Defects in cell polarity underlie TSC and ADPKD-associated cystogenesis

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Clinical trials are underway for the treatment of tuberous sclerosis (TSC)-associated tumours using mTOR inhibitors. Here, we show that many of the earliest renal lesions from Tsc1+/− and Tsc2+/− mice do not exhibit mTOR activation, suggesting that pharmacological targeting of an alternative pathway may be necessary to prevent tumour formation. Patients with TSC often develop renal cysts and those with inherited co-deletions of the autosomal dominant polycystic kidney disease (ADPKD) 1 gene (PKD1) develop severe, early onset, polycystic kidneys. Using mouse models, we showed a genetic interaction between Tsc1 and Tsc2 with Pkd1 and confirmed an mTOR-independent pathway of renal cystogenesis. We observed that the Tsc and Pkd1 gene products helped regulate primary cilia length and, consistent with the function of this organelle in modulating cell polarity, found that many dividing pre-cystic renal tubule and hepatic bile duct cells from Tsc1, Tsc2 and Pkd1 heterozygous mice were highly misoriented. We therefore propose that defects in cell polarity underlie TSC and ADPKD-associated cystic disease and targeting of this pathway may be of key therapeutic benefit.

INTRODUCTION

Tuberous sclerosis (TSC) (MIM 191100) is an autosomal dominant disorder caused by germline mutations in either TSC1 (MIM 605284) or TSC2 (MIM 191092) and characterized by the development of benign hamartomatous growths in multiple organs and tissues (1). Patients with TSC often develop renal cysts and those with contiguous germline deletions of TSC2 and PKD1 develop severe infantile polycystic kidney disease (MIM 600273) (2), suggesting a functional co-operation between their gene products. Hamartin, the TSC1 gene product, and tuberin, the TSC2 gene product, function as a complex within the PI3K (phosphoinositide 3-kinase)-Akt-mammalian target of rapamycin (mTOR) pathway and regulate nutrient and growth factor signalling to mTOR (3). Many lesions from patients with TSC exhibit activation of mTOR and clinical trials are underway for the treatment of these tumours using mTOR inhibitors (4,5).

The primary cilia is a microtubule-based sensory organelle that receives both mechanical and chemical signals from other cells and the environment, and transmits these signals to the nucleus to elicit a cellular response (6). Primary cilia are anchored to the cell via the basal body. Hamartin has been localized to the basal body (7) and tuberin interacts with the autosomal dominant polycystic kidney disease (ADPKD1) gene product, polycystin-1 (MIM 601313) (8), which has been localized to the primary cilium (9). Numerous other proteins associated with cystic kidney disease have also been localized to the renal cilium or basal body including the ADPKD2 protein polycystin-2 (MIM 173910) (9,10), the product of the human autosomal recessive polycystic kidney disease gene (PKHD1), fibrocystin (MIM 606702) (11) and polaris and cystin, which are mutated in two mouse models of polycystic kidney disease (9). Mice with mutant polaris develop shortened cilia or no cilia in kidney epithelia (12) and PCK rats (an orthologous model for PKHD1) (13) have cilia that are abnormal and shortened (14).

Renal primary cilia monitor urinary flow through kidney tubules via the mechanotransduction properties of polycystin-1 and -2 (15), and the ciliary protein inversin acts as a molecular switch from the canonical to the non-canonical/planar cell polarity (PCP) Wnt signalling pathways by targeting cytoplasmic dishevelled (Dsh) for degradation (16). Furthermore, mutations in Kif3a, Ift88 and Ofd1, which

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disrupt ciliogenesis, restrict the activity of the canonical Wnt pathway with loss of Kif3a causing constitutive phosphorylation of Dsh (17). Interestingly, hamartin and tuberin associate with the GSK3/axin complex to promote β-catenin degradation and inhibit canonical Wnt-signalling (18), and tuberin also interacts with Dsh upon Wnt stimulation (19). The lengthening of developing renal tubules is associated with the mitotic orientation of cells along the tubule axis demonstrating intrinsic PCP (20), and this oriented cell division is thought to dictate the maintenance of constant tubule diameter during tubular lengthening, thereby preventing renal cyst formation (21). Defects in this process have been found in mice with a renal-specific inactivation of Tcf2, a transcription factor essential for the expression of genes involved in polycystic kidney disease (20), the PCK rat (20) and Pkd1 – deficiency (27). We found that these were >200% longer than primary cilia from pre-cystic renal tubule cells from wild-type, Tsc1/÷ or Tsc2/÷ mice (P < 0.001) (Table 1 and Fig. 2). We investigated whether hamartin and tuberin may play a role in the active maintenance of cilia via the modulation of intraflagellar transport (IFT). Mice with mutations in proteins necessary for cilia formation often have an absence or malfunction in nodal cilia in E7.5–E8.5 embryos which prevent the generation of the leftward nodal flow of extra-embryonic fluid required for the activation of the molecular signals in the left side of the body and, as a consequence, develop situs inversus (26). However, we failed to find any differences in nodal cilia from Tsc1/÷, Tsc1/÷, Tsc2/÷, Tsc2/÷ or wild-type embryos (Fig. 3), nor did we (23) or others (25), find any evidence of situs inversus or defects in cardiac tube position, suggesting that hamartin and tuberin may not play a direct role in IFT.

