Lowering of Pkd1 expression is sufficient to cause polycystic kidney disease

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Autosomal dominant polycystic kidney disease (ADPKD) is a major cause of renal failure and is characterized by the formation of many fluid-filled cysts in the kidneys. It is a systemic disorder that is caused by mutations in PKD1 or PKD2. Homozygous inactivation of these genes at the cellular level, by a ‘two-hit’ mechanism, has been implicated in cyst formation but does not seem to be the sole mechanism for cystogenesis. We have generated a novel mouse model with a hypomorphic Pkd1 allele, Pkd1nl, harbouring an intronic neomycin-selectable marker. This selection cassette causes aberrant splicing of intron 1, yielding only 13–20% normally spliced Pkd1 transcripts in the majority of homozygous Pkd1nl mice. Homozygous Pkd1nl mice are viable, showing bilaterally enlarged polycystic kidneys. This is in contrast to homozygous knock-out mice, which are embryonic lethal, and heterozygous knock-out mice that show only a very mild cystic phenotype. In addition, homozygous Pkd1nl mice showed dilatations of pancreatic and liver bile ducts, and the mice had cardiovascular abnormalities, pathogenic features similar to the human ADPKD phenotype. Removal of the neomycin selection-cassette restored the phenotype of wild-type mice. These results show that a reduced dosage of Pkd1 is sufficient to initiate cystogenesis and vascular defects and indicate that low Pkd1 gene expression levels can overcome the embryonic lethality seen in Pkd1 knock-out mice. We propose that in patients reduced PKD1 expression of the normal allele below a critical level, due to genetic, environmental or stochastic factors, may lead to cyst formation in the kidneys and other clinical features of ADPKD.

INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is characterized by the formation of multiple fluid-filled cysts in the kidneys, ultimately leading to renal failure and a need for renal replacement therapy in most patients. 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 are frequently observed (1).

ADPKD has a prevalence of 1:1000, and the majority of patients, ~85%, have a mutation in the PKD1 gene (2,3). This gene encodes a large protein, polycystin-1, which forms multiprotein complexes at the cell membrane and is thought to function in cell–cell/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 cation channel (5–7). Renal cysts in ADPKD patients arise from the nephrons and collecting ducts owing to alterations in the epithelium. Although all cells carry the same germ-line mutation, only a minority of nephrons form cysts. As an explanation for focal cyst development, a ‘two-hit’ hypothesis was proposed analogous to hereditary types of cancer (8). Indeed, somatic mutations resulting in homozygous inactivation of PKD1 or PKD2 were identified in a subset of renal and hepatic cysts, as well as in clonal expansion of cyst-lining cells (9,10).
Additional support was provided by Pkd2 mice carrying one null allele and one unstable Pkd2 allele, which are prone to develop cysts (11). However, the presence of polycystin-positive cysts in ADPKD patients and cyst formation in mice overexpressing Pkd1 suggest that cystogenesis is due to a more complex mechanism than recessive inactivation at the cellular level (12,13).

In this study, we investigated the effect of a reduced dosage rather than a lack of Pkd1 expression in a novel mouse model.

Mice with only 13–20% of correctly spliced Pkd1-transcripts are viable showing polycystic kidneys and a variety of extra-renal manifestations observed in ADPKD-patients.

**RESULTS**

**Polycystic kidneys in Pkd1nl/nl mice**

By gene targeting we generated a mutant Pkd1 allele, Pkd1nl, harbouring a selection cassette containing the neomycin resistance gene (neo) flanked by loxP sites in intron 1 of Pkd1. A third loxP site was inserted into intron 11 to allow deletion of exons 2–11 (Fig. 1). Since it is known that the presence of a selection cassette may interfere with RNA processing (14), we intercrossed heterozygous mutant mice and analyzed the offspring. Mice homozygous for the Pkd1nl allele were born at the expected frequency (Table 1). Homozygous Pkd1nl/nl mice, however, showed growth retardation and early mortality. The severity of the symptoms varied among these mice. The majority of the Pkd1nl/nl mice had distended abdomens and a ~40% reduction in body weight at weaning compared with their littermates. These mice died within 1–2 months after birth. Sacrificed 1-month-old mice had pale, massively enlarged cystic kidneys (Fig. 2A). Notably, ~25% of the homozygous mice manifested a less severe phenotype and became fertile adults. Their body weights at age of weaning were ~10 g and these mice showed less enlarged kidneys (Table 2). Three animals (~10%) stayed alive for at least 1 year, the eldest mouse was sacrificed at 15 months of age.

