Human Molecular Genetics, 2004, Vol. 13, No. 24 **3069–3077**
doi:10.1093/hmg/ddh336
Advance Access published on October 20, 2004

Lowering of *Pkd1* expression is sufficient to cause polycystic kidney disease

Irma S. Lantinga-van Leeuwen[1], Johannes G. Dauwerse[1], Hans J. Baelde[3], Wouter N. Leonhard[1], Annemieke van de Wal[3], Christopher J. Ward[4], Sjef Verbeek[1], Marco C. DeRuiter[2], Martijn H. Breuning[1], Emile de Heer[3] and Dorien J.M. Peters[1,*]

[1]Center for Human and Clinical Genetics and [2]Department of Anatomy and Embryology, Leiden University Medical Center, 2333 AL Leiden, The Netherlands, [3]Department of Pathology, Leiden University Medical Center, 2300 RC Leiden, The Netherlands and [4]Division of Nephrology, Mayo Clinic, Rochester, MN 55905, USA

Received July 9, 2004; Revised October 5, 2004; Accepted October 12, 2004

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, *Pkd1*^{nl}, 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 *Pkd1*^{nl} mice. Homozygous *Pkd1*^{nl} 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 *Pkd1*^{nl} 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).

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*To whom correspondence should be addressed at: Center for Human and Clinical Genetics, Leiden University Medical Center, Sylvius Laboratory, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands. Tel: +31 715276048; Fax: +31 715276075; Email: d.j.m.peters@lumc.nl

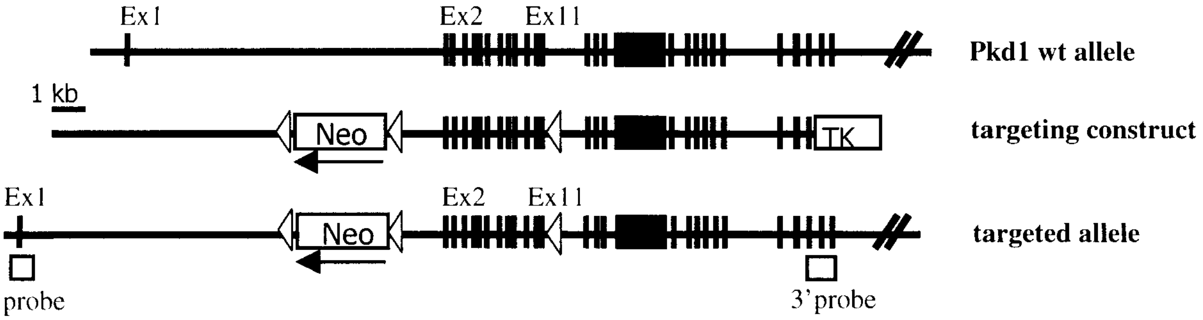

Figure 1. Generation of the *Pkd1* hypomorphic allele. A fragment of the genomic wild-type *Pkd1* locus is shown. The *Pkd1* targeting vector was constructed by inserting a *PGK* promoter-driven neomycin-resistance gene flanked by *loxP* sites into intron 1 and a third *loxP* site into intron 11. A 5′-probe covering exon 1, and a 3′-probe covering exons 24–26 from the *Pkd1* gene were used for genotyping by Southern blotting. PGK, phosphoglycerate kinase gene; Neo, neomycin phosphotransferase gene; TK, thymidine kinase gene; Ex, exon.

Table 1. Offspring of *Pkd1* mutant mice

	Pkd1$^{\mathrm{wt/wt}}$	*Pkd1*$^{\mathrm{wt/nl}}$	*Pkd1*$^{\mathrm{nl/nl}}$	Total
n (%)	36 (27%)	67 (50%)	32 (24%)	135

Genotypes of offspring from *Pkd1*$^{\mathrm{wt/nl}}$ × *Pkd1*$^{\mathrm{wt/nl}}$ intercrosses.

Table 2. Phenotypes of *Pkd1*$^{\mathrm{nl/nl}}$ mice

ID no.	Age (weeks)	Kidney weight (KW) (g)	Body weight (BW) (g)	KW/BW (%)	Weight at weaning ≥10 g	Wild-type/ total Pkd1 RNA (%)
269	3.5	2.8	8.9	33.3	No	13%
102	4	1.4	7.5	18.7	No	–
278	4	0.8	11.4	7.0	Yes	20%
279[a]	4	2.1	NA	NA	No	20%
503	4	1.8	7.5	23.2	No	–
506	4	2.1	9.5	22.3	No	–
508	4	1.9	9.2	20.3	No	–
520	4	1.2	12.8	9.7	Yes	13%
76	4.5	1.0	8.3	12.4	No	17%
50	5	1.1	6.3[b]	17.5	No	–
41	5.5	2.0	8.0	25.2	No	–
254	7	1.4	10.0	14.0	No	–
256	7	NA	8.6	–	No	18%
258	7	1.2	19.8	5.8	Yes	20%
242[a]	8	1.3	8.0	16.2	No	–
155	13 months	1.0	36.9	2.7	Yes	38%
146	15 months	0.6	27.3	2.2	Yes	48%
Controls	4–8	0.2–0.3	15–21	1–2	Yes	100%