**RESULTS**

**Investigating the role of mTOR activation in TSC-associated renal cystogenesis**

We have previously engineered Tsc1/÷– mice by deleting part of exon 6 through to exon 8 of Tsc1 with the concurrent insertion of a reporter/selection cassette (23). Tsc1/÷– mice develop renal cysts which progress into cystadenomas and renal cell carcinomas (RCCs). Thirty-seven percent of cysts from these mice do not stain for phosphorylated S6 ribosomal protein (pS6), a downstream marker of mTOR activation, whereas almost all advanced lesions (cystadenomas and RCCs) do exhibit mTOR activation (24). Here, we studied renal lesions from Tsc1/÷–, Tsc2/÷– and Pkd1/÷– mice. We discovered an mTOR-independent mechanism of cystogenesis and demonstrated for the first time that hamartin, tuberin and polycystin-1 play a fundamental role in maintaining cell polarity in pre-cystic renal and hepatic cells.

**Investigating the role of mTOR activation in ADPKD-associated cystogenesis**

We studied two murine models of Pkd1-deficiency. Pkd1del17–21/÷ mice (hereafter termed Pkd1/÷– mice) have exons 17–21 of the Pkd1 gene replaced with a lacZ–neomycin fusion gene and develop a mild renal phenotype with microscopic cysts in ~50% of mice by 9 months of age (27) and occasional hepatic cysts. Pkd1nl/nl mice carry a hypomorphic Pkd1 allele that yields only 13–20% normally spliced Pkd1 transcripts in the majority of homozygotes and develop a severe renal phenotype of bilaterally enlarged polycystic kidneys (28). We investigated whether renal and hepatic cysts from these mice developed via an mTOR-independent pathway. We studied forty-two renal cysts and ten hepatic cysts from the Pkd1nl/nl mice, we found that significantly less small (<50 μm) cysts stained (56%, 168/300, Fig. 1) when compared with large (>200 μm) cysts (85%, 93/110; P < 0.001).

**Understanding the relationship between TSC and ADPKD**

We tested for a genetic interaction between Tsc1, Tsc2 and Pkd1 by crossing Tsc1/÷– and Tsc2/÷– mice with Pkd1/÷– mice. We found that Tsc1/÷–Pkd1/÷– mice had significantly more renal lesions (an average of 33.2 microscopic lesions per mouse) when compared with either Pkd1/÷– (5.2 lesions per mouse, P = 0.01) or Tsc1/÷– (10 lesions per mouse, P = 0.01) mice at 9–12 months. In terms of the type of lesion, Tsc1/÷–Pkd1/÷– mice had significantly more cysts and...
cystadenomas when compared with either Pkd1\(^{+/−}\) (\(P = 0.02\) and \(P < 0.01\), respectively) or Tsc1\(^{+/−}\) mice (\(P = 0.01\) and \(P = 0.009\), respectively). We also found that Tsc2\(^{+/−}\)Pkd1\(^{+/−}\) mice had more renal lesions (228.8 lesions per mouse), which were more advanced, when compared with Tsc2\(^{+/−}\) mice (152 lesions per mouse) at 15–18 months (\(P = 0.03\)). Hepatic cysts were rare in all mice studied (an average of 2.7 lesions per Pkd1\(^{+/−}\) mouse, 2.25 lesions per Tsc1\(^{+/−}\)Pkd1\(^{+/−}\) mouse and 1.7 lesions per Tsc2\(^{+/−}\)Pkd1\(^{+/−}\) mouse, at 15–18 months). In terms of pS6 staining, we found that 47% (27/58) of renal cysts from Tsc1\(^{+/−}\)Pkd1\(^{+/−}\) mice failed to stain, whereas 93% (26/28) of advanced renal lesions from these mice did stain (\(P < 0.001\)), and, in Tsc2\(^{+/−}\)Pkd1\(^{+/−}\) mice, significantly fewer renal cysts stained when compared with advanced lesions (128/163 versus 42/42, \(P < 0.001\), Fig. 1). We did not find any hepatic cysts from Tsc1\(^{+/−}\)Pkd1\(^{+/−}\) mice (eight cysts studied) and Tsc2\(^{+/−}\)Pkd1\(^{+/−}\) mice (seven cysts studied) that stained for pS6 (Fig. 1). These data suggest a functional co-operation between both hamartin and tuberin with polycystin-1 in an mTOR-independent manner.

