**ABSTRACT**

Autosomal dominant polycystic kidney disease, the most common monogenetic disorder, is characterized by gradual replacement of normal renal parenchyma by fluid-filled cysts. Mutations in either PKD1 or PKD2 cause autosomal dominant polycystic kidney disease. Pkd1<sup>-/-</sup> or Pkd2<sup>-/-</sup> mice develop rapid renal cystic disease and exhibit embryonic lethality; this supports the “two-hit” hypothesis, which proposes that a germline mutation in PKD1 (or PKD2) followed by a second somatic mutation later in life is responsible for the phenotype. Here, for investigation of the loss of Pkd1 at specific times of development, an inducible Pkd1-knockout mouse model was generated. Inactivation of Pkd1 in 5-wk-old mice resulted in formation of only focal renal cysts 6 to 9 wk later but in a severe polycystic phenotype nearly 1 yr later. Cysts derived from either collecting tubules or distal tubules but not from proximal tubules, which correlated with sites of Cre-mediated recombination. Inactivation of Pkd1 in 1-wk-old mice, however, resulted in massive cyst disease 6 wk later, despite a similar pattern of Cre-mediated recombination between 1- and 5-wk-old kidneys. Moreover, a germline heterozygous Pkd1 mutation facilitated cyst formation when a somatic Pkd1 mutation was induced. A marked increase in proliferating cell nuclear antigen expression was observed in cyst-lining epithelia and in normal-looking tubules adjacent to but not in those distant from cysts. These data suggest that Pkd1 inactivation is not sufficient to initiate the cell proliferation necessary for cyst formation; a paracrine mechanism may account for focal cell proliferation and regional disease progression. We propose that an additional genetic or nongenetic “third hit” may be required for rapid development of cysts in polycystic kidney disease.

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Autosomal dominant polycystic kidney disease (ADPKD) is the most common life-threatening genetic disease and is characterized by fluid-filled kidney cysts and increased epithelial cell proliferation. Cysts are seen less frequently in the liver (approximately 80%) and pancreas (approximately 10%). Most ADPKD cases result from mutations in either PKD1 (approximately 85%) or PKD2 (approximately 15%), the genes encoding for polycystin-1 (PC1) and polycystin-2 (PC2), respectively. Both PKD1 and PKD2 are widely distributed in various organs and tissues. Initially, cysts are connected to the tubule with which they share a common lumen; however, they usually become separated when they are more than approximately 2 mm in size. Continuation of cyst growth is dependent on (at least) proliferation, transepithelial fluid secretion, and dedifferentiation. The severity of renal disease is highly variable even within the same family, ranging from rare in utero cases with massively enlarged cystic kidneys through more typical adult presentations with ESRD in the sixth decade, to cases with adequate kidney function into old age; therefore, it has
been suggested that environmental risk factors and/or modifier genes influence the progression of PKD.1 Several lines of mice with targeted mutations in the mouse Pkd1 gene have been generated. Homozygous deletion of Pkd1 (Pkd1<sup>del34/del34</sup> and Pkd1<sup>null/null</sup>) is embryonically lethal, with numerous large cysts in kidney and pancreas, further supporting the “two-hit” hypothesis, which proposes that a germline mutation followed by a somatic mutation later in life is responsible for the phenotype.2–5 Pkd1<sup>L/L</sup> embryos die primarily of cardiovascular defects (e.g., edema, vascular leaks, rupture of blood vessels) with renal and pancreatic cystic development by embryonic day 15.5 (E15.5) and E13.5, respectively.6 Investigation of the role of PC1 in the adult mouse has been hampered by the embryonic lethality of the homozygous deletion of Pkd1.

In contrast, homozygous Pkd1<sup>L</sup> mice with a germline hypomorphic allele of Pkd1 show cystic kidneys in adulthood; however, because severity of this phenotype varies widely (some die at 1 mo of age with massively enlarged kidneys, others with less severe cystic disease survive to 1 yr), this mouse model might have limited usefulness for therapeutic interventions.7 Studies of another homozygous Pkd1<sup>L3</sup> mouse with a hypomorphic allele of Pkd1 revealed that the Pkd1<sup>L3/L3</sup> kidneys appeared normal at birth but enlarged rapidly during the first 30 d and gradually decreased in volume by >50% afterward.8 This phenotypic course may more closely resemble autosomal recessive PKD than ADPKD. A floxed Pkd1 mouse line that develops renal and hepatic cysts when crossed with transgenic mice expressing Cre recombinase under the control of MMTV promoter (Pkd1<sup>flox/flox MMTVCre</sup>) was generated; however, the described renal phenotype is very mild: The kidney has a few visible cysts on its surface beginning in the 20th week of life.9 Our inducible Pkd1 knockout mouse line overcomes these limitations. In this mouse model, the Mx1Cre transgenic mouse was used to induce somatic inactivation of Pkd1 at specific time points, which allowed us to study the role of PC1 in quiescent cells of the adult kidney and in precystic kidney.

Results from a series of experiments suggest that inactivation of both Pkd1 alleles initiates cell proliferation sufficient to lead to cyst formation in the postnatal developing kidney but not in the developed kidney. Consequently, we propose that a “third hit” that triggers the reactivation of the developmental program or cell proliferation in adult life is required for rapid cyst development in adult kidneys.

