RESEARCH ARTICLE

Notch signaling is required for the formation of mesangial cells from a stromal mesenchyme precursor during kidney development

Scott C. Boyle*, Zhenyi Liu and Raphael Kopan‡,§

ABSTRACT

Mesangial cells are specialized pericyte/smooth muscle cells that surround and constrain the vascular network within the glomerulus of the kidney. They are derived from the stromal mesenchyme, a progenitor population distinct from nephron stem cells. Whether mesangial cells have a distinct origin from vascular smooth muscle cells (VSMCs) and the pathways that govern their specification are unknown. Here we show that Notch signaling in stromal progenitors is essential for mesangial cell formation but is dispensable for the smooth muscle and interstitial cell lineages. Deletion of RBPjk, the common DNA-binding partner of all active Notch receptors, with *Foxd1^tgCre* results in glomerular aneurysm and perinatal death from kidney failure. This defect occurs early in glomerular development as stromal-derived, desmin-positive cells fail to coalesce near forming nephrons and thus do not invade the vascular cleft of the S-shaped body. This is in contrast to other mutants in which the loss of the mesangium was due to migration defects, and suggests that loss of Notch signaling results in a failure to specify this population from the stroma. Interestingly, Pdgfrb-positive VSMCs do not enter the vascular cleft and cannot rescue the mesangial deficiency. Notch1 and Notch2 act redundantly through γ-secretase and RBPjk in this process, as individual mutants have mesangial cells at birth. Together, these data demonstrate a unique origin of mesangial cells and demonstrate a novel, redundant function for Notch receptors in mesangial cell specification, proliferation or survival during kidney development.

KEY WORDS: Kidney, Mesangium, Notch, Progenitors, Foxd1, Mouse

INTRODUCTION

Development of nephrons, the functional units of the kidney, is driven by reciprocal interactions between distinct progenitor populations. The best characterized of these is the relationship between the cap mesenchyme (CM) and the ureteric bud, which give rise to all of the epithelial cells of the nephron and collecting system, respectively (Boyle et al., 2008; Kobayashi et al., 2008; reviewed by Costantini and Kopan, 2010; Dressler, 2009). Less well understood are the molecular interactions that regulate development of the stromal mesenchyme (SM) and endothelial precursors.

Department of Developmental Biology, Washington University School of Medicine, St Louis, MO 63110, USA.
*Present address: Department of Pharmacy, St Louis Children's Hospital, St Louis, MO 63110, USA. ‡Present address: Division of Developmental Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229, USA.

§Author for correspondence (raphael.kopan@cchmc.org)

The SM and CM are both derived from a common, Osr1-expressing progenitor pool whose fate becomes progressively restricted as development proceeds (Mugford et al., 2008). The SM progenitor population surrounds the CM at the periphery of the developing metanephric kidney and requires expression of the transcription factor Foxd1 (Levinson et al., 2005). Despite their common developmental origin, each gives rise to vastly different cell types: whereas the CM produces about 15 specialized epithelial cell types along the nephron, the SM gives rise to supporting cells including interstitial fibroblasts, vascular smooth muscle cells (VSMCs) and mesangial cells (Humphreys et al., 2010; Sequeira Lopez and Gomez, 2011). Mesangial cells are a specialized type of pericyte/smooth muscle cell that wrap endothelial cells within the glomerulus, contributing to the architecture of the capillary loop structure and overall function of the filtration apparatus (Quaggin and Kreidberg, 2008). Mesangial and endothelial cells first come together within the vascular cleft of the S-shaped body (SSB), a CM-derived epithelial structure with proximal/distal polarity (Dressler, 2009). Podocyte precursors located in the proximal tail of the SSB express Vegf, which attracts endothelial progenitors into the cleft (Eremina et al., 2006). These endothelial cells then secrete platelet derived growth factor B (Pdgfb) polypeptide, drawing Pdgf-receptor β (Pdgfrb) expressing mesangial precursors into the cleft (Lindahl et al., 1998). Mutants for any of these ligands or receptors results in failed formation of the mesangium due to impaired chemotaxis of mesangial precursors, which accumulate outside the cleft of SSB but still express the definitive markers of mesangium: Pdgfrb and desmin (Eremina et al., 2006; Lindahl et al., 1998). These podocyte, endothelial and mesangial precursors organize to form a characteristic 'cup' structure where poorly understood interactions build the glomerular architecture. It is unclear whether VSMC or naïve SM cells attracted by Pdgfb become mesangial cells *in situ* or mesangial progenitors specified from the SM are the only cells capable of recognizing Pdgfb as a chemotactic signal.

Involvement of Notch in this process was initially suggested by a study of mice homozygous for a hypomorphic *Notch2* allele, which suffer from hypoplastic kidneys, glomerular aneurysm and perinatal death (McCright et al., 2001), and by expression of some Notch targets in the mesangium (Yu et al., 2012). Whereas hypoplasia is most likely caused by reduced *Notch* signals in CM derivatives (Cheng et al., 2007; Surendran et al., 2010), glomerular aneurysm would predict defects in vasculature (where Notch2 is not known to play a role) or in formation of the podocyte-endothelial-mesangial cooperative that provides structure and filtration capacity to the glomerular interior. Notch receptors are highly conserved regulators of a myriad of processes during development, adult tissue homeostasis and disease (Gridley, 2010; Koch and Radtke, 2010; Liu et al., 2010; MacGrogan et al., 2010). To test the hypothesis that

Notch signaling is involved in development of cells arising from the SM, we used activation-specific fate mapping (Liu et al., 2011; Vooijs et al., 2007) and identified patterns of Notch1 and Notch2 activity in SM-derived structures, including labeling of the mesangium by both receptors. We then used Foxd1^{tgCre} to delete RBPjk (Rbpj – Mouse Genome Informatics) in SM progenitors during the initial stages of metanephric kidney development. RBPjk is a nuclear DNA binding protein that mediates the transcriptional activity of all Notch receptors. We show that RBPjk activity in the SM is essential for normal glomerular development. RBPjk-deficient animals die within 48 hours of birth with microaneurisms evident on the kidney surface. This defect is localized to glomeruli, and immunohistological analysis demonstrates the absence of mesangial cells within the filtration apparatus. Pdgfrb-expressing SM-derived cells are present but fail to enter the vascular cleft of the SSB, and desmin+ cells are absent from the area surrounding the forming nephrons. Upstream of RBPjk, Notch1 and Notch2 fulfill this function redundantly in a γ-secretase-dependent manner, consistent with a canonical Notch signal. These studies demonstrate a function for Notch signaling during formation of a definitive mesangial progenitor population outside of the developing glomerulus, which then migrate into the vascular cleft towards a Pdgfb source. It also demonstrates that renal smooth muscle cells are independent of Notch signaling in the developing kidney and are unable to substitute for the lost mesangial progenitors. This study uncovers a Notch-dependent, mesangial progenitor specification program acting throughout nephrogenesis and raises the possibility that Notch signals continue to act in maintenance of the mature mesangium.

