Activation of Notch3 in Glomeruli Promotes the Development of Rapidly Progressive Renal Disease

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ABSTRACT

Notch3 expression is found in the glomerular podocytes of patients with lupus nephritis or focal segmental GN but not in normal kidneys. Here, we show that activation of the Notch3 receptor in the glomeruli is a turning point inducing phenotypic changes in podocytes promoting renal inflammation and fibrosis and leading to disease progression. In a model of rapidly progressive GN, Notch3 expression was induced by several-fold in podocytes concurrently with disease progression. By contrast, mice lacking Notch3 expression were protected because they exhibited less proteinuria, uremia, and inflammatory infiltration. Podocyte outgrowth from glomeruli isolated from wild-type mice during the early phase of the disease was higher than outgrowth from glomeruli of mice lacking Notch3. In vitro studies confirmed that podocytes expressing active Notch3 reorganize their cytoskeleton toward a proliferative/migratory and inflammatory phenotype. We then administered antisense oligodeoxynucleotides targeting Notch3 or scramble control oligodeoxynucleotides in wild-type mice concomitant to disease induction. Both groups developed chronic renal disease, but mice injected with Notch3 antisense had lower values of plasma urea and proteinuria and inflammatory infiltration. The improvement of renal function was accompanied by fewer deposits of fibrin within the glomeruli and by decreased peritubular inflammation. Finally, abnormal Notch3 staining was observed in biopsy samples of patients with crescentic GN. These results demonstrate that abnormal activation of Notch3 may be involved in the progression of renal disease by promoting migratory and proinflammatory pathways. Inhibiting Notch3 activation could be a novel, promising approach to treat GN.


GN represents the third leading cause of ESRD worldwide. Because of the lack of efficient treatment, there is an urgent need to understand the underlying mechanisms in order to propose early markers and/or potential therapeutic targets. In this study, we investigated the role of Notch3 in the pathogenesis of GN.

Notch receptors are highly conserved type I transmembrane glycoproteins and are encoded by four genes: Notch1, Notch2, Notch3, and Notch4. They are composed of a Notch extracellular domain (NECD) and a Notch intracellular domain (NICD). The activation of Notch receptors is induced by the interaction of NECD with one of the canonical Notch ligands, which are also type I transmembrane proteins and are encoded by Jagged1 and Jagged2 and Dll1, Dll3, and Dll4 genes.1 NECD binding to Notch ligands leads to the cleavage of Notch and to the release of NICD. The NICD then translocates to the nucleus.

where it interacts with the RBPJκ cofactor and initiates the transcription of Notch target genes, most belonging to the Hairy Enhancer of Split (HES) and/or Hairy Enhancer of Split-Related Genes (HEY) gene families.2–3 Several structural differences between the intracellular domains of Notch1–Notch4 receptors have been evidenced, which may account for their functional divergence and differential activation of Notch target genes. For example, the Notch3 extracellular domain specifically lacks EGF repeat 21 and parts of EGF repeats 2 and 3, which other Notch receptor subtypes possess.4 In addition, mutations in specific Notch receptors result in distinct pathologies, such as the mutation of the Notch3 gene resulting in cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) syndrome.5

Most studies on Notch in kidney diseases have primarily focused on Notch1 or on Notch1-induced signaling.6–8 In vitro investigations suggested that an abnormal activation of the Notch pathway in podocytes promotes cytoskeleton disruption and death by mitotic catastrophe, and that inhibition of the Notch pathway in mouse models of FSGS improved proteinuria and reduced podocyte loss during the initial phases of glomerular injury.9 However, few studies have specifically examined the role of Notch3 in renal physiology and pathophysiology. The expression of Notch3 is undetectable in the glomeruli of normal kidneys, but is clearly evident in nuclei of podocytes within the glomeruli of patients with lupus nephritis or FSGS.9 We recently reported that Notch3 is involved in the regulation of renal vascular tone.10 In subsequent studies, we showed that Notch3 is induced in renal tubular epithelial cells subjected to ureteral obstruction and promotes renal inflammation and fibrosis.11

In this study, we examined the role of Notch3 in a model of rapidly progressive renal disease and found that activation of Notch3 in podocytes induces phenotypic changes associated with cell migration, inflammation, proteinuria, and renal function loss. Inversely, mice lacking Notch3 expression were protected from the decline of renal function. Moreover, we used a pharmacogenetic strategy (administration of antisense) oligodeoxynucleotides (ODNs) to block the de novo activation of Notch3 and found that specific inhibition of Notch3 expression preserved podocyte structure and renal function. Our results suggest that abnormal activation of Notch3 in glomeruli promotes renal disease and that specifically targeting Notch3 neactivation can be a promising therapy for glomerular diseases.

RESULTS

Notch3 Receptor and HeyL Expressions Are Increased in Glomeruli of Mice Injected with Nephrotoxic Sheep Serum

Transcriptional analyses of Notch receptors in kidneys 5 and 9 days after nephrotoxic sheep serum (NTS) administration showed that only Notch3 mRNA was upregulated at these time points (Figure 1A). Moreover, quantification of the canonical Notch target genes Hey1, Hey2, HeyL, and Hes1–Hes5 showed that only HeyL was upregulated. HeyL expression was induced at day 5 and increased at day 9 (Figure 1B). In terms of Notch ligands, only Jagged 2 showed a small but significant upregulation (Supplemental Figure 1). Immunostaining confirmed previous results that Notch3 is expressed in vascular smooth muscle cells under normal conditions (Figure 1C).10 However, an extensive glomerular staining was observed in mice after NTS administration. To localize the glomerular cell type in which this de novo Notch3 expression occurred, we used confocal microscopy and found that Notch3 colocalized with nestin, suggesting a specific expression of Notch3 in podocytes after injury (Figure 1D). The activation of Notch3 appears to be experimental-model dependent because its expression increased in the NTS, ureteral obstruction, and ischemia-reperfusion models, but not in the hypertensive RenTg model (Supplemental Figure 2).

