Kidney-specific inactivation of the *Pkd1* gene induces rapid cyst formation in developing kidneys and a slow onset of disease in adult mice

Irma S. Lantinga-van Leeuwen[1], Wouter N. Leonhard[1], Annemieke van der Wal[2],
Martijn H. Breuning[1], Emile de Heer[2] and Dorien J.M. Peters[1],*

[1]Department of Human Genetics and [2]Department of Pathology, Leiden University Medical Center,
Leiden 2300RC, The Netherlands

Autosomal dominant polycystic kidney disease, caused by mutations in the *PKD1* gene, is characterized by progressive deterioration of kidney function due to the formation of thousands of cysts leading to kidney failure in mid-life or later. How cysts develop and grow is currently unknown, although extensive research revealed a plethora of cellular changes in cyst lining cells. We have constructed a tamoxifen-inducible, kidney epithelium-specific *Pkd1*-deletion mouse model. Upon administration of tamoxifen to these mice, a genomic fragment containing exons 2–11 of the *Pkd1*-gene is specifically deleted in the kidneys and cysts are formed. Interestingly, the timing of *Pkd1*-deletion has strong effects on the phenotype. At 1 month upon gene disruption, adult mice develop only a very mild cystic phenotype showing some small cysts and dilated tubules. Young mice, however, show massive cyst formation. In these mice, at the moment of gene disruption, cell proliferation takes place to elongate the nephron. Our data indicate that *Pkd1* gene deficiency does not initiate sufficient autonomous cell proliferation leading to cyst formation and that additional stimuli are required. Furthermore, we show that one germ-line mutation of *Pkd1* is already associated with increased proliferation.

INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is a common genetic disease characterized by progressive development of fluid-filled cysts in both kidneys. The formation of numerous cysts together with interstitial fibrosis usually causes chronic renal failure in 50% of patients by the age of 60 years (1). The kidney is the most severely affected organ, but the disease is systemic and extra-renal manifestations such as cyst formation in liver and pancreas, hypertension and cerebral aneurysms also can occur (2).

The majority of patients, 85%, carry a mutation in the *PKD1* gene (3). The protein encoded by the *PKD1* gene, polycystin-1, forms multiprotein complexes at the cell membrane and functions in cell–cell and cell–matrix interactions, signal transduction and in mechanosensation (4). The C-terminal region of polycystin-1 has been shown to interact with the *PKD2* gene product, polycystin-2, which is mutated in ∼15% of the

ADPKD patients, and functions as a Ca^{2+}-permeable cation channel (5–7). Both polycystins have been found in primary cilia. Several proteins in this cell organelle have been reported to be associated with cystic diseases and are probably participating in sensing fluid flow or shear stress (8).

ADPKD patients carry one germ-line mutation and one normal *PKD1* allele. Two genetic mechanisms for cyst formation have been postulated, e.g. somatic inactivation of *PKD1* or *PKD2* by a 'two-hit' mechanism has been shown for cysts in *PKD1* and *PKD2* kidneys (9,10) but reduced levels of polycystins may already be sufficient to cause polycystic kidney disease (11,12). In addition, cysts formation in mice overexpressing *Pkd1* suggest that a balanced polycystin-1 and -2 dosage seem to be critical to maintain an intact renal epithelial architecture (13,14).

A plethora of cellular changes in cyst-lining cells have been observed, but the precise mechanisms of how cysts arise and

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*To whom correspondence should be addressed. Tel: +31 715269490; Fax: +31 715268285; Email: d.j.m.peters@lumc.nl

grow are still not well understood. To study the pathogenesis of ADPKD several mouse models have now been generated. Homozygous *Pkd1* and *Pkd2* knock-out mice die at embryonic stages, around E15.5, or directly after birth due to severe cystic disease, vascular defects and/or abnormalities of the placental labyrinth (15–20). Heterozygous knock-out mice showed minimal renal cyst formation later on in life (21) and are therefore not very suitable to test therapeutic interventions. Hypomorphic *Pkd1* models, on the other hand, are viable and have polycystic kidney disease just as *Pkd2* mutant mice carrying one null allele and one unstable *Pkd2* allele (10,11,22). These mice also show extra-renal manifestations like cysts in liver, pancreas and/or develop aortic aneurysms (10–12,23).

We now have generated a kidney-specific and tamoxifen-inducible *Pkd1* mutant mouse line, using Cre/loxP recombination. Not hampered by early death due to malfunctioning of other organs these mice are highly suitable for long-term studies on the pathogenesis of polycystic kidney disease, for testing therapeutic interventions, and for studying the initial steps of the disease. We show that the proliferative status of the renal epithelium, at the time of *Pkd1* inactivation, has a major effect on the development of PKD.