Histological analysis of 1-month-old Pkd1nl/nl mice showed kidneys with dilated and cystic tubules and some small areas of mild fibrosis and mild inflammation. In the renal cortex of the most severely affected mice large cysts replaced most of the normal renal parenchyma (Fig. 3A and B).
15-month-old homozygous mouse also exhibited a bilaterally cystic phenotype, although the majority of the cysts were rather small. Kidneys of age-matched heterozygous and wild-type littermates showed no abnormalities. In addition, we observed no cysts in old heterozygous Pkd1<sup>nl/w</sup> mice (n = 7, age > 13 months) (Table 3).

Most cysts arise from the loops of Henle, distal tubules and collecting ducts, as defined by the segment-specific markers.
Tamm-Horsfall protein and Dolichos biflorus, respectively. Less than 10% of the cysts stained with proximal tubule marker megalin, and glomerular cysts were rarely identified (Fig. 3C–E).

Aberrant splicing in Pkd1

To assess whether the Pkd1 allele is a hypomorphic allele due to interference with normal splicing, we carried out reverse transcriptase–PCR analyses on RNA isolated from renal tissues, with primers specific for exons 1 and 2. A larger amplification product of 579 bp was observed in homozygous and heterozygous mice, whereas the normal littermates revealed only the expected 81 bp-sized PCR product (Fig. 2B). cDNA sequencing revealed that a 498 bp fragment was included by the use of cryptic splice sites from the neo cassette (Fig. 2C). We also identified two additional splice donor sites. All splice-variants use the same cryptic acceptor site, resulting in a frameshift and predicted to result in a premature translational stop (Fig. 2D).

To confirm that the phenotype of Pkd1

Homozygous offspring showed normal growth and no histological abnormalities up to 15 months of age (n = 5), thus eliminating the possibility that the pathogenic properties of the Pkd1

Intercrossing mice with a deletion of both the selection-cassette and the floxed part of the Pkd1 gene (exons 2–11) produced no viable offspring homozygous for the deletion, as expected from previously described mutant Pkd1-

Residual wild-type expression in Pkd1

Wild-type transcript was also present in Pkd1

Table 3. Cystic phenotype of Pkd1 mutant mice

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Kidney cysts</th>
<th>Liver cysts</th>
<th>Pancreatic cysts</th>
</tr>
</thead>
</table>
| Pkd1

+++, severe; +++, less severe; ±, mild or negative; −, negative; NA, not analyzed.

Figure 3. Renal and extrarenal pathology in Pkd1

Representative sections from homozygous 4–7-weeks-old Pkd1

Kidney sections from homozygous 4–7-weeks-old Pkd1

Liver sections stained with haematoxylin and eosin (HE) show many cysts. (A) 4-weeks-old mouse with a severe phenotype. (B) 4-weeks-old mouse with a severe phenotype. (C–E) Segment identity was characterized by staining with lectin Dolichos biflorus (C), as a marker for collecting tubules, and by using antisera directed against Tamm-Horsfall protein (D), as a marker for thick ascending limb and distal convoluted tubules, and collecting ducts. (F) Expression of polycystin-1 was detected in a subset of cysts using the monoclonal antibody PKS-A (12). (G) Pancreas and (H) liver sections stained with HE show the presence of bile duct dilatations and small cysts (cy) in Pkd1

Consecutive transverse sections of the descending aorta showed an intramural bleeding. Sections are stained with HE (I) and resorcin fuchsin to detect elastic lamels (J). The elastic lamels diverge by the intramural bleeding (asterisks).
transcript in the severe and less severely affected mice (Table 2). However, the few homozygous mice that stayed alive for more than 1 year showed distinct higher levels of normal Pkd1 expression (38% and 48%, Table 2), consistent with their mild cystic phenotype and extended lifespan.

