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Pkd1 Inactivation Induced in Adulthood Produces Focal Cystic Disease

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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*$^{-/-}$ or *Pkd2*$^{-/-}$ 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

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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*$^{del34/del34}$ and *Pkd1*$^{null/null}$) 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*$^{L/L}$ 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*^{nl} 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*^{L3} mouse with a hypomorphic allele of *Pkd1* revealed that the *Pkd1*$^{L3/L3}$ 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*$^{cond/cond}$ *MMTVCre*) 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.

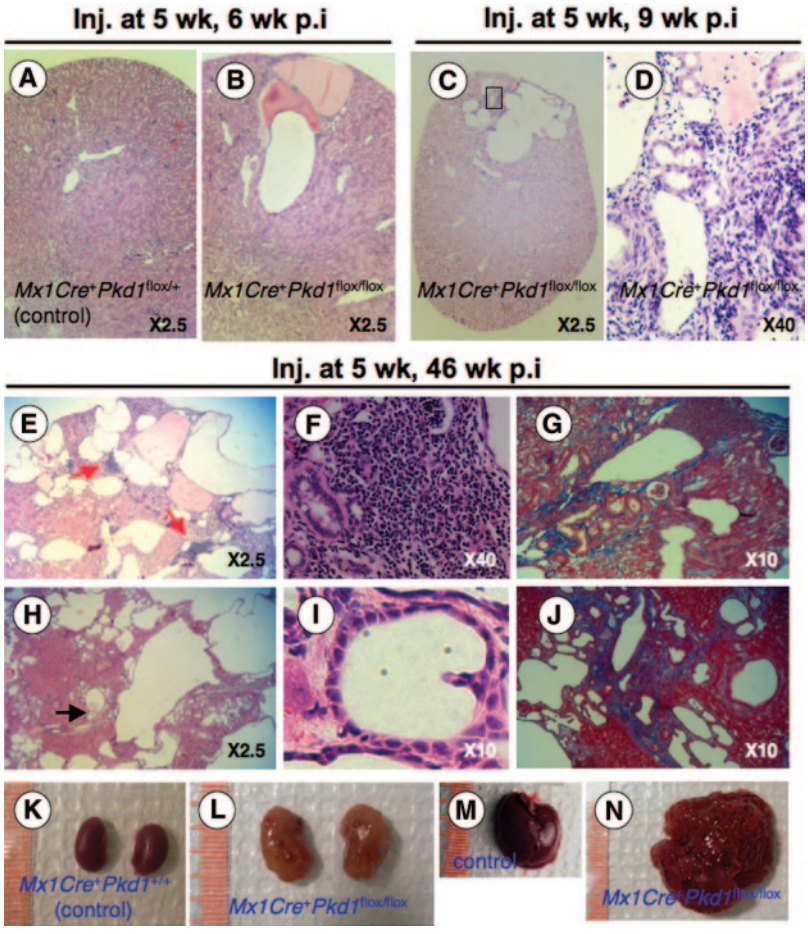

Figure 1. Induction of *Pkd1* mutation in adult kidney causes focal cyst formation. (A) *Mx1Cre*$^{+}$*Pkd1*$^{flox/flox}$ and control littermate mice were administered injects of pI:pC at 5 wk of age. Control (*Mx1Cre*$^{+}$*Pkd1*$^{flox/+}$) mouse showed normal kidney morphology. (B through D) *Mx1Cre*$^{+}$*Pkd1*$^{flox/flox}$ 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*$^{+}$*Pkd1*$^{+/+}$ mice showed normal kidney (K) and liver (M). (G and J) Fibrosis in *Mx1Cre*$^{+}$*Pkd1*$^{flox/flox}$ 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.

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 *loxP* sites.[10] This allows the creation of a null allele at a specific time point by crossing *Pkd1*$^{flox/flox}$ mice with a transgenic mouse line that expresses *Cre* recombinase under the control of an interferon (IFN)-inducible *Mx1* promoter (*Mx1Cre* mouse). Because ADPKD is an adult disease, we decided to inactivate *Pkd1* in young adult mice. *Mx1Cre*$^{+}$*Pkd1*$^{flox/flox}$

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*$^{+}$*Pkd1*$^{flox/+}$ mice 6 wk after injection of pI:pC (Figure 1A) had normal renal histology. Only focal cysts were seen in *Mx1Cre*$^{+}$*Pkd1*$^{flox/flox}$ 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*$^{+}$*Pkd1*$^{flox/flox}$ kidney and liver showed massive enlarged cysts (Figure 1, E through J, L, and N), whereas control kidney and liver from *Mx1Cre*$^{+}$*Pkd1*$^{+/+}$ 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 cyst 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*$^{+}$*Pkd1*$^{flox/flox}$ and control mice at 1 wk of age, when the mouse kidney is still developing. *Mx1Cre*$^{+}$*Pkd1*$^{flox/flox}$ 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*$^{flox/flox}$ mice (Figure 2, A and C), littermate *Mx1Cre*$^{+}$*Pkd1*$^{flox/flox}$ 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.