RESULTS

Generation of inducible and kidney-specific *Pkd1* mutant mice

To get more insight into cyst formation and cyst progression, we created a conditional and inducible *Pkd1* deletion mouse model. For this model we have generated mouse lines in which exons 2-11 of *Pkd1* are either deleted ($Pkd1^{del2-11}$) or flanked by *loxP* sites ($Pkd1^{lox2-11}$ hereafter $Pkd1^{lox}$) (Fig. 1). Homozygous $Pkd1^{lox,lox}$ mice are viable and have no discernable phenotype analyzed up to 15–17 months of age ($n = 5$). Homozygous $Pkd1^{del2-11,del2-11}$ mice die embryonically as reported for conventional knock-out mice (15–17). In order to selectively knock-out genes in renal epithelium we also created tamoxifen-inducible tam-*KspCad-CreER*^{T2} (*Cre*) mice
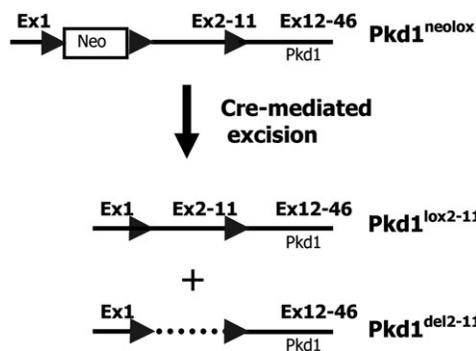
with *Cre*-recombinase expression in renal epithelium and capable to delete the floxed fragment of *Pkd1* (24). The transgenic mice, *Cre;Pkd1*$^{del2-11,lox}$ or *Cre;Pkd1*^{lox,lox}, were viable and fertile and showed no histological abnormalities compared with littermates without the *Cre*-transgene and the same *Pkd1*-genotype.

As expected, tamoxifen administration resulted in formation of the deleted allele, as shown by allele-specific PCR on genomic DNA isolated from the kidneys (Fig. 2).

Inactivation of the *Pkd1* gene in kidneys causes cyst formation

When we treated transgenic *Cre;Pkd1*$^{del2-11,lox}$ mice at 3–6 month of age with tamoxifen, pathologic examination of these mice revealed a mild cystic phenotype in the kidneys. At 1 month upon tamoxifen administration dilated tubules and 1–5 microscopic cyst per cross-section could be detected ($n = 4$) and after 3 months, ~10 microscopic renal cysts per cross-section and occasionally a small macroscopic cyst (cyst area 0.8 mm^2) could be seen ($n = 4$) (Fig. 3A). Cysts were detected bilaterally, in the cortex and the medulla.

In contrast, when 4-days old mice (PN4) received tamoxifen via breast feeding by the weaning mother, massive cyst formation was observed when sacrificed 1 month later ($n = 9$, Fig. 3B). In these mice after 13 days small cysts can sometimes be observed ($n = 15$) (Fig. 3C). The majority of cysts arises from distal parts of the nephron and are Uromodulin-positive (Fig. 3D). Dilated collecting ducts were also frequently observed. Transgenic mice with two floxed *Pkd1* alleles ($n=3$), showed a slightly milder cystic phenotype compared with *Cre;Pkd1*$^{del2-11,lox}$ littermates ($n = 3$) (Fig. 3E). No liver or pancreatic cysts were detected in the young conditional knock-out mice. Adult tamoxifen-treated *Cre;Pkd1*$^{del2-11,lox}$ animals also showed no pancreatic cysts but in the liver 0–5 microscopic and occasionally a macroscopic cyst per cross-section could be observed, similar as in *wt;Pkd1*$^{del2-11,+}$ and *wt;Pkd1*$^{del2-11,lox}$ mice of 4–11 months. These findings support previous data that deletion

Figure 1. Generation of *Pkd1* mutant mice. A *Pkd1*-targeted mouse line containing a *PGK-neo*-cassette flanked by two loxP sites in intron 1 of the *Pkd1* gene and a third loxP in intron 11 was generated previously (11). *Cre*-mediated excision of the floxed sequences results in mouse lines in which exons 2–11 of *Pkd1* are either deleted ($Pkd1^{del2-11}$) or flanked by *loxP* sites ($Pkd1^{lox2-11}$). PGK, phosphoglycerate kinase gene; Neo, neomycin phosphotransferase gene; Ex, exon.
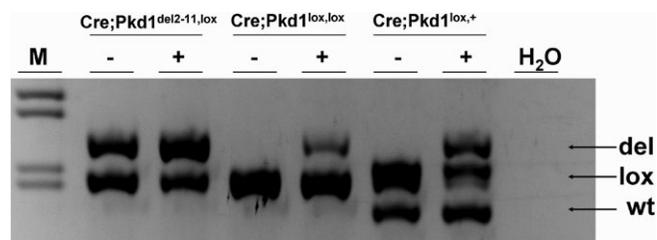