We found that the lengths of primary cilia from pre-cystic renal collecting tubule cells from Pkd1\(^{+/−}\) mice were 5% longer than those found in wild-type animals (\(P = 0.02\)) (Table 1). Interestingly, the lengths of primary cilia from pre-cystic collecting tubule cells from Tsc1\(^{+/−}\)Pkd1\(^{+/−}\) and Tsc2\(^{+/−}\)Pkd1\(^{+/−}\) mice were also significantly longer than those found in Tsc1\(^{+/−}\) and Tsc2\(^{+/−}\) mice (\(P < 0.001\) for both) and were of a similar length to those found in Pkd1\(^{+/−}\) mice (Table 1). Conversely, we found that the lengths of primary cilia from pre-cystic liver cholangiocytes from Pkd1\(^{+/−}\), Tsc1\(^{+/−}\)Pkd1\(^{+/−}\) and Tsc2\(^{+/−}\)Pkd1\(^{+/−}\) mice were all significantly shorter (17.6, 18.1 and 17.3% shorter, respectively) than those found in wild-type animals (\(P = 0.02\)) and were of a similar length to those found in Tsc1\(^{+/−}\) and Tsc2\(^{+/−}\) mice (Table 1). These data suggest that polycystin-1 plays a role in modulating primary cilium length, but there are clear tissue-specific differences. We also noted that primary cilia from epithelial cells lining cysts from Tsc1\(^{+/−}\)Pkd1\(^{+/−}\) and Tsc2\(^{+/−}\)Pkd1\(^{+/−}\) mice were 61–66% shorter than those found in cysts from Tsc1\(^{+/−}\) and Tsc2\(^{+/−}\) mice (\(P < 0.001\) for both, Fig. 2), supporting a functional relationship between polycystin-1 and hamartin/tuberin that affects renal primary cilium length.

**Investigating the role of hamartin, tuberin and polycystin-1 in cell polarity**

We sought defects in cell polarity in our mice by assessing the mitotic orientations of dividing pre-cystic cells from the renal proximal tubule, collecting duct and loop of Henle/distal convoluted tubule from mice at 48 h of age. For wild-type mice, we found that 78% of dividing cells from the proximal tubule, 82% from the collecting duct and 78% from the loop of Henle/distal convoluted tubule divided within 10° of the longitudinal axis, demonstrating that, in agreement with others (20), oriented cell division is tightly regulated during tubule lengthening (Fig. 4). In contrast, we found significant defects in the mitotic orientations of dividing cells from Tsc1\(^{+/−}\), Tsc2\(^{+/−}\) and Pkd1\(^{+/−}\) mice. For Tsc1\(^{+/−}\) mice, we found that 41% of dividing cells from the proximal tubule, 45% from the collecting duct and 53% from the loop of Henle/distal convoluted tubule divided within 10° of the longitudinal axis (\(P = 0.002\), 0.003 and 0.039, respectively, compared with wild-type), for Tsc2\(^{+/−}\) mice, we found that 46% of dividing cells from the proximal tubule, 27% from the collecting duct and 44% from the loop of Henle/distal convoluted tubule divided within 10° of the longitudinal axis (\(P = 0.003\), <0.001 and 0.009, respectively, compared with wild-type) and for Pkd1\(^{+/−}\) mice, we found that 61% of...
Figure 2. SEM examination of renal primary cilia in pre-cystic collecting tubule cells (A and B) and epithelial cells lining renal cysts (C–F). Primary cilia from pre-cystic cells from Tsc2⁺/− mice (B, mean length 2.016 μm) were 10% shorter compared with those from wild-type littermates (A, mean 2.223 μm, \( P \), 0.001). Primary cilia from epithelial cells lining renal cysts from (C) Tsc1⁺/− (mean 5.157 μm) and (E) Tsc2⁺/− (mean 5.091 μm) mice were 200% longer than primary cilia from pre-cystic tubule cells from wild-type, Tsc1⁺/− or Tsc2⁺/− mice (\( P \), 0.001). Pkd1-haploinsufficiency significantly reduced the length of the primary cilia from epithelial cells lining cysts from Tsc1⁺/− or Tsc2⁺/− mice: (D) Tsc1⁺/− Pkd1⁺/− mice, mean 3.384 μm and (F) Tsc2⁺/− Pkd1⁺/− mice, mean 3.091 μm (\( P \) < 0.001 for both). Scale bars: 5 μm.

