Pax2 gene dosage influences cystogenesis in autosomal dominant polycystic kidney disease

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Mutations in PKD1 cause dominant polycystic kidney disease (PKD), characterized by large fluid-filled kidney cysts in adult life, but the molecular mechanism of cystogenesis remains obscure. Ostrom et al. [Dev. Biol., 219, 250–258 (2000)] showed that reduced dosage of Pax2 caused increased apoptosis, and ameliorated cystogenesis in Cpk mutant mice with recessive PKD. Pax2 is expressed in condensing metanephrogenic mesenchyme and arborizing ureteric bud, and plays an important role in kidney development. Transient Pax2 expression during fetal kidney mesenchyme-to-epithelial transition, as well as in nascent tubules, is followed by marked down-regulation of Pax2 expression. Here, we show that in humans with PKD, as well as in Pkd1del34/del34 mutant mice, Pax2 was expressed in cyst epithelial cells, and facilitated cyst growth in Pkd1del34/del34 mutant mice. In Pkd1del34/del34 mutant kidneys, the expression of Pax2 persisted in nascent collecting ducts. In contrast, homozygous Pkd1del34/del34 fetal mice carrying mutant Pax2 exhibited ameliorated cyst growth, although reduced cystogenesis was not associated with increased apoptosis. Pax2 expression was attenuated in nascent collecting ducts and absent from remnant cysts of Pkd1del34/del34/Pax21Neu/+ mutant mice. To investigate whether the Pkd1 gene product, Polycystin-1, regulates Pax2, MDCK cells were engineered constitutively expressing wild-type Pkd1; Pax2 protein levels and promoter activity were both repressed in MDCK cells over-expressing Pkd1, but not in cells without transgenic Pkd1. These data suggest that polycystin-1-deficient tubular epithelia persistently express Pax2 in ADPKD, and that Pax2 or its pathway may be an appropriate target for the development of novel therapies for ADPKD.

INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common monogenic diseases in humans, with an incidence of 1:500 to 1:1000 in the general population. Affected individuals normally present in the third or fourth decade of life, although presentation in infancy or in utero has been reported (1,2). Typically, ADPKD is characterized by progressive bilateral cyst formation in the kidney, while cysts also commonly form in other organs such as the pancreas, liver and intestine. Intracranial aneurysm, mitral valve prolapse and intestinal diverticula are also associated with this disease [reviewed in (3,4)]. ADPKD accounts for ~5% of all patients on renal replacement therapy.

ADPKD is genetically heterogeneous with 85–90% of ADPKD patients harboring mutations in the PKD1 gene, encoding Polycystin-1 (PC-1), while most of the remaining patients have mutations in PKD2, encoding polycystin-2 (PC-2) and a third locus PKD3 is thought to exist because PKD segregates independently of PKD1 or PKD2 in a small number of families (3). In addition to genetic heterogeneity, there is phenotypic variability with respect to the severity of the disease, age of onset of end-stage renal failure and extra-renal manifestations, which vary widely between affected individuals (5). This notable phenotypic variability is likely to be due to the influence of specific additional genetic loci modifying the rate of onset and/or severity of disease.
Several lines of mice have been reported with targeted mutations in the mouse Pkd1 gene produced by homologous recombination (4). The first of these Pkd1 ‘knockout’ mice was the del34 Pkd1 knockout mouse, carrying a disruption of exon 34 of the mouse Pkd1 gene, mimicking a mutation in human PKD1, predicted to result in truncation of PC-1 (6). The del34 Pkd1 heterozygous mutant mice progressively developed scattered renal and hepatic cysts in a late onset manner, similar to that seen in human ADPKD. In addition, mislocalization of the epidermal growth factor receptor (EGFR) to the apical membrane of the cystic epithelia, which is a feature of human ADPKD, was demonstrated in the Pkd1del34/+ heterozygous mutant mice.

Homozygous Pkd1 mutant mice seldom survived to term, exhibiting perinatal lethality and a progressive severe renal cystic phenotype, which commenced at embryonic day 15.5 (E15.5). Histologically, the kidneys developed normally until E14.5, with microscopic dilatation of tubules appearing at E15.5. The number and size of cysts then increased progressively with age, resulting in full-term conceptuses with massively enlarged cystic kidneys, distended abdomens and gross edema.