Mice Lacking Notch3 Expression Are Protected from NTS-Induced Renal Injuries

Both Notch3 knockout (KO) mice and their wild-type (WT) littermates developed renal failure after NTS injections. The quantity of injected sera, evaluated by ELISA, was similar in strains (Supplemental Figure 3). However, proteinuria and uremia were significantly decreased in KO mice (Figure 2, A and B). Western blotting showed that nephrin expression decreased significantly in NTS/WT mice, whereas it remained preserved in NTS/KO mice (Figure 2, C and D). Histologic evaluation confirmed this protection because crescentic glomeruli (Figure 2, E and F) and fibrin deposits (Figure 2F) were decreased in NTS/KO mice compared with NTS/WT mice. Quantification of HeyL expression by Western blotting indicated that the NTS-induced upregulation of HeyL disappeared in Notch3 KO mice (Figure 2, H and I), confirming a specific activation of the Notch3-HeyL pathway.

Notch3 Deletion Reduces Renal Inflammation and NF-κB Activation in Glomeruli

Evaluation of macrophage and monocyte infiltrates, by performing F4/80 staining, indicated that the NTS/KO group had fewer interstitial and periglomerular infiltrates (Figure 3, A and B). CD68 staining to detect active macrophages confirmed intraglomerular infiltration in WT and their reduced presence in Notch3 KO mice (Supplemental Figure 4). Accordingly, the induction of inflammatory markers such as vascular cell adhesion molecule-1 and monocyte chemoattractant protein-1 (MCP-1) was significantly reduced in NTS/KO mice (Figure 3, C and D). Because the NF-κB pathway is activated in experimental and clinical GN,12,13 we performed protein expression analysis for the p65 and phospho-Ser536-p65 subunits (Figure 4). Both are faintly expressed in normal conditions, but were highly induced after NTS treatment in WT mice (Figure 4, A and B). This upregulation was significantly diminished in mice lacking Notch3 expression. Immunofluorescence staining showed that p65 activation was localized within podocytes, stained with nestin (Figure 4C).
Podocytes Acquire a Migratory Phenotype upon Induction and Activation of the Notch3 Pathway

To investigate the underlying cellular mechanisms of Notch3 activation in glomeruli during the progression of renal disease, podocytes were infected with an adenovirus expressing the Notch3 constitutively active intracellular domain (Notch3-ICD-IRES-EGFP [N3ICD]). A change in podocyte shape was observed after activation of the Notch3 pathway (Figure 5A). Phalloidin staining indicated a reorganized actin cytoskeleton, which appeared more elongated, a feature corresponding to a migratory phenotype (Figure 5B). Interestingly, the NF-κB subunit p65 was induced in podocytes expressing N3ICD, but not in controls (Figure 5C). Infection with an adenovirus expressing β-galactosidase (Ad-βGal) was used as a control (Figure 5, D–F). N3ICD overexpression increased expressions of HeyL, MCP-1, and Snail1 with concomitant decrease of podocin (Figure 5G, Supplemental Figure 5).

To confirm the migratory phenotype of podocytes after expression of N3ICD, a wound-healing assay was performed (Figure 6, A and B). After 16 hours, the scraped zone had decreased by 25% in podocytes expressing N3ICD, whereas it remained the same in control (Ad-βGal) podocytes. Inhibition of the NF-κB pathway blunted the migration of podocytes expressing N3ICD, whereas it had no effect in podocytes expressing only βGal.

To test whether a similar phenomenon occurs in vivo, we measured podocyte outgrowth in glomeruli freshly isolated from WT and KO mice in the early phase of disease (4 days). We found that the outgrowth area of glomeruli of mice lacking

Figure 1. Notch3 receptor expression is induced during progressive GN and localized mainly in podocytes after injury. (A) Among Notch receptors, Notch3 is upregulated in the kidney of NTS-injected mice at the transcriptional level as soon as day 5. (B) Among Notch target genes, only HeyL is significantly upregulated. (C) Immunostaining shows that under normal conditions, Notch3 is expressed in renal resistance vessels in WT mice (left panels), whereas it is strongly induced in glomeruli after NTS administration (middle and right panels). *P<0.05 and **P<0.01 compared with vehicle-treated mice (n=6 and 9 mice per strain for the vehicle- and NTS-treated groups, respectively). (D) A representative example of a confocal microscopy experiment showing that Notch3 (green) is absent in glomeruli under normal conditions (upper panels). By contrast, a strong de novo expression of Notch3 appears and colocalizes with nestin (red) after induction of the glomerular disease (lower panels). Photos of the left panels are at a magnification of ×63, whereas the right panels are zoomed images of the glomeruli of the left panels. D5, day 5; D9, day 9; Veh, vehicle.
Notch3 expression was significantly lower compared with that of WT mice (Figure 6, C and D). The migrating cells highly express nephrin (Figure 6E, Supplemental Figure 6).

**In Vivo Administration of Notch3 Antisense Blunts the Development of GN**

Next, we investigated the effects of inhibiting Notch3 expression in adult WT mice during the progression of GN. To this end, antisense oligonucleotides and their scrambled sequences as controls were administered daily in NTS-treated mice (Supplemental Figure 7). Once again, we observed that among the different members of the Notch family, only Notch3 and HeyL expressions were induced during the progression of the disease (Figure 7). Antisense treatment significantly decreased Notch3 and HeyL expressions to their normal levels. Scrambled ODN administration had no effect on Notch3-HeyL activation (Figure 7), demonstrating the efficiency and specificity of Notch3 antisense treatment.

Antisense, but not scrambled, treatment attenuated NTS-induced proteinuria and uremia (Figure 8, A and B). This functional protection was accompanied with an overall preservation of renal structure (Figure 8C), and decreased numbers of both crescents (Figure 8D) and fibrin deposits (Figure 8E) within glomeruli. Mice treated with antisense showed fewer interstitial and periglomerular macrophage infiltrates than NTS/scrambled mice (Figure 9, A and B). In addition, Notch3 antisense inhibited the NTS-induced activation of PDGF and EGF pathways (Figure 9C).

