Reduced Ciliary Polycystin-2 in Induced Pluripotent Stem Cells from Polycystic Kidney Disease Patients with PKD1 Mutations

Benjamin S. Freedman,* Albert Q. Lam,*† Jamie L. Sundsbak,‡ Rossella Iatrino,*§ Xuefeng Su,* Sarah J. Koon,‡ Maoqing Wu,* Laurence Daheron,† Peter C. Harris,‡ Jing Zhou,*† and Joseph V. Bonventre*†

*Renal Division and Harvard Center for Polycystic Kidney Disease Research, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts; †Harvard Stem Cell Institute, Harvard University, Cambridge and Boston Massachusetts; ‡Mayo Translational Polycystic Kidney Disease Center, Division of Nephrology and Hypertension, Mayo Clinic, Rochester, Minnesota; and §Nephrology, Dialysis, and Transplant Division, Policlinico Universitario di Modena, Modena, Italy

ABSTRACT
Heterozygous mutations in PKD1 or PKD2, which encode polycystin-1 (PC1) and polycystin-2 (PC2), respectively, cause autosomal dominant PKD (ADPKD), whereas mutations in PKHD1, which encodes fibrocystin/polyductin (FPC), cause autosomal recessive PKD (ARPKD). However, the relationship between these proteins and the pathogenesis of PKD remains unclear. To model PKD in human cells, we established induced pluripotent (iPS) cell lines from fibroblasts of three ADPKD and two ARPKD patients. Genetic sequencing revealed unique heterozygous mutations in PKD1 of the parental ADPKD fibroblasts but no pathogenic mutations in PKD2. Undifferentiated PKD iPS cells, control iPS cells, and embryonic stem cells elaborated primary cilia and expressed PC1, PC2, and FPC at similar levels, and PKD and control iPS cells exhibited comparable rates of proliferation, apoptosis, and ciliogenesis. However, ADPKD iPS cells as well as somatic epithelial cells and hepatoblasts/biliary precursors differentiated from these cells expressed lower levels of PC2 at the cilium. Additional sequencing confirmed the retention of PKD1 heterozygous mutations in iPS cell lines from two patients but identified possible loss of heterozygosity in iPS cell lines from one patient. Furthermore, ectopic expression of wild-type PC1 in ADPKD iPS-derived hepatoblasts rescued ciliary PC2 protein expression levels, and overexpression of PC1 but not a carboxy-terminal truncation mutant increased ciliary PC2 expression levels in mouse kidney cells. Taken together, these results suggest that PC1 regulates ciliary PC2 protein expression levels and support the use of PKD iPS cells for investigating disease pathophysiology.

Polycystic kidney disease (PKD) is associated with defects of primary cilia and replacement of the normal kidney parenchyma with tubular epithelial cysts and fibrosis, leading to progressive deterioration of kidney function. PKD is among the world’s most common life-threatening genetic diseases, affecting approximately 1 in 600 people, and it is a significant contributor to CKD. Autosomal dominant PKD (ADPKD) causes end stage kidney disease by the age of 60 years in approximately 50% of adults with the disease, whereas autosomal recessive PKD (ARPKD) is a more rare form that typically presents earlier in life and causes significant childhood mortality. PKD may be considered a developmental disorder, with renal cysts becoming detectable in utero even in ADPKD.1

Received November 16, 2012. Accepted April 22, 2013.
B.S.F. and A.Q.L. contributed equally to this work.
Published online ahead of print. Publication date available at www.jasn.org.
Correspondence: Joseph V. Bonventre, Renal Division, Brigham and Women’s Hospital, Harvard Medical School, Harvard Institutes of Medicine, Room 576, 4 Blackfan Circle, Boston, MA, 02115. Email: joseph_bonventre@hms.harvard.edu
Copyright © 2013 by the American Society of Nephrology
In addition to kidney cysts, hepatic involvement is common, with liver cysts developing in many ADPKD patients and congenital hepatic fibrosis being a hallmark of ARPKD.\textsuperscript{1,2} ADPKD is inherited as heterozygous mutations in \textit{PKD1} or \textit{PKD2}, whereas ARPKD is caused by biallelic mutations in \textit{PKHD1} (polycystic kidney and hepatic disease 1). These three genes encode transmembrane proteins, known as polycystin-1 (PC1), polycystin-2 (PC2), and fibrocystin/polyductin (FPC), respectively. PC1, PC2, and FPC form a receptor channel complex in membrane compartments including the primary cilium,\textsuperscript{3,4} a sensory organelle on the apical cell surface, and loss of this localization pattern has been observed in cystic renal epithelia from humans.\textsuperscript{5,6} Mutations in more than 50 gene products associated with the cilium cause a spectrum of related diseases known as the ciliopathies, most of which feature cystic kidneys.\textsuperscript{7} Ciliary trafficking signals have recently been identified at the carboxyl terminus of PC1 and the amino terminus of PC2, but the extent to which PC1 is involved in PC2 trafficking is not yet clear.\textsuperscript{8–11} The abnormal phenotype in ADPKD has been attributed to loss of epithelial cell heterozygosity as a result of an additional somatic mutation or environmental insult (the two-hit hypothesis), although there is also genetic evidence for a haploinsufficiency model.\textsuperscript{12–15}