Each of the murine models of ADPKD strongly support the two-hit hypothesis of ADPKD pathogenesis in that cysts form when kidneys carrying one germline Pkd1 or Pkd2 mutant allele acquire a second somatic mutation in either of these genes [reviewed in (7)]. Further support for this theory comes from the identification of mutations in and/or loss of a second allele of PKD1 or PKD2 in isolated human cyst epithelial cells. Similarly, in a genetically unstable Pkd2 knockout mouse, the second allele of Pkd2 is lost somatically in a stochastic fashion (through recombination). These Pkd2 mutant mice develop a more severe cystic phenotype more rapidly than stable Pkd1 and Pkd2 knockouts, although not as severe as the phenotype of homozygous mutants. Thus, in Pkd1 homozygous mice the ‘knockout’ of the Pkd1 gene most likely recapitulates in the whole animal events that occur in individual cysts of heterozygous mutants, leading to functional loss of Pkd1.

Mutations in the developmental gene, Pax2, are associated with renal hypoplasia, and renal cysts have occasionally been noted as part of the Pax2-mutant phenotype (8,9). Pax2 is critically required for kidney development, as Pax2 null mutant mice completely lack urogenital tracts, including absent kidneys and ureters (10). In contrast, over-expression of Pax2 in transgenic mice leads to multicystic kidney disease (11). Moreover, Pax2 expression has been observed in the cystic epithelia of several types of PKD, suggesting that the expression of Pax2 may be of fundamental importance during cystogenesis (12,13). Ostrom et al. (14), examined the role of Pax2 in cystogenesis in Cpk mice, which carry a mutation in cystin, an autosomal recessive PKD gene. By crossing Pax2 mutant mice with Cpk mice, a significant inhibition of renal cyst growth in fetal kidneys of the double mutant offspring was observed, which was apparently due to increased apoptosis in the cyst epithelium (14). Therefore, reduced Pax2 dosage was able to modulate the cystic phenotype in Cpk mice, a recessive model of PKD.

To determine whether Pax2 gene dosage influences the severity of ADPKD, we crossed Pkd1 mutant mice with Pax2 mutant mice, and examined the effect on cystogenesis in the offspring. Homozygous Pkd1del34/+ Pkd1mutant mice carrying a heterozygous Pax21Neu/+ mutation showed a marked reduction in cyst number and size compared with homozygous Pkd1 mutants carrying a wild-type Pax2 gene. The reduction in cyst size was not accompanied by alterations in levels of either apoptosis or proliferation. Endogenous Pax2 expression and a Pax2–promoter–reporter construct were repressed in MDCK cells overexpressing PC-1, as compared with non-overexpressing cells. Taken together, these data suggest that Pax2 expression plays an important role in ADPKD.

RESULTS

Pax2 is expressed in the cystic renal epithelium of human and mouse ADPKD

To determine whether PAX2 is expressed in cystic renal epithelial cells, sections from kidneys of human ADPKD and Pkd1del34/+ Pkd1del34/+ mutant mice (a mutant mouse model of ADPKD) were stained with rabbit polyclonal anti-Pax2 antibody. Positive staining was detected in the cyst epithelial cells of human ADPKD kidney, and in mouse cystic homozygous mutant Pkd1del34/+ kidney, while the medullary regions of non-cystic adult human kidney from an unaffected individual, and from non-cystic adult mouse heterozygous mutant Pkd1del34/+ kidney, or from wild-type kidney of a normal adult mouse showed low levels of PAX2 immunoreactivity, suggesting that Pax2 is constitutively expressed in the epithelium of kidney cysts, but not in normal adult kidney medulla (Fig. 1).

Renal cysts in Pkd1del34/+ heterozygous mutant mice occur infrequently. For this reason kidney sections from E18.5 fetal mice carrying a homozygous Pkd1del34/+ mutation were used for further studies. These fetal kidneys contained much greater number of cysts, and Pax2 immunoreactivity was observed in the cyst epithelium, as well as in newly forming nephrons, including bifurcating ureteric buds and condensing mesenchyme. In these kidneys, cysts were observed to derive from multiple parts of the nephron, including glomeruli, proximal and distal tubules, collecting ducts and ureteric bud. However, not all cysts in the Pkd1del34/+ kidneys stained with the Pax2 antibody. Anti-aquaporin antibodies, used as epithelial markers in conjunction with Pax2 antibodies during immunohistochemistry, showed that Pax2 positive cysts were generally derived from Pax2 positive epithelium, and cysts that did not express Pax2 were derived from Pax2 negative epithelial structures (data not shown).

