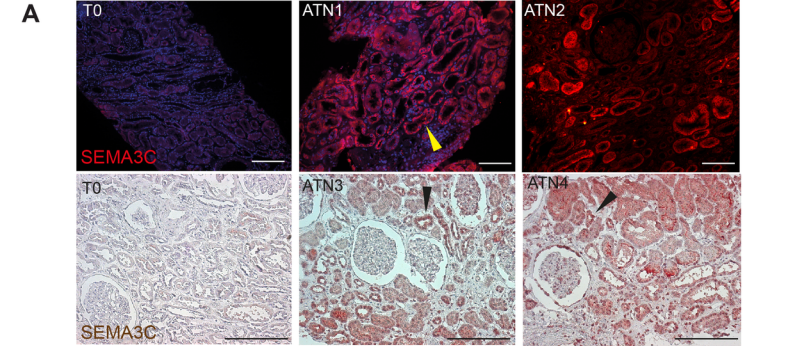
Figure 7. SEMA3C is a vascular permeability factor of the peripheral (skin) vasculature.

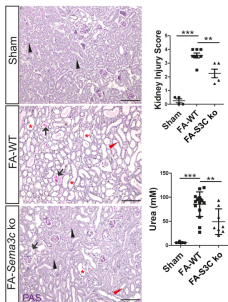
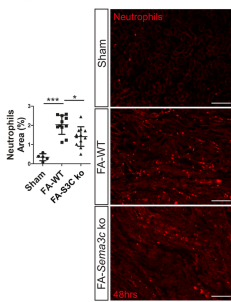
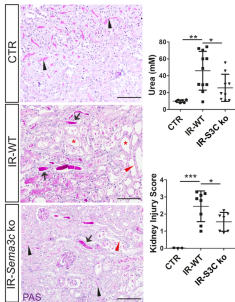
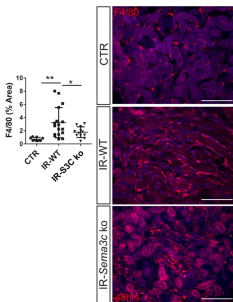
Representative images of Mile's assays performed on shaved dorsal skin of wildtype mice. 50μl intradermal punctures were realised with PBS, SEMA3C (2μg), SEMA3A (2μg), or VEGF-A (0.5μg); EB dye extravasation was quantified 30mins post injection. Results are expressed as OD₆₀₀ per 10⁻³ kg of tissue weight (n=4). Scale Bars: 5mm ***P≤ 0.001

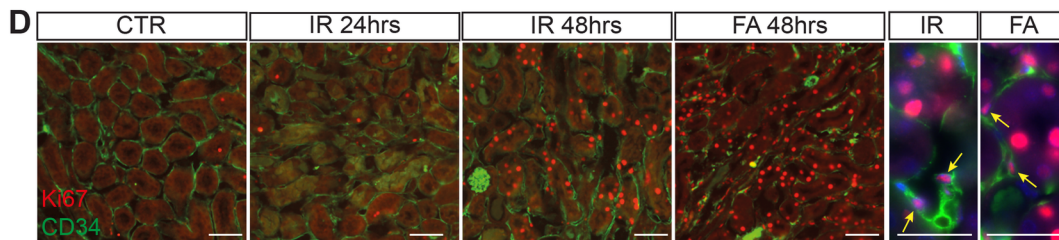
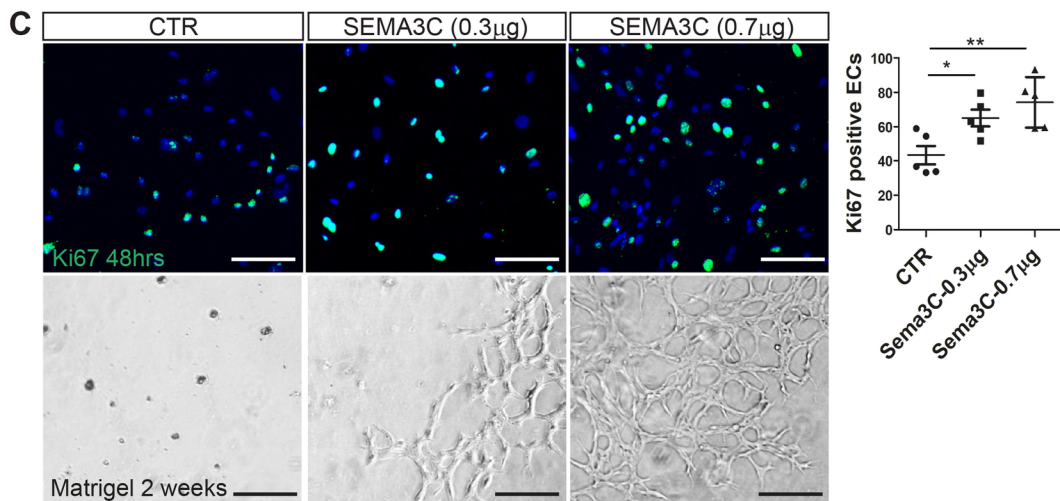
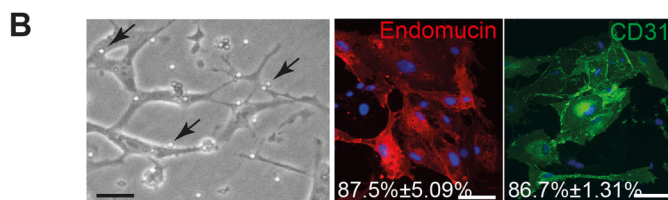
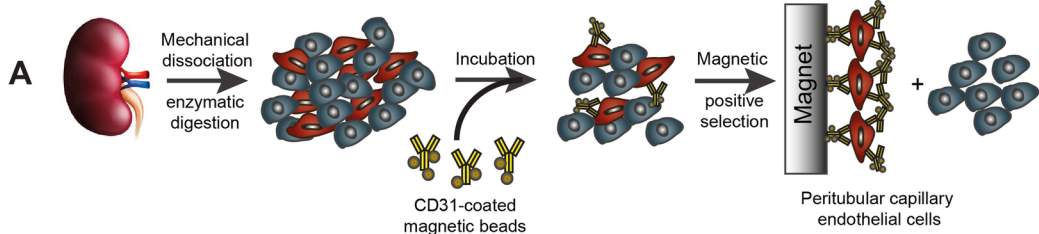
Figure 8. SEMA3C is a vascular permeability factor of the internal (renal) vasculature.