Table 1. Measurements of primary cilia length (μm) from pre-cystic renal tubule cells, epithelial cells lining renal cysts and pre-cystic hepatic cholangiocytes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Pre-cystic renal tubule cells, mean (and SD)</th>
<th>Epithelial cells lining renal cysts, mean (and SD)</th>
<th>Pre-cystic hepatic cholangiocytes, mean (and SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>2.233 (0.449), ( n = 205 )</td>
<td>n/a(^a)</td>
<td>7.342 (1.436), ( n = 72 )</td>
</tr>
<tr>
<td>Tsc1⁺/−</td>
<td>2.122 (0.537), ( n = 126 )</td>
<td>5.157 (3.059), ( n = 442 )</td>
<td>6.182 (1.495), ( n = 87 )</td>
</tr>
<tr>
<td>Tsc2⁺/−</td>
<td>2.016 (0.410), ( n = 255 )</td>
<td>5.091 (2.921), ( n = 128 )</td>
<td>6.404 (1.439), ( n = 94 )</td>
</tr>
<tr>
<td>Pkd1⁺/−</td>
<td>2.333 (0.399), ( n = 285 )</td>
<td>n/a(^b)</td>
<td>6.050 (1.448), ( n = 102 )</td>
</tr>
<tr>
<td>Tsc1⁺/− Pkd1⁺/−</td>
<td>2.389 (0.456), ( n = 269 )</td>
<td>3.384 (1.404), ( n = 157 )</td>
<td>6.011 (1.196), ( n = 134 )</td>
</tr>
<tr>
<td>Tsc2⁺/− Pkd1⁺/−</td>
<td>2.356 (0.589), ( n = 261 )</td>
<td>3.091 (1.351), ( n = 106 )</td>
<td>6.066 (1.764), ( n = 92 )</td>
</tr>
</tbody>
</table>

Measurements of pre-cystic tubule cells and cholangiocytes were taken from 3-month-old mice. \( n \) values denote number of primary cilia measured. n/a, not applicable.

\(^a\)Wild-type animals do not develop renal cysts.

\(^b\)No renal cysts were found from Pkd1⁺/− mice for SEM analysis.
dividing cells from the proximal tubule, 47% from the collecting duct and 44% from the loop of Henle/distal convoluted tubule divided within 10° of the longitudinal axis (P = 0.133, 0.001 and 0.002, respectively, compared with wild-type) (Fig. 4). Within each genotype, we observed no significant difference between the mitotic orientations of dividing cells from the different tubule segments.

We also observed that in Tsc1+/− mice, 21–30% of dividing cells from the proximal tubule, collecting duct and loop of Henle/distal convoluted tubule showed an ‘extreme’ dysregulation of mitotic orientation (with divisions between 60° and 90° to the tubule axis) and, similarly, in Tsc2+/− mice, 28–49% of dividing cells displayed this severe phenotype (Fig. 4). Such dysregulation was less frequently observed in dividing cells from Pkd1+/− mice (9–21% of cells depending upon tubule segment) and was rarely observed in wild-type mice (2–8% of cells) (P < 0.05 compared with both Tsc1+/− and Tsc2+/− cells). We also studied dividing cells from mice at 10 days of age and observed identical results to our studies at 48 h of age, with significant differences in the mitotic orientations of dividing cells in all regions of the kidney tubule from Tsc1+/−, Tsc2+/− and Pkd1+/− mice when compared with wild-type littermates (P < 0.05 for each genotype, Supplementary Material, Fig. S1). By 15 days of age, the number of dividing tubule cells had dramatically reduced in all mice regardless of genotype and by

Figure 3. SEM examination of nodal cilia from E8.5 embryos. (A) Lower magnification view of the node region (arrow) and notochordal plate (arrow head). We observed no difference in the length or structure of nodal cilia from wild-type (B), Tsc1+/− (C), Tsc1−/− (D), Tsc2+/− (E) and Tsc2−/− (F) embryos. Scale bars: (A) 100 μm, (B–F) 10 μm.
20 days of age, no dividing cells were observed indicating the completion of tubule development.