Homozygous pkd1del34/+ mutant mice develop a rapidly progressive PKD

Cysts were observed in fetal homozygous Pkd1del34/+ mutant kidneys from embryonic day 16.5 (E16.5) onwards, but were not yet visible at E15.5. By E18.5 cysts were numerous (Fig. 2). Homozygous Pkd1del34/+ mutant mice survived until approximately E19 to birth, indicating a late embryonic or early perinatal mortality, yet in E18.5 fetal mice, the expected genotypes were represented at near expected Mendelian ratios (Supplementary Material,
to enhanced apoptosis in the ureteric bud epithelium between E16.5 and E18.5 of kidney development (16–18). Accordingly, either Pax2 expression or enhanced cell survival within the arborizing fetal ureteric bud was sufficient to promote increased branching morphogenesis and enhanced nephrogenesis in fetal kidneys (16,17,19–21).

To investigate the effect of Pax2 haploinsufficiency on cystogenesis in Pkd1 mutant mice Pkd1<del34/+> heterozygous mutant mice on a C3H background were crossed with Pax2<1Neu/+> heterozygous mutant C3H mice to generate compound heterozygous mutants. The compound heterozygous mutant mice were then back-crossed with Pkd1<del34/+> mice to generate Pkd1 homozygous mutant mice (Pkd1<del34/del34>) containing either a wild-type (Pax2<2+/>) Pax2 allele, or a heterozygous (Pax2<1<Neu/+>) Pax2 mutation. The resulting litters were dissected at E15.5, E16.5, E17.5 and E18.5, weighed and kidneys dissected for morphometric and immunohistochemical studies.

A clear reduction in the appearance of cysts in Pkd1<del34/del34>Pax2<1<Neu/+> double mutant kidneys was visible in H&E stained sections of E18.5 fetal mice when compared with Pkd1<del34/del34> homozygous mutant kidneys at the same age (Fig. 3A). The total cyst and lumen volume in Pkd1<del34/del34>Pax2<1<Neu/+> double mutant kidneys was on average less than half that in Pkd1<del34/del34> homozygous mutant kidneys (P = 0.021), and similar to that of wild-type kidneys when measured using stereological methods (Fig. 3B).

At E18.5 Pkd1<del34/del34> kidneys weighed approximately twice as much as wild-type kidneys (Fig. 4). Non-cystic kidneys from mice with a Pax2<1<Neu/+> heterozygous mutation were on average 30% lighter than wild-type kidneys, and were much smaller than the homoygous mutant kidneys (P < 0.001). Pkd1<del34/del34>Pax2<1<Neu/+> double mutant kidneys were much lighter than Pkd1<del34/del34> kidneys (P < 0.001), and were instead similar in weight to Pkd1<del34/del34>Pax2<1<Neu/+> heterozygous mutant kidneys (Fig. 4). Similar results were obtained when kidney weight to body weight ratios were calculated (data not shown). At E17.5, a similar profile of kidney weights to that at E18.5 was obtained, while at E15.5 the differences in kidney mass between different genotypes due to cystogenesis were not yet discernable (Supplementary Material, Fig. S1 A,B).

**Pax2 haploinsufficiency reduces cyst size in Pkd1<del34/del34>Pax2<1<Neu/+> double mutant mice**

Humans and mice with only one functional copy of Pax2 exhibit haploinsufficiency, in that they present a mutant phenotype in the heterozygous state (8–10,15,16). Pax2<1<Neu/+> mutant mice have previously been shown to express equivalent levels of both mutant and wild-type Pax2 mRNA, yet the reduced Pax2 gene dosage in Pax2<1<Neu/+> mutant mice was associated with reduced nephrogenesis and smaller kidneys, and immunohistochemical staining revealed less intense Pax2 immunostaining in Pax2<1<Neu/+> mutant kidneys than in wild-type kidneys (16). The mechanism of reduced nephrogenesis in Pax2<1<Neu/+> mutants was shown to be due
Neither cell proliferation nor apoptosis was altered in cystic kidneys from Pax2<sup>1Neu<sup>+/+</sup>/Pkd1<sup>del34/del34</sup> mice when compared with Pkd1<sup>del34/del34</sup> mice

