Conditional Mutation of Pkd2 Causes Cystogenesis and Upregulates β-Catenin

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ABSTRACT

Loss of polycystin-2 (PC2) in mice (Pkd2<sup>−/−</sup>) results in total body edema, focal hemorrhage, structural cardiac defects, abnormal left-right axis, hepatorenal and pancreatic cysts, and embryonic lethality. The molecular mechanisms by which loss of PC2 leads to these phenotypes remain unknown. We generated a model to allow targeted Pkd2 inactivation using the Cre-loxP system. Global inactivation of Pkd2 produced a phenotype identical to Pkd2<sup>−/−</sup> mice with undetectable PC2 protein and perinatal lethality. Using various Cre mouse lines, we found that kidney, pancreas, or time-specific deletion of Pkd2 led to cyst formation. In addition, we developed an immortalized renal collecting duct cell line with inactive Pkd2; these cells had aberrant cell-cell contact, ciliogenesis, and tubulomorphogenesis. They also significantly upregulated β-catenin, axin2, and cMyc. Our results suggest that loss of PC2 disrupts normal behavior of renal epithelial cells through dysregulation of β-catenin-dependent signaling, revealing a potential role for this signaling pathway in PC2-associated ADPKD.


Autosomal dominant polycystic kidney disease (ADPKD) with an incidence of 1 in 400 to 1 in 1000 live births, is one of the most common monogenic disorders and is characterized by numerous fluid-filled renal cysts.1–3 ADPKD is a genetically heterogeneous disease resulting from mutations in at least two genes, PKD1 and PKD2. A mutation in PKD1, which is located on chromosome 16p13.3, is responsible for approximately 85% of families with ADPKD. A mutation in PKD2, which maps to chromosome 4q21, causes approximately 15% of familial ADPKD. There are also a few families with ADPKD that are not linked to the PKD1 or PKD2 locus, suggesting other ADPKD causal genes may be present.4

PKD1 encodes a 4303-amino-acid integral membrane protein (polycystin-1, PC1) with 11 putative transmembrane domains. A large extracellular domain at the PC1 amino (NH<sub>2</sub>)-terminus can be released by cleavage at the G-protein-coupled receptor proteolytic site, and after cleavage the N-terminal fragment may serve as a ligand for other proteins.5

PKD2 encodes a 968-amino-acid protein that is predicted to be an integral membrane protein with six transmembrane domains and intracellular NH<sub>2</sub>- and carboxy (COOH)-termini.6 PC2 is a receptor-operated, nonselective cation channel7,8

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that has a modest degree of amino-acid similarity to the transient receptor potential channel, and as a member of the transient receptor potential superfamily is often referred to as TRP2.9,10

The NH2- and COOH-termini of PC2 have been reported to contain several functional motifs through which PC2 acts during embryogenesis and organogenesis. In the PC2 COOH-terminus these sequentially are a single calcium-binding motif consisting of a helix, calcium-binding loop, and second helix referred to as the “EF hand,” which may involve calcium-modulated cation channel activity;6 an endoplasmic reticulum retention domain, which includes an acid patch (a motif in which DxD cluster together to form a highly acidic surface patch that appears to bind the sorting proteins, phosphofurin acidic cluster sorting protein 1 (PACS1) and 2 (PACS2), and which disrupts abrogates the interaction between PC2 and the PACS proteins and alters PC2 localization;11,12 and a helical PC1 interaction domain (PC1-ID) that associates with a putative coiled-coil domain on PC1 to form a PC1/2 heterodimer complex or PC2 homodimer that together can serve as a cation channel.8,13–15 At the NH2-terminus of PC2 resides a ciliary transport motif (RVxP, residues 5 to 8), and disruption of this motif arrests PC2 trafficking to the primary cilium of renal epithelial cells.16 Recently, our group identified a fibrocystin/polyductin (FPC) binding domain (FBD, residues 90 to 139) at the intracellular NH2-terminus of PC2. Loss of this domain results in instability of PC2 expression in vitro and in vivo.17,18

PC2 mediates diverse signal transduction events, playing a functional role in cell differentiation, proliferation, apoptosis, and polarization.19 Two important glycogen synthase kinase phosphorylation sites have been identified in PC2 (phosphoserine Ser812 at the intracellular COOH-terminus, and phosphoserine Ser76 at the intracellular NH2-terminus). Both phosphorylation sites may function in sorting PC2 to the plasma membrane.11,12 Li et al. reported that Id2, an inhibitor of the basic helix-loop-helix transcription factors, binds to the COOH-terminus of PC2, promoting activity of the cell cycle regulator p21 and leading to reduction of cell proliferation via downregulation of cyclin-E and cyclin-dependent kinase activity.21 Other studies have demonstrated that stimulation of EGF receptor induces PC2-associated channel activity through activation of RhoA with subsequent activation of mammalian diaphanos-related formin 1.22,23 cAMP has also been linked to a PC2 because some cystic cells exhibit abnormally high cAMP levels.3 It has been proposed that PC2-associated Ca2+ may inhibit adenylyl cyclase IV activity, thus suppressing conversion of ATP to cAMP. Downregulation of PC2 reduces intracellular Ca2+ release and increases cAMP levels through enhanced adenylyl cyclase IV activity.24,25 Furthermore, high levels of cAMP activity can disrupt the mitogen-activated protein kinase/extracellular signal-regulated kinase signaling pathway, which is recognized for regulating cell proliferation, and this dysregulation in turn results in aberrant cyst-cell proliferation.26 A recent study demonstrated that PC2 can also regulate cyst-cell proliferation via eIF2α phosphorylation, and this response is mediated by pancreatic endoplasmic-reticulum-resident eIF2α kinase.27 Interestingly, a recent report indicates that overexpression of PC2 may also result in cystic kidneys.28 Although the findings have provided insights into the signaling networks involved in ADPKD cyst formation, the precise underlying molecular mechanisms by which dysfunction of PC2 induces cystogenesis are still not fully understood.

To explore the functional role of PC2 in vivo, gene targeting mouse models for Pkd2 have been generated.29–31 Mice homozygous for Pkd2 exhibit embryonic lethality at E12.5 to birth and display total body edema, focal hemorrhage, cardiac structure defects, abnormal left-right axes, and cystic kidneys/pancreas, suggesting PC2 is required for embryogenesis and organogenesis.30,31 Because embryonic lethality occurs in null-Pkd2 mice and an unpredictable Pkd2-hypermutable allele appears in WS25 mice,29 the currently available Pkd2 mouse models are limited in their ability to fully assess PC2 functions during mouse development and cystogenesis. To overcome these limitations, we have generated a mouse model in which Pkd2 can be conditionally inactivated using a Cre-loxp system. Mice with Pkd2/D3/D3 alleles, in which both Pkd2-exon-3 are flanked by two loxp sites, are able to escape embryonic lethality with near-native PC2 expression. Under mediation of Cre recombinase, mice with Pkd2/D3/D3 alleles can be produced in which exon 3 of both Pkd2 alleles have been excised. Pkd2/D3/D3 mice exhibit embryonic lethality with undetectable PC2 expression by Western blot and show cyst formation in the kidney and pancreas. Through a crossmapping strategy, tissue-specific Cre-mediated Pkd2/D3/D3 mice were generated and cyst phenotypes observed in Pkd2/D3/D3 mice are expressed by Western blot and show cyst formation in the kidney and pancreas. Through a crossmapping strategy, tissue-specific Cre-mediated Pkd2/D3/D3 mice were generated and cyst phenotypes observed in Pkd2/D3/D3 mice. Using a Pkd2/D3/D3–/mouse (Im::Pkd2/D3/D3–/mouse), we developed a panel of immortalized renal collecting duct cell lines that harbor a temperature-sensitive SV40 large T antigen. By infection with AdCre virus, one of the renal collecting duct cell lines (D3) was induced to produce the daughter cell lines B2 and E8, which bear two null-Pkd2 alleles (Pkd2/D3/D3–/). These null-Pkd2 cell lines display aberrant cell-cell contact, ciliogenesis, and tubulomorphogenesis compared with their mother cell line D3. Interestingly, null-Pkd2 cells express significantly high levels of β-catenin, axin2, and cMyc. These results suggest that PC2 may regulate tubulomorphogenesis as well as other cellular behaviors (e.g., proliferation and polarization) through a β-catenin-dependent signaling pathway.