There is a need for human disease-specific laboratory models for PKD to better understand disease and develop therapies, because animal models may not fully genocopy or phenocopy the human disease.\textsuperscript{16,17} Primary cells taken from nephrectomized ADPKD kidneys have been linked to various epithelial cell phenotypes, but because these cells are derived from kidneys with advanced disease, it remains unclear whether these characteristics represent primary defects central to PKD etiology or secondary consequences of injury or de-differentiation.\textsuperscript{6,18–21} A powerful new technology, induced pluripotent stem (iPS) cells are adult somatic cells which have been reprogrammed into an embryonic pluripotent state.\textsuperscript{22,23} The result is a next generation cell culture model that can differentiate into diverse cell types and complex tissues for the purposes of regenerative therapies or investigating disease. As for other hereditary diseases, iPS cells from patients with PKD can be examined for disease-specific abnormalities to better understand the pathophysiology of clinical mutations and screen for potential therapeutics.\textsuperscript{7,24} PKD iPS cells derived from unaffected cell types, such as fibroblasts, might be expected to have fewer secondary phenotypes compared with cystlining epithelial cells, and they could be used to investigate PKD during development, when PKD disease genes are most highly expressed.\textsuperscript{1,16,21,25} Their intrinsic pluripotency, ability to self-renew indefinitely, and immunocompatibility also make PKD iPS cells an attractive potential source for renal replacement tissue. As a first step in this direction, generation of iPS cells from one ADPKD patient was recently reported, although no disease phenotypes were described.\textsuperscript{26} In our study, we generate iPS cell lines from ADPKD, ARPKD, and healthy control patients and evaluate their ability to ciliate, proliferate, and express PKD disease genes to establish a system \textit{in vitro} for investigating human PKD. We identify reduced levels of PC2 at the primary cilium in undifferentiated iPS cells, differentiated somatic epithelial cells, and hepatoblasts as a consistent phenotype in three ADPKD patients with \textit{PKD1} mutations but not in ARPKD patients. Furthermore, we have found using ADPKD iPS-derived hepatoblasts and cultured kidney cells that wild-type but not mutant PCI promotes PC2 localization to cilia.

### RESULTS

#### Generation and Characterization of iPS Cells from Patients with PKD

Dermal fibroblasts were obtained from three patients clinically diagnosed with ADPKD and two newborns with ARPKD.

### Table 1. Patient clinical data at the time of dermal biopsy

<table>
<thead>
<tr>
<th>PKD Type</th>
<th>Patient Number</th>
<th>Age at Skin Biopsy</th>
<th>Sex</th>
<th>Clinical Features</th>
<th>Serum Creatinine (mg/dl)</th>
<th>Estimated GFR (ml/min per 1.73 m\textsuperscript{2})</th>
<th>Dialysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dominant</td>
<td>1</td>
<td>60 yr</td>
<td>Man</td>
<td>Numerous kidney and liver cysts; family history (brother and niece)</td>
<td>4.37</td>
<td>14</td>
<td>No</td>
</tr>
<tr>
<td>Dominant</td>
<td>2</td>
<td>44 yr</td>
<td>Man</td>
<td>Numerous kidney and liver cysts; no family history</td>
<td>7.41</td>
<td>8</td>
<td>No</td>
</tr>
<tr>
<td>Dominant</td>
<td>3</td>
<td>62 yr</td>
<td>Woman</td>
<td>Numerous kidney and liver cysts; paternal family history (father, brother, grandmother, two uncles, and two cousins)</td>
<td>3.69</td>
<td>12</td>
<td>No</td>
</tr>
<tr>
<td>Recessive</td>
<td>4</td>
<td>1 d (expired)</td>
<td>Boy</td>
<td>Bilateral flank masses; oversized kidneys with many elongated cortical cysts/dilated tubules surrounded by normal glomeruli; no hydroureter or hydronephrosis; liver portal fibrosis with cystically dilated biliary ducts; oligohydramnios; pulmonary hypoplasia; bilateral leg contractures at the knees; minor external ear abnormality; no family history</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recessive</td>
<td>5</td>
<td>6 d (expired)</td>
<td>Girl</td>
<td>Renal failure and pulmonary insufficiency; kidney showed cystic tubular dilatation extending to surface; liver showed portal fibrosis with hamartomatous tortuous bile ducts; nephromegaly with sponge appearance; hyponatremia; hypotension; hyperbilirubinemia; no family history</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
All patients had cystic kidneys and livers. Genetic sequencing of PKD1 and PKD2 in the parental fibroblasts revealed that all the ADPKD patients possessed heterozygous mutations in PKD1 ranging from likely to definitely pathogenic (Table 2) based on computational analysis and similar mutations in the ADPKD database (www.pkdb.mayo.edu). These mutations included a novel point mutation, C39Y, in a conserved residue near the leucine-rich repeat region at the amino terminus, a novel truncating nonsense mutation, E1929X, in the PKD domains upstream of the transmembrane and intracellular domains, and a point mutation, R2051P, in the PKD domains similar to one previously described in the database. No pathogenic mutations were identified in PKD2.

One of the ARPKD patients had two missense mutations in PKHD1 likely to be pathogenic: W2736G, a previously described mutation (www.humgen.rwth-aachen.de) at an invariant site in FPC orthologs, and T36M, which is the most common mutation associated with ARPKD. The other patient was born with enlarged cystic kidneys, biopsy-confirmed hepatic fibrosis, pulmonary insufficiency, and oligohydramnios, and therefore, the patient fitted the clinical profile of ARPKD but had no obvious PKHD1 mutations. PKHD1 mutations are not always found by sequencing, and it is possible that this patient had mutations in noncoding regions of PKHD1, which were not examined, or rare mutations in another ciliopathy gene.

Fibroblasts from each patient were reprogrammed into distinct PKD iPS cell lines by retroviral transduction of OCT4 (octamer binding transcription factor 4), SOX2 (SRY [sex determining region Y]-box 2), KLF4 (Kruppel-like factor 4) and c-MYC (the avian myelocytomatosis virus oncogene cellular homolog). Representative PKD iPS cell lines from each patient were confirmed to resemble federally approved embryonic stem (ES) cell lines with respect to expression of pluripotency markers and alkaline phosphatase (Figure 1A), normal karyotype (Figure 1B), and in vivo and in vitro differentiation into cell types in teratomas and cell marker studies representing the three embryonic germ layers (Figure 1, C and D).