To investigate whether the Pax2 mutation in double mutant Pkd1<sup>del34/del34</sup>/Pax2<sup>1Neu<sup>+/+</sup> mice caused reduced cyst size by either reducing cell proliferation, or by elevating levels of apoptosis in the cyst epithelium, fetal kidneys of mice at E17.5 carrying the four different genotypes were analyzed using either BrdU incorporation to examine the number of cells undergoing DNA synthesis, or TUNEL staining to examine apoptosis. For the proliferation analysis, fetal mice were exposed in utero to a pulse of BrdU, their kidneys dissected at E17.5 and the kidneys processed using unbiased stereological techniques. The results from each stage showed that the presence of a Pax2<sup>1Neu</sup> heterozygous mutation together with the Pkd1<sup>del34/del34</sup> homozygous mutation did not significantly alter the rate of either cell division or apoptosis in the kidneys compared with the rate in cystic Pkd1<sup>del34/del34</sup> kidneys that lacked the Pax2 mutation (Supplementary Material, Fig. S2 A,B).

**Pax2 is persistently expressed in Pkd1<sup>del34/del34</sup> renal tubules**

The observation that Pax2 is expressed in the cystic epithelium could reflect an abnormal Pax2 expression pattern. To investigate this possibility, Pax2 immunohistochemistry was carried out on E18.5 double mutant Pkd1<sup>del34/del34</sup>/Pax2<sup>1Neu<sup>+/+</sup> mouse kidneys (Fig. 5). As indicated previously (e.g. Fig. 3A), cystogenesis was ameliorated in these sections. The expression of Pax2 protein was remarkably attenuated in the remnant cyst-like structures when compared with adjacent normal nephrogenic structures, except for those derived from the glomerular Bowman’s capsule, where Pax2 expression persisted in squamous epithelial cells of Bowman’s capsule cyst epithelium. These results suggest that reduced Pax2 gene dosage led to attenuation of Pax2 expression in collecting duct epithelia prior to cyst formation. However, Pax2 protein was still expressed in the normal developing structures of the nephrogenic zone of these kidneys (Fig. 5A).

We then hypothesized that during cystogenesis, Pax2 expression may persist longer in Pkd1<sup>del34/del34</sup> mutant kidneys when compared with wild-type mouse kidneys, where Pax2 expression patterns may persist longer (e.g. Fig. 3A). Cystogenesis was ameliorated in these sections. The expression of Pax2 protein was remarkably attenuated in the remnant cyst-like structures when compared with adjacent normal nephrogenic structures, except for those derived from the glomerular Bowman’s capsule, where Pax2 expression persisted in squamous epithelial cells of Bowman’s capsule cyst epithelium. These results suggest that reduced Pax2 gene dosage led to attenuation of Pax2 expression in collecting duct epithelia prior to cyst formation. However, Pax2 protein was still expressed in the normal developing structures of the nephrogenic zone of these kidneys (Fig. 5A).

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the length of the ureteric buds at levels similar to that in normal cells, and unlike wild-type or Pkd1del34/+ kidneys, remained expressed in Pkd1del34/del34 ureteric buds even as they developed into collecting ducts, some of which could be seen to be in the process of cyst formation (Fig. 5B–D).

**Pax2 is repressed by human PC-1 in MDCK cells**

We reasoned that PC-1 must repress the expression of Pax2 in nascent nephrons and tubules during normal renal development, and that by lowering the level of Pax2 in cystic kidneys using the Pax21Neu/+ mutation, we may be restoring the attenuation of Pax2 expression observed during tubule maturation in wild-type kidneys. To examine whether the expression of PC-1 would indeed repress Pax2 in collecting duct cells, we compared endogenous Pax2 protein levels in MDCKZeo cells lacking endogenous PC-1 to that in MDCKZeo cells stably transfected with a full-length human PKD1 cDNA (MDCK Pkd1Zeo clone C6/68). PKD1 protein and mRNA were easily identified in MDCK Pkd1Zeo cells by western immunoblotting and RT–PCR, respectively, but were undetectable in the control cells [Boletta et al. (22) and RT–PCR, data not shown]. Both cell lines expressed PC-2 protein, required for PC-1 function (23). As shown in Fig. 6A, MDCK Pkd1Zeo cells showed significantly less (43.7%, \( P = 0.003 \)) Pax2 protein on western immunoblots normalized for actin, compared with the control MDCKZeo cells.

