Trans-heterozygous Pkd1 and Pkd2 mutations modify expression of polycystic kidney disease

Guanqing Wu\(^1\), Xin Tian\(^1\), Sayoko Nishimura\(^1\), Glen S. Markowitz\(^4\), Vivette D’Agati\(^4\), Jong Hoon Park\(^5\), Lili Yao\(^1\), Li Li\(^5\), Lin Geng\(^1\), Hongyu Zhao\(^2,3\), Winfried Edelmann\(^6\) and Stefan Somlo\(^1,3,\)*

\(^1\)Department of Internal Medicine, \(^2\)Department of Epidemiology and Public Health and \(^3\)Department of Genetics, Yale University School of Medicine, New Haven, CT, USA, \(^4\)Department of Pathology, College of Physicians and Surgeons, Columbia University, New York, NY, USA and \(^5\)Department of Medicine and \(^6\)Department of Cell Biology, Albert Einstein College of Medicine, Bronx, NY, USA

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Autosomal dominant polycystic kidney disease (ADPKD) occurs by germline mutation in PKD1 or PKD2. Evidence of homozygous inactivation of either gene in human cyst lining cells as well as in mouse knockout models strongly supports a two-hit mechanism for cyst formation. Discovery of trans-heterozygous mutations in PKD1 and PKD2 in a minority of human renal cysts has led to the proposal that such mutations also can play a role in cyst formation. In the current study, we investigated the role of trans-heterozygous mutations in mouse models of polycystic kidney disease. In Pkd1\(^+/\)-, Pkd2\(^+/\)- and Pkd1\(^+/\)-; Pkd2\(^+/\)- mice, the renal cystic lesion was mild and variable with no adverse effect on survival at 1 year. In keeping with the two-hit mechanism of cyst formation, approximately 70% of kidney cysts in Pkd2\(^+/\)- mice exhibited uniform loss of polycystin-2 expression. Cystic disease in trans-heterozygous Pkd1\(^+/\)-; Pkd2\(^+/\)- mice, however, was notable for severity in excess of that predicted by a simple additive effect based on cyst formation in singly heterozygous mice. The data suggest a modifier role for the ‘trans’ polycystin gene in cystic kidney disease, and support a contribution from threshold effects to cyst formation and growth.

**INTRODUCTION**

Autosomal dominant polycystic kidney disease (ADPKD) is associated with germline mutations in PKD1 or PKD2 (1,2). The respective gene products, polycystin-1 and polycystin-2, act non-redundantly as a receptor-channel complex in a signaling pathway that is essential to the formation and maintenance of the highly differentiated state of lumen-forming epithelia in the kidney, liver and pancreas (3–5). Alterations in polycystin signaling that result in polycystic kidney disease affect proliferation, apoptosis, polarization and transcellular transport properties of renal epithelial cells (6). Molecular genetic analyses of cyst lining cells from kidneys and livers of ADPKD patients have suggested that cysts arise by clonal expansion of single cells that undergo somatic second hits leading to homozygous inactivation of either PKD1 or PKD2 (7–10). In support of this hypothesis, homozygous inactivation of either gene in mice results in cyst formation that supplants normal tubular elongation during kidney embryogenesis (11,12). Direct evidence of the causative role of second hits in cyst formation comes from the occurrence of ADPKD in compound heterozygous adult mice carrying a null allele for Pkd2 in combination with a Pkd2 allele that is prone to inactivation by genomic rearrangement (13).

Cellular recessive homozygous inactivation of one polycystin gene may not be the sole mechanism by which cysts form. Evidence of continued polycystin-1 immunoreactive protein expression in the majority of cysts (14), coupled with the notion that detection of loss of heterozygosity in end-stage human cystic tissue does not prove cause and effect, has been used to call into question the primacy of the two-hit mechanism (15). The occurrence of cysts in mice overexpressing polycystin-1 has been interpreted as supporting a role for the level of polycystin-1 expression in the human disease (16). A trans-heterozygous variant of the two-hit mechanism, with germline mutation at one polycystin gene coupled with somatic mutation

*To whom correspondence should be addressed: Boyer Center for Molecular Medicine, Yale University School of Medicine, 295 Congress Avenue, New Haven, CT 06519-1418, USA. Tel: +1 203 737 2974; Fax: +1 203 737 5313; Email: stefan.somlo@yale.edu
Correspondence may also be addressed to Guanqing Wu, Department of Medicine, Vanderbilt University, 2215 Garland Avenue, Nashville, TN 37232-0275, USA. Tel: +1 6159361761, Fax: +1 6159362661, Email: guanqing.wu@vanderbilt.edu.
The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.
at the other, has also been proposed (7). This hypothesis is supported by observations of somatic PKD1 mutations in kidney cysts of patients with germline PKD2 mutations, and vice versa (17,18).