### Table 2. Patient genetic information

<table>
<thead>
<tr>
<th>PKD Type</th>
<th>Patient Number</th>
<th>Harris ID/Coriell ID</th>
<th>Gene</th>
<th>Exon</th>
<th>Mutation Call</th>
<th>Mutation</th>
<th>SIFT Score</th>
<th>A-GVGD Score</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dominant 1</td>
<td>OX3502/—</td>
<td>PKD1</td>
<td>1</td>
<td>C39Y</td>
<td>Highly likely pathogenic</td>
<td>No</td>
<td>C65</td>
<td>APF 0.00</td>
<td>Conserved residue in LRR</td>
</tr>
<tr>
<td>Dominant 2</td>
<td>OX3503/—</td>
<td>PKD1</td>
<td>15</td>
<td>R2051P</td>
<td>Likely pathogenic</td>
<td>Yes</td>
<td>C15</td>
<td>APF 0.03</td>
<td>Not very well conserved site but nonconservative change</td>
</tr>
<tr>
<td>Dominant 3</td>
<td>OX3504/—</td>
<td>PKD1</td>
<td>15</td>
<td>E1929X</td>
<td>Definitely pathogenic</td>
<td>No</td>
<td>Unnecessary</td>
<td>APF 0.00</td>
<td>Conserved in ortholog and fibrocyt-L protein</td>
</tr>
<tr>
<td>Recessive 4</td>
<td>OX3687/GM10287</td>
<td>PKHD1</td>
<td>3</td>
<td>T36M</td>
<td>Definitely pathogenic</td>
<td>Yes</td>
<td>C65</td>
<td>APF 0.00</td>
<td>Most common PKD1 mutation</td>
</tr>
<tr>
<td>Recessive 5</td>
<td>OX3688/GM12607</td>
<td>PKHD1</td>
<td>52</td>
<td>W2736G</td>
<td>Highly likely pathogenic</td>
<td>Yes</td>
<td>Unnecessary</td>
<td>APF 0.00</td>
<td>Conserved in ortholog and fibrocyt-L protein</td>
</tr>
</tbody>
</table>

ID, identification; A-GVGD, align Grantham variation Grantham deviation; SIFT, sorting intolerant from tolerant; APF, affect protein function; LRR, leucine rich repeats.

PKD iPS Cells Express PKD Disease Genes

We found that iPS cells express PC1, PC2, and FPC proteins and we evaluated the effect of PKD mutations on expression characteristics of these proteins. Immunoblots for PC1 in undifferentiated ES and iPS cell lines revealed a set of overlapping bands at ~450 kDa corresponding to the wild-type protein (Figure 2A). In the ADPKD patient harboring a truncating mutation (patient 3), an additional band was consistently observed at ~250 kDa, possibly representing truncated PC1 expressed from the mutant allele (Figure 2A). PC2 immunoblots revealed single bands corresponding to the 110 kDa PC2 monomer and a higher-molecular mass multimer, which were of similar intensity in all of the lines (Figure 2B). To further confirm the specificity of our antibodies, human ES cells were compared side by side with IMCD3 (inner medullary collecting duct 3) cells, which were either untransfected or

(Table 1).
Figure 1. iPS cell lines derived from patients with PKD are pluripotent and karyotypically normal. (A) Immunocytochemistry of pluripotency markers for representative lines from each patient on feeder layers. (B) Corresponding normal karyotypes of iPS lines. (C) Hematoxylin and eosin-stained teratoma sections showing pigmented epithelium (ectoderm), cartilage (mesoderm), and glandular epithelium (endoderm). Similar results were obtained for all control lines. (D) Directed differentiation of iPS lines into lineages expressing
transfected with full-length PKD1. The PC1 antibody recognized a doublet at ~450 kDa in IMCD3 cells, consistent with reports in other kidney cell lines, which increased in intensity after transfection with PKD1 and comigrated with ES cell PC1 (Figure 2C).15 PC2 immunoblots revealed bands at 110 kDa in both IMCD3 and ES cells, which were not increased after transfection with PKD1 (Figure 2D). FPC was also expressed in ES and iPS cell lines, with a major band at ~450 kDa that comigrated with exogenously expressed human FPC (Figure 2E). No obvious or consistent differences were observed in the expression level of FPC in wild-type versus ARPKD cell lines. These results indicate that PKD disease proteins are expressed in undifferentiated pluripotent stem cells and that naturally occurring mutations in these proteins do not grossly impact their levels or stability in these cells.

Proliferation, Apoptosis, and Ciliogenesis in PKD iPS Cells

We tested representative PKD iPS lines for differences in rates of proliferation and cell death, which have been linked to PKD.1,2,11 Bromodeoxyuridine (BrdU) incorporation, histone H3 phosphorylation, and cleaved caspase-3 expression occurred at statistically indistinguishable rates in undifferentiated PKD and control iPS cell lines (Figure 3, A and B). We next investigated the ability of both PKD and control iPS cells to elaborate primary cilia, which has been previously reported in ES cells.29 Unstarved, undifferentiated OCT4-positive ES or iPS cells cultured on glass for 7 days in pluripotent stem cell media elaborated short primary cilia containing acetylated α-tubulin (Figure 3C). Quantitative comparison revealed similar numbers of ciliated cells among ES, control iPS, ADPKD, and ARPKD iPS cells (Figure 3D).

Reduced Ciliary PC2 in ADPKD iPS Cells

Localization of polycystins to the cilium is suggested to be defective in cyst-lining epithelia.5,6 In undifferentiated iPS cell cultures, PC2 localized prominently to the primary cilium in a proportion of the ciliated cell population and more faintly to the plasma membrane (Figure 4A). Quantitative analysis revealed an ~75% reduction in the number of cells with detectable PC2 at the cilium in iPS lines from the three ADPKD patients compared with healthy control lines or ARPKD iPS lines (Figure 4, A and B). Using confocal z stacks, we also measured the length of the cilary shaft in all of the lines, and found no differences that might account for the difference in PC2 fluorescence (Figure 4, C and D). Line scans drawn perpendicular to and through the center of individual ciliary shafts in these z stacks confirmed that the average signal intensity ratio of PC2 to acetylated α-tubulin was ~75% reduced in ADPKD cells compared with controls (Figure 4, C and E).

In contrast to cilia, localization of PC2 within the cell body basal to the cilium seemed similar in PKD iPS cells and controls (Figure 5A). Cytoplasmic PC2 colocalized with KDEL (the peptide sequence lysine-aspartic acid-glutamic acid-leucine), a marker of the endoplasmic reticulum, in all lines (Figure 5B). Immunodetection of PC1 is known to be problematic,30 and we were unable to immunolocalize endogenous or exogenous PC1 to cilia using anti-PC1 antibodies (Figure 5, C and D).