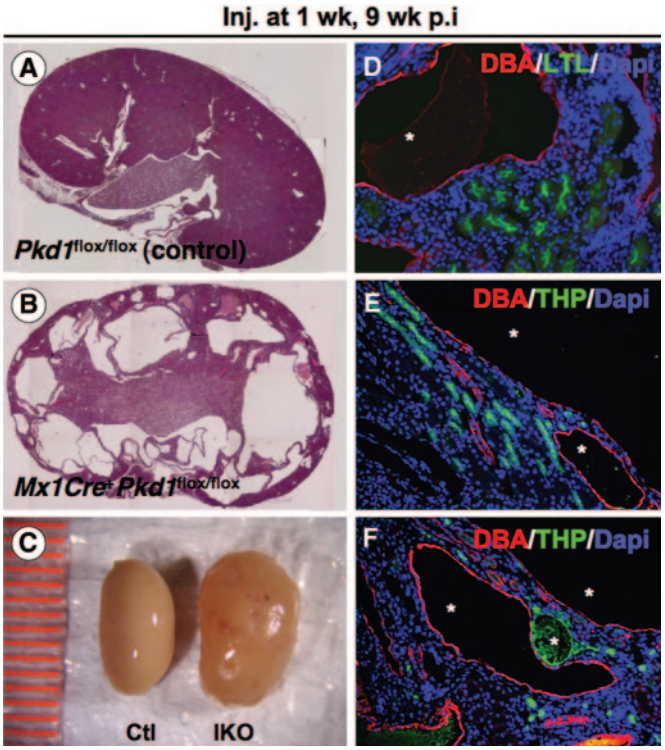

Figure 2. Cyst formation and enlargement are accelerated in mice with *Pkd1* inactivation induced in postnatal developing kidney. *Mx1Cre*$^{+}$*Pkd1*$^{flox/flox}$ and control littermate mice were administered injections of pI:pC at 1 wk of age and dissected 9 wk later. (A through D) Control *Pkd1*$^{flox/flox}$ kidney showed normal histology, whereas *Mx1Cre*$^{+}$*Pkd1*$^{flox/flox}$ 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.

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*$^{null/flox}$ mice that also carry the *Mx1Cre* transgene (*Mx1Cre*$^{+}$*Pkd1*$^{null/flox}$ mice) by injection with pI:pC at 1 wk of age and dissected them 6 or 9 wk later. Extensive cyst formation was seen in these mice (Figure 3, A through C) but not in littermate controls (Figure 3A). These compound heterozygotes had increased kidney weight (403.5 $\pm$ 222.8 [null/flox] *versus* 199.4 $\pm$ 51.7 [flox/flox] mg; $P < 0.006$; Figure 3D) and kidney/body weight ratio (16.3 $\pm$ 8.2 [null/flox] *versus* 7.9 $\pm$ 1.1 [flox/flox] mg/g; $P < 0.003$; Figure 3E) compared with *Mx1Cre*$^{+}$*Pkd1*$^{flox/flox}$ mice. Whereas only 21% of

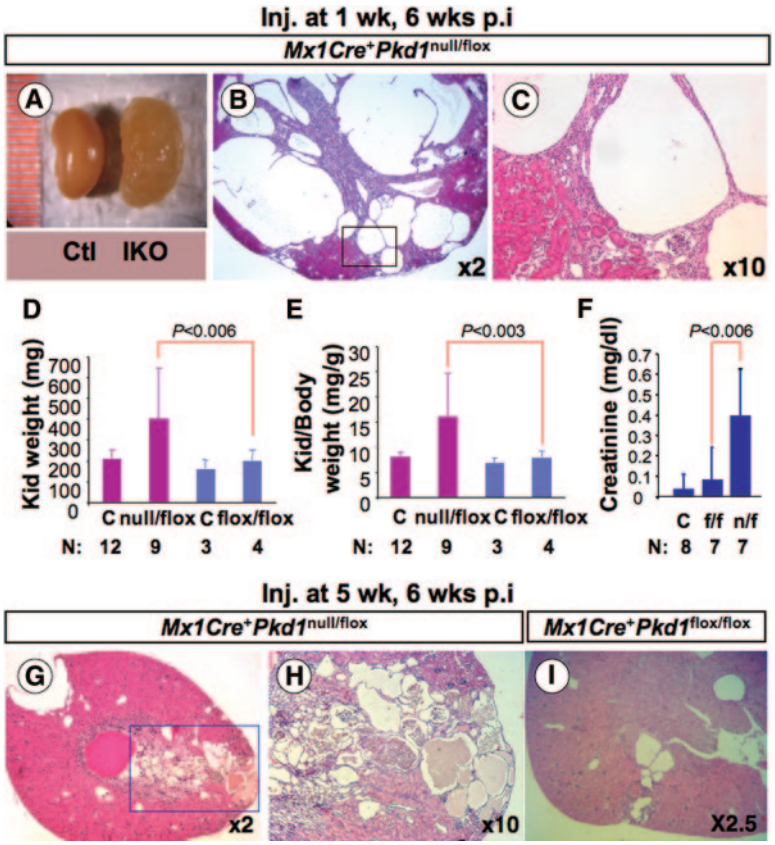

Figure 3. Germline *Pkd1* mutation facilitates cyst formation. (A through I) *Mx1Cre*$^{+}$*Pkd1*$^{null/flox}$ and littermate control mice were administered injects of h pI:pC at 1 wk (A through F) or 5 wk (G through I) of age and dissected 6 wk later (A through E) or 9 wk later (F). Extensive cyst formation was seen in *Mx1Cre*$^{+}$*Pkd1*$^{null/flox}$ kidney. (C) Normal-looking cortical tubules were seen around cysts. (D and E) *Mx1Cre*$^{+}$*Pkd1*$^{null/flox}$ mice have significantly higher kidney weight ratio (403.5 ± 222.8 [null/flox] *versus* 199.4 ± 51.7 [flox/flox] mg; $P < 0.006$; D) and kidney/body weight ratio (16.3 ± 8.2 [null/flox] *versus* 7.9 ± 1.1 [flox/flox] mg/g body wt; $P < 0.003$; E) compared with *Mx1Cre*$^{+}$*Pkd1*$^{flox/flox}$ and control mice. (F) *Mx1Cre*$^{+}$*Pkd1*$^{null/flox}$ (n/f) mice have also significantly higher serum creatinine levels compared with *Mx1Cre*$^{+}$*Pkd1*$^{flox/flox}$ (f/f) and control mice. c, control. (G through I) Injection at 5 wk of age produced a much milder phenotype than injection at 1 wk of age (G and H), but the compound heterozygous phenotype was more advanced than that produced in *Mx1Cre*$^{+}$*Pkd1*$^{flox/flox}$ mice (I). The genotype of control shown here is *Pkd1*$^{flox/+}$ (A, left).

Mx1Cre^{+}*Pkd1*$^{flox/flox}$ mice (three of 14) were found to have widespread cystic kidney 9 wk after injection, this phenotype was detected in 58% of *Mx1Cre*$^{+}$*Pkd1*$^{null/flox}$ 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*$^{+}$*Pkd1*$^{flox/flox}$ (0.4 ± 0.2 [null/flox] *versus* 0.086 ± 0.157 [flox/flox] mg/dl; $P < 0.006$) and their littermate control mice (0.4 ± 0.2 [null/flox] *versus* 0.038 ± 0.074 [control] mg/dl; $P < 0.0005$; Figure 3F).