**Notch3 Expression in Human Biopsies**

To investigate whether Notch3 induction is relevant in human glomerular diseases, we performed Notch3 immunostaining in
human kidney biopsies from three patients with extracapillary GN. A strong staining of the N3ICD was observed within all glomeruli of the three patients tested showing an activation of the Notch3 pathway (Figure 10). Nephrin staining was negligible in these glomeruli probably due to the advanced degree of the disease and the structural decomposition in these patients (Supplemental Figure 8).

DISCUSSION

The aim of our study was to investigate the role of Notch3 receptor activation in a model of progressive and severe nephropathy. To our knowledge, we provided evidence for the first time that an abnormal activation of the Notch3 pathway occurred in glomeruli after administration of nephrotoxic serum, specifically in injured podocytes. This activation was associated with major alterations of glomerular filtration and renal structure. By contrast, the renal function of mice lacking Notch3 expression was relatively preserved, because these mice showed less proteinuria and inflammation accompanied with decreased formation of crescents and fibrin deposits. A similar protection was found when WT animals were treated with specific Notch3 antisense ODNs.

Crescent formation is a common feature of rapidly progressive GN. One of the causes of crescent formation is podocyte migration to the urinary space.14 Developing new therapeutic approaches to interfere with the events associated with this pathologic process is still considered as one of the major challenges in the field. To this end, identification of new

Figure 3. Mice lacking Notch3 expression exhibit less renal inflammation. (A) Representative examples of monocyte/macrophage infiltration visualized by F4/80 staining in the renal cortex of WT and Notch3 KO mice with or without NTS administration. (B) Quantification of F4/80-positive infiltrating cells shows statistically significant protection of the Notch3 KO mice. (C and D) Accordingly, the activation of other inflammatory markers such as VCAM-1 and MCP-1 are lower in Notch3 KO mice. *P<0.05; **P<0.01; ***P<0.001 (n=6 and 9 mice per strain for the vehicle- and NTS-treated groups, respectively). VCAM-1, vascular cell adhesion molecule-1; Veh, vehicle.
key regulators involved in podocyte detachment from capillaries is essential in order to develop new therapeutic approaches to improve or even reverse this process. In this regard, the results with the Notch3 antisense treatment suggest that a future therapy could target the activation of the Notch3 pathway.

Our results are consistent with the notion that Notch3 is a new upstream actor in podocyte detachment. The glomerular outgrowth mimics crescent formation with podocyte detachment and migration. We found that podocytes acquire a migratory phenotype upon activation of the Notch3 pathway and we showed that Notch3 deficiency after NTS injection is associated with a decreased degree of glomerular outgrowth (Figure 6). These experiments strongly support the involvement of Notch3 activation in podocyte motility and in crescent formation. Several investigators have proposed the activation of growth factor receptor pathways, such as PDGF or EGF, as hallmarks of podocyte-activation and crescent formation during GN.15–17 Because Notch3 was found to induce activation of these growth factor receptors in lung cancer and in smooth muscle cells,18,19 it is possible that a similar interaction occurs in the activated podocytes. In agreement with this hypothesis, Notch3 antisense delivery inhibited the NTS-induced activation of these pathways in our study (Figure 5).

In the NTS model, renal injury is initiated in the glomeruli but it progresses and affects all renal compartments. Even when macrophage infiltration is absent in glomeruli, tubulointerstitial damage occurs characterized by tubular dilation and atrophy, interstitial infiltration, and fibrosis.20,21 It is probable that damaged glomeruli secrete chemokines and cytokines that can favor infiltration in the interstitium. Our hypothesis is that Notch3 activation mediates the initiation of glomerular injury and the subsequent progression of renal disease. Blocking this activation will alleviate the initiation of the pathologic mechanism and will protect against the further development of the nephropathy.

In addition, we found that Notch3 is involved in NF-κB pathway activation, especially in the p65 subunit in podocytes. A previous study indicated that the p65 subunit is localized in crescents from patients with RPGN.13 A recent work showed

Figure 4. Notch3 activates the NF-κB pathway during progressive GN. (A and B) Examples of Western blot experiments indicating that the p65 and phospho-p65 subunits are upregulated during NTS-induced renal disease in WT, but not in Notch3 KO mice. (C) A representative example of a confocal microscopy experiment shows that the p65 subunit (green) is highly detected in the glomeruli, especially in the podocytes (marked by nestin in red) of WT mice after NTS administration, whereas it is present to a much lesser degree in Notch3 KO mice after NTS administration. Nuclei are stained by TO-PRO-3 in blue. *P<0.05; **P<0.01 (n=3 mice per strain for the vehicle- and NTS-treated groups, respectively). Veh, vehicle.
that the genetic deletion of the NF-κB pathway preserved foot process integrity in an experimental model of GN. Moreover, it has been shown that the inhibition of Jagged1 in astrocytes inhibited Notch3 expression and decreased the LPS-induced translocation of NF-κB p65. This suggests that Jagged1-Notch3 sustains the inflammation through NF-κB in these cells. In this study, we provide several elements showing the interaction between Notch3 and inflammation.

We observed that NTS induces a de novo expression and activation of the Notch3 receptor in glomeruli and podocytes, which is concomitant to macrophage infiltration as well as to the increase of adhesion molecule and chemokine expression (Figure 3, Supplemental Figure 4). By contrast, genetic (KO) or pharmacogenetic (antisense ODNs) inhibition of Notch3 is accompanied by decreased macrophage infiltration and decreased expression of adhesion molecules and chemokines (Figures 3 and 9). In addition, we show a local activation of the NF-κB pathway, which is blunted in mice lacking Notch3 (Figure 4). Overexpression of N3ICD in podocytes is accompanied by increased cell motility, whereas inhibition of the NF-κB cancels this effect (Figure 6). This overexpression of N3ICD is accompanied by an important increase of HeyL and MCP-1 expressions. ***P<0.001.