Figure 2. Tam-*KspCad-CreER*^{T2}-mediated recombination of the floxed *Pkd1* allele. Agarose gel analysis of PCR products amplified from genomic DNA isolated from kidneys of adult *Cre;Pkd1*$^{del2-11,lox}$, young *Cre;Pkd1*^{lox,lox} and adult *Cre;Pkd1*$^{lox,+}$ mice treated (+) or not treated (−) with tamoxifen. In DNA from the *Cre;Pkd1*$^{del2-11,lox}$ mouse a slight increase in the 366-bp deletion-specific PCR product and a slight decrease in the 296-bp floxed-allele-specific product was observed in tamoxifen-treated compared with untreated animals. In *Cre;Pkd1*^{lox,lox} and *Cre;Pkd1*$^{lox,+}$ mice the deletion-specific product was only present in tamoxifen-treated animals. In treated and untreated *Cre;Pkd1*$^{lox,+}$ mice the 250 bp wild-type (wt)-specific product is present.
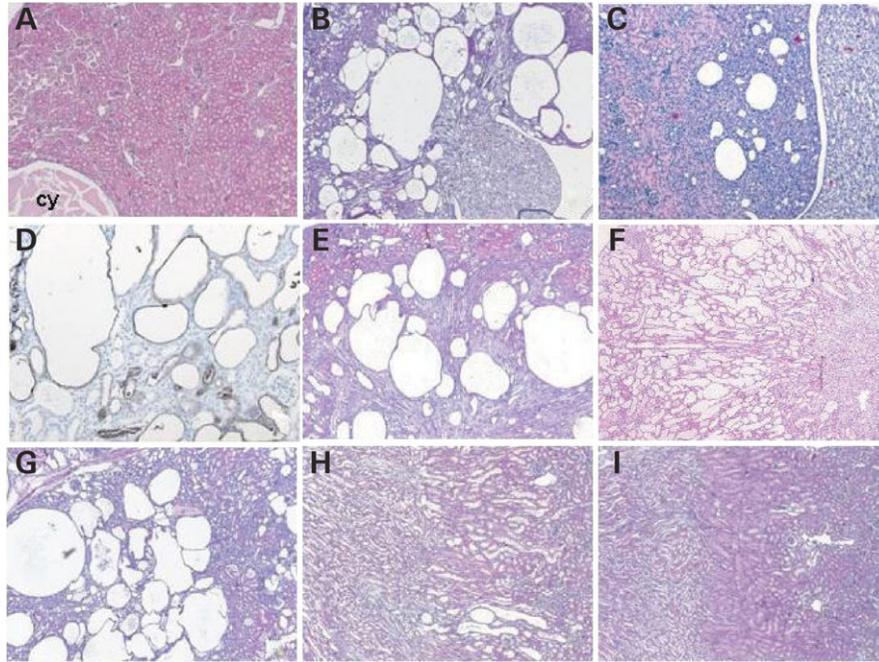
Figure 3. Age-dependent renal cyst development in tamoxifen-induced triple transgenic mice. *Pkd1* deletion induced in adult *Cre*;*Pkd1*[del2−11,lox] mice results in a minor cystic phenotype. (**A**) Grossly normal renal tissue with a cyst (cy) in a mouse treated with tamoxifen at age 3 months and sacrificed 3 months later (50×, f); (**B**) *Pkd1* deletion induced in newborn *Cre*;*Pkd1*[del2−11,lox] mice (PN4) causes massive cyst formation after 1 month (50×, f); (**C**) already 13 days after the inactivation of the *Pkd1* gene in newborn *Cre*;*Pkd1*[del2−11,lox] mice, cysts can be detected (100×, f); (**D**) the majority of cysts arise from distal parts of the nephron and are Uromodulin positive: PN4-treated *Cre*;*Pkd1*[del2−11,lox] mouse sacrificed after 1 month; (**E**) *Pkd1* deletion induced in a newborn *Cre*;*Pkd1*[lox,lox] mouse sacrificed 1 month later, showing a somewhat milder phenotype (50×, f); (**F**) in time, 5 months after tamoxifen-treatment, adult-induced conditional *Cre;Pkd1*[lox,lox] mice develop polycystic kidneys (25×, m); (**G−I**) phenotypic differences in conditional *Pkd1* mothers and pups: tamoxifen-treated pup, showing massive cyst formation, while her triple transgenic tamoxifen-treated mother shows only a few cysts; (G) PN4-treated *Cre*;*Pkd1*[del2−11,lox] pup sacrificed after 1 month (50×, f); (H) *Cre*;*Pkd1*[del2−11,lox] mother, treated at 6 months of age, and sacrificed 5 months later (50×); (I) female control littermate of the mother, also treated with tamoxifen and sacrificed at 11 months of age (wt; *Pkd1*[del2−11,lox]) (50×).

of the floxed *Pkd1* gene mediated by our KspCad-CreER[T2] mouse line is restricted to the kidneys (24).

Prolonged follow-up of adult-induced mice revealed that in time these animals do develop more severe PKD. Mice sacrificed at 5 months after tamoxifen-administration showed kidneys that were paler and were more or less enlarged compared with controls. Four out of five mice had kidney/body weight ratios above 2%, up to 9.5% (Controls 1.3−1.8%, $n = 5$). Male mice tend to manifest a more severe cystic phenotype while the recombination efficiency was similar in female and male animals. Histological examination revealed many dilated tubules and microcysts, especially at the medullar-cortical boundaries (Fig. 3F). Staining with kidney-segment markers showed that dilated tubules originated from all nephron-segments and collecting ducts and that many cysts showed no staining.

Maternal administration of tamoxifen induces a progressive cystic phenotype in newborn mutant mice but a slow disease onset in the mutant mothers

In the experiments described above, adult mice had received tamoxifen via a feeding needle, while young mice received tamoxifen via the milk from the mother who was treated. In most of these experiments the mother herself did not have the triple mutant phenotype. To minimize, however, the possibility that a technical artifact was underlying the phenotypic differences observed between adult and young mice, we treated three *Cre*;*Pkd1*[del2−11,lox] mothers with tamoxifen and carefully analyzed the pathology in mothers and progeny. In agreement with the phenotypes found in unrelated young and adult mice, the *Cre*;*Pkd1*[del2−11,lox] progeny sacrificed after 1 month showed a much more severe polycystic phenotype than their mothers sacrificed after 1−5 months (Fig. 3G and H).