Loss of Heterozygosity in ADPKD iPS Cells

Reduced ciliary PC2 in cyst-lining epithelia has been previously correlated with complete loss of PKD1 but also reported in heterozygous cells.6,12 Because iPS cell lines have a high mutational load,31 we investigated the possibility that they may have undergone a loss of heterozygosity during the reprogramming process. For each of the ADPKD patients, several individual colonies were isolated from one iPS cell line and expanded. In patients 2 and 3, sequencing of the regions containing the pathogenic PKD1 mutations revealed each colony to be heterozygous, similar to the original donor fibroblasts (Figure 5E). In patient 1, sequencing of the C39Y mutation site revealed an apparent decrease in signal from the wild-type allele in multiple colonies of iPS cells compared with the parental fibroblasts, suggesting a loss of heterozygosity (Figure 5E). Colonies from a second iPS cell line from this patient showed an even more dramatic loss of heterozygosity at the same mutant locus in multiple colonies (Figure 5F). Single nucleotide polymorphisms further 3’ in PKD1 in patient 1 remained heterozygous, indicating that heterozygosity was not lost in the entire allele (Figure 5G). The results in patients 2 and 3 suggest that a second-hit loss of heterozygosity is not a prerequisite for reduced ciliary PC2; however, the results in patient 1 indicate that such mutations may occur at an increased rate in culture during the generation or selection of iPS cells and possibly contribute to this phenotype.

Reduced Ciliary PC2 in ADPKD Somatic Epithelial Cells and Hepatoblasts

Having established reduced ciliary PC2 as a reproducible phenotype in different ADPKD iPS cell lines, we investigated this phenomenon in somatic cell types with potentially greater relevance to human disease. In one approach, ES and iPS cell lines were stochastically differentiated into embryoid bodies (EBs) for 2 weeks and then replated onto gelatin for an additional 1 week (Figure 6A). In an alternative approach, cells were treated with a stepwise regimen of defined factors to promote efficient differentiation into hepatoblasts (Figure 6B), the progenitors of cholangiocytes that give rise to liver cysts in PKD patients.32 Somatic epithelial cells expressing zonula occludens 1 (ZO-1) or hepatoblast monolayers

markers of the endoderm (SOX17), mesoderm (forkhead box protein F1 [FOXF1]), and ectoderm (NESTIN). Scale bars, 200 μm. AP, alkaline phosphatase; SSEA-3, stage-specific embryonic antigen 3; TRA-1-60, tumor rejection antigen 1–60.
coexpressing α-fetoprotein (AFP), cytokeratin 19 (CK19), and hepatocyte nuclear factor 4-α (HNF4) were then costained for acetylated α-tubulin and PC2 (Figure 6, C and D). PC2 was more frequently observed at the plasma membrane in stochastically differentiated epithelial cells than hepatoblasts or undifferentiated cells (Figures 3A and 6, C and D). In both hepatoblasts and EB epithelial cells, quantitative analysis revealed that PC2 localized to the primary cilium in ~40% of control and ARPKD iPS cells but only ~20% of ADPKD iPS cells (Figure 6, E and F). These differences in PC2 localization were not caused by changes in ciliary length, which was similar between ADPKD and other iPS lines under both differentiation protocols (Figure 6, G and H). PC2 fluorescence intensities at the plasma membrane seemed similar between the lines, which was quantified relative to the plasma membrane marker ZO-1 in somatic epithelial cells (Figure 6, C, I, and J). Somatic cells, thus, exhibit a defect in trafficking PC2 to the cilium similar to the defect observed in the undifferentiated iPS cells from which they derive.

**Overexpression of Wild-Type PC1 Enhances PC2 Ciliary Localization**

Our experiments suggested that mutations in PC1 contributed to a loss of PC2 at the cilium in PKD iPS lineages. To test this suggestion, we examined the effects of overexpressing full-length PC1 on the localization of endogenous PC2. Confluent hepatoblasts derived from ADPKD iPS cells were successfully transfected with a human *PKD1* construct tagged with green fluorescent protein (GFP), albeit at very low efficiencies of only 10–20 cells per confluent well of a 24-well plate (Figure 7A). PC2 signal intensity was increased at the cilium of the rare GFP+ cells compared with untransfected cells in the same cultures that did not express GFP (Figure 7B). This increase could be quantified by averaging the intensity profiles of line scans drawn through individual cilia and was approximately twofold in magnitude (Figure 7, C and D). These results indicated that exogenous expression of wild-type PC1 can rescue ciliary localization of PC2 in ADPKD cells.

This principle was investigated further in IMCD3 cells, a mouse kidney cell line more amenable to transfection with long *PKD1* constructs. IMCD3 cells were transfected with yellow fluorescent protein (YFP) -tagged constructs encoding either wild-type murine PC1 or, alternatively, a truncation mutant that has been linked to defective ciliary localization of PC1.5,6 Transfection efficiencies in IMCD3 cells were 1%–2% (Figure 8A), which was significantly greater than in hepatoblasts. Because endogenous PC2 localized to the cilium even in untransfected IMCD3 cells in ~75% of the population, these transfection efficiencies did not noticeably affect the number of PC2-positive cells (Figure 8B). However, overexpression of wild-type PC1 increased the endogenous PC2 signal at the cilium three- to fivefold, which was measured by the averaged

