Perinatal lethality with kidney and pancreas defects in mice with a targetted *Pkd1* mutation

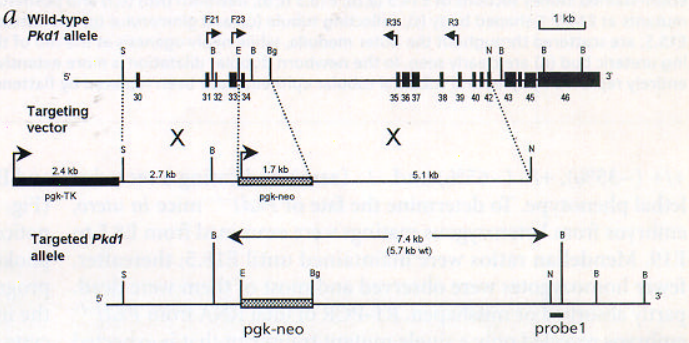
Weining Lu[1], Bernard Peissel[1], Hermik Babakhanlou[1], Anna Pavlova[1], Lin Geng[1], Xiaohong Fan[1], Cecilia Larson[2], Gregory Brent[2] & Jing Zhou[1]

***PKD1* is the most common site for mutations in human autosomal dominant polycystic kidney disease (ADPKD). ADPKD is characterized by progressive replacement of kidney tissue by epithelial cysts and eventual renal failure. Hepatic and pancreatic cysts are also common[1]. The PKD1 protein, polycystin, is a cell-surface protein[2,3] of unknown function that is widely expressed in epithelia and in vascular smooth muscle and myocardium[4–9]. None of the genetic forms of murine polycystic disease map to the murine *Pkd1* locus. We introduced into mice by homologous recombination a *Pkd1* truncation mutation, *Pkd1*$^{-}$, that mimics a mutation found in ADPKD. *Pkd1*$^{-}$ heterozygotes have no discernible phenotype, whereas homozygotes die during the perinatal period with massively enlarged cystic kidneys, pancreatic ductal cysts and pulmonary hypoplasia. Renal cyst formation begins at embryonic day 15.5 (E15.5) in proximal tubules and progresses rapidly to replace the entire renal parenchyma. The timing of cyst formation indicates that full-length polycystin is required for normal morphogenesis during elongation and maturation of tubular structures in the kidney and pancreas.**

Most of the known *PKD1* mutations are predicted to cause major disruption of the structure of polycystin[10]. We previously characterized a mutation that introduces a stop at codon 3532 of *PKD1*, and is predicted to generate a truncated polycystin lacking several transmembrane domains and the entire intracellular domain[11]. To mimic the effects of this mutation in the mouse, we created, by homologous recombination in embryonic stem cells, a mutant allele (*Pkd1*$^{-}$) in which exon 34 of *Pkd1* is replaced by a cassette expressing the selectable marker neomycin transferase (Fig 1*a*). *Pkd1*$^{-}$ contains a reading frameshift and encodes a polypeptide truncated by 836 residues. Four recombinant embryonic stem-cell clones were found to be heterozygous for *Pkd1*$^{-}$. Three clones produced chimaeric mice that were crossed with C57BL/6 or BALB/C mice to produce *Pkd1*$^{+/-}$ progeny. The phenotypes described below were not significantly influenced by genetic background.

More than 150 *Pkd1*$^{+/-}$ mice followed for seven months did not exhibit any discernible phenotype. Examination of brain, heart, lung, pancreas, kidney, liver, major vessels and spleen from the oldest mice (220 days) revealed normal anatomy and histology. No cysts were observed on serial section of entire kidneys from four mice.