We also transiently transfected MDCKZeo and MDCKPkd1Zeo cells with a luciferase reporter vector driven by a 4.5 kb portion of the 5′-UTR upstream of the human PAX2 gene to examine the effect of PC-1 on transcriptional activity of the PAX2 promoter (Fig. 6B). Cells were co-transfected with an SV40-driven renilla luciferase vector to control for transfection efficiency. Luciferase activity was significantly (\( P < 0.0001 \)) lower in MDCK Pkd1Zeo cells (0.33 luciferase/renilla units) than in control cells lacking PC-1 (1.36 luciferase/renilla units). These results suggest that PC-1 represses PAX2 expression transcriptionally in collecting duct cells.

**DISCUSSION**

Our data show that Pax2 gene dosage markedly influences renal cyst formation in a mouse model of ADPKD. Kidney mass and cyst size in double mutant Pkd1del34/del34/Pax21Neu/+ mutant mice were markedly reduced in the presence of the heterozygous Pax2 mutation when compared with Pkd1del34/del34 mice without a mutation in Pax2. Renal cyst formation is the major determinant of end-stage renal disease in ADPKD, therefore strategies to prevent cyst formation could significantly improve morbidity and mortality in patients with ADPKD.

Pkd1del34/+ heterozygous mutant mice did not develop cysts until ~18 months of age, and the cysts were very sparsely distributed in the collecting ducts and distal tubules. In contrast,
cyst formation in homozygous Pkd1<sup>del34/del34</sup> mutant mice was very marked and 100% penetrant at E18.5, with cysts occurring in most epithelial structures, including the Bowman’s capsule of glomeruli (6). The kidney cysts in Pkd1<sup>del34/del34</sup> homozygous mutants were observed in both Pax2 positive and Pax2 negative tubules suggesting that Pax2 expression was not a requirement for cyst formation. Nonetheless, the observation that reduced cystogenesis occurred in all parts of the nephron in Pkd1<sup>del34/del34</sup>Pax2<sup>+/Neu</sup> double mutant kidneys suggests that Pax2 expression per se was a critical factor in cystogenesis in ADPKD. Moreover, the observation that Pax2 was not expressed in the nascent collecting ducts or cysts of double mutant kidneys, but was strongly expressed in cysts of Pkd1<sup>del34/del34</sup> kidneys, suggests that the persistent expression of Pax2 was important in facilitating cystogenesis. It is assumed that cysts that lacked Pax2 in double mutant mice derived originally from nephron progenitors that once expressed Pax2.

An important consideration in interpreting the effect of a Pax2 mutation on cyst formation is whether the effect of the heterozygous Pax2<sup>1Neu</sup> mutation reflects haploinsufficiency rather than a gain-of-function. Haploinsufficiency is considered most likely because the phenotypic effect of the Pax2<sup>1Neu</sup> mutation in mice was very similar to that observed in heterozygous Pax2 knockout mice (10,15). The presence of both mutant and wild-type mRNA has been demonstrated in Pax2<sup>1Neu</sup> kidneys (16,24), indicating that the alteration in gene dosage is at the level of protein production. However, Pax2 is expressed in specific cell types in the developing kidney, and although it is expected that total Pax2 protein level would be halved in mice on a Pax2<sup>1Neu</sup> background, this has not yet been demonstrated experimentally. In the present study, expansion of cysts in some genotypes distorts the population of cells, and so a comparison of the level of Pax2 protein in individual cells between the different genotypes is even more technically challenging. Nevertheless, the effect of Pax2 haploinsufficiency has been clearly documented (8–10,14–17,20,21,24).