Efficient Cre-mediated deletion of the floxed *Pkd1* allele

To ascertain that recombination of the floxed allele had occurred in tamoxifen-treated triple transgenic mice, we performed a quantitative PCR on genomic DNA, isolated from the kidneys. Essentially, we analyzed the increase in deletion allele using primers in intron 1 and intron 11, flanking the deletion. In addition kidneys of tamoxifen-treated *Cre*;*Pkd1*[lox,+] mice were analyzed as well. Since *Cre*;*Pkd1*[lox,+] mice do not show a phenotype, we assume that the percentage of deletion allele is not affected by pathologic alterations of the kidneys. Adult mice show 20−25% deletion allele upon tamoxifen-administration, indicating that recombination in renal tissues had occurred in up to 40−50% of the cells. In mice treated at postnatal day 4 (PN4), we measured a lower but distinct increase in deletion of 4−12% indicating that the

Pkd1 gene was knocked out in ∼10–20% of the renal cells. In triple transgenic *Cre;Pkd1*$^{del2-11,lox}$ mice, which have already one deleted *Pkd1* allele, 54–59% deletion was measured in young mice and 61–85% deletion in adult mice (Fig. 4). From these experiments we conclude that in the triple transgenic mice the *Pkd1*-gene is deleted in a substantial number of cells. Even more, young mice that have a much more severe renal cystic phenotype compared with adult mice, show a slightly lower percentage of *Pkd1*-deletion. No deletion allele was measured in liver or in renal cells of non-treated *Cre;Pkd1*$^{lox,+}$ mice.

Since the lower percentage of recombination in the young mice might be explained by lower activity of the Ksp-Cadherin promoter at the time-point of tamoxifen administration, postnatal days 4–6, we measured *Cre* expression in 5-days-old mice and in adult mice by real time RT–PCR. *Cre* expression is ∼2-fold lower in young mice (PN5) compared with adult mice (mean 0.50 ± 0.06 versus mean 0.93 ± 0.18; median adult set at 1.0, $n = 6$ for both groups).

Cell proliferation differs in newborn and adult mice

We hypothesized that the proliferation status of the tissue may underlie the observed pathogenic differences between the adult and newborn tamoxifen-treated triple mice. Therefore, we stained kidney sections with the proliferation marker Ki-67. A substantial number of proliferating cells can be observed in newborn wild-type mice, while in adult wild-type controls Ki-67 positive nuclei can hardly be found, indicating that at the moment of tamoxifen treatment the renal epithelium is proliferative in young mice but scarcely in adults (Fig. 5).

The mean proliferation index (PI) in kidneys of young mice (postnatal week 1) was 8.2% (range 4.4–16%, $n=4$) while in adult mice (>3 months) the mean PI was around 0.2% (range 0.1–0.4, $n=3$) (Table 1).

Pkd1 haploinsufficiency is associated with increased renal epithelial cell proliferation

In cystic kidneys, the epithelia of dilated tubules and small cysts show strong proliferation, as many nuclei are Ki-67 positive. Large cysts incidentally show a positive nucleus (Fig. 5). An association between haploinsuffiency of *Pkd* genes and an increased proliferating index has been postulated for renal tissues of ADPKD patients and adult heterozygous *Pkd2* mice compared with controls (25). To address this issue in our mouse models we measured the PI of precystic tubular epithelial cells in different *Pkd1* mutant mice and controls. Adult mice that carry only one *Pkd1* allele show a higher PI compared with mice that carry no deletion allele (mean 1.2% versus mean 0.3%, $P < 0.01$), suggesting that a germ-line mutation of *Pkd1* results in increased proliferation (Table 2). The adult conditional knock-out mice show roughly a 5–20-fold increase in Ki-67 index compared with mice that carry no deletion allele. These mice were sacrificed at 3 or 5 months after tamoxifen-treatment and displayed variable cystic phenotypes. In the group of mice sacrificed 3 months after treatment, which show grossly normal tubules, the PI was not significantly different from mice with one deletion allele (compare mean PI 1.9%, $n = 5$, to mean 1.2%, $n = 8$; $P = 0.14$). In the mice sacrificed 5 months after treatment,
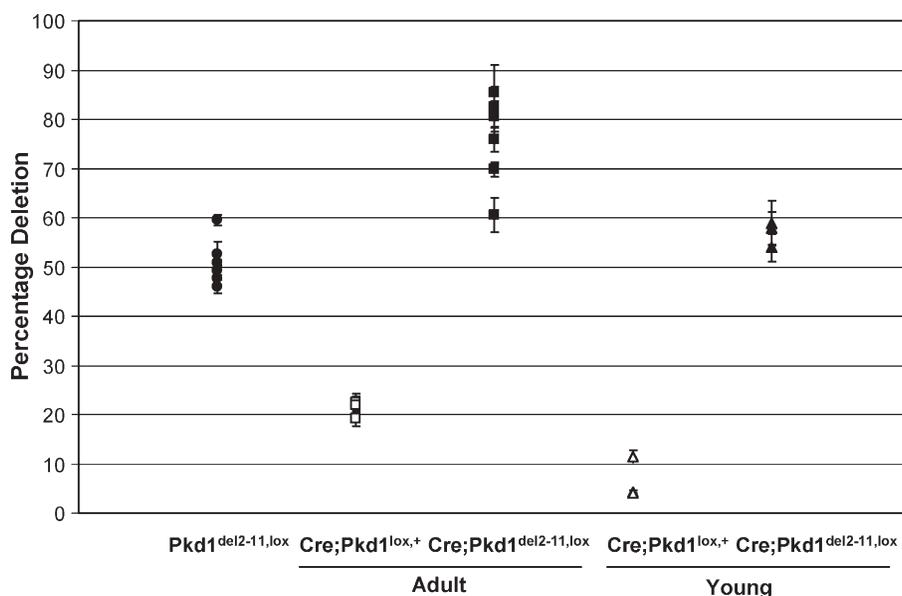

