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Pkd1 is Required for Male Reproductive tract Development

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Abstract

Reproductive tract abnormalities and male infertility have higher incidence in autosomal dominant polycystic kidney disease (ADPKD) patients than in general population. In this work, we revealed that *Pkd1,* whose mutations account for 85% of ADPKD cases, is essential for male reproductive tract development. Disruption of *Pkd1* caused a spectrum of defects in the murine male reproductive tract. The earliest visible defect in $Pkd1^{-/-}$ reproductive tract was cystic dilation of the efferent ducts, which are derivatives of the mesonephric tubules. Epididymis development was delayed or arrested in the $Pkd1^{-/-}$ mice. No sign of epididymal coiling was seen in the *Pkd1* null mice. Disruption of *Pkd1* in epithelia alone using the *Pax2-cre* mice was sufficient to cause efferent duct dilation and coiling defect in the epididymis, suggesting that *Pkd1* is critical for epithelial development and maintenance in male reproductive tract. In-depth analysis showed that *Pkd1* is required to maintain tubulin cytoskeleton and important for *Tgf-β/Bmp* signal transduction in the epithelia of male reproductive tract. Altogether, our results provide the first direct evidence for developmental roles of *Pkd1* in male reproductive tract and provide new insights in reproductive tract abnormalities and infertility in ADPKD patients.

Keywords

Polycystin-1; polycystic kidney disease; epididymis; efferent duct; mice

Introduction

The male reproductive tract is a ductal system consisting of a set of sex accessory tissues, including the efferent ducts, epididymis, and vas deferens. These sex accessory tissues are primarily derived from the Wolffian duct, which is an epithelial tube formed bilaterally from the intermediate mesoderm via mesenchyme to epithelium transformation during early organogenesis (E8.5 to E9.5 in mice) (Hannema and Hughes, 2007; Staack et al., 2003; Wilhelm and Koopman, 2006). The intermediate mesoderm also forms a urogenital ridge, from which three sets of tubular nephric structures develop during embryonic development. The pronephros is the first of the three embryonic kidneys to be established. The pronephros degenerates shortly after being formed, while the mesonephros and metanephros are formed at the intermediate and caudal portions of the intermediate mesoderm (Hannema and

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Hughes, 2007; Joseph et al., 2009). At E10.5, an epithelial bud outgrows from the caudal end of the Wolffian duct, which is the ureteric bud, and later invades into the metanephric mesenchyme to form the renal collecting duct system through continuous branching morphogenesis (Sakurai, 2003; Staack et al., 2003). The extra-renal segment of the ureteric bud, together with its associated mesenchyme, forms the ureter. The Wolffian duct also connects with the newly formed tubules in the mesonephros (Hannema and Hughes, 2007). Meanwhile, another pair of epithelial structures, the Müllerian ducts, are being formed parallel to the Wolffian ducts by invagination of the coelomic epithelium (Klattig and Englert, 2007; Orvis and Behringer, 2007). During gender specification, the Wolffian ducts and mesonephric tubules degenerate in females, and the Müllerian ducts form the sex accessory tissues including oviducts, uterus, cervix and vagina (Hannema and Hughes, 2007; Klattig and Englert, 2007).

In males, however, the Müllerian ducts degenerate (Klattig and Englert, 2007; Wilhelm and Koopman, 2006). The mesonephric tubules, which attach to the Wolffian duct, form the efferent ducts via a remodeling process connecting the testis with the epididymis. At E15, as the testes descend, the Wolffian duct elongates and twists into two major segments, the segment for epididymis and the segment for vas deferens. The epididymal primordium is further specified into caput, corpus, and cauda segments (Cornwall, 2009; Joseph et al., 2009). The epithelial duct of the caput epididymis starts to coil during E15.5 to E16.5, controlled by testicular testosterone signaling that is relayed by *Tgf-β* signaling in the epididymis (Tomaszewski et al., 2007). Later, coiling also occurs in other segments of the epididymis, and finally a spatially complex 3-dimensional epithelial duct system is formed (Cornwall, 2009; Joseph et al., 2009). At the caudal end of each Wolffian duct, the seminal vesicle will be formed via epithelial outgrowth during postnatal development.

Sperm are generated in the testes and mature while migrating through the tortuous and lengthy reproductive tract. Necrospermia and reproductive tract cysts have been detected in a significant number of patients with autosomal dominant polycystic kidney disease (ADPKD) and infertility in male ADPKD patients is not uncommon (Belet et al., 2002; Kanagarajah et al., 2011; Manno et al., 2005; Torra et al., 2008; Vora et al., 2008), implying that the ADPKD genes, *Pkd1* and *Pkd2,* might be involved in reproductive system development and homeostasis. ADPKD is one of the most common inherited genetic diseases, affecting up to 1 in 500 people. It is characterized by progressive development of renal cysts, enlargement of kidneys, and gradual loss of renal function. Over half of the cases proceed to end-stage renal disease by the fifth and sixth decades. Mutations in the *Pkd1* gene account for over 85% of the ADPKD cases, and the remaining cases are attributable to mutations in *Pkd2*.

The *Pkd1* gene encodes polycystin1 (PC1), a large membrane-spanning glycoprotein with 4303 amino acids and an estimated molecular mass of 460-kDa. The predicted structure of polycystin1 includes a large extracellular N terminus, 11 membrane-spanning domains, and a short cystoplasmic C-terminus. The large extracellular N terminus contains two complete leucine-rich repeat motifs flanked by cysteine-rich sequences, a C-type lectin domain, 16 copies of unique Ig-like PKD domains, an acid cysteine-rich low-density lipoprotein related motif, and a region of homology with a sea urchin receptor for egg jelly (Drummond, 2011; Sandford et al., 1997; Wilson, 2001). These extracellular domains play established roles in

protein-protein interactions (Drummond, 2011; Wilson, 2001). The PC1 C-terminus tail contains a G protein binding domain and a coiled-coil domain, which are thought to transduce extracellular signaling into intracellular signaling through activation of G-protein or interactions with cytoplasmic molecules at the coiled-coil domain (Delmas et al., 2002; Qian et al., 1997; Wilson, 2001; Xu et al., 2003). The function of PC1 is critical to maintain structural integrity in a variety of tissues and organs. Although a large number of studies have been conducted to investigate the functions of the ADPKD genes, it is still unclear if ADPKD genes have a developmental role for the reproductive system. In this work, we found that *Pkd1* is essential for normal male reproductive tract development. In the absence of *Pkd1*, the efferent ducts were severely dilated and the epithelial duct of the epididymis failed to elongate and coil. In-depth analysis suggests that *Pkd1* is critical for maintaining developmental signaling in male reproductive tract.