Kidney and body weights of homozygous *Pkd1* mice. Mouse numbers 278, 520, 258, 155 and 146 showed a less severe growth retardation. In these mice the kidney weight (KW) as percentage of body weight (BW) was <10%, and body weight at weaning (3–4 weeks) was ≥10 g. Wild-type ($n = 4$) and heterozygous ($n = 6$) *Pkd1* littermates had normal KW/BW ratios of ~1.5%.

[a] Mice analyzed for the presence of aneurysms.

[b] Dead at time of analysis.

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 *Pkd1*$^{\mathrm{nl/nl}}$ mice

By gene targeting we generated a mutant *Pkd1* allele, *Pkd1*$^{\mathrm{nl}}$, 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 *Pkd1*$^{\mathrm{nl}}$ allele were born at the expected frequency (Table 1). Homozygous *Pkd1*$^{\mathrm{nl/nl}}$ mice, however, showed growth retardation and early mortality. The severity of the symptoms varied among these mice. The majority of the *Pkd1*$^{\mathrm{nl/nl}}$ 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 *Pkd1*$^{\mathrm{nl/nl}}$ 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). The

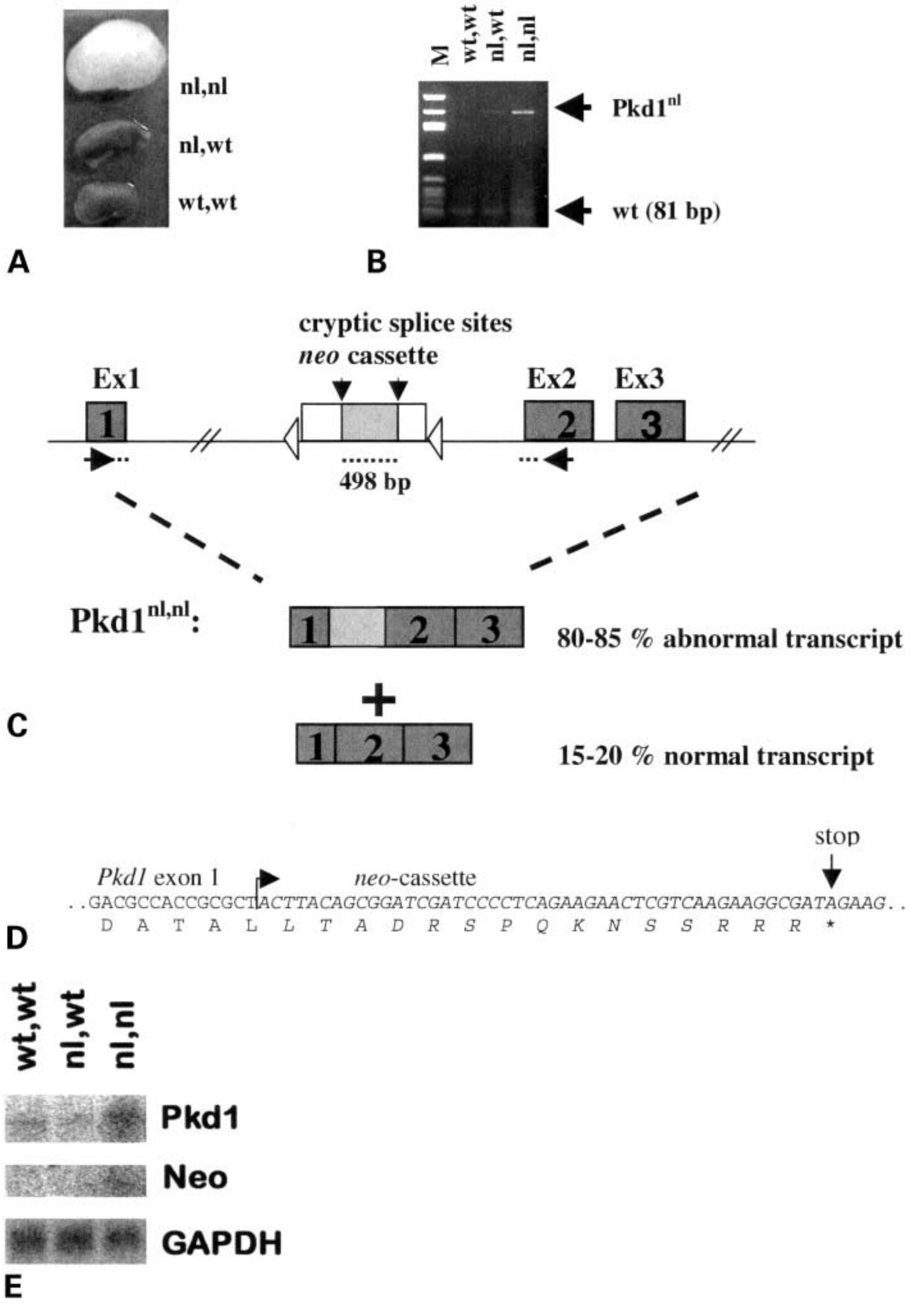

Figure 2. Polycystic kidneys and structure of *Pkd1* transcripts in *Pkd1*$^{nl/nl}$ mice. (**A**) Kidney from a 1-month-old *Pkd1*$^{nl/nl}$ mouse is enlarged, pale, and cystic, compared with kidneys from heterozygous and wild-type littermates. (**B**) Schematic representation of *Pkd1* transcripts in *Pkd1*$^{nl/nl}$ mice. RT-PCR on renal RNA with primers in *Pkd1* exons 1 and 2 showing the expected 81 bp fragment in normal mice and an additional 579 bp fragment, containing 498 bp of the selection cassette sequence, in heterozygous and homozygous *Pkd1*^{nl} mice. M, marker. (**C**) Alternative splicing of *Pkd1* results in a mixture of wild-type and mutant transcripts. Ex, exon (only three of the 46 *Pkd1* exons are shown). (**D**) Nucleotide and predicted amino acid sequences of the mutant *Pkd1* transcripts that result in an early protein translation stop (asterisk). (**E**) Northern blot analysis using a *Pkd1* exon 15 probe demonstrated the ~14 kb *Pkd1* transcript in 7-weeks-old wild-type (lane 1), heterozygous (lane 2) and homozygous (lane 3) littermates. Hybridization of the same filter with a *neo* cDNA probe showed the inclusion of *neo*-cassette-derived sequences in the *Pkd1* transcript of mutant mice. Glyceraldehyde 3-phospate dehydrogenase (GAPDH) was used as a loading control.

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*$^{nl/+}$ 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

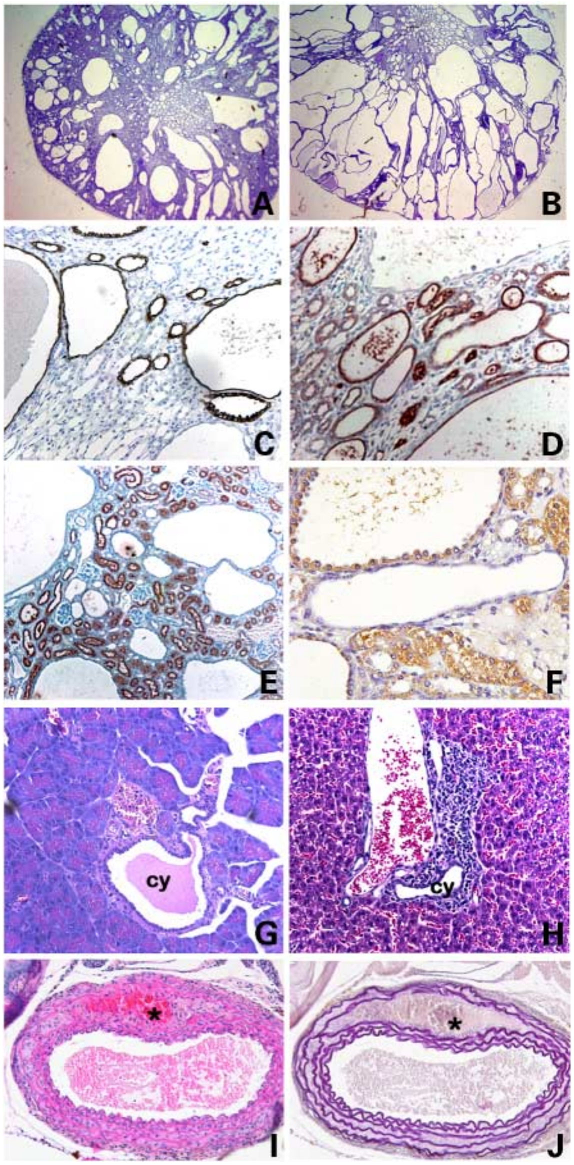

Figure 3. Renal and extrarenal pathology in *Pkd1*$^{\mathrm{nl/nl}}$ mice. (**A–F**) Kidney sections from homozygous 4–7-weeks-old *Pkd1*$^{\mathrm{nl/nl}}$ mice. Sections stained with haematoxylin and eosin (HE) show many cysts. (A) 4-weeks-old mouse with a less severe phenotype. (B) 4-weeks-old mouse 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 megalin (E), a proximal tubule marker. The majority of the cysts are derived from loops of Henle, distal-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*$^{\mathrm{nl/nl}}$ mice. (**I** and **J**) Consecutive transverse sections of the descending aorta showed an intramural bleeding. Sections are stained with HE (I) and resorchin fuchsin to detect elastic lamels (J). The elastic lamels diverge by the intramural bleeding (asteriks).

