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 transcrip-
tion 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 in-
facts 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 pharmacoge-
etic strategy (administration of antisense) oligodeoxynu-
cleotides (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 dis-
ease 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 nephrotic 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 af-
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 glo-
meruli (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 per-
foming 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 con-
firmed intraglomerular infiltration in WT and their reduced presence in Notch3 KO mice (Supplemental Figure 4). Accord-
ingly, the induction of inflammatory markers such as vascu-
lar 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 sub-
units (Figure 4). Both are faintly expressed in normal condi-
tions, but were highly induced after NTS treatment in WT mice (Figure 4, A and B). This upregulation was significantly dimin-
ished in mice lacking Notch3 expression. Immunofluorescence staining showed that p65 activation was localized within po-
docytes, 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
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. 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
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.\textsuperscript{12} 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.\textsuperscript{22} 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 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.
(UVO) 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 (Supplemental Figure 2). 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 in 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

![Graph A](image1.png)

**Figure 7.** Renal expression of Notch family and their target genes in vehicle- or NTS-injected WT mice treated with Notch3 antisense or scrambled ODN. Scrambled injections do not alter the NTS-induced increase in Notch3 (A) and HeyL (B) expressions. *P<0.05 versus vehicle; **P<0.01 versus scrambled (n=5 mice per condition received vehicle, and n=9 mice per condition received NTS injections). SCB, scrambled; Veh, vehicle.

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 existent 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.

Figure 8. Treatment with Notch3 antisense ODN preserves renal function and structure and prevents the progression of renal disease. (A and B) The NTS-induced increase of proteinuria and BUN is attenuated in WT mice treated with Notch3 antisense. (C) Representative examples of Masson’s trichrome staining. (D and E) Quantification of crescents and fibrin deposits confirmed the protection of renal structure in animals treated with Notch3 antisense ODN. *P<0.05; **P<0.01; ***P<0.001 (n=5 and 9 mice per treatment for the vehicle- and NTS-treated groups, respectively). AS, antisense; SCB, scrambled; Veh, vehicle.
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 line28 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)22 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. A previous study10 demonstrated that 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-guage 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 fitted with a 26-guage needle 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-Notch3 (ab78048, 1:1000; Abcam, Inc.), and anti-F4/80 (MCA497R, 1:200, 90 minutes at 37°C; AbdSerotec). Images were obtained on the Tenon Hospital confocal microscopy platform (Carl Zeiss inverted confocal microscope).
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 CGAGGTAGCAAACAAGT</em>C</td>
</tr>
<tr>
<td>Notch3 scrambled 1</td>
<td>C<em>C</em>C<em>C CGGCCCGCGCAGCCTGGG</em>C</td>
</tr>
<tr>
<td>Notch3 antisense 1</td>
<td>C<em>T</em>A<em>T TAGCGACGTAGGAT</em>A G*C</td>
</tr>
<tr>
<td>Notch3 scrambled 2</td>
<td>C<em>T</em>A<em>T CGGCCCGCGCAGCCTGGG</em>C</td>
</tr>
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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−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.

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

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Sense</th>
<th>Antisense</th>
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<tbody>
<tr>
<td>Hsp90</td>
<td>TACTCGGCTTCTCGGTCA</td>
<td>TGAAAGGCAAAAGGTCCTCA</td>
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<tr>
<td>Notch1</td>
<td>ACTATCTGGCGGCGCTTTC</td>
<td>GCCACTGTTGACTCTCTTCT</td>
</tr>
<tr>
<td>Notch2</td>
<td>TGCTGTGTGACAATGTGAGT</td>
<td>GTGTGCTGCACAGTATTTGCTAT</td>
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<tr>
<td>Notch3</td>
<td>AGCTGGGTCTCTGAGGAT</td>
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<td>Notch4</td>
<td>GACGCTTGTGCAACCCTTC</td>
<td>CCTACAGAGCGCTCCCTCT</td>
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<tr>
<td>PDGFR b</td>
<td>TCAAGCTGCAAGCTAACATG</td>
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<tr>
<td>PDGFB</td>
<td>CGCGCTTGTAGCTAGAGTCC</td>
<td>GAGCTTAGGCGCTTGG</td>
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<td>Hey1</td>
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<td>CGCGCAGACTCAAGTTTCCC</td>
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<tr>
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<td>GGTGGAGGGAGGAAAGATTAA</td>
<td>GTTGTGGTGAATTTGGACCT</td>
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<tr>
<td>HeyL</td>
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<td>HB EGF</td>
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<td>CACGGCACCCTCTCAGCTTCTT</td>
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<td>VCAM-1</td>
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<td>CCCAGATGTTGTCATCTTCTT</td>
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<tr>
<td>MCP-1</td>
<td>GTGGGCTCAGCCAGATGCA</td>
<td>AGCCCTACATTGGGATCATCTT</td>
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</table>

VCAM-1, vascular cell adhesion molecule-1.
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DISCLOSURES

None.

REFERENCES


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