The Pathogenic Role of Notch Activation in Podocytes

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Abstract
Podocytes play a key role in the maintenance of the glomerular filtration barrier. Depletion or dysregulative mechanisms of podocytes can lead to the development of glomerulosclerosis. Signaling pathways that control these processes in podocytes are not fully understood. Recent studies from our and other laboratories found that genes that belong to the Notch pathway are regulated in patients and in animal models of renal disease. Genetic studies performed on mice with conditional expression of active Notch1 protein showed massive albuminuria, glomerulosclerosis, and ultimately renal failure and death of the animals. γ-Secretase inhibitors and genetic deletion of Notch transcriptional binding partner (Rbpj) protected animals from nephrotic syndrome. Further studies are needed to define whether the activation of Notch pathway in podocytes represents a common pathomechanism in glomerular injury, and its potential to be a therapeutic target for the treatment of chronic kidney disease.

Podocytes Play a Critical Role in Glomerular Diseases

Glomerular visceral epithelial cells (podocytes) are located at the outer surface of the glomerular basement membrane (GBM), and their foot processes are anchored to the GBM [1, 2]. Neighboring foot processes are connected by a specialized cell-cell junction, the glomerular slit diaphragm. Alterations of the foot process and slit diaphragm configuration result in effacement of foot processes and lead to the development of proteinuria. Podocyte injury plays an important role in glomerular diseases and progressive nephron loss. Pavenstadt et al. [3] described 3 mechanisms of podocyte injury: the dysregulative, the inflammatory and the degenerative mechanisms.

The collapsing form of focal segmental glomerulosclerosis (FSGS) is a typical example of the dysregulative type of podocyte damage. In this case, dedifferentiation of podocytes leads to regulatory defect. This is followed by cell proliferation in the Bowman's space, which leads to the collapse of the glomerulus [3]. During various forms of inflammatory glomerulonephritis, abnormal activity of podocytes results in the formation of cellular crescents [3]. In models of degenerative glomerular disease, the
progressive loss of glomerular podocytes is the culprit behind the disease. Depletion of glomerular podocytes leads to the fixation of the parietal cells to the GBM followed by the establishment of tuft adhesions to the Bowman’s capsule. In the case of healing by fibrosis, the lesion turns into segmental glomerulosclerosis. Toxic and metabolic glomerular injury models – including the puromycin aminonucleoside (PAN)-, doxorubicin- and diabetes-induced nephrotic syndrome models – lead to progressive renal damage via degenerative mechanisms [3].

**Podocyte Depletion in Renal Disease**

Detachment of glomerular epithelial cells has been documented in cases of FSGS and membranous nephropathy in humans [4]. A recent study using streptozotocin-treated rats suggests that podocytes may detach from the GBM in this experimental diabetic nephropathy (DNP) model, leading to loss of podocytes into the urinary space [5]. These investigators were able to culture cells from the urine that expressed typical podocyte markers, indicating that some podocytes may remain viable after detachment [4, 6].

Apoptosis of resident glomerular podocytes has recently been proposed as a cellular mechanism that may underlie podocyte loss in nondiabetic glomerulopathies [7, 8] in cases of progressive glomerulosclerosis in TGF-β1 transgenic mice [9], the CD2AP+/− mice [10] and the PAN nephropathy model [11]. Interestingly, podocyte apoptosis precedes endocapillary and tubular epithelial apoptosis in these models. Since glomerular epithelial cells are unable to divide, apoptosis and/or detachment of the cells will result in depletion of glomerular podocytes. However, it is unclear whether there is a relationship between apoptosis and detachment as generally apoptotic cells might detach from the basement membrane and cells that detach from a GBM usually die [12, 13].

**The Notch Signaling Pathway**

The Notch signaling pathway was initially identified in *Drosophila* when a mutation in Notch was found to cause an increase in the number of neuroblasts at the expense of epidermal cells [14]. This pathway is present in all metazoan, and it functions as one of the major pathways that determine cell identity during development [15]. Notch is a transmembrane protein that interacts with ligands of the Jagged and Delta family [16]. There are 4 Notch members in mammals (Notch 1–4), 2 Jagged (Jag), and 4 Delta-like genes [17]. Each of these proteins shows a cell type- and tissue-specific expression during development. Notch is made in the endoplasmic reticulum as pre-Notch. O-fucosyltransferase functions as a chaperone to transport Notch from the endoplasmic reticulum to the Golgi for glycosylation and fucosylation. A furin-like convertase cleaves pre-Notch into intracellular and extracellular domains. This protein is then transported to the plasma membrane. Interaction of these ligands with Notch triggers a series of proteolytic cleavage, initially by ADAM proteases (S2) and finally by the γ-secretase complex. This final cleavage releases the Notch intracellular domain (ICN), which is a transcription factor. Transport of ICN to the nucleus allows it to bind to other transcriptional activators (including RBP-J, MAML, p300) and the complex then mediates the transcription of various genes including Hes and Hey family members, which are transcription factors and mediate the program of cell identity (fig. 1) [18–21]. In the absence of ICN, RBP-J binds to co-repressor molecules that repress transcription from the DNA bound to RBP-J [22]. ICN later undergoes ubiquitination- and proteosome-mediated degradation.