---

**Figure 2.** PKD iPS cells express PKD disease genes. Representative immunoblots of (A) PC1 and (B) PC2 with β-actin loading control in undifferentiated ES and iPS cell lines. A putative truncated PC1 band is observed for ADPKD patient 3 (arrowhead). (C) Immunoblots for PC1 and (D) PC2 with α-tubulin loading control for both in IMCD3 cells transfected with either empty vector negative control (−) or GFP-tagged full-length human PKD1 (+) compared with untransfected H9 human ES cells. (E) Representative immunoblot of FPC in ES and iPS lines. A small volume of lysate from a porcine renal tubular epithelial cell line stably expressing myc-tagged, human fibrocytin (hFPC-myc) was loaded three lanes to the right as a positive control. The extreme right lane is a mirrored, shorter exposure of the hFPC-myc lane that was superimposed onto the film. BJ, iPS cells derived from human foreskin fibroblasts; F5, hfib2-iPS5; HDF, iPS cells derived from adult human dermal fibroblasts.
wide-field fluorescence intensity of line scans through ~75 individual cilia, compared with untransfected cells or cells transfected with the PC1 truncation mutant (Figure 8, C–E). Importantly, a corresponding difference was observed in the localization of the exogenous PC1: wild-type PC1 localized efficiently to the cilium, which was detected by anti-YFP immunofluorescence, whereas the truncation mutant was observed in the cytoplasm but was generally absent from cilia (Figure 8, C and D). We also investigated these populations using confocal microscopy. Measurement of ciliary shaft length in confocal z stacks found no change between untransfected cells and cells overexpressing YFP-PC1 (Figure 8F), whereas differences in YFP and PC2 intensity along the cilium were clearly evident (Figure 8G).
was required to detect this YFP signal, which was not detected in unstained cells (Figure 8, H and I). Furthermore, in control cultures transfected with PC1 wild-type but not incubated with primary antibody against PC2, no ciliary signal for PC2 was observed (Figure 8, H and I). These immunofluorescence controls eliminated the possibility that the increase in PC2 signal intensity at the cilium in the red channel was caused by bleed-through from YFP-PC1 in the green channel. These results

Figure 4. PC2 is reduced at primary cilia in ADPKD iPS cells. (A) Colocalization of PC2 with acetylated α-tubulin (AcT) and the tight junction marker ZO-1 in representative ES or iPS lines. Insets show a close-up of a single cilium. (B) Percentage of cilia positive for PC2; ~1,000 cells were counted per condition per experiment (n=3 experiments). (C) Representative confocal x and y planes and corresponding z stacks of cells from these populations. Crosshairs in the merge illustrate the x and y planes shown in the z stack. (D) Ciliary lengths in these cell populations averaged from confocal z stacks. (E) Averaged intensity profiles of line scans through ~45 individual cilia from these populations pooled from two to three different experiments. Data points represent averaged raw fluorescence intensity values from many images taken with identical exposures. Line scans were 16 μm in length. White dotted vertical line overlay in C illustrates how line scans were drawn. Scale bars, 10 μm. Error bars indicate SEM. P value, t test.
Figure 5. Reduced ciliary PC2 is not due to a global loss of PC2 or PC1. Colocalization of PC2 with (A) acetylated α-tubulin and the tight junction marker ZO-1 or (B) the endoplasmic reticulum marker KDEL in iPS lines in a focal plane basal to the primary cilium. (C) Representative image showing lack of 7e12 antibody anti-PC1 immunofluorescence in H9 ES cells. Inset shows close-up view of one cilium. (D) Representative image of IMCD3 cells transfected with GFP-tagged human PC1. Transfected cells express variable levels of PC1. Whereas anti-GFP identifies PC1 in both the cell body and cilia, anti-PC1 only recognizes PC1 in the cell body. A close-up view of the red dotted rectangular region is shown below for each channel. The yellow dotted circle indicates a representative cilium. Untransfected cells (arrowhead) appear negative for PC1 staining. (E) PKD1 chromatograms representing wild-type, ADPKD fibroblasts, and derived PKD iPS colonies. Base pair positions are indicated relative to the start of the coding sequence, and amino acids are shown in blue shading. Red arrows highlight point mutations. (F) Chromatogram of a second iPS cell line (cell line 1B) derived from patient 1 showing loss of heterozygosity at the mutant locus. (G) Single nucleotide polymorphisms (SNPs; red arrows) in this iPS cell line. Exons and SNP identifiers are provided above the chromatograms. Scale bars, 10 μm.
Figure 6. Ciliary PC2 is reduced in ADPKD somatic cells and hepatoblasts. Schematic of protocols for (A) stochastic differentiation into embryoid bodies or (B) directed differentiation into hepatoblasts. Markers used to identify stages of differentiation are shown in red. (C) PC2 localization in EB outgrowth epithelial cells expressing ZO-1 at the membrane and AcT at the cilium. Insets show close-up of a representative cilium. (D) Representative immunofluorescence images of hepatoblast marker expression (column 1) and PC2 localization to cilia (columns 2–5). Average percentage of HNF4+ cells coexpressing AFP and CK19 is displayed at lower right of column 1 images. (E) Quantification of PC2 positive cilia in EB outgrowths or (F) hepatoblast monolayers (~300 cells counted per condition per experiment; n=3 experiments per line). (G) Corresponding ciliary length measurement for EB outgrowths or (H) hepatoblasts. (I) Averaged intensity profiles of line scans through ~40 cell–cell junctions from somatic epithelial cells pooled from two to three different experiments. Data points represent averaged raw fluorescence intensity values from images taken with identical exposures. Line scans were 16 μm in length. Red dotted line overlay in C illustrates how line scans were drawn. (J) Average PC2/ZO-1 fluorescence ratios for the points at the apex of the curves shown in I. Patient 1 iPS lines were prone to fibroblastic differentiation under feeder-free conditions and, therefore, unavailable for hepatoblast differentiation. Scale bars, 10 μm. Error bars indicate SEM. P value, t test between the two pooled groups in brackets. BMP, bone morphogenetic protein.
indicated that wild-type PC1 but not mutant PC1 promotes localization of PC2 to the cilium, consistent with our findings that ciliary localization of PC2 is reduced in human iPS cells with \textit{PKD1} mutations.