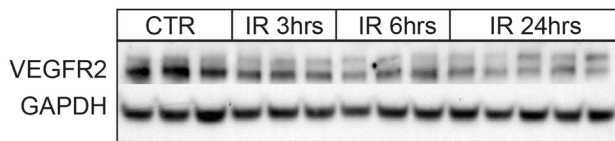
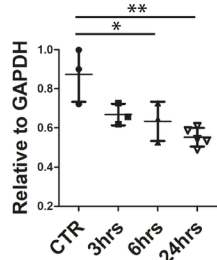
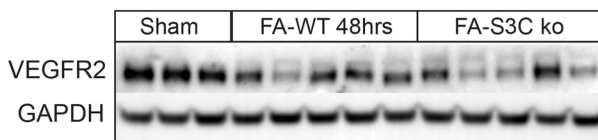
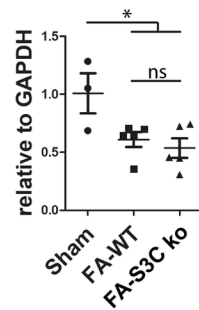
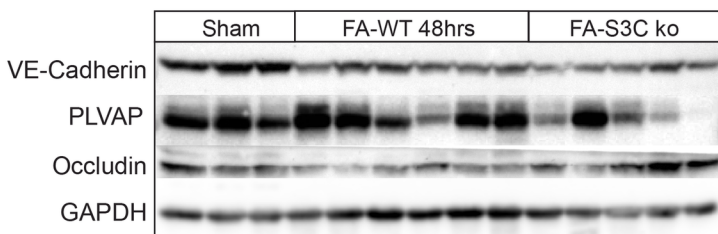
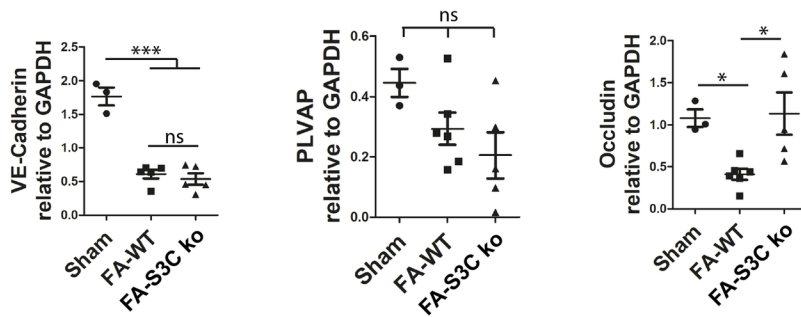
(A) Representative intravital microscopy images of mouse kidneys acquired at the indicated time post retro-orbital injection of FITC-Albumin mixed with either 0.9% NaCl (control, CTR) (n=5), 5μg VEGF-A (n=3), or 5μg SEMA3C (n=8). Yellow arrowheads indicate the presence of FITC-Albumin positive casts in VEGF-A and SEMA3C injected mice. Red arrowheads points to FITC-positive dots that are interpreted as extravasates at capillary branching sites. The boxed area at t=12.5min is shown at higher magnification. (B) Tiling acquisition performed 35mins post retro-orbital injection of FITC-Albumin mixed with either 0.9% NaCl (control, CTR) (n=5) or 5μg SEMA3C (n=8). Scale bars: 100 μm"

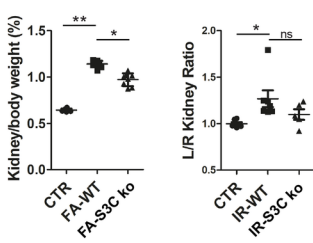
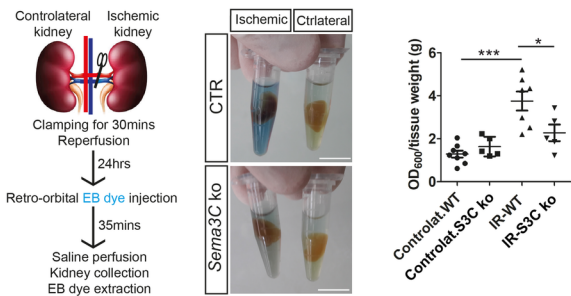
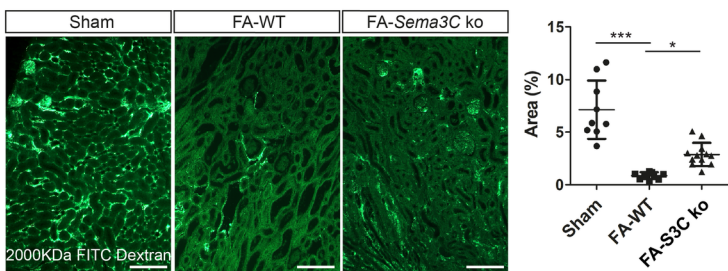




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