RESULTS

Cells derived from the stromal mesenchyme experience Notch1 and Notch2 activation during kidney development

To determine if nonepithelial lineages experience Notch activation during development we utilized $Notch1^{IP-CreLO}$ and $Notch2^{IP-CreLO}$ reporter mice (Liu et al., 2013; Liu et al., 2011; Morimoto et al., 2010; Vooijs et al., 2007). Briefly, these mice were engineered to replace one copy of the Notch intracellular domain with Cre recombinase containing a C-terminal 6-Myc Tag (Cre-6MT). When ligand binds to the Notch::Cre-6MT hybrid receptor, Notch cleavage by Adam10 and γ-secretase releases Cre from the membrane. Cre6MT can then recombine a reporter allele and allow its expression from the $Rosa26$ locus, irreversibly labeling cells and all of their descendants that experience Notch activation during kidney development (Soriano, 1999). Analyses of postnatal day 0 (P0) kidneys from $Notch1^{IP-CreLO}$ $Rosa^{+/CAG-eYFP}$ and $Notch2^{IP-CreLO}$ $Rosa^{+/CAG-eYFP}$ (Madisen et al., 2010) mice reveled that $Notch1^{IP-CreLO}$ labels the entire vascular endothelium (Fig. 1A-A″, asterisks), consistent with reports suggesting high and repeated activation of Notch1 in these cells (Gridley, 2010). Only occasional VSMCs are labeled in these vessels (Fig. 1A-A″, arrowheads). By contrast, cells experiencing $Notch2^{IP-CreLO}$ activation abound in the VSMC compartment but are never found in the endothelium (Fig. 1C-C″, arrowheads). Within glomeruli, we frequently observe labeled Pdgfrb+ mesangial cells in both lines, indicating that they have experienced Notch1 and Notch2 activation during development (Fig. 1B-B″, D-D″, arrowheads). By contrast, the interstitium only experiences Notch2 activation (Fig. 1B-B″,D′,D″, arrows). These data demonstrate that although Notch1 and Notch2 are coactivated in some cell types, they are individually active in subsets
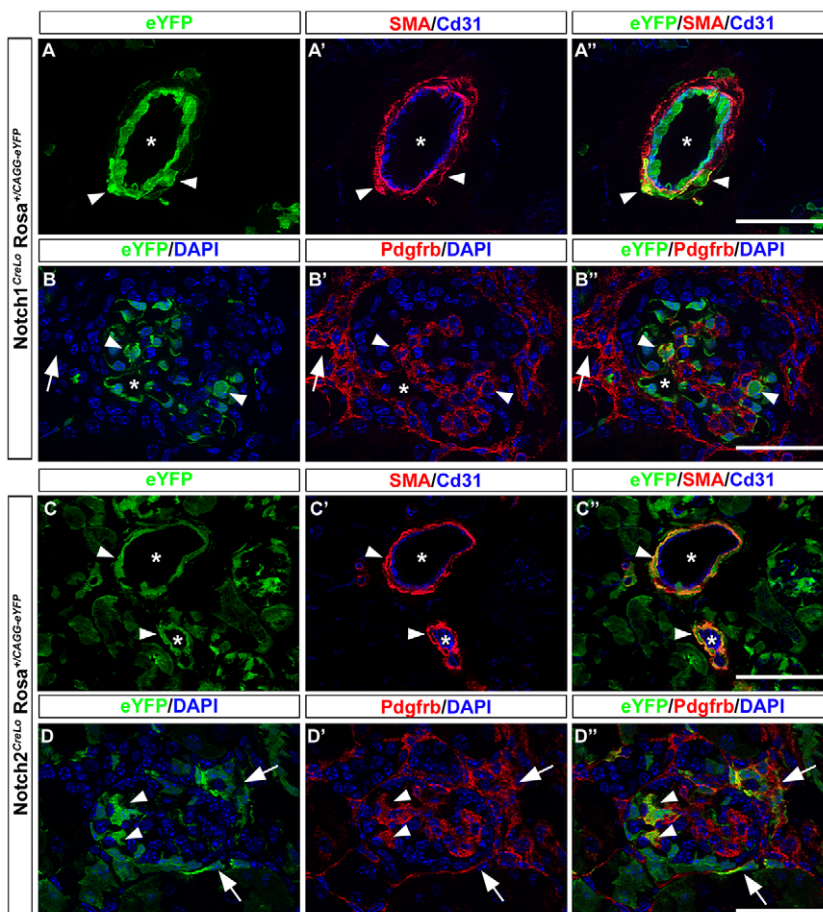
Fig. 1. Cells derived from the stromal mesenchyme experience Notch activation during development. The stromal mesenchyme gives rise to VSMCs, mesangial cells and interstitial fibroblasts. P0 kidneys carrying the $Rosa^{+/CAG-eYFP}$ reporter from $Notch1^{IP-CreLO}$ (A-B″) and $Notch2^{IP-CreLO}$ (C-D″) mice were used to examine lineages arising from the stroma that had experienced Notch activation (eYFP-green). (A-A″) The entire vascular endothelium (CD31, blue) is composed of cells that have experienced Notch1 activation, demonstrating the fidelity of the system. Labeled VSMCs are infrequently found in $Notch1^{IP-CreLO}$ kidneys (SMA-Red, arrowheads). (B-B″) Within glomeruli, mesangial cells (Pdgfrb-Red, arrowheads) are lineage positive for Notch1 activity. The majority of Notch1 lineage+ cells are endothelial cells within capillary loops (*). Interstitial fibroblasts (Pdgfrb+, outside of glomerulus, arrows) that activated Notch1 during development are not observed. (C-C″) The entire VSMC compartment is composed of cells that experienced Notch2 activation during development (SMA-Red); Notch2 cells are never found in the endothelium. (D-D″) Mesangial cells (Pdgfrb-Red, arrowheads) are also Notch2 lineage positive and interstitial fibroblasts experience Notch2 activation during development (arrows). Scale bars: 50 μm.

of stromal-derived lineages during differentiation, consistent with possible additional roles for Notch signaling outside of the nephron proper during kidney development.