RESULTS

Generation of a Mouse Model with a Floxed Mutant Allele at Pkd2

Analysis of the organization of the Pkd2 gene showed that excision of exon 3 would lead to a frame shift and induction of a premature termination codon 23 amino acids downstream of exon 2. Using this information, we produced a mouse model with the Pkd2f3 allele (Figure 1A).18 By crossmating with
ACTB-Flpe mice (Flpe transgene is under the control of human β-actin promoter), the pGKNeo cassette at the Pkd2<sup>floxed</sup> allele is embryonically excised by mediation of Flpe recombinase, resulting in the Pkd2<sup>fl</sup> allele in which two loxP sites flank exon 3 of Pkd2 (Figure 1AII, B).

Mice with Pkd2<sup>fl</sup> alleles develop normally and do not show embryonic lethality (Table 1). Compared with wild type, there is neither obvious reduction of Pkd2 mRNA (Figure 1C) nor significantly decreased PC2 protein expression in the Pkd2<sup>fl</sup> embryos (Figure 1D). In addition, careful examination of at least five 8-wk-old Pkd2<sup>fl</sup> mice showed no obvious cystic phenotypes were present (grossly or histologically) in the kidneys and liver. These results indicate that the Pkd2<sup>fl</sup> allele does not significantly interrupt normal Pkd2 expression.

Once we obtained mice with the Pkd2<sup>fl</sup> allele (Figure 1AII), we crossed our Pkd2<sup>fl</sup> mice with early virus gene promoter (Elia)-Cre transgenic mice, in which Cre recombinase is expressed in early-stage embryogenesis under the control of the early virus gene promoter Elia<sup>32,33</sup> to produce mice with a Pkd2 exon-3-deletion allele (Pkd2<sup>del</sup>) (Figure 1AIII). Cross-mating of Pkd2<sup>del</sup> with Elia-Cre mice allowed us to identify Pkd2<sup>del</sup> mice by PCR genotyping (Figure 1E). Pkd2<sup>del</sup> mice show perinatal lethality and total body edema in their embryos (Table 2) similar to that observed for previously generated Pkd2<sup>−/−</sup> (Pkd2<sup>del</sup>, Pkd2<sup>−/−</sup> mice). To test if Pkd2<sup>del</sup> is a null-Pkd2 allele, we performed quantitative real-time PCR using total RNA from E13.5 embryos with wild-type, Pkd2<sup>−/−</sup>, Pkd2<sup>del</sup>, Pkd2<sup>−/−</sup>, and Pkd2<sup>deletion</sup> alleles. The level of Pkd2 mRNA in Pkd2<sup>del</sup> embryos (Figure 1C) is equivalent to that of our previously generated Pkd2<sup>−/−</sup> mice, indicating that the Pkd2<sup>del</sup> allele disrupts normal Pkd2 mRNA expression. In addition, an anti-PC2 antibody, hPKD2-Cm1A11, which recognizes the intracellular COOH-terminal portion of PC2, was used to detect PC2 in E13.5 embryos with wild-type, Pkd2<sup>−/−</sup>, Pkd2<sup>del</sup>, and Pkd2<sup>−/−</sup> alleles by Western blots. PC2 is absent in embryos with Pkd2<sup>del</sup> alleles, whereas there is no significant PC2 expression change in either Pkd2<sup>−/−</sup> or Pkd2<sup>del</sup> compared with wild-type littersmates. (E) PCR genotyping mice with Pkd2<sup>del</sup> heterozygous and homozygous alleles. A 540-bp PCR band was observed for the Pkd2<sup>del</sup> allele whereas a 350-bp PCR band was observed for the wild type.

**Table 1. Genotyping analysis of mice with Pkd2<sup>del</sup> allele**

<table>
<thead>
<tr>
<th>Age</th>
<th>Pkd2&lt;sup&gt;+/+&lt;/sup&gt; n (%)</th>
<th>Pkd2&lt;sup&gt;++/+&lt;/sup&gt; n (%)</th>
<th>Pkd2&lt;sup&gt;del&lt;/sup&gt; n (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;3 wk</td>
<td>9 (29)</td>
<td>13 (42)</td>
<td>9 (29)</td>
<td>31</td>
</tr>
</tbody>
</table>

n = number of mice

**Figure 1.** Pkd2 conditional knockout construct and molecular analysis of the specific targeting event at the Pkd2 locus. (A) Schematic representation of Cre-mediated gene-targeting strategy. (I) Pkd2<sup>del</sup> allele: There is a pGKNeo cassette flanked by two FRT sites at intron 2 of Pkd2, whereas Pkd2-exon-3 is flanked by two loxP sites. (II) Pkd2<sup>del</sup> allele: The pGKNeo cassette at the Pkd2<sup>del</sup> allele was excised under mediation of Flpe recombinase and converted to the Pkd2<sup>del</sup> allele, in which there are only two loxP sites flanking Pkd2-exon-3. (III) Pkd2<sup>del</sup> allele: Pkd2-exon-3 is excised by mediation of Cre recombinase to produce Pkd2<sup>del</sup> allele. X, XbaI; B, BglII. (B) Tail biopsy DNAs from mice with Pkd2<sup>fl</sup> allele(s) were digested with BglII and hybridized with probe B from outside the targeted region (Figure 1A). The expected 15-kb wild-type band was observed in wild-type and Pkd2<sup>del</sup> heterozygous and homozygous mice. (C) Quantitative PCR to test Pkd2 mRNA expression level of E13.5 embryos with Pkd2<sup>−/−</sup> (Pkd2<sup>del</sup>, Pkd2<sup>del</sup>, Pkd2<sup>del</sup>), and Pkd2<sup>del</sup> alleles compared with a wild-type embryo. No significant change was observed between Pkd2<sup>del</sup> and wild-type alleles. Virtually no Pkd2 mRNA was seen in Pkd2<sup>del</sup> or Pkd2<sup>del</sup> embryos. (D) A monoclonal anti-PC2 antibody, hPKD2-Cm1A11, which recognizes the intracellular COOH-terminal portion of PC2, was used to detect PC2 in E13.5 embryos with wild-type, Pkd2<sup>−/−</sup>, Pkd2<sup>del</sup>, and Pkd2<sup>del</sup> alleles by Western blots. PC2 is absent in embryos with Pkd2<sup>del</sup> alleles, whereas there is no significant PC2 expression change in either Pkd2<sup>−/−</sup> or Pkd2<sup>del</sup> compared with wild-type littersmates. (E) PCR genotyping mice with Pkd2<sup>del</sup> heterozygous and homozygous alleles. A 540-bp PCR band was observed for the Pkd2<sup>del</sup> allele whereas a 350-bp PCR band was observed for the wild type.

