Acute kidney injury and aberrant planar cell polarity induce cyst formation in mice lacking renal cilia

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Polycystic kidney disease (PKD) is an inherited disorder that is characterized by the accumulation of cysts in the renal parenchyma and progressive decline in renal function. Recent studies suggest that PKD arises from abnormalities of the primary cilium. We have previously shown that kidney-specific inactivation of the ciliogenic gene Kif3a during embryonic development produces kidney cysts and renal failure. Here, we used tamoxifen-inducible, kidney-specific gene targeting to inactivate Kif3a in the postnatal mouse kidney. Kidney-specific inactivation of Kif3a in newborn mice resulted in the loss of primary cilia and produced kidney cysts primarily in the loops of Henle, whereas inactivation in adult mice did not lead to the rapid development of cysts despite a comparable loss of primary cilia. The age-dependence and locations of the cysts suggested that cyst formation required increased rates of cell proliferation. To test this possibility, we stimulated cell proliferation in the adult kidney by inducing acute kidney injury and tubular regeneration. Acute kidney injury induced cyst formation in adult Kif3a mutant mice. Analysis of pre-cystic tubules in Kif3a mutant mice showed that the loss of cilia did not stimulate cell proliferation but instead resulted in aberrant planar cell polarity as manifested by abnormalities in the orientation of cell division. We conclude that primary cilia are required for the maintenance of planar cell polarity in the mammalian kidney and that acute kidney injury exacerbates cystic disease.

INTRODUCTION

Polycystic kidney disease (PKD) is the most common genetic cause of end-stage renal failure in humans (1). PKD is characterized by enlargement of the kidneys and progressive decline in renal function due to the accumulation of cysts in the renal parenchyma. The cysts originate from the renal tubules and are lined by epithelial cells that exhibit abnormalities in cell proliferation, apoptosis, fluid secretion, cell signaling and extracellular matrix production. The autosomal dominant form of PKD (ADPKD) usually presents in middle-aged adults with bilateral enlarged kidneys and decline in GFR. Extrarenal manifestations include liver cysts, cardiac valve abnormalities, hypertension and intracranial aneurysms. ADPKD is caused by mutations of PKD1 or PKD2, which encode the proteins polycystin-1 and polycystin-2, respectively. The autosomal recessive form of PKD (ARPKD) primarily affects infants and children and is characterized by the association of cysts in the renal collecting ducts with biliary dysgenesis and congenital hepatic fibrosis. The severe perinatal form of ARPKD presents with bilateral kidney enlargement, intratuerine renal failure, oligohydramnios and pulmonary hypoplasia. Affected individuals may also present later in life with chronic kidney disease and portal hypertension due to congenital hepatic fibrosis. ARPKD is caused by mutations of PKHD1, which encodes a membrane protein called fibrocystin (polyductin, tigmin).

Although the genes that are mutated in PKD have been identified, the pathogenetic mechanisms that initiate cyst formation remain poorly understood. Recent studies suggest that PKD arises from abnormalities of the primary cilium (2). The primary cilium is a solitary, hair-like organelle that

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projects from the surface of most cells (3,4). Most primary cilia are non-motile and are composed of an axoneme comprising nine microtubule doublets (9+0 pattern) surrounded by the cilary membrane. The primary cilium is anchored in the cell by the basal body, a microtubule-based structure that also functions as one of the centrioles during cell division. In the kidney, a single primary cilium is found on the apical surface of most tubular epithelial cells composing the nephrons and collecting ducts. Renal cilia project into the tubule lumen and bend in response to fluid flow. Bending of the cilium stimulates an increase in cytosolic calcium concentration (5). These findings suggest that primary cilium function as mechanosensors of urine flow in the renal tubules. Primary cilia have also been implicated in cell cycle regulation, sonic hedgehog signaling and Wnt signaling (6). Three lines of evidence suggest that PKD arises from abnormalities in the structure and/or function of primary cilia: First, polycystin-1, polycystin-2 and fibrocystin are found in primary cilia in renal epithelial cells (4). The protein products of genes that are mutated in other cystic kidney diseases, such as nephronphthisis and Bardet-Biedl syndrome, are also localized to the primary cilium and/or basal body (7,8). Second, mutations of polycystin-1 and blocking Abs against polycystin-2 inhibit the increase in cytosolic calcium that is induced by fluid flow, which suggests that the polycystins are involved in ciliary signaling (9). Third, mutant mice that contain shortened or absent primary cilia develop kidney cysts (4).