It is noteworthy that in compound heterozygous *Pkd1*$^{null/flox}$ 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,

Table 1. Number of *Pkd1*-inactivated and control mice administered injections of pI:pC[a]

Time after Injection (wk)	Injection Age (Dosage)							
	5 Wk (250 μg)				1 Wk (62.5 μg)			
	Mx1Cre^{+}*Pkd1*$^{flox/flox}$		*Mx1Cre*$^{+}$*Pkd1*$^{null/flox}$		*Mx1Cre*$^{+}$*Pkd1*$^{flox/flox}$		*Mx1Cre*$^{+}$*Pkd1*$^{null/flox}$	
	No. of IKO	No. of Control	No. of IKO	No. of Control	No. of IKO	No. of Control	No. of IKO	No. of Control
6	4	1[c]	4	1[d]	6	5[e]	12 (7[b])	4[c], 4[d], 2[f]
7	1							
9	6		1	1[c], 1[d], 3[f]	14 (3[b])	15[e]	4 (3[b])	3[c], 4[d], 3[f]
11			2	2[f]			3[b]	2[d], 1[g]
12 to 29	3	1	4	1, 2[d], 1[f]	7	7[e]	6[b]	3[d], 2[e], 1[f]
42	1	1[h]						
46	1[b]	1[h]						
Total	15	3	8	9	27	27	25	29

[a]IKO, inducible knockout.
[b]Entire cystic kidney.
[c]*Mx1Cre*$^{+}$*Pkd1*$^{flox/+}$.
[d]*Pkd1*$^{flox/+}$.
[e]*Pkd1*$^{flox/flox}$.
[f]*Pkd1*$^{null/flox}$.
[g]*Pkd1*$^{null/+}$.
[h]*Mx1Cre*$^{+}$*Pkd1*$^{+/+}$.

consistent with data obtained with *Mx1Cre*$^{+}$*Pkd1*$^{flox/flox}$; however, renal histology (Figure 3, G and H) reveals that these compound heterozygotes develop more striking cystic disease than *Mx1Cre*$^{+}$*Pkd1*$^{flox/flox}$ mice (Figure 3I). These data suggest that a germline mutation has an effect on cyst development.

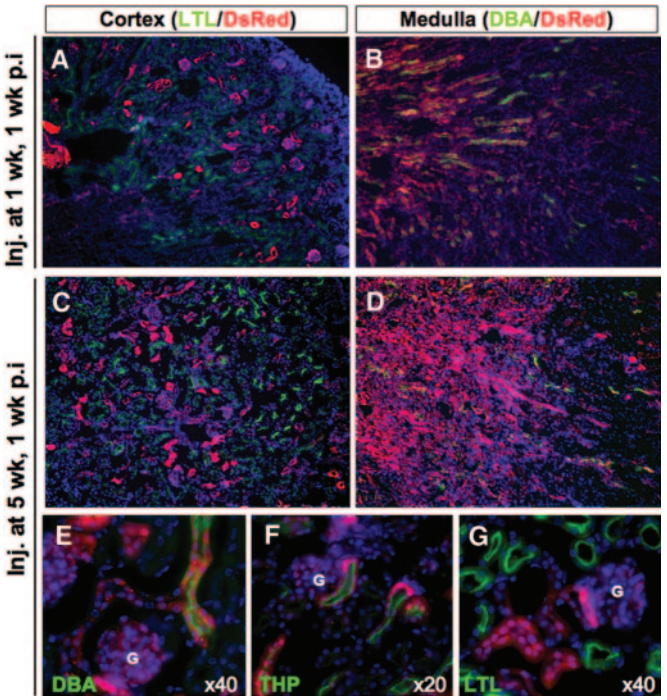

Figure 4. Cre-mediated recombination in developing and developed kidneys. *Mx1Cre*$^{+}$*DsRed*$^{+}$ mouse kidneys were injected with pI:pC at 1 and 5 wk of age and harvested 1 wk later. In this system, the expression of *Cre*-recombinase allows the DsRed (red fluorescence) protein to be expressed. DsRed signals were directly visualized in kidney cryosections. (A and C) Sporadic DsRed signals were seen in the cortex but with no overlap with LTL. (B and D) Widespread DsRed signals were seen throughout medulla in structures also labeled with DBA in both 1- and 5-wk-injected mice. (E through G) Strong Ds-Red signals in DBA-positive (E) and THP-positive (F) but not LTL-positive (G) tubules are also shown at a higher magnification. G, glomerulus.

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 *Mx1Cre*-mediated genetic recombination, we crossed *Mx1Cre* mice with a reporter strain (*DsRed* mice) in which *DsRed* is expressed only after Cre-mediated recombination. *Mx1Cre* mice with *DsRed* reporter transgene (*Mx1Cre*$^{+}$ *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). *Mx1Cre*-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 *Mx1Cre* 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

