Lineage Specification of Parietal Epithelial Cells Requires $\beta$-Catenin/Wnt Signaling

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ABSTRACT

$\beta$-Catenin/Wnt signaling is essential during early inductive stages of kidney development, but its role during postinductive stages of nephron development and maturation is not well understood. In this study, we used Pax8Cre mice to target $\beta$-catenin deficiency to renal epithelial cells at the late S-shaped body stage and the developing collecting ducts. The conditional $\beta$-catenin knockout mice formed abnormal kidneys and had reduced renal function. The kidneys were hypoplastic with a thin cortex; a superficial layer of tubules was missing. A high proportion of glomeruli had small, underdeveloped capillary tufts. In these glomeruli, well differentiated podocytes replaced parietal epithelial cells in Bowman’s capsule; capillaries toward the outer aspect of these podocytes mimicked the formation of glomerular capillaries. Tracing nephrogenesis in embryonic conditional $\beta$-catenin knockout mice revealed that these “parietal podocytes” derived from precursor cells in the parietal layer of the S-shaped body by direct lineage switch. Taken together, these findings demonstrate that $\beta$-catenin/Wnt signaling is important during the later stages of nephrogenesis and for the lineage specification of parietal epithelial cells.


Development of the mammalian kidney is initiated by mutual interactions of the outgrowing ureteric bud and the metanephric blastema. The latter induces branching of the ureteric bud leading to formation of the collecting duct system, whereas the tips of the ureteric bud induce epithelial transformation of the metanephric mesenchymal cells. The induced cells of the metanephric blastema aggregate to form renal vesicles and upon successive invaginations become S-shaped bodies, which give rise to several distinct cell types of the nephron including parietal and visceral glomerular epithelial cells. On invasion by blood vessels, parietal and visceral epithelial cells build up the renal corpuscle. Whereas the visceral epithelial cells become podocytes, parietal epithelial cells form the parietal layer of Bowman’s capsule.

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Wnt molecules play important roles in the development of many organs using different signaling pathways (reviewed in Refs. 1, 2). Among these, the canonical pathway has attracted exceptional attention. Canonical Wnt signaling triggers stabilization of β-catenin, its nuclear translocation, and the formation of active transcriptional complexes using members of the T cell factor (TCF)/Lef family of transcription factors as binding partners.3 Using genetic mouse models, Wnt signaling has been shown to be functionally involved in different aspects of kidney development (reviewed in Ref. 4). During renal vesicle formation, Wnt9b expression in the ureteric bud is required for induction of the mesenchymal–epithelial transition of metanephric blastemal cells.5 Similarly, metanephric blastemal cells of mice lacking Wnt4 do not undergo epithelial transformation.6 Because Wnt4 can induce renal vesicle formation in vivo and explain culture even in Wnt9b mutant metanephric mesenchyme, it was suggested that Wnt4 acts in an autocrine fashion downstream of the paracrine Wnt9b factor.5,7 The blockage of mesenchymal-to-epithelial transformation results in rudimentary kidneys in both Wnt9b and Wnt4 knockout mice. Secondary to the absence of nephrogenesis, ureteric branching was also reduced, although to a larger extent in Wnt9b than in Wnt4 knockout mice.5,6 Both Wnt9b and Wnt4 signaling activities may primarily involve canonical Wnt signaling. Park et al.8 used Six2Cre mice to remove β-catenin activity specifically from the cells of the metanephric blastema. These β-catenin loss-of-function mutants lacked early and late markers of an inductive response, including Wnt4 expression; they did not form nephrons and showed early cessation of ureteric branching thus mimicking the phenotype of Wnt9b knockout mice.8 Furthermore, activation of a β-catenin gain-of-function mutation initiated a full inductive response in the metanephric mesenchyme even in a Wnt9b mutant background.8 Evidence that linked β-catenin function to Wnt4 signaling was provided by the demonstration of Lhx1 activation in Wnt4 mutants upon activation of β-catenin in the metanephric mesenchyme.8 Lhx1 expression has previously been shown to be dependent on Wnt4.9 Wnt4 signaling during epithelial-to-mesenchymal transformation may also involve noncanonical calcium/nuclear factor of activated T cells signaling.10,11 Canonical Wnt/β-catenin signaling has also been implicated in ureteric branching directly. β-Catenin reporter genes in transgenic mice have shown strong activity in the tips and stalks of the branching ureteric bud.12,13 Using Hoxb7Cre mice to remove β-catenin from the developing ureteric bud, it was found that β-catenin is required for ureteric branching.14,15 Finally, Wnt7b/β-catenin signaling has been shown to direct collecting duct growth and extension of the loop of Henle in the medullary zone.16 When Wnt7b was removed from the collecting duct system by Sox2Cre-mediated recombination, the renal medulla failed to form. However, in this case, the ligand Wnt7b signals in a paracrine fashion to interstitial cells of the surrounding mesenchyme. Accordingly, removal of β-catenin from the interstitium but not from the collecting duct mimicked the phenotype of mice lacking Wnt7b.16 In contrast, the Pax8Cre-mediated knockout of dkk1, an antagonist of Wnt signaling, resulted in an overgrown papilla.17 This suggests that Wnt7b along with dkk1 coordinate proper formation of the renal papilla in vivo.