**Flxed Pkd2-Exon-3 Allele Can Be Conditionally Induced by Mediation of Cre Recombinase to Functionally Inactivate PC2 Expression In Vivo**

Our previous findings indicate that somatic inactivation of Pkd2 is able to induce renal cyst formation.<sup>29</sup> To ensure complete excision of exon 3 in both Pkd2 alleles, we proposed a
mouse model with one of its alleles as null (Pkd2\(^{-/-}\)) and the other with floxed exon 3 (Pkd2\(^{f3/-}\)). This combination of alleles will ensure complete knockout of Pkd2 in the targeted tissues upon Cre activation. Using a mating strategy, we generated Pkd2\(^{f3/-}\) mice with Cre-recombinase transgene and assessed if renal cysts occurred in Pkd2\(^{f3/-}\) alleles locally converted into Pkd2\(^{d3/-}\) alleles through Cre mediation.29 For these experiments we used γGt-Cre mice, in which Cre recombinase is controlled by the type I promoter of a rat gamma glutamyl transpeptidase (γGt)34 and is expressed in renal tubular epithelial cells35,36 to produce γGt-Cre::Pkd2\(^{f3/}\) mice (Supplemental Table 1). By dissecting a 9-wk-old γGt-Cre::Pkd2\(^{f3/}\) mouse, we observed gross and histologic cystic kidneys (Figure 2A versus B, F versus G), suggesting that Pkd2\(^{f3/}\) alleles can be converted into Pkd2\(^{d3/}\) alleles under mediation of Cre recombinase in vivo.

To test whether Pkd2\(^{d3/}\) alleles can be induced from Pkd2\(^{f3/}\) alleles in a temporally inducible manner, Mx1-Cre mice were used to generate Mx1-Cre::Pkd2\(^{f3/}\) mice. In Mx1-Cre mice, Cre recombinase is controlled by a promoter from the intracellular anti-influenza virus protein (Mx1)37 and the Mx1 promoter can be triggered by inducing reagents IFN or the IFN inducer pl-pC (polynosinic-polycytidylic acid), which is a synthetic double-stranded RNA. Cre recombinase can be induced and expressed in various promoter-driven tissues and organs by application of pl-pC, including renal and hepatic epithelia.38,39 Using nine 4- to 10-wk-old Mx1-Cre::Pkd2\(^{f3/}\) mice (Supplemental Table 2), we intraperitoneally injected six with pl-pC-solvent (500 μg/d) for 3 d; the other three mice were used as controls and injected with 0.9% saline/d for 3 d. All of the mice were sacrificed 8 wk after the last injection. The control mice did not display any gross cystic phenotypes in the liver or kidneys. The Mx1-Cre::Pkd2\(^{f3/}\) mouse injected with pl-pC at 4 wk showed gross cysts in the kidney, liver, and pancreas. Of the four mice in the 6-wk-old pl-pC injection group, two mice exhibited gross cysts in the kidney (Figure 2, C versus D), liver, and pancreas; and two mice exhibited only gross liver cysts. The mouse with pl-pC injection at the age of 10 wk did not show any gross cysts (Supplemental Table 2). Histologic examination of mice receiving pl-pC injections revealed renal and hepatic cysts (Figure 2, H and I), with varying degrees of severity.

![Figure 2](https://www.jasn.org/images/20:2556-2569,Fig2.jpg)

**Figure 2.** Temporal inactivation of the floxed Pkd2 allele induces cyst formation in the kidneys, liver, and pancreas. (A) A gross cystic kidney (arrow) is seen in a 9-wk-old γGt-Cre::Pkd2\(^{f3/}\) mouse. (B) A wild-type gross kidney from its littermate acts as a normal control. (C) A 6-wk-old Mx1-Cre::Pkd2\(^{f3/}\) mouse received intraperitoneal injections of 500 μg pl-pC for 3 consecutive days and was dissected and examined after 8 wk. Cysts (arrow) are seen in the kidney of the Mx1-Cre::Pkd2\(^{f3/}\) mouse. (D) A wild-type gross kidney from its littermate acts as a normal control. (E) A 6-wk-old Pdx1-Cre::Pkd2\(^{f3/}\) mouse received intraperitoneal injections of 1 mg Tamoxifen for 5 consecutive days and the pancreas was removed and examined 4 wk after injection. Multiple gross cysts (arrow) in the mouse pancreas were observed. Kidney sections, which correspond to (A through C), are shown in (F through H), respectively. (I) The same mouse shown in C also presented a great number of cysts in the liver (arrow). (J) There are multiple pancreatic cysts in the histologic section (arrow). Bars represent 0.2 mm in A and B and F through H; 0.5 mm in C through E; 5 μm in I; and 20 μm in J.

### Table 2. Genotyping analysis of mice with Pkd2\(^{d3/}\) allele

<table>
<thead>
<tr>
<th>Age</th>
<th>Pkd2(^{f3/}) n (%)</th>
<th>Pkd2(^{d3/}) n (%)</th>
<th>Total</th>
<th>Edema</th>
<th>Autolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>E13.5</td>
<td>3 (30)</td>
<td>5 (50)</td>
<td>2 (20)</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>At birth</td>
<td>6 (25)</td>
<td>22 (69)</td>
<td>2 (6)</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>&gt;3 wk</td>
<td>22 (45)</td>
<td>27 (55)</td>
<td>0 (0)</td>
<td></td>
<td>49</td>
</tr>
</tbody>
</table>

\(n\) = number of mice.
### Table 3. Summary of *Pkd2* mutant models described in the study