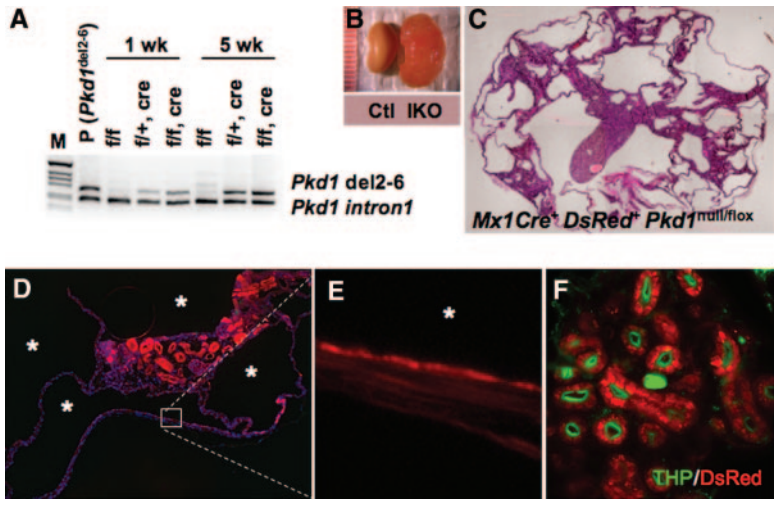

Figure 5. Cyst lining epithelial cells express DsRed reporter gene. (A) Genomic DNA was isolated from control (f/f), *Mx1Cre*$^{+}$*Pkd1*$^{flox/+}$ (f/+, Cre), and *Mx1Cre*$^{+}$*Pkd1*$^{flox/flox}$ (f/f, Cre) kidneys injected with pI:pC at 1 or 5 wk of age. PCR was performed using specific primers located in introns 1 and 6 of *Pkd1* to amplify the deleted fragment in *Pkd1* locus. Primers located in intron 1 of *Pkd1* were used as DNA quantity control. Cre-mediated recombination efficiency in 5-wk-old kidney was even higher, compared with that in 1-wk-old kidney. (B through F) Twelve-week-old *Mx1Cre*$^{+}$*DsRed*$^{+}$*Pkd1*$^{null/flox}$ kidneys with pI:pC injection at 1 wk of age. The representative gross kidneys (B) and a hematoxylin- and eosin-stained cystic section (C) are shown. C was digitally assembled to show the whole kidney. Seven-micron cryosections of cystic kidneys were directly visualized for DsRed signals (D and E) or stained with THP (F). Endogenous DsRed signal (red) was seen in both cyst-lining epithelium and noncystic tubules. *Cyst lumen.

Table 2. *Cre*-mediated recombination efficiency calculated as a ratio of deleted/total *Pkd1* floxed alleles (%)

Parameter	$Pkd1^{del2-6/+}$	1 Wk		5 Wk	
		$Mx1Cre^{+}Pkd1^{f/+}$	$Mx1Cre^{+}Pkd1^{f/f}$	$Mx1Cre^{+}Pkd1^{f/+}$	$Mx1Cre^{+}Pkd1^{f/f}$
Semiquantitative PCR	100	34.6	29.8	63.1	58.0
quantitative real-time PCR	100	34.6	35.6	53.9	48.6

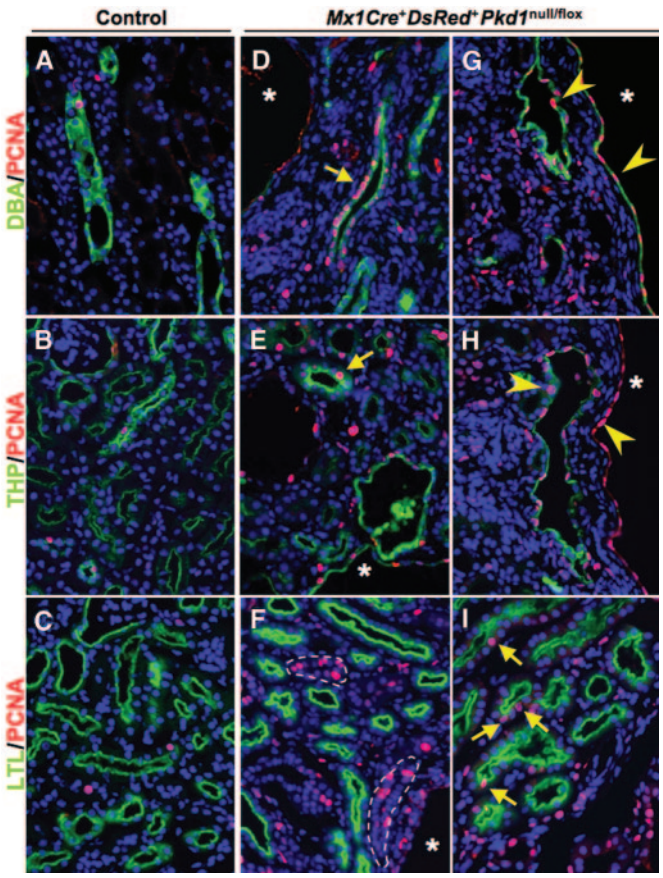

Figure 6. A marked increase in PCNA expression in cysts and normal-looking tubules around cysts. (A through I) Control $Pkd1^{flox/+}$ (A through C) and $Mx1Cre^{+}DsRed^{+}Pkd1^{null/flox}$ (D through I) littermate mice were administered injections of pI:pC at 1 wk of age, and kidneys were harvested 11 wk later. Four-micron paraffin-embedded sections were co-stained with PCNA (red) and DBA (A, D, and G), THP (B, E, and H), or LTL (C, F, and I). A dramatic increase in PCNA expression was observed in normal-looking tubules (arrows) or dilated tubules (arrowheads) adjacent to the cysts and in cyst-lining epithelial cells (arrowheads) from $Mx1Cre^{+}DsRed^{+}Pkd1^{null/flox}$ kidney with extremely cystic phenotype.

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 ($Pkd1^{del2-6}$) 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 $Mx1Cre^{+}Pkd1^{flox/+}$ and $Mx1Cre^{+}Pkd1^{flox/flox}$ 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 $Mx1Cre^{+}Pkd1^{flox/flox}$ 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 $Mx1Cre^{+}Pkd1^{flox/+}$ kidneys, the deletion efficiency calculated as deleted/total *Pkd1* floxed alleles was comparable to that in the flox homozygotes (Table 2). Germline $Pkd1^{del2-6/+}$ 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 $Mx1Cre^{+}DsRed^{+}Pkd1^{null/flox}$ 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 $Mx1Cre^{+}Pkd1^{null/flox}$ mice carrying the *DsRed* reporter gene ($Mx1Cre^{+}DsRed^{+}Pkd1^{null/flox}$ mice). In these mice, recombination must occur at the *loxP* sites flanking the stop codon preceding *DsRed* to remove the stop codon, in addition to a set of *loxP* sites at the *Pkd1* locus to delete exons 2 through 6 required for cyst development, thereby activating DsRed expression and inactivating *Pkd1*. DsRed signals should be detected in cyst-lining epithelial cells. Three-month-old $Mx1Cre^{+}DsRed^{+}Pkd1^{null/flox}$ mice with *Cre* expression induced at 1 wk of age had bright red kidneys, massively enlarged and cystic (Figure 5, B and C), indicating Cre-mediated recombination at both *DsRed* and *Pkd1* 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).