Notably, real-time RT–PCR detected a 2–3-fold increase in the total amount of Pkd1 transcripts in the 4–8-weeks-old Pkd1nl nl mice compared with Pkd1+/+ controls (2.69 ± 0.67 versus 1.00 ± 0.40; P < 0.001), whereas no significant differences were found in heterozygous mice (1.08 ± 0.17) (Fig. 4B). Northern blot analysis confirmed the elevated expression of total Pkd1 in homozygous mice Pkd1nl nl, and the inclusion of neo-cassette-derived sequences in the Pkd1 transcripts (Fig. 2E). The upregulation may represent a continued immaturity of the epithelium or loss of terminal differentiation of the epithelium in the cystic kidneys in Pkd1nl nl mice. The latter seems more likely, as expression of Pkd2 was not increased in the cystic kidneys (1.13 ± 0.22; P = 0.21), while both Pkd1 and Pkd2 expression are highest in embryonic and neonatal kidneys (20). Notably, in human PKD1 kidneys upregulation of the normal transcript has also been reported (12).

Polycystin-1 expression in normal and cystic tubules

Consistent with the residual wild-type expression of the Pkd1nl allele, we observed staining for polycystin-1 in kidneys from Pkd1nl nl mice, using the monoclonal antibody PKS-A (12). Although the majority of the cysts in Pkd1nl nl mice were virtually negative for polycystin-1 staining, small and large polycystin-1 positive cysts were detected as well (Fig. 3F). A few cysts exhibited a heterogeneous staining pattern indicating variation in polycystin-1 expression. This patchy staining has been observed in human cystic kidneys as well and suggested to be dependent on the stage of cell division or other intracellular factors (21).

Extrarenal abnormalities in Pkd1nl nl mice

Only mild cystogenesis was observed in liver and pancreatic sections of Pkd1nl nl mice, concordant to human ADPKD. In the livers, either no cysts (6 of 10) or small cysts and bile duct dilatations (4 of 10) were detected, and in the pancreas, duct dilatation of ducts and occasionally a cyst (8 of 10) were detected (Fig. 3G and H) (Table 3).

Cardiovascular abnormalities, such as cardiac valve abnormalities, saccular intracranial aneurysms and dissecting thoracic aortic aneurysms are also associated with human ADPKD (22). Accordingly, cardiac septation defects and vascular fragility have been reported for Pkd1 and Pkd2 knockout embryos (17,18,23). Therefore, we analyzed the abdominal and thoracic aorta from six Pkd1nl nl animals, two of them presented in Table 2. All mice showed dissecting aneurysms, often accompanied with large intramural bleedings (Fig. 3i and j).

DISCUSSION

Our findings provide the first evidence that decreased levels of wild-type Pkd1 could lead to the clinical features of ADPKD. We showed that the intronic neomycin-insertion into Pkd1 had a profound effect on splicing efficiency, resulting in a mixture of mutant and wild-type sequences. Approximately 15–20% of the Pkd1 transcripts were correctly spliced in the majority of the homozygous Pkd1nl nl mice. These hypomorphic mice show that low levels of wild-type Pkd1 expression can overcome the embryonic lethality seen in knock-out mice where Pkd1 protein is absent. Although the hypomorphic Pkd1nl nl mice survive, they develop severe cystic kidneys as well as extrarenal features of ADPKD. Although the severity of the symptoms varied among the Pkd1nl nl mice, there was no apparent correlation between the phenotypic features and the
PKD2 has also been suggested with somatic mutations in part by acquired missense mutations resulting in mutant proteins that are still expressed (12). A classical two-hit model has some shortcomings, such as failure to identify somatic mutations in a significant number of cysts somatic mutations occurred via a separate event. The classical two-hit hypothesis is supported by studies showing loss of, or mutations in, the normal allele in renal cysts, leading to chronic renal failure in 50% of patients. However, no hot-spot for missense mutations has been identified (36). In the PKD2 mouse model develop aortic aneurysms only upon the induction of systemic hypertension (36). In the Pkd1nl/nl mice, aortic aneurysms are spontaneously formed, strongly suggesting that Pkd1-gene expression indeed is crucial for development and maintenance of the structural integrity of the blood vessel wall.