The formation of primary cilia is dependent on intraflagellar transport (IFT), a process in which large protein complexes are transported along the cilary axoneme by specialized motor proteins (4,10). Anterograde IFT from the cell body towards the tip of the cilium is mediated by kinesin-II, whereas retrograde IFT is mediated by cytoplasmic dynein. Kinesin-II is a trimer composed of two motor subunits, KIF3A and KIF3B, and an adapter subunit, KAP3 (11). Knockout mice that lack KIF3A are unable to form primary cilia, indicating that IFT is essential for ciliogenesis. The absence of primary cilia results in severe developmental abnormalities, laterality defects and embryonic lethality (12,13). To circumvent embryonic lethality and to elucidate the function of primary cilia in the kidney, we have previously generated kidney-specific Kif3a knockout mice using Cre/loxP recombination (14). Ksp/Cre transgenic mice, in which Cre recombinase is expressed specifically in renal tubular epithelial cells, were crossed with mice carrying loxP sites flanking an essential exon of Kif3a. Bitransgenic Ksp/Cre;Kif3a<sup>lox<sup>−−</sup></sup> mice were viable at birth but developed kidney cysts beginning at P5 and renal failure by 2–3 weeks of age. Analysis of the cystic kidneys revealed that primary cilia were absent from the apical surface of the cyst epithelial cells. Mutations of other proteins that are involved in IFT also produce ciliary cystic kidneys revealed that primary cilia were absent from the apical surface of the cyst epithelial cells. Mutations of other proteins that are involved in IFT also produce ciliary abnormalities and kidney cysts (15). These results support the role of primary cilia in the pathogenesis of PKD.

In Ksp/Cre;Kif3a<sup>lox<sup>−−</sup></sup> mice, inactivation of Kif3a by Cre/loxP recombination occurs during embryonic development. To elucidate the roles of primary cilia in the kidney after birth, we used tissue-specific, inducible gene targeting to inactivate Kif3a in the kidneys of neonatal and adult mice. Our results indicate that postnatal cyst formation in Kif3a mutant mice is dependent on cell proliferation and is associated with abnormalities in planar cell polarity.

**RESULTS**

**Generation of Ksp/Cre<sup>ERT2</sup> transgenic mice**

The role of Kif3a in the neonatal and adult mouse kidney was studied using kidney-specific, inducible gene targeting. First, we generated transgenic mice that expressed a Cre<sup>ERT2</sup> fusion protein under the control of the Ksp-cadherin (Cdh16) gene promoter. The Cre<sup>ERT2</sup> protein contains Cre recombinase fused in-frame to a modified human estrogen receptor and is normally retained in the cytosol in an inactive state (16). Upon binding to 4-hydroxytamoxifen, the fusion protein translocates to the nucleus where it mediates Cre/loxP recombination. By crossing Cre<sup>ERT2</sup> transgenic mice with mice containing essential exon(s) of a gene of interest flanked by loxP sites, gene targeting can be temporally regulated. To control tissue-specificity, the expression of Cre<sup>ERT2</sup> was placed under the control of the Ksp-cadherin gene promoter. In the adult mouse, the Ksp-cadherin promoter directs gene expression exclusively in tubular epithelial cells in the kidney (17,18). Expression is detectable in all segments of the nephron with the highest levels of expression in the collecting ducts and distal tubules. To generate Ksp/Cre<sup>ERT2</sup> mice, the 1.3-kb Ksp-cadherin promoter and a β-globin TATA box were linked to the Cre<sup>ERT2</sup> coding region, and transgenic mice were produced by pronuclear microinjection. Transgenic founders were identified and bred to generate permanent lines. One line (no. 24) that highly expressed Cre<sup>ERT2</sup> in the kidney and that showed no recombination in the absence of tamoxifen was selected for further study. A similar mouse strain has also been produced by others (19).