We generated more comprehensive, three-dimensional (3D) images of the aberrant mitotic orientations in the kidney using confocal microscopy. In agreement with our previous results, we found that only 44, 40 and 50% of dividing cells from Tsc1<sup>+/−</sup>, Tsc2<sup>+/−</sup> and Pkd1<sup>+/−</sup> mice, respectively, divided within 10° of the longitudinal tubule axis, as compared to 80% of dividing cells from wild-type mice (P = 0.023, 0.01 and 0.037, respectively, Fig. 5). We also found that 39 and 40% of dividing cells from Tsc1<sup>+/−</sup> and Tsc2<sup>+/−</sup> mice, respectively, showed an extreme dysregulation of mitotic orientation (divisions between 60° and 90° to the tubule axis) and this was only found in 5% of dividing cells from wild-type mice (P = 0.01 and 0.008, respectively, Fig. 5). These data clearly suggest that defects in cell polarity are present in pre-cystic cells of Tsc1<sup>+/−</sup>, Tsc2<sup>+/−</sup> and Pkd1<sup>+/−</sup> mice. We found no difference between Tsc1- and Tsc2-associated polarity defects. As expected, we did find significant differences in the orientations of dividing tubule cells from Tsc1<sup>+/−</sup>-Pkd1<sup>+/−</sup> and Tsc2<sup>+/−</sup>-Pkd1<sup>+/−</sup> mice when compared with their wild-type littermates, but did not observe any differences between Tsc1<sup>+/−</sup>-Pkd1<sup>+/−</sup> and Tsc2<sup>+/−</sup>-Pkd1<sup>+/−</sup> mice, or their corresponding single heterozygote littermates (all were similarly misoriented; data not shown).

We also addressed whether defects in cell polarity were present in hepatic tissues from our mouse models. We assessed the mitotic orientations of dividing pre-cystic cells from the hepatic bile ducts of mice at 10 days of age. For wild-type mice, we found that 94% (16/17) of cells from the bile duct divided within 10° of the longitudinal axis, demonstrating that, similar to the developing renal tubule, oriented cell division is tightly regulated during bile duct lengthening (Fig. 6). In contrast, we found significant defects in the mitotic orientations of dividing hepatic cells from Tsc1<sup>+/−</sup>, Tsc2<sup>+/−</sup> and Pkd1<sup>+/−</sup> mice. For Tsc1<sup>+/−</sup> mice, we found that only 33% (7/21) of cells divided within 10° of the longitudinal axis, for Tsc2<sup>+/−</sup> mice, we found that 29% (6/21) of cells divided within 10° of the longitudinal axis and for Pkd1<sup>+/−</sup> mice, we found that 45% (9/20) of cells divided within 10° of the longitudinal axis (P < 0.001 for all, compared with wild-type).

Further investigating the role of hamartin, tuberin and polycystin-1 in PCP

The most distinct example of vertebrate PCP is the uniform orientation of stereociliary bundles, consisting of a single specialized primary cilium and multiple stereocilia, at the apices of sensory hair cells in the mammalian auditory sensory organ (29). We analysed stereociliary bundles from Tsc1<sup>+/−</sup>, Tsc2<sup>+/−</sup>, Pkd1<sup>+/−</sup>, Tsc1<sup>+/−</sup>-Pkd1<sup>+/−</sup> and Tsc2<sup>+/−</sup>-Pkd1<sup>+/−</sup> mice by SEM to search for abnormalities in PCP but found no differences between these animals and their wild-type littermates. Regardless of genotype, all turns of the cochlea (from the apex through to the base) appeared normal in both structure and length (data not shown) and showed a uniform arrangement of three rows of outer hair cells, with a row of inner hair cells underneath (Fig. 7).

**DISCUSSION**

Clinical trials using mTOR inhibitors have produced promising results in the treatment of TSC-associated renal angiomyolipomas, often with a reduction in tumour volume immediately...
after treatment (4,5). Similar trials are underway for patients with ADPKD. Here, we found that many of the earliest renal lesions from \(\text{Tsc}1^{+/−}\), \(\text{Tsc}2^{+/−}\) and \(\text{Pkd}1^{+/−}\) mice did not exhibit activation of mTOR and other investigators have also found that \(~70\%\) of cysts from patients with ADPKD had weak or absent pS6 staining (7). These data suggest that although mTOR inhibitors may be an effective treatment for the advanced stages of TSC and ADPKD-associated kidney disease, they may have little effect in preventing initial cyst/tumour formation. Indeed, rapamycin has already been shown to have no effect on the number of microscopic precur-sor kidney lesions that develop in a rat model of \(\text{Tsc}2\)-inactivation (30).