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).

Table 3. Cystic phenotype of *Pkd1* mutant mice

	Kidney cysts	Liver cysts	Pancreatic cysts
$\mathrm{Pkd1}^{\mathrm{nl/nl}}$ 4–8 weeks	++/+++ ($n = 15$) all bilateral	± ($n = 10$)	± ($n = 10$)
$\mathrm{Pkd1}^{\mathrm{wt/nl}}$ >13 months	−($n = 7$)	NA	NA

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

Aberrant splicing in *Pkd1*$^{\mathrm{nl/nl}}$ mice

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*$^{\mathrm{nl/nl}}$ mice is due to aberrant splicing of *Pkd1*, we removed the *neo* cassette by crossbreeding the mice with EIIa-Cre mice (15). These mice mosaically express Cre-recombinase in the germ-line, such that partial or complete excision of loxP-flanked DNA sequences occurs. In this way, we obtained mice in which the *neo* cassette was removed but leaving *Pkd1* unchanged. 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*$^{\mathrm{nl/nl}}$ mice are due to mutations introduced during the ES cell-targeting. 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*-mice (16–19).

Residual wild-type expression in *Pkd1*$^{\mathrm{nl/nl}}$ mice

Wild-type transcript was also present in *Pkd1*$^{\mathrm{nl/nl}}$ mice, showing that natural splice sites are also used. We quantified the proportion of wild-type *Pkd1* transcripts with a real-time PCR assay. We designed a PCR primer pair in the 5′ part of the *Pkd1* gene (5′PCR) that specifically amplified wild-type transcripts and a second PCR primer pair in the 3′ part of the *Pkd1* gene (3′PCR) that amplified both wild-type and splice-variant transcripts. The 3′PCR versus 5′PCR ratio's showed that in the majority of *Pkd1*$^{\mathrm{nl/nl}}$ mice 13–20% of the *Pkd1* transcripts are spliced as in wild-type mice (Fig. 4A; Table 2). Consistently, heterozygous mice have an average of 55% correctly spliced *Pkd1* transcripts. In general, there was no apparent correlation between the phenotypic features and the relative levels of normal *Pkd1*

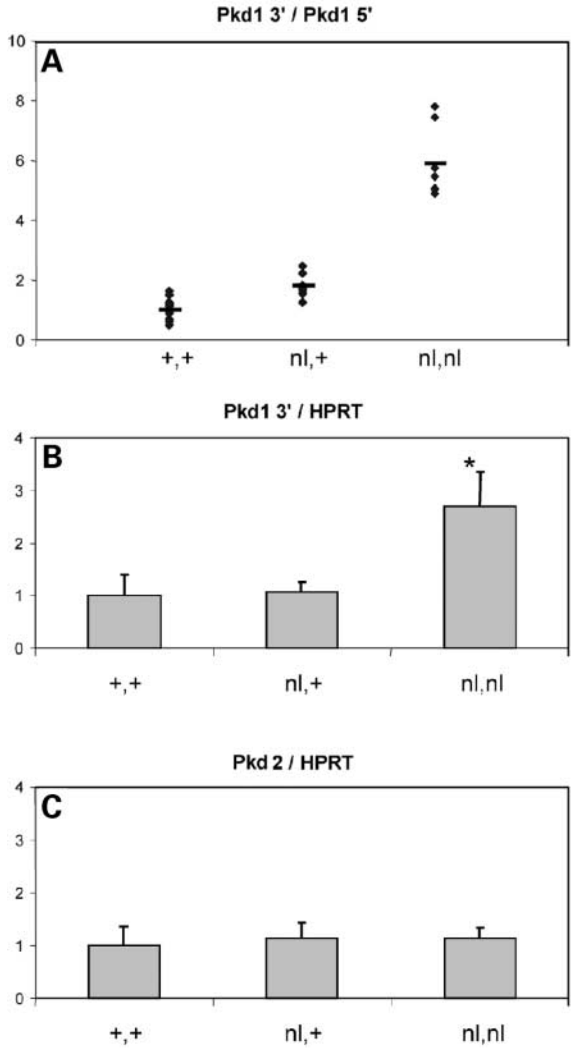

Figure 4. Real-time PCR analysis of renal RNA samples of 4–8-weeks-old *Pkd1*$^{nl/nl}$ mice. Means of duplicate measurements are shown. (**A**) Wild-type *Pkd1* transcripts relative to total *Pkd1* mRNA. The ratio of *Pkd1* 3′PCR versus *Pkd1* 5′PCR is normalized to the mean ratio of 10 wild-type littermates, which has been set to 1. Homozygous *Pkd1*$^{nl/nl}$ mice show an ∼6-fold increase in ratio, indicating that one-sixth (∼17%) of the transcripts are correctly spliced. (**B**) *Pkd1* (*Pkd1* 3′PCR) and (**C**) *Pkd2* expression normalized to expression of the *HPRT* gene that served as reference. Error bars represent standard error. The data represent the mean ± SE of 7–10 animals. Asteriks (*) indicate significant difference from normal, $P < 0.05$ (Student's *t*-test).