Results

Pkd1 expression in the developing male reproductive tract

To know which tissue requires *Pkd1* during reproductive tract development, we first examined *Pkd1* expression by performing LacZ staining on $Pkd1^{LacZ/+}$ and $Pkd1^{LacZ/LacZ}$ mice. Consistent with a previous study (Chauvet et al., 2002), *Pkd1* expression was detected in the Wolffian duct and mesonephric tubules starting as early as E11.5 to E12.5 (Figure 1A, B). As the $Pkd1^{LacZ/LacZ}$ mice have two copies of LacZ genes, staining of $Pkd1^{LacZ/LacZ}$ tissues was much more intense than that of the $Pkd1^{LacZ/+}$ mice. Later, after sex determination initiated by gonadal differentiation, *Pkd1* was seen in the epididymis (Figure 1C, D). Histological examination of the stained tissue revealed that *Pkd1* expression shifted from the epithelium to the mesenchyme in the epididymis as development proceeded (Figure 1D). The spatiotemporal dynamics of *Pkd1* expression suggests that it regulates both epithelium and mesenchyme development in the developing epididymis.

Cystic dilation of efferent ducts in $Pkd1^{-/-}$ mice

A majority of $Pkd1^{-/-}$ mice died at E15.5 to E16.5 with multiple system defects, including hydrops fetalis and cysts in the kidneys and pancreas, similar to other *Pkd1* knockout mouse models (Guay-Woodford, 2003; Wilson, 2008). We found that the reproductive system in heterozygous mice was comparable to that of wild type mice, but the $Pkd1^{-/-}$ mice displayed severe defects in the male reproductive tract.

From E15.0, we detected cystic dilation of the efferent ducts in the $Pkd1^{-/-}$ mice (Figure 2A, B), and at E15.5 and E16.5, efferent duct dilation became severe (Figure 2C, D; E, F). At E16.5, transformation from cuboidal cells to flat cells was seen in the cystic epithelium (Figure 2G-J). The basement membrane, formed and maintained by both overlying epithelial cells and underlying mesenchymal cells, is an important structure to maintain epithelial integrity. Alterations in basement membrane have been detected in ADPKD patients and in mouse models of polycystic kidney diseases (Calvet, 1993; Grantham et al., 2011). Further, mice with a mutation in laminin alpha5 develop polycystic kidneys (Shannon et al., 2006). We thus examined basement membrane development by performing laminin staining. Results showed that the basement membrane was thickened and laminated in dilated efferent

ducts of the *Pkd1*$^{-/-}$ mice (Figure 3A, B; C, D). We further performed apoptosis and proliferation assays by TUNEL and Ki67 staining at the stage of E16.5. Apoptosis was undetectable in the efferent ducts in both mutant and control mice (data not shown). However, proliferation rate was significantly increased in the mutant efferent ducts compared to the control (Figure 3E-I). Altogether, these alterations suggest a phenotypic change in efferent duct epithelium of the *Pkd1*$^{-/-}$ mice.

Epididymis defects in *Pkd1*$^{-/-}$ mice

The epididymis is a derivative of the Wolffian duct, and shows high expression of *Pkd1* (Chauvet et al., 2002). Normal epididymis development is characterized by continuous elongation and coiling. Coiling was evident at E16.5 in the control mice, initiating from the caput epididymis (Figure 4A, C). By contrast, there was no sign of coiling in the *Pkd1*$^{-/-}$ epididymis at this stage; a straight epithelial tube was seen in the mutant epididymis (Figure 4B, D). Ki67 staining showed that proliferation rate was significantly decreased in the epithelium of mutant epididymis in comparison to the control at E16.5 (Figure 4E, F, G). Moreover, the adjacent mesenchyme was also poorly formed in the mutants, and showed a significant decrease in cell proliferation (Figure 4D, F, H). We failed to detect abnormal apoptosis in the mutant epididymis (data not shown). Altogether, these results indicate that both epithelium and mesenchyme were defective in the *Pkd1*$^{-/-}$ epididymis.

Epithelial-specific disruption of *Pkd1* causes efferent duct dilation similar to that of the *Pkd1*$^{-/-}$ mice

Since *Pkd1* expression was seen in both mesenchyme and epithelium, we sought to dissect the roles of *Pkd1* in these different tissues by using a conditional knockout approach. We used an epithelial-specific Cre strain for the reproductive tract, the *Pax2-cre* mice (Ohyama and Groves, 2004). We first confirmed that expression of the *Pax2-cre* transgene in the reproductive tract is exclusively in the epithelium by performing LacZ staining of the reproductive system of the *Pax2-cre; Rosa26R* mice. Consistent with previous publications (Airik et al., 2006; Ohyama and Groves, 2004), *Pax2-cre* specifically targeted the epithelium of the mesonephric tubules, the Wolffian ducts, and the Müllerian ducts, but was absent from the testes and the mesenchyme in the reproductive tract of both male and female mice (Figure 5A-D).

We then crossed *Pax2-cre;Pkd1*$^{LacZ/+}$ mice with *Pkd1*$^{LoxP/LoxP}$ mice to obtain *Pax2-cre;Pkd1*$^{LacZ/LoxP}$ mutant mice, which contain a *Pkd1* germline mutation in one copy and a pair of *LoxP* sites in the other copy that would enable *Pkd1* deletion by *Cre* expression at *Pax2* domains. The *Pax2-cre;Pkd1*$^{LacZ/LoxP}$ mutant mice died in the first few days after birth. No mutant mice survived beyond 3 days post-partum (dpp). Kidney cystic dilation was detected at E15.5 (data not shown), and was severe at E16.5 (Figure 5E, F). Efferent duct dilation in the *Pax2-cre;Pkd1*$^{LacZ/LoxP}$ mice was first detected at E15.5 and became obvious from E16.5 (Figure 5E, F; G, H), similar to that seen in the *Pkd1*$^{-/-}$ mice. This result suggests that epithelial *Pkd1* plays a critical role to maintain structural integrity in the efferent ducts. At postnatal stages, efferent duct dilation became very severe and most of the efferent ducts were dilated (Figure 5I-N). The cells of the cystic epithelium were mostly transformed to flat cells as demonstrated by pan-cytokeratin staining (Figure 5I, J). Some epithelial cells

were also detached, losing cell-cell adhesion (Figure 5I, J). However, cell proliferation rate in the epithelium of the dilated efferent ducts was comparable to the control at this stage, suggesting cell proliferation slows after significant dilation (Figure 5K, L). Up to the last stage of mutant mouse survival, there was no dilation in the vas deferens (Figure 5M, N).