**DISCUSSION**

Despite decades of research into PKD, it remains unclear which cellular characteristic \textit{in vitro}, if any, recapitulates the pathobiology of PKD common to many patients.\textsuperscript{1,5,6,21} To personalize the study of PKD pathobiology, we established and characterized five PKD iPS lines from unrelated patients. We discovered that iPS cells themselves express PC1, PC2, FPC, and elaborate primary cilia, making this cell type a convenient starting point for investigation. Because no verified protocol currently exists for directed differentiation of human pluripotent stem cells into kidney tubular epithelial cells that give rise to kidney cysts, we instead differentiated the iPS cells into two populations: ciliated somatic cells, which express PC2 and embryoid body epithelial cells, and hepatoblasts, which give rise to liver cysts in many PKD patients.\textsuperscript{1,2} We found in multiple patients and cell types a significant reduction in the number of PC2-positive cilia specifically in cell lines from ADPKD patients but not ARPKD patients or healthy controls. A similar phenotype has been reported in ADPKD human cystic kidney epithelial cells with \textit{PKD1} mutations, although such cells are derived from tissues with advanced disease and have other cellular phenotypes, making it difficult to compare them directly with healthy epithelium.\textsuperscript{5,6,19} Because our data reveal that the defect was also observed in heterozygous iPS cells as well as their differentiated descendants, which seemed normal.
with respect to cell cycle, ciliogenesis, and differentiation characteristics, it seems likely that ciliary PC2 mislocalization is an early and highly penetrant metric of ADPKD.

With the emerging centrality of the primary cilium for PKD, the interdependence of PC1, PC2, and FPC for trafficking and localization to this organelle is a topic of acute interest and

Figure 8. Wild-type but not mutant PC1 promotes ciliary localization of endogenous PC2 in IMCD3 cells. (A) Transfection efficiencies (fraction of cells that expressed YFP) for YFP-tagged murine PC1 wild-type and mutant constructs. (B) Total number of cells with detectable PC2 at the cilium in untransfected (control) cultures or those cells transfected with murine PC1 constructs. (C) PC2 and AcT colocalization in IMCD3 cells expressing YFP-tagged wild-type murine PC1 full-length (mPC1), PC1 C-terminal truncation mutant (mPC1 mutant), or untransfected control cells from the same cultures. Merge is pseudocolored to highlight PC2/AcT colocalization. (D) Averaged intensity profiles of line scans through ~75 individual cilia from these populations pooled from three different experiments. Data points represent averaged raw fluorescence intensity values from images taken with identical exposures. Line scans were 16 μm in length. Red dotted line overlay in C illustrates how line scans were drawn. (E) Average PC2/AcT fluorescence ratios for the points at the apex of the curves shown in D. (F) Ciliary lengths in these cell populations averaged from confocal z stacks. (G) Representative confocal x, y planes and corresponding z stacks of cells from these populations. Crosshairs in the merge illustrate the x and y plane shown in the z stack. (H) 4× and (I) 40× resolution bleed-through controls. Representative images of IMCD3 cells transfected with the wild-type construct of YFP-mPC1 incubated with primary antibodies against GFP and PC2 and subsequently incubated with both secondary antibodies (stained), no secondary antibodies (unstained), or only the secondary antibody to GFP (488 only) are shown. Corresponding fourth-channel images of AcT show cilia. Identical exposures are shown for all images. Scale bars, 5 μm in C, G, and I; 50 μm in H. Error bars in bar graphs and line scan time points indicate SEM. P values, t test.
Clinical relevance.9,17 In our PKD iPS cell cultures, the proportion of cells expressing PC2 at the cilium is reduced but not completely lost. Because the mutations in all of our ADPKD lines were in PKD1 and not PKD2 and because overexpression of wild-type PC1 significantly enhanced ciliary PC2 localization in both ADPKD iPS-derived hepatoblasts and IMCD3 mouse kidney cells, our results are consistent with a model where mutations in PC1 lead to reduced quantities of PC2 at the cilium (Figure 9).5,6 PC1 and PC2 interact with each other through their carboxyl termini, providing a plausible mechanism for codependency of localization.4,17 Although some studies suggest that PKD2 may be less sensitive to dosage effects in the presence of wild-type PKD1,33–35 changes in PC2 localization in the company of PC1 mutations may have a pathogenic role. Although in mouse cells, PC2 has been reported to traffic to the cilium in Pkd1−/− homozygotes,9 a quantitative comparison of PC2 ciliary trafficking between mutant and wild-type PC1 in the mouse has not been defined. Furthermore, others have argued that PC1 is necessary for PC2 localization to cilia and other cellular membranes.6,10,11,20 One possible resolution of these findings is that PC1 can quantitatively enhance ciliary trafficking of PC2, even if it is not absolutely required.

In vivo, ADPKD takes decades to markedly impair kidney function, leading to the proposal that a second genetic hit and/or a third hit related to environmental stress is required for cystogenesis.12,36,39 However, other evidence in animal models suggests that subthreshold polycystin expression levels may be sufficient to cause disease.13–15 iPS cells from two of our ADPKD patients did not lose heterozygosity at the mutation site, similar to a previous report on iPS cells generated from one ADPKD patient.26 In a third ADPKD patient, two independent iPS cell lines exhibited an underrepresentation or close to complete loss of the wild-type allele specifically at the site of a germ-line C39Y mutation. Notably, the iPS cell reprogramming and selection process has previously been observed to introduce novel coding mutations and fix rare mutations present in a minority of the original fibroblast population, with a total mutagenesis rate of approximately six coding mutations per exome.31 The fact that the mutation in patient 1 iPS cells occurred specifically to encompass the second allele of the C39Y locus suggests that the mutation site might constitute a genetic hotspot within PKD1 or that PKD1 may be more prone to mutation than other genes. No obvious difference was observed in PC2 ciliary localization in the two lines from this patient compared with lines from the other two ADPKD patients who did not lose heterozygosity at their disease loci. Additional research will be necessary to understand the genetic mechanisms underlying ADPKD iPS cell second hits in vitro, which might provide insight into disease mechanisms in vivo.

These data neither prove nor preclude the possibility that second-hit mutations are required to cause cystogenesis in vivo, and they do not directly address the role of impaired PC2 ciliary localization in vivo. They do, however, suggest that reductions in functional PC1 levels are sufficient to cause reductions in PC2 levels in cilia, which may play a role in the pathogenesis of PKD. For example, subthreshold PC1 levels could synergistically reduce PC2 levels at the cilium, which would further decrease the stoichiometric likelihood of forming functional PC1–PC2 complexes. These iPS and descendant cell models may serve as tools to find new therapeutic agents that can rescue the PC2 phenotype and may be therapeutic in vivo. The ability of pluripotent stem cells to provide novel mechanistic insights into the cellular pathophysiology of PKD coupled with their unique capacity to generate complex tissues and all somatic cell types make them a powerful new resource for PKD research.