In the current study, we investigated the role of trans-heterozygous mutations at Pkd1 and Pkd2 in mouse models of polycystic kidney disease. We used mice with true null alleles that do not express detectable transcripts for either Pkd1 or Pkd2 to determine the phenotypic effects of heterozygosity at either or both disease genes. In all genotypes, including Pkd1<sup>+/−</sup>: Pkd2<sup>+/−</sup>, the renal cystic lesion was generally mild and involved a minority of tubules. There were no adverse effect on survival up to 12 months. This suggests that trans-heterozygous inactivating mutations alone are not sufficient to cause microscopically visible cyst formation. However, semiquantitative analysis of cyst occurrence among wild-type, Pkd1<sup>+/−</sup>: Pkd2<sup>+/−</sup> and Pkd1<sup>+/−</sup>: Pkd2<sup>+/−</sup> mice showed that Pkd1<sup>+/−</sup>: Pkd2<sup>+/−</sup> mice develop more renal cysts than predicted by a simple additive effects model. These findings are most consistent with the hypothesis that the expressivity of cystic disease resulting from somatic mutation at one polycystin gene is modified by the expression level of the other polycystin gene.

**RESULTS**

**Comparison of Pkd1 and Pkd2 knockout mice**

We inactivated Pkd1 by inserting a selectable neo<sup>+</sup> cassette into the AscI site at codon 4 in exon 1 in the same transcriptional orientation as Pkd1 (Fig. 1A). Genotyping of serial staged embryos show that Pkd1<sup>−/−</sup> mice die in mid-gestation, beginning at E12.5 (Table 1) (11,19–21) in a manner very similar to Pkd2<sup>−/−</sup> mice (12). A minority of Pkd1<sup>−/−</sup> embryos show edema at E12.5; by E14.5, virtually all non-resorbing embryos are edematous (Table 1) and exhibit diffuse hemorrhages (data not shown) (19). Viable Pkd1<sup>−/−</sup> embryos are rare after E16.5, although three pale and small Pkd1<sup>−/−</sup> fetuses were live-born, only to die in the immediate perinatal period (Table 1).

Northern hybridization failed to detect a Pkd1 transcript in Pkd1<sup>−/−</sup> embryos using a downstream hybridization probe (exons 31–34) (Fig. 1B) as well as an upstream probe (exons 2 and 3; data not shown). This finding was confirmed in RT–PCR experiments on E15.5 mouse kidneys (Fig. 1C). Our Pkd1<sup>−/−</sup> knockout allele differs from others published to date (11,19,20) in that it produces no detectable transcript and truncates any potential partial protein product at codon 4, in the putative leader sequence. As such, it is an isolated loss-of-function allele without potentially confounding effects of stable partial protein products. There are no differences in expression of the Pkd2 transcript in Pkd1<sup>−/−</sup> or Pkd1<sup>+/−</sup> embryos compared with controls (Fig. 1B). Conversely, there are no differences in expression of the Pkd1 transcript in Pkd2<sup>−/−</sup> and Pkd2<sup>+/−</sup> embryos and controls (Fig. 1D). There were also no differences in polycystin-2 protein expression detected by immunoblotting in Pkd1<sup>−/−</sup> and Pkd1<sup>+/−</sup> embryos and controls (data not shown) (20). We have previously shown that Pkd2<sup>+/−</sup> mice express half-normal levels of polycystin-2 (13), suggesting that, at least for polycystin-2, transcript dosage corresponds to protein dosage.

Pkd1<sup>−/−</sup> mice develop cysts in the pancreas by E14.5 and in the kidney beginning at E15.5 (Fig. 2). The livers appear normal during gestation. Consistent with a defect in tubular maturation and elongation in null animals, many early kidney cysts contain glomerular tufts (Fig. 2) similar to those seen in Pkd2<sup>−/−</sup> (12) and other Pkd1 mutant models (19). This differs from adult polycystic mice, where cysts affect discrete segments of the mature tubule (13), suggesting that they arise by a change in the state of terminal differentiation. Serial transverse four-chamber views of the hearts from Pkd1<sup>−/−</sup> embryos after E12.5 (n = 3) did not show defects in cardiac septation comparable to those observed in Pkd2<sup>−/−</sup> mice (12). The concordance of the phenotypes in the kidney and pancreas in our Pkd1 and Pkd2 knockouts that do not produce detectable transcripts of either gene supports the view that the polycystins function in a common pathway (5). The discordance of the cardiac phenotypes in our true null Pkd1 and Pkd2 mutants suggests that the inter-relationship of polycystin-1 and -2 function is more complex in cardiac development (12).