Phenotypic severity in homozygous mice was variable, with kidney/body weight ratios ranging from 2 to 33% and death occurring as early as day 25 and as late as 15 months. Death may be caused by renal failure. However, as we found aortic aneurysms in mice sacrificed at 4–8 weeks of age, rupture of aneurysms could also be the cause of early mortality. The mixed genetic background, 129Ola and C57Bl/6, is likely to contribute to the phenotypic variations, as genetic background has been reported to modulate cystic pathology in PKD mouse models (37). Stochastic fluctuations in gene expression, however, may also contribute to the

Table 4. Primer and probe sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ – 3’)</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-PCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pkd1 Forward</td>
<td>ACGCTAGGGCGCGATCTGCTG</td>
<td>Exon 2</td>
</tr>
<tr>
<td>Pkd1 Reverse</td>
<td>TATGTCCAGCGCTACAGCAGCTG</td>
<td>Exons 1–2</td>
</tr>
<tr>
<td>Pkd1 Taqman Probe</td>
<td>GCCACCCCGCTAGACCTG</td>
<td>Exons 1–2</td>
</tr>
<tr>
<td>Pkd1 Taqman Probe</td>
<td>TACGAAACAGCGCTTCCTTCTTAAATG</td>
<td>Exons 3</td>
</tr>
<tr>
<td>Pkd1 Taqman Probe</td>
<td>GTCTAACGTCAGCTGGAGCTG</td>
<td>Exons 3</td>
</tr>
<tr>
<td>Pkd1 Taqman Probe</td>
<td>CCGCTCCCGCTCACCCAGG</td>
<td>Exons 3</td>
</tr>
<tr>
<td>Pkd2 Forward</td>
<td>GCAGAAGCTATCTCCAGTG</td>
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</tr>
<tr>
<td>Pkd2 Reverse</td>
<td>TAGCTGCAATGGATGGG</td>
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<td>HpRT Forward</td>
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<td>HpRT Reverse</td>
<td>AACCTTTATGTCCGCCGTTG</td>
<td>Exon 4</td>
</tr>
<tr>
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<td>Exon 3</td>
</tr>
<tr>
<td>Pkd2 Taqman Probe</td>
<td>CTGTAGATTCTTATACGCTAAGACTGCTAAGCTTCTGTAATGCAA</td>
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<td>Pkd1 Taqman Probe</td>
<td>CACGCGGCCGCTACAGCTGG</td>
<td>Exons 13–14</td>
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<td>Pkd1 Taqman Probe</td>
<td>TGCCTTCCCGCTCACCCAGG</td>
<td>Exons 13–14</td>
</tr>
</tbody>
</table>

During life-time, ADPKD patients acquire 100–1000 of renal cysts, leading to chronic renal failure in 50% of patients by the age of 60 years (24). Two proposed mechanisms for focal cyst development are the occurrence of somatic mutations in the normal alleles (second hits) and gene-dosage effects. The second-hit hypothesis is supported by studies showing loss of, or mutations in, the normal allele in epithelial cells of a subset of cysts (9,10). In all those studies showing loss of, or mutations in, the normal allele in expression results in structural weakness of the arterial wall.

Relative levels of the normally spliced Pkd1 transcript except for the few animals with a much milder phenotype. It may be, however, that at the time of cyst-initiation the relative expression levels showed more variation due to stochastic fluctuations.
phenotypic variation. Interestingly, patients with ADPKD also show diversity in disease progression and in extra-renal manifestations. Although the factors that affect disease progression are not clear, modifying genes, environmental factors as well as stochastic effects on gene expression are probably involved (38).

Given the similarities to human polycystic kidney disease, Pkd1<sup>nl/nl</sup> mice may serve as an attractive model to study cyst and aneurysm formation and the effects of therapy. In particular, as Pkd1 knock-out mice are embryonic lethal and heterozygous knock-out mice show only a mild cystic phenotype at old age. Diets or drugs tested as potential therapeutic interventions sometimes show opposing effects, depending on the animal model used. For instance EGF-R antagonists have opposing effects in the HAN:SPRD-rat and in the PCK-rat models for PKD, emphasizing the need for models that mimic human disease (39,40). Promising therapeutic results have been obtained with a vasopressin V2 receptor antagonist. This drug, OPC31260, has now been evaluated in animal models for recessive PKD, and most recently in a Pkd2 mouse model but not yet in a Pkd1 mouse model (41,42).

Transgenic mice overexpressing Pkd1 also show cyst formation, although mainly of glomerular origin (13). Hence, a balanced Pkd1-dosage seems critical to establish and maintain intact renal epithelial architecture. Our findings suggest that in addition to somatic mutations, reduced Pkd1 expression of the normal allele due to genetic, environmental or stochastic factors may lead to clinical features of ADPKD in patients. Because steady-state expression levels of Pkd1 need to be retained within predetermined boundaries, we doubt whether modulation of PKD1 gene expression may ever become sufficiently precise to be successful as a treatment modality for ADPKD. Manipulation of downstream effects of aberrant PKD1 expression is likely to be more promising to slow down or prevent cyst growth.

