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Repair Problems in Podocytes: Wnt, Notch and Glomerulosclerosis

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Abstract

Wnt/Ctnnb1 and Notch signaling play key roles in kidney development and epithelial cell specification. Recent reports suggest that these pathways are reactivated in response to injury and in different disease conditions. Studies using genetically modified animal models demonstrated that sustained activation of Notch and Wnt signaling in podocytes are causally related to albuminuria and glomerulosclerosis development. Here, we discuss the role and regulation of Wnt/Ctnnb1 and Notch signaling in podocytes.

Kidney injury and repair from fish to mice

Toxic/anoxic insults to tubular epithelial cells cause cell death followed by the dedifferentiation, proliferation and redifferentiation of epithelial cells (1, 2). Following an acute insult, kidney function usually returns to normal and no gross histological abnormalities observed in the human kidney, indicating the repair of the tubular compartment (3, 4).

Skate and zebrafish are also capable to repair the glomerular compartment by growing new glomeruli after nephron removal (5) (Figure 1). Conversely, in the mammalian kidney new nephrons do not form once nephrogenesis is complete. The mammalian kidney mainly responds to nephron loss by compensatory growth, characterized by glomerular enlargement and tubular epithelial cell proliferation and enlargement (Figure 1). Glomerular epithelial cells (podocytes) are terminally differentiated cells and they are unable to undergo cell division (6). Targeted deletion of glomerular epithelial cells of up to 20% (using a rat model of diphtheria toxin-induced cell depletion) induces transient albuminuria, afterwards no major structural changes can be observed in the glomerulus (7). As podocytes are unable to divide, it appears that they enlarge in response to podocyte injury, potentially via regulation of the mTOR pathway (6, 8, 9). Careful molecular studies indicate that if podocyte loss exceeds 20%, remaining cells are unable to compensate for the loss. A maladaptive form of

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repair response is initiated, causing further podocyte destruction, dedifferentiation and glomerulosclerosis development (Figure 2).

What is the mechanism of organ regeneration? In the Hydra and Urodele Amphibians (Newt, Salamander), cell death (apoptosis) itself is the trigger for regeneration and repair (5). Caspase activation triggers Wnt3 release from apoptotic cells, inducing proliferation and regeneration (10, 11) (Figure 1). A similar mechanism has been proposed in the mammalian liver and kidney following ischemia-reperfusion injury (12). For example, in the kidney the release of Wnt7b from inflammatory macrophages plays key role in orchestrating the repair and regeneration of tubular epithelial cells, following ischemic injury (13). Wnt/ β -catenin (Ctnnb1) and Notch activation play important role in liver regeneration (Figure 1).

Wnt/Ctnnb1 signaling

Mammalian Wnt signaling was first reported in 1987 by identifying the *int1* oncogene, which turned out to be the mammalian homolog of the *Drosophila* gene *wingless* (*Wg*) (14, 15). The name “Wnt” was coined by combining “int” and “wingless”. The *Drosophila* Armadillo and its mammalian homologue Ctnnb1 was then identified as the major effector of the *Wg*/Wnt pathway (16). In mammals, 19 different Wnts and 10 Wnt receptors (Frizzled) have been discovered thus far (17).

In the absence of Wnt ligands (“off-state”) cytoplasmic Ctnnb1 levels are low as cytoplasmic Ctnnb1 is constantly recruited to a multi-protein “destruction complex” for N-terminal phosphorylation, followed by polyubiquitination and proteasomal destruction (Figure 3). Therefore, in the “off-state”, cytoplasmic and nuclear Ctnnb1 levels are low. In the presence of Wnt ligands (“on-state”) the Wnt/Ctnnb1 signal is initiated. Extracellular Wnt binds to specific receptors, including Frizzled and Lrp5/6, which induces the disassembly of the destruction complex. In the “on-state”, Ctnnb1 enters the nucleus, where it forms a transcriptional complex with the TCF/LEF family (Figure 3). The classic target genes of Ctnnb1 include *Axin2*, *Lef1*, *Dkk1* and *Cd44*, however, they show high cell type specificity (18). In addition to the canonical (Ctnnb1-mediated) pathway, Wnt-signaling can trigger Ctnnb1-independent (non-canonical) pathways as well. The most studied non-canonical pathways are the regulation of intracellular Ca^{2+} signaling and planar cell polarity/convergent extension (PCP/CE) pathways (19).