Epithelial-specific disruption of *Pkd1* is sufficient to cause epididymis coiling defect

Coiling defect was also present in the epididymis of the *Pax2-cre;Pkd1*$^{LacZ/LoxP}$ mice. At E16.5, there was no coiling in the *Pax2-cre;Pkd1*$^{LacZ/LoxP}$ epididymis, similar to that seen in the *Pkd1*$^{-/-}$ mice (data not shown). Up to E18.5, only a few rounds of coiling events were seen in the mutant caput epididymis; by contrast, in the control, a highly coiled epithelial duct was already present in the caput epididymis (Figure 6A, B). Ki67 staining showed that while proliferation in the mesenchyme was still present, cell proliferation ceased in the mutant epithelium, suggesting epithelial growth arrest at this stage (Figure 6C, D). At postnatal stages, coiling was apparently arrested after initiation in the mutant mice, and was absent in the corpus and cauda regions of the epididymis, whereas a highly coiled duct was seen in all segments of the epididymis in the control (Figure 6E-L). Histologic examination of the epididymis showed that highly condensed mesenchyme was present around the mutant epithelium at P1 (Figure 6G, H), suggesting that the mesenchyme was not significantly affected by epithelial disruption of *Pkd1* at this stage. Of note, mesenchyme of the mutant epididymis was more condensed than that of the heterozygous and wildtype controls (Figure 6G, H). This was likely due to lack of epithelial elongation and coiling in the mutant epididymis. The mutant corpus epididymis was often dilated at postnatal stages (Figure 6K, L). Laminin staining suggested that basement membrane was still present in the mutant epididymis (Figure 6M, N). However, pan-cytokeratin staining demonstrated that cell shape changed in the mutant epithelium (Figure 6O, P; Q, R). Epithelial changes were seen before dilation was evident (Figure 6I, J). At P2, we also detected abnormal apoptosis in the mutant epididymis (Figure 6S, T). Altogether, the results showed that coiling was initially delayed in the mutant epididymis and was eventually arrested in the absence of epithelial *Pkd1*.

Disruption of *Pkd1* induces tubulin-cytoskeleton change and affects *Tgf-β/Bmp* signaling in male reproductive tract

Cell shape change in the *Pkd1*$^{-/-}$ epithelium suggests *Pkd1* might be involved in regulating cytoskeleton dynamics. Indeed, previous studies showed that *Pkd1*-encoded PC1 regulates cytoskeleton arrangement in renal epithelial cells (Boca et al., 2007; Xu et al., 2001). Here, we examined tubulin cytoskeleton in male reproductive tract of the *Pax2-cre;Pkd1*$^{LacZ/LoxP}$ mutant mice by immunostaining for α-tubulin, a subunit for microtubule assembling. Results showed that while α-tubulin expression levels were comparable between the mutant and control at most embryonic stages, expression was markedly reduced at postnatal stages in mutant epithelium of the efferent ducts and epididymis (Figure 7A-D), suggesting that tubulin cytoskeleton was affected by loss of *Pkd1*.

Given that *Tgf-β/Bmp* signaling pathways are critical for ductal system development and maintenance in male reproductive tract, and disrupting *Tgf-β* signaling abolishes coiling in the epididymis (Di Giovanni et al., 2011; Tomaszewski et al., 2007), we tested if disruption

of *Pkd1* affects *Tgf-β/Bmp* signaling in the male reproductive tract by performing Smad2/3 and phospho-Smad1/5 staining, which are the intracellular mediators of *Tgf-β* and *Bmp* signaling respectively. As has been described previously (Tomaszewski et al., 2007), Smad2/3 level was high in the epididymis epithelium, indicative of a high requirement of *Tgf-β* signaling by the epithelial cells (Figure 7E). However, in the *Pkd1*$^{-/-}$ epididymis epithelium, Smad2/3 level was markedly reduced beginning in late gestation (Figure 7F, and data not shown), suggesting that *Tgf-β* signaling was disrupted in the mutant epididymis. Similarly, the level of Smad2/3 was also reduced in the cystic lining epithelium of mutant efferent ducts (Figure 7G, H).

In contrast to Smad2/3, which was predominantly present in the epithelium, phospho-Smad1/5 was detected at a high level in both epithelium and mesenchyme in the efferent duct and epididymis of control mice. However, the ratio of cells positive for phospho-Smad1/5 was significantly decreased in mutant epithelium but not in mesenchyme in *Pkd1* epithelial-specific knockout mice in both efferent ducts (Figure 8A-E) and epididymis (Figure 8F-J). This was more obvious in severely dilated ducts, in which positive cells were very few (Figure 8D). Altogether, these results suggest a link between PC1 and *Tgf-β*/*Bmp* signaling pathways in the male reproductive tract. Since *Tgf-β*/*Bmp* ligands are mainly secreted by local mesenchyme cells that were not affected by epithelial *Pkd1* disruption, epithelial responsiveness to *Tgf-β*/*Bmp* ligands was likely changed in the mutant (Tomaszewski et al., 2007).

Pkd1$^{-/-}$ epididymis fails to respond to Tgf-β1 in culture

After 3 days of culture, the epididymis began to show signs of coiling in the control but not in the mutant, consistent with the *in vivo* observation in the *Pkd1*$^{-/-}$ mice (Figure 9A, and data not shown). Addition of Tgf-β1 enhanced epididymal coiling in cultures of control epididymis (Figure 9B), suggesting that Tgf-β1 can activate the intracellular Tgf-β signalling cascade in culture. However, Tgf-β1 failed to rescue epididymal coiling in the *Pkd1*$^{-/-}$ mice in the culture system (Figure 9C), supporting the notion that epithelial responsiveness to growth factors was defective in the mutant epididymis.