The regulation of the Notch pathway is complex and occurs at many different levels. The most important of all appears to be the ligand binding, followed by the γ-secretase-mediated cleavage. In recent years the γ-secretase complex received significant attention as a potential therapeutic target for Alzheimer’s disease and cancer. Multiple different compounds have been developed that target the γ-secretase complex. They have been extensively tested in animals, and some of them are in Phase III clinical trials for the treatment of Alzheimer’s disease and breast cancer [23].

**The Notch Pathway Controls Cell Differentiation, Proliferation and Apoptosis in a Cell-Context-Dependent Manner**

The Notch signaling pathway plays a critical role in cellular differentiation and organ development (including the kidneys, pancreas, etc.). In a diverse developmental context, Notch signaling has been associated with amplification of some somatic stem cells, such as the neural and hematopoietic stem cells. The function of Notch is highly context dependent [24–27]. For example, within the hematolymphoid compartment, constitutively overactive Notch signaling can be observed in large propor-
The Pathogenic Role of Notch Activation in Podocytes

**Fig. 1.** Schematic representation of the Notch signaling pathway. Upon Delta or Jagged binding, the Notch receptor undergoes proteolytic cleavage dependent on γ-secretase activity. Notch intracellular domain (ICN) translocates to the nucleus, and binding to Rbpj, activates the dissociation of co-repressor complex resulting in induction of Hes and other target genes. ICN1 later undergoes ubiquitination- and proteosome-mediated degradation.

**Fig. 2.** The pathogenic activation of Notch in podocytes. TGF-β1 leads to Notch pathway activation in podocytes via directly inducing Notch ligand Jag1. a Active Notch1 expression in podocytes leads to podocyte apoptosis via inducing p53. b Conditional expression Notch intracellular domain in vivo in podocytes leads to apoptosis and increased TGF-β and VEGF expression, and it is sufficient to induce foot process effacement albuminuria and glomerulosclerosis. c Blocking Notch activation with γ-secretase inhibitors (GSI) ameliorates albuminuria and renal damage in animal models.
tion of T cell malignancies, and recent data identified Notch-activating mutations in more than 50% of human T cell leukemias [28]. In malignant T cells, Notch signaling induces proliferation, differentiation and survival [29, 30]. However, the Notch receptor expressed in malignant B cells resulting in constitutive Notch signaling leads to growth inhibition and apoptosis [31].

Experimental evidence supports the idea that signaling pathways essential for embryonic development also have a role in regulating self-renewal in tissues [32, 33]. Mutations in these pathways (such as TGF-β, Wnt, and ErbB) often lead to tumorigenesis, as is also true for Notch. An interesting aspect of Notch is its apparently opposite functions in tumor development, because it can act as an oncogene or as a tumor suppressor. Several studies suggest that Notch activation plays an important oncogenic role in breast and intestinal cancer development [17, 33–35]. Notch plays differential roles in 2 types of skin cancer. Notch inhibits the development of keratinocyte-derived cancer [36], while it has an oncogenic role in melanomas [37]. In neuronal cells, Notch1 signaling inhibits growth through induction of cell cycle arrest and apoptosis via upregulation of proapoptotic proteins, including p53 and the activation of JNK kinase [38].

**Role of the Notch Signaling Pathway during Renal Development**

Detailed analyses of the expression pattern of Notch and related genes during nephrogenesis have been performed [39–41]. Recently, these studies were excellently summarized by Kopan et al. [42]. Notch1, Notch2, Dll1 and Jag1 mRNA are detected in the renal vesicle and its derivatives. Expression of Notch1 partially overlapped with Notch2 in the S-shaped body [41]. Notch2 and Jag1 were also expressed in the collecting duct. Notch3 expression has been reported in the distal portion of the S-shaped body [39]. Notch4 was mainly detected in endothelial cells.