Foxd1tgCre RBPjkF/F mice die after birth with glomerular aneurisms and nonfunctional kidneys

To test the hypothesis that Notch signaling is required in stromal derivatives during development, we used a transgenic *Foxd1tgCre* line that expresses Cre recombinase specifically in the SM during gestation to conditionally delete *RBPjk*, the obligate DNA-binding partner of all notch intracellular domains (NICD; reviewed in Kopan and Ilagan, 2009). *Foxd1+* progenitors give rise to the interstitium, VSMC and mesangial cells (Humphreys et al., 2010). *Foxd1tgCre RPBjkF/F Rosa+/eYFP* mice are born at the expected Mendelian ratios, but die within 48 hours of birth with little or no urine in the bladder and blood spots visible on the surface of the kidneys, indicative of defects in vasculature or associated structures (Fig. 2A,B). Mutant kidneys are smaller with normal gross architecture and patterning, but histology reveals that they have reduced proximal tubule (PT) density, demonstrated by Haematoxylin and Eosin (H&E) and Lotus tetragondobus lectin (LTL) staining (Fig. 2C-E). At higher magnification, the mutant kidneys contained many blood-filled glomeruli with little or no internal structure (Fig. 2G,H). To quantify the nephron endowment and glomerular aneurysm defects we used serial sections to count total and blood-filled glomeruli from control

(*Foxd1tgCre RPBjk+/F Rosa+/eYFP*, *n*=8) and mutant (*Foxd1tgCre RPBjkF/F Rosa+/eYFP*, *n*=12) animals (Fig. 2I). We saw a small, but nonsignificant, decrease in total glomerular count in mutant animals despite the reduction in PT density we observed. In control animals, we never saw glomeruli containing aneurism, while >80% of glomeruli were dilated and blood filled in mutant animals. These data demonstrate that RBPjk is required in the stromal mesenchyme or its derivatives for proper formation of glomeruli and functional kidneys.

Mutant glomeruli contain endothelial cells and podocytes, but lack mesangial cells

Next, we investigated fully formed glomeruli in control and *Foxd1tgCre RPBjkF/F; Rosa+/eYFP* mice at P0. Normally, glomeruli are composed of a tightly organized network of endothelial capillary loops (CD31+; Pecam1+ – Mouse Genome Informatics) surrounded by a ring of podocytes (WT1+, Fig. 3A-A″). Mutant kidneys display a normal podocyte distribution and endothelial cells are present, but instead of capillary loops a single, endothelium-lined dilated space forms (Fig. 3B′,B″, asterisks). In the wild-type glomerulus, we find that *Foxd1tgCre* labels mesangial precursors during prenatal development (Humphreys et al., 2010), with an extremely rare podocyte being labeled, despite the apparent presence of Foxd1 mRNA in all podocytes (Brunskill et al., 2011) (see Discussion). By contrast, we noted a striking absence of enhanced yellow fluorescent
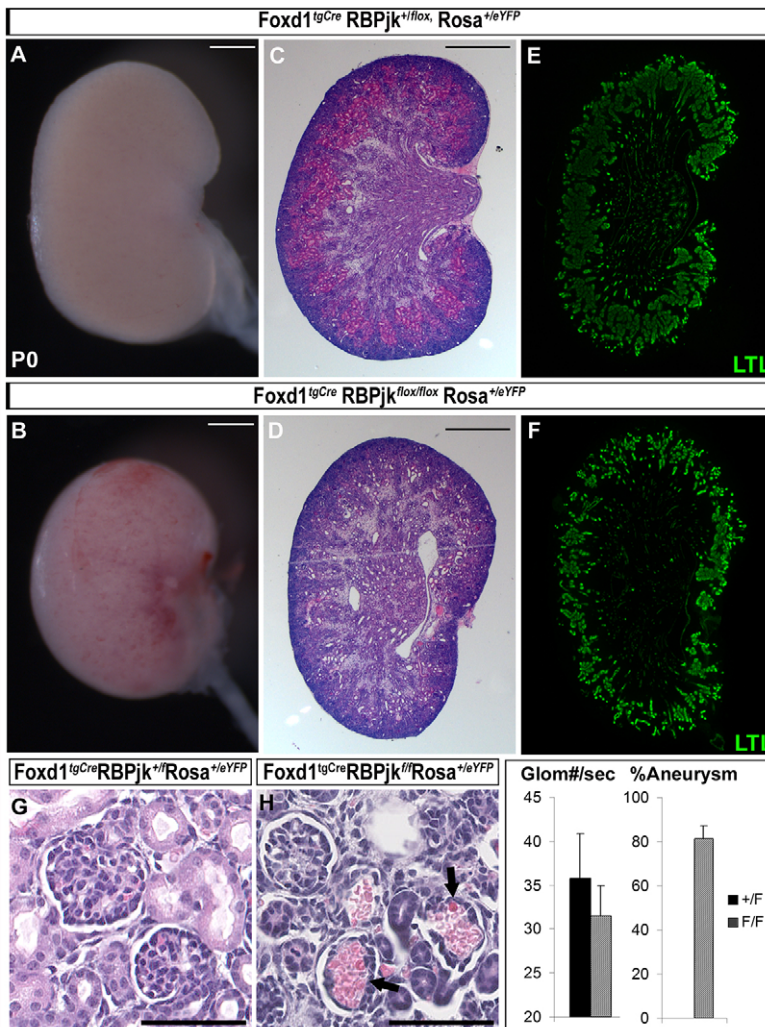
Fig. 2. RBPjk is required in the stromal mesenchyme and its derivatives for proper glomerular formation. *Foxd1tgCre* was used to delete RBPjk from the stromal mesenchyme during kidney development. (A,B) Whole-mount images of control (A) and mutant (B) kidneys demonstrates marked blood spots visible on the surface of mutant kidneys. (C-F) H&E (C,D) and LTL (E,F) staining reveals grossly normal kidney architecture in mutants, but a reduction in proximal tubule density in the cortex. (G,H) Higher magnification of H&E staining shows aneurysm within glomeruli in mutant kidneys. (I) Quantification of glomerular number and percentage of glomeruli that have aneurysm in control and mutant kidneys. Arrowheads, normal glomeruli; arrows, hemorrhaged glomeruli. Panels A-H are representative images across multiple experiments in which the same observations were made. Control, *n*=8; mutant, *n*=12. Scale bars: 500 µm in A-D; 50 µm in G,H.
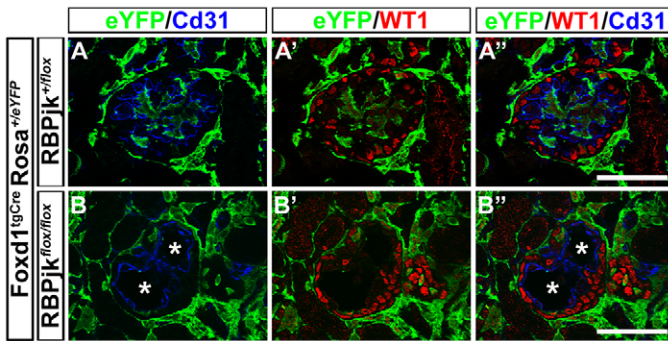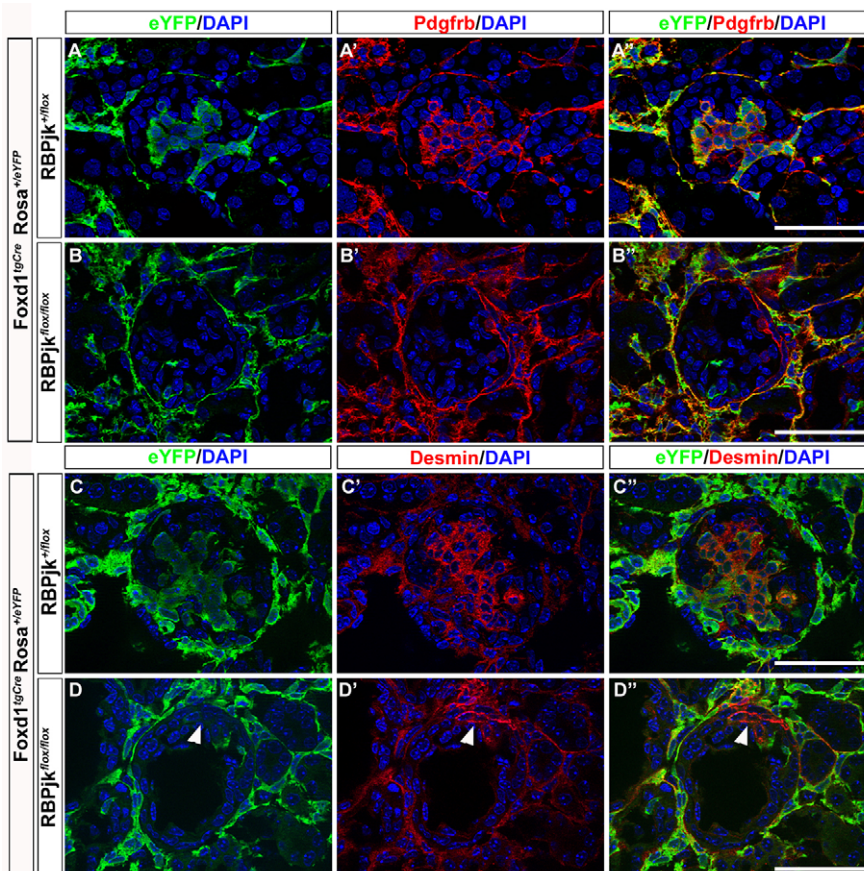
Fig. 3. *Foxd1^tgCre* RPBjk^F/F* Rosa^+/eYFP* glomeruli contain podocytes and severely dilated endothelial capillary loops. (A-A″) *RPBjk^+/flox* kidneys contain glomeruli with a highly organized vascular tree (CD31, blue) surrounded by podocytes (WT1, red). Stromal derived cells are found within glomeruli (YFP, green). (B-B″) *RPBjk^F/F* glomeruli have normal podocyte distribution surrounding a completely dilated vascular endothelium with no capillary loop organization (*). Stroma-derived cells are absent from glomeruli. Scale bars: 50 μm.

