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Pax2 gene dosage influences cystogenesis in autosomal dominant polycystic kidney disease

Cherie Stayner[1,3], Diana M. Iglesias[2], Paul R. Goodyer[2], Lana Ellis[1], Greg Germino[3], Jing Zhou[4] and Michael R. Eccles[1,*]

[1]Developmental Genetics Laboratory, Department of Pathology, University of Otago, PO Box 913, Dunedin, New Zealand, [2]Montreal Children's Hospital Research Institute, 4060 St Catherine Street West, Westmount, Quebec, Canada H3Z 2Z3, [3]Department of Molecular Biology and Genetics, Johns Hopkins Medical Institute, Baltimore, USA and [4]Brigham and Women's Hospital, Harvard Medical School, Boston, USA

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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 *Pkd1*$^{del34/del34}$ mutant mice, *Pax2* was expressed in cyst epithelial cells, and facilitated cyst growth in *Pkd1*$^{del34/del34}$ mutant mice. In *Pkd1*$^{del34/del34}$ mutant kidneys, the expression of *Pax2* persisted in nascent collecting ducts. In contrast, homozygous *Pkd1*$^{del34/del34}$ 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 *Pkd1*$^{del34/del34}$/*Pax2*$^{1Neu/+}$ 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.

*To whom correspondence should be addressed. Tel: +64 34797878; Fax: +64 34797136; Email: michael.eccles@stonebow.otago.ac.nz

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 $Pkd1^{del34/+}$ 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 $Pkd1^{del34/del34}$ mutant mice carrying a heterozygous $Pax2^{1Neu/+}$ 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 $Pkd1^{del34/del34}$ 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 $Pkd1^{del34/del34}$ kidney, while the medullary regions of non-cystic adult human kidney from an unaffected individual, and from non-cystic adult mouse heterozygous mutant $Pkd1^{del34/+}$ 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 $Pkd1^{del34/+}$ heterozygous mutant mice occur infrequently. For this reason kidney sections from E18.5 fetal mice carrying a homozygous $Pkd1^{del34/del34}$ 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 $Pkd1^{del34/del34}$ 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 epithelia, and cysts that did not express Pax2 were derived from Pax2 negative epithelial structures (data not shown).

Homozygous $pkd1^{del34/del34}$ mutant mice develop a rapidly progressive PKD

Cysts were observed in fetal homozygous $Pkd1^{del34/del34}$ 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 $Pkd1^{del34/del34}$ 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,