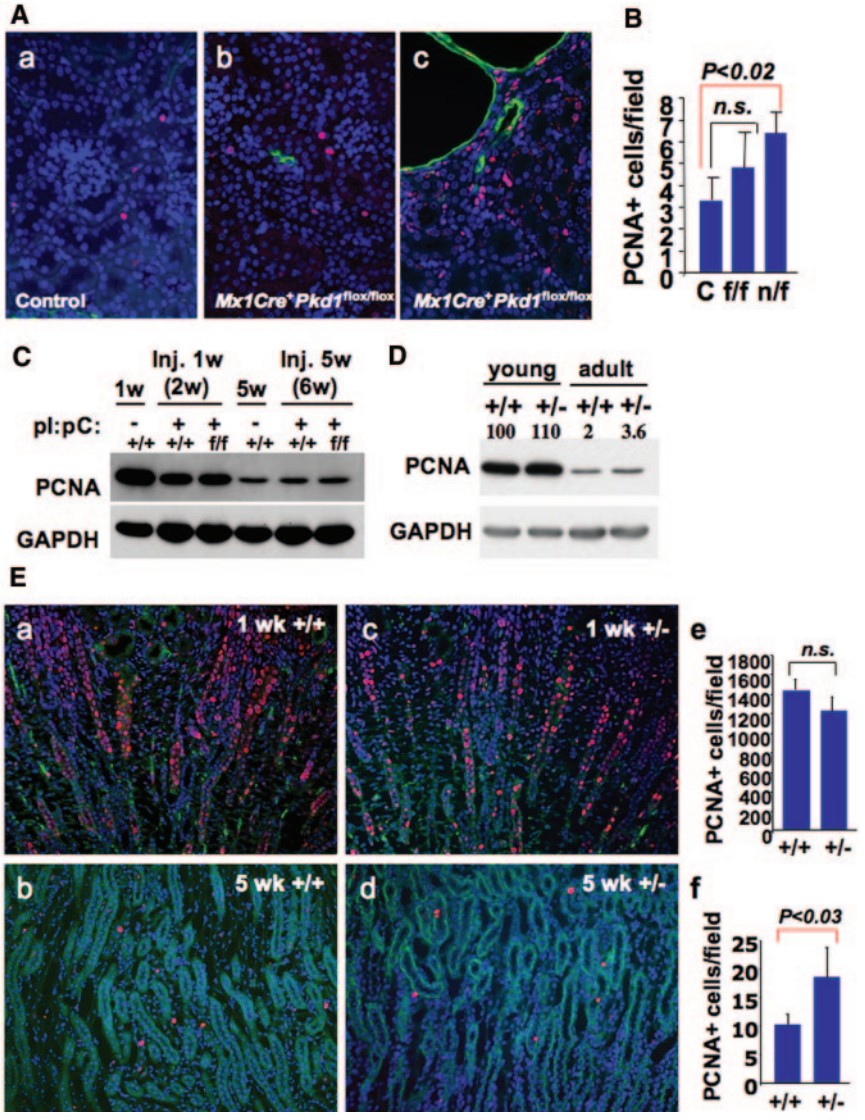

Figure 7. PCNA expression in normal-looking tubules distant from cysts. (A) Control (*Pkd1*$^{flox/flox}$) (a) and *Mx1Cre*$^{+}$*Pkd1*$^{flox/flox}$ (b and c) paraffin-embedded kidney sections were stained with PCNA (red) and DBA (green). (B) In regions farther from the cysts, there was a two-fold increase in PCNA-positive cells in *Mx1Cre*$^{+}$*Pkd1*$^{null/flox}$ (6.37 ± 1.00 [null/flox] *versus* 3.30 ± 1.00 [control] cells/field; $n \geq 15$; data are means ± SD with three mice with each genotype; $P < 0.02$) but little increase in those in *Mx1Cre*$^{+}$*Pkd1*$^{flox/flox}$ 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; $n \geq 25$; data are means ± SD with three mice in each genotype; $P < 0.02$).

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*$^{+}$*DsRed*$^{+}$*Pkd1*$^{null/flox}$ 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*$^{+}$*Pkd1*$^{null/flox}$, *Mx1Cre*$^{+}$*Pkd1*$^{flox/flox}$, 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*$^{+}$*Pkd1*$^{null/flox}$ compound heterozygous mice compared with those in their littermate controls ($P < 0.02$; Figure 7, Aa, Ab, and B); however, only a

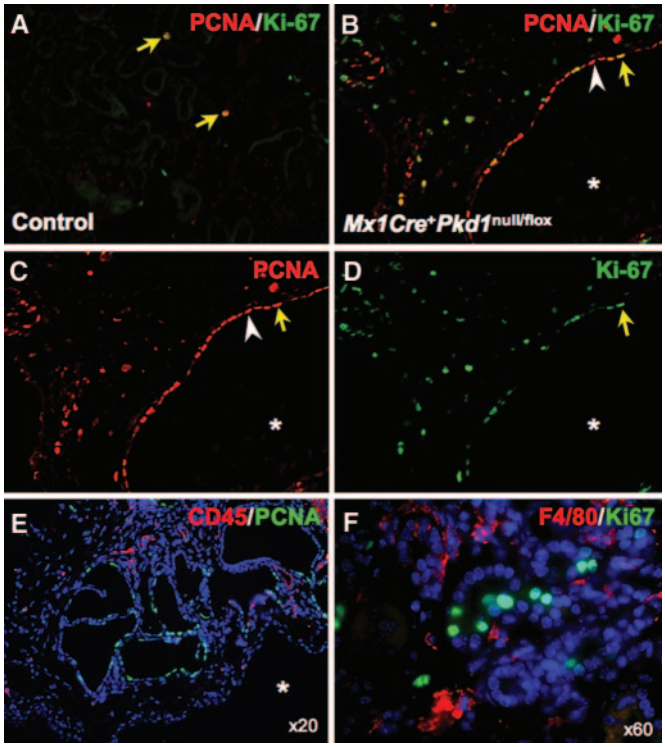

Figure 8. PCNA and Ki-67 double labeling in normal-looking tubules adjacent to cysts. (A through F) Control *Pkd1*$^{flox/+}$ littermate (A) and *Mx1Cre*$^{+}$*DsRed*$^{+}$*Pkd1*$^{null/flox}$ (B through F) mice were administered injections of pI:pC at 1 wk of age, and the kidneys were harvested 11 wk 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*$^{+}$*Pkd1*$^{null/flox}$ 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.