Ksp/Cre<sup>ERT2</sup> transgenic mice were crossed with R26R-EYFP mice, which carry an enhanced yellow fluorescent protein (EYFP) reporter gene that is activated by Cre/loxP recombination (20). Adult (5-week-old) bitransgenic Ksp/Cre<sup>ERT2</sup>;R26R-EYFP progeny were treated with tamoxifen (4 mg/40 g body weight, i.p.) or vehicle for 5 days, and tissues were examined for expression of EYFP 1 week later. Ab staining of the kidneys revealed that the Cre<sup>ERT2</sup> fusion protein was localized to the cytosol in vehicle-treated animals and translocated to the nucleus following treatment with tamoxifen (Fig. 1A, upper panels). Treatment with tamoxifen induced Cre/loxP recombination, as indicated by expression of the EYFP reporter gene (Fig. 1A, lower panels). Similar results were obtained following treatment with 4-hydroxytamoxifen (data not shown). The induction of EYFP expression was verified by immunoblot analysis (Fig. 1B, lower panel). No EYFP expression was observed in male mice that were treated with vehicle alone, indicating that recombination was tamoxifen-dependent. A low frequency of EYFP expression was observed in female mice 8 weeks after treatment with vehicle, which probably reflected induction by endogenous estrogens (data not shown). To avoid background recombination, male mice were used in all subsequent experiments. To assess tissue-specificity, immunoblot analysis was performed on various tissues following treatment with tamoxifen. Expression of EYFP was detected in the...
Kidneys of tamoxifen-treated Ksp/CreERT2;R26R-EYFP mice but not in any other organs, indicating that recombination was kidney-specific (Fig. 1B). Immunoblot analysis of tissues from tamoxifen-treated Ksp/CreERT2;R26R-EYFP mice revealed expression of EYFP only in the kidney, indicating that Cre/loxP recombination was tissue-specific (upper panel). Immunoblot analysis of kidneys from Ksp/CreERT2;R26R-EYFP mice that were treated with tamoxifen or vehicle revealed that Cre/loxP recombination was tamoxifen-dependent (lower panel). Ksp/Cre;R26R-EYFP mouse kidneys were used as positive controls (+), and Ksp/Cre mouse kidneys were used as negative controls (−). Immunoblotting with anti-tubulin Ab was used as a loading control. (C) To identify the nephron segments in which Cre/loxP recombination was induced, Ksp/CreERT2;R26R mice were injected with tamoxifen and the kidneys were co-stained with an Ab against β-galactosidase (red) and markers of specific nephron segments (green). Staining with LTA, anti-NKCC2, anti-NCX1 and anti-AQP3 revealed that lacZ was expressed in proximal tubules (pt), thick ascending limbs of loops of Henle (tal), distal convoluted tubule (dt), and collecting duct (cd), respectively. Arrows indicate co-localization. Scale bars: 20 μm. (D) Efficiency of tamoxifen-inducible Cre/loxP recombination in different nephron segments. Adult Ksp/CreERT2;R26R mice (n = 3) were treated with tamoxifen for 5 days, and 2 weeks later the kidneys were removed and co-stained with an Ab to β-galactosidase and markers of specific nephron segments. Histogram shows the percentage of lacZ-positive cells in the proximal tubules (pt), thick ascending limbs of loops of Henle (tal), distal convoluted tubule (dt) and collecting ducts (cd). Error bars indicate SD.