The signaling mechanisms that control later stages of nephrogenesis such as differentiation of parietal and visceral epithelial cells of the glomerulus have not yet been defined. The essential requirement for β-catenin during earlier stages of kidney development and the lack of appropriate genetic tools to target specifically later stages of nephrogenesis have prevented any such analysis. In this study, we report the phenotype of conditional β-catenin knockout mice in which Ctnmb1 (mouse β-catenin gene) was removed during later stages of nephrogenesis through the use of Pax8Cre deleter mice. The conditional β-catenin knockout mice were found to be viable; however, renal function was impaired. Several kidney abnormalities were observed including lack of the outermost cortex, presence of glomerular cysts, and underdeveloped glomerular capillary tufts. Notably, podocytes were located in an abnormal parietal position replacing normal flat parietal epithelial cells. We conclude that β-catenin/Wnt signaling is required for proper cell fate decision of parietal epithelial cells and is responsible for maintaining the gross architecture of the mouse renal corpuscle in vivo.

RESULTS

We have previously analyzed canonical Wnt signaling activity during nephrogenesis using TCF/lacZ reporter mice and noted that specific β-galactosidase expression was occasionally found in a parietal position of developing glomeruli (Figure 1A and Ref. 13). We therefore reasoned that activation of the Wnt signaling pathway in parietal epithelial cells may be involved in their differentiation. To examine a potential role for β-catenin during late stages of nephrogenesis, mice with a floxed β-catenin allele18 were crossed with mice that express Cre recombinase under control of the Pax8 promoter.19 Using this approach, β-catenin deficiency can be targeted to the cells of the late S-shaped body stage and ureteric bud cells, whereas cells of the metanephric blastema are not affected.19 We confirmed the known pattern of activity of Pax8Cre mice by using β-galactosidase staining of embryonic kidneys derived from double transgenic Pax8Cre/Rosa26 lacZ reporter mice (Figure 1B). Both visceral and parietal epithelial cells of maturing glomeruli as well as ureteric bud cells revealed Pax8Cre activity (Figure 1B).

Next, we used Pax8Cre mice to remove β-catenin from developing kidneys. β-Catenin protein depletion was widespread in the adult kidneys of β-catenin–deficient mice (lox/lox Pax8Cre) (Figure 1, C–H). Collecting duct, proximal and distal tubular epithelial cells, and parietal epithelial cells were essentially negative for β-catenin expression. Only individual cells, scattered throughout the kidney in a mosaic fashion, had escaped the β-catenin knockout (Figure 1, D, F, and H). These findings prove the efficiency of the β-catenin knockout in our system.
We did not observe prenatal or perinatal lethality until 2 weeks of age. β-Catenin-deficient mice, however, showed significantly reduced survival time and kidney function (Supplemental Figure 1, A and B). Their kidneys appeared hypoplastic with the outermost cortex deleted and with the presence of glomerular cysts (Supplemental Figure 1, C and D).