**RESULTS**

**Somatic Inactivation of Pkd1 in Adult Kidney Causes Focal Cyst Formation**

Both Pkd1 and Pkd2 are widely distributed in various organs and tissues. It has been shown that conventional homozygous deletion of either Pkd1 or Pkd2 is embryonically lethal. To overcome this problem and to study the effect(s) of homozygous deletion of these genes in adult animals, we generated conditional knockout (CKO) mice of Pkd1 by flanking exons 2 through 6 with lox<sup>P</sup> sites.10 This allows the creation of a null allele at a specific time point by crossing Pkd1<sup>flox/flox</sup> mice with a transgenic mouse line that expresses Cre recombinase under the control of an interferon (IFN)-inducible Mxi1 promoter (Mx1Cre mouse). Because ADPKD is an adult disease, we decided to inactivate Pkd1 in young adult mice. Mx1Cre<sup>+</sup> Pkd1<sup>flox/flox</sup>

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**Figure 1.** Induction of Pkd1 mutation in adult kidney causes focal cyst formation. (A) Mx1Cre<sup>−</sup> Pkd1<sup>flox/flox</sup> and control littermate mice were administered injects of pi:pC at 5 wk of age. Control (Mx1Cre<sup>−</sup> Pkd1<sup>flox/flox</sup>) mouse showed normal kidney morphology. (B through D) Mx1Cre<sup>−</sup> Pkd1<sup>flox/flox</sup> mice developed focal cysts 6 wk (B) and 9 wk (C and D) after injection. (E through M) Extensive cyst formation in kidney (E through G and L) and liver (H through J and N) was observed 46 wk later, whereas control Mx1Cre<sup>−</sup> Pkd1<sup>+/−</sup> mice showed normal kidney (K) and liver (M). (G and J) Fibrosis in Mx1Cre<sup>−</sup> Pkd1<sup>flox/flox</sup> kidney and liver was detected by Masson’s trichrome staining showing collagen deposition. (E, F, and I) In addition, local leukocyte infiltration was observed in the interstitium of the kidney (E and F, arrow), and dilation of biliary ducts was seen (I). Magnifications indicated in all figures are original magnifications.
and littermate control mice were administered an injection of 250 μg of polyinosinic-polycytidylic acid (pI:pC) every day for 5 d beginning at 5 wk of age. Kidneys from control Mx1Cre<sup>+</sup> Pkd1<sup>flox/+</sup> mice 6 wk after injection of pI:pC (Figure 1A) had normal renal histology. Only focal cysts were seen in Mx1Cre<sup>+</sup> Pkd1<sup>flox/flox</sup> kidney (Figure 1B). Furthermore, fibrosis and leukocyte infiltration were seen around cysts 9 wk after injection (Figure 1, C and D). Similar to what is seen in humans, the cyst-lining epithelial cells are flattened. To investigate whether the cystic phenotype would be more severe later in life, we kept mice for nearly 1 yr. When we dissected mice 46 wk after injection of pI:pC, Mx1Cre<sup>+</sup> Pkd1<sup>flox/flox</sup> kidney and liver showed massive enlarged cysts (Figure 1, E through J, L, and N), whereas control kidney and liver from Mx1Cre<sup>+</sup> Pkd1<sup>+/+</sup> mice were normal (Figure 1, K and M). Fibrosis and leukocyte infiltration were seen in both the kidney and the liver, as indicated by Masson’s trichrome staining (Figure 1, G and J). Pancreatic cysts were seen occasionally in older mice (data not shown).

Cyst Formation and Enlargement Are Accelerated in Mice with an Induced Pkd1 Mutation in Postnatal Developing Kidney

It has been shown that conventional homozygous deletion of Pkd1 results in formation at E15.5 in proximal tubules followed by rapid cyst development in the collecting system and cystic replacement of the entire renal parenchyma, which indicates that Pkd1 is required for kidney morphogenesis during late embryogenesis; however, because these mice do not survive after birth, the role of PC1 in postnatal developing kidney remains unclear. We therefore induced Cre expression in Mx1Cre<sup>+</sup> Pkd1<sup>flox/flox</sup> and control mice at 1 wk of age, when the mouse kidney is still developing. Mx1Cre<sup>+</sup> Pkd1<sup>flox/flox</sup> and littermate control mice were injected with pI:pC at 1 wk of age and dissected 9 wk later. Because 1-wk-old mice are approximately one quarter the size of 5-wk-old mice, we used one quarter the dose used at 5 wk of age. Compared with control Pkd1<sup>flox/flox</sup> mice (Figure 2, A and C), littermate Mx1Cre<sup>+</sup> Pkd1<sup>flox/flox</sup> mice developed enlarged kidneys with multiple cysts (Figure 2, B and C); however, proximal tubules, identified by the presence of brush border membranes, seemed unaffected (data not shown).

Cysts are Derived from Either Collecting Tubules or Distal Tubules

For identification of the origin of the renal tubular cysts, nephron segment–specific markers were used. When labeled with the lectin Dolichos biflorus agglutinin (DBA), which is specifically expressed in the collecting duct, most of the cysts were highlighted (Figure 2, D through F). In contrast, none of the cysts was labeled with Lotus tetragonolobus lectin (LTL) for proximal tubule (Figure 2D). In addition, some cysts were positively labeled with the antibody directed against Tamm-Horsfall protein (THP), a thick ascending limb marker (Figure 2, E and F). THP-positive cysts were usually smaller than DBA-positive cysts (Figure 2F). A fraction of cysts were negative for all three markers. This pattern of tubular origin is similar to that seen in cysts in human ADPKD.