<table>
<thead>
<tr>
<th>Genotypes (allele nomenclatures)</th>
<th>Mutations</th>
<th>PC2 Expression Level</th>
<th>Phenotypes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pkd2</em>+/− (Pkd2&lt;sup&gt;tm2som&lt;/sup&gt;)</td>
<td>One allele contains a Neo cassette at exon 1 of <em>Pkd2</em></td>
<td>Approximately one-half of the normal level</td>
<td>No obvious cystic phenotype in the kidney, liver, and pancreas. Mouse development seems normal.</td>
<td>57,58</td>
</tr>
<tr>
<td><em>Pkd2</em>−/− (Pkd2&lt;sup&gt;tm2som&lt;/sup&gt;)</td>
<td>Both alleles contain Neo cassettes at exon 1 of <em>Pkd2</em></td>
<td>No PC2 detected by Western blotting</td>
<td>The mice are embryonic lethality at E12.5 to birth, along with total body edema, focal hemorrhage, cardiac structure defects, abnormal left-right axis, and hepatorenal/pancreatic cysts.</td>
<td>33,57,58</td>
</tr>
<tr>
<td><em>Pkd2</em>&lt;sup&gt;n3nf3&lt;/sup&gt;</td>
<td>Both alleles harbor a Neo cassette at intron 2 of <em>Pkd2</em></td>
<td>&lt;1/3 normal level of PC2 detected by Western</td>
<td>The mice are not embryonic lethal and develop cystic phenotype in the kidney, liver, and pancreas around the age of 12 mo. Ciliary defects were seen in renal epithelial cells. Fifteen mice showed growth retardation.</td>
<td>17 and Figure 5E of this study</td>
</tr>
<tr>
<td><em>Pkd2</em>&lt;sup&gt;33/33&lt;/sup&gt;</td>
<td>Both alleles of <em>Pkd2</em> exon 3 are flanked by the floxP sites</td>
<td>Normal level of PC2 detected by Western</td>
<td>No obvious cystic phenotype in the kidney, liver, and pancreas. Mouse development seems normal.</td>
<td>Table 1 in this study</td>
</tr>
<tr>
<td><em>Pkd2</em>&lt;sup&gt;d3/d3&lt;/sup&gt;</td>
<td>Both alleles of <em>Pkd2</em> exon 3 are excised to produce truncated PC2</td>
<td>No PC2 detected by Western</td>
<td>Similar to <em>Pkd2</em>−/− mice</td>
<td>Table 2 in this study</td>
</tr>
<tr>
<td>γGT-Cre::<em>Pkd2</em>&lt;sup&gt;0/−&lt;/sup&gt;</td>
<td>One allele is <em>Pkd2</em>&lt;sup&gt;tm2som&lt;/sup&gt;; another allele is <em>Pkd2</em> with exon 3 flanked by the floxP sites</td>
<td></td>
<td><em>Pkd2</em>-exon-3 floxed allele can be excised under control of a noninducible type I promoter of a rat γGt. The mouse exhibits cystic kidney at 9 mo of age.</td>
<td>Figure 2 and Supplemental Table 1 of this study</td>
</tr>
<tr>
<td>Mx1-Cre::<em>Pkd2</em>&lt;sup&gt;0/−&lt;/sup&gt;</td>
<td>The same as above</td>
<td></td>
<td><em>Pkd2</em>-exon-3 floxed allele can be excised under control of a pl-pC-inducible promoter from the intracellular Mx1. Some of the mice showed cystic phenotype in the kidney, liver, and pancreas.</td>
<td>Figure 2 and Supplemental Table 2 of this study</td>
</tr>
<tr>
<td>Pdx1-Cre::<em>Pkd2</em>&lt;sup&gt;0/−&lt;/sup&gt;</td>
<td>The same as above</td>
<td></td>
<td><em>Pkd2</em>-exon-3 floxed allele can be excised by a Tamoxifen-induced Cre-ER&lt;sup&gt;TM&lt;/sup&gt; transgene under control of a <em>Pdx1</em> promoter. The mice showed massive pancreatic cysts.</td>
<td>Figure 2 and Supplemental Table 3 of this study</td>
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dissolved in 200 μl of corn oil daily for 5 d. The other four mice were injected with vehicle alone daily for 5 d and used as controls. Four weeks after the last injection was given, the mice were sacrificed. The control mice did not display any obvious pancreatic cystic phenotypes, whereas the mice receiving Tamoxifen injections exhibited gross and histologic pancreatic cysts (Figure 2, E and J). Taken together, our results from three independent Cre-mediated transgene systems provide unequivocal evidence that the Pkd2-floxed allele can be temporally induced to a null-Pkd2 allele, thus inactivating PC2 expression in vivo.

Establishment of Renal Collecting Duct Cell Lines with Null-Pkd2 Alleles from Pkd2<sup>3/−</sup> Mutant Kidneys

Pkd2<sup>3/−</sup> mice with C57Bl/6 congenic background were crossbred with congenic Immortomice (Im<sup>1</sup>) to obtain Im::Pkd2<sup>3/−</sup> mice. To establish null-Pkd2 cell lines, two kidneys from an 8-wk-old Im::Pkd2<sup>3/−</sup> mouse were removed and minced finely with a scalpel. A Dolichus biflorus agglutinin (DBA)-based isolation approach was used to develop immortalized renal collecting duct cell lines from the kidneys. After limiting dilution, at least 48 immortalized renal collecting duct cell colonies were isolated from the Im::Pkd2<sup>3/−</sup> cell pool. We used E-cadherin and cytokeratin as epithelial markers and DBA as the collecting duct marker to identify their origin. Using these biomarkers, 28 collecting duct cell lines with Im::Pkd2<sup>3/−</sup> alleles were selected from the Im::Pkd2<sup>3/−</sup> cell pool (Figure 3A). One cell line (D3) was selected for infection with Cre-expressing adeno virus (AdCre)<sup>42</sup> to induce Im::Pkd2<sup>3/−</sup> alleles into Im::Pkd2<sup>4/−</sup> alleles. AdCre-infected D3 cells were then cloned by the aforementioned approach. Thirteen clones were isolated from AdCre-infected D3 cell pool, and at least four were positive for Pkd2<sup>4/−</sup> alleles by PCR genotyping (Figure 3B). To verify that the cell lines with Pkd2<sup>4/−</sup> genotypes resulted in a null-Pkd2 allele, an anti-PC2 antibody, hPKD2-Cm1A11, was used to detect PC2 expression levels in the cloned Pkd2<sup>4/−</sup> cell lines by Western blot. The Western blot results were consistent with the PCR genotyping results, showing that cell lines with Pkd2<sup>4/−</sup> alleles (i.e., E8, C1, and B2) did not have any detectable PC2 expression (Figure 3C).

These results provide strong evidence that the cell lines we have generated with Pkd2<sup>4/−</sup> alleles are null-Pkd2 cells.

Loss of PC2 Expression Impairs Tubulomorphogenesis In Vitro

To characterize cellular behaviors of the null-Pkd2 cell lines, we performed three-dimensional (3D) cultures to test whether loss of PC2 could induce abnormal tubulomorphogenesis in vitro. We used the D3 (Pkd2<sup>3/−</sup>) cell line and its Cre-mediated daughter cell line E8 (Pkd2<sup>4/−</sup>) in 3D Matrigel culture experiments, the protocols of which have been described previously.<sup>43</sup> Although D3 cells have a heterozygous allele for Pkd2, it seems that most of the cells are able to form normal tubular structures in the 3D cultures (Figure 4A, left), with tubulogenesis failure (Figure 4A, right) only present in approximately 2% of the cells. In sharp contrast, tubulogenesis failure was present in approximately 75% of the E8 cells in 3D Matrigel cultures (Figure 4B). Moreover, if one examines the number of tubular branches that reach five and over, none are observed with the E8 cell cultures whereas approximately 35% are noted for D3 cells (Figure 4B). These results indicate that loss of PC2 interrupts normal tubulomorphogenesis in vitro.