The remaining tubular system presented the different segments with intact morphology. The proximal tubules consisted of a regular columnar epithelium with clearly detectable brush borders in periodic acid–Schiff (PAS) staining and electron microscopy (data not shown). The loop of Henle and distal tubules were morphologically normal, and connecting tubules and collecting ducts were positive for aquaporin 2 expression (Figure 1, E–H).

In β-catenin–deficient mice, most of the glomeruli had an abnormal morphology (Figure 2, A and B). They frequently showed underdeveloped capillary tufts and cystic dilation of Bowman’s capsule, which was properly connected to the proximal tubule (Figure 2B). Notably, juxtaglomerular glomeruli, which represent the earliest developing nephrons, mostly appeared normal (Figure 2A).

A striking phenotype at the cellular level was the regular presence of podocytes in a parietal position (Figure 2C). At the ultrastructural level, these parietal podocytes were fully differentiated and apparently

![Figure 1](image-url)  
**Figure 1.** TCF and Pax8Cre activity are found in presumptive parietal epithelial cells during glomerulogenesis. (A) Enzymatic β-galactosidase staining of embryonic kidneys (embryonic day 15.5 [E15.5]) derived from TCF/lacZ reporter mice. Specific β-galactosidase expression is seen in parietal epithelial cells of maturing glomeruli. (B) Enzymatic β-galactosidase staining of cryosections of embryonic kidneys (E15.5) derived from Pax8Cre/Rosa26R mice. β-Galactosidase positivity is seen in all epithelial cells of the kidney including cells of the S-shaped body (SS), collecting duct (CD), visceral epithelial cells (VECs), and parietal epithelial cells (PECs) of a maturing glomerulus and tubules (T). Cells of the metanephric blastema (MB), interstitial cells, and blood vessels are negative. (C and D) Immunostaining for β-catenin in control (lox/lox) and β-catenin–deficient (lox/lox Pax8Cre) mice at 3 months of age. (C) Control kidneys show β-catenin expression in all tubular epithelial cells and parietal epithelial cells (arrow) at the basolateral surface. Aquaporin 2 is detected at the apical surface of collecting duct cells. UP, urinary pole. (F) In kidneys of β-catenin–deficient mice, β-catenin is efficiently removed in nearly all epithelial cells. (arrow = parietal epithelial cells). A few epithelial cells show persistent β-catenin expression (arrowhead). (G) Aquaporin 2 positive tubules in control kidneys show strong β-catenin expression. (H) In β-catenin–deficient mice, aquaporin 2 expression is similar in distribution and intensity compared with those in control mice. A few epithelial cells show persistent β-catenin expression (arrowhead). Original magnifications: ×500 in A; ×125 in B; ×500 in C and D; ×750 in E–H.
had attracted parietal capillaries (Figure 2D). A three-layered capillary-to-urinary space barrier similar to the glomerular filtration barrier was formed. It consisted of a fenestrated capillary endothelium (the fenestrae were mostly bridged by diaphragms) followed by a thick basement membrane consisting of a lamina rara interna, densa, and rara externa like the glomerular basement membrane at the tuft, finally by a layer of interdigitating podocyte foot processes with slit membranes indistinguishable from those of visceral podocytes proper (Figure 2E). Occasionally, parietal capillaries protruded into Bowman’s space (Figure 2, F, G, and H). Like glomerular capillaries within the tuft, parietal capillaries formed a capillary neck that was filled with arborized cells reminiscent of mesangial cells. Their processes were studded with actin filaments, and they were connected to the turning points of the peripheral endothelial basement membrane into the parietal basement membrane (Figure 2I).