Germline Pkd1 Mutation Facilitates Cyst Formation

In human ADPKD, all cells in renal tubules carry a germline-mutated allele. Cyst formation begins after the second somatic mutation in the normal allele (two-hit hypothesis). To examine the impact of a germline mutation of Pkd1 on cyst formation, we induced somatic inactivation of Pkd1 in compound heterozygous Pkd1<sup>null/flox</sup> mice that also carry the Mx1Cre transgene (Mx1Cre<sup>+</sup> Pkd1<sup>null/flox</sup> mice) by injection with pI:pC at 1 wk of age and dissected them 6 or 9 wk later. (A through D) Control Pkd1<sup>flox/flox</sup> kidney showed normal histology, whereas Mx1Cre<sup>+</sup> Pkd1<sup>flox/flox</sup> mice exhibited enlarged cystic kidneys (B and C). Most of the cysts were derived from DBA-positive (red) tubules (A through C) but not from LTL-positive (green) tubules (D). (E and F) THP-positive (green) cysts were sometimes seen and were much smaller than DBA-positive (red) cysts. *Cyst lumen. A and B were digitally assembled to show the whole kidney.
Mx1Cre<sup>+</sup>Pkd1<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup> mice (three of 14) were found to have widespread cystic kidney 9 wk after injection, this phenotype was detected in 58% of Mx1Cre<sup>+</sup>Pkd1<sup>null/fl<sup>ox</sup></sup> mice (seven of 12) as early as 6 wk after injection (Table 1). These compound heterozygous mice also have significantly higher levels of serum creatinine than the Mx1Cre<sup>+</sup>Pkd1<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup> (0.4 ± 0.2 [null/fl<sup>ox</sup>] versus 0.086 ± 0.157 [fl<sup>ox</sup>/fl<sup>ox</sup>] mg/dl; P < 0.006) and their littermate control mice (0.4 ± 0.2 [null/fl<sup>ox</sup>] versus 0.038 ± 0.074 [control] mg/dl; P < 0.0005; Figure 3F).

It is noteworthy that in compound heterozygous Pkd1<sup>null/fl<sup>ox</sup></sup> mice, injection with pI:pC at 5 wk of age also caused milder phenotype (Figure 3, G and H) than injection at 1 wk of age, but the compound heterozygous phenotype was more advanced than that produced in Mx1Cre<sup>+</sup>Pkd1<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup> mice (I). The genotype of control shown here is Pkd1<sup>fl<sup>ox</sup>/+</sup> (A, left).

Table 1. Number of Pkd1-inactivated and control mice administered injections of pI:pC<sup>a</sup>

<table>
<thead>
<tr>
<th>Time after Injection (wk)</th>
<th>5 Wk (250 µg)</th>
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<td>Injection Age (Dosage)</td>
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<td>Mx1Cre&lt;sup&gt;+&lt;/sup&gt;Pkd1&lt;sup&gt;fl&lt;sup&gt;ox&lt;/sup&gt;/fl&lt;sup&gt;ox&lt;/sup&gt;&lt;/sup&gt;</td>
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<td>Total</td>
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<sup>a</sup>IKO, inducible knockout.

<sup>b</sup>Entire cystic kidney.

<sup>c</sup>Mx1Cre<sup>+</sup>Pkd1<sup>fl<sup>ox</sup>/+</sup>.

<sup>d</sup>Pkd1<sup>fl<sup>ox</sup>/+</sup>.

<sup>e</sup>Pkd1<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup>.

<sup>f</sup>Pkd1<sup>null/fl<sup>ox</sup></sup>.

<sup>g</sup>Pkd1<sup>fl<sup>ox</sup>/null</sup>.

<sup>h</sup>Mx1Cre<sup>+</sup>Pkd1<sup>fl<sup>ox</sup>/+</sup>.</p>

![Figure 3](https://www.jasn.org)
consistent with data obtained with \( Mx1\text{Cre}^{+}\ Pkd1^{\text{floxed/floxed}} \); however, renal histology (Figure 3, G and H) reveals that these compound heterozygotes develop more striking cystic disease than \( Mx1\text{Cre}^{+}\ Pkd1^{\text{floxed/floxed}} \) mice (Figure 3I). These data suggest that a germline mutation has an effect on cyst development.

**Patterns of Cre-Mediated Recombination Events Are Similar in 1- and 5-Wk-Old Kidneys**

To investigate whether the patterns of Cre-mediated recombination events are similar between 1- and 5-wk-old kidneys and to confirm the renal tubular segment distribution of \( Mx1\text{Cre} \)-mediated genetic recombination, we crossed \( Mx1\text{Cre} \) mice with a reporter strain (\( DsRed \) mice) in which \( DsRed \) is expressed only after Cre-mediated recombination. \( Mx1\text{Cre} \) mice with \( DsRed \) reporter transgene (\( Mx1\text{Cre}^{+}\ DsRed^{+} \) mice) and injected with pI:pC at 1 or 5 wk of age for 5 consecutive days were dissected 1 d after the last injection. Direct visualization with an epifluorescent microscope revealed that expression of the \( DsRed \) transgene was restricted to a subset of kidney tubules (Figure 4, A through D). \( Mx1\text{Cre} \)-mediated recombination was found throughout the outer medulla and sporadically in the cortex in both 1- and 5-wk-old mice (Figure 4, A through D), suggesting that Cre excision efficiency is similar between these stages. Labeling of cryosections with specific kidney tubule markers revealed that \( DsRed \) was easily detected in collecting tubule/ducts (Figure 4, B, D, and E) and distal tubules (Figure 4F); however, \( DsRed \) signal was undetectable in proximal tubules (LTL labeled; Figure 4, A, C, and G). In the glomerulus, \( DsRed \) was found in all cellular compartments (Figure 4, E and F). In contrast, pI:pC injection of mice carrying the \( DsRed \) reporter gene but not the \( Mx1\text{Cre} \) transgene did not elicit \( DsRed \) signals (data not shown).

