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Research Article

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The Role of Core Planar Cell Polarity Vangl2 Gene in the Renal Tubule Development in Mice

Abstract

Background: Polycystic kidney disease (PKD) is a common kidney disease that affects the development and maintenance of renal tubules, leads to cyst formation, and often progresses to end-stage kidney disease. It has been postulated that defective planar cell polarity (PCP) signaling contributes to initiation of cyst formation in PKD via controlling both convergent extension (CE, a process of directional cell movements) and oriented cell division (OCD, a process of directional cell divisions during tubular elongation post-natally). Indeed, mutations of the key PCP gene, Van Gogh-like 2 (Vangl2), lead to abnormal renal tubules in murine embryonic kidneys, correlating with the original postulate.

Methods: In order to further understand the influence of the Vangl2 gene on renal morphogenesis and cystogenesis, control and Vangl2 mutant embryos—as well as post-natal Vangl2 mice with conditional excision of the Vangl2 gene in renal collecting tubules—were generated, then analyzed using immunostaining and fluorescence microscopy.

Results: Our results show that Vangl2 plays a role in CE and apical constriction (AC) during embryonic stage of tubulogenesis. Compared to control animals, mutant Vangl2$^{\Delta/\Delta}$ and conditional Vangl2$^{\Delta/CD}$ embryos displayed: i) a significant dilation in the diameter of renal tubules seen as an increased tubule cross-section area and a larger number of cells per cross-section; and ii) changes in cell shape indicative of defective AC. Surprisingly, post-natal mice showed virtually no difference in any of these aspects comparing to control mice, suggesting that other pathways may compensate for the lack of PCP signaling in maintenance of the tubule architecture.

Limitations: a) The analysis of the renal tubules at the specific time points does not account for the dynamics of tubular movement and growth in real time; b) a mechanistic and morphological distinction between mice and humans may exist in the renal collecting duct tubules, pertaining to the Vangl2 gene's influence in the PCP pathway; and c) the degree of mosaicism resulting from the gene excision by Cre-recombinase may correlate with the severity of the phenotype.

Conclusion: We conclude that the PCP pathway is required for normal tubule development during embryogenesis. Our results, however, indicate that the cystogenesis seen in PKD postnatally may not be directly attributed to the disrupted PCP signaling, and requires the derangement of additional pathways.

Introduction

Polycystic kidney disease (PKD) is characterized by the formation and progressive enlargement of cysts in the kidney, contributing to an impairment of renal function and eventual end-stage kidney disease.(1) Patients with autosomal dominant polycystic kidney disease (ADPKD), the most common form of PKD, experience a significantly reduced quality of life, and most require extensive therapy, dialysis, and kidney replacement by the age of 55-60.(2) Indeed, ADPKD is the most common potentially lethal single-gene disorder, occurring on average in 1 per 600 PKD cases worldwide.(2-4) There is presently no cure for PKD, which highlights the necessity of research on the mechanisms underlying the development and maintenance of renal tubules.

Formed through mesenchymal-to-epithelial transition and branching morphogenesis, kidney tubules are integral to the proper hemodynamics in the body.(5) Upon its formation, the ureteric bud (UB) receives signals from the mesenchyme, which dictates the branching of UB into structures such as the ureter and, more pertinent to this article, the system of collecting duct tubules of the kidney.(6) In order to promote the branching and tubular growth/elongations, the cells undergo organized, directional intercalation during embryonic development, known as convergent extension (CE), and oriented cell division (OCD), which, in rodents, occurs in the first two weeks after birth.(7,8) Cell proliferation within the tubules is therefore directly linked to the proper elongation of the tubules and an establishment and the maintenance of tubular diameter.

In effect, it is critical for cells to exhibit polarity, as they migrate and differentiate into various tissues, including renal tubules. Throughout several embryonic processes, two polarity axes play a role in defining the proper orientation of epithelial cells. The first, apical-basal polarity, refers to the orientation of the cells perpendicularly to the basal lamina, which demonstrates adhesive properties that signal for the formation of an apical surface at the opposite end of the cell. The second axis of polarity, known as planar cell polarity (PCP), defines cell polarity within a tissue axis (e.g. along a tubule) perpendicular to the apical-basal polarity.(9) Typical examples of PCP are the patterns of *Drosophila* wing hairs and stereocilia in the inner ear of mammals.(10,11) However, it is the role of the PCP pathway in the formation of a uniform kidney tubule diameter that is of greatest concern in this paper.