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 *Pkd1*$^{nl/nl}$ 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 *Pkd1*$^{nl/nl}$, 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 *Pkd1*$^{nl/nl}$ 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 *Pkd1*^{nl} allele, we observed staining for polycystin-1 in kidneys from *Pkd1*$^{nl/nl}$ mice, using the monoclonal antibody PKS-A (12). Although the majority of the cysts in *Pkd1*$^{nl/nl}$ 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 *Pkd1*$^{nl/nl}$ mice

Only mild cystogenesis was observed in liver and pancreatic sections of *Pkd1*$^{nl/nl}$ 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 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 *Pkd1*$^{nl/nl}$ 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 *Pkd1*$^{nl/nl}$ 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 *Pkd1*$^{nl/nl}$ mice survive, they develop severe cystic kidneys as well as extrarenal features of ADPKD. Although the severity of the symptoms varied among the *Pkd1*$^{nl/nl}$ mice, there was no apparent correlation between the phenotypic features and the

Table 4. Primer and probe sequences

Primer	Sequence (5′ − 3′)	Position
Real-time PCR		
5′Pkd1 Forward	GCCACCGCGCTAGACCTG	Exons 1–2
5′Pkd1 Reverse	TAGCAAACACGCCTTCTTCTAATG	Exon 3
5′Pkd1 Taqman Probe	GCTTAAGTCCAGCTCCACCAGTGCTGAAAG	Exons 2–3
3′Pkd1 Forward	TCAAGGAGTTCCGCCACAAAG	Exons 45–46
3′Pkd1 Reverse	CTGGACGAGGTGGATGGGT	Exon 46
3′Pkd1 Taqman Probe	TGCCTTCCCGCTCATCCAGGG	Exon 46
Pkd2 Forward	GGGAAGCATCTCCAGTGGG	Exon 13
Pkd2 Reverse	GATGCTGCCAATGGAGTGC	Exon 14
Pkd2 Taqman Probe	CCACGCGCCTCACCAGTACTTGGAA	Exons 13–14
HPRT Forward	GGCTATAAGTTCTTTGCTGACCTG	Exon 3
HPRT Reverse	AACTTTTATGTCCCCCGTTGA	Exon 4
HPRT Taqman Probe	CTGTAGATTTTATCAGACTGAAGAGCTACTGTAATGACCA	Exon 3
RT-PCR		
Pkd1 Forward	ACGCTAGGGCCGAGTCTG	Exon 1
Pkd1 Reverse	TATGTCCAGCGTCTGAAGTAGG	Exon 2

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.

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 the epithelial cells of a subset of cysts (9,10). In all those cysts somatic mutations occurred via a separate event. The model, however, has some shortcomings, such as failure to identify somatic mutations in a significant number of cysts and expression of polycystin-1 in a subset of cysts in kidneys of *PKD1*-patients. These findings may be explained in part by acquired missense mutations resulting in mutant proteins that are still expressed (12). A *trans*-heterozygous two-hit model has also been suggested with somatic mutations in the *PKD2* gene in a *PKD1* kidney, and vice versa (25,26). However, no hot-spot for missense mutations has been identified and *trans*-heterozygous mutations have been identified in only a small fraction of cysts. In addition, severely affected cystic kidneys in a group of early-onset ADPKD patients would require a large number of second hits within the first few months of life (21,27).

The alternative hypothesis for cystogenesis implies that mutations in one allele may be sufficient to cause cyst formation. This gene-dosage-effect is called haploinsufficiency. In general, gene expression levels show considerably more variation in the presence of only one functional allele (28). Therefore, stochastic fluctuations in *Pkd1*-gene dosage, below a tissue-specific threshold, may interfere with the control of processes involved in maintenance of the renal epithelial architecture. Interestingly, a growing number of reports are supporting the notion of haploinsufficiency in tumorigenesis for a variety of tumors, in addition to Knudson's classical two-hit model. Like cystogenesis in ADPKD, these tumours have a relatively late age of onset (29–31).

Consistent with human ADPKD, most cysts arise from the loops of Henle, distal tubules and collecting ducts, as defined by segment specific markers, and glomerular cysts were only rarely observed. Mice also showed mild cyst formation in the pancreas and liver. In contrast, massive cystic dilatation of pancreatic ducts has been reported for homozygous *Pkd1* knock-out embryos, and these pancreatic cysts occurred well before the renal cysts appeared (16,19).