**Cre-Mediated Pkd1 Excision Efficiency in 1- and 5-Wk-Old Kidneys**

Although the pattern of Cre-mediated activation of \( DsRed \) was similar in 1- and 5-wk-old kidneys, the activation of \( DsRed \) occurs as a \( Pkd1 \)-independent Cre-mediated recombination event. To exclude the possibility that \( Pkd1 \) inactivation efficiency in 5-wk-old kidney is lower than that in 1-wk-old kidney, which in turn is responsible for the milder phenotype, we...
performed quantitative real-time PCR as well as semiquantitative PCR on genomic DNA isolated from mice 1 wk after injection of pI:pC. Recombination at the targeted Pkd1 locus (Pkd1del2−6) would allow the amplification of an approximately 280-bp band with primers located in introns 1 and 6 of the Pkd1 gene. Primers located in intron 1 of Pkd1, which amplifies an approximately 170-bp band, were used as an internal control. As expected, we were able to detect the 280-bp del2−6 band in Mx1Cre+ Pkd1flox/+ and Mx1Cre+ Pkd1flox/flox mice but not in wild-type mice (Figure 5A). The quantitative real-time PCR and semiquantitative PCR analysis revealed that, after normalization with Pkd1 intron 1 PCR products, the deletion efficiency in the Mx1Cre+ Pkd1flox/flox kidneys calculated as deleted/total Pkd1 floxed alleles was approximately 35 and 50% in 1- and 5-wk-old kidneys, respectively (Table 2). In the Mx1Cre+ Pkd1flox/+ kidneys, the deletion efficiency calculated as deleted/total Pkd1 floxed alleles was comparable to that in the flox homozygotes (Table 2). Germline Pkd1del2−6/− heterozygous DNA in which the recombination rate at the deletion allele is 100% was used as a control. Because Mx1Cre is expressed only in distal nephron segments, not in proximal tubules, these data indicate excellent recombination efficiency at the Pkd1 locus, and this efficiency is even higher in 5-wk-old kidney, compared with that in 1-wk-old kidney.

**Cre-Mediated Recombination Is Seen in Normal-Looking Tubules and Cysts in Mx1Cre+ DsRed+ Pkd1null/flox Mice**

Why does germline Pkd1 mutation facilitate cyst formation? There are two possibilities: one is Cre excision efficiency, because there is only one set of loxP sites for excision in compound heterozygotes in contrast to two sets of loxP sites in flox/flox mice. The other is that a germline mutation may predispose the kidney tubular epithelial cells to form a cyst. The analyses of semiquantitative PCR and quantitative real-time PCR on genomic DNA showed that a similar efficiency of Pkd1 deletion for both flox/+ and flox/flox genotypes (Figure 5A, Table 2). We also generated Mx1Cre+ Pkd1null/flox mice carrying the DsRed reporter gene (Mx1Cre+ DsRed+ Pkd1null/flox mice). In these mice, recombination must occur at the loxP sites flanking the stop codon preceding DsRed loci. The periodate-lysine-paraformaldehyde (PLP)-fixed, OCT-embedded tissues were then labeled with DBA, LTL, or anti-THP antibody. Interestingly, we observed DsRed signals in cyst-lining epithelial cells and in noncystic tubules (Figure 5, D through F). Most DsRed-expressing normal-looking tubules were stained with THP (Figure 5F). Occasionally they were stained with DBA but never with LTL (data not shown).
Striking Increase in Proliferating Cell Nuclear Antigen Expression in Normal-Looking Tubules Adjacent to Cysts as Well as Cyst-Lining Epithelial Cells from Pkd1- Inactivated Mice

Because DsRed signals were detected in a number of noncystic tubules adjacent to the cysts, we wondered whether these tubules had normal cell cycles. We performed immunohistochemical analysis using an antibody against proliferating cell nuclear antigen (PCNA). We used paraffin-embedded tissue sections instead of cryosections to mask the signal of DsRed so that red fluorescence could be used for double staining. Double labeling of anti-PCNA antibody with DBA, THP, or LTL showed that PCNA-positive cells were seldom seen in any tubules in normal 12-wk-old kidneys (Figure 6, A through C). Of interest, numerous PCNA-positive cells were detected around the cysts as well as in cyst-lining epithelial cells of Mx1Cre+/H11001DsRed+/H11001Pkd1null/flox kidney (Figure 6). Large numbers of PCNA-positive cells around cysts were found in either DBA- or THP-positive normal-looking tubules or dilated tubules (Figure 6, D, E, G, and H). Surprisingly, there were some PCNA-positive cells around cysts in LTL-positive tubules, in which only one allele of Pkd1 is inactivated (Figure 6, F and I, arrows). Western analyses confirmed an increase in cellular PCNA levels in these compound heterozygous kidneys (data not shown).

Modest Increase in PCNA Expression in Normal-Looking Tubules Distant from the Cysts