**Phenotypic consequences of trans-heterozygous Pkd1 and Pkd2 mutations**

The main purpose of the present study was to evaluate the role of trans-heterozygous mutations at Pkd1 and Pkd2 on cyst formation in the kidney. We excluded a role for inactivation of either Pkd gene in the transcription of the other gene, and extended this to the level of protein expression in the case of polycystin-2 in Pkd1<sup>−/−</sup> embryos (Fig. 1B and C and data not shown). To examine the phenotypic effects of trans-heterozygosity at Pkd1 and Pkd2, we intercrossed our Pkd1<sup>+/+</sup> and Pkd2<sup>+/−</sup> strains to produce cohorts of age-matched, littermate mice. The genetic backgrounds of both the Pkd1<sup>+/−</sup> and Pkd2<sup>−/−</sup> parental strains were approximately 40% 129/Sv and 60% C57Bl/6J. Expected Mendelian ratios were observed for the four genotypes (Pkd1<sup>+/−</sup>: Pkd2<sup>−/−</sup>; Pkd1<sup>+/−</sup>: Pkd2<sup>−/−</sup> and wild-type) in live-born progeny, and no deaths were observed in any group during the study. Cohorts were sacrificed at 3, 6, 9 and 12 months. Gross kidney surface cysts were most commonly observed in 12-month Pkd1<sup>+/−</sup>: Pkd2<sup>−/−</sup> mice [4 of 14 kidneys (29%)]; additionally, one gross cyst, each, was observed in Pkd1<sup>+/−</sup>: Pkd2<sup>−/−</sup> at 9 months (6%) and in Pkd1<sup>+/−</sup> at 12 months (10%). Liver weights were uniform for all groups at all time points (data not shown). Gross liver cysts occurred in three of seven (43%) Pkd1<sup>+/−</sup>: Pkd2<sup>−/−</sup> mice at 12 months, but were also seen in one of five (20%) and two of seven (29%) Pkd2<sup>−/−</sup> mice at 9 and 12 months, respectively. Grossly visible surface liver cysts were not observed in Pkd1<sup>+/−</sup> mice at any time point.

For histologic analysis, all kidneys from study animals were fixed in 10% formalin, hemisected in the sagittal plane and embedded in paraffin. Two non-contiguous sections of equal, maximal area from each kidney (four sections per study animal) were stained with hematoxylin–eosin and scored for number of microscopic cysts by two renal pathologists (G.S.M. and V.D.) blinded to the genotypes. Cysts were defined as tubules with three or more times normal proximal tubular diameter. The sum of the number of cysts in four sections from
each animal was used as an index of cystic disease severity. Histologic examination found cysts distributed within the cortex and outer medulla (Fig. 3C–G). Most cysts were lined by cuboidal or flattened epithelium devoid of brush border, consistent with distal tubular origin. Rare proximal tubular and glomerular cysts were also seen. The majority of cysts were microscopic, ranging from three to five times the normal proximal tubular diameter. Some cysts were lined by...
hyperplastic epithelium (Fig. 3F). All cases with cysts also had scattered dilated tubules, possibly representing incipient cysts (Fig. 3C). There were no obvious histologic differences in the appearance of cysts with differing genotypes or at varying ages (Fig. 3).

The observed numbers of cysts for each strain at each time point are summarized in Table 2 and Figure 3A. After multiple comparison adjustments, no significant differences between genotypes were found in cyst number at 3 months. However, Pkd1+/−: Pkd2+/− mice had statistically significant differences in cyst occurrence when compared with Pkd1+/− (P = 0.02), Pkd2+/− (P = 0.01) and wild-type (P = 0.03) at 6 months, with wild-type (P = 0.04) at 9 months, and with Pkd2+/− (P = 0.005) and wild-type (P = 0.007) at 12 months (Fig. 3A). Since our data did not detect age dependence in cyst occurrence, we also pooled the cyst number by genotype across all age groups. Pkd1+/−: Pkd2+/− mice had significant differences in cyst occurrence compared with Pkd1+/− (P = 0.006), Pkd2+/− (P = 0.0001) and wild-type (P = 0.0001) when analyzed in this manner (Fig. 3A). To assess whether heterozygosities at Pkd1 and Pkd2 have simple additive effects on cyst occurrence, we treated the number of cysts in a given mouse as a Poisson random variable. Under the hypothesis of additive effects, the means of the number of cysts in the Pkd1+/−, Pkd2+/− and Pkd1+/−: Pkd2+/− mice were modeled as being μ1, μ2 and μ1 + μ2, respectively; without this additive effects constraint, the means of the number of cysts were modeled as being μ1, μ2 and μ3, respectively. When likelihood-ratio tests were performed to test the additive effects of Pkd1+/− and Pkd2+/− in Pkd1+/−: Pkd2+/−, the null hypothesis of additive effects was rejected at all time points (P < 0.001).