In 2006, Fischer et al. detected a randomized OCD in two independent postnatal rodent models of polycystic kidney disease. This led others to postulate that defective PCP signaling is causative of cystic initiation, although, until recently, extensive research has not been done to investigate a potential mechanism.(12,13) Mutations of key components in the PCP pathway have been linked to a renal cystic phenotype; for example, an increased tubule diameter in embryonic kidneys of Fat4 (PCP-gene) homozygous mutants was attributed to OCD disruption.(14) Fat4-/- mice

carrying one disrupted allele of Vangl2 exhibited an exacerbated dilatation of renal tubules comparing to Fat4-/-, particularly in the medullary zone that mostly consists of collecting ducts bundles.(14) One recent paper describes the genetic interactions between known PKD-related gene, Pkd1, and Vangl2 in the brain ventricles.(15) Loss of Pkd1 affects planar orientation and position of basal bodies in the multiciliated ependymal cells in the brain; in compound heterozygous mice for Pkd1 and Vangl2, this phenotype was exacerbated. Therefore, there is some evidence that disruption of PCP gene Vangl2 may contribute to PKD, including genetic interaction between the two pathways.

In *Xenopus*, Vangl2 has specifically been linked to the regulation of apical constriction (AC) during gastrulation, an early morphogenetic process which facilitates the proper arrangement of cell layers in the embryo.(16) Ossipova et al. showed that loss of Vangl2 affected cell wedging and recruitment of specific acto-myosin regulators that drive the shortening of the apical surface length (apical constriction).(16) Despite the reports of dilated tubules in embryonic Vangl2 mutant kidneys, the process by which Vangl2 regulates tubular diameter and cystogenesis remains unknown.

In the research presented here, we studied the role of Vangl2 in renal tubulogenesis during embryonic stages, as well as during early postnatal stages. From our results, we report that a loss of Vangl2 leads to tubular dilatation and cystic transformation during embryonic kidney development via the control of the processes of convergent extension and apical constriction. However, architecture of tubules in postnatal mouse kidneys is not affected by the loss of Vangl2. Thus, we conclude that the relationship between defective PCP signaling and PKD is incorrectly labelled as cause-and-effect.

Materials and Methods

Generation of Vangl2 mutant mice

We previously reported the Vangl2 mouse with a Floxed allele which contains LoxP sites flanking Exon 4 ($Vangl2^{FL/FL}$).(17) The generation of the Vangl2 mice with a ubiquitous excision of Exon4 ($Vangl2^{\Delta/\Delta}$ null allele) was previously described.(17) For this study, $Vangl2^{\Delta/\Delta}$ mutant mice were generated by brother–sister mating. These mice are embryonically lethal at around embryonic day (E) 17.5-18.5. Age matched $Vangl2^{FL/FL}$ embryos were used as controls. Hoxb7-Cre mice (expressing Cre-recombinase transgene in the collecting duct tubule (18)) were kindly provided by Dr. Carlton Bates (University of Pittsburg, PA). To generate Vangl2 mice with a conditional excision of Vangl2 in the collecting ducts, series of breedings were undertaken to obtain Hoxb7-$CreV2^{\Delta/CD}$ (conditional mutant $Vangl2^{\Delta/CD}$) mice. $Vangl2^{FL/+}$ mice were used as controls, with intact Floxed alleles due to the lack of Cre allele. The mouse DNA was isolated from a tail biopsy and was analyzed by PCR. All experiments with $Vangl2^{\Delta/\Delta}$ and a matching control were conducted on E17.5 embryos. Post-natal mice at P7 of genotypes $Vangl2^{\Delta/CD}$ and $Vangl2^{FL/+}$ were generated, and the kidneys were excised and preserved for further analysis. All animal work was conducted per the Canadian Animal Care Guidance with approval by the Animal Care Committee, McGill University.