protein (eYFP)+ stromal derivatives in the interior of *Foxd1^tgCre* *RPBjk^F/F; Rosa^+/eYFP* glomeruli. These observations strongly suggest that mesangial cells are absent in *Foxd1^tgCre* *RPBjk^F/F* *Rosa^+/eYFP* animals.

To determine whether mesangial cells were indeed absent from glomeruli in *Foxd1^tgCre* *RPBjk^F/F; Rosa^+/eYFP* mice, we stained for two independent mesangial markers: Pdgfrb (Lindahl et al., 1998) and desmin (Hölthofer et al., 1995). In control littermates, both Pdgfrb and desmin are expressed in eYFP-labeled stromal derivatives within glomeruli (Fig. 4A-A″,C-C″). By contrast,

desmin and Pdgfrb expressing cells were absent from the vast majority of mutant glomeruli (Fig. 4B′,B″,D-D″). On the rare occasions when we did observe Pdgfrb+ (not shown) or desmin+ (Fig. 4D″, arrowhead) cells in dilated glomeruli, the cells were eYFP negative, presumably because they failed to express Cre and therefore still had functional RBPjk protein. Alternatively, a subpopulation of mesangial cells may arise from outside of the Foxd1 lineage, although we did not find compelling evidence for this (i.e. a mixture of labeled and unlabeled mesangial cells) in control glomeruli. Together, these data demonstrate that RBPjk is required for the formation of the glomerular mesangium. The absence of these cells compromises capillary loop organization and structure, resulting in glomerular aneurysm and nonfunctional kidneys. It is also possible that glomerular aneurysm reflects abnormalities in one or both of the other two primary cell types (endothelium and podocytes) that join with the mesangium to compose the interior of the mature glomerulus.

To examine if loss of fenestrated endothelial cells or loss/effacement of podocyte foot processes has occurred in mesangium-deficient glomeruli, we analyzed mutant glomeruli by transmission electron microscopy in P0 mice. Despite the fact that mutant mice have a single, dilated glomerular space, the endothelial cells clearly have fenestrations (supplementary material Fig. S1, arrows), basement membrane is normally deposited and podocytes form a morphologically normal slit diaphragm (supplementary material Fig. S2, arrowheads). These data demonstrate that mesangial cells are not required for acquisition of fenestrations by glomerular endothelium, that podocytes form normally in *Foxd1^tgCre* *RPBjk^F/F; Rosa^+/eYFP* mice and that the primary defect is loss of mesangial construction, which is necessary for the organization and architecture of capillary loops.

Fig. 4. Mesangial cells are absent from *Foxd1^tgCre* RPBjk^F/F* Rosa^+/eYFP* glomeruli. Two independent mesangial markers, Pdgfrb and desmin, were used to examine glomeruli from *RPBjk^+/F* and *RPBjk^F/F* mice. Pdgfrb (A-B″, red) and desmin (C-D″, red) label stroma-derived (YFP, green) mesangial cells within glomeruli and interstitial fibroblasts outside glomeruli. (A-A″,C-C″) In control animals the stereotypical mesangial tree is seen and the interstitial space is filled with fibroblasts. (B-B″,D-D″) In kidneys with *RBPjk* deleted from stromal mesenchyme derivatives the interstitium forms normally, but the mesangium is completely absent. Occasionally, desmin+ cells were found within glomeruli, but these were invariably eYFP–, indicative of 'escapers' that did not express Cre (D-D″, arrowheads). Scale bars: 50 μm.

VSMCs and interstitium are present in *Foxd1^tgCre RPBjk^F/F Rosa^+/eYFP* kidneys

To determine if RBPjk is required only to establish the mesangial fate from stromal progenitors, or is necessary for stromal differentiation to any lineage, we asked whether removal of RBPjk from the stromal mesenchyme impairs differentiation of VSMCs and/or interstitium, derived from the same progenitors. In both control and mutant kidneys, CD31+ vessels were surrounded by eYFP+ cells (supplementary material Fig. S2A,B). To ask if these cells were differentiated VSMC, we performed double labeling for eYFP/smooth-muscle α-actin (αSMA) and eYFP/desmin. These studies confirmed that RBPjk-deficient VSMCs were specified from the SM and differentiated normally to surround the vessels in mutant kidneys (supplementary material Fig. S2C-F). In addition, although we cannot rule out subtle functional defects in interstitium that would have been revealed with aging, we never saw any evidence for abnormal formation or distribution of interstitial fibroblasts in mutant pups (Figs 3-5).