Pkd1$^{nl/nl}$ mice also develop aortic aneurysms, frequently accompanied by intramural bleedings. In ADPKD patients, aortic aneurysms have also been reported (32). However, the prevalence of cerebral aneurysms has been studied more extensively and is increased ~10-fold compared with the general population (33–35). It seems that reduced gene expression results in structural weakness of the arterial wall that, influenced by hemodynamic factors, cause aneurysm formation. Homozygous *Pkd1* and *Pkd2* knock-out mice die *in utero* having massive oedema and focal haemorrhages (16,23), and a heterozygous *Pkd2* mouse model develop aneurysms only upon the induction of systemic hypertension (36). In the *Pkd1*$^{nl/nl}$ 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 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*$^{nl/nl}$ 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 of the recessive form of 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*$^{nl/nl}$ mice

A *Pkd1* genomic clone was isolated from a mouse 129/SV PAC library (RPCI-21) and a 30 kb *Sac*II 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 *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. 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 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*$^{nl/+}$ ES cells into C57BL/6 blastocysts, and two independently targeted ES clones (#4 and #18) were found to transmit the *Pkd1*^{nl} 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$^{nl/nl}$ 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 resorchin 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-phospate dehydrogenase probes.

ACKNOWLEDGEMENTS

We thank Yvonne de Jong, Jos van der Kaa and Jill Claassen for their technical assistance, and Stefan White for reading the manuscript. This research was supported by grants from the Dutch Kidney Foundation (98.1745) and the Netherlands Organisation for Scientific Research (NWO) (VIDI 016.036.353), and the Center of Medical System Biology (CMSB) established by the Netherlands Genomics Initiative/Netherlands Organisation for Scientific Research (NGI/NWO).