It was not practical to demonstrate that Pax2 gene dosage had an effect on cystogenesis in Pkd1<sup>del34/del34</sup> homozygous mice, because cystogenesis was too infrequent. However, the influence of reduced Pax2 gene dosage on cystogenesis in heterozygous Pkd1 mutants would be expected to be similar to that observed in Pkd1<sup>del34/del34</sup> homozygotes. Cysts that form in heterozygous Pkd1 mutant mice and humans with ADPKD are thought to contain a somatic mutation in the remaining allele of the Pkd1 gene. Therefore, in homozygous mice the ‘second hit’ mutation in Pkd1 is thought to be a stochastic somatic event, whereas in homozygous mutant mice the ‘second hit’ is inherited through the germline. Although cystogenesis was ameliorated in homozygous Pkd1<sup>del34/del34</sup> mice carrying a Pax2<sup>1Neu</sup> mutation, these mice did not exhibit greater survival than Pkd1<sup>del34/del34</sup> mice, probably because homozygous Pkd1<sup>del34/del34</sup> mutant mice died as a result of polyhydramnios, hydrops fetalis, spina bifida occulta, otocephalodrondyplasia or other uncharacterized abnormalities that were not influenced by the Pax2 mutation (6).

Cyst formation in recessive Cpk poly cystic kidney mouse was ameliorated by Pax2 haploinsufficiency similar to that in Pkd1<sup>del34</sup> mice (14). However, contrary to our expectations, and in contrast to the observation that increased apoptosis caused by Pax2 haploinsufficiency in the cystic kidneys reported in Ostrom et al. (14), the presence of a Pax2 mutation did not markedly reduce cell proliferation or elevate apoptosis levels in the cysts of Pkd1 homozygous mutant kidneys between E15.5 and E17.5. These data suggest that significantly altered levels of apoptosis or proliferation during this window of time in embryonic development was not the mechanism by which Pax2 haploinsufficiency inhibited cyst formation in Pkd1 mutant mice, although the possibility that Pax2 haploinsufficiency led to more subtle effects on apoptosis or proliferation over a longer period of time, which then impacted on cystogenesis, could not be ruled out.

It would appear, however, that unattenuated expression of Pax2 in ureteric bud and epithelia derived from mesenchymal condensates during kidney development contributed to cystogenesis in Pkd1 mutant mice. Our data are consistent with the notion that Pax2 expression persists in the cyst epithelium from the time of fetal kidney development. Indeed, renal cysts in humans with ADPKD are thought to begin as early as in utero (2), which argues that the cysts could develop in the same fashion as in Pkd1 homozygous mutant mice, although over a longer time, and long after the normal cessation of Pax2 expression in nephrogenesis.

Pax2 has been implicated in mesenchymal to epithelial transition, and the acquisition of the renal epithelial phenotype. Moreover, it is known that prior to terminal differentiation of renal epithelial cells, it is necessary for Pax2 expression to be down-regulated, since de-regulated constitutive expression of Pax2 in adult mouse kidney under the control of the CMV promoter led to multi-cystic kidney disease (11). Therefore, persistent Pax2 expression resulting from
the absence of a functional Pkd1 gene product could be sufficient to maintain epithelial cells in a replication-competent pre-terminal differentiated state, although the exact role that Pax2 plays in promoting the cyst growth remains unknown.

Unattenuated expression of PAX2 has been shown in other cystic renal cell diseases in humans, such as medullary cystic disease (12), and in cysts of patients with juvenile nephronophthisis (13). It will therefore be of interest to determine whether strategies to limit Pax2 gene dosage would ameliorate cystogenesis in other cystic kidney diseases in the same way as observed so far in Cpk and Pkd1 mice. If so, a more general role for Pax2 in kidney cystogenesis might be implied. With regard to ADPKD in humans, better targets for the design of therapeutic agents to treat PKD.