Stromal progenitor cells do not assume the mesangial cell fate in *Foxd1^tgCre RPBjk^F/F Rosa^+/eYFP* mice

Next, we wanted to determine whether mesangial cells formed but simply failed to migrate into the nascent glomerulus or were never specified from the SM in *Foxd1^tgCre RPBjk^F/F Rosa^+/eYFP* animals. We performed Pdgfrb/eYFP staining at embryonic day 17.5 (E17.5) and examined the nephrogenic zone where glomeruli are forming and early epithelial structures are present. Although all cells exiting the SM compartment express some Pdgfrb, cells closest to forming nephrons express higher levels, and these Pdfgrb^HI cells were routinely found near the lip of the vascular cleft in control comma-shaped bodies (Fig. 5A-A″, arrowheads). In S-shaped bodies, Pdgfrb^HI/eYFP double-positive cells were always found invading the vascular cleft and subsequently pushing into immature glomeruli during the cup stage (Fig. 5C-C″,E). In *Foxd1^tgCre RPBjk^F/F Rosa^+/eYFP* kidneys, cells still expressed Pdgfrb, but invading Pdgfrb^HI cells were conspicuously absent. Instead of bright Pdgfrb^HI cells surrounding comma- and S-bodies, Pdgfrb staining was uniform and Pdgfrb+ or eYFP+ cells did not invade the vascular cleft at any stage of development (Fig. 5B-B″,D-D″, arrowheads). Further, glomerular cups were devoid of eYFP+ cells and vascular dilation was already evident (Fig. 5F). These data indicate that the absence of mesangial cells in RBPjk mutants is the result of an early defect that either prevents precursors from entering the vascular cleft of the S-shaped body, affects their proliferation/survival or prevents their specification from the SM.

To determine if removal of RBPjk impacted proliferation or survival of cells that have the potential to become mesangium, we examined phospho-histone H3 (PHH3) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) status in eYFP+ cells in control and *Foxd1^tgCre RPBjk^F/F Rosa^+/eYFP* kidneys at E18.5. This analysis is complicated by the fact that there are no known markers for cells specifically fated to become mesangial. To examine only those cells that had the potential to become mesangial, we only interrogated cells located in the peripheral stromal compartment or in the nephrogenic zone. An average of ~5.5 eYFP+/PHH3+ cells per section were observed in both mutant and control kidneys (supplementary material Fig. S3A,B, *P*=0.798). Similarly, TUNEL analysis did not detect an increase in the number of TUNEL+ cells with an average of ~3 cells in each 20x section (supplementary material Fig. S3C-D, *P*=0.87). These data demonstrate that the failure of mesangial cells to form in *Foxd1^tgCre RPBjk^F/F Rosa^+/eYFP* animals is not the result of overt
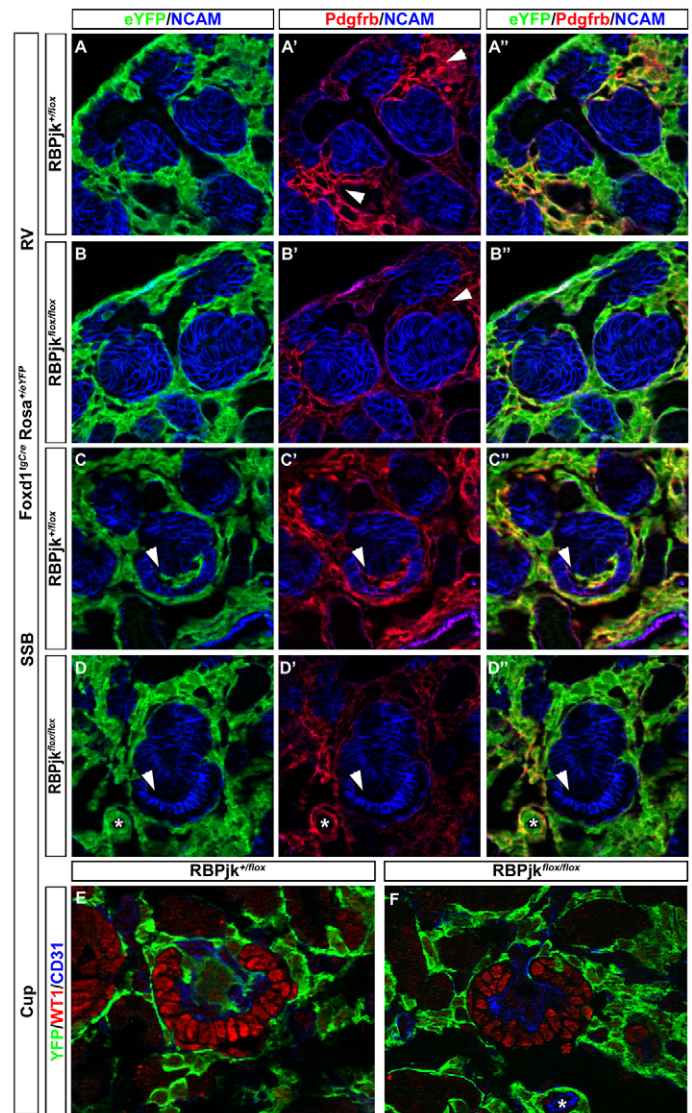

Fig. 5. Stromal derived cells fail to enter the vascular cleft in *Foxd1^tgCre RPBjk^F/F Rosa^+/eYFP* mice. (A-B″) In control kidneys cells expressing high levels of Pdgfrb (arrowheads) are found around early epithelial structures. These Pdgfrb 'high' cells are not evident in *Foxd1^tgCre RPBjk^F/F Rosa^+/eYFP* kidneys. (C-D″) Pdgfrb+, eYFP+ stromal derived cells are absent from the vascular cleft of SSBs (arrowheads). (E,F) At the 'cup' stage normal podocyte patterning and vascular endothelial cells are evident in mutant kidneys, but YFP+ stromal derived cells are not found in the interior of developing glomeruli.

changes is either proliferation or apoptosis in the SM or SM-derived cells.