REFERENCES

1. Gabow, P.A. (1990) Autosomal dominant polycystic kidney disease: more than a renal disease. *Am. J. Kidney Dis.*, **16**, 403–413.
2. Peters, D.J.M. and Sandkuijl, L.A. (1992) Genetic heterogeneity of polycystic kidney disease in Europe. In Breuning, M.H., Devoto, M., Romeo, G. (eds). *Contributions to Nephrology*: *Polycystic Kidney Disease* Karger, Basel, Vol. 97, pp. 128–139.
3. The European Polycystic Kidney Disease Consortium (1994) The polycystic kidney disease 1 gene encodes a 14 kb transcript and lies within a duplicated region on chromosome 16. *Cell*, **77**, 881–894.
4. Wilson, P.D. (2004) Polycystic kidney disease. *N. Engl. J. Med.*, **350**, 151–164.
5. Tsiokas, L., Kim, E., Arnould, T., Sukhatme, V.P. and Walz, G. (1997) Homo- and heterodimeric interactions between the gene products of PKD1 and PKD2. *Proc. Natl Acad. Sci. USA*, **94**, 6965–6970.
6. Gonzalez-Perrett, S., Kim, K., Ibarra, C., Damiano, A.E., Zotta, E., Batelli, M., Harris, P.C., Reisin, I.L., Arnaout, M.A. and Cantiello, H.F. (2000) Polycystin-2, the protein mutated in autosomal dominant polycystic kidney disease (ADPKD), is a Ca2 + -permeable nonselective cation channel. *Proc. Natl Acad. Sci. USA*, **98**, 1182–1187.
7. Hanaoka, K., Qian, F., Boletta, A., Bhunia, A.K., Piontek, K., Tsiokas, L., Sukhatme, V.P., Guggino, W.B. and Germino, G.G. (2000) Co-assembly of polycystin-1 and -2 produces unique cation-permeable currents. *Nature*, **408**, 990–994.
8. Reeders, S.T. (1992) Multilocus polycystic disease. *Nature Genet.*, **1**, 235–237.
9. Qian, F.J., Watnick, T.J., Onuchic, L.F. and Germino, G.G. (1996) The molecular basis of focal cyst formation in human autosomal dominant polycystic kidney disease. *Cell*, **87**, 979–987.
10. Pei, Y., Watnick, T., He, N., Wang, K., Liang, Y., Parfrey, P., Germino, G. and St George-Hyslop, P. (1999) Somatic PKD2 mutations in individual kidney and liver cysts support a ‘two-hit’ model of cystogenesis in type 2 autosomal dominant polycystic kidney disease. *J. Am. Soc. Nephrol.*, **10**, 1524–1529.
11. Wu, G., D’Agati, V., Cai, Y., Markowitz, G., Park, J.H., Reynolds, D.M., Maeda, Y., Le, T.C., Hou, H., Jr, Kucherlapati, R. *et al.* (1998) Somatic inactivation of Pkd2 results in polycystic kidney disease. *Cell*, **93**, 177–188.
12. Ward, C.J., Turley, H., Ong, A.C.M., Comley, M., Biddolph, S., Chettty, R., Ratcliffe, P.J., Gattner, K. and Harris, P.C. (1996) Polycystin, the polycystic kidney disease 1 protein, is expressed by epithelial cells in fetal, adult and polycystic kidney. *Proc. Natl Acad. Sci. USA*, **93**, 1524–1528.
13. Pritchard, L., Sloane-Stanley, J.A., Sharpe, J.A., Aspinwall, R., Lu, W., Buckle, V., Strmecki, L., Walker, D., Ward, C.J., Alpers, C.E. *et al.* (2000) A human PKD1 transgene generates functional polycystin-1 in mice and is associated with a cystic phenotype. *Hum. Mol. Genet.*, **9**, 2617–2627.
14. Lewandoski, M. (2001) Conditional control of gene expression in the mouse. *Nat. Rev. Genet.*, **2**, 743–755.
15. Lakso, M., Pichel, J.G., Gorman, J.R., Sauer, B., Okamoto, Y., Lee, E., Alt, F.W. and Westphal, H. (1996) Efficient *in vivo* manipulation of mouse genomic sequences at the zygote stage. *Proc. Natl Acad. Sci. USA*, **93**, 5860–5865.
16. Lu, W., Peissel, B., Babakhanlou, H., Pavlova, A., Geng, L., Fan, X., Larson, C., Brent, G. and Zhou, J. (1997) Perinatal lethality with kidney and pancreas defects, in mice with a targetted *Pkd1* mutation. *Nature Genet.*, **17**, 179–181.
17. Kim, K., Drummond, I., Ibraghimov-Beskrovnaya, O., Klinger, K. and Arnaout, M.A. (2000) Polycystin 1 is required for the structural integrity of blood vessels. *Proc. Natl Acad. Sci. USA*, **97**, 1731–1736.
18. Boulter, C., Mulroy, S., Webb, S., Fleming, S., Brindle, K. and Sandford, R. (2001) Cardiovascular, skeletal, and renal defects in mice with a targeted disruption of the Pkd1 gene. *Proc. Natl Acad. Sci. USA*, **98**, 12174–12179.