### CONCISE METHODS

**Generation, Maintenance, and Characterization of iPS Cells**

Three anonymous patients with a clinical diagnosis of ADPKD based on imaging and family history provided written informed consent and underwent skin biopsies in a protocol approved by the Brigham and Women’s Hospital Institutional Review Board. Fibroblasts ADPKD 1–3, ARPKD 4 and 5 (Coriell GM10287 and GM12607), human foreskin fibroblasts (ATCC CRL-2522), and adult human dermal fibroblasts (InVitrogen C-013–5C) were reprogrammed by two rounds of overnight transduction with murine stem cell virus-interna...
Ribosome entry site-green fluorescent protein encoding retroviruses for OCT4, SOX2, KLF4, and c-MYC produced in 293FT cells (Invitrogen). Multiple lines from each patient were karyotyped (Cell Line Genetics) and frozen. Lines were maintained on mouse embryonic fibroblast feeders (Global Stem Cell GSC-6001G) in 20% Knock Out Serum Replacement, 1X nonessential amino acids, 1X Glutamax, 1X penicillin-streptomycin, 50 μM β-mercaptoethanol, DMEM-F12, and 10 ng/ml human fibroblast growth factor 2 (FGF2; Invitrogen) and restarted every ~50 passages. Teratomas were generated by implantation of one six-well plate of confluent iPSCs on feeders underneath the kidney capsule or neck scruff of nonobese diabetic-severe combined immunodeficiency mice, which were euthanized ~8 weeks later in adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Estimated glomerular filtration rates in ADPKD patients were calculated using the Modification of Diet in Renal Disease formula.

**Genetic Sequencing of PKD1, PKD2, and PKHD1**

The Purelink Genomic DNA Mini Kit was used according to instructions (Invitrogen) to prepare genomic DNA from confluent monolayers of parental fibroblasts or feeder-free iPSC cultures expanded from individual colonies. DNA was subjected to exon sequencing as described,27 and align Grantham variation Grantham deviation and sorting intolerant from tolerant scores were calculated. Loss of heterozygosity was evaluated manually based on the chromatograms.

**Immunoblot**

Confluent feeder-free iPSCs from one well of a six-well plate were scraped into 100 μL RIPA buffer plus protease inhibitors (150 mM NaCl, 1% nonidet-P40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris [pH 8.0], 2 μg/ml aprotinin, leupeptin, pepstatin, and 1 mM PMSF) and sonicated briefly. Laemmli buffer was added to 14,000 g supernatant, which was heated only to 37°C. Proteins were electrophoresed in a Mini-Protein TGX gradient gel (Bio-Rad), transferred overnight at 35 V in 25 mM Tris, 192 mM Glycine, and 10% methanol for PC2 or 25 mM Tris, 192 mM Glycine, and 0.1% SDS for PC1 to Immobilon-P (Millipore), blocked with 5% milk, Tris-buffered saline, and 0.1% Tween-20 (Sigma), and incubated in block with antibodies against PC2 (ab90648; Abcam), PC1 (7e12; Santa Cruz), fibrocytin (4883; with human recombinant FPC from a porcine renal tubular epithelial cell line stably expressing FPC as a positive control),28 or β-actin (Cell Signaling). Detection was performed using horseradish peroxidase-conjugated secondary antibodies (Dako) and Western Lightning (Perkin-Elmer) according to instructions.

**Immunohistochemistry**

Feeder-free cultures were passaged on 3% Reduced Growth Factor GelTrex plus 1X Pen-Strep (Invitrogen) in Modified Tennelle’s Special Recipe 1 (mTeSR1, Stem Cell Technologies). Cells were dissociated with Accutase (Stem Cell Technologies), plated in mTeSR1 with 10 μM Rho-kinase inhibitor Y27632 (Stemgent) at 60,000 cells/24 well, grown feeder-free for 2 days, and then pulsed for 2 hours with 10 μM BrdU (Sigma). Alternatively, confluent colonies on feeders were split 1:12 onto 3% GelTrex (Invitrogen) -coated CC-2 chamber slides (Lab-Tek) for 7 days in mTeSR1 to form cilia or 24-well plates for 5 days for pluripotency markers. For transfection experiments, 200,000 IMCD3 cells were plated onto 12-mm coverslips in 10% FBS/DMEM, transfected the next day with 1 μg amino-terminal YFP-tagged Pkd1 wild-type or carboxy-terminal truncation mutant in pcDNA4.1 (X. Su and J. Zhou, unpublished observations) using Lipofectamine LTX (Invitrogen), and cultured for 72 hours. Cells were washed in PBS, fixed in 4% paraformaldehyde for 15 minutes at room temperature, washed, blocked in 5% donkey serum (Millipore), 0.3% Triton-X-100, and PBS, incubated overnight in 3% BSA/PBS with primary antibodies, washed, incubated with secondary antibodies (A31570, A21206, and A11039; Invitrogen; 705–605–147 and 715–175–151; Jackson ImmunoResearch), washed, and mounted in Vectorshield H-1200. Primary antibodies included OCT4 (sc-5279; Santa Cruz), NANOG (RCAB0014PF; Cosmobio), stage-specific embryonic antigen 3 (MAB4303; Millipore), tumor rejection antigen 1–60 (MAB4360; Millipore), PC2 (sc-25749; Santa Cruz), acetyl-tubulin (051M4770; Sigma or 53S5; Cell Signaling), ZO-1 (33910; Invitrogen), BrdU (Santa Cruz), phospho-H3 (sc-8656-R; Santa Cruz), brachyury (sc-17745; Santa Cruz), SOX17 (AF1924; R&D), forkhead box protein F1 (AF4798; R&D), AFP (A0008; Dako), CK19 (M0888; Dako), HNF4 (sc-6556; Santa Cruz), Nestin (AB5922; Millipore), KDEL/GRP78 (SPA-827; Assay Designs), and GFP (A10262; Invitrogen). For BrdU incorporation studies, cells were treated with 2 N hydrochloric acid for 1 hour before blocking. The Alkaline Phosphatase Detection Kit (Millipore) was used according to instructions. Fluorescence images were captured using Nikon epifluorescence 90-I and C-1 confocal (upright) or Eclipse Ti (inverted) microscopes with identical exposures for each cell line. BrdU incorporation and histone H3 phosphorylation were imaged at 4X and scored automatically using a Cellprofiler algorithm; 40× fields containing cilia were chosen at random, imaged in the acetylated α-tubulin channel, reimaged in the same plane for PC2, refocused, and imaged for nuclei. Transfected cells were identified by GFP staining of the N-terminal PC1 fusion tag.