Figure 5. Notch3-induced phenotypic changes in cultured podocytes. (A–C) Representative examples of phase-contrast microscopy experiments showing that podocytes infected with Ad-N3ICD show a more elongated shape (A) associated with a major actin cytoskeleton reorganization (B) and induction of the p65 NF-κB subunit in nuclei (pointed out by white arrows) (C). (D–F) Podocytes transfected with a control Ad-βGal virus do not show any of the above-described alterations. (G) This overexpression of N3ICD is accompanied by an important increase of HeyL and MCP-1 expressions. ***P<0.001.
(UOO) model, a classic model of tubular inflammation. Recent studies observed an interaction between Notch3 and p65/NF-κB to regulate T cell function under control conditions or in disease. A pathogenic role for the Notch3 receptor was first described in CADASIL syndrome. CADASIL syndrome is a systemic arteriopathy attributed to Notch3 mutations that induce an accumulation of the extracellular domain of Notch3 in the vascular smooth muscle cells of the media layer of arteries. However, most studies have examined the role of Notch3 either during vascular development or during tumor growth. Regarding the involvement of the Notch family in CKD, the majority of existing studies have focused on the role of Notch1. It was shown that conditional induction of the intracellular domain of Notch1 expression in podocytes induces apoptosis through p53, which results in glomerulosclerosis and proteinuria. Notch1 upregulation was found in patients with diabetic nephropathy and FSGS. Notch1 was also found to be induced in tubular epithelial cells, promoting tubulointerstitial fibrosis through HeyL.

A few studies have only recently explored the involvement of Notch3 in renal physiopathology. In the model of folic acid–induced renal disease, the expressions of all Notch receptors were increased and Notch1 protein expression was found in the tubular epithelial and interstitial cells. A significant increase of Notch3 was observed in NTS, UUO, and ischemia-reperfusion models. By contrast, Notch1 mRNA expression was only increased, to a much lesser extent than Notch3, in the UUO model. Thus it appears that Notch1 and Notch3 are not redundant and are selectively activated in a cell- and model-specific way. We found that Notch3 participates in the maintenance of renal autoregulation by contributing to the normal development of renal resistance vessels during early life. Other investigations proposed that after injury, activation of Notch3 is essential for differentiation of parietal epithelial cell progenitors into podocytes. In our study, we did not detect a significant Notch3 staining in parietal epithelial cells of WT mice after administration of NTS. In the anti-Thy1.1 model of renal disease,
Notch3 was found to be induced in mesangial cells and this de novo expression was associated with the induction of Hey2 and Hes2 target genes.25 We recently showed that Notch3-HeyL pathway activation promotes tubulointerstitial fibrosis and inflammation by increasing MCP-1 synthesis in the UUO model.11 These findings suggest that activation of the Notch3-HeyL pathway can be a common renal pathogenic mechanism promoting glomerulosclerosis, tubulointerstitial fibrosis, and renal inflammation. It was also recently shown that the inhibition of HeyL induces the decrease of proinflammatory cytokines in breast cancer cells.26 The abnormal overexpression of Notch3 in glomeruli of patients with Goodpasture’s syndrome indicates the relevance of Notch3 activation in renal disease. The potential of a therapy based on specific targeting of the Notch3-HeyL pathway is supported by our results concerning in vivo administration of Notch3 antisense ODNs (Figures 8–10). These mice were protected from disease progression in all examined functional, structural, and inflammatory parameters compared with the scrambled-treated littermates. These results also demonstrate that only the neoactivation of the Notch3 pathway is detrimental and that it is sufficient to inhibit this neoexpression to obtain beneficial effects. Of course, the ODN delivery is systemic and affects all sites of Notch3 expression. The antisense strategy was used to replace a pharmacologic approach (Notch3-specific inhibitors are not available) and to provide a first element of proof of the concept that specifically blocking Notch3 activation can have beneficial effects in a renal disease. Thus, the validation of a therapy based on Notch3 inhibition would require the synthesis of specific pharmacologic inhibitors, because the existing inhibitors, such as γ-secretase inhibitors, lack specificity and the use of long-lasting treatments with ODN antisense in patients with chronic renal disease can have adverse effects.

In conclusion, we demonstrate that the Notch3 receptor is induced in podocytes after glomerular aggression. This abnormal expression is associated with the transition toward a migratory and proinflammatory phenotype that promotes crescent formation, proteinuria, and renal inflammation and the decline of renal function. Genetic deletion or pharmacogenetic inhibition of Notch3 protects animals against the development of renal disease by preserving podocytes. These results imply that Notch3 is a new key regulator of the pathophysiologic processes occurring during the progression of renal disease and that blocking its activation could be a new therapeutic approach for this incurable pathology.

CONCISE METHODS

Animals
All mice were handled in strict accordance with good animal practice as defined by the relevant national animal welfare bodies of France, and all animal work was approved by the appropriate committee of the National Institute for Health and Medical Research (INSERM) and the Pierre and Marie Curie University (Paris, France). Animals were housed at constant temperature with access to water and food ad libitum. Mice lacking the Notch3 gene (KO) and their WT littermates on the Sv129 background were used. Initially, the KO animals were provided by Dr. Anne Joutel in a B6/C57 background.10,11 These animals were backcrossed 10 times in a Sv129 background in our animal facility. Decomplemented NTS was prepared as previously described.27 Female mice (aged 3 months) received intravenous injections of a total 30 μL of NTS/g body weight over 3 consecutive days (days 0, 1, and 2) to induce crescentic GN. Control mice were injected with PBS. None of the animals died before the end of the protocol. Mice were euthanized 9 days after the first injection. Blood, urine, and renal...
tissues were collected for subsequent analyses. Nine mice per strain were injected with NTS and six mice per strain were used as vehicle-injected controls. For additional details for experimental protocols see the supplemental material.

Administration of Antisense ODNs Targeting Notch3
Because the dose of NTS used in the above-described series of experiments produced severe proteinuria, we preferred to use a dose of NTS producing milder proteinuria in this set of experiments. In this series, the serum was diluted by one-half compared with the previous series. The choice was based on preliminary experiments testing proteinuria at different NTS dilutions.