We rationalized that loss of PC2 may disrupt the normal cell-cell contact leading to dysregulation of normal tubulomorphogenesis. To test this hypothesis, D3 (Pkd2<sup>3/−</sup>) cells and daughter null-Pkd2 E8 cells were stained with an antibody against ZO-1, which is a putative marker for tight junctions.<sup>44</sup> Although ZO-1 was predominantly found at cell-cell junctions in D3 cells (Figure 4C, left), junctional staining of ZO-1 in E8

![Figure 3. Isolation of Pkd2<sup>4/−</sup> renal collecting duct cell lines and generation of the cell lines with null-Pkd2 alleles (Pkd2<sup>4/−</sup>).](https://www.jasn.org) A. Pkd2<sup>4/−</sup> cell line D3 was characterized by staining with the epithelial cell markers (a) cytokeratin and (b) E-cadherin and a collecting duct cell marker (c) DBA. Positive staining is seen in cultured D3 cell line for all three makers. (B) After AdCre infection, several single clones were isolated from the AdCre-infected D3 cell pool. The D3 daughter cell lines E8, C1, and B3 were genotyped by PCR, in which a pair of primers is anchored outside regions of the loxP-exon3-loxP cassette (Figure 1AII). A 550-bp PCR band can be detected in cell lines with the wild-type 350-bp product and mutant 170-bp product (lower panel in B). C. Western blot analyses confirmed the genotypes of these cell lines. Cell lines with Pkd2<sup>4/−</sup> alleles (such as mother cell line D3 and its daughter cell line B3 with its remaining Pkd2<sup>2/−</sup> allele) showed positive immunoactivity for PC2 (approximately 110 kD), whereas no PC2 band was detected in cell lines with Pkd2<sup>4/−</sup> alleles (i.e., E8, C1, and B2). Bar represents 5 μm in A.
cells showed a discontinuous and diffuse submembranous distribution pattern (Figure 4C, right). Further examination utilizing E-cadherin staining to detect cell-cell adherens junctions indicated E-cadherin was predominantly observed at the cell-cell junctions in D3 cells (Figure 4D, left); whereas in the E8 cells, E-cadherin junctional staining exhibited more diffuse and cytosolic distribution (Figure 4D, right). The results provide evidence that loss of PC2 disrupts the normal structure of tight junctions and impairs the formation of adherens junctions in vitro.

Null-Pkd2 Cells Demonstrate Fewer and Shorter Ciliary Structures

To determine whether loss of PC2 results in a defect in the primary cilium of renal epithelial cells, we compared the D3 (Pkd2f3/−) cell line to its daughter E8 (Pkd2f3/−) cell line using an anti-acetylated α-tubulin antibody along with immunofluorescent (IF) staining to examine the number and morphology of primary cilia. Compared with D3 cells, there were far fewer primary cilia in cultured E8 cells. The anti-acetylated α-tubulin antibody stained approximately 40% of D3 cells (Figure 5, A and C), whereas fewer than 20% of E8 cells were stained (Figure 5, B and C) (P < 0.05). Moreover, the mean length of primary cilia was 3 μm in cultured D3 cells, whereas that of the daughter E8 cells was less than 1 μm (P < 0.05) (Figure 5D). These in vitro results indicate that loss of PC2 induces ciliary defects in renal epithelial cells.

To confirm these observations in vivo, we used a similar IF staining approach to examine the number and morphology of primary cilia between corresponding regions of 12-mo-old wild-type and Pkd2nf3/nf3 littermate kidneys. Pkd2nf3/nf3 mice have hypomorphic alleles for Pkd2 with subsequent down-regulation of PC2 to approximately two thirds of the normal expression level,18 resulting in kidneys that are globally (rather than focally) lacking in PC2. This provides us with a superior experimental platform for ciliary comparison. We used this PC2-deficient mouse model to examine if the lack of PC2 interrupts the ciliary structure of the renal epithelial cells in vivo. Compared with their wild-type littermates, there were far fewer primary cilia in the cortical (Figure 5, Ea versus Eb) and medullary (Figure 5, Ec versus Ed) regions of Pkd2nf3/nf3 kidneys. Thus, ciliary examination of our in vitro and in vivo model systems indicate that PC2 deficiency results in fewer and shorter ciliary structures in renal epithelial cells.

Lack of PC2 Increases Proliferation and Apoptosis of Renal Epithelial Cells

Renal cyst formation is closely associated with proliferation and apoptosis of tubular epithelial cells.45,46 To characterize these cellular behaviors in our null-Pkd2 cell lines, we performed a proliferation assay on Pkd2f3/− (D3 and B3) and Pkd2d3/− (E8 and B2) cell lines. Compared with Pkd2f3/− cells, Pkd2d3/− cells demonstrated a significant increase in tritiated thymidine uptake (P < 0.05), suggesting that loss of PC2 increases cell proliferation (Figure 6A).

In addition, we also examined the apoptosis rates for the D3/B3 and E8/B2 cell lines using TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling) assays. Under routine culture conditions, approximately 5 to 10% of D3 and E3 cells were apoptotic, whereas under the same conditions more than 25% of the null-Pkd2 cells were undergoing programmed cell death (P < 0.05) (Figure 6B). This result suggests that normal PC2 expression can prevent programmed cell death of the renal epithelial cells. The cellular characteristics of increasing proliferation and apoptosis that we observed with our Pkd2 mutant cell lines corroborate the work of many other investigators.45,47,48

Abnormal Cellular Phenotypes in Null-Pkd2 Cells May Be Induced by Upregulated β-Catenin Expression

Some reports have indicated that renal cystic disease associates with dysregulation of β-catenin-dependent Wnt signaling.49,50

Figure 4. Loss of PC2 impairs tubulomorphogenesis and disrupts normal cell-cell contact in vitro. (A) Phase contrast photomicrographs were taken from examples of Pkd2f3/− D3 cells and Pkd2f3/− E8 cells cultured for 7 d in 3D Matrigel gels. Tubulomorphogenesis was seen in 3D cultured Pkd2f3/− D3 cells (left), but not in its daughter Pkd2f3/− E8 cells (right). (B) Quantification of the tubulomorphogenic results from 3D cultured Pkd2f3/− D3 and Pkd2f3/− E8 cells. Approximately 35% of Pkd2f3/− D3 cells generate five or more tubular branches and less than 2% exhibit cell aggregation. Under similar conditions, 75% of Pkd2f3/− E8 cells display cell aggregation and none formed five or more tubular branches. (C) IF staining of ZO-1 (green) showed a nearly normal view of tight junctions in cultured Pkd2f3/− D3 cells (left), whereas this normal tight junction is disrupted in Pkd2f3/− E8 cells (right). A ciliary marker anti-acetylated α-tubulin antibody (red) was used for IF co-staining. (D) Cultured Pkd2f3/− D3 and Pkd2f3/− E8 cells were stained with an anti-E-cadherin antibody (red). Compared with D3 cells (left), a diffuse E-cadherin distribution was seen in cultured E8 cells (right). DAPI dye (blue) was utilized for nucleic acid staining. Bar represents 20 μm in A and 5 μm in C through D.
Figure 5. Loss of PC2 induces aberrant ciliogenesis in null-Pkd2 renal cells and tissues. (A) A common ciliary marker anti-acetylated α-tubulin antibody was used for IF staining of the Pkd2^SS/−_D3 cell line. Confocal images show ciliary structures (arrows) in top (upper in A) and lateral views (lower in A). (B) Simultaneous IF staining of the Pkd2^SS/−_cell line E8, results in ciliary structures that were shorter in size and fewer in number than Pkd2^SS/−_D3 cells (arrows). (C) One hundred individual cells in five random high-power fields (1000×) from cell lines D3 and E8 were numbered, and the numbered cells were rated for the presence or absence of cilium-staining. In Pkd2^SS/−_D3 cells, 40% of cells stained positive for cilia, compared with 20% of Pkd2^SS/−_E8 cells (*P < 0.05). (D) The length of 50 individual primary cilia of these cultured cells from three random high-power fields was measured using lateral views of the confocal images; the average length of the primary cilia was calculated. The primary cillum length is approximately 3 μm in Pkd2^SS/−_D3 cells and approximately 1 μm in Pkd2^SS/−_D3 cells (*P < 0.05). (E) An anti-acetylated α-tubulin antibody was used to stain kidney sections from 12-mo-old Pkd2^Nf3/Nf3 mice and its wild-type littermates. Ciliary structures (arrows) were abundantly observed in wild-type kidneys (a and c). Cilia were fewer and shorter (arrows) in the corresponding medullary region of Pkd2^Nf3/Nf3 kidneys (a versus b). Similar reductions in number and length of ciliary structures (arrows) were observed in the corresponding medullary region of Pkd2^Nf3/Nf3 kidneys (c versus d). Bars represent 5 μm in A, B, Ea, and Eb; and 10 μm in Ec and Ed.