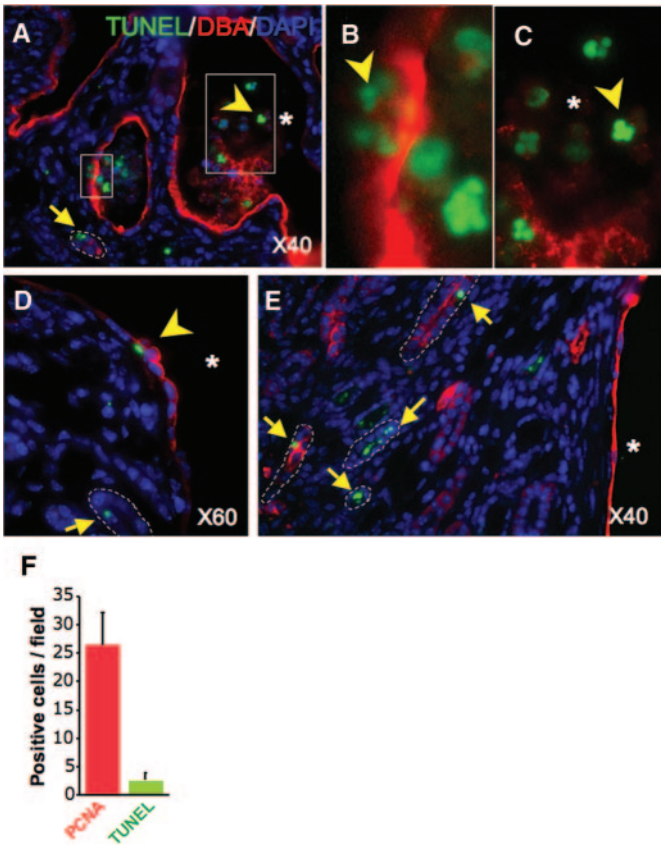

Figure 9. An increase in apoptosis in normal-looking tubules adjacent to cysts. TUNEL assay was carried out on extremely cystic kidney sections (*Mx1Cre*$^{+}$*DsRed*$^{+}$*Pkd1*$^{null/flox}$) labeled with DBA (red). (A through E) The TUNEL-positive (green) cells were detected in normal-looking tubules 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.

subtle increase in PCNA expression was seen in *Mx1Cre*$^{+}$*Pkd1*$^{flox/flox}$ 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 labeling is in striking contrast to the dramatic increase of PCNA-positive cells in normal-looking tubules adjacent to cysts in extremely cystic kidneys (Figure 6), as well as in kidneys with mild cystic disease (Figure 7Ac). Western blot analyses of kidneys 1 wk after *Pkd1* inactivation at either 1 or 5 wk of age showed similar levels of PCNA expression between wild-type and *Mx1Cre*$^{+}$*Pkd1*$^{flox/flox}$ mice (Figure 7C), although there is a significant increase in more cystic kidneys (data not shown), which suggests increased cell proliferation is not an immediate event. To examine the effect of a germline *Pkd1* mutation on cell proliferation in developing and mature kidneys, we performed Western blotting and immunostaining of PCNA on *Pkd1*^{null} heterozygous mice. Whereas the levels of PCNA expression (Figure 7D) and numbers of PCNA-positive cells (Figure E, a, c, and e) were similar between *Pkd1*$^{null/+}$ and wild-type littermate mice at 1 wk of age, we detected a statistically significant increase in 5-wk-old *Pkd1*^{null} 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

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[2]: 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 kidney[11] and liver cysts[12] in human tissues with ADPKD support this hypothesis. Consistent with the two-hit hypothesis, when we induced the somatic mutation in the $Mx1Cre^{+}Pkd1^{null/flox}$ 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 $Mx1Cre^{+}DsRed^{+}Pkd1^{null/flox}$ 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 $Mx1Cre^{+}Pkd1^{flox/flox}$ (Figure 7A) 6 wk after *Pkd1* inactivation at 5 wk of age. In $Mx1Cre^{+}Pkd1^{null/flox}$ 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 germline *Pkd1* haploin-

sufficiency similar to that in *Pkd2* heterozygous mice.[17] 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[18–22] (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 this model, we conclude that not all *Pkd1* haploinsufficient PCNA-positive cells will proceed to become cyst-lining cells. Because PCNA labels cells in the late G1 and S phases of the cell cycle, whereas Ki-67 labels cells in all phases of the cell cycle except the G_0 phase, we performed double staining with PCNA and Ki-67. Interestingly, we detected fewer cells brightly labeled for Ki-67 than PCNA (Figure 8). It is known that the level of Ki-67 antigen in the late G1 and very early S-phase cells is only slightly above the background,[23] and the half-life of Ki-67 antigen is much shorter than that of PCNA; therefore, the data suggest that a portion of the *Pkd1* mutant cells may have prolonged G1 and S phase of the cell cycle. The possibility that *Pkd1* mutation specifically affects PCNA expression remains to be excluded.