Notch3 expression was inhibited with a cocktail of two different sequences of ODNs specifically targeting Notch3 mRNA, designed using IDT DNA (Integrated DNA Technologies). Sequences were modified with phosphorothioate to prevent their in vivo hydrolysis by exonucleases (Sigma-Aldrich, St. Quentin Fallavier, France, Table 1). The absence of cross-reactivity with related sequences in GenBank was checked. Control groups were treated with a cocktail of two scrambled sequences. The antisense or scrambled ODNs were diluted in saline sodium chloride and administrated by intraperitoneal injections every 24 hours (100 pmol ODN per injection), with a pre-injection 48 hours before the administration of nephrotoxic serum injections. Five mice were used in each vehicle-injected group and nice mice were utilized in each NTS-injected group.

Proteinuria and BUN Measurements
Urine was collected at days 0, 4, and 9. Proteinuria was measured using utilizing a Konelab automater (Thermo Fisher Scientific, Waltham, MA), and was normalized to urine creatinine. BUN levels were measured with an enzymatic method (Konelab automater) and expressed in millimoles per liter.
Evaluation of Histologic Parameters

Half of the kidney from each animal was fixed in alcohol/formalin/acetic acid, dehydrated, and embedded in paraffin. Sections (4-μm thick) were placed onto glass slides heated at 56°C for 2 hours and were stained with Masson’s trichrome solution.

Crescents were quantified by examining microphotographs of random, nonoverlapping fields. A minimum of 30 glomeruli per mouse were evaluated. Results were expressed as the percentage of crescentic glomeruli per evaluated glomeruli.

Fibrin deposition was evaluated by counting the number of individual deposits per glomerulus and was expressed as the average of the number of individual deposits per glomerulus. Crescents and fibrin deposits were evaluated blindly.

In Vitro Experiments with Cultured Podocytes

A previously described, conditionally immortalized mouse podocyte cell line was maintained in RPMI 1640 (Gibco BRL) supplemented with 10% FBS, 100 U/ml penicillin/streptomycin (Gibco BRL), and 10 U/mL recombinant mouse γ-IFN (Peprotech, Rocky Hill, NJ) to induce synthesis of the immortalizing T antigen. Cells were stored in humidified incubators with air and 5% CO₂ at 33°C. Subcultures were obtained with trypsin after cells had reached confluence. To initiate differentiation, cells were thermoshifted to 37°C and maintained in medium without γ-IFN for 2 weeks.

Podocytes were serum starved for 24 hours and infected with adenoviruses expressing either β-galactosidase-GFP (Ad-βGal) or Notch3 intracellular domain Notch3 ICD-EGFP (Ad-Notch3 ICD) at a moi of 10. Infection efficiency was controlled by counting GFP-positive versus non-GFP-positive cells. The cells were harvested, and total RNA was extracted. Before the scratch assay, cells were infected with Ad-βGal or Ad-N3ICD for 32 hours. The scratch was then made and images were taken 16 and 24 hours later. The NF-κB inhibitor used was BAY11-7082 (Calbiochem) dissolved in DMSO at a concentration of 1 μM. Nontreated wells received the same concentration of DMSO.

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In this concentration, the NF-κB inhibitor had no toxicity to the cells (Supplemental Figure 9).

Figure 9. Notch3 antisense treatment decreases interstitial and periglomerular inflammatory infiltrates and the activation of promigratory growth factor receptor pathways. (A) Representative examples of monocyte/macrophage infiltration (F4/80 staining) in the renal cortex of mice treated with Notch3 antisense or scrambled ODN with or without NTS administration. (B) Quantification of F4/80-positive infiltrating cells shows a statistically significant protection of Notch3 antisense-treated mice. (C) The NTS-induced activation of promigratory pathways such as PDGF and EGF is inhibited in the Notch3 antisense-treated mice. *P<0.05; **P<0.01; (n=5 and 9 mice per treatment for the vehicle- and NTS-treated groups, respectively). AS, antisense; SCB, scrambled; Veh, vehicle.
Isolation of Glomeruli and Outgrowth Experiments

Glomeruli were isolated by two-step sieving of renal cortices 4 days after NTS injection in WT and KO groups (three mice per strain). Kidneys were decapsulated, cut into small pieces using a scalpel, and digested in 1 mg/ml collagenase for 3 minutes at 37°C. The tissue was then passed through a 100-μm filter fitted on a 50-ml tube. The filter was flushed with complete medium (RPMI 1640, supplemented with 10% FBS and 100 U/ml penicillin/streptomycin), and then discarded. Next, the 50-ml tube containing tubules and glomeruli was gently shaken several times and passed through a 40-μM filter. The filter was flushed with medium using a 20-ml syringe and 18-gauge needle. The filter, now containing glomeruli, was turned upside down on a clean 50-ml tube and flushed with complete medium using a 20-ml syringe in order to remove glomeruli. The remaining solution containing the glomeruli was centrifuged at 1000 rpm for 2 minutes. Complete medium was added to the pellet and glomeruli were distributed in cell-cultured plates. Isolated glomeruli were maintained in RPMI medium for 4 days. Podocyte outgrowth was quantified by measuring the area of the ellipse.

Western Blot Analyses

Proteins were extracted from half kidneys using a RIPA lysis buffer supplemented with sodium orthovanadate, PMSE, a protease inhibitor cocktail (Tebu Bio, Le Perray en Yvelines, France), and sodium fluoride. Total protein concentrations were measured using the Bradford assay. Twenty micrograms of protein was loaded on NuPAGE 4/12% electrophoresis gels (Invitrogen), and then transferred onto a PVDF membrane (Immobilon-p; EMD Millipore, St Quentin en Yvelines, France). Immunoblotting was performed for HeyL (ab78048, 1:1000; Abcam, Inc.), nephrin H300 (Santa Cruz Biotechnology), and p65 (ab16502, 1:1000; Abcam, Inc.). β-actin (1:5000; Imgenex, San Diego, CA) was used as the loading control.