If the absence of a mesangial cells is strictly a chemotactic defect caused by the failure of cells to elevate Pdgfrb and migrate into the cleft, Pdgfrb/desmin-expressing cells should be present near the SSB cleft as seen in Pdgfβ ligand and receptor mutants. In control kidneys, we could readily identify desmin+, eYFP+ cells entering the vascular cleft (Fig. 6A-A″). By contrast, desmin+ cells were absent from both the vascular cleft and from the area around mutant SSBs (Fig. 6B-B″). Combined, these data demonstrate that RPBjk-deficient progenitors fail to produce desmin-positive cells capable of assuming the mesangial fate, and that VSMC cells are not able to substitute for the lost mesangial progenitors. These analyses support the conclusion that RBPjk is required specifically in the mesangial
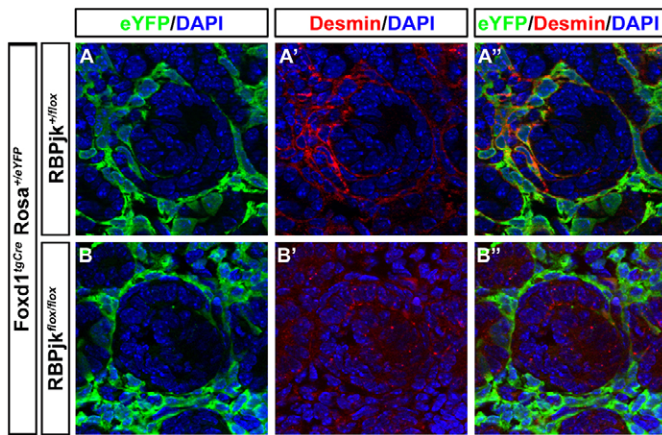
Fig. 6. Desmin+ cells are absent from the area around the SSB in ***Foxd1^{tgCr}e RPBjk^{F/F} Rosa^{+/eYFP}*** **animals.** To distinguish between a mesangial migration defect (as in seen in *Pdgfb/Pdgfrb* mutants) and a failure of formation, we looked for desmin+ cells around developing nephrons. (A-A″) In control animals desmin+ (arrowheads) cells can be found around comma-shaped bodies and streaming into the vascular cleft. (B-B″) Desmin+ cells are absent in the area around comma-shaped bodies and from the vascular cleft in *Foxd1^{tgCre} RPBjk^{F/F} Rosa^{+/eYFP}* kidneys.
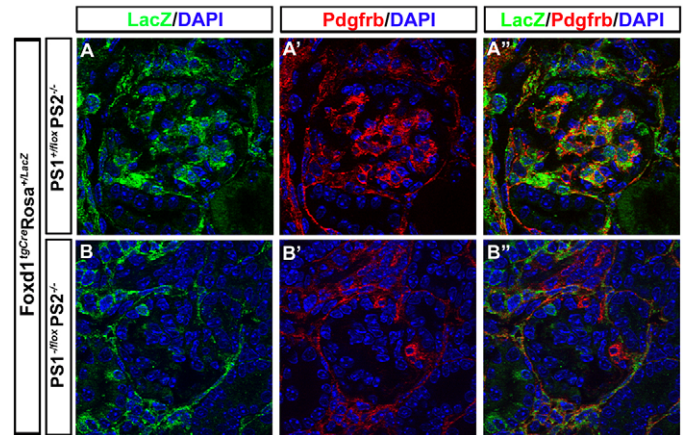
Fig. 7. Formation of the mesangium requires the canonical Notch signaling pathway. All Notch receptors require γ-secretase cleavage for activation. To determine if mesangial cell specification occurs through the canonical pathway or if RBPjk functions independently of Notch receptor activation we deleted PS1 and PS2, the catalytic subunits of γ-secretase. (A-A″) Mesangial cells form normally in *Foxd1^{tgCre}; PS1^{+/F}; PS2^{−/−}; Rosa^{+/R26R}* mice. (B-B″) Deletion of both PS1 and PS2 in *Foxd1^{tgCre}; PS1^{F/F}; PS2^{−/−}; Rosa^{+/R26R}* mice blocks mesangial cell development and phenocopies deletion of RPBjk. Scale bars: 50 μm.

lineage, and not for the general differentiation of all stromal derivatives.

Formation of the mesangium requires the canonical Notch signaling pathway

Given that all of our experiments thus far have utilized RBPjk conditional alleles, it is formally possible that we are documenting a Notch-independent but RBPjk-dependent role (Johnson and Macdonald, 2011) during mesangial cell specification. To establish if mesangial fates are Notch dependent, we removed presenilin 1 and 2 (PS1 and PS2; Psen1 and Psen2 – Mouse Genome Informatics) from stromal derivatives. Presenilins contain the catalytic site of the γ-secretase enzyme, a protease that cleaves a number of Type I proteins within their transmembrane domain and are required for the activation of all four Notch receptors (Kopan and Ilagan, 2009). One copy of PS1 in the absence of PS2 is sufficient to fulfill this function. If Notch proteins were involved in this process, we would expect the phenotype to be identical to the RBPjk mutants. Conversely, if RBPjk functioned in a Notch-independent manner, we would expect these mutants to have no mesangial cell phenotype. *Foxd1^{tgCre} PS1^{Δ/f} PS2^{−/−} Rosa^{+/R26R}* mice had smaller kidneys at P0 and the majority of their glomeruli did not contain mesangial cells (Fig. 7). The frequency of glomerular aneurysm and the survival time of PS mutant mice were more variable than those of RBPjk mutants, presumably owing to the fact that a catalytic activity may perdure longer post-deletion than a downstream effector. Nonetheless, we consistently observe that a majority of glomeruli lack a mesangium. These data indicate that RBPjk is functioning through the actions of Notch receptors during the specification of mesangial cells.

Notch receptors act redundantly in mesangial cell specification

We have shown here that both Notch1 and Notch2 are activated in the mesangium (Fig. 1). To determine if they act redundantly or, as we have demonstrated previously, one receptor has a dominant role (Cheng et al., 2007; Liu et al., 2013), we used *Foxd1^{tgCre}* to delete

conditional alleles of either *Notch1* or *Notch2* individually. *Foxd1^{tgCre} Notch1^{F/F} Rosa^{+/eYFP}* and *Foxd1^{tgCre} Notch2^{F/F} Rosa^{+/R26R}* animals were born at the expected Mendelian ratios. Unlike *Foxd1^{tgCre} RPBjk^{F/F} Rosa^{+/eYFP}* mice, they survived the perinatal period and matured normally. Examination of glomeruli in these lines with Pdgfrb and eYFP staining revealed a mesangial tree indistinguishable from wild-type controls. These data indicate that either Notch1 or Notch2 can satisfy the requirements for mesangial cell specification. Although at P0, eYFP+, Pdgfrb– podocytes in either mutant or wild-type glomeruli are extremely rare (supplementary material Fig. S4, arrow), by P28 *Foxd1^{tgCre} Rosa^{+/eYFP}* animals had both their podocytes and mesangial cells labeled, indicating that *Notch1* and *Notch2* would eventually be deleted in both the mesangium and in podocytes in the adult. The confounding impact of Notch loss in both cell types prevented us from analyzing the impact of Notch deletion in aging glomeruli.