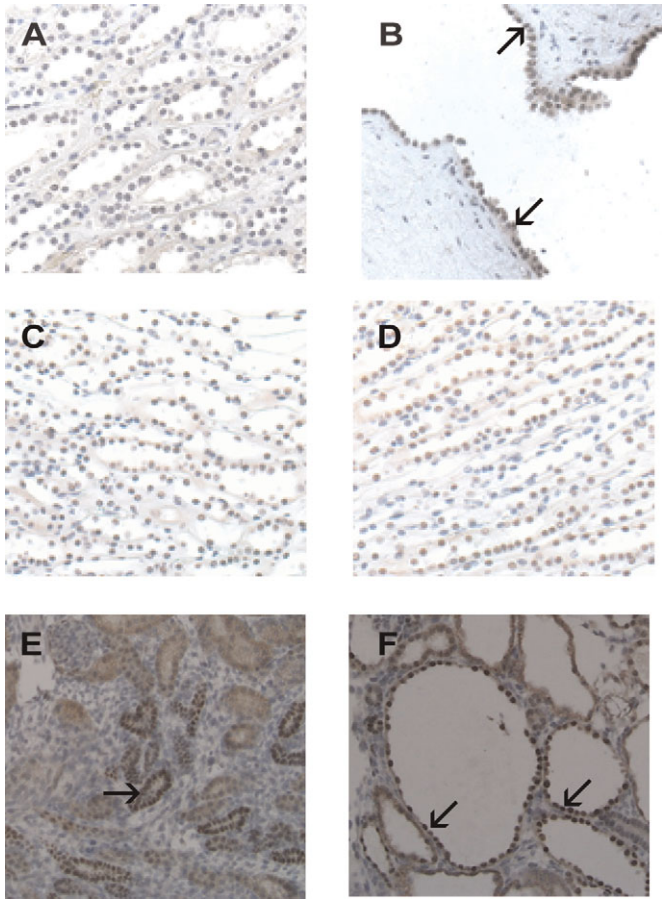

Figure 1. Expression of PAX2 in human and mouse ADPKD cyst epithelia. (**A**) Immunohistochemistry performed using a Pax2 antibody on normal human adult kidney medulla and (**B**) section of kidney from a patient with ADPKD showing nuclear PAX2 expression in the cyst epithelial cells (arrows). (**C**) Immunohistochemistry using a Pax2 antibody on a section of wild-type kidney of a normal adult mouse and (**D**) non-cystic adult mouse heterozygous mutant *Pkd1*$^{del34/+}$ kidney. (**E**) Immunohistochemistry using a Pax2 antibody on kidney medulla from a wild-type E18.5 fetal mouse showing nuclear Pax2 expression in inner medullary collecting ducts (arrow) and (**F**) on a section of cystic E18.5 fetal mouse kidney from a mouse with a homozygous *Pkd1*$^{del34/del34}$ mutation, showing nuclear Pax2 expression in the cyst epithelial cells (arrows).

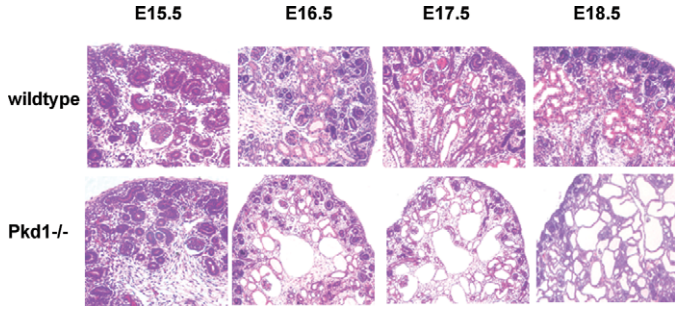

Figure 2. Cystogenesis in *Pkd1*$^{del34/del34}$ kidneys when compared with wild-type kidneys. Paraffin-embedded sections from E15.5, E16.5, E17.5 and E18.5 kidneys stained with haematoxylin from wild-type mice, and *Pkd1*$^{del34/del34}$ homozygous mutant mice (*Pkd1*$^{-/-}$) showing the progressive generation of numerous cysts in *Pkd1*$^{-/-}$ kidneys in comparison with the wild-type kidneys.

Table S1). Extra-renal defects in homozygous *Pkd1*$^{del34/del34}$ mutants probably accounted for the perinatal lethality.

Pax2 haploinsufficiency reduces cyst size in *Pkd1*$^{del34/del34}$/*Pax2*$^{1Neu/+}$ 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*^{1Neu}/+ 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*$^{1Neu/+}$ mutant mice was associated with reduced nephrogenesis and smaller kidneys, and immunohistochemical staining revealed less intense Pax2 immunostaining in *Pax2*$^{1Neu/+}$ mutant kidneys than in wild-type kidneys (16). The mechanism of reduced nephrogenesis in *Pax2*$^{1Neu/+}$ mutants was shown to be due 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*$^{+/+}$) *Pax2* allele, or a heterozygous (*Pax2*$^{1Neu/+}$) *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*$^{1Neu/+}$ 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*$^{1Neu/+}$ 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*$^{1Neu/+}$ heterozygous mutation were on average 30% lighter than wild-type kidneys, and were much smaller than the homozygous mutant *Pkd1*$^{del34/del34}$ kidneys ($P < 0.001$). *Pkd1*$^{del34/del34}$/*Pax2*$^{1Neu/+}$ double mutant kidneys were much lighter than *Pkd1*$^{del34/del34}$ kidneys ($P < 0.001$), and were instead similar in weight to *Pax2*$^{1Neu/+}$ 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 the different genotypes due to cystogenesis were not yet discernable (Supplementary Material, Fig. S1 A,B).