Immunohistochemistry and Immunofluorescence in Mice

Immunohistochemistry was performed on 4-μm-thick sections from paraffin-embedded tissue with the following conditions for the detection of F4/80 for the staining of monocytes and macrophages (MCA497R, 1:200, 90 minutes at 37°C; AbdSerotec) An appropriate secondary antibody was used (Microm Microtech, France). AEC (Clinisciences, France) was used as substrate and hematoxylin QS (Vector Laboratories, Burlingame, CA) was used for counterstaining. F4/80 staining was quantified. Briefly, five photographs per animal were used and results were expressed as the percentage of staining-positive area of the total tissue area.

Images were obtained on the Tenon Hospital confocal microscopy platform (Carl Zeiss inverted confocal microscope).

Immunofluorescence experiments in mice were performed as follows. Tissue sections were fixed in acetone for 10 minutes, permeabilized with 0.1% Triton for 45 minutes, blocked with 2% BSA for 1 hour, and incubated with the following: anti-p65 (ab16502, 1:200, 2 hours at 37°C; Abcam, Inc.), anti-NICD3 (23426, D200, 2 hours at 37°C; Abcam, Inc.), anti-NICD3 (23426, D200, 2 hours at 37°C; Abcam, Inc.).

Figure 10. Notch3 staining in biopsies from patients with Goodpasture’s syndrome. Representative examples of confocal microscopy images showing a strong Notch3-intracellular domain expression (green) within glomeruli of patients with extracapillary GN.
**Table 1. ODN sequences**

<table>
<thead>
<tr>
<th>Gene</th>
<th>DNA Antisense Sequence</th>
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<tr>
<td>Notch3 antisense 1</td>
<td>G<em>T</em>T<em>C</em>CGAGGTAGCAGAAA<em>G</em>T*A</td>
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<tr>
<td>Notch3 antisense 1</td>
<td>C<em>C</em>C<em>CGGCCCCCAGCCCCCAGTGGC</em>G<em>G</em></td>
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<tr>
<td>Notch3 scrambled 1</td>
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<tr>
<td>Notch3 scrambled 2</td>
<td>C<em>G</em>C<em>ACGGCCCGTCCCGAGCAGCCC</em>G<em>C</em></td>
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Inc., and anti-nestin (1:200, 2 hours at 37°C; BD Pharmingen). Nuclei were stained with TO-PRO-3 iodide (D1000, 15 minutes at room temperature; Life Technologies). Alexa Fluor secondary antibodies were used for detection.

For *in vitro* immunofluorescence experiments, cells were fixed in acetone, permeabilized with 0.1% Triton, and incubated with p65 (ab16502, 1:200, 37°C; Abcam, Inc.). To reveal the staining Alexa Fluor 647, goat anti-rabbit IgG (A-21244; Invitrogen) was used as a secondary antibody. For actin cytoskeleton staining, phalloidin-TRITC (P1951, 1:400; Sigma-Aldrich) was used.

For the immunofluorescence in the outgrowth experiments, isolated glomeruli were maintained in RPMI medium for 4 days. At 4 days, glomeruli were fixed with 4% paraformaldehyde for 15 minutes, permeabilized with 0.1% Triton, and then blocked with 2% BSA for 30 minutes. Glomeruli were then incubated with an anti-Nephrin antibody (AF3159; R&D Systems) for 2 hours at 37°C. Alexa Fluor 488 secondary antibody was used for detection, followed by staining of nuclei by TO-PRO-3 iodide (diluted 1000×, 15 minutes at room temperature; Life Technologies). Images were obtained on the Tenon Hospital confocal microscopy platform (Carl Zeiss inverted confocal microscope).

**Immunofluorescence Staining in Human Biopsies**

Renal biopsies from patients were retrospectively analyzed. Informed consent was given by the patients for use of part of the biopsy for scientific purposes. All procedures and use of tissue were performed according to the national ethical guidelines and were in accordance with the Declaration of Helsinki. Cellular crescents contained three or more layers of cells without interposition of extracellular matrix. Experiments were performed on biopsies from patients with Goodpasture’s syndrome with extracapillary proliferation and proteinuria of 1 g per 24 hours. Patients (aged 65–83 years) had glomerular lesions in 100% of the examined glomeruli, 50%–60% crescentic glomeruli, interstitial inflammatory infiltration, and GFR values between 6–12 ml/min, and were hemodialyzed. Sections were fixed in acetone for 10 minutes, permeabilized with 0.1% Triton for 45 minutes, blocked with 2% BSA for 45 minutes, and stained with anti-N3ICD (23426, D200, 2 hours at 37°C; Abcam, Inc.); nuclei were stained with TO-PRO-3 iodide (D1000, 15 minutes at room temperature; Life Technologies). Alexa Fluor secondary antibodies were used for detection (1:1000). A total of four patient’s biopsies were used for this set of experiments.

**Quantitative Real-Time PCR**

RNA was extracted from podocytes using EZ Spin columns (Fermentas, Saint Léon-Rot, Germany) and from the renal cortex using TRI Reagent (Euromedex, Mundolsheim, France), cDNA was synthesized from 1 μg RNA using the Fermentas H Minus First-Strand cDNA Synthesis Kit according to the manufacturer’s instructions. Real-time PCR was performed with the Roche Light Cycler 480 sequence detection system using SYBR Green PCR Master Mix (Qiagen). Specific primers for target mRNAs were designed using the Roche Universal Probe Library website (Table 2) under the following program: 95°C for 5 minutes, 45 cycles at 95°C for 15 seconds and 60°C for 15 seconds, and 72°C for 15 seconds. Results are expressed as 2-ΔΔCt, where Ct is the cycle threshold number normalized to the mean 2-ΔCt for each corresponding control group. Dissociation curves were analyzed after each run for each amplicon in order to determine the specificity of quantification when using SYBR Green.

**Statistical Analyses**

Data are expressed as mean values±SEM. Data were analyzed using one-way ANOVA followed by a Fisher’s test. Values of *P*<0.05 were considered significant.