Defects in the structure or function of primary cilia are thought to underlie numerous disorders associated with cystic kidneys and Hartman et al. (7) recently described a ciliary disrup-tion in TSC with enhanced cilia development in \(\text{Tsc}1\) and \(\text{Tsc}2\) null mouse embryonic fibroblasts which manifests in an mTOR-independent mechanism. In our study, we found that hamartin, tuberin and polycystin-1 all played a role in maintaining the length of primary cilia in pre-cystic renal tubule cells and hepatic cholangiocytes, but found no evidence for a direct role of these proteins in IFT. It is unclear whether these subtle (yet statistically significant) changes in primary cilia length have any pathophysiological consequence(s); however, this organelle is thought to modulate PCP (16) and we did observe aberrant cell polarity in pre-cystic renal and hepatic cells from our \(\text{Tsc}1^{+/−}\), \(\text{Tsc}2^{+/−}\) and \(\text{Pkd}1^{+/−}\) mice.

PCP is a common feature of many epithelia and is perpendicular to the apical/basal (A/B) polarity axis. Although the apical localization of PCP determinants such as Frizzled (Fz1) is critical for their function, the link between A/B polarity and PCP is poorly understood. Recent studies have shown that dPatj, which plays a key role in A/B polarity, binds to the cytoplasmic tail of Fz1 which recruits aPKC, and in turn phosphorylates and inhibits Fz1, thereby providing a direct link between A/B polarity and PCP (31). Accordingly, components of the aPKC complex and dPatj produce PCP defects in the \(\text{Drosophila}\) eye. Interestingly, tuberin has been found to directly interact with PATJ (32) and \(\text{Drosophila}\) with mosaic \(\text{Tsc}1\) mutant cells in their eyes exhibit ommatidial misrotations (33). Close examination of the misoriented tubule cells from \(\text{Tsc}1^{+/−}\), \(\text{Tsc}2^{+/−}\) and \(\text{Pkd}1^{+/−}\) mice revealed that most of these cells were dividing in a plane perpendicular to the epithelial sheath (Fig. 5), consistent with defects in A/B polarity. Given that primary cilia are the most apical structures in a cell, this raises the possibility that the defects that we observed in primary cilium length may in fact be secondary consequences of perturbed A/B polarity. Consistent with this hypothesis, loss of the PCP effector genes \(\text{fuzzy}\) and \(\text{inturned}\), leads to the disruption of the cytoskeleton and ciliogenesis defects (34). Such secondary effects on primary cilium may help to explain why we observed inconsistencies in cilium length from \(\text{Pkd}1^{+/−}\) mice in pre-cystic renal tubules (5% longer) and hepatic cholangiocytes (17.6% shorter than those from wild-type littermates).

![Figure 5](image-url)

**Figure 5.** Three-dimensional reconstruction of the mitotic orientations of dividing pre-cystic renal tubule cells using confocal microscopy. (A) Graph showing the distribution of the mitotic angles in \(\text{Tsc}1^{+/−}\) (light grey bar), \(\text{Tsc}2^{+/−}\) (dark grey bar) and \(\text{Pkd}1^{+/−}\) mice (black bar) compared with wild-type littermates (white bar). Significantly fewer cells from \(\text{Tsc}1^{+/−}\), \(\text{Tsc}2^{+/−}\) and \(\text{Pkd}1^{+/−}\) mice divided within \(10°\) of the tubule axis compared with wild-type littermates (\(P < 0.038\)). Confocal fluorescent micrographs showing examples of the orientation of dividing cells in wild-type (B), \(\text{Tsc}1^{+/−}\) (C), \(\text{Tsc}2^{+/−}\) (D) and \(\text{Pkd}1^{+/−}\) (E) mice using anti-H3pS10 to stain for dividing chromosomes (red) and THP to stain for tubules (green). Scale bars: 10 \(\mu\text{m}\).
It has recently been shown by others that hamartin and tuberin play a role in neuronal polarity (35) and here, we show a role for these proteins in both renal tubule and hepatic bile duct polarity. Since we did not find any obvious defects in the orientations of the stereociliary bundles from our mice, we suggest that hamartin, tuberin and polycystin-1 are not ‘classical’ PCP proteins and that the associated defects in polarity are tissue or cell-type specific.