Among 71 fully weaned mice from heterozygous intercrosses, distribution of genotypes differed significantly from expectation:



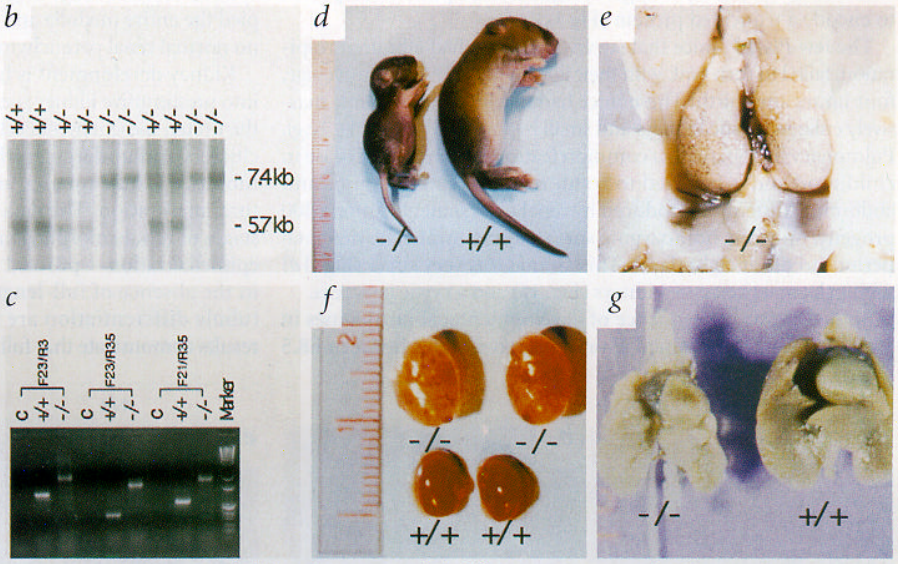


Fig. 1 Targetted disruption of *Pkd1* and generation of *Pkd1*$^{-/-}$ mice. ***a***, Structures of wild-type and targetted *Pkd1* alleles. Exon 34 and parts of introns 33 and 34 of *Pkd1* were replaced by a phosphoglycerate kinase (pgk) promoter-driven neomycin (neo)-resistant gene. Single arrows indicate the direction of transcription. TK, thymidine kinase; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; N, *Nae*I; S, *Spe*I. F21, F23, R3 and R35 are primers used to detect transcripts shown in *c*. ***b***, DNA isolated from F2 littermates was digested with *Bam*HI and hybridized with a 0.2-kb genomic fragment (probe1). The 5.7-kb and 7.4-kb bands correspond to the wild-type and mutant alleles, respectively. ***c***, RT-PCR of *Pkd1* transcripts in wild-type (+/+) and homozygous (–/–) whole embryos using primers F21, F23, R3 and R35, whose positions are shown in *a*. C, control without reverse transcriptase. DNA sequencing of the PCR products revealed that the mutant transcript contains a stop codon just beyond exon 34. ***d***,***f***, Eight-day littermates showing reduced body size and enlarged kidneys in –/– mutants. ***e***,***g***, Gross appearances of the kidney and lungs, respectively, of newborn mutants. Note the cystic kidneys and smaller lungs in –/– mutants.

[1]*Renal and* [2]*Thyroid Divisions, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, Massachusetts 02115, USA. Correspondence should be addressed to J.Z. e-mail: zhou@mbcrr.harvard.edu*