To determine whether excess PCNA expression is an early event in cystogenesis, we looked at normal regions in much less severe cystic kidney tissues. Paraffin kidney sections from Mx1Cre+/H11001Pkd1null/flox, Mx1Cre+/H11001Pkd1flox/flox, and control mice killed 6 wk after pI:pC injection at 5 wk of age were stained with anti-PCNA antibody. There was a statistically significant increase in the number of PCNA-positive cells in noncystic tubules distant from cysts in Mx1Cre+/H11001Pkd1null/flox (6.37 ± 1.00 [null/flox] versus 3.30 ± 1.00 [control] cells/field; n ≥ 15; data are means ± SD with three mice with each genotype; P < 0.02) but little increase in those in Mx1Cre+/Pkd1flox/flox kidney, compared with those in control kidney. All mice in A and B were littermates administered injections of pI:pC at 5 wk of age, and their kidneys were harvested 6 wk later. (C) The effect of somatic inactivation of Pkd1 on PCNA expression was analyzed by Western blotting of kidney lysates. Mice of indicated genotypes were administered injections of pI:pC for 5 consecutive days at 1 or 5 wk of age. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. (D and E) Pkd1 haploinsufficiency on cell proliferation at 1 and 5 wk of age was analyzed by Western blotting (D) and immunostaining (E, a through d). Quantification is shown in e and f. There was a significant increase in PCNA-positive cells in 5-wk-old but not 1-wk-old Pkd1+/− kidneys, compared with those in Pkd1+/+ (18.6 ± 5.2 versus 10.1 ± 2.0; n ≥ 25; data are means ± SD with three mice in each genotype; P < 0.02).
subtle increase in PCNA expression was seen in Mx1Cre+/H11001/Pkd1flox/flox homozygous mice when compared with controls (Figure 7, B and C). These data suggest an effect of germline Pkd1 haploinsufficiency. This subtle increase in PCNA expression in noncystic tubules adjacent to cysts, the disease progression is slow, and tubules retained normal-looking morphology for months. We wondered whether these PCNA-positive cells are

PCNA expression (Figure 7D) and numbers of PCNA-positive cells (Figure E, a, c, and f) were similar between Pkd1null/+ and wild-type littermate mice at 1 wk of age, we detected a statistically significant increase in 5-wk-old Pkd1null heterozygous mouse kidneys (P < 0.03; Figure 7, D and E, b, d, and f). As expected, we observed a striking drop in PCNA expression in 5-wk-old compared with 1-wk-old kidneys (Figure 7, D and E).

Abnormal Expression of Cell-Cycle Markers in Noncystic Tubules Adjacent to the Cyst
Although there was a marked increase in PCNA expression in noncystic tubules around the cysts, the disease progression is slow, and tubules retained normal-looking morphology for months. We wondered whether these PCNA-positive cells are

Figure 8. PCNA and Ki-67 double labeling in normal-looking tubules adjacent to cysts. (A through F) Control Pkd1flox/+ littermate (A) and Mx1Cre+/DsRed+/Pkd1null/flox (B through F) mice were administered injections of pl:pC at 1 wk of age, and the kidneys were harvested 11 wk later. Four-micron paraffin-embedded sections were co-stained with PCNA (red) and Ki-67 (green).

Co-localization of PCNA and Ki-67 was usually seen in normal control kidneys (A, arrow). A dramatic increase in Ki-67 and PCNA expression was seen in Mx1Cre+/DsRed+/Pkd1null/flox kidneys (B through D) compared with controls. Cells labeled only with PCNA were frequently seen in cysts (B and C, arrowhead). (E and F) An increase in PCNA (green) or Ki-67 (green) was observed in dilated or normal-looking tubules but not in infiltration cells, as indicated with either CD45 or F4/80 in red.

Figure 9. An increase in apoptosis in normal-looking tubules adjacent to cysts. TUNEL assay was carried out on extremely cystic kidney sections (Mx1Cre+/DsRed+/Pkd1null/flox) labeled with DBA (red). (A through E) The TUNEL-positive (green) cells were detected in normal-looking tubules adjacent to the cyst (A, D, and E, arrow). Cyst-lining epithelial cells (A, B, and D, arrowhead) and detached cells from basement membrane in cysts (A through C, arrowhead) were also occasionally positive for TUNEL. Dashed line shows the outline of tubules. *Cyst lumen. (F) The number of PCNA-positive cells and TUNEL-positive cells were counted and compared (26.5 ± 5.6 versus 2.6 ± 1.2; data are means ± SD with sections from three mice). A total of 15 randomly selected areas from three mice (two sections per mouse) were counted for each marker.
actively proliferating or arrested in the cell cycle; therefore, we double-labeled PCNA with another cell proliferation marker, Ki-67, in normal control kidneys, focal cystic kidneys, and extremely cystic kidneys. We found that most PCNA-positive cells co-localized with Ki-67 in normal control kidneys (Figure 8A) and in noncystic tubules distant from the renal cysts in focal cystic kidneys (data not shown). To our surprise, we saw many cyst-lining epithelial cells that were brightly labeled by PCNA but not by Ki-67 (Figure 8, B through D, arrowhead). Cells around the cysts showed relatively similar expression pattern between PCNA and Ki-67, although there were also fewer Ki-67-positive cells (Figure 8, B through D, arrow). The majority of PCNA- or Ki-67-positive cells were not stained with the leukocyte marker CD45 or the macrophage marker F4/80 (Figure 8, E and F), excluding them as infiltrating cells.

**Increase in Apoptosis in Normal-Looking Tubules Adjacent to Cysts as Well as Cyst-Lining Epithelial Cells from Pkd1-Inactivated Mice**

To investigate whether there is an increase in apoptosis in normal-looking tubules adjacent to cysts, we performed a terminal uridine deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay to detect DNA fragmentation resulting from apoptotic signaling cascades. We found TUNEL-positive cells with nuclear condensation and/or fragmentation in both normal-looking tubules adjacent to the cyst (Figure 9, A, D, and E, arrows) as well as cyst-lining epithelial cells (Figure 9, A and D). Many cyst luminal cells presumably detached from the cyst wall are also TUNEL positive (Figure 9, A through C, arrowheads). Although the number of TUNEL-positive cells is only approximately one tenth of PCNA-positive cells (Figure 9F), the number of cells undergoing apoptosis could be higher than what we detected by TUNEL assay, because apoptotic cells are rapidly phagocytosed by their neighbors. The increase in apoptosis in normal-looking tubules may compromise, to some degree, the expansion of proliferating Pkd1-deficient cells and contribute to the normal morphology of Pkd1-deficient kidneys.