In conclusion, we show that hamartin, tuberin and polycystin-1 play a key role in maintaining cell polarity. We propose that dysregulation of the A/B polarity (and consequently PCP) pathway initiates cystogenesis in TSC and ADPKD, and subsequent activation of mTOR promotes cyst expansion (in PKD) and tumour progression (in TSC). Therefore, pharmacological targeting of the A/B polarity pathway may be necessary to prevent the initiating stage of cystogenesis in both of these diseases. Several issues still require further investigation. First, although we demonstrated aberrant polarity in renal tubule cells and hepatic bile ducts from Tsc1<sup>−/−</sup>, Tsc2<sup>−/−</sup> and Pkd1<sup>−/−</sup> mice at 2–10 days of age, these animals ultimately develop tubules and bile ducts that are structurally indistinguishable from wild-type littermates and, in the case of the Tsc models, do not develop renal cysts for many months and never develop liver cysts. Although we have identified dilated renal tubules (which may result from aberrantly dividing tubule cells and subsequently develop into cysts) in Tsc1<sup>−/−</sup> and Tsc2<sup>−/−</sup> mice as early as 1 month of age, these were rare. We therefore propose that the aberrantly dividing cells remain ‘dormant’ until further cellular and/or molecular events trigger cyst formation. Interestingly, it has recently been shown that the phenotypic consequences of Pkd1-inactivation are defined by a developmental switch that signals the end of the terminal renal maturation process (36). Such developmental switches may also modulate Tsc-associated cyst development and explain the tissue-specific differences that we observed. Secondly, we hypothesize, but have not yet proven, that the activation of mTOR occurs after somatic inactivation of the wild-type Tsc1 or Tsc2 allele. In support of this hypothesis, we have previously shown that somatic Tsc1 mutations are infrequent in cysts but common in advanced lesions from Tsc1<sup>−/−</sup> mice (24). Further studies are therefore warranted to unravel the exact nature and sequence of events that occur after the initial aberrant cell divisions to promote renal tumourigenesis in TSC.

**MATERIALS AND METHODS**

Genotyping and immunohistochemistry

All procedures with animals were carried out in accordance with Home Office guidelines. PCR genotyping of DNA from tail tips and yolk sacs was performed by amplification of the wild-type and mutant Tsc1, Tsc2 or Pkd1 alleles using the following primers in a 35 cycle PCR reaction with AmpliTaq gold DNA polymerase (Applied Biosystems): Tsc1 wild-type, Ex8F 5′-TGCTGGAGGCCCACTGGT-3′ and Ex8R 5′-TGCAAGGCCCACCTGTT-3′ (183 bp product), Tsc1 mutant, TSC1HETF2 5′-AGGTGCTAACCAGTATA-3′ and TSC1HETR2 5′-TGATGCTAACCAGTATA-3′ (183 bp product), Tsc2 wild-type, Ex8F 5′-TGCTGGAGGCCCACTGGT-3′ and Ex8R 5′-TGCAAGGCCCACCTGTT-3′ (183 bp product), Tsc2 mutant, TSC1HETF2 5′-AGGTGCTAACCAGTATA-3′ and TSC1HETR2 5′-TGATGCTAACCAGTATA-3′ (183 bp product), and Pkd1 wild-type, Ex8F 5′-TGCTGGAGGCCCACTGGT-3′ and Ex8R 5′-TGCAAGGCCCACCTGTT-3′ (183 bp product), Pkd1 mutant, TSC1HETF2 5′-AGGTGCTAACCAGTATA-3′ and TSC1HETR2 5′-TGATGCTAACCAGTATA-3′ (183 bp product).
TT-3' and TSC1HETR 5'-CCAATGGGCTCATTACTCTCA-3' (268 bp product), Tsc2 wild-type, genF 5'-AATCGCCTCCGAATGATAGG-3' (658 bp product), Pkd1 wild-type, PKD1WTF 5'-GCTCGCACTTTCAGCAATAAGAC-3' and PKDWTR 5'-CAGGATTTCCACTGGGTTCT-3' (661 bp product), Pkd1 mutant, PKDNEOF 5'-AGCGTTGGCTACCCGTGATATTG-3' and PKDEXON21R 5'-GTCTCCGTGATGTTCTTACGCATT-3' (731 bp product). Products were analysed on 2% agarose gels. Genotyping for the Pkd1nl/nl mice was carried out as previously described (28). We determined the average number of microscopically visible kidney and liver lesions per mouse and performed immunohistochemistry using anti-pS6 (Ser240/244; Cell Signalling Technologies, Beverly, MA, USA) on both kidneys and livers from five mice per genotype, as previously described (23).