At the molecular level, parietal podocytes were found to be positive for Wilms’ tumor suppressor gene 1 (WT1) protein (Figure 2, J and K), vascular endothelial growth factor (VEGF) (Figure 2, L and M), and synaptopodin (Figure 2, N and O). Notably, parietal capillaries were in continuity with peritubular capillaries (Figure 3A). Furthermore, affixation of the efferent arteriole to the outer aspect of Bowman’s capsule was frequently observed leading to efferent arterioles that ran alongside Bowman’s capsule for half (or even more) of its circumference (Figure 3B). As a consequence, the glomerular...
ureteric bud (Figure 4A). During maturation of the glomeruli, β-catenin expression in embryonic kidneys. In the renal vesical stage, similar to presumptive visceral podocytes (Supplemental Figure 2A). With cells at the parietal cell layer that were morphologically similar; the vascular pole in between appears to be unorganized. (E) Cells of the proximal tubule (left) appear cuboidal with microvilli at the apical side forming the brush border. They are densely packed with mitochondria. Parietal podocytes (right) are flat and characterized by foot processes. Between those cell types, there is always a morphologically different epithelial cell (EC), which has none of the morphological characteristics of the neighboring cell types. Original magnifications: ×500 in A, B, and D; ×5000 in C; ×2500 in E.

vascular pole became misshapen with widely separated afferent and efferent arterioles. Parietal podocytes were not only associated with capillaries but also with arterioles (Figure 3C). A slim glomerular stalk was lacking; instead, capillaries were associated with parietal podocytes and Bowman’s capsule. Note the continuous endothelium and the many profiles of smooth muscle cells (asterisks). G, glomerulus. (D) Glomerular profile with a misshapen vascular pole. The afferent (a) and efferent (e) arterioles are widely separated; the vascular pole in between appears to be unorganized. (E) Cells of the proximal tubule (left) appear cuboidal with microvilli at the apical side forming the brush border. They are densely packed with mitochondria. Parietal podocytes (right) are flat and characterized by foot processes. Between those cell types, there is always a morphologically different epithelial cell (EC), which has none of the morphological characteristics of the neighboring cells.

The origin of parietal podocytes can be explained by at least two different scenarios. Either the precursors of the parietal epithelial cells have chosen a different (visceral-like) cell fate or parietal epithelial cells died and the parietal compartment has been subsequently invaded by visceral epithelial cells. To address this question, we studied early glomerulogenesis. Histologic analysis of developing metanephros showed normal progression of nephroneogenesis at all stages from comma-shaped bodies to capillary loop stages until neonatal day 6. From neonatal day 6 on, however, we observed immature cells at the outer cortex in the kidneys of β-catenin–deficient mice. The glomerular anlagen of the last generations of developing nephrons were unusually organized with cells at the parietal cell layer that were morphologically similar to presumptive visceral podocytes (Supplemental Figure 2A).

Using immunohistochemistry, we assayed β-catenin expression in embryonic kidneys. In the renal vesical stage, β-catenin expression was found in all epithelial cells including ureteric bud (Figure 4A). During maturation of the glomeruli, β-catenin expression declined in presumptive podocytes but remained high in parietal epithelial cells (Figure 4, B and C). Staining of β-catenin–deficient mice showed that β-catenin deficiency started in late S-shaped bodies and was complete in the maturing glomerulus stage (Figure 4, D and E) affecting presumptive parietal and tubular epithelial cells. We did not detect any change in E-cadherin and zonula occludens-1 protein (ZO-1) expression in β-catenin–deficient mice (Supplemental Figure 2, B and C). In control and β-catenin–deficient mice, visceral and parietal epithelial cells did not express E-cadherin in the S-shaped body stage.