**DISCUSSION**

We successfully generated a new inducible Pkd1 knockout mouse model using the Mx1Cre transgene, whose expression is inducible by pI:pC. This novel mouse model recapitulates many facets of human PKD.

In human ADPKD, cysts form focally in only approximately 5% of the nephrons. A two-hit hypothesis has been proposed to explain the focal nature of cyst development: A germline mutation (first hit) in one of the two copies (“alleles”) of, for example, PKD1 in a given cell is not sufficient to alter the phenotype of that cell and initiate cyst formation, because the second allele is functioning normally. Cystogenesis occurs when a somatic mutation knocks out the “normal” allele (second hit), neither allele is functional, and no polycystin function remains. A cyst then forms through proliferation of the cell that has received two hits. Indeed, somatic PKD1 or PKD2 mutations found in kidney11 and liver cysts12 in human tissues with ADPKD support this hypothesis. Consistent with the two-hit hypothesis, when we induced the somatic mutation in the Mx1Cre+/Pkd1null/+/H11001 mice in developing kidney (1 wk old), we saw severe polycystic kidneys (Figure 3, A through C) 1 to 2 mo later. In fact, macroscopic cysts were seen as early as 1 wk after induction (data not shown). Inactivation of both Pkd1 alleles in 1-wk-old developing kidney also resulted in rapid extensive cyst formation (Figure 2B). By contrast, Pkd1 inactivation in 5-wk-old mature kidneys results in focal cyst formation 1 to 2 mo later with regional disease progression (Figures 1, B through D, and 3, G through I). This focal/regional nature of cystic development cannot be explained by the two-hit hypothesis, because all cells carry an inherited mutation, and almost all distal nephron segments have received a somatic hit (Figure 4); therefore, we propose that an additional trigger (third hit) in a cell is necessary for rapid cyst formation, in addition to the two hits in Pkd1.

It is noteworthy that PC1 expression level in mouse kidney parallels kidney development; it is high during late embryogenesis and remains high until 2 wk after birth, when kidney maturation is complete.13 Only a low level of PC1 is maintained in adult kidneys.13 This developmentally regulated expression pattern led us to propose that PC1 plays a role in tubule elongation and maturation during development and in maintenance of the mature state during adult life.13 Studies showed that PC1 and PC2 modulate cell-cycle progression.14,15 A major feature in 1-wk-old compared with 5-wk-old kidneys is postnatal renal development with rapid tubule growth and maturation. Our PCNA study showed that renal cell proliferation in the 5-wk-old mouse was approximately 50-fold lower than in the 1-wk-old mouse (Figure 7, C and D). To understand the difference in disease phenotype (widespread versus focal and regional), we went on to investigate the pattern of cell proliferation in Pkd1-inactivated kidneys and to determine whether inactivation of Pkd1 is sufficient to trigger cell proliferation.

We found numerous PCNA-positive cells among cyst-lining epithelial cells in Mx1Cre1/DsRed2/Pkd1null/+/H11001 adult kidneys, whereas tubules in littermate control mice had only a few PCNA-positive cells, as expected (Figure 8). Increased PCNA staining has been widely used as a marker for cell proliferation and has been reported in end-stage human ADPKD kidneys.16 Our adult-onset PKD model also allows us to determine the effects of Pkd1 inactivation in pre- to early cystic tissues. In striking contrast to what is seen in end-stage human ADPKD kidneys, neither tubular epithelia nor interstitial cells in normal areas farther away from cysts exhibited discernible increased cell proliferation, as indicated by PCNA staining in Mx1Cre1/Pkd1null/+/H11001 (Figure 7A) 6 wk after Pkd1 inactivation at 5 wk of age. In Mx1Cre1/Pkd1null/+/H11001 mouse kidneys of the same age, we observed a two-fold increase in PCNA-positive cells compared with the kidneys of control littermates (Figure 7B). This increase likely results from germine Pkd1 haploin-
sufficiency similar to that in Pkd2 heterozygous mice. These data suggest that somatic inactivation of Pkd1 in mice with a germline mutation (two hit) is still not sufficient to initiate enough cell proliferation for cyst formation. Western blotting of PCNA in kidneys 1 wk after Pkd1 inactivation at either 1 or 5 wk of age further supports this notion (Figure 7C).

Given the developmentally regulated expression of PC1, it is tempting to speculate a need for PC1 when the renal developmental program is reactivated during kidney regeneration in response to renal injury or other genetic or nongenetic insults. The resulting phenotype from loss of PC1 would depend on when the kidney receives such a hit/stimulus. The number of hits and their effects would accrue with time. This may in part explain why somatic inactivation of Pkd1 in adult mice causes only a mild cystic phenotype; these experimental mice, unlike humans, are kept in a nearly “sterile” environment with standard food and are not exposed to any toxins.