**Loss of polycystin-2 as a mechanism of cyst formation**

Somatic inactivation of functional PKD1 or PKD2 alleles has been implicated as the mechanism of cyst formation in ADPKD (7,13). Most somatic second hits in mice result from loss of the entire chromosome (22–24). Since the germline mutation in our Pkd2 knockout mouse produces no immunoreactive protein (12,13), we expected that the majority of cysts arising from homozygous inactivation of Pkd2 would exhibit absence of immunoreactive polycystin-2 in cyst lining cells. Cysts resulting from homozygous loss of polycystin-1 or from trans-heterozygous mutations to polycystin-1 and polycystin-2 without somatic loss of either would continue to show immunoreactive polycystin-2 in cyst lining cells.

We investigated these possibilities using immunocytochemical detection of polycystin-2 with polyclonal antisera directed against the cytosolic C terminus of the protein. These antisera, YCC2, have been used to localize polycystin-2 in paraffin sections (13,25–29), but the distribution of polycystin-2 in cryosections of adult mouse kidney has not been reported. Initially, we examined the immunocytochemical distribution of polycystin-2 in perfusion-fixed cryosections of wild-type kidneys from C57BL/6J mice. Nephrin segments were defined by co-staining with monoclonal antibodies to sodium proton (Na+/H+) exchanger 3 (NHE3) and Tamm–Horsfall protein as markers for proximal tubules and for thick ascending limb and distal convoluted tubules, respectively, and with Dolichus biflorus agglutinin (DBA) as a marker for collecting tubules (Fig. 4A). Under these conditions, polycystin-2 shows diffuse cytoplasmic staining in renal tubular cells in all cortical nephrin segments (Fig. 4A). Glomeruli do not have immunoreactive polycystin-2 (Fig. 4A). Medullary thick limbs of the loop of Henle as well as medullary collecting ducts are positive, while thin limbs of Henle are negative (data not shown). The segment-specific distribution of polycystin-2 is identical to that previously reported (26,27). The diffuse cellular staining pattern in cryosections is consistent with the pattern of expression of polycystin-2 in cultured epithelial cells (25) and with biochemical evidence that polycystin-2 is abundantly expressed in the endoplasmic reticulum (ER) of native kidney tissue as well (30). We further confirmed that this staining pattern is consistent with that previously reported for our Pkd2<sup>W25S</sup>/− adult mouse model of polycystic kidney disease (13). Consistent with the loss of polycystin-2 expression in cyst lining cells, the cyst lining in Pkd2<sup>W25S</sup>/− mice is negative for polycystin-2, while surrounding tubules show a diffuse cytoplasmic staining pattern (Fig. 4A).

To assess polycystin-2 expression in cyst-lining cells, kidneys from a pair of mice of each genotype (Pkd1+/−, Pkd2+/− and Pkd1+/−: Pkd2+/−) at 10–12 months of age were cut into four equal pieces in the sagittal plane, and cryosections were cut from each of 16 kidney pieces per genotype. A total of 20–25 non-contiguous sections from each genotype were examined by immunostaining with YCC2 (Fig. 4B). Among