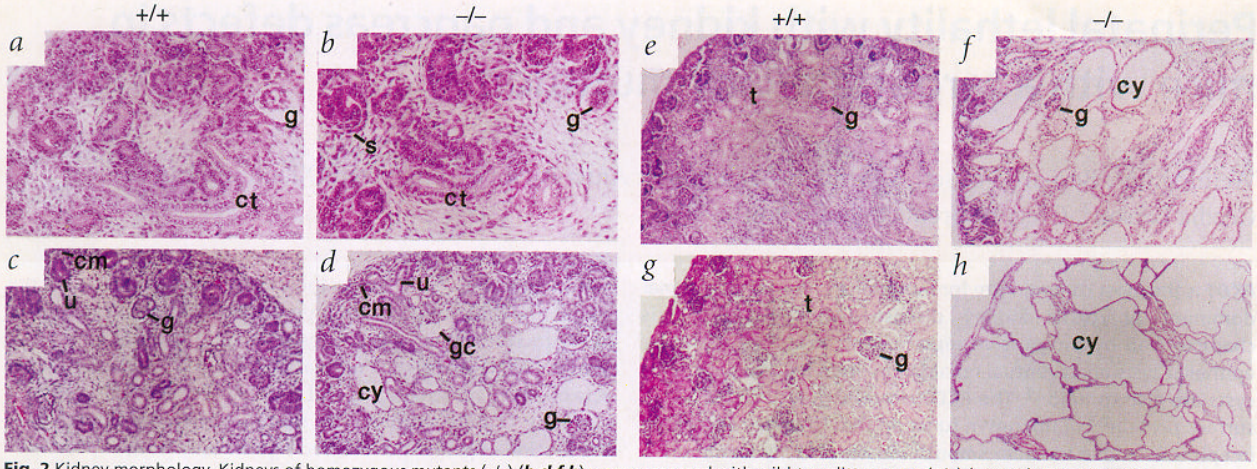


Fig. 2 Kidney morphology. Kidneys of homozygous mutants (–/–) (***b,d,f,h***) were compared with wild-type littermates (+/+) (***a,c,e,g***). Haematoxylin-eosin–stained kidney sections of E14.5 (*a,b*), E15.5 (*c,b*), newborn (NB) (*e,f*) and postnatal day 8 (P8) (*g,h*) revealed normal kidney development in *Pkd1*$^{-/-}$ mutants at E14.5: S-shaped body (s), collecting tubule (ct) and glomerulus (g) are histologically normal. Tubular and periglomerular cysts (cy, gc), first noted at E15.5, are scattered throughout the outer medulla, while nephrogenesis at the rim of the kidney proceeds normally. Condensed mesenchyme (cm) and branching ureteric bud (u) are clearly seen. In the newborn, tubular dilatation is more extensive, affecting most of the kidney. By P8, renal parenchyma is almost entirely replaced by cysts, and cuboidal tubular epithelia have been replaced by flattened cyst-lining epithelia. Magnification in *a,b* ×100; *c–h* ×50.

+/+ (~35%), +/– (~65%) and –/– (zero), indicating a recessive lethal phenotype. To determine the fate of *Pkd1*$^{-/-}$ mice *in utero*, embryos from heterozygous matings were examined from E8.5 to E19. Mendelian ratios were maintained until E18.5; thereafter, fewer homozygotes were observed and most of them were dead, partly absorbed or misshapen. RT-PCR of total RNA from *Pkd1*$^{-/-}$ embryos revealed only a single mutant transcript that is expected to encode a truncated protein (Fig 1*c*).

The few *Pkd1*$^{-/-}$ mice that survived to term had laboured respiration, failed to turn pink with their littermates and died, on average, four hours after birth. These mice had distended abdomens, massively enlarged cystic kidneys and small hypoplastic lungs (Fig. 1*e,g*). Pulmonary hypoplasia is a common feature of polycystic disease in childhood and is believed to result from oligohydramnios and abdominal distention produced by renal enlargement. Among 400 progeny, only one homozygote survived the immediate neonatal period (to die at eight days). This animal was very small (Fig. 1*d*) with pale, massive cystic kidneys (Fig. 1*f*) and a cystic pancreas.

To determine the sequence of developmental abnormalities in *Pkd1*$^{-/-}$ mice, we examined whole embryo sections between E8.5 and E19. Histologically, the kidneys developed normally until E14.5 (Fig. 2*a,b*). Multifocal microscopic dilatation of tubules was first noticed in proximal tubules of the outer medulla, as defined by morphology and lectin profiling, at E15.5 (Fig. 2*d*). This was followed by progressive dilatation and cyst formation in collecting tubules of the inner medulla and cortex (Fig. 2*f,h*). The number and size of cysts increase with age (Fig. 2). In newborns, epithelial cysts occupied the entire medulla and most of the cortex. By postnatal day 8, no normal renal parenchyma remained in the sole survivor.