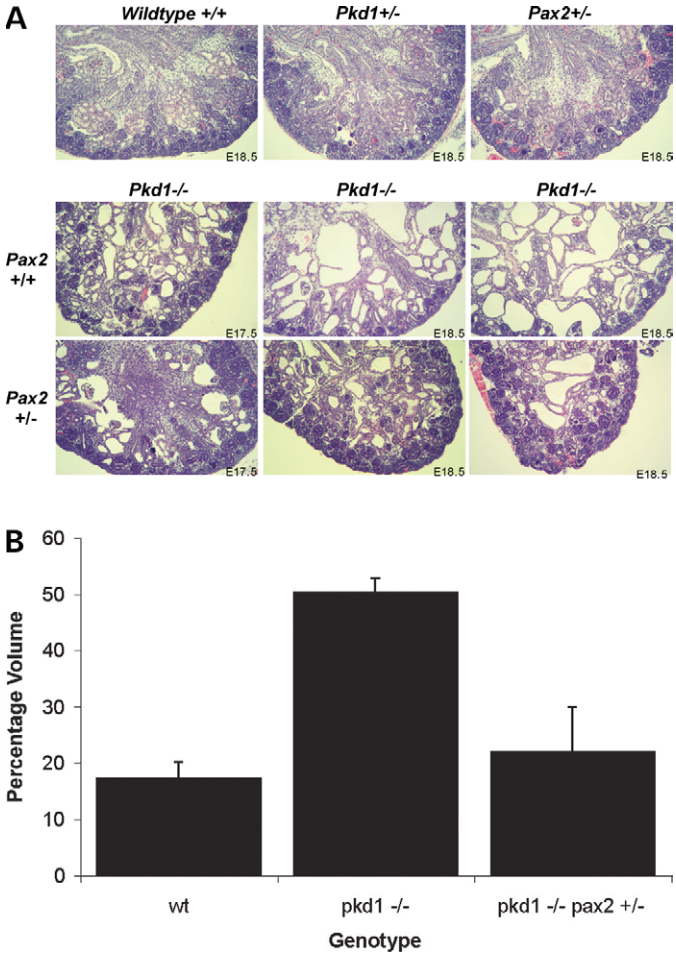

Figure 3. Inhibition of cystogenesis in *Pkd1* mutant mice carrying a heterozygous *Pax2* mutation. (**A**) Paraffin-embedded sections cut in the same plane from E17.5 or E18.5 kidneys were stained with haematoxylin. In the top three panels, the phenotypes of wild-type *Pkd1* heterozygous mutant (Pkd1 +/−) and *Pax2* heterozygous mutant (Pax2 +/−) kidneys are shown. No cystic disease is observed in these panels. In the bottom six panels, the effect of the presence of a heterozygous *Pax2* mutation on cyst formation (as seen in the lower three panels), is shown by comparison to the effect of absence of the *Pax2* mutation (upper three panels) in *Pkd1* homozygous mutant kidneys. Reduced cyst formation is observed in the presence of a *Pax2* heterozygous mutation when compared with the panels with absence of a *Pax2* heterozygous mutation. (**B**) Percentage lumen or cyst in E18.5 kidneys. The graph shows the percentage lumen or cyst present in wild-type kidneys (wt), $Pkd1^{del34/del34}$ kidneys (*Pkd1* −/−) and $Pkd1^{del34/del34}/Pax2^{1Neu/+}$ kidneys (*Pkd1* −/− *Pax2* +/−), after analysis using stereological methods. A significantly greater percentage of the kidney volume was cyst or lumen in the *Pkd1* −/− kidneys when compared with either wt ($P = 0.016$) or to *Pkd1* −/− *Pax2* +/− ($P = 0.021$). No significant difference was observed between wt and *Pkd1* −/− *Pax2* +/− ($P = 0.499$). Error bars represent SD.

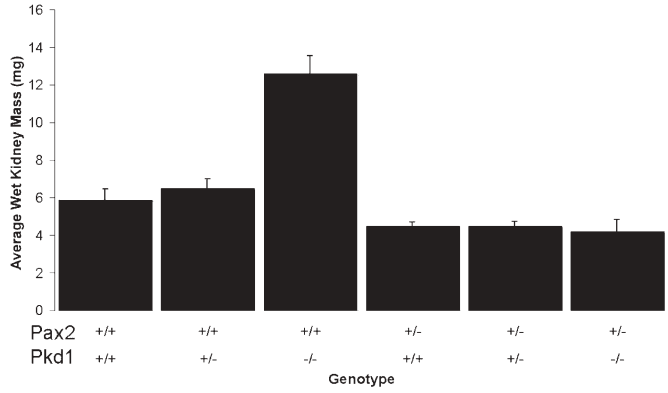

Figure 4. Effect of *Pax2* gene dosage on kidney weight in E18.5 *Pkd1*/*Pax2* crosses. Relationship between kidney mass and genotype in E18.5 fetal mice. The difference between kidney mass in *Pax2* +/+ *Pkd1* −/− fetal mice and kidney mass in *Pax2* +/− *Pkd1* −/− fetal mice was highly significant ($P < 0.001$). Error bars represent SD.