Scanning electron microscopy

At least five mice of each genotype were culled and perfused transcardially with 40 ml of phosphate buffered saline at pH 7.4 followed by 50 ml of phosphate buffered 4% formaldehyde/0.2% glutaraldehyde (PBFG). The kidneys and liver were removed, bisected longitudinally, post-fixed for 24 h in PBFG and infiltrated with 2.3 M sucrose in Tris-buffered saline (TBS). Kidneys and livers were frozen and the tubule lumens and bile ducts exposed by sectioning with a freezing stage sledge microtome. Cochleas encased in the temporal bone were dissected from 4-week-old mice and immersed in PBFG overnight at 4°C. The temporal bone and vestibular and tectorial membranes were then removed and the cochleas placed in fixative overnight and transferred to TBS. Embryos were removed from their extra-embryonic membranes and fixed in PBFG overnight. All specimens were then dehydrated using the hexamethyldisilazane method (37), mounted on aluminium stubs using carbon paint, sputter coated with gold using an EMscope vacuum coater (EMScope, Ashford, Kent, UK) and viewed at 5 kV in a JEOL 840A SEM (JEOL, Tokyo, Japan). Primary cilia lengths were measured using analySIS software (Soft Imaging System GmbH, Münster, Germany) by an observer blinded to genotype.

Orientation of cell division

Fluorescent microscopy. Kidney sections from mice at 48 h, 10, 15 and 20 days of age and liver sections from mice at 10 days of age were stained with anti-phospho-histone H3 (Ser\(^{\text{10}}\)) antibody (anti-H3pS10) (1:50, Cell Signalling Technologies) to label the chromosomes of dividing cells in late anaphase and telophase. Rehydrated 4 \(\mu\)m kidney and liver sections were boiled in 10 mM citrate buffer (pH 6.0) for 10 min and incubated overnight at 4°C with anti-H3pS10 and further incubated for 30 min at room temperature (RT) in the dark with tetramethyl rhodamine isothiocyanate (TRITC) conjugated goat anti-rabbit IgG (H+L) (1:300, Che-
micon International/Millipore). Sections were counterstained with either fluorescein isothiocyanate (FITC) 
Lotus tetragonolobus lectin (LTL) (1:100, Vector Laboratories, Burlingame, CA, USA) for the proximal kidney tubule, FITC Dolichos biflorus agglutinin (1:100, Vector Laboratories) for the collecting duct or immunostained overnight at 4°C with Tamm–Horsfall glycoprotein (THP) (1:150, Santa Cruz Biotechnology Inc., CA, USA) for the thick limb of the loop of Henle/distal convoluted tubule or cytookeratin 19 (CK19) (1:100, Santa Cruz Biotechnology Inc.) for cholangiocytes lining the bile duct, followed by FITC conjugated chicken anti-goat IgG (H+L) (1:200, Chemicon International/Millipore). All staining with fluorescent labelled reagents was performed in the dark for 30 min at RT. Slides were mounted with ProLong® Gold anti-fade reagent with DAPI (Invitrogen, Eugene, Oregon) and examined using an Olympus BX51 microscope (Olympus Optical, London, UK). Images were acquired using a Zeiss Axioscam digital camera and analysed with AxioVision software (both from Carl Zeiss Vision, Hallbergmoos, Germany). The orientation of cell division was determined by measuring the angle between the mitotic spindles of dividing cells and the longitudinal axis of the kidney tubules or bile ducts, using five mice per genotype for the kidneys and eight mice per genotype for the livers (the observer was blinded to genotype). Metaphase chromosomes were ignored to avoid the measurement of spindles that had not yet reached their definitive orientation.

Confocal microscopy. Thirty micron thick kidney sections from mice at 48 h of age were stained with anti-H3pS10 and THP, as described above, and imaged using a Leica TCS SP2 AOBS spectral confocal laser scanning microscope (Leica Microsystems, Heidelberg). Dividing pre-cystic cells from the loop of Henle/distal convoluted tubule were scanned using ×40 and ×63 oil immersion objective lenses using appropriate excitation and emission settings for sequential recordings of FITC (Ex[max]: 494 nm; Em[max]: 518 nm) and TRITC (Ex[max]: 555 nm; Em[max]: 580 nm). Z-stacks of optical sections (512 × 512 pixels) were taken through the tissue at a step size of 0.4 μm and these were used to create Maximum intensity-type 3D reconstructions using Leica Confocal software. Mitotic orientations were determined as described above.

Statistical analyses
We used two-sample t-tests or Mann–Whitney confidence interval tests to compare lesion counts and primary cilium lengths between genotypes and the χ²-test to compare the proportions of lesions staining for pS6 and the distributions of mitotic angles between genotypes.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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