Discussion

This work describes for the first time that *Pkd1* is critical for normal male reproductive tract development, and provides new insights into reproductive tract abnormalities and infertility in ADPKD patients. A striking finding in male reproductive tract of *Pkd1*$^{-/-}$ mice was cystic dilation of the efferent ducts. However, initial mesonephric tubulogenesis prior to differentiation into a male reproductive phenotype was normal in the *Pkd1*$^{-/-}$ mice, suggesting that *Pkd1* is not critical for early tubule morphogenesis in the mesonephros. We detected dysregulated proliferation and dedifferentiation in epithelial cells of mutant efferent ducts, which are reminiscent of the kidney cystic changes in ADPKD (Chapin and Caplan, 2010; Chapman, 2007) and indicate that a common mechanism might underlie cystogenesis of mesonephric and metanephric-derived tissues.

Tissue specific deletion of *Pkd1* from epithelium alone caused efferent duct defects recapitulating those seen in global knockout mice, implying that epithelial expression of

Pkd1 plays a central role in maintaining structural integrity in the efferent ducts. Normally, epithelial cells of the efferent ducts, like that of the nephric tubules, absorb significant amounts of fluid in physiological conditions. Phenotypic changes of the efferent duct epithelia might affect fluid absorption and might, as in polycystic kidney epithelium, even cause fluid secretion (Sullivan and Grantham, 1996; Sullivan et al., 1998). If so, the epithelial changes will facilitate fluid buildup and cystic dilation in the efferent duct system by defective fluid regulation.

Another major discovery of this study is that *Pkd1* is required for epididymis development, which is characterized by continuous epithelial elongation and coiling producing an epithelial duct of up to 1 meter in length in mice (Hinton et al., 2011; Joseph et al., 2009). Coiling and spatial arrangement of epididymal duct are governed by a complex signaling network involving testosterone signaling and a number of developmental signaling pathways (Cornwall, 2009; Di Giovanni et al., 2011; Hsia and Cornwall, 2004; Joseph et al., 2009; Kitagaki et al., 2011; Tomaszewski et al., 2007; Xu et al., 2010). Here we showed that *Pkd1* is also an essential player in this regulatory network. In the absence of *Pkd1*, both the epithelium and associated mesenchyme became defective, and epithelial elongation and coiling were disturbed in the epididymis. The data from the epithelial-specific knockout mice further demonstrated that *Pkd1* has primary functions for epididymis development, as in this model the testes that produce testosterone and the epididymal mesenchyme that is the primary target of the testicular testosterone in embryonic epididymis were unaffected by epithelial *Pkd1* deletion (Cooke et al., 1991). In this epithelial-knockout model, the epithelial duct of the epididymis not only failed to elongate and coil but also displayed cystic dilation starting from late gestation.

Multiple lines of evidence indicate that PC1 regulates cytoskeleton dynamics (Boca et al., 2007; Wilson, 2001; Wilson, 2004; Xu et al., 2001). Cell shape change in the $Pkd1^{-/-}$ epithelium in both efferent ducts and epididymis indicates that PC1 is involved in cytoskeleton control in these tissues. Here, we revealed that PC1 regulates dynamics of α-tubulin cytoskeleton in the epithelium of male reproductive tract. Reduced expression level of α-tubulin in the $Pkd1^{-/-}$ epithelia indicates a reduction of microtubules, which are essential for intracellular transport, mitosis and other cellular processes. Furthermore, microtubules are the key cytoskeleton component for the cilium, an important organelle coordinating signaling pathways during development and in tissue homeostasis. Microtubule reduction also indicates that cilia in the $Pkd1^{-/-}$ epithelia might also change. Our results also suggest that in the male reproductive tract PC1 activity is linked to signal pathways that control epithelial development. Indeed, deficiency of PC1 compromised *Tgf-β/Bmp* signal pathways, which are essential for ductal system development and epididymal coiling in male reproductive tract (Di Giovanni et al., 2011; Tomaszewski et al., 2007). However, molecular pathways linking PC1 activity with developmental pathways and cytoskeleton regulation remain to be determined.

We noted dramatically different effects of loss of *Pkd1* function on cell proliferation in different tissues. In the epididymis, deficiency of PC1 caused a significant decrease in cell proliferation, whereas in the efferent ducts and renal epithelium the presence of PC1 appeared to restrict cell proliferation (Bhunia et al., 2002; Nishio et al., 2005). These results

indicate that the effect of PC1 on cell proliferation depends on the specific developmental context. Dysregulated proliferation in male reproductive tract of the *Pkd1*$^{-/-}$ mice might be a prerequisite for cystic dilation upon mechanical stimulation from fluid flow. It is possible that PC1 serves as a negative regulator for stress-induced proliferation in a physiological setting but is essential for cell proliferation in response to developmental signaling.

Fluid flow from the testes not only produces mechanical stimulation but also carries various growth factors to the tissues of male reproductive tract, regulating epithelium development and maintenance (Cornwall, 2009; Joseph et al., 2009; Xu et al., 2011; Xu et al., 2010). The epididymal lumen opens at E13.5 to E14.5, and fluid is present from this stage (Cornwall, 2009; Joseph et al., 2009). Efferent duct dilation and fluid buildup prevent testicular fluid from flowing into the epididymal lumen and also disrupt fluid flow dynamics in the epididymis. Furthermore, fluid regulation defect (absorption/secretion) in the efferent ducts might cause severe dilution of luminal factors important for epididymis development.

In summary, our data for the first time show that *Pkd1* is essential for male reproductive tract development. PC1 regulates cellular activity by linking extracellular environmental signaling, including mechanical stress and extracellular matrix signaling, to a set of critical signaling pathways. This mechanism might also exist in adult tissues. Alteration in PC1 signaling by *Pkd1* loss of function or gain of function mutations might lead to dysregulation of gene expression in adult tissues, and abnormally activate or inactivate critical signaling pathways causing cystogenesis. Finally, it should be noted that the male reproductive system continues to develop and mature at postnatal stages until puberty. Genital structures, such as the seminal vesicles, ejaculatory ducts and prostate, are still to be developed even at the most advanced stage of our conditional knockout mice survival. Furthermore, maintenance of reproductive organs may require *Pkd1* in adulthood. In future studies, inducible Cre systems may be helpful to test these hypotheses and to examine roles of *Pkd1* in maintenance of male reproductive system.

Experimental procedures

Mice

Animal use protocol is approved by Johns Hopkins University Animal Care and Use Committee. *Pkd1*$^{LacZ/+}$(*Pkd1*$^{+/-}$), and *Pkd1*$^{LoxP/LoxP}$ mice were provided by the Polycystic Kidney Disease Core at Johns Hopkins University (Bhunia et al., 2002; Piontek et al., 2004). *Pax2-cre* and *Rosa26R* mice were described previously (Ohyama and Groves, 2004; Soriano, 1999). Mice were genotyped by PCR. All the mice were maintained in a mixed background.