Kidney development is initiated by growth of the ureteric bud into presumptive kidney mesenchyme. In response to a signal from the ureter, mesenchymal cells condense, aggregate into pretubular clusters and undergo epithelial conversion, generating simple tubules[12]. These tubules undergo further functional and structural maturation. The data presented here show that the earliest stages of tubule morphogenesis—nephrogenic condensation and epithelialization—proceed normally in *Pkd1*$^{-/-}$ embryos. Indeed, in the absence of full-length polycystin, lumen formation and tubule differentiation are normal until as late as E15.5. These results demonstrate that full-length polycystin is not required dur-

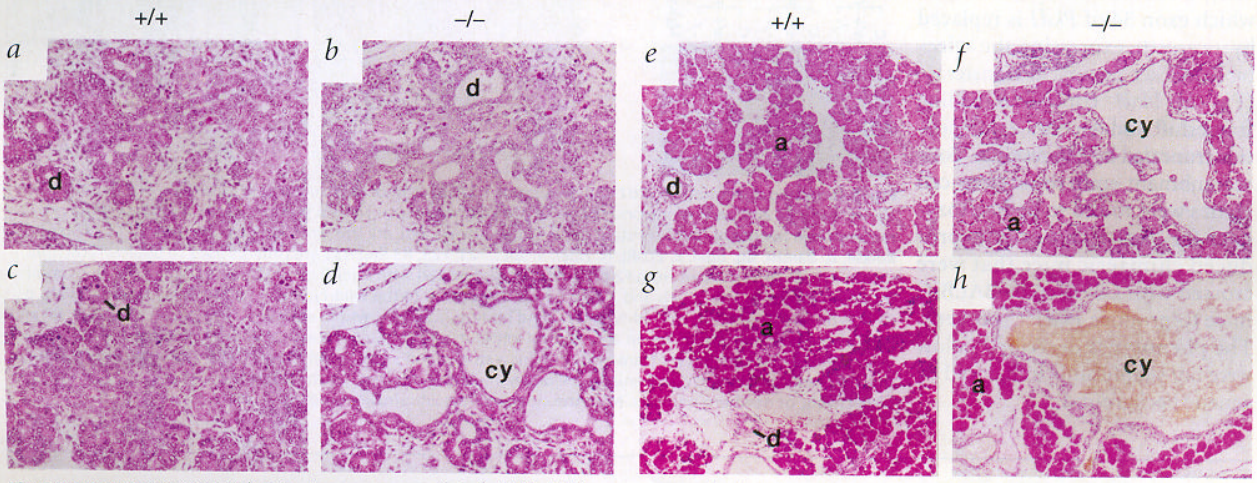


Fig. 3 Pancreatic cysts in *Pkd1*$^{-/-}$ mice. Homozygous mutants (–/–) (***b,d,f,h***) were compared with wild-type littermates (+/+) (***a,c,e,g***). Pancreas sections at E13.5 (*a,b*), E14.5 (*c,d*), E17.5 (*e,f*) and newborn (NB) (*g,h*) revealed dilatation of pancreatic ducts (d) in –/– mice as early as E13.5. The cysts (cy) enlarge with age; in the newborn, a single huge cyst was seen. Acini (a) appear to develop normally. Magnification in *a–d* ×100; *e–h* ×50.

ing nephrogenic induction but is critically important in the establishment of normal tubular architecture. This conclusion is consistent with studies showing that polycystin expression in renal epithelia peaks between E16 and E19 and remains elevated until tubular maturation is complete in neonatal life[5]. Thereafter, expression declines to lower levels in the adult, and polycystin may play a role in maintaining tubular architecture[4–7].