### Table 1. Embryonic survival of Pkd1 mutant mice

<table>
<thead>
<tr>
<th>Age</th>
<th>Pkd1+/+ n (%)</th>
<th>Pkd1+/− n (%)</th>
<th>Pkd1−/− n (%)</th>
<th>Edema</th>
<th>Autolysis</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>E 10.5</td>
<td>4 (15)</td>
<td>14 (54)</td>
<td>8 (31)</td>
<td>0</td>
<td>0</td>
<td>26</td>
</tr>
<tr>
<td>E 11.5</td>
<td>6 (21)</td>
<td>15 (52)</td>
<td>8 (27)</td>
<td>0</td>
<td>0</td>
<td>29</td>
</tr>
<tr>
<td>E 12.5</td>
<td>11 (28)</td>
<td>21 (53)</td>
<td>8 (19)</td>
<td>1</td>
<td>1</td>
<td>40</td>
</tr>
<tr>
<td>E 13.5</td>
<td>8 (23)</td>
<td>21 (60)</td>
<td>6 (17)</td>
<td>1</td>
<td>1</td>
<td>35</td>
</tr>
<tr>
<td>E 14.5</td>
<td>5 (25)</td>
<td>12 (60)</td>
<td>3 (15)</td>
<td>2</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>E 15.5</td>
<td>10 (33)</td>
<td>16 (53)</td>
<td>4 (14)</td>
<td>3</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>E 16.5</td>
<td>8 (28)</td>
<td>17 (59)</td>
<td>4 (13)</td>
<td>3</td>
<td>1</td>
<td>29</td>
</tr>
<tr>
<td>E 17.5</td>
<td>9 (35)</td>
<td>15 (58)</td>
<td>2 (7)</td>
<td>1</td>
<td>1</td>
<td>26</td>
</tr>
<tr>
<td>At birth</td>
<td>10 (23)</td>
<td>30 (70)</td>
<td>3 (7)</td>
<td>1</td>
<td>0</td>
<td>43</td>
</tr>
<tr>
<td>3 weeks</td>
<td>24 (22)</td>
<td>86 (78)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>110</td>
</tr>
</tbody>
</table>

Genotypes of offspring from Pkd1+/− × Pkd1+/− intercross.

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a total of 22 cysts found in kidneys from \textit{Pkd1}^{+/−}/C0 mice, 17 (77%) stained positive for polycystin-2 (Fig. 4B) and 5 (23%) could not be evaluated owing to the markedly attenuated cellular profiles of lining cells. No cysts in \textit{Pkd1}^{+/−} mice showed absent staining for polycystin-2, confirming the presence of polycystin-2 immunoreactive protein in all cysts that could be evaluated. In contrast, in 21 cysts examined in the \textit{Pkd2}^{+/−} mice, 14 (67%) cysts showed uniform absence of polycystin-2 immunoreactivity (Fig. 4B), 4 (19%) showed persistent polycystin-2 immunoreactivity and 3 (14%) could not be evaluated. Finally, among 171 cysts observed in \textit{Pkd1}^{+/−}/\textit{Pkd2}^{+/−}/C0 mice, 160 (93%) showed uniformly positive staining for polycystin-2, 8 (5%) were negative for polycystin-2 and 3 (2%) could not be evaluated (Fig. 4B). In all cases, cyst lining cells were either uniformly positive or negative for polycystin-2; no cysts exhibited a heterogeneous staining pattern.

**DISCUSSION**

The functional inter-relationship of polycystin-1 and polycystin-2 is well established (3–5). In the present study, we sought to investigate the role of the combination of mutations at these genes on kidney cyst formation. In doing so, we used a pair of mutant mouse lines engineered to insure that no partial protein products were produced by either mutant allele. We compared exon 1 truncations of \textit{Pkd1} and \textit{Pkd2} in similar genetic backgrounds, and found very similar patterns of embryo survival, edema and hemorrhage and identical cystic phenotypes in the kidney and pancreas for both strains. In contrast, despite the similar nature of the mutations and the similar genetic backgrounds, we did not observe the defects in cardiac septation that are universally present in \textit{Pkd2} knockouts (12) in our \textit{Pkd1} knockouts. Defects in the cardiac conotruncus have been reported in one \textit{Pkd1} mutant line (20), but not in others (19,21). Therefore, cardiac defects in \textit{Pkd1} knockouts appear to be qualitatively different from those in \textit{Pkd2} knockouts, and may be influenced by genetic background or the nature of the germline mutation, perhaps through a dominant-negative mechanism.

The similarity of critical components of the null phenotypes for \textit{Pkd1} and \textit{Pkd2} suggests that the proteins act in a common genetic pathway in the affected organs. This is consistent with the findings in \textit{Caenorhabditis elegans}, where mutations to either or both polycystin homologues cause identical phenotypes (5). The course of ADPKD in humans is milder with germline mutations in \textit{PKD2} than with mutations in \textit{PKD1} (31). One implication of the similarity that we found in \textit{Pkd1} and \textit{Pkd2} knockouts is that the increased severity of human ADPKD observed in patients with mutations in \textit{PKD1} (31) is unlikely to be related to any inherently more severe phenotypic effect of loss of \textit{PKD1} compared with loss of \textit{PKD2}. This indirectly supports the notion that the increased severity of ADPKD in patients is at least in part a consequence of more frequent second hits at the larger, more complex \textit{PKD1} gene. In keeping with this hypothesis, our \textit{Pkd1}^{+/−} mice had a tendency to have more cysts than \textit{Pkd2}^{+/−} carrying the comparable null mutation.