Sectioning of Tissues

Embryonic E17.5 and P7 tissues were fixed overnight in 4% Paraformaldehyde/Phosphate Buffered Saline, pH 7.4, at 4°C. Then, they were dehydrated in various concentrations of ethanol/Phosphate Buffered Saline, embedded in paraffin (McGill University Health Center Research Institute histology service) and sectioned at 4 μm on the microtome. Each section was viewed on a light microscope to assess the depth of sectioning. The sections were mounted on slides (2 per slide) and dried in the incubator overnight at 37°C.

Immunofluorescence Staining

The E17.5 and P7 sections were de-paraffinized by heat at 55°C in a dry incubator and treated with xylene, according to standard protocol. The sections were rehydrated in the ethanol solutions of progressively decreasing concentrations in the Phosphate Buffered Saline, pH 7.4. The epitope retrieval procedure was performed by immersing slides in the 10 nM Na-Citrate buffer which was pre-heated at 100°C (brought to a boil) (Vector Laboratories, CA). The slides were then incubated in a microwave for 20 min at maximum heating. Special care was taken to ensure that the boiling buffer was covering the sections at all times. The immunostaining was conducted as follows: first, the tissues were blocked overnight at 4°C with a solution containing 3% Bovine Serum Albumin, 5% Normal Goat Serum, 5% Normal Donkey Serum, 0.1% TritonX, and 0.05% Sodium Dodecyl Sulphate in the PBS (pH 7.4). The tissues were then stained with anti-Calbindin (the specific marker of collecting ducts, 1:500, D-28K) and anti-E-Cadherin (a marker of epithelial cells, 1:500) antibodies followed by the incubation with secondary donkey anti-mouse IgG Alexa 546 and goat- anti-rabbit IgG Alexa 488 antibodies. The nuclei were visualized by staining with 4',6-diamidino-2-phenylindole (DAPI). Fluorescence microscopy was performed using the Zeiss AxioObserver Z1 inverted fluorescence microscope.

Morphological analysis

Low Magnification Analysis: Low magnification images were compared by looking at three renal morphological features: a) the collecting duct tubules; b) the cortico-medullary definition; and c) the overall size of the kidney, in relation to the scale bar. Dilated tubules were defined by their larger lumen, whereas cystic tubules displayed an aggravated dilatation and were lined by a flattened and disorganized epithelium.

Convergent Extension Analysis: CE analysis was performed on the images using the Zeiss ZEN disk 2012 (Blue Edition) program (Zeiss, Germany). Analysis consisted of measuring the cross-sectional area of the tubule, as well as its width. We defined the 'width' as the longest axis seen in the tubular cross section. The perpendicular axis to this width was also measured and the ratio of the two diameters was calculated. Only the cross-sections with measurements within 10% of each other were qualified as "perfectly" transverse and used for the CE analysis. For E17.5 embryos and P7 mice, a minimum of 20 perfect transverse structures were used for every animal to calculate the tubular area and width. The cross-sections used for the CE analysis were also used to count the number of nuclei. Four embryos and three P7 mice per genotype were analyzed.

Apical Constriction Analysis: Apical constriction (AC) analysis was performed on the transverse "perfect" tubule sections of the E17.5 kidneys by measuring the apical and the basal lengths of each cell (visualized with anti-Cadherin antibody). These apical and basal lengths were defined as the size of the most apical and basal surfaces of the cell, spanning from one lateral membrane to the other. The ratio of apical-to-basal lengths was calculated for each cell. The measurements were conducted on at least 60 transverse tubule sections in 4 embryos per genotype.

Statistical Analysis

To test for significant differences (p-value) between the results, the Student's t-test with an equal two-tailed distribution and an unequal variance was used. A p-value smaller than 0.05 was considered as statistically significant. Standard mean errors are shown in each bar graph.