Kidney Development and Ctnnb1

Together with other secreted factors such as fibroblast growth factor (*FGF*), transforming growth factor- β (*TGF- β*) and *hedgehog* protein, *Wnt* proteins play a key role in kidney development. *Wnt4* is expressed in the early condensate and pretubular aggregates. *Wnt4* null mutants have severely hypoplastic kidneys, indicating that *Wnt4* is necessary for tubulogenesis especially for mesenchymal-epithelial transformation (20). In the absence of *Wnt4*, the metanephric mesenchyme condenses normally and the early induction markers *Wt1*, *Pax2* and *N-myc* are increased, but the mesenchyme fails to generate pretubular aggregates or to undergo subsequent tubulogenesis. *Wnt4* is involved in the transition of the tubular mesenchyme to epithelium, and is required for tubulogenesis both *in vivo* and *in vitro*. Cell lines expressing *Wnt1*, *Wnt4*, *Wnt6* and *Wnt7b* can induce tubule formation of the kidney mesenchyme, indicating the pivotal role of Wnt signaling in mesenchymal to epithelial transition (MET) induction during kidney development (21).

Ctnnb1 in podocytes

Ctnnb1 is a multifunctional protein; it interacts with structural proteins including cadherins. In the absence of Wnt signaling, Ctnnb1 forms a stable complex with E-cadherin and

establishes a link with the actin cytoskeleton and participates in adherence junction formation (22). The role and regulation of *Ctnnb1* with cadherins and actin cytoskeletal proteins have not been fully explored in podocytes, however, it could be an interesting research area. Once MET and kidney development is complete, little Wnt activity can be observed in the adult kidney (23). Podocyte culture experiments indicate that *Ctnnb1* is expressed diffusely in cell-cell contact sites at baseline (24). There is also an indication that it can form a complex with the podocyte slit diaphragm protein densin (25).

Increased Wnt activation and *Ctnnb1* nuclear translocation have been reported upon *in vitro* and *in vivo* podocyte injury. Puromycin (PAN) administration to cultured podocytes induces the nuclear translocation of *Ctnnb1* in an integrin-linked kinase dependent manner (26) (25). TGF- β and adriamycin also regulate the Wnt/Ctnnb1 pathway *in vitro* (27). Increased podocyte Wnt/Ctnnb1 signaling was reported in murine models of diabetic nephropathy and focal segmental glomerulosclerosis (FSGS) (24, 27). The Wnt/Ctnnb1 pathway was one of the most consistently up-regulated pathway in microarray studies performed on microdissected human DKD, FSGS and IgA nephropathy samples (28, 29). These results indicate consistency in the activation of Wnt/Ctnnb1 pathway in podocytes in response to injury and disease.

What is the consequence of Wnt/Ctnnb1 activation in podocytes?

The first report describing the consequence of *Ctnnb1* activation in podocytes comes from the Liu group (27). Dai et al. injected Wnt1-expressing plasmid intravenously into mice and successfully induced *Ctnnb1* accumulation in podocytes. While *Wnt1* alone did not induce albuminuria, animals developed high-grade proteinuria when they were injected with adriamycin, even though they were not on an adriamycin-sensitive background (27). Interestingly, they also reported that intravenous injection of lithium chloride into mice immediately induced transient albuminuria and foot process effacement in wild type mice (27). Lithium is an inhibitor of glycogen synthase beta (GSK- β) therefore capable to stabilize *Ctnnb1*. Consistent with this data, viral expression or infusion of Dkk1 –an inhibitor of wnt signaling-into transgenic mice expressing the telomerase component TERT –a mouse model of glomerulosclerosis and HIV-associated nephropathy (HIVAN)-also ameliorated podocyte injury and proteinuria (58). In contrast, Lin et al. reported that systemic activation of Wnt signaling ameliorated diabetes-related albuminuria in mice (30). In summary, the renal effects of systemic manipulation of the Wnt/Dkk1 system appear to be highly context and maybe disease dependent.