Figure 6. Characteristics of proliferation and apoptosis in the null-Pkd2 renal epithelial cell lines. (A) Pkd2^SS/−_cell lines D3 and B3 and Pkd2^SS/−_B2 and E8 were incubated with 3H-thymidine after which the rate of 3H-thymidine incorporation was determined as described in the Concise Methods section. Null-Pkd2 cells (B2 and E8) showed significantly higher 3H-thymidine values than Pkd2^SS/−_cells (D3 and B3) (*P < 0.05), a result analogous to previous observations that loss of PC2 promotes renal epithelial proliferation. (B) A TUNEL assay was also used to assess apoptosis. Null-Pkd2 cells (B2 and E8) showed a significantly higher percentage of cells undergoing apoptosis than Pkd2^SS/−_cells (D3 and B3) (*P < 0.05).

In addition, a β-catenin overexpression mouse model exhibits renal cystic disease. Therefore, we hypothesized that cyst formation induced by loss of PC2 might be associated with upregulation of β-catenin expression. To test this premise, lysates of cultured E8 and B2 cells (Pkd2^SS/−_) and cultured D3 and B3 cells (Pkd2^SS/−_) were examined by Western blots using an anti-β-catenin antibody. Significantly higher β-catenin levels were detected in the null-Pkd2 cells (E8 and B2) than were observed for the heterozygous cells (D3 and B3) (P < 0.05) (Figure 7, A and B, left panel). The results suggest that lack of PC2 leads to upregulation of β-catenin expression. To further substantiate this finding, we tested another putative β-catenin associated factor, axin2, using a similar approach. Lysates from the same panel of cell lines were tested by Western blot using an anti-axin2 antibody. Similar to what was observed when examining β-catenin expression, we detected significantly higher levels of axin2 expression in cultured null-Pkd2 cells (E8 and B2) than the Pkd2 heterozygous cells (D3 and B3), indicating that loss of PC2 upregulates axin2 expression levels (P < 0.05) (Figure 7, A and B, right panel). The significantly higher levels of β-catenin and axin2 in null-Pkd2 cells suggest that these important proteins may contribute to the abnormal cellular behaviors (i.e., cell proliferation and apoptosis) demonstrated in this study.

To further evaluate the role of β-catenin, we used the ligand Wnt3a, which is a putative upstream regulator of β-catenin. When Wnt3a was added to the culture medium to stimulate β-catenin-dependent signaling, we found the Pkd2 heterozygous cell lines D3 and B3, had a temporally induced upregulation of β-catenin expression. To test this premise, lysates of cultured E8 and B2 cells (Pkd2^SS/−_) and cultured D3 and B3 cells (Pkd2^SS/−_) were examined by Western blots using an anti-β-catenin antibody. Significantly higher β-catenin levels were detected in the null-Pkd2 cells (E8 and B2) than were observed for the heterozygous cells (D3 and B3) (P < 0.05) (Figure 7, A and B, left panel). The results suggest that lack of PC2 leads to upregulation of β-catenin expression. To further substantiate this finding, we tested another putative β-catenin associated factor, axin2, using a similar approach. Lysates from the same panel of cell lines were tested by Western blot using an anti-axin2 antibody. Similar to what was observed when examining β-catenin expression, we detected significantly higher levels of axin2 expression in cultured null-Pkd2 cells (E8 and B2) than the Pkd2 heterozygous cells (D3 and B3), indicating that loss of PC2 upregulates axin2 expression levels (P < 0.05) (Figure 7, A and B, right panel). The significantly higher levels of β-catenin and axin2 in null-Pkd2 cells suggest that these important proteins may contribute to the abnormal cellular behaviors (i.e., cell proliferation and apoptosis) demonstrated in this study.

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Figure 7. Loss of PC2 induces cyst formation by dysregulating β-catenin-dependent signaling. (A) Western analyses showed that β-catenin and axin2 were significantly upregulated in null-Pkd2 cells (B2 and E8), as compared with their mother Pkd2+/− cell line D3. An anti-PC2 antibody, hPKD2-Cm1A11, was used to evaluate the level of PC2 expression in the various cell lines. (B) A normalized quantitative analysis of the densitometry values of β-catenin and axin2 observed from B2/E8 or D3/B3 cell lines using results from at least three Western blots. The statistical analysis indicates that loss of PC2 significantly upregulates β-catenin expression levels (*P < 0.05). There is also a significant increase in axin2 expression in null-Pkd2 B2/E8 cell lines compared with Pkd2+/− D3/B3 cell lines (*P < 0.05). (C) Wnt3a was added to culture medium for 0, 24 (1 d), or 48 (2 d) h. Equal amounts of cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes. Western blots analysis using antibodies directed toward axin2, β-catenin, and cMyc indicate that an inducible upregulated pattern is observed for axin2, β-catenin, and cMyc proteins in the Pkd2+/− D3 cell line. In contrast, the Pkd2−/− B2 cell line has a high constitutive level of axin2, β-catenin, and cMyc that is not altered with application of Wnt3a. (D) A panel of Western blots was performed for detection of cytosolic β-catenin (upper panel) and axin2 (lower panel). Compared with the Pkd2−/− D3 cell line, both Pkd2+/− cell lines (B2 and E8) showed an increased expression for β-catenin and axin2. (E) A normalized quantitative analysis for cytosolic β-catenin and axin2 expression levels in Pkd2+/− D3 and Pkd2−/− B2/E8 cell lines indicates that loss of PC2 significantly upregulates cytosolic β-catenin and axin2 (*P < 0.05). (F) IHC staining of β-catenin, axin2, and cMyc expression in the kidney of E13.5 embryonic littersmates. A β-catenin antibody was used to stain the kidneys of (a) wild type and (b) Pkd2+/− cell lines (E8 and B2), as compared with a Pkd2−/− cell line D3 (*P < 0.05) (Figure 7, D and E). IHC staining of β-catenin, axin2, and cMyc expression observed from B2/E8 or D3/B3 cell lines (*P < 0.05) (Figure 7, D and E). The results provide further evidence that loss of PC2 disturbs β-catenin expression and likely alters β-catenin-dependent signaling.

To determine whether dysregulation of β-catenin and associated factors occurred in vivo, we used a panel of antibodies against β-catenin, axin2, and cMyc to perform IHC staining in a pair of the kidneys from E13 wild-type and Pkd2+/− littermates and in adult littermates with pI−pC-induced Mx1-Cre::Pkd2+/− and Pkd2−/− alleles. Mild-to-moderate positive staining of β-catenin was observed in the epithelial cells of Pkd2−/− kidneys compared with wild-type embryos (Figure 7F, a and b), as well as in pI−pC-induced Mx1-Cre::Pkd2−/− adult kidneys compared with its Pkd2+/− littermates (Figure 7G, a and b). Similar results were also noted.
in immunohistochemical staining of axin2 (Figure 7F, c and d, and 7G, c and d) and cMyc (Figure 7F, e and f, and 7G, e and f) when comparing epithelial cells of Pkd2d3/d3 and Pkd1-null systems. 