Seventy percent of cysts in \textit{Pkd2}^{+/−} mice showed uniform absence of polycystin-2 immunoreactivity in cyst lining cells. The few cysts that continued to show immunoreactivity for polycystin-2 also did so in a uniform manner. Absence of
Figure 3. The cystic phenotype of Pkd1<sup>+/−</sup>:Pkd2<sup>+/−</sup> mice. (A) Cyst number presented as mean values ± SD for four genotypes at varying ages and in aggregate across all ages (n, number of animals in each group). Statistical analyses are presented in the text. (B–G) Representative H&E sections. The renal cortex of wild-type mice is devoid of cysts (B). Cysts (cy) lined by flattened epithelium are identified in Pkd1<sup>+/−</sup> (C), Pkd2<sup>+/−</sup> (D) and Pkd1<sup>++</sup>:Pkd2<sup>++</sup> (E) kidneys. Lesser degrees of tubular dilatation (dl) are also apparent (C). Some tubular cysts are lined by hyperplastic epithelium with enlarged, hyperchromatic nuclei with prominent nucleoli (Pkd1<sup>+/−</sup>, F). Cysts were identified as early as 3 months (Pkd1<sup>++</sup>:Pkd2<sup>++</sup>; G). (B–E, G: ×125 and F: ×250)
polycystin-2 in the majority of Pkd2+/− cysts is consistent with loss of the normal Pkd2 allele, while the uniformity of the absent staining within individual cysts supports their clonal origin. The minority of cysts showing apparent polycystin-2 expression may arise from cells sustaining second hits that resulted in a non-functional, but immunoreactive, polycystin-2 protein. In contrast, all cysts that could be evaluated in Pkd1+/−:Pkd2+/− mice showed uniformly positive staining for polycystin-2 indistinguishable from surrounding normal tubules, suggesting that polycystin-2 expression is not altered in Pkd1 cysts. This is in keeping with null embryos that also showed no differences in expression of the normal polycystin gene.

The absence of increased mortality or massive polycystic kidney disease in Pkd1+/−:Pkd2+/− mice effectively excludes the possibility that trans-heterozygous loss-of-function mutations in Pkd1 and Pkd2 are sufficient to cause cyst formation by a simple compound haploinsufficiency threshold model. This is in keeping with an instance of human bilineal ADPKD in which two individuals with an unknown mutation in PKD1 and a premature termination mutation in PKD2 had renal survival to approximately 50 years of age (32). As would be predicted by the two-hit mechanism, both polycystin-2 expressing and non-expressing cysts were found in Pkd1+/−:Pkd2+/− mice, but with a large preponderance (approximately 95%) of the former. The most likely explanation of these findings is that the expression or function of the normal polycystin-1 allele has been affected in the majority of cysts in trans-heterozygous animals.

The most striking feature of cyst formation in this study is the greater-than-additive effect of trans-heterozygous mutations in Pkd1 and Pkd2 on the number of microscopic cysts observed. This finding was consistently observed in the cohort study at all time points, was apparent in the six animals used in the immunostaining studies and could not have been predicted from human studies (32). The observation of increased numbers of microscopically visible cysts in Pkd1+/−:Pkd2+/− mice reflects an increased number of cystogenic events, an increased rate of cyst growth after such events or some combination thereof. Neither stochastic variation in the occurrence of second hits nor environmental factors can explain the significant variation in the trans-heterozygous versus singly heterozygous mice, leaving genetic modifier effects as the most likely cause. Two general classes of modifiers can be considered—those distinct from the polycystin genes (‘genetic background’) or the polycystin genes themselves. The difference in cyst number observed between Pkd1+/− or Pkd2+/− and Pkd1+/−:Pkd2+/− in littersmates is unlikely to be explained simply by genetic background effects. This conclusion is supported by the consistency of the observed extra-additive effect in multiple animals and at multiple time points. Therefore, the polycystin genes themselves or, nominally, genes very closely linked to the polycystin genes, are most likely to account for the more-than-additive effects of trans-heterozygous mutations.