DISCUSSION
Precursors of stroma-derived lineages experience Notch signaling

The Notch signaling pathway plays an important role in nephron development, but nonepithelial cells within the metanephros also experience Notch signaling based on conditional fate-mapping studies that utilize hybrid receptors to release Cre instead of NICD when bound by ligand. Important caveats should be noted when interpreting the results obtained with these animals. We have found that the C-terminal 6MT substantially reduces the activity of Cre, and this effect will be described in detail in an upcoming manuscript. In addition, these knock-in alleles compete with endogenous Notch receptors for ligand, and each activated Notch::Cre receptor releases one molecule of Cre. Thus, multiple receptors must be activated within a cell for recombination to occur, reducing the likelihood of labeling cells with nonphysiological levels of Notch activation. As a result, *Notch^{IP-CreLO}* alleles preferentially label cells experiencing repeated or high levels of activation; not all Notch-dependent cells within a given lineage will label. Despite these caveats, we see evidence
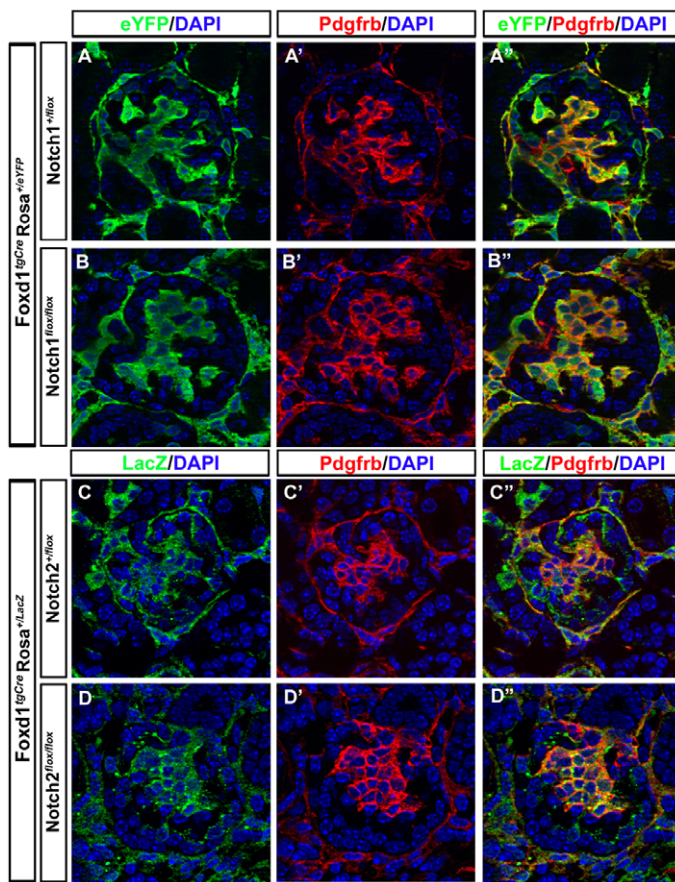
Development

Fig. 8. Notch receptors act redundantly during mesangial cell formation. To determine if Notch1 or Notch2 plays a dominant role during mesangial development, we deleted each receptor individually from the SM. (A-B″) Mesangial trees in *Foxd1tgCre; Notch1F/F; Rosa+/eYFP* glomeruli (B-B″) are indistinguishable from control littermates (*Foxd1tgCre; Notch1+/F; Rosa+/eYFP*, A-A″). (C-D″) Mesangial cells form normally in *Foxd1tgCre; Notch2+/F; Rosa+/eYFP* (C-C″) and *Foxd1tgCre; Notch2F/F; Rosa+/eYFP* glomeruli (D-D″). Scale bars: 50 μm.

for Notch activity in collecting ducts, endothelium, VSMCs, the mesangium, and in many nephron epithelia (the specific contribution of cells experiencing Notch activation will be reported elsewhere). The involvement of Notch in each of these lineages has been previously noted, but a role for Notch signaling in the mesangium has escaped notice due to the severe defects caused when Notch signaling is disrupted.

Mesangial cell specification requires canonical Notch signals, mediated redundantly by Notch receptors

Deletion of γ-secretase or RBPjk resulted in a specific loss of a Pdgfrb[HI], desmin+ cell population that is visible near forming SSBs, which we identified as presumptive mesangial progenitors. Although these cells share the same markers as VSMCs, and both populations detect their target vasculature via Pdgf signaling (Hellström et al., 1999; Hölthofer et al., 1995; Lindahl et al., 1998), it appears that VSMC and mesangial populations are distinct, homing to distinct targets. *Notch2* is expressed in stromal progenitors, but *Notch1* expression is detected only after stromal cells move out of the stromal mesenchyme (Liu et al., 2013). Based on this, we wished to determine whether *Notch2* was dominant in SM derivatives as it is in the CM-derived tissue (Liu et al., 2013).

Our genetic analysis identified no evidence that *Notch1* or *Notch2* were dominant in directing mesangium production from SM progenitors. That was a bit surprising given that a hypomorphic *Notch2* allele produced glomerular aneurysm (McCright et al., 2001), consistent with our phenotype. We think it is most likely that this germline *Notch2Del1* allele used by McCright et al. produced this phenotype as a secondary consequence of the requirement for Notch2 in podocytes and subsequent improper glomerular formation and patterning. Alternatively, the lack of similar phenotype in our experiment may be the result of genetic background. Based on our own unpublished observations, and those of others, the Bl6 background contains unknown modifiers that promote Notch phenotypes in the kidney and elsewhere (McCright et al., 2002). Combined with the expression and activation patterns, our data suggest that the decision is not made in the renal capsule but rather among bipotential (mesangial, VSMC) or tripotential (interstitium, mesangium, VSMC) SM cells migrating out from the renal capsule in a manner that can be fulfilled by either Notch1 or Notch2 alone or with help from Notch3 or 4. The effects of constitutive and persistent Notch activation in stromal progenitors (Boyle et al., 2011) were difficult to interpret because NICD activation leads to loss of Foxd1 expression (supplementary material Fig. S5). Foxd1 loss has a striking impact on the differentiation of the stroma and the CM (supplementary material Fig. S5) (Levinson et al., 2005), confounding the analysis.

Unlike cardiac and pulmonary VSMC (High et al., 2008; Morimoto et al., 2010), Notch signaling is clearly not necessary for Pdgfrb expression in SM derived cells; whether the sole function of Notch is to elevate Pdgfrb levels in a subset of VSMC cells, or activate other targets/receptors that drive cells towards the SSB and the mesangial fate, remains to be determined. In this respect, renal VSMC may resemble the bronchial SMC: both populations form independently of Notch signaling (Morimoto et al., 2010), but neither can replace a vacancy in a nearby and closely related niche. These observations suggest that despite their many superficial similarities, smooth muscle cells form distinct and nonoverlapping populations, some that require Notch signaling and others that do not. It remains to be determined whether elevating Pdgfrb in SMCs will rescue the loss of the mesangium. Further, the identity and location of the Notch ligand(s) required for mesangial cell fate determination remains a mystery.

The impact of SM derivatives on adjacent populations

Signaling from a cell in a developing population to its neighbors is an indispensable part of organogenesis, and is the foundation of kidney development. In addition to the well-characterized interactions between the ureteric bud and the CM that drive branching morphogenesis and nephron differentiation (Costantini and Kopan, 2010), the stromal progenitors play an important, yet poorly understood, role in regulating differentiation of the CM (Levinson and Mendelsohn, 2003). Another regulatory role for a SM-derived population, specifically the interstitium, was uncovered in Wnt7b mutants (Yu et al., 2009).