Histology and immunohistochemistry

Tissue preparation for histology and immunohistochemistry was performed using standard procedures. Paraffin sections were used for histologic examination and immunostaining. Anti-laminin (Sigma), pan-cytokeratin (Sigma), α-tubulin (Neomarkers), Smad2/3 and phospho-Smad1/5 antibodies (Cell Signalling Technology) were used for immunodetection

on sections. Secondary antibodies were peroxidase-conjugated (Sigma). DAB was used for peroxidase-mediated color reaction. Hematoxylin was used for counterstaining.

Apoptosis and Proliferation assays

Paraffin sections were used for TUNEL staining and proliferation assay. TUNEL staining for apoptosis was performed using DeadEnd Colorimetric Apoptosis Detection System (Promega). The Ki67 antibody (Millipore, AB9260) was used for proliferation assay. Serial sections at intervals of 35μm were used for comparison of proliferation rates on each genotype. Three samples were used for each genotype. The number of Ki67+ cells relative to total number of cells was calculated and used for proliferation rate.

LacZ staining

Urogenital system primordia were dissected out from mouse embryos, fixed with 4% paraformaldehyde in 1× PBS for 1 hour at 4 °C, rinsed three times in 1× PBS, and stained in X-gal reaction solution overnight at room temperature. Stained tissues were visualized in 80% glycerol or sectioned for examination.

Tissue culture

Embryonic genital organ system was dissected out and cultured at the air-medium interface on a Transwell filter system (Corning Incorporated) for 3 days (Nie et al., 2011). DMEM containing 10% FBS, 2 mM glutamine, 100 U penicillin/ml, and 0.1 mg/ml streptomycin was changed every two days. Tgf-β1 protein (Millipore, GF111) was used at a concentration of 200ng/ml.

Statistical analysis

Statistical analysis was performed using two tailed t test.

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Abbreviations

ADPKD	autosomal dominant polycystic kidney disease
Ig	immunoglobulin
PC1	polycystin1
PKD	polycystic kidney disease

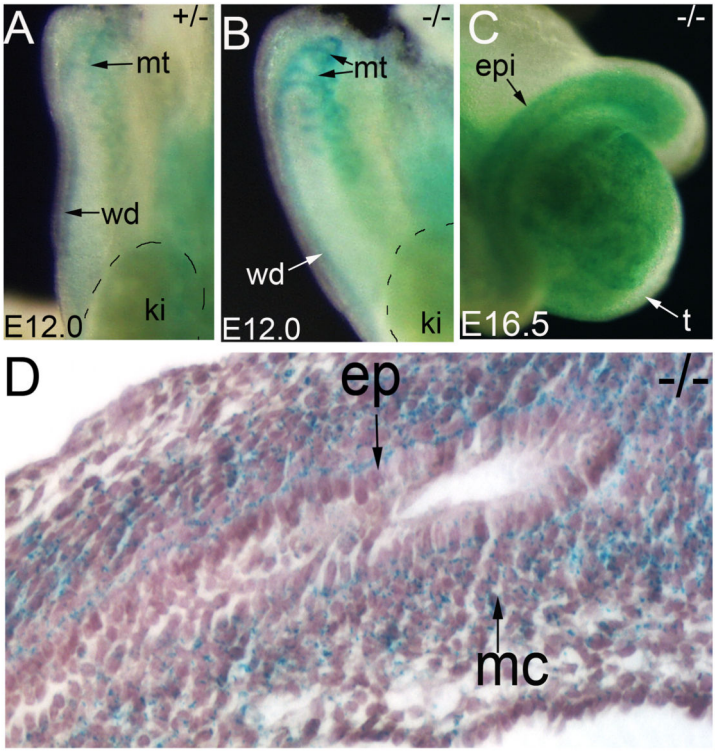

Figure 1. Constitutive *Pkd1* expression in male reproductive tract

(A, B) LacZ staining of the urogenital primordia at E12.0, showing *Pkd1* expression in the mesonephric tubules and the Wolffian duct. (C) Whole mount LacZ staining of *Pkd1*$^{-/-}$ epididymis at E16.5 (dorsal view). (D) Sectional view of the LacZ stained *Pkd1*$^{-/-}$ epididymis at E16.5. ep: epithelium; epi: epididymis; ki: kidney; mc:mesenchyme; mt; mesonephric tubules; wd: Wolffian duct;