Neither cell proliferation nor apoptosis was altered in cystic kidneys from $Pax2^{1Neu/+}/Pkd1^{del34/del34}$ mice when compared with $Pkd1^{del34/del34}$ mice

To investigate whether the *Pax2* mutation in double mutant $Pkd1^{del34/del34}/Pax2^{1Neu/+}$ 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 $Pax2^{1Neu/+}$ heterozygous mutation together with the $Pkd1^{del34/del34}$ homozygous mutation did not significantly alter the rate of either cell division or apoptosis in the kidneys compared with the rate in cystic $Pkd1^{del34/del34}$ kidneys that lacked the *Pax2* mutation (Supplementary Material, Fig. S2 A,B).

Pax2 is persistently expressed in $Pkd1^{del34/del34}$ 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 $Pkd1^{del34/del34}/Pax2^{1Neu/+}$ 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 Bowmans capsule, where Pax2 expression persisted in squamous epithelial cells of Bowmans 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 $Pkd1^{del34/del34}$ ureteric bud epithelia than normal, and might fail to attenuate in the mature collecting duct epithelia of $Pkd1^{-/-}$ kidneys, thereby leading to cystogenesis. A detailed examination of Pax2-stained kidney sections from homozygous $Pkd1^{del34/del34}$ mutant mice showed that, when compared with wild-type mice, Pax2 protein was indeed expressed persistently along

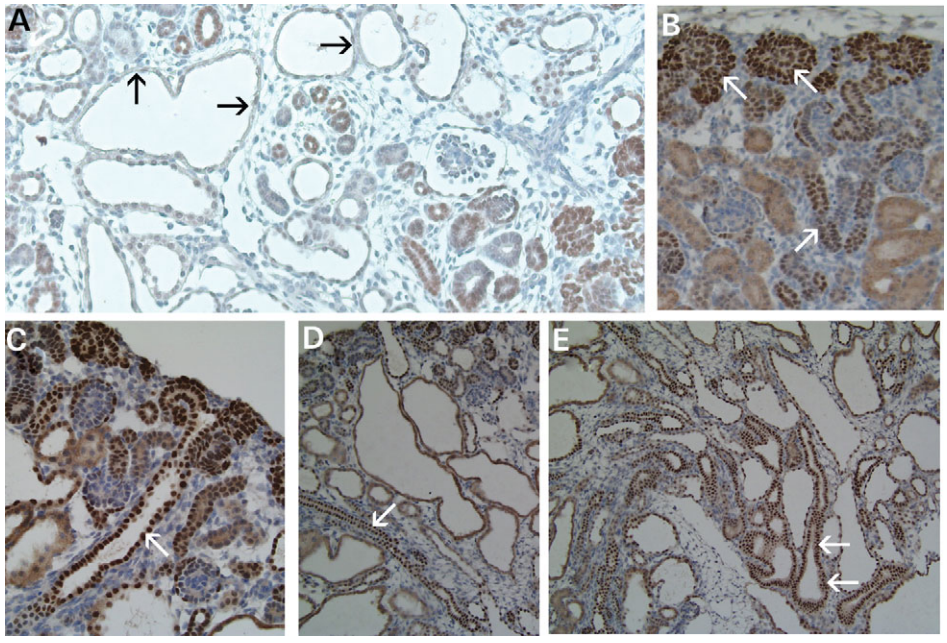

Figure 5. Pax2 is persistently expressed in ADPKD renal cysts. (**A**) In double mutant *Pkd1*$^{del34/del34}$/*Pax2*$^{1Neu/+}$ kidneys, Pax2 expression in the remaining small cysts (arrows) was very low or absent in comparison with the early nephrogenic structures at the right side of the image, as detected by immunohistochemistry with a Pax2 antibody. (**B**) Pax2 immunohistochemistry in wild-type E18.5 fetal kidney revealed strong Pax2 expression in the nephrogenic zone (arrows) in the early nephrogenic structures. Soon after fusing with the nephrogenic mesenchyme, Pax2 expression in the ureteric buds becomes attenuated, and Pax2 levels in the prospective collecting ducts are reduced in intensity by comparison with the early nephrogenic structures (arrow). (**C**–**E**) In contrast, Pax2 expression in prospective collecting ducts in *Pkd1*$^{del34/del34}$ homozygous mutant kidneys remain comparatively strongly expressed (C) (arrows). Persistent expression of Pax2 is observed in collecting ducts reaching deep into the medullary zone (D) (arrow). The expression of Pax2 in the ureteric bud and collecting ducts is continuous with the renal cystic epithelia (E) (arrows). Magnification: (A, D, E) 20×, (B, C) 40×.