Notch pathway proteins are not only expressed in the developing kidney, but they seem to play an important role in cellular differentiation. Humans haploinsufficient for Jag1 [43] are prone to Alagille syndrome, one symptom of which can result in the development of renal abnormalities, whereas abnormal glomerulogenesis is also observed when Notch2 activity is reduced [44, 45]. Mouse metanephiroi cultured in the presence of a γ-secretase inhibitor (DAPT), to block Notch signaling, resulted in reduced ureteric bud branching, but normal mesenchymal condensation and expression of markers, indicating that mesenchyme induction had occurred. However, fewer renal epithelial structures were observed, with a severe deficiency in proximal tubules and glomerular podocytes, which are derived from cells in which activated Notch1 is normally present. Distal tubules were present, but in reduced numbers, and this was accompanied by an increase in intervening nonepithelial cells [46]. Recently, Cheng et al. [47] showed that both Notch1 and Notch2 are detected in the early renal vesicle; however, Notch2 acts nonredundantly to control the processes of nephron segmentation through an Rbpj-dependent process. These observations suggest that the Notch pathway activation is required for the progression of renal vesicles to comma- and S-shaped bodies and determining the proximal tubule and podocyte fates.

**Role of Notch in the Pathogenesis of Glomerulosclerosis**

In order to better understand the mechanism of glomerulosclerosis, we performed gene expression studies (using microarrays) on kidney samples obtained from animal models of diabetes and DNP, and also on kidney samples from patients with renal disease [49, 50]. With a combined expression and pattern recognition analysis, we found that genes that belong to the Notch pathway are regulated in glomeruli of mice with diabetic renal disease and in patients with DNP and FSGS [51]. This observation led us to further study the potential role of the Notch pathway in glomerular disease. We found increased expression of Notch intracellular domain in podocytes of patients and mice with DNP and FSGS. A similar analysis performed by Walsh et al. [52] also detected increased Notch, Jagged and TGF-β expression in the tubulo-interstitial compartment of human DNP samples, when they compared these to control human kidney samples.

In order to study the in vivo function of increased Notch expression in glomerular podocytes, we used a unique in vivo podocyte-specific titratable active Notch overexpression system. This was achieved by intercrossing of 2 already available and validated mouse models, the podocin rTA (reverse tetracycline transactivator; podrTA) mice that express the tetracycline-inducible transactivator gene under the podocin promoter [53] with the tet-O-ICN1 mice [35]. By intercrossing these 2 mouse strains, Notch intracellular domain can be specifically induced in podocytes by tetracycline. Upon administration of tetracycline or doxycycline, the rTA (in
podocytes) binds to the tetracycline-responsive elements in the tet-O-ICN1 mice and regulates the expression ICN1. ICN1 can be turned on and off conveniently with the administration or withdrawal of doxycycline (fig. 2).

Renal development was normal in the PodrTA/Tet-O-ICN1 mice; we did not observe albuminuria, glomerular or tubular abnormalities, even at 10 weeks of age without doxycycline administration. Doxycycline-containing food was administered starting at 3–4 weeks of age, after completion of kidney development [51].

As early as 7 days following the initiation of doxycycline treatment, the podrTA/Tet-O-ICN1 mice developed proteinuria. After 2 weeks treatment with doxycycline-containing chow, animals developed severe albuminuria reaching 5,000 μg/mg albumin/creatinine. Albuminuria was around 50 μg/mg albumin/creatinine in doxycycline-treated wild-type, tet-O-ICN1 or podrTA mice or podrTA/tet-O-ICN1 mice that were not treated with doxycycline. Histological examination of the kidneys showed severe glomerular abnormalities. The early lesions were characterized by diffuse mesangial matrix accumulation in the glomerulus. More advanced lesions showed segmental sclerosis of some of the glomeruli, most similar to FSGS of humans. Control animals showed no histological abnormalities. Immunohistochemistry showed a severe reduction (almost absence) in podocyte-specific markers, i.e. nephrin and WT-1, indicating a severe decrease in podocyte numbers or the loss of specific markers on existing podocytes. Therefore, electron microscopy (EM) was performed to evaluate the podocytes. EM showed a severe reduction in podocyte numbers and almost complete effacement of podocyte foot processes. Endothelial cells appeared normal and fenestrated. No immune deposits were observed in the glomerulus. Interestingly, we also observed remnants of dead cells occasionally. These cell remnants showed nuclear condensation, with a perinuclear rim, which could be consistent with apoptotic cell bodies. In order to further evaluate whether podocyte apoptosis occurs in this animal model, we performed TUNEL staining in control and double transgenic animals. We found an increase in TUNEL-positive staining in nuclei of glomerular podocytes of doxycycline-treated double-transgenic animals compared to wild-type mice. We also found that the expression of proapoptotic genes, including trp53, Bax and Apaf, were significantly increased in double-transgenic animals. In separate cell culture experiment, we confirmed the proapoptotic role of Notch1 in podocytes. This proof of principle study established that Notch activation in podocytes is ‘pathogenic’, and that it is alone sufficient to induce podocyte foot process effacement, podocyte depletion and subsequently albuminuria and glomerulosclerosis (fig. 2).