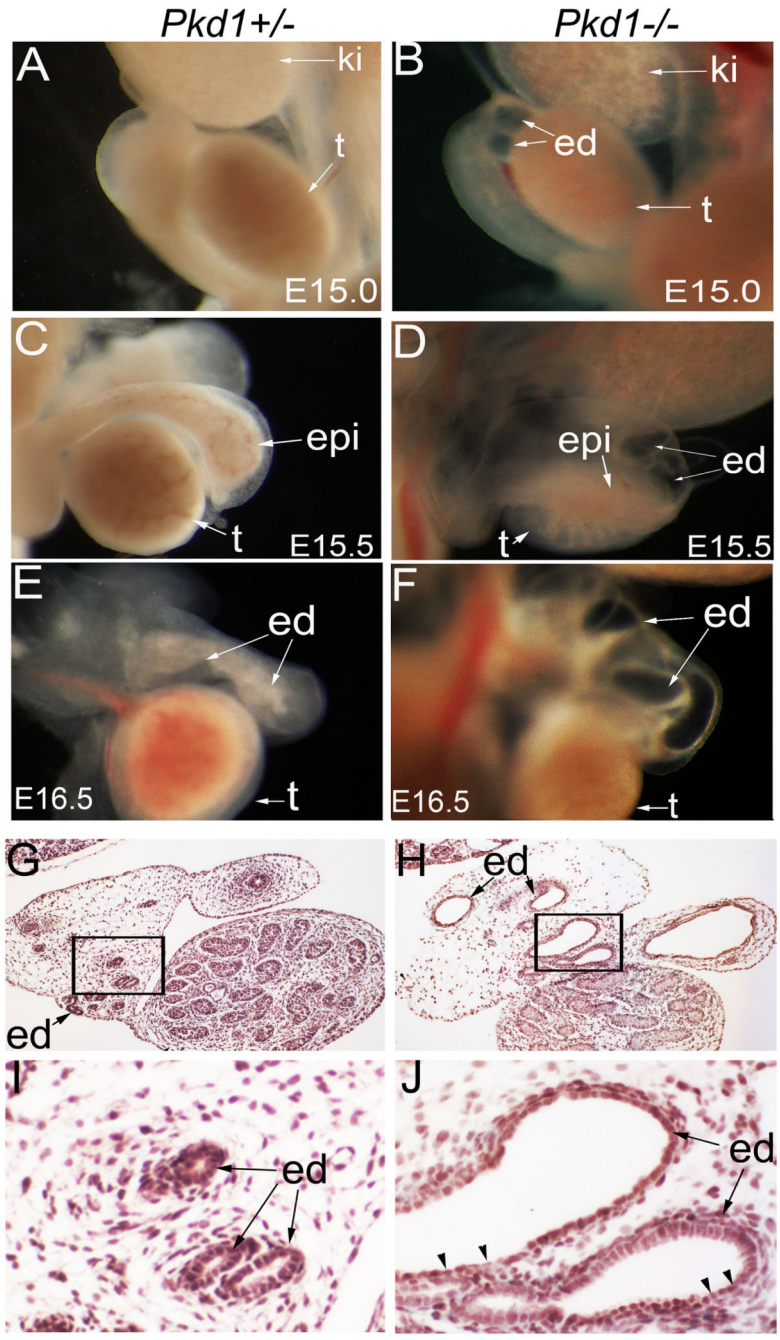

Figure 2. Efferent duct dilation in the *Pkd1*$^{-/-}$ mice

(A, B) Male reproductive system at E15.0. Note efferent duct dilation in the *Pkd1*$^{-/-}$ mutant before testes fully descended. (C, D) Male reproductive system at E15.5. (E, F) Male reproductive system at E16.5, showing severe dilation of mutant efferent ducts. (G, H) Histology of the male reproductive system at E16.5, showing dilated efferent ducts in the *Pkd1*$^{-/-}$ mutant. (I, J) High magnification view of the framed areas in (G, H), arrow heads indicate flat epithelial cells. ed: efferent duct; epi: epididymis; ki: kidney; t: testis; wd: Wolffian duct

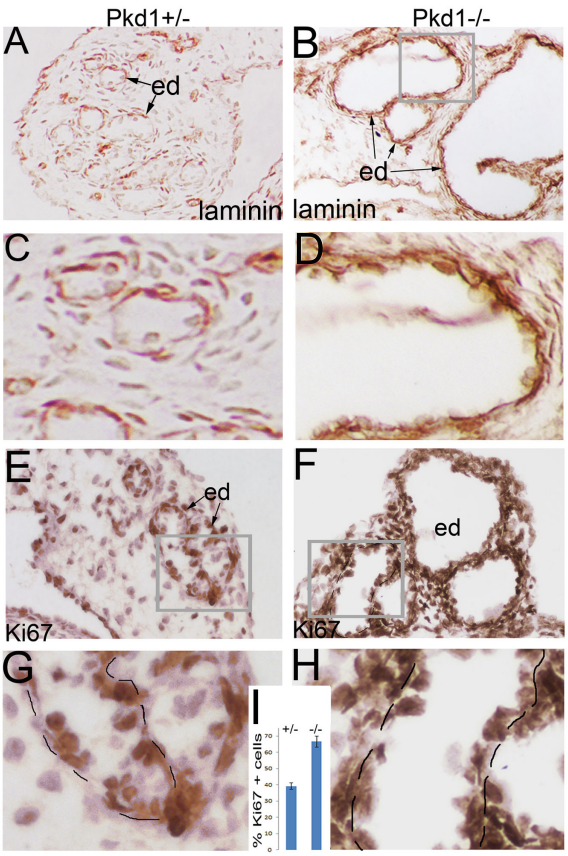

Figure 3. Laminin and Ki67 staining of the efferent ducts

(A, B) Laminin staining of the efferent ducts at E16.5. (C, D) High magnification view of the framed areas in (A, B). (E, F) Ki67 staining of the efferent ducts. (G, H) High magnification view of the framed areas in (E, F). (I) Histograms showing proliferation rates in the efferent duct epithelia. ed: efferent duct

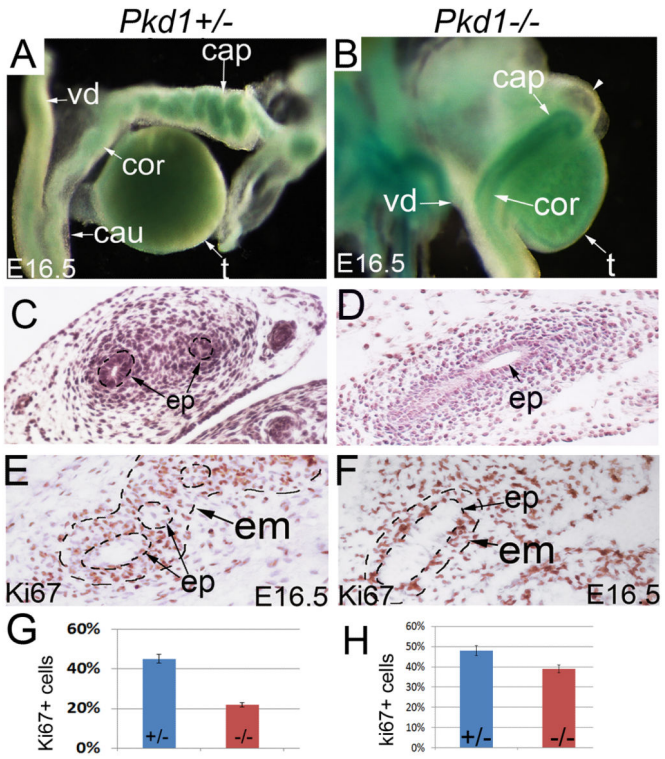

Figure 4. Epididymis defects in *Pkd1*$^{-/-}$ mice

(A, B) LacZ staining of E16.5 male reproductive system. Dorsal view showing coiling defect of epithelial duct in the mutant epididymis. (C, D) Histology of E16.5 epididymides. (E, F) Ki67 staining of the epididymis at E16.5. (G) Histograms showing proliferation rates in *Pkd1*$^{+/-}$ and *Pkd1*$^{-/-}$ epididymis. cap: caput epididymis; cau: cauda epididymis; cor: corpus epididymis; ed: efferent duct; em: epididymal mesenchyme; ep; epithelium; t: testis; vd: vas deferens