DISCUSSION

Although we have previously generated Pkd2 knockout mouse models by which some knowledge regarding the mechanism of cyst formation has been garnered,29–31 the models are unacceptable for studying the full spectrum of PC2 functions because of the embryonic lethality.30 To bypass this limitation, we generated a mouse model in which Pkd2 can be conditionally inactivated using a Cre-loxP system. Using this mouse model, we generated a collecting duct epithelial cell line (D3) from 8-wk-old Pkd2<sup>d3</sup>/<sup>d3</sup> mice kidneys. By infection of the D3 cells with AdCre virus, we converted the Pkd2<sup>d3</sup>/<sup>d3</sup> cells to Pkd2<sup>d3</sup>/<sup>d3</sup> cells in which Pkd2 is fully lost (B2/E8). Through characterization of these new cell lines, we found upregulation of the β-catenin expression was associated with loss of PC2. The observed dysregulation of β-catenin may be the underlying mechanism for aberrant tubulomorphogenesis, primary ciliogenesis, cell-cell contacts, and cell proliferation. The study presented here provides evidence that loss of PC2 upregulates β-catenin, axin2, and cMyc expression in <em>in vitro</em> and <em>in vivo</em> Pkd2-null systems, suggesting there may be a disruption in canonical Wnt signaling.

Although many inducible knockout mice of Pkd1 have been produced, no inducible knockout mouse model for Pkd2 has been reported until now. We previously generated a mouse model with a hypermutable allele for Pkd2 (Pkd2<sup>W525</sup>) that escapes the embryonic lethality and enables maturation of homozygous mice into adulthood. Using this novel model, we generated a mouse model in which PC2 expression can be functionally inactivated under mediation of Cre recombinase to produce mouse or cells with null-Pkd2 (Pkd2<sup>d3/d3</sup> or Pkd2<sup>d3/d4</sup>) alleles. Generation of this inducible Pkd2 mutant model is not only beneficial for the establishment of null-Pkd2 cell lines, but it also provides a powerful tool to gain insights into PC2 tissue-specific functions during embryogenesis and organogenesis.

Several studies have demonstrated that the disruption of ciliary formation in renal epithelia induces renal cystogenesis.55,56 We recently reported that downregulation of Pkd1 significantly decreases formation of cilia in cultured Pkd1-silenced inner medullary collecting duct cells, implicating downregulation of FPC in the disruption of ciliogenesis in renal epithelial cells.43 A similar observation was made <em>in vivo</em> because we noted fewer and shorter primary cilia in renal epithelial cells of Pkd1<sup>−/−</sup> mice than their wild-type littermates. Together with our evidence that FPC and PC2 physically interact and lack of FPC downregulates PC2 expression,17,18 we hypothesized that PC2 deficiency may also result in abnormal ciliogenesis in renal epithelial cells. Because previous ADPKD models were embryonic lethal (Pkd2<sup>−/−</sup> and Pkd1<sup>−/−</sup> mice<sup>30,57</sup>) or displayed focal cystic lesion in the affected kidneys (Pkd2<sup>W525</sup> mice<sup>29</sup> and Cre-mediated inducible Pkd1-mutant models<sup>36,53,54</sup>) it has been difficult to precisely identify corresponding disease lesions between adult mutant and wild-type kidneys to conclude ciliary defects in ADPKD. Our generation of null-Pkd2 cell lines (E8 and B2) provides a valuable tool for investigations aimed at determining the role of PC2 in ciliogenesis <em>in vitro</em>. For example, our observations indicate that loss of PC2 can induce aberrant ciliogenesis, a result similar to that reported for Pkd1<sup>−/−</sup> cultured cells.43

In addition, our Pkd2<sup>W525/loxp</sup> mouse model (Figure 1A1) can be used to investigate the role of PC2 on ciliary defects <em>in vivo</em> because of their downregulation of PC2 to two thirds of wild-type levels. This PC2 deficiency is unique in that it is universal rather than focal.18 Renal focal cysts are not suitable for <em>in vivo</em> comparison of ciliary defects because cilia length changes along the nephron. It is very difficult to identify identical nephron segments in different individual kidneys to provide accurate tubule comparisons. We therefore used the Pkd2<sup>W525/loxp</sup> mouse model to perform corresponding regional comparisons of kidneys. By comparing the ciliary morphology between kidneys of Pkd2<sup>W525/loxp</sup> and wild-type littermates, we conclude that lack of PC2 induces ciliary defects in renal epithelial cells <em>in vivo</em>

Although our null-Pkd2 cell lines are derived from renal collecting ducts, we can not conclude that the ciliary defects exclusively occur in collecting duct cells. Our <em>in vivo</em> data (Figure 5E) indicate that cilia defects are observed in medullary and cortical regions of the kidney. We therefore believe that loss of PC2 may induce cilia defects in all renal epithelial segments, rather than just collecting ducts. However, whether the loss of PC2 directly affects ciliary assembly will require further investigations.

We also found that normal E-cadherin-mediated cell-cell contacts are disrupted in cultured null-Pkd2 collecting duct cells. Because E-cadherin-mediated cell-cell adhesion is an initial trigger for signaling to assemble intercellular junctions, and because intercellular junctions are essential in epithelial polarity and tubule formation,44,58,59 disruption of normal E-cadherin distribution might induce abnormal cytosolic β-catenin levels and impede renal epithelial polarization.43,60 The polarity defect may result in aberrant renal epithelial or-
ganization and tubulogenesis and, in turn, induce cystogenesis in the kidneys.

Recent studies have demonstrated that human cystic disease may involve Wnt signal transduction. Romaker et al. reported that dysregulation of a secreted Frizzled-related protein 4 antagonizes Wnt signaling and induces cyst formation in ADPKD. Interestingly, a previous study indicated that the PKD1 promoter may actually serve as a target of the Wnt signaling pathway, with the PKD1 promoter being highly activated by administration of β-catenin. In addition, transgenic mouse models for β-catenin or cMyc cause severe PKD phenotypes, providing additional evidence that upregulation of β-catenin or cMyc alone is able to induce cyst formation in the kidney. Finally, a recent report demonstrated that PC1, the gene product of PKD1, can interact with β-catenin to inhibit canonical Wnt signaling by dysregulation of β-catenin. Given that PC1 physically interacts with PC2, we examined the role of PC2 in canonical Wnt signaling. Our observation that loss of PC2 significantly upregulates β-catenin expression supports the hypothesis that cystogenesis in PC2-associated ADPKD may be the result of dysregulation of β-catenin-dependent Wnt signaling.

In summary, we have generated a mouse model with a functional floxed Pkd2 allele and established a panel of Pkd2-deficient renal collecting duct cell lines from this mouse model. These animal and cell systems will provide a valuable platform to further study PC2 functions and its role in the pathogenesis of ADPKD. By characterizing the mouse model and its resulting Pkd2δ3/− and Pkd2δ3/− cell lines, we conclude loss of PC2 induces ciliary defects in renal epithelial cells, impairs renal tubulomorphogenesis, and disrupts normal cell-cell contacts. It may be that the observed changes in dysregulation of β-catenin in Pkd2δ3/− cell lines leads to some, or all, of these pathogenic changes. Taken together, our findings indicate a functional role of β-catenin in PC2-associated ADPKD. It is hoped that this finding may give rise to a new spectrum of molecular targets for therapeutic intervention of ADPKD.