**Table 2. Primer sequences used for RT-PCR**

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<th>Antisense</th>
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<td>Notch4</td>
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<td>MCP-1</td>
<td>GTGGGGCTACCGCACTGATGA</td>
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VCAM-1, vascular cell adhesion molecule-1.
ACKNOWLEDGMENTS

The authors thank Drs. Nicole Endlich, Isabelle Limon, and Eric Rondeau and Jean-Jacques Boffa for providing the podocyte cell line, the adenosviruses, and the human biopsies, respectively. This work was supported by grants from INSERM and Agence Nationale de la Recherche. F.E.M. and Z.K. received Ecole Doctorale 394 fellowships from Pierre and Marie Curie University as well as additional financial support from the French Society of Nephrology (to F.E.M.) and INSERM (to Z.K.).

DISCLOSURES

None.

REFERENCES


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Supplementary Figure 1. Notch ligands expression in the NTS model.
Supplementary Figure 2. Notch1 and Notch3 expressions in models of kidney injury.
Supplementary Figure 3. Anti sheep IgG levels in NTS treated WT and Notch3 KO mice.
Supplementary Figure 4. Anti CD68 staining in renal cortex of NTS-treated mice showing the presence of macrophages into the glomeruli of WT mice (left). Macrophages were substantially decreased in the glomeruli of Notch3 KO mice (right panels).
Supplementary Figure 5. Changes in marker expression in podocytes overexpressing N3ICD.
Supplementary Figure 6. Additional figures showing nephrin staining in the outgrowth experiment (at higher magnifications from the Figure 6E).
Supplementary Figure 7. Antisense ODN presence (spots) in the glomeruli of NTS-treated mice.
Supplementary Figure 8. Nephrin staining in biopsies of Goodpasture’s (left) and minimal change nephropathy (right) patients.
Supplementary Figure 9. Cytotoxicity test of the NFκB inhibitor BAY11.
SUPPLEMENTARY FIGURE LEGENDS

**Suppl Figure 1.** Expression of Notch ligands in the NTS model. The mRNA transcript expressions of Notch ligands were evaluated in mice sacrificed 9 days after NTS injections. Kidneys were collected, decapsulated, and the cortex was dissected from the medulla. The cortical tissue was then used for RNA extraction and subsequent RTqPCR analysis of Jagged1, Jagged2, Delta1 and Delta4. *, p <0.05; **, p <0.01.

**Suppl Figure 2.** Notch1 and Notch3 mRNA transcript expression in models of kidney injury. Notch1 and Notch3 mRNA expressions were analyzed in different models of kidney injury including NTS (A), Unilateral Ureteral Obstruction (UUO; B), Ischemia Reperfusion (IR; C) and mice over-expressing renin (RenTg (D). Mice were sacrificed at various time points detailed in the materials and methods. Kidneys were collected, decapsulated and snap frozen. Total mRNA was extracted and reverse transcriptase was performed. Notch1 and Notch3 expression levels were analyzed using RTqPCR. Results are expressed as a fold-induction of the mRNA level of WT control mice and represent the mean and standard deviation of 4-5 mice per group. *, p <0.05; **, p <0.01; ***, p <0.001.

**Suppl Figure 3.** Anti sheep IgG levels in NTS-treated WT and Notch3 KO mice. There is no difference in anti-sheep IgG levels between NTS wild type and NTS Notch3 KO mice.

**Suppl Figure 4.** Anti-CD68 staining in glomeruli of NTS-treated mice. Note the presence of active macrophages within glomeruli of WT mice (left panel). Their presence was reduced in Notch3 KO mice (right panels).

**Suppl Figure 5.** Changes in marker expression in podocytes overexpressing N3ICD.

**Suppl Figure 6.** Additional figures at higher magnification showing that cells migrating from glomeruli in the outgrowth experiments express nephrin.

**Suppl Figure 7.** Detection of Notch3 antisense oligonucleotides in the glomeruli of NTS-treated mice.

**Suppl Figure 8.** Nephrin immunostaining disappears in patients with Goodpasture’s syndrome. Nephrin expression was tracked using immunofluorescence in patients with Goodpasture’s syndrome (left images) and in patients with minimal change nephropathy (right images). Nephrin staining can be easily detected in the minimal change nephropathy, but is negligible in the Goodpasture’s patients indicating that the podocytes of these biopsies have lost the expression of characteristic proteins. Representative images of two patients per disease are shown.

**Suppl Figure 9.** Cytotoxicity test of the NFκB inhibitor BAY11 is podocytes. Triton used as a positive control, increased LDH levels, whereas BAY11 had similar to untreated control levels.
MATERIALS AND METHODS

Unilateral Ureteral Obstruction
After induction of general anesthesia (intraperitoneal injection of 50 mg/kg of pentobarbital) the left ureters of 3 month-old WT mice were ligated at two separate points through a left flank incision. Non-obstructed sham kidneys were used as controls. Four mice from each group were sacrificed after 7 days of obstructive nephropathy. Kidneys were collected, decapsulated, and the cortex was dissected from the medulla. The cortical tissue was then used for RNA extraction and subsequent RTqPCR analysis.

Renin Transgenic Mice
Experiments were performed using RenTg mice backcrossed in the genetic background Sv129 as already described (1). These mice express renin ectopically at a constant high level in the liver leading to elevated mRNA (2.7±0.3 renin/18S versus 0 in RenTg and WT mice respectively) and protein levels of active renin into the blood stream (1, 2). Thus, RenTg mice are hypertensive as endogenous synthesis of angiotenin II is increased. At 12 months of age 4 mice from each group were sacrificed. Kidneys were collected, decapsulated, and the cortex was dissected from the medulla. The cortical tissue was then used for RNA extraction and subsequent RTqPCR analysis.

Ischemia Reperfusion
After induction of general anesthesia (intraperitoneal injection of 50 mg/kg of pentobarbital) a posterior subcostal incision was made on the left side, and the renal pedicle was dissected and occluded with a small vascular clamp. After 45 min, the clamp was removed. Mice were sacrificed 6, 24 and 48 hours after clamp removal. Kidneys were collected, decapsulated, and the cortex was dissected from the medulla. The cortical tissue was then used for RNA extraction and subsequent RTqPCR analysis.