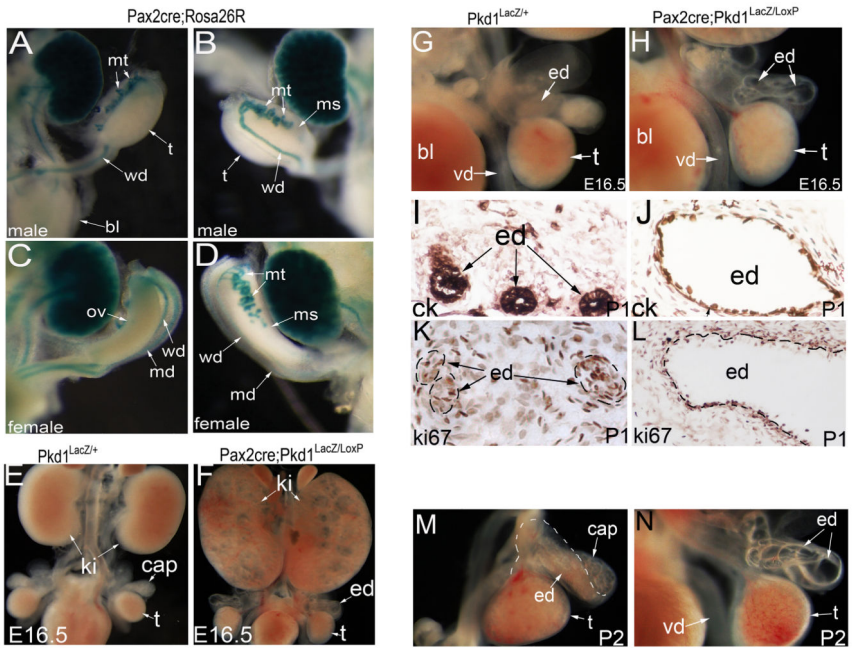

Figure 5. Efferent duct defects in *Pax2-cre;Pkd1*$^{LacZ/LoxP}$ mice

(A, B) LacZ staining of male reproductive system in *Pax2-cre;Rosa26R* mice at E13.5: ventral view(A) and dorsal view (B). (C, D) LacZ staining of female reproductive system in *Pax2-cre;Rosa26R* mice at E13.5: ventral view (C) and dorsal view (D). (E, F) Urogenital organs in *Pkd1*$^{LacZ/+}$ control and *Pax2-cre;Pkd1*$^{LacZ/LoxP}$ mutant mice at E16.5. (G, H) Ventral view of the genital organs at E16.5, showing dilation of the efferent ducts in the mutant. (I, J) Pan-cytokeratin staining of the efferent ducts at P1. (K, L) Ki67 staining of the efferent ducts at P1. (M, N) Ventral view of the genital organs at P2, showing severe dilation of the efferent ducts in the mutant. bl: bladder; cap: caput epididymis; ck: pan-cytokeratin; ed: efferent duct; ki: kidney; md: Müllerian duct; ms: mesonephros; mt: mesonephric tubules; ov: ovary; t: testis; vd: vas deferens; wd: Wolffian duct

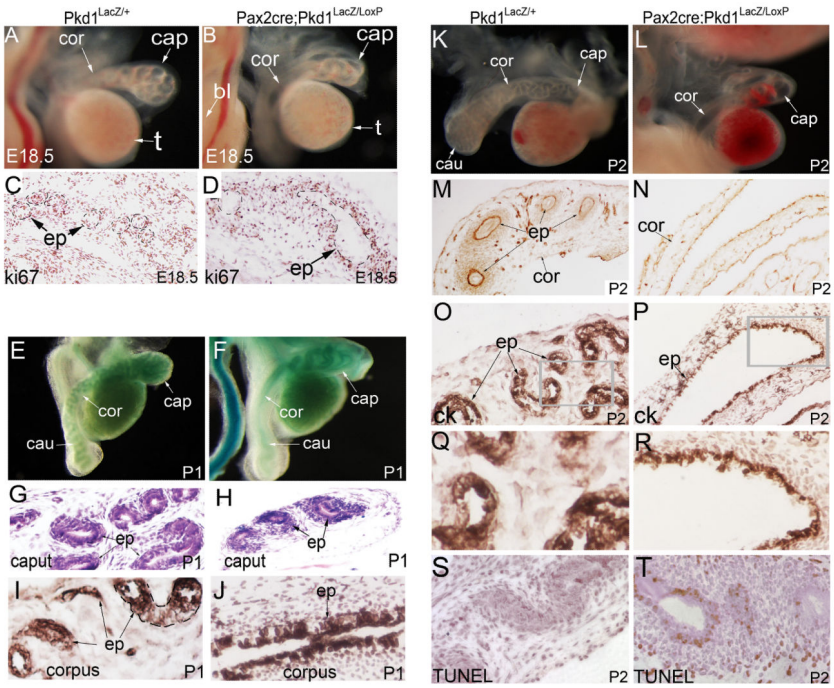

Figure 6. Epididymis defects in *Pax2-cre;Pkd1*$^{LacZ/Loxp}$ mice

(A, B) Dorsal view of E18.5 male reproductive system showing dilation and coiling defect in the mutant epididymis. (C, D) Ki67 staining of E18.5 epididymis. Note low proliferation in the mutant epithelium. (E, F) LacZ staining of male genital system at P1. Dorsal view revealed coiling defect in the mutant epididymis. (G, H) Histology of the caput epididymis at P1. (I, J) Pan-cytokeratin staining of the corpus epididymis at P1. (K, L) Dorsal view of the epididymis at P2, showing coiling defect and dilation in the mutant epididymis. (M, N) Laminin staining of corpus epididymis at P2. (O, P) Pan-cytokeratin staining of the corpus epididymis at P2. (Q, R) Magnification view of the framed areas in (O and P). Note abnormal epithelium in mutant. (S, T) TUNEL staining of epididymis at P2. bl: bladder; cap: caput epididymis; cau: cauda epididymis; ck: pan-cytokeratin; cor: corpus epididymis; ed: efferent duct; ep: epithelium; LM: laminin