Early and massive dilatation of pancreatic ducts is part of the phenotype in homozygous mutants (Fig. 3). We observed cysts in major pancreatic ducts as early as E13.5, well before renal cysts appeared (Fig. 3*b*). Newborn and eight-day pancreases contained massive yellow fluid-filled cysts (Fig. 3*h*). It has been shown that polycystin is abundant in fetal pancreatic ducts, but not in exocrine and endocrine cells[4]. In homozygous mutants, acini appear to develop normally and islets of Langerhans were evident, albeit reduced in number compared to those in normal littermates (data not shown). Thus, these structures, which are believed to derive from proto-differentiated progenitor cells of ductal origin[13], develop independently of polycystin.

Liver cysts occur in about 30% of patients with ADPKD; yet, surprisingly, no hepatic cysts were observed in *Pkd1*$^{-/-}$ mutants. Other organs in which epithelial ducts are found (salivary glands, testis and ovary) and other structures in which polycystin is expressed (heart and skin) also develop normally. These findings suggest that full-length polycystin is not critical for normal morphogenesis of these structures during fetal life. It is conceivable that *Pkd1*$^{-/-}$ mutants elaborate a truncated form of polycystin that partly rescues the null phenotype.

The 'two-hit hypothesis' of ADPKD pathogenesis proposes that cysts form when one *PKD1* allele contains a germline mutation and the other acquires a somatic mutation[14]. This hypothesis has received support from the detection of loss of heterozygosity in some cysts[15,16], but Ward *et al.* found that the *PKD1* allele without the germline mutation was overexpressed, suggesting that a second (somatic) hit was not the primary event in cyst formation[6]. Our data strongly support the two-hit hypothesis. First, there was no evidence of tubular dilatation in fetal or early adult life in heterozygotes, as would be expected if somatic mutations accrued over time. Second, as expected, all tubules in homozygous mutant mice are severely affected during development.

In summary, we have shown that full-length polycystin is required for normal renal and pancreatic development. The lethal phenotype in homozygous mouse embryos may explain the absence of surviving human homozygotes despite the prevalence of *PKD1* mutations in the human population.

Methods

Targetting vector. To construct the targeting vector, a 190-bp mouse genomic *Pkd1* DNA probe containing exon 38 was used to isolate P1 clones from a 129/Sv strain mouse genomic library. An 8.8-kb *Spe*I–*Nae*I *Pkd1* fragment was sub-cloned into vector litmus 28. A 0.8-kb *Eco*RI–*Bgl*II fragment that contains exon 34 was replaced by a pgk-neo selection cassette in the same orientation. The pgk-TK cassette was then cloned into the 5′ *Sna*BI polylinker site. The resulting targeting vector contained 2.7 kb of homologous sequence on the short arm and 5.1 kb on the long arm (Fig. 1*a*).

Generation of *Pkd1*$^{-}$ mutant mice. To generate *Pkd1*$^{-}$ mutant mice, 129/Sv J1 ES cells (a gift from E. Li) were electroporated and selected according to standard procedures. Positive clones were injected into C57BL/6 and BALB/C blastocysts to generate chimaeras. Tail and yolk-sac DNA isolation and Southern-blot analysis of genomic DNA were performed.

Southern-blot analysis of genotype ES cells and mice. To identify the targetted mutant ES cells and to genotype the mice, genomic DNA was digested with *Bam*HI and analysed by Southern blotting. A 0.2-kb *Nae*I–*Bam*HI fragment (probe1) that detects a 5.7-kb fragment in wild-type DNA and 7.4-kb fragment in targeted clones was used (Fig. 1*a*,*b*). A 0.3-kb *Sty*I–*Spe*I genomic fragment (probe2), located immediately upstream of the short arm *Spe*I site, was used to confirm the homologous recombination in the 5′ homology region. The wild-type *Apa*I fragment detected by this probe is 8.2 kb, and the mutant fragment is 12.7 kb (data not shown). The homologous recombination frequency was 1%.