Results

Analysis of Embryonic (E17.5) Mouse Kidney

In order to ascertain the role of Vangl2 in embryonic renal tubular development, embryonic day (E) 17.5 sections of the control ($Vangl2^{FL/FL}$), null mutant ($Vangl2^{\Delta/\Delta}$, with ubiquitous loss of Vangl2 in all cells), and conditional ($Vangl2^{\Delta/CD}$ Cre+) embryos were analyzed. Embryonic $Vangl2^{\Delta/\Delta}$ kidneys displayed the following abnormalities: i) hypoplasia (at low magnification, mutant maximal kidney sections fit into the 'image frame', whereas a larger number of images were required to capture the entire area of the maximal control kidney, indicative of hypoplasic mutant kidneys); ii) a disorganized tubular architecture with a profound loss of the medullary zone; and iii) the presence of dilated and cystic

tubules (Fig. 1). Vangl2$^{\Delta/CD}$ kidneys also displayed an abnormal renal morphology, albeit the degree of abnormalities was more moderate. Control kidneys had a clearly demarcated cortico-medullary division, and did not display any dilated tubules or cystic structures.

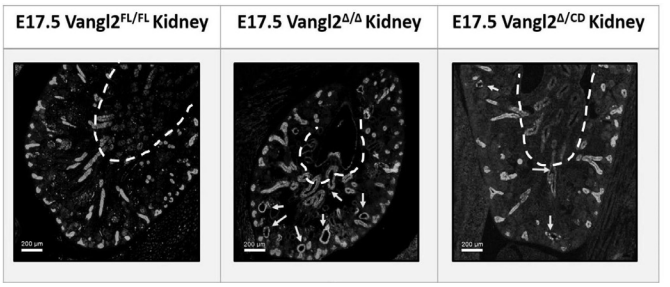

Fig. 1. Morphology of E17.5 control Vangl2FL/FL, null Vangl2$^{\Delta/\Delta}$, and conditional Vangl2$^{\Delta/CD}$ (HoxB7CreV2$^{\Delta/CD}$) kidneys (50X magnification). Kidneys immunostained with anti-Calbindin (green, collecting duct marker), anti-E-Cadherin (red, epithelial tubular cells marker) antibodies and DAPI (blue, nuclei marker) stained. Dotted lines indicate the cortico-medullary definition. Vangl2$^{\Delta/\Delta}$ kidneys are hypodysplastic, with dilated cystic collecting ducts (arrows). Vangl2$^{\Delta/CD}$ kidneys feature dilated tubules and occasional cystic phenotype.

During embryogenesis, renal tubule formation has been shown to be controlled by convergent extension, regulated by PCP signaling.(19) To assess CE (regulated by the PCP pathway), we measured the cross-sectional area and additionally counted the average number of cells in the "perfect" collecting duct cross sections (differences of two perpendicular axes are less than 10%). We found that E17.5 Vangl2$^{\Delta/\Delta}$ and Vangl2$^{\Delta/CD}$ renal tubules had a cross-sectional area that was significantly larger than that of the controls, indicative of the tubular dilatation (Fig. 2). We likewise observed that the average number of cells surrounding the tubular lumen (visualized by the DAPI-stained nuclei) was statistically higher in both null and conditional kidneys, which was another manifestation of the failed CE that led to an excessively dilated tubular phenotype (Fig. 2).

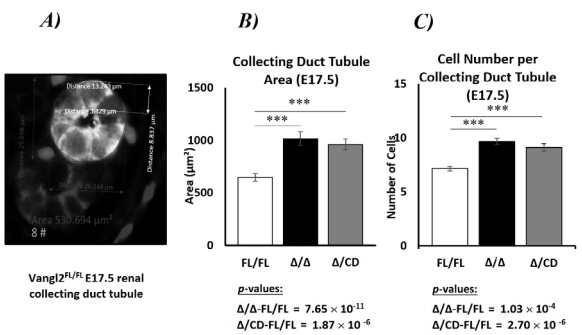

Fig. 2. Convergent extension (CE) analysis in control, null, and conditional renal collecting ducts in embryonic E17.5 kidneys. A) Perfect cross-section of a collecting duct tubule at 40X (perpendicular measurements in red); B) Statistical analysis of the collecting ducts area.; C) Statistical analysis of the cell number per collecting duct (Vangl2$^{FL/FL}$; N=87. Vangl2$^{\Delta/\Delta}$; N=85. Vangl2$^{\Delta/CD}$; N=113). ***p<0.001.