Waters et al. [54] performed a similar experiment using different mouse strains, when the expression of active NOTCH1 domain was turned on in podocytes during the capillary loop stage of development. They used the nephrincre and STOP-NOTCH-IC mice to achieve their goal. Histological and molecular analyses revealed normal glomerular morphology and expression of podocyte markers in newborn podocyte-specific NOTCH-IC-expressing mice. At 2 weeks of age, animals developed profound albuminuria, glomerulosclerosis and early mortality due to end-stage renal failure. Upon histological analysis of this model, they found severe foot processes effacement, loss of expression of Wt1, nephrin and podocin, and the expression of Pax2 was increased. The renal histology was more consistent with diffuse mesangial-sclerosis-like disease. In addition, in contrast to our studies, they found that podocytes expressing active Notch1 undergo massive proliferation. The damaging effects of NOTCH-IC expression were prevented in transgenic mice after simultaneous conditional inactivation of Rbpj in murine podocytes using the Rbpjflox mice.

There are many similarities in the 2 studies described above, including the rapid development of albuminuria, foot process effacement, loss of Wt1, nephrin and podocin expression, and the development of glomerulosclerosis in both models. However, it appears that while active Notch1 expression induced apoptosis in mature podocytes, it led to their proliferation if it was expressed prior to their full differentiation. It is not clear whether this differential response was induced by the different degree of Notch1 expression or by the different timing of Notch1 expression. Nevertheless, it appears that Notch1 can act on different target genes in developing and in mature (fully differentiated) podocytes and emphasize the critical spatial and temporal role of this pathway.

At present, there is no specific cure for most forms of acquired chronic kidney disease. Therefore, we explored whether inhibition of the Notch pathway can influence glomerular disease development. The critical and regulated step in Notch activation is the γ-secretase-complex-mediated cleavage of the receptor. During the last few decades, many different inhibitors have been developed that block this enzyme complex. Many of these compounds are tested in early phase I and II trials, and appear to be relatively safe in humans.

Chronic GSI (DBZ) treatment of mice and rats is shown to be safe and relatively well tolerated [55]. For these experiments, we used the PAN-induced albumin-
albuminuria model of rats. PAN treatment of rats leads to the development of nephrotic syndrome, and it is a well established model of podocyte apoptosis, detachment and podocyte depletion [11].

When we examined mRNA levels of Notch pathway genes in glomerular extracts of PAN-treated rats, we found a significant increase in Notch1, 2 and Notch target genes Hes1, 5, Hey1 levels in glomeruli, 6 days following single 20 mg/kg PAN injection of Sprague-Dawley rats. The mRNA increase was more pronounced in this model, and we observed an approximately 10- to 15-fold increase in Notch1 levels compared to control rats. Val-1744Notch1 and Hes1 immunostaining was also increased in glomeruli of PAN-treated rats. Most of the staining appeared to be in the podocytes.

PAN-treated rats developed albuminuria starting from day 4. Chronic DBZ treatment blocked the increase in Notch pathway gene expression in isolated glomeruli, indicating the effectiveness of the drug. Rats treated with DBZ had significantly less albuminuria than control animals. DBZ treatment also effectively protected rats from the development of podocyte foot process effacement as shown by EM analysis [51]. We also performed TUNEL staining to examine podocyte apoptosis. Our results indicate that PAN + DBZ-treated rats had less podocyte apoptosis compared to PAN-treated animals. DBZ treatment was not only effective when it was initiated before the PAN injection, but it effectively reduced albuminuria after the development of nephrotic syndrome (fig. 2).

While our studies show a strong correlation between DBZ treatment and Notch activation, from the current studies we could not exclude the possibilities that other γ-secretase-dependent mechanisms are responsible for the effect of DBZ, including potential effects on CD44, erbB4 and N-cadherin cleavage, are currently being addressed in our laboratory [56–58]. It should also be noted, however, that currently no information is available regarding the role of any of these pathways in podocytes and in nephrotic syndrome.

To address these issues we performed studies on mice with podocyte-specific genetic deletions of Rbpj (thereby Notch signaling). Diabetic mice with podocyte-specific deletions of Rbpj showed lower proteinuria and less podocyte damage when compared to wild-type diabetic mice. These studies again confirm that podocyte-specific Notch activation plays a critical role in the development of proteinuria and podocyte damage (fig. 2).

**Conclusion**

In summary, we can conclude that γ-secretase- and Rbpj-dependent mechanisms play a critical role in the development of podocyte damage and proteinuria, suggesting that Notch activation and the development of proteinuria and podocyte damage are causally linked. The Notch, γ-secretase pathway appears to be a useful novel therapeutic option for the cure of podocyte damage causing proteinuria.

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**References**

The Pathogenic Role of Notch Activation in Podocytes