Figure 4. Percentage deletion of the floxed *Pkd1* allele in young weaning and in adult tamoxifen-induced *Cre;Pkd1*$^{del2-11,lox}$ mice and *Cre;Pkd1*$^{lox,+}$ control mice. Quantitative real-time PCR performed on genomic DNA isolated from kidneys. Mean with standard error are shown for each animal. Controls, *Pkd1*$^{del2-11,+}$ mice ($n = 6$) with one deleted allele, show on an average 50% *Pkd1*-deletion, indicating that in 100% of cells one allele is deleted. Adult *Cre;Pkd1*$^{lox,+}$ mice ($n = 3$) show 19–22% *Pkd1* deletion (mean 20%) indicating that in ∼40% of cells one allele is deleted. Adult *Cre;Pkd1*$^{del2-11,lox}$ mice ($n = 6$) show 61–85% *Pkd1* deletion (mean 76%), indicating that in ∼52% of cells both alleles are deleted. At young-age treated *Cre;Pkd1*$^{lox,+}$ mice ($n = 3$) and *Cre;Pkd1*$^{del2-11,lox}$ mice ($n = 3$) show 4–12% (mean 7%) and 54–59% *Pkd1* deletion (mean 57%), respectively, suggesting that in ∼10–20% of cells Cre-mediated recombination of the floxed *Pkd1* allele has occurred. Significant higher percentage of deletion was measured in adult *Cre;Pkd1*$^{del2-11,lox}$ mice compared with young *Cre;Pkd1*$^{del2-11,lox}$ mice (Mann–Whitney test, $P = 0.024$).
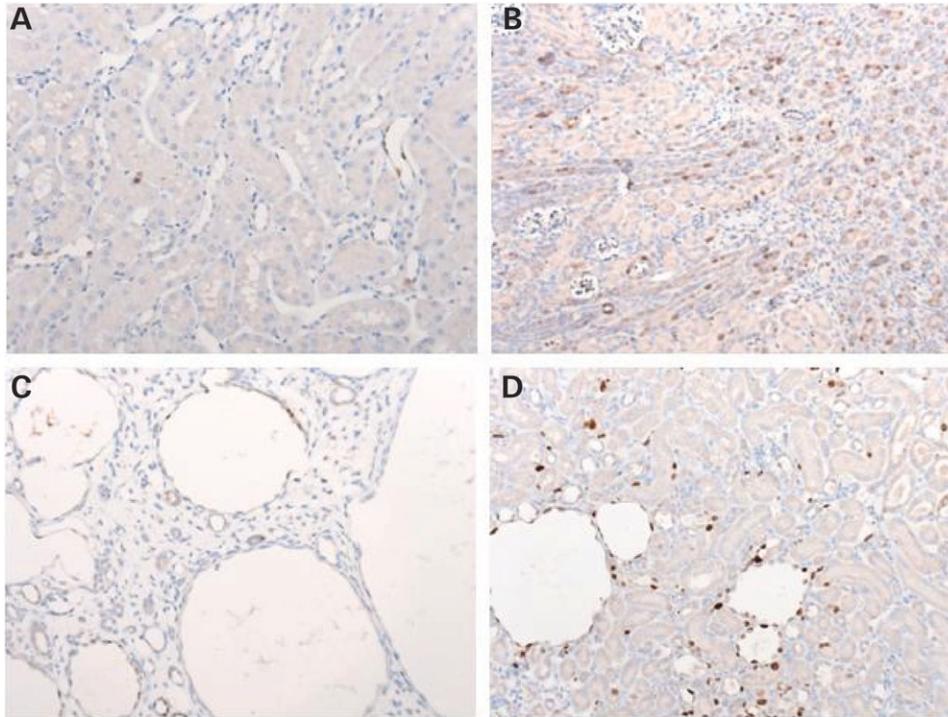
Figure 5. Cell proliferation in normal and cystic kidneys. Only a few Ki-67-positive nuclei are detected in kidneys of a 3-month-old adult wild-type mouse (**A**). In contrast, multiple Ki-67-positive nuclei are seen in tubular cells of a 7-days-old wild-type mouse (**B**). In cystic *Cre;Pkd1*^{del2 – 11,lox} kidneys occasionally Ki-67-positive nuclei can be observed in large cysts (**C**) whereas multiple Ki-67-positive cells were found in dilated tubules and small cysts (**D**) (A–D, 20×).

Table 1. Tubular cell proliferation index (PI) at the time-points of tamoxifen-induction

	Postnatal week 1 (wild-type)	3 months (wild-type)
Mean PI $\pm$ SD (%)	8.2 $\pm$ 5.4	0.2 $\pm$ 0.2
Number of mice	4	3

which show many dilated tubules, a significant increase in Ki-67 index was observed (mean PI 5.2%, $n = 5$; $P = 0.02$).

In the young-age-treated mice the increase in PI upon *Pkd1* disruption, was not significant (Table 2), either because there is not yet an increased PI or because base line proliferation of renal cells in young mice is too high to demonstrate any additional increase. All together these data support the idea that deletion of *Pkd1* results in increased renal epithelial proliferation with conditional knock-outs displaying cystic changes showing the highest levels of proliferation.