It is interesting that we also found a number of PCNA-positive epithelial cells in normal-looking tubules and interstitial cells surrounding the cysts (Figures 6 and 7Ac). Combined with our observation of a regional effect of cyst formation (Figure 3, G through I) and that cystic fluids in ADPKD are rich in growth factors and other mitogenic factors (presumably produced by cyst-lining epithelial cells), it is tempting to suggest the presence of a chemical gradient in cells surrounding cysts that would have a paracrine effect and would facilitate cyst formation in adjacent normal-looking Pkd1-deficient tubules. It is also possible that the physical expansion of a cyst puts pressure and causes local ischemia on cells in the surrounding tissues, which respond with an increase in cell proliferation. Because PCNA-positive cells are also seen among tubular epithelial cells with a germline mutation positive for LTL, a proximal tubule marker (Figure 6I), yet no cysts were labeled with LTL in kidneys of compound heterozygous mice with a germline mutation positive for LTL, a proximal tubular epithelial cell marker (Figure 6I), yet no cysts were labeled with LTL in compound heterozygous mice. These results in mice carrying a DsRed reporter transgene as well as at the Pkd1 locus (Figure 5); therefore, we believe that germline mutation facilitates cyst formation, probably by exposing the cells to a lower dosage of PC1 during development and programming the cells to be more susceptible to other injuries. Indeed, germline Pkd1 heterozygotes (Pkd1 \textsuperscript{del34/+} or Pkd1 \textsuperscript{null/+}) also develop renal cysts but at a much later stage when compared with compound heterozygotes. These data support the hypothesis that two hits are required for cyst formation in PKD.

Recent studies have suggested significant roles for polycystins in primary cilia and for primary cilia in the pathogenesis of polycystic kidney disease. PC1-deficient cells cannot carry out fluid flow shear stress–induced Ca\textsuperscript{2+} signaling through their primary cilia.\textsuperscript{25} Consistent with the results described here, inactivation of several genes required for ciliogenesis, such as Tg737 and Kif3A, in the developing mouse kidney leads to the rapid development of cystic disease,\textsuperscript{26} whereas inducible knockout of Tg737 in adulthood results in slow and late onset of cystic phenotype. These data, together with findings from this study, suggest that the disruption of neither the polycystin protein complex nor the primary cilia is sufficient to trigger cell proliferation leading to rapid cyst formation. A third hit is required for cystogenesis in mature kidneys.

Renal cysts in ADPKD may arise from all segments of the nephron and collecting ducts; however, immunohistochemistry of adult ADPKD kidney has revealed that most cysts originate in collecting ducts.\textsuperscript{24,27,28} Mx1Cre-mediated genetic recombination occurs in distal nephron including thick ascending limbs and collecting tubules/ducts, resulting in the inactivation of Pkd1 in these tubular segments. Consistent with studies of human ADPKD,\textsuperscript{29,30} we observed that most cysts are of collecting tubule/duct origin and are larger than those derived from other tubular segments such as thick ascending limbs, further supporting the contention that our inducible Pkd1 knockout mouse model is well suited for the study of ADPKD.

After the submission of this work for publication, two tamoxifen-inducible (Pkd1\textsuperscript{del12–11} and Pkd1\textsuperscript{del2–4}) mouse models that inactivate Pkd1 in either distal nephron segments\textsuperscript{31} or all nephron segments\textsuperscript{32} were reported. A consensus finding among all models is that postdevelopmental inactivation of Pkd1 results in slow onset of cystic disease, whereas inactivation in developing kidney causes rapid onset of the disease; however, the slow and focal cyst development and regional progression of cystic disease in mature kidneys (which prompted us to propose the third-hit hypothesis described...
here) were not reported elsewhere. The stage of phenotyping could be a contributing factor. We detected cysts as early as 6 wk after inactivation, in contrast to the absence of cyst in Pkd1<sup>del2–4</sup> mice 3 mo after by tamoxifen-induced inactivation. Numerous small cysts are seen in TmCrePkd1<sup>del2–4</sup> mice, probably as a result of Pkd1 inactivation in proximal tubules, because proximal tubule cysts are often smaller and tend to reduce in size with time. The effects of germline mutation in compound heterozygotes described here were not reported by Pontiek et al. Consistent with this study, Lantinga et al. reported an increase in cell proliferation associated with haplinsufficiency; however, phenotypic studies were limited in that report. This study also documents for the first time that there is a gradient distribution of cell proliferation markers around the cysts, which correlates with regional progression of disease and suggests that increased cell proliferation may be a nonautonomous cellular process, and a paracrine mechanism may be involved in regional disease progression.

**CONCISE METHODS**

**Generation of Inducible Pkd1 Knockout Mice**

The Pkd1<sup>lox</sup> allele has been described elsewhere. Briefly, the selection marker neo cassette flanked by twoloxP sites was inserted into intron 1 of the Pkd1 locus, and the thirdloxP site was inserted into intron 6 of the Pkd1 locus. To obtain mice with a Pkd1 allele whose exons 2 through 6 are flanked withloxP sites (Pkd1<sup>lox/lox</sup>+ mice) and does not have a neo cassette, and mice with a Pkd1 allele with neither neo cassette nor exons 2 through 6 (Pkd1<sup>lox/lox−</sup>+ mice), we crossed mice with germline transmission of the trilox construct with germline Cre-expressing mice. Heterozygous Pkd1<sup>lox/lox−</sup>+ mice, which do not carry germline Cre transgenes, were crossed with a transgenic mouse line that expresses Cre recombinase under the control of an IFN-inducible Mxi1 promoter (Mxi1Cre mice) to generate Pkd1<sup>lox/lox−</sup>+ mice carrying the Mxi1Cre transgene (Mxi1Cre<sup>+</sup>Pkd1<sup>lox/lox−</sup>+ mice). Then Mxi1Cre<sup>+</sup>Pkd1<sup>lox/lox−</sup>+ mice were bred with either Pkd1<sup>lox/lox−</sup>+ or Pkd1<sup>lox/−</sup>+ to generate Mxi1Cre<sup>+</sup>Pkd1<sup>lox/lox−</sup> mice (Mxi1Cre is hemizygous) or Mxi1Cre<sup>+</sup>Pkd1<sup>lox/lox−</sup> mice, respectively. The genotypes of Mxi1Cre<sup>+</sup>Pkd1<sup>lox/lox−</sup> were used as controls.