Mice with podocyte-specific stabilized *Ctnnb1* have been generated by crossing the Podocin-Cre (NPHS2^{Cre}) mice with animals in which the third exon of *Ctnnb1* is flanked by LoxP sites (Ctnnb1^{FloxE3}) (31). Serine/threonine residues that are key targets for phosphorylation (and degradation) are located on the third exon of the *Ctnnb1* gene. By deleting this phosphorylation site, *Ctnnb1* becomes stabilized and acts as a dominant active form. Mice heterozygous for the stabilized *Ctnnb1* allele in podocytes (NPHS2^{Cre}/Ctnnb1^{FloxE3/WT}) developed mild mesangial expansion and albuminuria by 20 weeks of age. Electron microscopy studies showed diffuse and irregular glomerular basement membrane thickening. Homozygous mutant mice developed severe GBM thickening, high-grade albuminuria and glomerulosclerosis (24). These studies thereby indicate that sustained *Ctnnb1* activation in podocytes causes albuminuria and glomerulosclerosis development.

What is the mechanism of Ctnnb1 induced changes in podocytes?

Mouse models and cultured cell lines were used to answer this question. Liu et al. proposed that stabilized expression of Ctnnb1 cause epithelial-to-mesenchymal-transition (EMT) of podocytes (27). The increased expression of Snai1 and the decreased expression of nephrin

were interpreted as evidence of podocyte EMT. Podocyte cell lines established from mutant mice or treated with various inhibitors of *GSK3 β* showed increased survival ability, but they exhibited decreased adhesion to several matrices and decreased migratory activity (24). Simultaneously, we were also able to detect detached podocytes in urine of mice with podocyte specific *Ctnnb1* activation. Our group interpreted these findings as that activation of podocyte *Ctnnb1* decreases podocyte adhesion, thereby causing albuminuria and glomerulosclerosis (24). This could be mediated by an interaction between Ctnnb1 and integrin β 1. It appears that motility changes and decreased adhesion is part of the Ctnnb1 induced phenotype. Whether or not EMT occurs in podocytes (which would include GBM degradation and invasion through the GBM) remains to be established.

Does Wnt/Ctnnb1 inhibition protect from albuminuria and glomerulosclerosis?

Since enhanced expression of Wnt/Ctnnb1 induced albuminuria and glomerulosclerosis, we would expect that inhibition of *Ctnnb1* would protect mice from glomerular disease. To answer this question three independent groups have generated mice with podocyte-specific *Ctnnb1* deletion (24, 27, 32). All three groups reported no major histological changes by light microscopy or albuminuria at baseline. This is consistent with the observation that the Wnt/Ctnnb1 pathway is not required for podocyte maintenance.

Dai and Heikkila groups used an Adriamycin-induced “acute nephrotic syndrome” in control and in mice with podocyte-specific *Ctnnb1* deletion (27, 32). Four days after the injection of Adriamycin, mice with podocyte specific *Ctnnb1* deletion showed less albuminuria and glomerular damage compared to wild type mice. Unfortunately, the adriamycin model did not allow for examination of the long-term implications of *Ctnnb1* deletion, including glomerulosclerosis. Interestingly, as opposed to the short-term adriamycin model, *Ctnnb1* knockout mice showed increased albuminuria and accelerated glomerulosclerosis in a long-term (20 weeks) type 1 diabetic nephropathy model (24). Similar results have been reported with the use of Dickkopf-related protein 1 (*Dkk1*), an inhibitor of canonical Wnt signaling by the virtue of its ability to bind to and block the LRP5/6 co-receptor (33). Dai et al reported that systemic administration of *Dkk1* reduced albuminuria in the short term ADR nephropathy model (27). We generated podocyte-specific inducible *Dkk1* transgenic mice by crossing podocyte-specific reverse tetracycline activator expressing mice (34) with mice carrying the tetracycline responsive *Dkk1* transgene (35). Just like the podocyte specific Ctnnb1 null mice the podocyte specific Dkk1 also did not show any baseline phenotype (histological lesions or albuminuria). Albuminuria and glomerular damage was highly increased in the *Dkk1* transgenic mice when they were made diabetic. Similarly, Lin et al observed amelioration of renal damage in diabetic mice injected with *Dkk1* antisense oligonucleotide (30). These discrepancies likely indicate the differential role of Wnt/Ctnnb1 in acute and chronic models of glomerular damage.