To analyze apical constriction, the same tubular cross sections chosen for convergent extension measurements were studied. The apical constriction describes the process of cellular 'wedging', where the apical surface of the cell is constricted due to the recruitment of specific proteins to that surface. This allows for a tighter cell packaging and the reduction of the diameter in developing tubules. The collecting ducts (identified by Calbindin staining) were stained with anti-E-cadherin to delineate lateral borders of each cell; the apical and basal lengths were measured and the apical-to-basal ratio was calculated. We observed that this ratio was larger in both null and conditional mutants (Fig. 3), indicating that the cells were more cuboidal. Although significantly different from controls, we consistently found that the tubular phenotype in conditional mice was milder than in null mutants, including fewer cells in the tubular cross-sections, as well as less profound changes in cell shape.

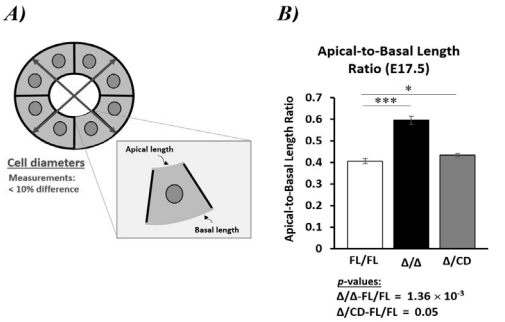

Fig. 3. Apical constriction (AC) analysis in control, null and conditional renal collecting ducts in embryonic E17.5 kidneys. A) Depiction of AC measurements: apical and basal surface lengths were measured in "perfect" tubule cross-sections and the ratios were calculated for each cell. B) Statistical analysis of AC in collecting ducts (Vangl2$^{FL/FL}$; N=87, Vangl2$^{\Delta/\Delta}$; N=85, Vangl2$^{\Delta/CD}$; N=113). ***p<0.001.

In summary, our results indicate that during the embryonic stage, defective CE and AC lead to dilated collecting ducts as well as cyst formation in the Vangl2 deficient tissues.

Analysis of Post-natal (P7) Mouse Kidneys

Vangl2$^{\Delta/\Delta}$ mice are embryonically lethal. To circumvent the embryonic lethality and to ascertain whether the PCP deficiency leads to cystogenesis post-natally, Vangl2$^{\Delta/CD}$ mutant mice were generated through Cre-recombinase knockout of the Vangl2 gene in collecting ducts. Cre-negative Vangl2$^{FL/+}$ mice were used as a control.

Morphological analysis of post-natal (P7) Vangl2$^{\Delta/CD}$ and control Vangl2$^{FL/+}$ kidneys revealed that, surprisingly, mutant kidneys were similar in size to control kidneys and lacked any discernable cystic phenotype (Fig. 4). Furthermore, when the average cross-sectional area of collecting ducts was analyzed at 400X, we observed no significant difference in the diameter width between the mutant and control kidneys, indicating a lack tubular dilatation.

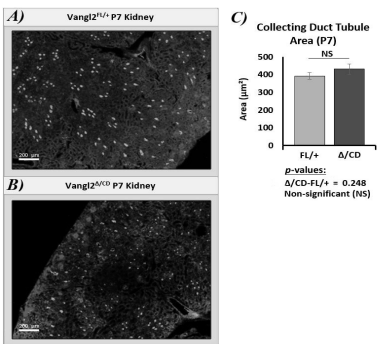

Fig. 4. Morphology of the postnatal day 7 (P7) Vangl2Δ/CD and Vangl2 FL/+ kidneys. A,B) Kidneys were immunostained with anti-Calbindin (green) and anti-ECAD (red), and stained with DAPI (blue), and imaged at 50X magnification; C) Statistical analysis of the cross-sectional area in the P7 Vangl2$^{\Delta/CD}$ (N=87) and Vangl2 $^{FL/+}$ (N=69) renal mouse collecting duct tubules.