MATERIALS AND METHODS

Mouse breeding, tissue isolation and genotyping

Heterozygous mutant Pkd1(-/-) and Pax2(+/+) mice were obtained on C57 backgrounds. The Pkd1(+/+) knockout mice had been back-crossed onto the C3H strain for at least 10 generations. Pax2(+/+) mice were crossed with Pkd1(-/-) mice to produce 25% of offspring carrying the genotype, Pkd1(+/+)Pax2(+/+)(1/2) and Pkd1(-/-)Pax2(+/+)(1/2), which were then back-crossed with Pkd1(-/-)Pax2(+/+) mice to produce the following genotypes: Pkd1(+/+)Pax2(+/+)(wild-type), Pkd1(-/-)Pax2(+/+)(Pax2 heterozygous), Pkd1(+/+)Pax2(-/-)(Pax2 homozygous mutant), Pkd1(-/-)Pax2(-/-)/Pkd1(+/+)Pax2(+/+)(Pkd1 homozygous mutant), Pkd1(-/-)Pax2(-/-)/Pkd1(+/+)Pax2(+/+)(Pkd1/Pax2 double mutant). Timed matings were identified by the presence of a vaginal plug, and designated as embryonic day 0.5. Fetal mice were dissected under a Leica dissecting scope, weighed and a piece of tail tissue removed for genotyping. Kidneys were dissected under a Leica dissecting microscope, weighed and one kidney fixed in 10% neutral buffered formalin (NBF) or 4% paraformaldehyde, while the contralateral kidney was frozen in liquid nitrogen. Following 24 h in 10% NBF, kidneys were transferred to 70% ethanol and fixed, embedded in paraffin and serial-sectioned through the whole kidney at a thickness of 4 μM. Six pairs of sections were selected in a systematic uniform manner starting from a randomly chosen first section. BrdU staining was carried out using a BrdU in situ detection kit following the manufacturer's recommendations (BD Pharmingen, San Diego, USA). BrdU-stained kidney sections were photographed, printed as montages and counting of positively stained nuclei was carried out as previously described (16). TUNEL labeling was carried out using an in situ cell death detection kit following the manufacturer's recommendations (Roche Diagnostics, Hoffman-La Roche Ltd., Basel, Switzerland). TUNEL-labeled sections were analyzed as digital images. A further set of eight sections, also with a randomly chosen first section, were selected systematically and analyzed using stereological grids to estimate the volume and also the luminal content of each kidney, using the Cavalieri method as previously described (24). The volume and luminal content was determined using the point-sample intercept method. To determine whether the total number of cells in kidneys from different genotypes was variable, an estimate of the tissue volume minus lumen content, as well as the cell density minus lumen content was made. To obtain total tissue volumes (minus the cyst or luminal volumes) of the kidney for each genotype, the luminal and/or cyst volumes were subtracted from total volumes of the E17.5 kidneys. Having done this it was found that the total tissue volumes of the E17.5 kidneys were essentially the same (0.7 ± 0.05 mm³) for each of Pkd1(+/+)Pax2(+/+), (wild-type), Pkd1(-/-)Pax2(+/+), (Pkd1 heterozygous), Pkd1(-/-)Pax2(-/-) and Pkd1(-/-)Pax2(+/+), (Pkd1 double mutant) kidneys. The corrected cell density was calculated as follows. Using a 5 cm × 5 cm counting frame on printed images of haematoxylin-stained kidney sections, all the nuclei were counted within the area. A stereology grid was then placed over the frame and used to estimate the percentage lumen in the area. The number of nuclei was then adjusted to that of the equivalent area with no luminal content. This was done five times on one section for each kidney.

Immunohistochemistry

Pax2, aquaporin 1 and aquaporin 2 staining was carried out as previously described (16). The Pax2 primary antibody was rabbit anti-Pax2 (Zymed, San Francisco, CA, USA) (1:50). The aquaporin primary antibodies (rabbit anti-rat) were a gift from Dr Jennifer Bedford. The secondary antibody was a biotinylated goat anti-rabbit (ABC kit, Vector Laboratories, Burlingame, CA, USA) and detection of immunohistochemical labeling was carried out using diaminobenzidine tetra-chloride detection as the chromagen, with haematoxylin counterstaining.