**EB and Hepatoblast Differentiation**

For EBs, cells on feeders were subjected to collagenase IV for 10 minutes, scraped off, resuspended in ES cell medium minus FGF2 on low-adhesion plates (Corning), fed every 2–3 days for 2 weeks, replated onto gelatin, and incubated in DMEM/10% FBS (HyClone) for 1 week to form ciliated outgrowths. Random fields containing ciliated ZO-1-positive epithelial cells were imaged for PC2 and scored manually. For hepatoblasts, 80,000 cells on a feeder-free 24 well were differentiated in Advanced RPMI, 1X Glutamax, and 1X penicillin-streptomycin (Invitrogen) with 5 μM CHIR99021 (Stemgent) for 24 hours, 100 ng/ml Activin A (R&D) for 2–3 days, 1X B27 supplement (Invitrogen), 20 ng/ml bone morphogenetic protein 4 (R&D), and 10 ng/ml FGF2 (Invitrogen) for 5 days, and 1X B27/10 ng/ml hepatocyte growth factor (Peprotech) for 5 days. Transfection was performed on day 2 of hepatocyte growth factor treatment with 1 μg GFP-WT PKD1 (21367; Addgene), fixed 72 hours after transfection, and stained for GFP, PC2, and acetylated α-tubulin.

**Germ Layer-Directed Differentiation**

For germ layer-directed differentiation experiments, human iPSC cells were dissociated with Accutase for 10 minutes and plated as a
single-cell monolayer onto 3% Geltrex in mTeSR1 medium at a density of 4×10^4 cells/cm². Cells were cultured under these conditions for 2 days before differentiation (15%–20% confluence). To generate definitive endoderm, cells were treated with Advanced RPMI (Invitrogen), 1× penicillin-streptomycin, 1× l-Glutamax (Invitrogen), 5 μM CHIR99021 (Stemgent), and 100 ng/ml Activin A (R&D). After 24 hours, the media was changed to Advanced RPMI, 1× penicillin-streptomycin, 1× l-Glutamax, and 100 ng/ml Activin A for 2 days. To generate mesoderm, cells were treated with Advanced RPMI, 1× penicillin-streptomycin, 1× l-Glutamax, and 5 μM CHIR99021 for 24 hours followed by Advanced RPMI, 1× penicillin-streptomycin, 1× l-Glutamax, and 50 ng/ml bone morphogenetic protein 4 (R&D) for 2 days. To generate neural precursor cells, we modified an existing protocol and treated cells with neural induction media containing Advanced DMEM/F12:Neurobasal (1:1), 1× N2 (Invitrogen), 1× B27 (Invitrogen), 1× l-Glutamax, 5 μg/ml BSA, and 10 ng/ml human leukemia inhibitory factor (Millipore) supplemented with 3 μM CHIR99021, 2 μM SB431542 (Stemgent), and 10 μM N-[3-(3,5-Di-fluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (Sigma) for 7 days.

Quantification and Statistical Analyses
Unmodified images were scored manually in a binary fashion for the presence or absence of detectable PC2 at the cilium. Single line scans were drawn through the center of randomly selected cilium in the acetylated α-tubulin channel using NIS Elements (Nikon); multi-channel fluorescence intensities were recorded, and the raw averages were plotted in charts. Values of the pixel at the center/apex of the line scan peaks were averaged for statistical comparisons. Ciliary shaft lengths were measured by drawing a line from the base to the tip of individual cilia in the acetylated α-tubulin channel. Statistical comparisons were made between sets of values indicated in brackets using a two-tailed t-test for two samples with unequal variance (heteroscedastic) calculated using Excel’s t.test function (=ttest([set1], [set2], 2, 3).

ACKNOWLEDGMENTS

We thank the groups of Jagesh Shah (Brigham and Women’s Hospital), Oxana Ibragimov-Beskrovnyaya (Genzyme), Christopher Ward (Mayo), and Seth Alper (Beth Israel Deaconess/Harvard) and members of the Harvard Center for Polycystic Kidney Disease Research, particularly those members of the laboratories of J.Z. and J.V.B., for helpful reagents and discussions. We thank Rachel Clark for dermal biopsies, the Harvard Stem Cell Institute iPSC Core Facility for deriving and providing ADPKD iPSC cell lines, George Daley (Childrens Hospital Boston) for generously providing control Hfib-2-iPS4 and -iPS5 lines, Paul Lerou (Brigham and Women’s Hospital) for help with teratoma assays, Craig Brooks for help with imaging, Gregory Germino for providing the GFP-WT PC1 construct and its sequence, the Coriell Institute for Medical Research for ARPKD fibroblast lines, and the National Institutes of Health Loan Repayment Program.

Studies were supported by National Institutes of Health Grants DK864406, DK39773, DK072381, DK092036, DK51050, DK53357, and DK090728, March of Dimes Grant 1-FY12-527, and Harvard Stem Cell Institute Grants DP-0096-11-01 and DP-0127-1300.

Parts of this work were presented in abstracts at the American Society of Nephrology Annual Meetings held November 8–13, 2011, in Philadelphia, Pennsylvania, and November 1–4, 2012, in San Diego, California.

DISCLOSURES
None.

REFERENCES


Reduced Ciliary PC2 in PKD iPSC Cells 1585