To trace podocyte-specific differentiation, we analyzed WT1 expression. At the S-shaped body stage, WT1 was expressed in both presumptive podocytes and cells of Bowman’s capsule (Figure 4F). Whereas WT1 expression remained high in the podocyte lineage in control mice during later stages of maturation, it faded out in the cells of Bowman’s capsule (Figure 4G). In β-catenin–deficient mice, however, WT1 expression remained persistently high in the parietal lineage throughout glomerulogenesis (Figure 4G); also, VEGF was expressed by these cells (Figure 4, H and I). Furthermore, electron microscopy revealed developing podocytes at the parietal cell layer during S-shaped body maturation from early S-shaped body to later stages (Figure 4, J–L). Those presumptive podocytes attracted capillaries already at these early stages (Figure 4, K and L). In maturing glomeruli, developing parietal podocytes formed foot processes and attracted capillaries (Figure 4, M and N). Using TUNEL staining, we did not observe parietal epithelial cells undergoing apoptosis (data not shown).

Together, our findings demonstrate a switch in lineage differentiation of parietal epithelial cells toward a podocyte-like cell fate when precursor cells of the late S-shaped body stage become deficient for β-catenin expression. Secondary changes included underdeveloped glomerular tufts and substantial reorganization of Bowman’s capsule by adopting a glomerulolike architecture apparently orchestrated by parietal podocytes.

DISCUSSION

As has previously been shown, the mesenchymal–epithelial transition of metanephric blastemal cells, a key step during nephron development, is strictly dependent on Wnt4 expression in cis and Wnt9b expression in trans (from the ureteric bud). Conditional knockout experiments have demonstrated that β-catenin is the likely intracellular mediator of these effects. Because nephroneogenesis was completely blocked at very
Figure 4. Parietal podocytes develop directly from the parietal layer of S-shaped bodies in β-catenin–deficient mice. (A) Immunostaining for β-catenin reveals β-catenin expression in all cells of the comma-shaped body in both control (lox/lox) and β-catenin–deficient (lox/lox Pax8Cre) mice. (B and C) At the S-shaped body stage, β-catenin is expressed in precursor cells of the tubules and the parietal epithelial cells (PECs) (arrowheads) but only weakly in visceral epithelial cells (VECs) (asterisks) (boxed areas enlarged in C). There is no difference between control and β-catenin–deficient mice at this stage. (D and E) In maturing glomeruli, β-catenin staining is only observed in PECs (arrowheads) of control mice. VECs (asterisks) are neither positive in control nor in β-catenin–deficient mice (boxed areas enlarged in E). (F) At the S-shaped body stage, WT1 stains positive in both VECs (asterisks) and PECs (arrowheads). (G) In maturing glomeruli of control mice, WT1 expression is still observed in VECs (asterisks) but not in PECs (arrowheads). In β-catenin–deficient mice, both VECs and PECs remain positive for WT1 expression. (H) At the S-shaped body stage, VECs (asterisks) are strongly positive for VEGF, whereas PECs (arrowheads) are only weakly positive. (I) In maturing glomeruli, positive VEGF staining is observed in VECs (asterisks) but not in PECs (arrowheads) in control mice. In β-catenin–deficient mice, both VECs and PECs remain positive for VEGF. (J through N) Transmission electron micrographs of developing glomeruli. (J–L) S-shaped body stage. (M and N) Capillary loop stage. At the S-shaped body stage, presumptive PECs (asterisks) of control mice are flat (J), whereas the presumptive PECs from β-catenin–deficient mice (K and L) are columnar in shape (asterisks) and associated with capillaries (arrows). At the maturing glomerulus stage from a β-catenin–deficient mouse (M) (accompanied by a schematic draft in N), PECs have developed foot processes lining up along the parietal basement membrane (blue in the scheme) as have the VECs on the glomerular basement membrane (red in the scheme). Large capillaries (lumina are orange in the scheme) are found at the outer side of the parietal basement membrane. E18.5. Original magnifications: ×750 in A–I; ×5000 in J; ×2000 in K and L; ×1500 in M.
early stages, however, no functional kidneys were formed in these previous studies. Our study was designed in such a way that β-catenin was removed from developing kidneys at later stages only. This was achieved by a specific Cre-deleter strain, Pax8Cre, which is active in late S-shaped bodies and developing collecting ducts but not within the metanephric blastema or the branching ureteric bud tips. This approach allowed us to study the role of β-catenin during late nephrogenesis and nephron maturation.