RT-PCR analysis. RT-PCR was carried out with 5 μg total RNA isolated from E12.5 embryos, using three sets of primers (Fig. 1*a*,*c*): set 1, F23 (exon 33)/R3 (exon 39), amplified a 1,680-bp fragment in *Pkd1*$^{-/-}$ RNA and a 900-bp fragment in *Pkd1*$^{+/+}$ RNA; set 2, F23/R35 (exon 35), amplified a 1,160-bp fragment in *Pkd1*$^{-/-}$ RNA and a 380-bp fragment in *Pkd1*$^{+/+}$ RNA; set 3, F21/R35, amplified a 1,385-bp fragment in *Pkd1*$^{-/-}$ RNA and a 605-bp fragment in *Pkd1*$^{+/+}$ RNA. Each mutant transcript was cloned and sequenced by the dideoxy chain termination method.

Histological analysis. Specimens were fixed in formalin, embedded in paraffin, sectioned at 4 μm and stained with haematoxylin-eosin.

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1. Gabow, P.A. Autosomal dominant polycystic kidney disease. *N. Engl. J. Med.* **329**, 332–342 (1993).
2. International PKD Consortium. Polycystic kidney disease: the complete structure of the *PKD1* gene and its protein. *Cell* **81**, 289–298 (1995).
3. Hughes, J. *et al.* The polycystic kidney disease 1 (PKD1) gene encodes a novel protein with multiple cell recognition domains. *Nature Genet.* **10**, 151–160 (1995).
4. Geng, L. *et al.* Identification and localization of polycystin, the *PKD1* gene product. *J. Clin. Invest.* **98**, 2674–2682 (1996).
5. Geng, L. *et al.* Distribution and developmentally regulated expression of murine polycystin. *Am. J. Physiol.* **272**, F451–F459 (1997).
6. Ward, C.J. *et al.* 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 (1996).
7. Griffin, M.D., Torres, V.E., Grande, J.P. & Kumar, R. Immunolocalization of polycystin in human tissues and cultured cells. *Proc. Assoc. Am. Physicians* **108**, 185–197 (1996).
8. Peters, D.J. *et al.* Adult, fetal, and polycystic kidney expression of polycystin, the polycystic kidney disease–1 gene product. *Lab. Invest.* **75**, 221–230 (1996).
9. Palsson, R. *et al.* Characterization and cell distribution of polycystin, the product of autosomal dominant polycystic kidney disease gene 1. *Mol. Med.* **2**, 702–711 (1996).
10. Peral, B. *et al.* Screening the 3′ region of the polycystic kidney disease 1 (*PKD1*) gene reveals six novel mutations. *Am. J. Hum. Genet.* **58**, 86–96 (1996).
11. Geng, L. *et al.* Gene structure, sequence, and a point mutation in the PKD1 gene, and search for point mutations. *J. Am. Soc. Nephrol.* **5**, 622 (1994).
12. Sorokin, L. & Ekblom, P. Development of tubular and glomerular cells of the kidney. *Kidney Int.* **41**, 657–664 (1992).
13. Madsen, O.D. *et al.* Pancreatic development and maturation of the islet B cell: studies of pluripotent islet cultures. *Eur. J. Biochem.* **242**, 435–445 (1996).
14. Reeders, S.T. Multilocus polycystic disease. *Nature Genet.* **1**, 235–237 (1992).
15. Qian, F., Watnick, T.J., Onuchic, L.F. & Germino, G.G. The molecular basis of focal cyst formation in human autosomal dominant polycystic kidney disease type I. *Cell* **87**, 979–987 (1996).
16. Brasier, J.L. & Henske, E.P. Loss of the polycystic kidney disease (PKD1) region of chromosome 16p13 in renal cyst cells supports a loss-of-function model for cyst pathogenesis. *J. Clin. Invest.* **99**, 194–199 (1997).