Polycystin-1 and polycystin-2 interact to form a macromolecular signaling complex (4). Since the null Pkd1 and Pkd2 alleles that we used do not produce detectable transcripts, the observed effects in trans-heterozygous mice cannot occur from either dominantly or dominant-negatively acting alleles. A simple threshold model due to compound haploinsufficiency (i.e. 50% reduction in both genes as opposed to 50% reduction of either gene) also does not apply, since essentially all tubules are normal even at 12 months. The sporadic and focal nature of cyst formation in trans-heterozygous mice suggests the continued requirement of somatic events to cause cysts. Since complete inactivation by a second hit at either polycystin gene is sufficient to initiate cyst formation, the only means by which haploinsufficiency at the other locus can result in the observed extra-additive effect after complete inactivation is by causing faster growth of cysts. However, if inactivation of either gene completely abrogates polycystin signaling (5), it is difficult to understand how such a growth rate effect could occur – although one cannot rule it out.

The observed extra-additive effects may result from a modified threshold effect with somatic mutations producing hypomorphic, as opposed to completely inactivated, alleles in either gene. Under such a model, a somatically acquired variant in one of the polycystin genes produces an allele, likely a missense variant, with reduced function that would result in either a very slow-growing or no cyst on a wild-type background. However, in the presence of reduced dosage of its partner polycystin protein, a critical threshold of activity is reached, resulting in cyst formation. In keeping with this, there was an apparent ‘under-representation’ of complete loss of polycystin-2 by immunostaining (approximately 5%) in our Pkd1+/−:Pkd2+/− cysts, leaving open the possibility that an increased proportion of cysts arise by second hits that retain immunoreactive protein (i.e. missense variants). This hypothesis is consistent with observations in human cysts that identified trans-heterozygous mutations (17,18). Those studies did not find loss of heterozygosity at either polycystin gene, but did not exclude second hits resulting in hypomorphic allelic variants. Either this mechanism would require that the hypomorphic variants actually be human polymorphisms or it would require two independent second hits – albeit not necessarily in the same cells. In the former case, the presence of a germline Pkd1 mutation and a ‘weak’ normal Pkd1 allele will allow somatic mutation to either Pkd1 (more common) or Pkd2 to give rise to cysts. In the latter case, a hypomorphic

Table 2. Descriptive summaries for number of cysts

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>PKD1+/−−/−:Pkd2+/−−</th>
<th>PKD1+/−−</th>
<th>PKD2+/−−</th>
<th>Wild-type</th>
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</thead>
<tbody>
<tr>
<td>3</td>
<td>20.5 (18.9) n = 4</td>
<td>7.3 (6.4) n = 3</td>
<td>1.0 (0.0) n = 2</td>
<td>0 (0) n = 3</td>
</tr>
<tr>
<td>6</td>
<td>23.0 (15.0) n = 5</td>
<td>2.8 (4.3) n = 4</td>
<td>0.8 (1.2) n = 6</td>
<td>0.5 (0.7) n = 2</td>
</tr>
<tr>
<td>9</td>
<td>7.0 (6.9) n = 8</td>
<td>0 (0) n = 3</td>
<td>0.8 (1.3) n = 5</td>
<td>0.5 (1.2) n = 6</td>
</tr>
<tr>
<td>12</td>
<td>13.0 (11.5) n = 7</td>
<td>5.4 (6.1) n = 5</td>
<td>1.55 (1.92) n = 11</td>
<td>0 (0) n = 5</td>
</tr>
</tbody>
</table>

*Average number of cysts, the standard deviation (in parentheses) and the number of mice analyzed (n).*
Figure 4. Polycystin-2 expression in cysts. (A) Polycystin-2 expression in the renal cortex is present in a diffuse homogeneous cellular pattern in all nephron segments, but is absent from glomeruli (*). Polycystin-2 immune (YCC2; top panels) and pre-immune (bottom right three panels) antiserum double-labeled with the tubular basement membrane marker laminin-γ1, the proximal apical membrane marker Na+/H+ exchanger 3 (NHE3), the medullary thick ascending limb/distal convoluted tubule marker Tamm–Horsfall protein and the cortical collecting duct marker Dolichos biflorus agglutinin (DBA). The lower left panel shows YCC2 staining of cystic Pkd2WS25/C0 kidney tissue counterstained with propidium iodide. Polycystin-2 expression is absent in cyst lining cells (arrows). (B) Polycystin-2 expression in cyst lining cells as a function of genotype. Cysts in Pkd1+/−/C0 kidneys express near-normal patterns of polycystin-2. In contrast, polycystin-2 expression is uniformly absent in cyst lining cells in Pkd2−−/− kidneys. Pkd1+/−:Pkd2+/− kidneys (four right panels) typically show near-normal patterns of polycystin-2 expression (left panels). In a minority of cysts, however, polycystin-2 expression is absent (right panels). Arrows, cyst lining cells. Nuclear counterstain with propidium iodide in three lower left panels.
second hit to the normal allele could cause a slow-growing, mildly proliferative cyst; when one of those cystic cells subsequently undergoes an inactivating mutation eliminating one copy of the other polycystin gene, that cell goes on to form a cyst through more rapid growth.