the length of the ureteric buds at levels similar to that in normal cells, and unlike wild-type or *Pkd1*$^{del34/+}$ kidneys, remained expressed in *Pkd1*$^{del34/del34}$ 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 *Pax2*$^{1Neu/+}$ 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 MDCK^{Zeo} cells lacking endogenous PC-1 to that in MDCK^{Zeo} 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 MDCK^{Zeo} cells.

We also transiently transfected MDCK^{Zeo} and MDCK^{Pkd1Zeo} 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 *Pkd1*$^{del34/del34}$/*Pax2*$^{1Neu/+}$ mutant mice were markedly reduced in the presence of the heterozygous *Pax2* mutation when compared with *Pkd1*$^{del34/del34}$ 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.

Pkd1$^{del34/+}$ 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,

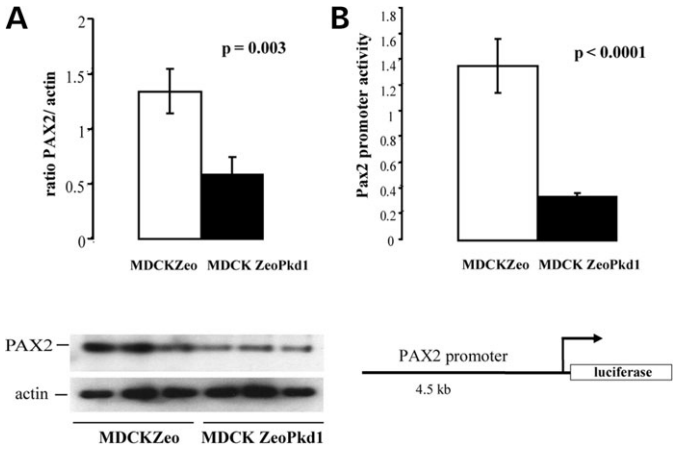

Figure 6. PC-1 suppresses *PAX2* expression in MDCK cells. (**A**) $MDCK^{Zeo}$ and $MDCK^{ZeoPkd1}$ cell lysates (30 μg protein) were resolved on 10% polyacrylamide gels. Pax2 and actin were detected by western immunoblotting. Band density was measured and the Pax2/actin ratios from three separate experiments were plotted in a bar graph. $MDCK^{ZeoPkd1}$ cells express only 46% as much endogenous Pax2 protein as control cells ($P = 0.003$). (**B**) $MDCK^{Zeo}$ and $MDCK^{ZeoPkd1}$ cells were co-transfected with a pRL-SV40 *renilla* plasmid and a plasmid containing the luciferase gene under the control of the h*PAX2* gene 5′-flanking sequence (4.5 kb). After 48 h, cell lysates were assayed for luciferase activity and normalized to *renilla* values. Results are expressed as luciferase/*renilla* absorbance units. *PAX2* promoter activity in $MDCK^{Zeo}$ cells was three times higher than in $MDCK^{ZeoPkd1}$ cells.

cyst formation in homozygous $Pkd1^{del34/del34}$ mutant mice was very marked and 100% penetrant at E18.5, with cysts occurring in most epithelial structures, including the Bowmans capsule of glomeruli (6). The kidney cysts in $Pkd1^{del34/del34}$ 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 $Pkd1^{del34/del34}/Pax2^{1Neu/+}$ 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 $Pkd1^{del34/del34}$ 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 $Pax2^{1Neu}$ mutation reflects haploinsufficiency rather than a gain-of-function. Haploinsufficiency is considered most likely because the phenotypic effect of the $Pax2^{1Neu/+}$ 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 $Pax2^{1Neu/+}$ 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 $Pax2^{1Neu/+}$ 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 $Pkd1^{del34/+}$ heterozygous 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 $Pkd1^{del34/del34}$ 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 heterozygous 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 $Pkd1^{del34/del34}$ mice carrying a $Pax2^{1Neu/+}$ mutation, these mice did not exhibit greater survival than $Pkd1^{del34/del34}$ mice, probably because homozygous $Pkd1^{del34/del34}$ mutant mice died as a result of polyhydramnios, hydrops fetalis, spina bifida occulta, osteochondrodysplasia or other uncharacterized abnormalities that were not influenced by the *Pax2* mutation (6).