The role of *Ctnnb1* has been further characterized *in vitro* with *Ctnnb1*-null podocytes. Decreased *Ctnnb1* in cultured podocytes was associated with enhanced expression of podocyte differentiation markers such as *Wt1* and podocin indicating that *Ctnnb1* silencing is necessary for podocyte differentiation. *Ctnnb1* silencing also increased cell adhesiveness to different matrices (24). *Ctnnb1* silencing-induced cell differentiation could be beneficial in glomerular injury models. However, we observed that the loss of *Ctnnb1* in podocytes increased their susceptibility to apoptosis, which could be responsible for the accentuated phenotype observed in the long-term glomerular disease models, consistent with the notion that Ctnnb1 acts as a major survival pathway. Consistent with these in studies, Grouls et al. showed that developmental deletion of Ctnnb1 from glomerular precursors induced the early differentiation and expression of podocyte markers. Interestingly, again similar to our in

vitro findings, parietal cell markers were absent in this model, indicating that the *Ctnnb1* pathway plays an important role in podocyte parietal cell lineage decision (59).

Downstream of Wnt signaling

To better understand the mechanism, by which Wnt signaling controls podocyte phenotype, we performed genome wide transcript analysis of isolated glomeruli from mice with podocyte specific deletion and stabilization of *Ctnnb1* (24). One of the pathways that appeared to be regulated by *Wnt* signaling was the Notch pathway. During development and differentiation these two pathways often interact and in the kidney they are responsible for the mesenchymal to epithelial transition and tubular epithelial cell development (20). It was initially thought that Notch acts downstream of *Wnt4* activation in the MET process. However, a recent study demonstrating Notch's ability to mediate MET independently of Wnt suggests otherwise (36).

Notch, a single transmembrane protein, can interact with ligands of the Jagged and Delta family. Mammals have four Notch receptors (Notch1-4), and five identified Notch ligands (Delta-like1, 3, and 4 and Jagged1 and 2) (Figure 3). Each of these proteins shows a cell type- and tissue-specific expression (37).

Notch signaling is initiated upon ligand binding to a Notch receptor on a neighboring cell. Ligand binding leads to a series of proteolytic cleavage steps, where Notch is initially cleaved by ADAM metalloproteases (at site S2) and subsequently by the gamma-secretase complex (at S3 site). This final cleavage releases the Notch intracellular domain (NICD), a transcription factor. In the nucleus, NICD binds to other transcriptional factors most importantly RBPj (also known as CSL), which converts RBPj from a transcriptional repressor to a transcriptional activator. As a result, the transcriptional complex induces the expression of Notch target genes such as *Hes* and *Hey* transcription factors (38, 39).

Notch Signaling in Podocytes

Notch signaling controls cell differentiation in diverse organ systems including the kidney and it is essential for proximal tubule development and podocyte specification (39). Once glomerular development is complete, Notch activity is largely decreased in murine and human glomeruli. Increased glomerular Notch activity has been reported in several different injury models and patients with chronic glomerular disorders, diabetes, HIV, FSGS (40) (41), and systemic lupus erythematosus (42).