To investigate the impact of a germline mutation of *Pkd1* on cyst formation, we generated $Mx1Cre^{+}Pkd1^{null/flox}$ compound heterozygous mice. Compared with $Mx1Cre^{+}Pkd1^{flox/flox}$ mice, compound heterozygous mice showed a more severe cystic phenotype when induced by pI:pC at either 1 or 5 wk of age. Kidney/body weight ratio approximately doubled when *Cre* expression was induced in 1-wk-old mice (Figure 3E). Renal function as reflected by serum creatinine is severely impaired (Figure 3F). Cysts were seen as early as 1 wk after injection. $Mx1Cre^{+}Pkd1^{null/flox}$ mice with *Pkd1* inactivation in 5-wk-old mature kidneys had more advanced cystic disease (albeit still restricted to a part of the kidney) when compared with the simple focal cysts in $Mx1Cre^{+}Pkd1^{flox/flox}$ mice at the same stage. The increased disease severity in compound heterozygous mice is more likely due to the presence of the germline mutation than to greater *Cre* excision efficiency, because we observed efficient *loxP* recombination events 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 ($Pkd1^{del34/+}$ or $Pkd1^{null/+}$) also develop renal cysts but at a much later stage when compared with compound heterozygotes.[4,24] 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^{2+} signaling through their primary cilia.[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,[26] whereas inducible knockout of *Tg737* in adulthood results in slow and late onset of cystic phenotype.[26] 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.[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,[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 ($Pkd1^{del2-11}$ and $Pkd1^{del2-4}$) mouse models that inactivate *Pkd1* in either distal nephron segments[31] or all nephron segments[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*$^{del2-4}$ mice 3 mo after by tamoxifen-induced inactivation.[32] Numerous small cysts are seen in *Tm*Cre*Pkd1*$^{del2-4}$ 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.[10] The effects of germline mutation in compound heterozygotes described here were not reported by Pontiek *et al.*[32] Consistent with this study, Lantinga *et al.*[7] reported an increase in cell proliferation associated with haploinsufficiency; 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*^{flox} allele has been described elsewhere.[10] Briefly, the selection marker *neo* cassette flanked by two *loxP* sites was inserted into intron 1 of the *Pkd1* locus, and the third *loxP* site was inserted into intron 6 of the *Pkd1* locus. To obtain mice with a *Pkd1* allele whose exons 2 through 6 are flanked with *loxP* sites (*Pkd1*$^{flox/+}$ mice) and does not have a neo cassette, and mice with a *Pkd1* allele with neither neo cassette nor exons 2 through 6 (*Pkd1*$^{del2\text{-}6/+}$ mice), we crossed mice with germline transmission of the trilox construct with germline *Cre*-expressing mice. Heterozygous *Pkd1*$^{flox/+}$ 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 *Mx1* promoter (*Mx1Cre* mice) to generate *Pkd1*$^{flox/+}$ mice carrying the *Mx1Cre* transgene (*Mx1Cre*$^{+}$*Pkd1*$^{flox/+}$ mice). Then *Mx1Cre*$^{+}$ *Pkd1*$^{flox/+}$ mice were bred with either *Pkd1*$^{flox/+}$ mice or *Pkd1*$^{null/+}$ mice to generate *Mx1Cre*$^{+}$*Pkd1*$^{flox/flox}$ mice (*Mx1Cre* is hemizygous) or *Mx1Cre*$^{+}$*Pkd1*$^{null/flox}$ mice, respectively. The genotypes of *Mx1Cre*$^{+}$ *Pkd1*$^{flox/+}$, *Pkd1*$^{flox/+}$, *Pkd1*$^{flox/flox}$, *Mx1Cre*$^{+}$ *Pkd1*$^{null/+}$, *Pkd1*$^{null/+}$, *Pkd1*$^{null/flox}$, and *Mx1Cre*$^{+}$*Pkd1*$^{+/+}$ 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 Carrying *loxP*-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 by *loxP* sequences. The chicken β actin promoter coupled with the cytomegalovirus is used for this transgene. DsRed mice were crossed with *Mx1Cre* mice to generate double-transgenic mice with *Mx1Cre* and DsRed. After injection with pI:pC to induce *Cre* recombinase, genetic recombination occurs at the two *LoxP* 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 of *lox*P Sites Flanking *Pkd1* Exons 2 through 6

Quantitative real-time PCR and semiquantitative PCR were performed on genomic DNA from kidneys of *Mx1Cre*$^{+}$*Pkd1*$^{flox/+}$, *Mx1Cre*$^{+}$*Pkd1*$^{flox/flox}$, 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 TCT GC-3′, 5′-GGC 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 GCG TGT GCC ACC ATG ATG-3′ and 5′-CCC TGA CAT CCA CAT GGT GGC TA-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% nonfat 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 glyceraldehyde-3-phosphate dehydrogenase (1:200; Santa Cruz) and secondary antibodies (1:5000).

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DISCLOSURES

None.