As expected, early steps of nephron development occurred normally in conditional β-catenin-deficient mice. In later steps, characteristic deficiencies were found. The main findings were a virtually normal collecting duct system demonstrating no absolute requirement for β-catenin in maintaining this compartment, a normal although shortened tubular system along with a lacking outermost cortex, and, most prominently, abnormal glomeruli with underdeveloped capillary tufts and transformation of parietal epithelial cells into podocytes. Whereas the lack of a superficial tubular layer and an overall shortening of the tubular system might reflect an intrinsic requirement for β-catenin for tubular cell proliferation, it could equally well result from the glomerular maldevelopment indicating simply the lack of downstream growth-promoting signals from the glomerulus to the tubule. It is unclear why the earlier generations of nephrons were not affected in a similar way, but similar observations have previously been made in a conditional knockout model of bone morphogenic protein-7.20

The prominent glomerular phenotype is difficult to understand in terms of simple β-catenin deficiency. First, endothelial cells are not targeted by Pax8Cre activity, which is specific for epithelial cells. Second, although podocytes will become recombined by Pax8Cre activity, because they lack β-catenin expression they are unlikely to become phenotypically changed. Accordingly, no phenotype has been observed in a podocyte-specific knockout model of β-catenin.21 Hence, the observed growth retardation of the capillary tuft can only be explained by indirect scenarios such as a distorted VEGF gradient caused by ectopic production of VEGF in the periphery of Bowman’s space, by altered glomerular flow caused by the parietal capillaries, or gross architectural constraints resulting from the inappropriate anchoring of the tuft to the extraglomerular mesangium. Alternatively, as it has been shown previously,23 a significant proportion of podocytes is recruited from the parietal cell compartment. As shown in this study, inactivation of β-catenin replaced parietal cells by podocytes, which can no longer undergo cellular division. Therefore, it can be speculated that only insufficient numbers of podocytes could be recruited onto the glomerular tuft, because the cellular pools on Bowman’s capsule were depleted prematurely. Insufficient recruitment of cells then resulted in a hypoplastic but intact capillary tuft. It can also be speculated that aberrant VEGF secretion by parietal podocytes in the periphery indirectly disturbed the gradient of VEGF seen by glomerular endothelial cells thereby negatively interfering with directed capillary outgrowth.

The parietal podocytes observed in our model were highly differentiated cells and almost indistinguishable from their visceral counterpart. At least three different possible scenarios may explain the origin of these parietal podocytes. Failure of normal parietal epithelial cells to develop and/or premature death followed by invasion of podocytes from the visceral pole into the unoccupied parietal compartment may provide one possible explanation. Second, transdifferentiation of mature parietal epithelial cells into podocytes may have occurred, or, third, a switch in cell fate decision from a parietal to a visceral lineage may have happened during early nephrogenesis. Strong evidence has been compiled from our studies in support of the latter. There was no loss of parietal cells apparent during early nephron development, and we could exclude transdifferentiation of mature parietal epithelial cells because podocyte-specific differentiation could be traced back to a parietal position at the earliest stages possible. Therefore, one can conclude that β-catenin is required in prospective parietal epithelial cells for proper differentiation and maturation. In the absence of β-catenin/Wnt signaling, however, prospective parietal epithelial cells acquire the phenotype of visceral epithelial cells, which seems to represent the default pathway of differentiation in these closely related cell types (Figure 5).