19. Wu, G., Tian, X., Nishimura, S., Markowitz, G.S., D’Agati, V., Park, J.H., Yao, L., Li, L., Geng, L., Zhao, H. *et al.* (2002) *trans*-Heterozygous *Pkd1* and *Pkd2* mutations modify expression of polycystic kidney disease. *Hum. Mol. Genet.*, **11**, 1845–1854.
20. Guillaume, R. and Trudel, M. (2000) Distinct and common developmental expression patterns of the murine *Pkd2* and *Pkd1* genes. *Mech. Dev.*, **93**, 179–183.
21. Ong, A.C., Harris, P.C., Davies, D.R., Pritchard, L., Rossetti, S., Biddolph, S., Vaux, D.J., Migone, N. and Ward, C.J. (1999) Polycystin-1 expression in PKD1, early-onset PKD1, and TSC2/PKD1 cystic tissue. *Kidney Int.*, **56**, 1324–1333.
22. Perrone, R.D. (1997) Extrarenal manifestations of ADPKD. *Kidney Int.*, **51**, 2022–2036.
23. Wu, G., Markowitz, G.S., Li, L., D’Agati, V.D., Factor, S.M., Geng, L., Tibara, S., Tuchman, J., Cai, Y., Park, J.H. *et al.* (2000) Cardiac defects and renal failure in mice with targeted mutations in Pkd2. *Nat. Genet.*, **24**, 75–78.
24. Hateboer, N., Dijk, M.A., Coto, E., Saggar-Malik, A.K., San Millan, J.L., Torra, R., Breuning, M. and Ravine, D. (1999) Comparison of phenotypes of polycystic kidney disease types 1 and 2. *Lancet*, **353**, 103–107.
25. Watnick, T., He, N., Wang, K., Liang, Y., Parfrey, P., Hefferton, D., St George-Hyslop, P., Germino, G. and Pei, Y. (2000) Mutations of *PKD1* in *ADPKD2* cysts suggest a pathogenic effect of *trans*-heterozygous mutations. *Nat. Genet.*, **25**, 143–144.
26. Koptides, M., Mean, R., Demetriou, K., Pierides, A. and Deltas, C.C. (2000) Genetic evidence for a *trans*-heterozygous model for cystogenesis in autosomal dominant polycystic kidney disease. *Hum. Mol. Genet.*, **9**, 447–452.
27. Brook-Carter, Ph.T., Peral, B., Ward, C.J., Thompson, P., Hughes, J., Maheshwar, M.M., Nellist, M., Gamble, V., Harris, P.C. and Sampson, J.R. (1994) Deletion of the *TSC2* and *PKD1* genes associated with severe infantile polycystic kidney disease—a contiguous gene syndrome. *Nat. Genet.*, **8**, 328–332.
28. Cook, D.L., Gerber, A.N. and Tapscott, S.J. (1998) Modeling stochastic gene expression: implications for haploinsufficiency. *Proc. Natl Acad. Sci. USA*, **95**, 15641–15646.
29. Knudson, A.G. (1971) Mutation and cancer: statistical study of retinoblastoma. *Proc. Natl Acad. Sci. USA*, **68**, 820–823.
30. Song, W.J., Sullivan, M.G., Legare, R.D., Hutchings, S., Tan, X., Kufrin, D., Ratajczak, J., Resende, I.C., Haworth, C., Hock, R. *et al.* (1999) Haploinsufficiency of CBFA2 causes familial thrombocytopenia with propensity to develop acute myelogenous leukaemia. *Nat. Genet.*, **23**, 166–175.
31. Fodde, R. and Smits, R. (2002) Cancer biology. A matter of dosage. *Science*, **298**, 761–763.
32. Torra, R., Nicolau, C., Badenas, C., Bru, C., Perez, L., Estivill, X. and Darnell, A. (1996) Abdominal aortic aneurysms and autosomal dominant polycystic kidney disease. *J. Am. Soc. Nephrol.*, **7**, 2483–2486.
33. Chapman, A.B., Rubinstein, D., Hughes, R., Stears, J.C., Earnest, M.P., Johnson, A.M., Gabow, P.A. and Kaehny, W.D. (1992) Intracranial aneurysms in autosomal dominant polycystic kidney disease. *N. Engl. J. Med.*, **327**, 916–920.
34. Huston, J. III, Torres, V.E., Sulivan, P.P., Offord, K.P., Wiebers, D.O. (1993) Value of magnetic resonance angiography for the detection of intracranial aneurysms in autosomal dominant polycystic kidney disease. *J. Am. Soc. Nephrol*, **3**, 1871–1877.
35. Wakabayashi, T., Fujita, S., Ohbora, Y., Suyama, T., Tamaki, N. and Matsumoto, S. (1983) Polycystic kidney disease and intracranial aneurysms. Early angiographic diagnosis and early operation for the unruptured aneurysm. *J. Neurosurg.*, **58**, 488–491.
36. Qian, Q., Hunter, L.W., Li, M., Marin-Padilla, M., Prakash, Y.S., Somlo, S., Harris, P.C., Torres, V.E. and Sieck, G.C. (2003) Pkd2 haploinsufficiency alters intracellular calcium regulation in vascular smooth muscle cells. *Hum. Mol. Genet.*, **12**, 1875–1880.