Cyst formation in recessive *Cpk* polycystic kidney mouse was ameliorated by *Pax2* haploinsufficiency similar to that in $Pkd1^{del34}$ 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 epithelia 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 characterization of the signaling pathways involving *PKD1* and *PAX2* may allow the identification of critical intermediates involved in renal cystogenesis, and potential molecular targets for the design of therapeutic agents to treat PKD.

MATERIALS AND METHODS

Mouse breeding, tissue isolation and genotyping

Heterozygous mutant *Pkd1*^{del34} and *Pax2*^{1Neu} mice were obtained on C3H backgrounds. The *Pkd1*^{del34} knockout mice had been back-crossed onto the C3H strain for at least 10 generations. *Pax2*$^{1Neu/+}$ mice were crossed with *Pkd1*$^{del34/+}$ mice to produce 25% of offspring carrying the genotype, *Pkd1*$^{del34/+}$/*Pax2*$^{1Neu/+}$, which were then back-crossed with *Pkd1*$^{del34/+}$ mice to produce the following genotypes: *Pkd1*$^{+/+}$/*Pax2*$^{+/+}$ (wild-type), *Pkd1*$^{+/+}$/*Pax2*$^{1Neu/+}$ (*Pax2* mutant), *Pkd1*$^{del34/del34}$/*Pax2*$^{+/+}$ (*Pkd1* homozygous mutant), *Pkd1*$^{del34/del34}$/*Pax2*$^{1Neu/+}$ (*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 by caesarian section at E15.5, E16.5, E17.5 or E18.5, the fetus 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 for paraffin embedding. Genotyping was carried out by PCR amplification of tail-tip DNA with primers that would allow detection of the *Pkd1*^{del34} transgene cassette, or the *Pax2*$^{1Neu/+}$ G insertion mutation. PCR products were then electrophoresed on 1% agarose gels (*Pkd1*^{del34}), or analyzed by DHPLC (*Pax2*^{1Neu}) to detect the respective genotypes. PCR primers used were as follows: *Pax2* forward primer, 5′-GGGCACGGGGGTGTGAACCAG-3′; *Pax2* reverse primer, 5′-CTGCCCAGGATTTTGCTGACACAGCC-3′; *Pkd1* intron 34 (reverse primer for mutant or wild-type allele), 5′-CTTTAATCCCTGCACTCAGGA-3′; *Pkd1* exon 34 (forward primer for wild-type allele), 5′-CTGATCCATC AGGTACTGGCT-3′; *Neo* Cassette (forward primer for mutant del34 allele), 5′-CAGCGCATCGCCTTCTATC-3′.

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 tetrachloride detection as the chromagen, with haematoxylin counterstaining.

Stereological analysis of mutant and wild-type kidneys

Whole fetal kidneys (*Pkd1*$^{+/+}$/*Pax2*$^{+/+}$, *Pkd1*$^{+/+}$/*Pax2*$^{1Neu/+}$, *Pkd1*$^{del34/del34}$/*Pax2*$^{+/+}$, *Pkd1*$^{del34/del34}$/*Pax2*$^{1Neu/+}$) 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 photomontages 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 lumenal 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 lumenal volumes) of the kidney for each genotype, the lumenal 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 mM^3) for each of *Pkd1*$^{+/+}$/*Pax2*$^{+/+}$ (wild-type), *Pkd1*$^{del34/del34}$/*Pax2*$^{+/+}$ (Pkd1 −/−) and *Pkd1*$^{del34/del34}$/*Pax2*$^{1Neu/+}$ (Pkd1 −/−, Pax2 +/−) kidneys. The *Pkd1*$^{+/+}$/*Pax2*$^{1Neu/+}$ (Pax2 +/−) kidneys were slightly smaller, as expected. There was also very little difference between corrected cell densities in the kidneys between the genotypes (127 ± 5 nuclei per frame). 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 lumenal 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 h*PAX2* 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 mM 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). The 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 lumenal 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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