**MATERIALS AND METHODS**

**Generation of Pkd1<sup>nl/nl</sup> mice**

A Pkd1 genomic clone was isolated from a mouse 129/SV PAC library (RPCI-21) and a 30 kb SacII fragment containing exon 1 was subcloned. A loxP-flanked neomycin resistance gene (neo) under the control of the phosphoglycerate kinase (PGK) promoter was cloned in antisense orientation into the Eco105I site in intron 1 and a synthetic double-stranded oligonucleotide containing a loxP site was cloned into the BglII site in intron 11. As a result, a 7 kb region containing exons 2–11 of the Pkd1 gene is flanked by loxP sites (Fig. 1). The targeting vector contained 8.5 kb of homologous DNA sequence on the short arm and 10 kb on the long arm. A thymidine kinase containing vector contained 8.5 kb of homologous DNA sequence on the short arm and 10 kb on the long arm. A thymidine kinase gene under the control of the PGK promoter was inserted at the end of the 3’-arm for counter-selection. The linearized targeting construct was electroporated into 129/Ola embryonic stem (ES) cells, which were then subjected to selection with G418. Homologous recombination was identified by Southern blot hybridizations in 10% of the G418-resistant clones using 5’- and 3’-external Pkd1 probes and a neo probe. Chimeric mice were obtained by injection of Pkd1<sup>nl/+</sup> ES cells into C57BL/6 blastocysts, and two independently targeted ES clones (#4 and #18) were found to transmit the Pkd1<sup>nl</sup> allele through the germline. The F1 and F2 progenies in mixed C57BL/6 × 129/Ola background were analyzed in this study. The genotypes were assessed by PCR analysis of tail genomic DNA.

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.

**Histological analysis**

Pkd1<sup>nl/nl</sup> mice and their heterozygous and wild-type littermates were sacrificed and their tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 4 or 5 μm and stained with haematoxylin and eosin, periodic acid-Schiff or resorcin fuchsin.

Kidney-segment identity was characterized using lectin Dolichos biflorus (Sigma-Aldrich, Zwijndrecht, The Netherlands), and antibodies against Tamm Horsfall protein (Cappel-Organon Teknika, Durham, NC, USA) and megalin (43). To detect polycystin-1, sections were stained with PKS-A, a monoclonal antibody directed to the C-terminal region of human polycystin-1 (12), in combination with the DAKO ARK animal research Kit (DAKO, Glostrup, Denmark).

**RNA analysis**

Tissues were snap-frozen in liquid nitrogen and stored at −80°C. We isolated total RNA from kidneys using TRIzol reagent (Invitrogen Life Technologies, Breda, The Netherlands) and cDNA was synthesized with SuperScript II (RNase H) reverse transcriptase (GibcoBRL, Breda, The Netherlands). An aliquot of 1 μg total RNA was reverse transcribed and amplified by PCR using Pkd1-specific and neo-cassette-specific primers. Each mutant transcript was sequenced.

Real-time PCR analysis of renal RNA samples was performed using TaqMan technology (PE Applied Biosystems, Nieuwerkerk a/d/ IJssel, The Netherlands) as published previously using oligo-dT and hexamer primers (44). We developed two Pkd1 PCR primer pairs: the 5’Pkd1 PCR specifically amplifies wild-type Pkd1 mRNA, and the 3’Pkd1 PCR amplifies both mutant and wild-type Pkd1 transcripts. The 5’ Pkd1 5’PCR forward primer was designed to span the exon 1–exon 2 boundary, and the reverse primer is situated in exon 3. The Pkd1 3’PCR forward primer spans the exon 45–exon 46 boundary, whereas the reverse primer is located in exon 46. Primer and probe sequences are presented in Table 4. The expression of the housekeeping gene HPRT (hypoxanthine guanine phosphoribosyl transferase) served as reference for gene expression. We performed all reactions in duplicate. For northern blot analysis, RNA (10 μg/lane) was separated on 1% agarose–formaldehyde gels, transferred to nylon membranes and hybridized to Pkd1, neo and glyceraldehyde-3-phosphate dehydrogenase probes.
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