Assessment of anti-sheep IgG titers in mice sera
Anti-sheep IgG titers were measured in mice sera by ELISA assay (Alpha Diagnostic International, San Antonio, TX, USA). Plates were coated with 20µg/mL of sheep IgG overnight at 4°C and then blocked using a 5% albumin solution. Serum was added at various dilutions according to the manufacturer’s instructions.

Antisense oligonucleotide detection
WT mice were injected with two different sequences of oligodeoxynucleotides (ODN) specifically targeting Notch3 mRNA, designed using IDT DNA (Integrated DNA Technologies). Sequences were modified with phosphorothioate to prevent their in vivo hydrolysis by exonucleases (Sigma-Aldrich, St. Quentin Fallavier, France) and included a fluorescein tag to allow detection by epi-fluorescence microscopy. The ODNs were diluted in saline sodium chloride and administrated by intraperitoneal injections of 100 pmol/ODN per mouse. Three mice were used and sacrificed 3 hours after injection. Kidneys were collected, snap-frozen and immediately sectioned. Cryosections were fixed in PFA4% and analyzed using epi-fluorescence microscopy.

Transfection of E11 podocytes
Cells were plated at 80% confluence and maintained in RPMI 1640 supplement with 10% fetal bovine serum, 100U/ml penicillin, and 0.1mg/ml streptomycin at 37°C. At 2 weeks of incubation at 37°C, an empty control vector pCDNA3 and the pUC57 vector containing the mouse Notch intracellular domain were transfected using the JetPei transfection reagent
(Polypus Transfection) according to the manufacturer's instructions. Cells were harvested 24 hours after transfection and total RNA was extracted for subsequent analysis by RTqPCR.

**Cytotoxicity test**
Cells were treated with 1µM NFκB inhibitor BAY11 in serum-free medium for 24 hours. Podocytes were treated with 1% Triton as a positive control. Supernatants were collected and lactate dehydrogenase (LDH) activity was detected with the LDH Cytotoxicity Detection Kit (Roche) according to the manufacturer’s instructions.

**Reverse transcriptase and RTqPCR**
RNA was extracted from podocytes using EZ Spin columns (Fermentas, Saint Léon-Rot, Germany) and from renal cortex using TRI Reagent (Euromedex, Mundolsheim, France). cDNA was synthesized from 1µg RNA using the Fermentas H minus First Strand cDNA synthesis kit according to the manufacturer’s instructions. Real time PCR was performed with the Roche Light Cycler 480 sequence detection system using SYBR Green PCR master mix (Qiagen). Specific primers for target mRNAs were designed using the Universal Probe Library Roche website (Table 1) under the following program: 95°C for 5 min, 45 cycles at 95°C for 15 s and 60°C for 15 s, and 72°C for 15 s was used. Results are expressed as 2-ΔCt, where Ct is the cycle threshold number normalized to the mean 2-ΔCt for each corresponding control group. Dissociation curves were analyzed after each run for each amplicon in order to determine the specificity of quantification when using SYBR Green. HPRT was used as the housekeeping reference gene.

**Glomerular isolation and immunofluorescence**
Glomeruli were isolated from WT mice by two-step sieving of renal cortices 4 days after NTS injection in WT and KO groups (3 mice per strain). Kidneys were decapsulated, cut into small pieces using a scalpel and digested in 1mg/mL collagenase for 3 minutes at 37°C. The tissue was then passed through a 100µm filter fitted on a 50mL tube. The filter was flushed with complete medium (RPMI 1640, supplemented with 10% fetal bovine serum, 100 U/ml penicillin/streptomycin), then thrown away. Next, the 50mL tube containing tubules and glomeruli was gently shaken several times and passed through a 40µm filter. The filter was flushed with medium using a 20cc syringe and 18G needle. The filter, now containing glomeruli, was turned upside down on a clean 50mL tube and flushed with complete medium using a 20cc syringe fitted with a 26G needle in order to remove glomeruli. The remaining solution containing the glomeruli was centrifuged at 1000rpm for 2 minutes. Complete medium was added to the pellet and glomeruli distributed on glass cover slips in cell culture plates. Isolated glomeruli were maintained in RPMI medium for 4 days. At four days glomeruli were fixed with 4% PFA for 15 minutes, permeabilized with 0.1% Triton then blocked with 2% BSA for 30 minutes. Glomeruli were then incubated with an anti-Nephrin antibody (R and D systems, AF3159) for 2 hours at 37°C. Alexa fluor 488 secondary antibody was used for detection, followed by staining of nuclei by ToPro3 iodide (Life Technologies, diluted 1000x, 15min at room temperature). Images were obtained at the Tenon hospital’s confocal microscopy platform (Zeiss inverted confocal microscope).

**Immunohistochemistry in mice**
Cryostat sections (4 µm thick) of renal cortex from WT controls and NTS-treated mice were fixed with paraformaldehyde 4% for 5 min and incubated with 10% FBS/0.1% Triton in PBS to block unspecific staining. Macrophages were detected by immunoperoxidase staining using an anti-CD68 antibody (1/100, Serotec).
**Immunofluorescence of human biopsies**

Renal biopsies from patients with minimal change nephropathy and Goodpasture's syndrome were analyzed for Nephrin staining. Sections were fixed in acetone for 10min., permeabilized with 0.1% Triton for 45min., blocked with BSA 2% for 45min., and stained with an anti-Nephrin antibody (R and D systems, AF3159). Alexa fluor 546 secondary antibody was used for detection, followed by staining of nuclei with TOPRO3 iodide (life technologies, D1000, 15min., RT). Images were obtained at the Tenon hospital’s confocal microscopy platform (Zeiss inverted confocal microscope). A total of 3 patient's biopsies from each group were used for this set of experiments.

**Table 1:** Primer sequences used for RT-PCR

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