DISCUSSION

In this paper we present the first study describing an inducible and kidney-specific *Pkd1* knock-out mouse model to study ADPKD. Our findings show that timing of *Pkd1* gene disruption has a major effect on the severity of cyst development. Adult mice showed a mild cystic phenotype 1 month after the tamoxifen-treatment that resulted in disruption of the *Pkd1* gene in a substantial number of cells. Newborn mice, in contrast, and in spite of a lower percentage of *Pkd1* del-

etion, showed rapid and massive cyst formation in the same timeframe, suggesting that in these mice renal tissue is highly susceptible to cystogenesis, while in adult mice, with a relatively mild phenotype, it is not. We hypothesized that the proliferation status of the tissue may underlie the observed pathogenic differences in adult and young mice. Indeed, staining with the proliferation marker Ki-67 showed a substantial number of proliferating cells in newborn wild-type mice, while in adult wild-type controls Ki-67 positive nuclei can hardly be found, indicating that at the moment of *Pkd1* gene inactivation by tamoxifen treatment the renal epithelium is proliferative in young mice but not in adults.

Dilated tubules and cysts originate from all nephron-segments, which is in line with the Cre expression pattern of our conditional knock-out mouse model (24). In the adult mice, 5 months upon the induction of *Pkd1*-gene disruption, the majority of cysts did not show staining for any of the segment-markers, whereas in young mice at 1 month upon *Pkd1*-gene disruption, cysts frequently showed staining for Uromodulin. Since especially the loops of Henle are proliferating and enlarging in the developing kidneys of young mice, a large proportion of cysts seem to originate from this segment. In adult mice dilatations have been observed in different parts of the nephron, but cysts seem to have lost expression of their renal segment-specific markers.

The observation that in adult mice the number of renal epithelial cells, in which both alleles of *Pkd1* are disrupted, does not rapidly expand and grows out to form cysts, suggests that in the context of the renal tissue *Pkd1* gene deficiency does not initiate autonomous cell proliferation beyond control. Our

Table 2. Tubular cell proliferation index (PI) in adult and young mutant *Pkd1* mice

	Adult mice						Young mice			
	No deletion allele (A)		One deletion allele (B)		Conditional knock-out (C)		No deletion allele (D)	One deletion allele (E)	Conditional knock-out (F)	
	wt;wt	wt;lox or wt;lox,lox	wt;del or wt;del,lox	Oil-treated Cre;del,lox	C1 at +3 months	C2 at +5 months	lox or lox,lox	del or del,lox	Cre; lox,lox	Cre; del,lox
Mean PI (%) ± SD	0.2 ± 0.2	0.4 ± 0.3	1.3 ± 0.8	1.0 ± 0.5	1.9 ± 0.9	5.2 ± 4.1	8.7 ± 4.0	15.2 ± 10.9	15.0 ± 2.3	16.8 ± 13.7
Number of mice	3	3	5	3	5	5	9	5	4	4
Total mean ± SD	0.3 ± 0.2 (n = 6)		1.2 ± 0.7 (n = 8)				8.7 ± 4.0 (n = 9)	15.2 ± 10.9 (n = 5)	15.9 ± 9.1 (n = 8)	

Mice with no deletion allele (A, D), one deletion allele (B, E), and conditional knock-out mice (C, F) have been compared. The tubular cell proliferation index (PI) was determined by counting Ki-67-positive cells in normal and dilated tubules. Adult conditional knock-out mice (age 6–13 months) were sacrificed 3 or 5 months after the tamoxifen-treatment; 3 months $Cre;Pkd1^{del2-11.lox}$ (n = 4) and $Cre;Pkd1^{lox,lox}$ (n = 1), 5 months $Cre;Pkd1^{lox,lox}$ (n = 5). Renal tissues showed grossly normal structure after 3 months (C1) whereas dilated tubules and small cysts are visible after 5 months (C2). Young conditional knock-out mice (19-days old) received tamoxifen via the mother at postnatal days 4, 5 and 6 and were sacrificed 13 days later. At that time-point major cystic changes are not yet visible. Tamoxifen-induced disruption of *Pkd1* results in increased PI values. Significant differences were observed between groups B and A, mice carrying one versus no deletion allele ($P \leq 0.01$), and between groups B and C2 ($P = 0.02$). Dividing the group of conditional knock-out mice, in mice sacrificed after 3 months and mice sacrificed after 5 months revealed only for the latter group a significantly increased PI compared with mice carrying one deletion allele (C1 versus B, $P = 0.14$; C2 versus B, $P = 0.02$). Young heterozygous *Pkd1* mice tend to show increased proliferation compared with controls but this difference is not significant (E versus D, $P = 0.24$). Deleting the second allele resulted not in a significantly further increased PI (F versus E, $P = 0.94$).

finding seems to be in agreement with the observation by Nishio *et al*. (26), who showed that cystic epithelial cells of *Pkd1* null mice do not grow in nude mice. Apparently, additional stimuli or events are required to trigger the process. *In vitro*, however, decreased levels of *Pkd1* gene expression induced an increase in cell proliferation but cell culture conditions are usually permissive for growth factor-induced cell proliferation (26,27). In the absence of polycystin-1, cells continuously proliferate since a negative regulatory signal, regulated by polycystin-1, is missing. In the developing kidneys of neonatal mice, proliferation is induced by growth factors required for elongation of the nephron and renal development.