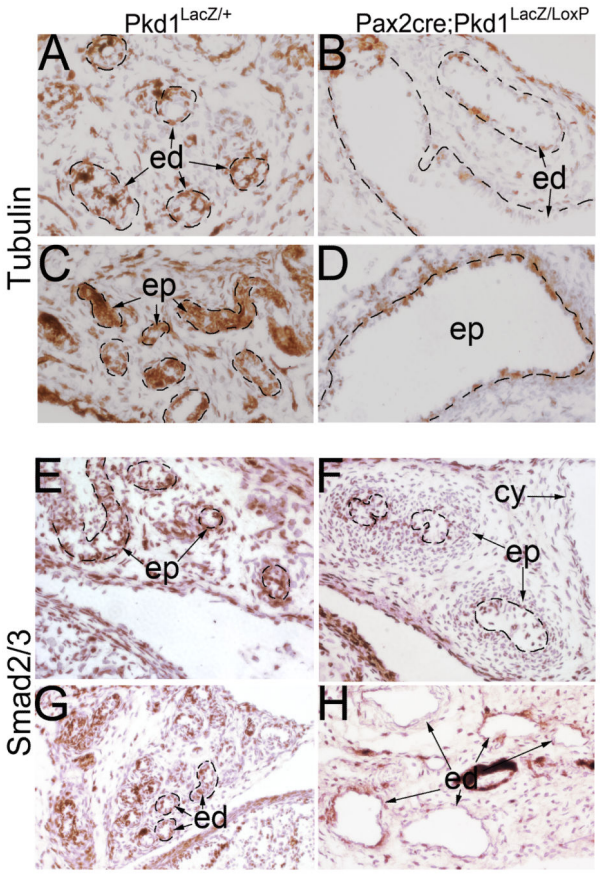

Figure 7. Reduced α-tubulin and Smad2/3 expression in male reproductive system of *Pax2-cre;Pkd1*$^{LacZ/Loxp}$ mice at P1

(A, B) α-tubulin staining of the efferent ducts, showing reduced α-tubulin expression in the mutant epithelium. (C, D) α-tubulin staining of the epididymis, showing reduced α-tubulin expression in the mutant epithelium. (E, F) Smad2/3 staining of the epididymis. (G, H) Smad2/3 staining of the efferent ducts. cy: cystic dilation; ed: efferent duct; ep: epithelium

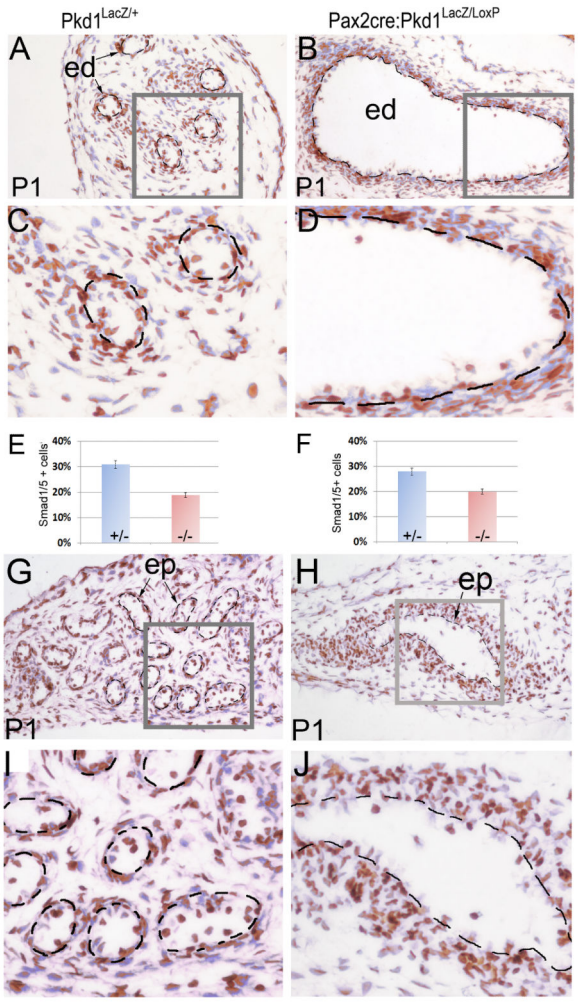

Figure 8. Reduced phospho-Smad1/5 in efferent ducts and epididymis of *Pax2-cre;Pkd1*$^{LacZ/Loxp}$ mice at P1

(A, B) Phospho-Smad1/5 staining of the efferent ducts in mutant and control. (C, D) Magnification view of the framed areas in (A, B). (E) Histograms representing the ratios of phospho-Smad1/5/8 positive cells in efferent duct epithelia of mutant and control. (F) Histograms representing the ratios of phospho-Smad1/5 positive cells in the epididymal epithelia of mutant and control. (G, H) Phospho-Smad1/5 staining of the epididymis in mutant and control. (I, J) Magnification view of the framed areas in (G, H). ed: efferent duct; ep: epithelium

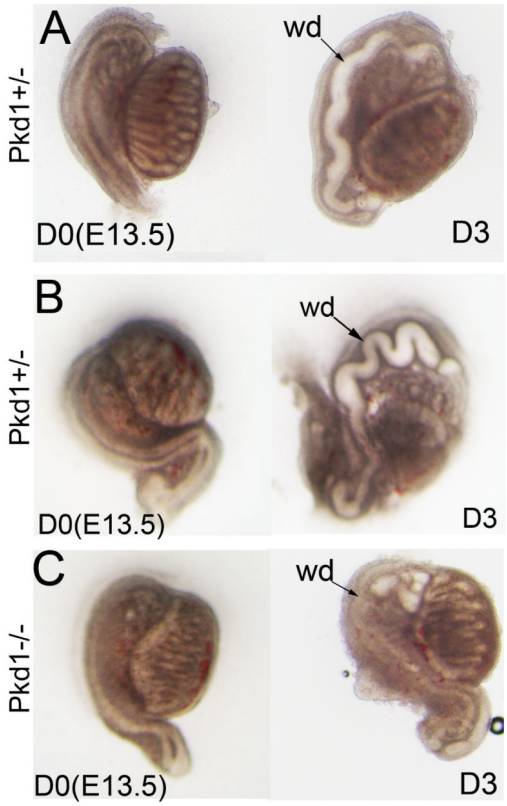

Figure 9. Male reproductive tract development in culture

(A) *Pkd1*$^{+/-}$ reproductive system at E13.5 in a Transwell culture system. (B) *Pkd1*$^{+/-}$ reproductive system at E13.5 in culture in the presence of Tgf-β1 protein. (C) *Pkd1*$^{-/-}$ reproductive system at E13.5 in culture in the presence of Tgf-β1 protein. D0: first day in culture; D3: third day in culture; wd: Wolffian duct