37. Guay-Woodford, L.M. (2003) Murine models of polycystic kidney disease: molecular and therapeutic insights. *Am. J. Physiol. Renal Physiol.*, **285**, F1034–F1049.
38. Peters, D.J.M. and Breuning, M.H. (2001) Autosomal dominant polycystic kidney disease: modification of disease progression. *Lancet*, **358**, 1439–1444.
39. Torres, V.E., Sweeney, W.E. Jr., Wang, X., Qian, Q., Harris, P.C., Frost, P. and Avner, E.D. (2003) EGF receptor tyrosine kinase inhibition attenuates the development of PKD in Han:SPRD rats. *Kidney Int.*, **64**, 1573–1579.
40. Torres, V.E., Sweeney, W.E., Wang, X., Qian, Q., Harris, P.C., Frost, P. and Avner, E.D. (2003) Inhibition of epidermal growth factor receptor tyrosin Kinase activity worsens the polycystic kidney disease in PCK rats. *J. Am. Soc. Nephrol.*, **14**, 109A–110A.
41. Gattone, V.H., Wang, X., Harris, P.C. and Torres, V.E. (2003) Inhibition of renal cystic disease development and progression by a vasopressin V2 receptor antagonist. *Nat. Med.*, **9**, 1323–1326.
42. Torres, V.E., Wang, X., Qian, Q., Somlo, S., Harris, P.C. and Gattone, V.H. (2004) Effective treatment of an orthologous model of autosomal dominant polycystic kidney disease. *Nat. Med.*, **10**, 363–364.
43. Verroust, P.J., Birn, H., Nielsen, R., Kozyraki, R. and Christensen, E.I. (2002) The tandem endocytic receptors megalin and cubilin are important proteins in renal pathology. *Kidney Int.*, **62**, 745–56.
44. Koop, K., Eikmans, M., Baelde, H.J., Kawachi, H., De Heer, E., Paul, L.C. and Bruijn, J.A. (2003) Expression of podocyte-associated molecules in acquired human kidney diseases. *J. Am. Soc. Nephrol.*, **14**, 2063–2071.