**CONCISE METHODS**

**Mouse Strains**

We previously generated Pkd2 mutant mice (Pkd2−/− i.e., Pkd2tm2swm). We also used a mouse model with a hypomorphic Pkd2 allele (Pkd2δ3) (Supplemental Figure 1) in which PC2 can be downregulated to two thirds of its normal expression level because of the positional effect of a pGKNeo cassette at the Pkd2-intron-2 locus. We crossmated Pkd2δ3 mice with ACTB-Flpe mice, in which Flpe recombinase is controlled by human β-actin promoter and Flpe recombinase can be activated at early embryogenesis to produce mice with a Pkd2δ3 allele in which the pGKNeo cassette is excised (Figure 1AII). We then crossmated Pkd2δ3 mice with Pkd2−/− mice and a spectrum of Cre mouse strains to produce mice with Cre::Pkd2δ3/− alleles, in which Cre::Pkd2δ3/− can be converted into a Cre::Pkd2δ3/− allele under mediation of Cre recombinase. The Cre mouse strains included γGt-Cre mice, in which Cre recombinase is controlled by the type I promoter of a rat γGt and is expressed primarily in renal tubular epithelial cells. Mx1-Cre mice, in which Cre recombinase is controlled by an IFN-γ promoter and can be induced by intraperitoneal injection of PI-pCIC and Pdx1-Cre mice, in which Cre recombinase can be induced by the pancreas duodenal homeobox gene promoter under control of an inducible Pdx1-Cre-ERTTM-loxP system. Pdx1-Cre involves pancreatic duct and endocrine cell development and the Pdx1-Cre-ERTTM-loxP system can be activated by the intraperitoneal injection of Tamoxifen. Elia-Cre transgenic mice, in which Cre recombinase is expressed in early-stage embryogenesis under the control of Elia12,33 to produce mice with Pkd2 exon-3-deletion allele (Pkd2δ3). All mouse models used in this study are from the C57/B16 congenic background and are listed in Table 3.

**Antibodies**

Our monoclonal antibody against the human PC2 COOH-terminus (hPKD2-Cm1A11) was described in previous studies. The following antibodies and staining materials were purchased: anti-acetylated α-tubulin, anti-γ-tubulin, anti-β-actin, and anti-cMyc antibodies (Sigma); anti-ZO-1 (Zymed Laboratories); anti-β-catenin and anti-E-cadherin (BD Transduction Laboratories); cytokeratin (Santa Cruz Biotechnology, Inc.); anti-axin2 (Cell Signaling Technology); fluorescein lotus tetragonolobus lectin and fluorescein DBA (Vector Laboratories); and fluorescein anti-Tamm–Horsfall glycoprotein (The Binding Site, Ltd.).

**Southern/Western Blotting and Quantitative PCR**

Southern and Western analyses were performed using protocols similar to those described in previous publications. Quantitative PCR was performed using the iCycler iQ real-time PCR detection system with the iQ SYBR Green Supermix kit (Bio-Rad). A pair of primers to detect Pkd2 were identical to those described in a prior publication.

**Histology, Immunofluorescence Staining, and Confocal Microscopy**

Detailed procedures for histology and immunofluorescence were published previously. For microscopic analysis, images were obtained using a Zeiss Axioplan 2IE research microscope system with objectives. For confocal microscopy, the images of antibody staining were collected using Z series sections on a Zeiss LSM 510 confocal system with 40 or 63 oil objectives. Multiple sections (0.3 μm in thickness) were projected onto one plane for presentation. The cells used to establish normal cell-cell contacts were plated and grown to confluence for 3 to 5 d on 24-well plates with a glass cover.

**Renal Epithelial Cell Lines and Their Cultures**

All renal epithelial cell lines reported in this study were generated from an 8-wk-old Pkd2δ3/− mouse. The kidneys were finely minced with a scalpel and the minced tissue was incubated with 0.5% collagenase type IV at 37°C for 45 min and pipetted vigorously. The undisgested tissue was removed by filtration through a 40-μm mesh filter. The remaining single cells and small organoids were washed three times with PBS containing 5 mM glucose. The cells were incubated
with 10 μg/ml biotinylated DBA (Vector, B-1035) at 4°C for 60 min. Then, the cells were washed again with PBS before incubation with 50 μl CELLeLectin Biotin binder Dynabeads (Dynal Biotech) at 4°C for 30 min. Because Dynabeads are superparamagnetic polystyrene beads, the incubated mixtures were washed twice with PBS containing 5 mM glucose using a magnetic rack. The cells were eluted with release buffer and were plated on 24-well dishes with LHC-9 Medium (Life Technologies) under 5% carbon dioxide at 37°C overnight. The cells were then changed to cultured medium containing 5 U/ml murine IFN-γ (PeproRech Inc.) and placed in a 33°C incubator for at least ten cell passages with media replacement every other day. After this, the culture medium was switched to 10% FCS MDEM/F12 (1:1) (Life Technologies) and the cells were cultured for at least another ten cell passages using the same culture conditions. The pool cells were cloned using a limited dilution method. The cloned cell lines were characterized by epithelial markers, and one of them (D3) with Pkd2f3/+ alleles was selected for further experiments.

The D3 cells were infected with AdCre virus and were cloned again by the same approach aforementioned. Cloned cell lines were isolated from the AdCre-infected D3 cell pool. Two D3 daughter cell lines with Pkd2f3/+ alleles (E8 and B2) and one daughter cell lines with Pkd2f3/+ alleles (B3) (non-AdCre-infected cells) were chosen for our further detailed cellular characterization and experiments. The use of 3D extracellular matrix gels for cultured cell lines has been previously described. Briefly, the cultured gel was made by 1:1 mixture of collagen I and Matrigel gels along with 10% FCS. The tubule formation was determined in five randomly picked fields.

Before performing any cell-based assays, all established cell lines were cultured under nonpermissive conditions (37°C without γ-INF) for at least 3 d to turn off SV40 transgene activities.

**Cell Proliferation and Apoptosis**

Cells (40,000 per well) were placed onto 24-well plates, and after 5 d in culture, cells were pulsed for 24 h with 3H-thymidine (1 μCi/well). The cells were then removed from the plates, dialyzed against PBS for 24 h to remove free 3H-thymidine, lysed in 1% SDS (100 μl final volume), and the lysates measured using a β counter.

For apoptosis studies, cells (40,000 per well) were placed onto 24-well plates and grown to subconfluence with 7% FCS DMEM/F-12 (1:1) medium (Life Technologies) under 5% CO2 at 37°C. The cells were incubated with 2 μM ionomycin (Alexis Corporation) under serum-free conditions. Six hours later, a TUNEL assay (DeadEndTM fluorometric TUNEL system, Promega) was performed per the manufacturer's instructions. The apoptotic cells were counted from three randomly picked high-power fields (40×).

**Statistics**

All biochemical assays were repeated at least twice and graph data were presented as the mean ± SD. Statistical analysis was performed where appropriate using the t test or one-way ANOVA followed by Tukey's multiple comparison test. Differences with P values <0.05 were considered statistically significant.

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**DISCLOSURES**

None.

**REFERENCES**