It is interesting to note that β-catenin-deficient mice showed a reduction but not a complete absence of renal function. We think that, in addition to the intact juxtaglomerular glomeruli, the abnormal midcortical and superficial glomeruli contributed to the overall filtration rate at a significant rate. It is interesting to discuss the potential functional role of parietal podocytes, which constituted a significant proportion of podocytes in this model. Parietal podocytes apparently had attracted capillaries and mesangial-like cells toward their outer aspect mimicking formation of glomerular capillaries. These findings suggest that formation of the filtration barrier and capillary loops may be entirely orchestrated by podocytes. Considering the likely differences in transmural pressure gradients between tuft and parietal capillaries (blood pressure in parietal capillaries should be much too low to generate an effective filtration pressure) and the different morphology of parietal capillaries (endothelial fenestrae having diaphragms), it is unlikely that parietal capillaries contribute to filtration at all. Whereas fenestrated capillaries without diaphragms constitute just a weak barrier, fenestrated capillaries with diaphragms will form a much stronger barrier. The reason why visceral capillaries develop fenestrae without diaphragms but parietal capillaries develop fenestrae with diaphragms is unclear.

The close developmental relationship of parietal and visceral epithelial cells becomes independently evidenced by the complete switch in cell fate decision seen in the β-catenin knockout mice presented in this study.

In the current study, we showed that Pax8Cre-directed knockout of β-catenin leads to developmental defects affecting the gross architecture of glomeruli. A reduced number of functional nephrons may lead to chronic renal disease and
eventually to renal failure. These findings prove an important role for β-catenin during late stages of nephrogenesis.

The frequent occurrence of parietal podocytes suggests that β-catenin is involved in the cell fate decision of parietal epithelial cells.

CONCISE METHODS

Transgenic Mice
Animal experimental procedures were performed according to German and European Legislation and approved by the Governing Office for Baden-Württemberg (Regierungspräsidium, Karlsruhe, Germany).

Mice carrying homozygote floxed alleles of the β-catenin gene\(^1^9\) (in this study referred to as β-catenin\(^{\text{fl}}\)/β-catenin\(^{\text{fl}}\)) were interbred with Pax8Cre mice\(^1^9\) generating mice heterozygous for Pax8Cre and β-catenin\(^{\text{fl}}\) (in this study referred to as Pax8\(^{\text{fl}}\)/β-catenin\(^{\text{fl}}\)/β-catenin\(^{\text{fl}}\)). Pax8\(^{\text{fl}}\)/β-catenin\(^{\text{fl}}\)/β-catenin\(^{\text{fl}}\) mice were interbred again creating mice heterozygous for Pax8Cre and homozygous for β-catenin\(^{\text{fl}}\) (in this study referred to as β-catenin\(^{\text{fl}}\)-deficient mice [lox/lox Pax8Cre]). Littermates were used as controls (in this study referred to as lox/lox Pax8Cre). Rosa26R is a Cre reporter line in which a gene encoding cytosolic β-galactosidase is under control of the ubiquitously active Rosa26 promoter.\(^2^3\) However, expression is conditional on the prior removal of a neomycin expression cassette that is flanked by loxP sites. Rosa26R mice were used as Cre recombinase reporter mice.

Animal Housing
We housed mice in groups of up to five. They received regular laboratory chow, and water was supplied ad libitum. We maintained a 12-hour day and night cycle. For timed pregnancies, it is assumed that the morning of plug detection corresponds with embryonic day 0.5. Embryo ages are rounded to the nearest half day.

Genotyping
For the identification of the β-catenin alleles (RM41/RM42 primers), the Pax8Cre transgene (LC-1 primers), and Rosa26R transgene (Rosa26R primers), DNA was isolated from tail biopsies of adults and embryos. After lysis in buffer containing proteinase K, genomic DNA was isolated using QIAamp DNA Mini Kit (Qiagen GmbH) and dissolved in TE buffer; 0.5 mg of genomic DNA was used for PCR.