REFERENCES

1. Rossetti S, Consugar MB, Chapman AB, Torres VE, Guay-Woodford LM, Grantham JJ, Bennett WM, Meyers CM, Walker DL, Bae K, Zhang QJ, Thompson PA, Miller JP, Harris PC: Comprehensive molecular diagnostics in autosomal dominant polycystic kidney disease. *J Am Soc Nephrol* 18: 2143–2160, 2007
2. Reeders ST: Multilocus polycystic disease. *Nat Genet* 1: 235–237, 1992
3. Lu W, Peissel B, Babakhanlou H, Pavlova A, Geng L, Fan X, Larson C, Brent G, Zhou J: Perinatal lethality with kidney and pancreas defects in mice with a targeted *Pkd1* mutation. *Nat Genet* 17: 179–181, 1997
4. Lu W, Shen X, Pavlova A, Lakkis M, Ward CJ, Pritchard L, Harris PC, Genest DR, Perez-Atayde AR, Zhou J: Comparison of Pkd1-targeted mutants reveals that loss of polycystin-1 causes cystogenesis and bone defects. *Hum Mol Genet* 10: 2385–2396, 2001
5. Muto S, Aiba A, Saito Y, Nakao K, Nakamura K, Tomita K, Kitamura T, Kurabayashi M, Nagai R, Higashihara E, Harris PC, Katsuki M, Horie S: Pioglitazone improves the phenotype and molecular defects of a targeted Pkd1 mutant. *Hum Mol Genet* 11: 1731–1742, 2002
6. Kim K, Drummond I, Ibraghimov-Beskrovnaya O, Klinger K, Arnaout MA: Polycystin 1 is required for the structural integrity of blood vessels. *Proc Natl Acad Sci U S A* 97: 1731–1736, 2000
7. Lantinga-van Leeuwen IS, Dauwerse JG, Baelde HJ, Leonhard WN, van de Wal A, Ward CJ, Verbeek S, Deruiter MC, Breuning MH, de Heer E, Peters DJ: Lowering of Pkd1 expression is sufficient to cause polycystic kidney disease. *Hum Mol Genet* 13: 3069–3077, 2004
8. Jiang ST, Chiou YY, Wang E, Lin HK, Lin YT, Chi YC, Wang CK, Tang MJ, Li H: Defining a link with autosomal-dominant polycystic kidney disease in mice with congenitally low expression of Pkd1. *Am J Pathol* 168: 205–220, 2006
9. Piontek KB, Huso DL, Grinberg A, Liu L, Bedja D, Zhao H, Gabrielson K, Qian F, Mei C, Westphal H, Germino GG: A functional floxed allele of Pkd1 that can be conditionally inactivated in vivo. *J Am Soc Nephrol* 15: 3035–3043, 2004
10. Starremans PG, Li X, Finnerty PE, Guo L, Takakura A, Neilson EG, Zhou J: A mouse model for polycystic kidney disease through a somatic in-frame deletion in the 5′ end of Pkd1. *Kidney Int* 73: 1394–1405, 2008
11. Qian F, Watnick TJ, Onuchic LF, Germino GG: The molecular basis of focal cyst formation in human autosomal dominant polycystic kidney disease type I. *Cell* 87: 979–987, 1996
12. Watnick TJ, Torres VE, Gandolph MA, Qian F, Onuchic LF, Klinger KW, Landes G, Germino GG: Somatic mutation in individual liver cysts supports a two-hit model of cystogenesis in autosomal dominant polycystic kidney disease. *Mol Cell* 2: 247–251, 1998
13. Geng L, Segal Y, Pavlova A, Barros EJ, Lohning C, Lu W, Nigam SK, Frischauf AM, Reeders ST, Zhou J: Distribution and developmentally regulated expression of murine polycystin. *Am J Physiol* 272: F451–F459, 1997
14. Bhunia AK, Piontek K, Boletta A, Liu L, Qian F, Xu PN, Germino FJ, Germino GG: PKD1 induces p21(waf1) and regulation of the cell cycle via direct activation of the JAK-STAT signaling pathway in a process requiring PKD2. *Cell* 109: 157–168, 2002
15. Li X, Luo Y, Starremans PG, McNamara CA, Pei Y, Zhou J: Polycystin-1 and polycystin-2 regulate the cell cycle through the helix-loop-helix inhibitor Id2. *Nat Cell Biol* 7: 1202–1212, 2005
16. Nadasdy T, Laszik Z, Lajoie G, Blick KE, Wheeler DE, Silva FG: Proliferative activity of cyst epithelium in human renal cystic diseases. *J Am Soc Nephrol* 5: 1462–1468, 1995
17. Chang MY, Parker E, Ibrahim S, Shortland JR, Nahas ME, Haylor JL, Ong AC: Haploinsufficiency of Pkd2 is associated with increased tubular cell proliferation and interstitial fibrosis in two murine Pkd2 models. *Nephrol Dial Transplant* 21: 2078–2084, 2006
18. Wilson PD: Aberrant epithelial cell growth in autosomal dominant polycystic kidney disease. *Am J Kidney Dis* 17: 634–637, 1991
19. Horie S, Higashihara E, Nutahara K, Mikami Y, Okubo A, Kano M, Kawabe K: Mediation of renal cyst formation by hepatocyte growth factor. *Lancet* 344: 789–791, 1994
20. Moskowitz DW, Bonar SL, Liu W, Sirgi CF, Marcus MD, Clayman RV: Epidermal growth factor precursor is present in a variety of human renal cyst fluids. *J Urol* 153: 578–583, 1995
21. Norman JT, Gatti L, Wilson PD, Lewis M: Matrix metalloproteinases and tissue inhibitor of matrix metalloproteinases expression by tubular epithelia and interstitial fibroblasts in the normal kidney and in fibrosis. *Exp Nephrol* 3: 88–89, 1995
22. Gardner KD Jr, Burnside JS, Elzinga LW, Locksley RM: Cytokines in fluids from polycystic kidneys. *Kidney Int* 39: 718–724, 1991
23. Bruno S, Darzynkiewicz Z: Cell cycle dependent expression and stability of the nuclear protein detected by Ki-67 antibody in HL-60 cells. *Cell Prolif* 25: 31–40, 1992
24. Geng L, Segal Y, Peissel B, Deng N, Pei Y, Carone F, Rennke HG, Glucksmann-Kuis AM, Schneider MC, Ericsson M, Reeders ST, Zhou J: Identification and localization of polycystin, the PKD1 gene product. *J Clin Invest* 98: 2674–2682, 1996
25. Nauli SM, Alenghat FJ, Luo Y, Williams E, Vassilev P, Li X, Elia AE, Lu W, Brown EM, Quinn SJ, Ingber DE, Zhou J: Polycystins 1 and 2 mediate mechanosensation in the primary cilium of kidney cells. *Nat Genet* 33: 129–137, 2003
26. Davenport JR, Watts AJ, Roper VC, Croyle MJ, van Groen T, Wyss JM, Nagy TR, Kesterson RA, Yoder BK: Disruption of intraflagellar transport in adult mice leads to obesity and slow-onset cystic kidney disease. *Curr Biol* 17: 1586–1594, 2007
27. Verani RR, Silva FG: Histogenesis of the renal cysts in adult (autosomal dominant) polycystic kidney disease: A histochemical study. *Mod Pathol* 1: 457–463, 1988

28. Torres VE, Harris PC: Polycystic kidney disease: genes, proteins, animal models, disease mechanisms and therapeutic opportunities. *J Intern Med* 261: 17–31, 2007
29. Osathanondh V, Potter EL: Pathogenesis of polycystic kidneys: Type 3 due to multiple abnormalities of development. *Arch Pathol* 77: 485–502, 1964
30. Heggo O: A microdissection study of cystic disease of the kidneys in adults. *J Pathol Bacteriol* 91: 311–315, 1966
31. Lantinga-van Leeuwen IS, Leonhard WN, van der Wal A, Breuning MH, de Heer E, Peters DJ: Kidney-specific inactivation of the Pkd1 gene induces rapid cyst formation in developing kidneys and a slow onset of disease in adult mice. *Hum Mol Genet* 16: 3188–3196, 2007
32. Piontek K, Menezes LF, Garcia-Gonzalez MA, Huso DL, Germino GG: A critical developmental switch defines the kinetics of kidney cyst formation after loss of Pkd1. *Nat Med* 13: 1490–1495, 2007