This is the first indication that cyst number or growth resulting from mutations in the genes that cause human ADPKD are subject to genetic modifier effects. Understanding how this process is modulated may lead directly to the discovery of therapies for ADPKD.

MATERIALS AND METHODS

Inactivation of Pkd1

An approximately 7 kb BamHI-HindIII fragment containing exon 1 was excised from mouse BAC 287A3 (RPCI 22 library) and subcloned. The targeting construct was generated by inserting a positive selection marker into the unique AscI site in exon 1 and a negative selection marker in the vector arm (Fig. 1A). The construct was electroporated into WW6 ES cells (33) and 24 positive clones were identified by PCR. The PCR genotyping primers were 5'-TGG CCG CTT TTC TGG ATT CAT C-3' (NeoF1) in the selectable marker cassette and 5'-CCA ACC CTG GTT GCC AAC ACT G-3' (B8R10) outside the 3' end of the targeting construct. Two of the positive clones, verified by Southern blotting (Fig. 1A), were passed through the germline to produce Pkd1+/– F1 progeny. RT–PCR was performed using primers (e31F1; exon 31) GGA CCA AAA CCC CAC ACC CA and (e34R1; exon 34) AAG CAG GTC TGT TTC TAG GAG GG for Pkd1 (427 bp); (MCF11; exon 3) GTC GAA AGA GAA GAA CAA C and (MCR2a; exon 6) TCC CTC TCA CTT GTG TAG AT for Pkd2 (651 bp). The Pkd2 null strain has been previously described (12). Trans-heterozygous animals were obtained by intercrossing Pkd1+/– and Pkd2+/– mice.

Histology and immunocytochemistry

For cyst histology, mouse kidneys were immersion-fixed in 10% phosphate-buffered formalin, hemisected in the midline sagittal plane, embedded in paraffin and stained with hematoxylin–eosin as previously described (13). For immunocytochemistry, mouse tissues were obtained by perfusion fixation. Mice were anesthetized and perfused with ~30 cm3 of PBS containing 0.4% lidocaine and 0.01% heparin followed by fixation with approximately 45 cm3 PLP (periodate–lysine–paraformaldehyde: 0.01 M NaIO4, 0.75 M lysine and 2% paraformaldehyde) in phosphate buffer. Kidneys were removed and incubated for 4 hours in PLP buffer at 4°C. Each kidney was cut into four pieces in the sagittal plane using a Stadie–Riggs tissue slicer (Thomas Scientific). Tissues were dehydrated in 30% sucrose in PBS for 12 hours at 4°C and embedded in OCT (Sakura). Cryosections (10 μm) were prepared from each piece of frozen kidney. Sections were preincubated for 15 minutes in PBS containing 0.1% bovine serum albumin and 10% goat serum, followed by incubation for 1 hour with either YCC2 (25) or preimmune serum, both diluted at 1:1000. The YCC2 antiserum is directed against the cytoplasmic C terminus of polycystin-2. Goat anti-rabbit IgG conjugated with Alexa Fluor 488 (A-11032) and anti-rat IgG (A-11007), all from Molecular Probes. Sections were examined and digital images acquired by confocal laser scanning microscopy (Zeiss, LSM510).

Statistical methods

For each trait of interest (number of cysts, ratio of kidney to body weight, and ratio of liver to body weight), we compared the differences among the four strains at each time point (3 months, 6 months, 9 months and 12 months) and in aggregate. Since six comparisons were made for each trait at each time point, adjustments were made for multiple comparisons in the assessment of statistical significance levels. In particular, we used PROC MULTTEST in SAS (1994) to perform all pairwise comparisons, and chose the PERMUTATION option (n=10,000) to avoid possible departure from asymptotic results due to small sample sizes and possible violations of normal trait distributions. To assess whether Pkd1 and Pkd2 have additive effects on the number of cysts, we treated the number of cysts in a given mouse as a Poisson random variable. Under the hypothesis of additive effects, the means of the number of cysts in the Pkd1+/–, Pkd2+/–, and Pkd1+/–:Pkd2+/– strains were modeled as being μ1, μ2, and μ1 + μ2, respectively, whereas, without this additive effects constraint, the means of the number of cysts in the Pkd1+/–, Pkd2+/– and Pkd1+/–:Pkd2+/– strains were modeled as being μ1, μ2, and μ3, respectively. Likelihood-ratio tests were then performed to test the hypothesis of additive effects.

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