Stereological analysis of mutant and wild-type kidneys

Whole fetal kidneys (Pkd1(+/+)Pax2(+/+), Pkd1(-/-)Pax2(+/+), Pkd1(-/-)Pax2(-/-), Pkd1(+/+)Pax2(+/+), Pkd1(-/-)Pax2(+/+)Pkd1(-/-)Pax2(-/-)) were fixed, embedded in paraffin and serial-sectioned through the whole kidney at a thickness of 4 μM. Six pairs of sections were selected in a systematic uniform manner starting from a randomly chosen first section. BrdU staining was carried out using a BrdU in situ detection kit following the manufacturer's recommendations (BD Pharmingen, San Diego, USA). BrdU-stained kidney sections were photographed, printed as montages and counting of positively stained nuclei was carried out as previously described (16). TUNEL labeling was carried out using an in situ cell death detection kit following the manufacturer's recommendations (Roche Diagnostics, Hoffman-La Roche Ltd., Basel, Switzerland). TUNEL-labeled sections were analyzed as digital images. A further set of eight sections, also with a randomly chosen first section, were selected systematically and analyzed using stereological grids to estimate the volume and also the luminal content of each kidney, using the Cavalieri method as previously described (24). The volume and luminal content was determined using the point-sample intercept method. To determine whether the total number of cells in kidneys from different genotypes was variable, an estimate of the tissue volume minus lumen content, as well as the cell density minus lumen content was made. To obtain total tissue volumes (minus the cyst or luminal volumes) of the kidney for each genotype, the luminal and/or cyst volumes were subtracted from total volumes of the E17.5 kidneys. Having done this it was found that the total tissue volumes of the E17.5 kidneys were essentially the same (0.7 ± 0.05 mm³) for each of Pkd1(+/+)Pax2(+/+), (wild-type), Pkd1(-/-)Pax2(+/+), (Pkd1 heterozygous), Pkd1(-/-)Pax2(-/-) and Pkd1(-/-)Pax2(+/+), (Pkd1 double mutant) kidneys. The corrected cell density was calculated as follows. Using a 5 cm × 5 cm counting frame on printed images of haematoxylin-stained kidney sections, all the nuclei were counted within the area. A stereology grid was then placed over the frame and used to estimate the percentage lumen in the area. The number of nuclei was then adjusted to that of the equivalent area with no luminal content. This was done five times on one section for each kidney.

Cell culture

MDCK (Madin Darby Canine Kidney cells) stably transfected with the full-length human PKD1 cDNA or the empty vector...
as a control, were grown in DMEM, 10% FBS, 1% penicillin/streptomycin, geneticin and zeocin. A detailed description of this cell line can be found elsewhere (22).

**Transient transfection assays**

A 4.5 kb hPAX2 promoter driving a luciferase expression vector (pGL2basic, Promega, Madison, WI, USA) and pGL2basic as a control were transfected into MDCK cells stably transfected with PKD1 cDNA and control cells. The transfections included the Renilla-luciferase expression vector, pRL-SV40 (Promega) as a control for transfection efficiency. Transfections were performed in triplicate in 24-well plates; each experiment was performed three times. At 90% confluency, cells were transfected with 200 ng of the corresponding plasmids using Fugene 6 (Roche) following procedures recommended by the manufacturer. Firefly-luciferase and Renilla-luciferase reporter activities were determined using Dual Luciferase Assay System reagents and protocol (Promega, Madison, WI, USA) and quantified in a MicroLumat Plus luminometer (EG&G Berthold, Salem, MA, USA).

**Western immunoblotting**

Proteins were isolated from transfected cells using a lysis buffer consisting of 8 m urea, 0.1 m EDTA, 4% SDS and 40 mM Tris–HCl of pH 6.8. Total protein lysate of 30 μg were diluted in 62.5 mM Tris–HCl of pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.004% bromophenol blue and boiled prior to loading into the gel. Samples were subjected to SDS–PAGE using a 10% separating gel. Samples were transferred to a nitrocellulose membrane, treated with blocking buffer (5% dry milk in PBS-Tween 0.1%), probed with rabbit polyclonal anti-murine Pax2 antibody (1:250) (Zymed). Membranes were washed and reprobed with peroxidase-conjugated goat anti-rabbit IgG antibody (1:1000) (Perkin-Elmer Life Science, Boston, MA, USA). Blocking membranes were then treated with enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech, Buckinghamshire, UK) and exposed to autoradiography film (Kodak Biomax MR Film). Membranes were stripped and reprobed with mouse monoclonal anti-actin antibody (1:5000) (Oncogene, Boston, MA, USA) as a control to ensure equal loading of protein in the wells. The pixel density of protein bands identified by immunoblotting was adjusted for the density of the control protein using MCID-M5 4.0 image analysis software.

**Statistical analysis**

For the percentage luminal volume calculations Tukey’s Honest Significant Difference test was used to assess all pairwise differences between the three genotypes based on fitting an ANOVA model to account for genotype, litter and litter–genotype interaction effects. For the kidney mass calculations, Pax2 and luciferase expression, and apoptosis calculations, two-sample t-tests (pooled variance) were used to compare between pairs of genotypes, or cell lines. For the BrdU-labeling calculations, a global F was used to test for equality of the means.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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