Discussion

In this study, we have shown that the PCP gene Vangl2 regulates the processes of convergent extension and apical constriction in the renal tubules during embryonic kidney development. These observations are novel and suggest a functional conservation of the PCP gene regulation of CE and AC in various tissues. However, we found that the post-natal P7 mutant kidney phenotype is indistinguishable from the control kidneys with regard to the size and shape of the collecting duct tubules. This observation is

rather unexpected. For over 10 years, the research community has strongly believed that the PCP pathway controls tubular diameter and, when deficient, contributes to the tubular dilation and cyst appearance featured in polycystic kidney disease.(12,13) Our observations are consistent with the notion that pathways others than PCP (e.g. the ones that are regulated by the ADPKD genes Pkd1 and Pkd2) are likely responsible for the control of tubular diameters during post-natal phase of tubular elongation and the onset of PKD.(20)

Kunimoto et al. recently reported a similar lack of cystic transformation postnatally in the double Vangl2/Vangl1 and Fz3/Fz6 double homozygous mutants.(8) Through a series of elegant experiments, the authors convincingly showed that the collecting duct tubules in the mutant 16 week-old mice were similar to that of controls, despite some statistically enlarged tubule diameters seen in mutant tissues during the embryonic stage. The authors, however, did not address the mechanisms of tubular dilatation in embryonic tissues nor did they describe cystic structures in the embryonic kidneys.

Both our own observations and those reported by Kunimoto suggest that there might be a switch in the regulating pathways that control the tubular diameter during embryonic kidney development and after birth. We have analyzed E17.5 (a highly statistically significant difference) and P7 (no difference) tissues. We, therefore, surmise that the "switch" occurs somewhere in between. In the future, it would be important to analyze kidneys at the intermediate stages to pinpoint the exact timing of the mechanistic changes that regulate collecting duct tubule diameter.

Our data is corroborated by the study of the inner ear development in the Vangl2 mutant.(21) Copley et al. detected a profound defect in the planar polarity of the stereociliary bundles on the sensory hair cells in the cochlea's organ of Corti in E18.5 mutants. However, 10 days after birth, Copley et al. observed a rescue of the PCP defect and the realignment of the stereociliary bundles. The authors concluded that the refinement process was Vangl2-independent. Our study of renal collecting duct tubules is also indicative of a possibility of such mechanistic switch or a 'refinement process'.

Cells rely on internal signals to intercalate in a highly organized and coherent manner, which Vangl2 mediates through CE and AC. In Vangl2$^{\Delta/\Delta}$ and Vangl2$^{\Delta/CD}$ embryos, this movement was clearly disrupted, as shown by the higher number of cells forming the circumference of the tubule, a larger diameter, and a cystic phenotype. The findings of defective apical constriction and the consequent tubule diameter dilation point towards Vangl2's essential role in renal physiology. The ideal 'wedged cell' shape normally arises from the constriction of the apical side due to the recruitment of proteins which regulate actomyosin and drive constriction of the apical cell surface. This leads to a smaller tubular diameter. The fact that the Vangl2$^{\Delta/\Delta}$ collecting duct cells have a larger apical-to-basal length ratio and a more cuboidal shape means that AC of the renal cells is partially controlled by the PCP pathway. Conditional mice showed a smaller apical-to-basal length ratio than the embryonic mutants, but still displayed a significant difference when compared to Vangl2$^{FL/FL}$. This statistical difference is biologically relevant despite the milder phenotype, since it might still contribute, at least partly, to tubular dilatation consistently seen in conditional embryos. Dysregulation of renal collecting duct tubule diameter might contribute to cystogenesis and as such, a small statistical difference is significant in the context of renal dysfunction and disease.

Our study has some limitations that must be considered. One limitation is that tubular kidney development was not assessed in a dynamic way. Indeed, the analysis involved taking pictures to document the progress (or lack thereof) of cyst formation at the specific time points. However, the process of tubulogenesis features continuous complex movements and 3-dimensional elongation, and our study did not account for this. Moreover, despite the fact that PCP-gene expression is well-conserved between vertebrates, potential mechanistic differences between mice and humans have not been taken into account. Milder phenotype of renal tubules in the E17.5 conditional Vangl2$^{\Delta/CD}$ tissues versus the null Vangl2$^{\Delta/\Delta}$ mutants can be explained by the Cre-recombinase mosaic excision.

Our mouse models paint a convincing image of the Vangl2 effects on renal tubulogenesis. PCP signaling is required for normal tubule development, but defective PCP is not solely culpable for the defective renal tubulogenesis and cytogenesis seen in PKD. Further research on the potential pathogenic signaling pathways leading to tubule dilation and cyst formation postnatally is necessary to fully understand the underlying mechanisms of PKD.

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