It is well documented that a number of signaling pathways, as Ras-Raf-ERK, JAK/STAT signaling and Akt/PI3 kinase activation can be induced by overexpression of full length or truncated *Pkd1* constructs (28–30). In addition, in immortalized renal cystic epithelial cells, with reduced levels of polycystin-1, a hyperproliferative response to IGF-1 is associated with enhanced activation of the Ras-Raf-ERK signaling system compared with normal cells, suggesting that cells with reduced levels of polycystin-1 are more sensitive to growth factor stimulation (31). However, the exact signaling cascades critical for the initiation of cyst formation are not completely known.

Cell proliferation is not only critical for the initiation of cyst formation but proliferation, in addition to extra cellular matrix remodeling and fluid accumulation, is contributing to cyst growth as well. Indeed the epithelia of dilated tubules and small cysts show increased proliferation, as many nuclei are Ki-67 positive. Large cysts, however, scarcely show positive nuclei, as described for renal cystic tissues from humans and the Han:SPRD-rats. Our data support the notion that proliferation is important in the early steps of cyst formation and cyst growth while at later stages epithelial stretch induced by increased luminal pressure may contribute to cyst growth (32,33). Promising results obtained by treatment of two non-orthologues murine models for polycystic kidney disease with the cyclin-dependent kinase inhibitor Roscovitine, also suggest that proliferation may be an important target for therapeutic interventions in ADPKD-patients (34). Likely, the drug will be more effective in not too advanced stages of the disease. These non-orthologous mouse and rat models have proven to be very useful to study the pathogenesis of polycystic kidney disease and to test potential therapeutic interventions. Diets or drugs that have been tested, however, sometimes had opposing effects, emphasizing the importance of using models that mimic the human disease (35–38).

Aberrant functioning of primary cilia may also be a crucial step in cystogenesis, since most if not all proteins encoded by genes related to cyst formation appear to be involved in ciliary or basal body function. Cilia of renal epithelial cells act as mechanosensors responding to flow by creating a Ca^{2+} influx, a process hampered in $Pkd1^{-/-}$ cells (8). Defective signaling may affect maintenance of the mature epithelial architecture and, indeed, in time the phenotype of adult mice becomes more severe, similar to the late-onset of PKD in human patients. Sensing the direction of urine flow by primary cilia may also be critical for proper orientation of tubular epithelial cells, e.g. planar cell polarity. Defects in

these processes are correlated with tubular dilation and cyst formation (39,40).

In most experiments we used triple transgenic mice with one floxed and one deletion allele, thus mimicking the process of somatic inactivation by a second hit. We also analyzed triple transgenic mice with two floxed *Pkd1* alleles, *Cre;Pkd1*^{lox,lox}. In general the phenotype of these mice was milder compared with *Cre;Pkd1*$^{del2-11,lox}$ mice sacrificed after similar time periods. Possible explanations are that Cre is either not always deleting both floxed *Pkd1* alleles, or the deletion of the second allele may occur with some delay. It may also take more time until the levels of polycystin-1, the protein encoded by *Pkd1*, fall below a critical threshold, or cells with one mutant *Pkd1*-allele may be more prone to Cre-mediated deletion of the other allele. In addition, haploinsufficiency of the surrounding cells may enhance cyst formation and progression.

Haploinsufficiency at *Pkd2* can induce phenotypic changes in vascular smooth muscle cells and influence the proliferative activity of renal tubular epithelium in *Pkd2* mutant mice of 9–12 months (12,25). It was hypothesized that the increase in cell proliferation may predispose the tubular cells to a somatic mutation, necessary for cystic changes (25). In support of this hypothesis, this study showed that heterozygous adult *Pkd1* mice, like the adult heterozygous *Pkd2* mice, had an ~4-fold increased PI compared with wild-type animals ($P < 0.05$), strengthening the possibility that haploinsufficiency of *Pkd1* may underlie the difference between *Cre;Pkd1*$^{del2-11,lox}$ and *Cre;Pkd1*^{lox,lox} mice and that haploinsufficiency of *Pkd* genes may predispose tubular cells to a somatic mutation. Inactivation of the second allele eventually results in cystic changes and after that time-period of ~5 months, a further increase in proportion of proliferating cells was observed in dilated tubules and starting cysts. Apparently, it takes time for additional stimuli to occur or to build up to sufficient levels for cystogenesis.

The difference in efficiency of Cre-mediated *Pkd1* gene disruption in young compared with adult mice may be explained by the 2-fold higher expression of Cre in kidneys of adult mice compared with 5-days-old mice. Additionally, reduced intake of tamoxifen may underlie lower recombination efficiency. In the case of the young mice a whole litter received tamoxifen via breast-feeding from the mother who was treated, whereas adult animals received tamoxifen individually and directly via oral administration. Nevertheless, it is clear that the model presented here, can serve to disrupt the *Pkd1* gene at different pre- and post-natal stages. Endogenous *Ksp-Cadherin* mRNA as well as a reporter gene under control of the *Ksp-Cadherin* promoter fragment have also been reported to be expressed in developing nephrons and in adult mice (41,42).