**Induction of Cre Expression**

Mice were administered an intraperitoneal injection of 62.5 or 250 μg of IFN inducer pI:pC (Sigma, St. Louis, MO) for 5 consecutive days at 1 or 5 wk of age, respectively.

**Mx1Cre Mice CarryingloxP-Flanked DsRed Reporter Gene**

Transgenic mice carrying DsRed.MST (DsRed mice) were obtained from the Jackson Laboratory (Bar Harbor, ME) and used as a reporter strain. Transcription of DsRed.MST transgene is suppressed by placement of a “stop” sequence and polyadenylation sites flanked byloxP sequences. The chicken β actin promoter coupled with the cytomegalovirus is used for this transgene. DsRed mice were crossed with Mxi1Cre mice to generate double-transgenic mice with Mxi1Cre and DsRed. After injection with pI:pC to induce Cre recombinase, genetic recombination occurs at the twoloxP sites, excising the intervening stop sequence and polyadenylation sites, thereby allowing red fluorescence protein variant DsRed to be expressed. DsRed fluorescence on cryosection of kidney was detected using a Nikon (Tokyo, Japan) microscope.

**Tissue Preparation, Histology, and Immunohistochemistry**

After mice were anesthetized with pentobarbital, the thoracic cavity was opened and the right atrium was cut. PBS was perfused through the left ventricle. PLP was then perfused to accomplish the perfusion fixation. Immediately, kidney and liver were harvested, weighed, and immersed in PLP solution. After replacement of PLP with 30% sucrose, tissues were then embedded in OCT compound (Sakura, Kobe, Japan) and stored at −80°C. Seven-micron cryosections were freshly cut and dried for 30 to 60 min at room temperature. After rehydration with PBS for 5 min, sections were postfixed with 2% paraformaldehyde for 10 min. After washing with PBS, sections were blocked with either 10% goat serum or donkey serum for 30 min at room temperature. Primary antibody was then applied to the sections and incubated overnight at 4°C. The anti-THP antibody was used at dilution 1:100 (MP Biomedical, Solon, OH). Lectin DBA and LTl were used at dilution 1:500 (Vector Laboratories, Burlingame, CA). Paraffin-embedded sections were used to detect PCNA. Briefly, paraffin sections (4 μm) were dewaxed, rehydrated through graded alcohols, and treated with boiled 10 mM citrate (pH 6.0; Vector) for 30 min. The staining dish was exposed to room temperature, and the slides were allowed to cool for 1 to 2 h. Sections were then incubated with 10% goat serum for 30 min and then incubated with anti-PCNA antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Ki-67 antibody (1:200; NeoMarkers, Fremont, CA), anti-CD45 antibody (1:200; eBioscience, San Diego, CA), and/or anti-F4/80 antibody (1:200; Caltag Laboratory, Burlingame, CA) overnight at 4°C or enzyme solution (Roche, Basel, Switzerland) for TUNEL assay for 1 h. After washing with PBS, sections were incubated with secondary antibody for 1 h at room temperature. After washing with PBS, sections were mounted with Prolong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA).

**PCR Detection for Recombination Events ofloxP Sites Flanking Pkd1 Exons 2 through 6**

Quantitative real-time PCR and semi-quantitative PCR were performed on genomic DNA from kidneys of Mxi1Cre<sup>+</sup>Pkd1<sup>lox/lox−</sup>+ Mxi1Cre<sup>+</sup>Pkd1<sup>lox/lox−</sup>+ and their littermate control mice that were injected with pI:pC at 1 and 5 wk of age. Cre recombinase-mediated exon 2 through 6 deletion events were detected by primers located in introns 1 and 6 of the Pkd1 gene (5′-GCT AGC AGG TTC CAG GGA-3′, 5′-GTC ATT GGG AAG GGA GGT ACT CA-3′). The following Pkd1 primers located in intron 1 were used as a DNA quantity control: 5′-AAG GGG TGT GCC ACC ATG ATG-3′ and 5′-CCC TGA CAT CCA CAT GGT GGC-3′.
Western Blotting
KIDNEYS WERE HOMOGENIZED WITH T-PER (PIERCE, ROCKFORD, IL) CONTAINING PROTEASE INHIBITORS (ROCHE). EQUAL AMOUNTS OF TISSUE LYSATES (100 µg) WERE SEPARATED ON 12% SDS-PAGE. THE PROTEINS WERE TRANSFERRED ONTO NITROCELLULOSE MEMBRANES (AMERSHAM, PISCATAWAY, NJ). THE MEMBRANES WERE THEN BLOCKED WITH 5% NONFOOD DRY MILK IN PBS FOR 30 MIN AND INCUBATED OVERNIGHT AT 4°C WITH ANTIBODY TO PCNA (1:200; SANTA CRUZ). AFTER THREE WASHINGS WITH PBS, THE MEMBRANES WERE INCUBATED WITH PEROXIDASE-CONJUGATED GOAT ANTI-MOUSE IgG (1:5000; AMERSHAM) FOR 1 H. FINALLY, THE BLOTS WERE DEVELOPED BY THE ENHANCED CHEMILUMINESCENCE METHOD. MEMBRANES WERE THEN STRIPPED OFF ANTIBODIES USING RESTORE WESTERN BLOT STRIPPING BUFFER (PIERCE) FOR 30 MIN AT 37°C. MEMBRANES WERE REPROBED WITH GLYCEROLALDEHYDE-3-PHOSPHATE DEHYDROGENASE (1:200; SANTA CRUZ) AND secondary antibodies (1:5000).

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DISCLOSURES
None.

REFERENCES