The mechanism of Notch activation *in vivo* is not fully understood. *In vitro*, transforming growth factor beta (TGF- β) treatment induces the Notch ligand *Jagged1* expression and downstream *Notch* activation. *In vivo*, we also observed the activation of Notch downstream of the Wnt/*Ctnnb1* pathways. As TGF β has been reported to induce Wnt, it is likely that these pathways are involved in a complex interaction (27) (Figure 3).

Two groups independently reported the consequences of Notch activation in podocytes using transgenic mice (43-45). Our group used a podocyte-specific inducible activation of Notch1 intracellular domain in mice at 4 weeks of age (43), while Waters et al. generated a podocin-Cre-induced NICD1 transgenic mice. Both models were characterized by rapid development of proteinuria and focal glomerulosclerosis-like phenotype, causing rapid death due to renal failure (45). Histological analyses, however, revealed some differences. Developmental activation of Notch induced podocyte proliferation and dedifferentiation. On the other hand, podocyte apoptosis and loss have been reported in mice when Notch was turned on in adult animals (43, 45). Two groups have reported that inhibition of Notch signaling by using blockers of the gamma secretase complex (an enzyme that is responsible

for Notch cleavage) or deletion of the Notch transcription binding partner Rbpj significantly ameliorated symptoms of diabetic nephropathy in mouse and rat model of diabetes (43, 46, 47). The concept of sustained Notch activation might again be consistent with an attempt of podocyte repair/regeneration. However, it appears that the capacity for podocyte reparation is limited and that sustained Notch expression in podocytes is maladaptive inducing glomerulosclerosis.

Podocyte repair: Wnt/Ctnnb1, Notch and glomerulosclerosis

During the last few years we learned that developmental pathways are re-activated in injured glomeruli; including the Wnt and Notch pathways. The reactivation of these pathways appears to recapitulate observations from phylogenetically lower species, where Wnt and Notch activation following injury play key roles in the regeneration process (Figure 1). Therefore it would seem logical that these pathways are activated in injury to mediate regeneration or repair. Regeneration either involves the proliferation and differentiation of the remaining cell or proliferation and differentiation of stem cells. As podocytes are terminally differentiated cells and unable to divide, it is unlikely that podocyte/glomerular repair occurs via podocyte proliferation. The presence and role of kidney stem cells has been debated (48-50). Recently, stem/progenitor cells have been described in the urinary pole of the Bowman's capsule. Notch and Wnt have been proposed to play roles in stem cell niches in renewing tissues (intestine, skin and hemopoietic system) (51-54). There are some indications that this could be the case in the podocyte/glomerulus as well. We observed that podocyte vs. parietal cell type marker expression was regulated by Wnt/Ctnnb1 activity; as increased Wnt/Ctnnb1 expression was associated with loss of podocyte differentiation markers and expression of parietal cell type specific markers, while deletion of *Ctnnb1* increased the expression of podocyte markers wt1 and podocin. The Romagnani group reported that Notch might be necessary for stem cell expansion but *Notch* silencing is critical for podocyte differentiation (42). Further studies will be needed to determine the role and contribution of parietal stem cells to podocytes in healthy and diseased glomeruli and the specific role of *Notch* and *Wnt* in this process. Studies are consistent that sustained activation of *Notch* and *Wnt* in differentiated podocytes is detrimental and sufficient to induce albuminuria and glomerulosclerosis.

What determines *Notch* and *Ctnnb1* target specificity in podocytes, parietal cells and during development? It appears that the signaling pathways that initiate injury, repair and development are very similar. One possibility includes that the targets and thereby outcome of Notch and Wnt activation is regulated by signal strength and signal duration. It appears that in the context of podocyte injury, Notch activation is sustained and increased by the lack of podocyte repair (Figure 2). Target activation and therefore the response to Notch and Wnt could be different in differentiated podocytes versus the developing mesenchyme due to differences in open chromatin sites as well. This would be typical and consistent for the action of morphogens like *Wnt* and *Notch*, and could contribute to the highly cell type- and context-dependent outcome.