The cystic phenotype in adult conditional knock-out mice is in line with the observation that heterozygous *Pkd1*$^{+,-}$ mice hardly develop cysts in contrast to ADPKD patients. In this respect, it may be good to realize that mice have a shorter life-span, are kept in a highly controlled environment and are not exposed to putative harmful factors like infections, or toxic agents. The kidney has the capacity to regenerate upon renal ischemia, inflammation and drug or toxicant exposure. Renal injury followed by repair processes including proliferation

and apoptosis, may be an important permissive condition for cystogenesis in adult mice and in patients.

In conclusion, we have generated the first inducible renal-specific knock-out mouse model for cystogenesis in ADPKD. We can selectively inactivate the *Pkd1* gene in renal epithelium in an allele-specific fashion, bypassing the embryonic or postnatal lethality observed in *Pkd1*-deficient mice. Conditional knock-out mice do not show extra-renal manifestations like cysts in liver and pancreas and vascular defects as did *Pkd1*$^{-/-}$ embryos and 4-week-old *Pkd1* hypomorphic mice (11,15,16). Not hampered by early death due to malfunction of other organs our mice are highly suitable for long-term studies of the pathogenesis of ADPKD and for testing therapeutic interventions. Deletion of *Pkd1* results in increased proliferation with conditional knock-outs displaying cystic changes showing the highest levels of proliferation. Kidney-specific inactivation of *Pkd1* in newborn mice caused massive renal cyst formation, showing that *Pkd1* is essential for nephron maturation in developing kidneys. In newborn mice kidney development is not yet completed and cell proliferation still occurs to elongate the nephron. Therefore, we hypothesize that proliferation in the absence of functional polycystins is a trigger for cyst formation.

MATERIALS AND METHODS

Generation of tam-*KspCad-Cre;Pkd1*$^{del2-11,lox}$ mice and tamoxifen administration

Mice with the *Pkd1*$^{del2-11}$ and *Pkd1*^{lox} allele were generated as described for the hypomorphic *Pkd1*^{nl} allele (11). In short, a *loxP*-flanked PGK-neo-poly(A) cassette in antisense orientation was inserted into the *Eco*105I site in intron 1 and a synthetic double-stranded oligonucleotide containing a *loxP* site was cloned into the *Bgl*II site in intron 11. The linearized targeting construct was electroporated into 129/Ola embryonic stem (ES) cells, and homologous recombination was identified using 5′- and 3′-external probes. Chimeric mice were obtained by injection of *Pkd1*$^{nl,+}$ ES cells into C57BL/6 blastocysts and clone no. 4 and no. 18 were found to transmit the mutant *Pkd1* allele through the germ line. Partial or complete excision of the *loxP*-flanked DNA sequences occurred by crossbreeding clone no. 18-derived mice with EIIaCre-mice (43), and the generated mice with the *Pkd1*^{nl}, the *Pkd1*^{lox}, and the *Pkd1*$^{del2-11}$ alleles were further bred (Fig. 1). Mice bred on a C57BL/6 background for more than eight generations were analyzed in this study. Genotypes were assessed by PCR analysis of tail genomic DNA using a reverse primer in intron 11 combined with a forward primer in intron 1 (del allele) or a forward primer in intron 11 (floxed and wild-type alleles).

Generation of tam-*KspCad-CreER*^{T2} mice and tamoxifen treatment have been described previously (24). Adult mice, 3–4 months of age, have been treated with 5 mg tamoxifen for three consecutive days, using a feeding needle. Weaning mothers, 3–6-months-old, received a similar treatment starting at postnatal day 4 (PN4) of the progeny, and these newborn mice received tamoxifen via breast-feeding from the mother.

All experiments using mice were approved by the local animal experimental committee of the Leiden University Medical Center and by the Commission Biotechnology in Animals of the Dutch Ministry of Agriculture.

Quantitative PCR to detect deletions and Cre expression

To quantify the deletion a real-time PCR was performed on genomic DNA isolated from the kidneys of tamoxifen-treated and control mice. The increase in 'deletion' was analyzed as described previously (23). Essentially, primers have been selected in intron 1 and intron 11, flanking the deletion. As reference PCR, a fragment from exon 36 of the undeleted distal part of the *Pkd1* gene was used. DNA isolated from heterozygous *Pkd1*$^{del2-11,lox}$ mice ($n = 6$), with 50% of *Pkd1* alleles containing a deletion, served as a 50% reference. All measurements were performed three times in triplicate.

RT–PCR measurements to quantify the Cre expression in PN5 ($n = 6$) and adult mice ($n = 6$) were expressed as mean $\pm$ SD, related to the median value of adult mice (set at 1.0). Measurements were done in triplicate and performed twice.

Histological analysis

Tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 4 or 5 μm and stained with hematoxylin-eosin (HE). Kidney-segment identity was characterized using lectin *Dolichos biflorus*, and antisera against Uromodulin, aquaporin-2 and megalin as described (11). Ki-67 (1 : 3000, Novocastra) staining was performed using standard protocol.

Proliferative index (PI)

The PI was assessed essentially as described by Chang *et al.* (25), by counting the percentage of Ki-67-positive renal cells in four to six randomly selected fields of a cross-section. Between 400 and 1300 tubular epithelial nuclei of normal and dilated tubules with a diameter of up to five times the normal tubule diameter were counted. Only clearly definable nuclei with heavy/or granular staining were identified as positive. Results are expressed as mean $\pm$ SD.

Statistical analysis

Statistical comparisons between groups were performed with the Mann–Whitney rank-sum test, at 95% confidence interval. A *P*-value of <0.05 was considered statistically significant.

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