Two cell types that we thought might be affected by the absence of a mesangium were podocytes and fenestrated endothelium. The mechanisms by which endothelial cells in the developing glomerulus acquire fenestrations are poorly understood. Fenestrations are the pores in endothelial cells that allow filtrate to pass from the capillary loop to the podocyte basement membrane. To our knowledge, no one has asked if signals from the invading mesangium during capillary loop formation are required for acquisition of fenestrations. Our mutant phenotype provides an ideal

platform to investigate this question because cup-stage glomeruli have endothelial cells but lack mesangial support. Our data show conclusively that both fenestrated endothelium and slit diaphragms form in mesangium-free glomeruli, demonstrating unequivocally that these features of the filtration apparatus do not depend on signals from the mesangium.

Finally, we note that in the absence of Notch signaling in the SM, kidney size and PT density was reduced without a dramatic change in glomerular numbers. This may reflect a Notch-dependent activity in the interstitium that regulates the proliferation/growth of nephron epithelia without being required for their differentiation. This possibility is reminiscent of the Wnt7b phenotype. It remains to be determined if Wnt7b expression is affected in these animals, if Notch activity is required in SM progenitors for expression of signals that regulate the CM or if the lack of filtration simply compromises the proper maturation of the kidney (Evan and Tanner, 1986; Tanner and Evan, 1989). Of note, all of the other segments we looked at including loop of Henle, thick-ascending limb and distal tubule were present in mutant kidneys (not shown).

Maintaining the mesangium in aging kidney may require Notch

Although the mesangium was not required for formation of the filtration apparatus (fenestrated endothelium, basement membrane and slit diaphragm), mesangial cells are nonetheless crucial for renal function, and are likely to play a role in the pathogenesis of renal disease. In aging adults, glomerulosclerosis (GS) is an important contributor to end-stage renal disease (ESRD), but the cellular origins and molecular evolution of this condition are still controversial. Because GS is primarily a disease of podocyte dysfunction (Wiggins, 2009), many believe that mesangial changes are simply secondary to podocyte loss. Other observations suggest that increased glomerular volume, perhaps driven by increased endothelial and mesangial cell numbers and/or matrix deposition, acts to expand glomerular basement membrane surface area and drive an increase in podocyte size (Alpers and Hudkins, 2011; Wiggins, 2009). This added stress on podocytes may eventually lead to their loss. To the best of our knowledge, mesangial cell turnover rates during normal aging and following injury have not been investigated. Further, it is unclear what specific contribution mesangial cells make to ESRD, and if targeting the mesangium will result in a therapeutic advantage. Data presented here and elsewhere strongly suggest that Notch signaling may play a role in development of GS. Unfortunately, mice allowing specific manipulation of the mesangium during development and in the adult have yet to be developed. Such tools would allow for detailed investigation of mesangial cell turnover during normal aging and disease and enable the separation of podocyte- and mesangial specific effects in GS.

MATERIALS AND METHODS
Mice
All mouse studies were carried out with the approval of the Washington University Division of Comparative Medicine, Protocol #2011013. *Foxd1^{tgCre}* [Tg(Foxd1-GFP/cre)^{1Amc} (Humphreys et al., 2010)], *Rosa^{eyfp}* [Gt(ROSA)26Sor$^{tm1(EYFP)Cos}$ (Srinivas et al., 1999)], *Rosa^{R26R}* [Gt(ROSA)26Sor^{tm1Sor} (Soriano, 1999)], *RPBjk$^{F/F}$* [Rbpj^{tm1Hon} (Tanigaki et al., 2002)], *Notch1$^{F/F}$* [Notch1^{tm1Rko} (Yang et al., 2004)], *Notch2$^{F/F}$* [Notch2^{tm1Grid} (McCright et al., 2006)], and *PS1$^{F/F}$; PS2$^{−/−}$* [Psen1^{tm1Shn} (Shen et al., 1997); Psen2^{tm1Haa} (Steiner et al., 1999)] alleles have all been previously described. Genotyping was performed using the universal PCR genotyping protocol (Stratman et al., 2003). Primer sequences are available upon request.

Immunofluorescence
Kidneys were dissected from mice at indicated ages, fixed in 4% paraformaldehyde/phosphate-buffered saline (PFA/PBS) with rocking for 4 hours at 4°C, subjected to 15% (4 hours) and then 30% (O/N) sucrose in PBS and embedded in optimal cutting temperature compound (OCT). Sections were cut at 8 μm. For staining, slides were thawed at room temperature (RT) for 10 minutes, washed 3 × 5 minutes in PBS and blocked with 10% donkey serum (DS) in PBS + 1% BSA + 0.2% milk + 0.3% Triton X-100 (PBS-BB) for 60 minutes. Primary antibodies were diluted in PBS-BB and incubated at 4°C overnight as follows: chick anti-GFP (Aves, 1:500), rat anti-Pdgfrb (1:500), rat anti-Cd31 (BD Biosciences, 1:200), mouse anti-smooth muscle α-actin (Sigma, 1:2500), rabbit anti-desmin (Cell Signaling 1:500), mouse anti-NCAM (Sigma, 1:250), rabbit anti-β-gal (Cappel, 1:5000), biotinylated LTL (Vector Labs, 1:250), rabbit anti-WT1 (Santa Cruz, 1:200). Slides were washed three times for 10 minutes in PBS and incubated in secondary antibody as 1:500 in PBS-BB for 1 hour at RT. All secondary antibodies were raised in donkey and purchased from Jackson ImmunoResearch. Slides were washed three times for 10 minutes in PBS and mounted with Vectashield containing 4′,6-diamidino-2-phenyldole (DAPI). Imaging was carried out with a Zeiss Axioimager equipped with an Apotome for optical sectioning. All control and mutant images were photographed using identical exposure times and capture settings.

Proliferation and apoptosis
Proliferation was examined with the above immunofluorescence protocol using dual labeling for αmouse PHH3 (Millipore, 1:500) and YFP. PHH3/YFP double-positive cells in the stromal mesenchyme and nephrogenic zone were counted on 4-6 20× sections from each control (*n*=4) or mutant (*n*=5) animal. Cells that had clearly assumed the interstitial, smooth muscle or mesangial fate were not counted. For apoptosis, we applied the same approach using the In Situ Cell Death Detection Kit (Roche) according to the manufacturer's directions.

Electron microscopy
For transmission electron microscopy, tissues were fixed, embedded in plastic, sectioned and stained as described previously (Noakes et al., 1995).

Glomerular number quantification
P0 kidneys were dehydrated, embedded in paraffin and sectioned at 7 μm through the entire kidney. Every tenth section (70 μm spacing) was collected and stained with H&E. Images were acquired by an automated digital slide scanner (NanoZoomer-XR C12000, Hamamatsu Photomics) at 20× magnification to facilitate uniform acquisition and processing of samples. Five total sections were counted for each kidney; the section most representative of the midline plane and the two sections to either side of the midline (70 and 140 μm). This was done in order to count the largest areas of each kidney and avoid the edges were counts can be more variable. Within this sample the fraction of glomeruli containing aneurysm was tabulated based on histology (see Fig. 1H as an example).

Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.100271/-/DC1

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