The PCR conditions were set as follows: 1 cycle of 3 minutes at 94°C, 35 cycles of 30 seconds at 94°C, 30 seconds at 52°C–60°C, 30 seconds at 72°C; 1 cycle of 7 minutes at 72°C. β-Catenin primers were as follows: RM41, 5′-AAGGTAGAGTGAAGATTGTGTT-3′; RM42, 5′-CACCAGTGCCTGTCTTATTC-3′; generating 324-bp and 221-bp products from the floxed and wild-type alleles. LC-1 primers were as follows: Cre3, 5′-TCGCTGATTACCGGTGATGC-3′; Cre4, 5′-CCATGAGTAGGAACGAACCTGGTCG-3′; Rosa26-R primers were as follows: Rosa26R-fwd1, 5′-AAAGTTGAGGTGAAGATTGTGTT-3′; Rosa26R-rev1, 5′-GGGAAGAGTTTGTTGCACC-3′.

β-Galactosidase Staining
To reveal β-galactosidase activity, we prepared dissected tissues. We washed the samples in PBS, fixed them in ice-cold 3% paraformaldehyde for 1 hour, washed them in PBS again, and incubated them overnight in 18% sucrose in PBS at 4°C. The next day, we froze the samples on dry ice and stored them at −80°C. We incubated 5-μm cryosections overnight in the dark in 5-bromo-4-chloro-3-indolyl β-d-galactopyranoside (X-gal) solution (50 mM Tris HCl pH 7.5, 2.5 mM potassium ferriyrocyanide, 15 mM NaCl, 1 mM MgCl\(_2\), and 0.5 mg/ml X-gal). We counterstained cryosections with eosin and mounted them in glycerol–gelatin.

Electron Microscopy
The mice were fixed by retrograde total body perfusion-fixation as previously described\(^2^3\) using 3% glutaraldehyde, 0.1 M cacodylate, and 0.1% picric acid as fixative. We processed tissue by standard procedures and embedded it into Epon. We cut 1-μm sections with an Ultratome microtome (Leica). Semithin sections were stained with methylene blue\(^2^5\) and examined with light microscopy; ultrathin sections were stained with uranyl acetate and lead citrate and studied with transmission electron microscopy.
For immunocytochemistry, hematoxylin and eosin staining, PAS staining, we perfused mice anterograde with 3% paraformaldehyde (PFA) in PBS and immersed the organs in the same fixative for 2–4 hours before embedding them in paraffin. We performed hematoxylin and eosin stains, PAS staining, and TUNEL staining according to standard protocols.

Immunocytochemical detection of β-catenin (monoclonal mouse anti-human β-catenin antibody; 1:100; BD Transduction Laboratories, BD Biosciences), wt1 (polyclonal rabbit anti-human WT-1 antibody; 1:100; Santa Cruz Biotechnology, Heidelberg, Germany), VEGF (polycional goat anti-human VEGF antibody; 1:140; R&D Systems), and E-cadherin (monoclonal mouse, anti-human E-cadherin c-terminal recombinant protein antibody; 1:125; BD Transduction Laboratories, BD Biosciences) was performed on 4-μm paraffin sections of PFA-perfused tissue according to standard protocol. For detection, we applied Vectastain Elite ABC-Kit (Vector Labs) and diaminobenzidine as substrate.

Blood and Urine Samples
We collected blood samples from the retro-orbital sinus into heparinized tubes after killing the mice with CO2. We centrifuged heparinized blood for 10 minutes at 1500 rpm and took the supernatant plasma. Urine probes were collected with metabolic cages over 72 hours.

Cortex Width
PFA-fixed and PAS-stained paramedian kidney sections were analyzed. The radial distances between the inner rim of juxtamedullary glomeruli and the cortex rim was measured 20 times at random locations, and the mean was set as the cortex width of a kidney.

Statistical Analyses
Results are reported as mean ± SD. Differences between the experimental and control animal in each group were tested with the unpaired t test. P<0.05 was considered statistically significant. The difference of survival between experimental and control animal was tested by Kaplan–Meier analyses. The statistical analyses were performed with GraphPad Prism 5.0.

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DISCLOSURES
None.

REFERENCES


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