It seems that as opposed to the fish, regeneration in the mammalian kidney is highly restricted and while the pathways that are reactivated in the context of injury appear similar, the response to Notch and Wnt signaling is a maladaptive one, inducing further podocyte damage. The mechanism of Notch and Wnt induced maladaptive repair and fibrosis development does not seem to be restricted to glomerular podocytes. A similar process has been described in the lung, liver and even in the skin, where sustained Notch and Wnt activation induce a maladaptive repair, ultimately inducing fibrosis and organ failure development (55-57).

As podocytes are unable to repair this leads to the activation of a self-perpetuating cycle where insults induce a maladaptive response, which is mediated by Wnt and Notch signaling. Activation of Wnt and Notch cause podocyte dedifferentiation, detachment and apoptosis perpetuating the damage and causing glomerulosclerosis. Limiting the activation of the Wnt and Notch signaling can halt this vicious cycle and could be an important therapeutic strategy.

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Figure 1. Comparing and contrasting regeneration and repair in different species and organs

A. In the hydra, amputation causes apoptosis and caspase activation, which is the signal for regeneration. Activation of the Wnt, Hh pathways induce cell proliferation and complete regeneration of organs. B. In the liver partial hepatectomy induces a caspase activation and Wnt and Notch mediated repair of the organ. C. Nephron removal in zebrafish prompts the growth of new nephrons and regeneration of kidney. D. New nephrons are not formed in mice and partial nephrectomy causes remaining cells to enlarge and tubular cells to proliferate and functional repair occurs. E. More than 20% podocyte loss causes sustained albuminuria and structural damage. Enhanced Notch and Wnt signaling in podocytes cause further damage, cellular dedifferentiation, apoptosis, causing glomerulosclerosis and functional deterioration of the kidney.

Figure 2. Podocyte injury causing further podocyte injury via activation of repair pathways Genetic mutations or environmental stressors (for example hyperglycemia, hypertension) cause podocyte stress. Podocyte stress activates a local vicious cycle causing the release of different damaging cytokines for example transforming growth factor beta and angiotensin. These pathways not only cause podocyte damage but they activate local repair pathways including Wnt/Ctnnb1 and Notch. Wnt/Ctnnb1 signaling induces podocyte dedifferentiation and detachment. Enhanced Notch activity can elicit further podocyte apoptosis. Both podocyte detachment and apoptosis lead to further podocyte loss, in turn decreasing VEGF levels, inducing capillary collapse and ultimately glomerulosclerosis. The cycle continues as podocyte loss will cause further stress to remaining podocytes and sustained activation of repair pathways ultimately causing the damage of the glomerulus. Red arrows denote potential therapeutic interventions: blocking TGF β or angiotensin action or by limiting the activation of repair pathways Wnt and Notch we can break the vicious cycle and halt glomerulosclerosis development.

Figure 3. Role of Notch and Wnt/Ctnnb1 in podocyte development and disease

Schematic representation of the activation of the Notch and the Wnt/Ctnnb1 pathways in podocytes. Expression of the ligand (Delta or Jagged family) on the ligand expressing cell induces proteolytic cleavage of the Notch receptor on the signal receiving cell, releasing the Notch intracellular domain (NICD). NICD travels to the nucleus and binds to other transcriptional regulators including Rbpj and regulates gene transcription. Binding of the Wnt ligand to its receptor (Lrp5/6 and Frizzled) causes the inhibition of the Ctnnb1 destruction complex, thereby stabilizing Ctnnb1. Ctnnb1 then travels to the nucleus, binds to other transcriptional regulators including proteins from the Tcf family and thereby regulate gene transcription. During development high Wnt/Ctnnb1 and Notch activity is necessary for podocyte development. Once development is complete both pathways are mainly silenced in adult podocytes. Reactivation of Notch and Wnt/Ctnnb1 can be observed in the context of albuminuria and glomerular disease. Increased Notch and Ctnnb1 activities contribute to proteinuria and glomerulosclerosis development.