Kidney-specific inactivation of Kif3a at P2 produces kidney cysts

To conditionally inactivate Kif3a in the kidney, we crossed Ksp/CreERT2 mice with Kif3a<sup>lox/+</sup> mice that contain loxP sites inserted into the genomic DNA flanking exon 2 of Kif3a. Since exon 2 encodes the essential motor domain, excision of exon 2 by Cre/loxP recombination leads to gene inactivation (21). Ksp/CreERT2 mice were crossed with Kif3a<sup>lox/lox</sup> and Kif3a<sup>+/−</sup> mice to generate Ksp/CreERT2;Kif3a<sup>lox/lox</sup> progeny that carried one floxed Kif3a allele and one null allele. An R26R reporter gene was also introduced into the cross so that Cre/loxP recombination could be monitored by expression of lacZ. Inactivation of Kif3a in newborn mice was achieved by administering tamoxifen to nursing dams for 3 days. Figure 2 shows the effects of tamoxifen treatment beginning at P2 and analysis 4 weeks later. Control mice (Ksp/CreERT2;Kif3a<sup>lox/lox</sup>;R26R) showed expression of lacZ in renal
Figure 2. Kidney-specific inactivation of Kif3a at P2 results in the loss of primary cilia and produces kidney cysts. Conditional Kif3a mutant mice (Ksp/CreER<sup>12</sup>;Kif3a<sup>lox/lox</sup>−;R26R) and littermate controls (Ksp/CreER<sup>12</sup>;Kif3a<sup>lox/lox</sup>−;R26R) were treated with tamoxifen from P2 to P4 and analyzed 4 weeks later. Kidney sections were stained with X-gal to identify lacZ-positive cells in which Cre/loxP recombination had been induced with tamoxifen. (A) Kidneys from tamoxifen-treated control mice showed normal histology of the renal cortex (co) and medulla (me). (B) A higher magnification image shows mosaic expression of lacZ in the renal tubules. (C) Conditional Kif3a mutant mice treated with tamoxifen developed kidney cysts (cy) near the cortico-medullary junction. (D) The kidney cysts were lined with lacZ-positive epithelial cells. (E and F) To identify the origins of the cysts, kidney sections were stained with markers of specific nephron segments. Staining with DBA (green) and anti-NKCC2 Ab revealed that the cysts originated from the loops of Henle (E) and the collecting ducts (F). (G) In control kidneys, primary cilia (arrows) were present on the luminal surface of lacZ-positive tubular epithelial cells. Inset shows higher magnification image of the primary cilia. (H) In the Kif3a mutant kidneys, cilia were absent from the apical surface (arrowheads) of lacZ-positive cyst epithelial cells. Arrows indicate cilia that were present in adjacent lacZ-negative, non-cystic tubules. Nuclei were counterstained with DAPI. Scale bars: 200 μm (A and C), 50 μm (B and D), 20 μm (E and F), 10 μm (H and I).

tubular epithelial cells, which indicated that administration of tamoxifen to nursing dams caused Cre-mediated recombination in the pups (Fig. 2A and B). The expression of lacZ in the tubules was mosaic, indicating that the recombination was not completely efficient. Histological analysis of the tamoxifen-treated control mice revealed normal kidney histology (Fig. 2A and B). In contrast, administration of tamoxifen to Ksp/CreER<sup>12</sup>;Kif3a<sup>lox/lox</sup>−;R26R mice (conditional Kif3a mutants) at P2 and analysis 4 weeks later revealed the presence of multiple cysts in the kidney (Fig. 2C). The cysts were located primarily at the cortico-medullary junction. X-gal staining of the Kif3a mutant kidneys revealed that most, but not all, of the cyst epithelial cells were lacZ positive indicating that the cysts were primarily lined by Kif3a mutant cells (Fig. 2D). The cyst index in the tamoxifen-treated Kif3a mutant kidneys was 434 ± 21 (SD) compared with a cyst index of 0 in the control kidneys.

To identify the origins of the cysts, kidney sections from tamoxifen-treated Kif3a mutant mice were labeled with markers of specific nephron segments. Most of the cysts (74 ± 1.3%) expressed the Na<sup>+</sup>/K<sup>+</sup>/2Cl<sup>−</sup> cotransporter type 2 (NKCC2) indicating that they originated from the thick ascending limbs of loops of Henle (Fig. 2E). The remainder (24 ± 2.1%) stained positive with Dolichos biflorus agglutinin (DBA) indicating that they originated from the collecting ducts (Fig. 2F). To verify that inactivation of Kif3a resulted in the loss of primary cilia, the kidneys were stained with an Ab against acetylated tubulin. The kidney sections were co-stained with an Ab against β-galactosidase to identify recombined cells. The lacZ-positive tubular epithelial cells in control mice contained primary cilia on their apical surface (Fig. 2G). In contrast, cilia were absent from the lacZ-positive cyst epithelial cells in the conditional Kif3a mutant mice (Fig. 2H). Primary cilia were present in adjacent lacZ-negative, non-cystic tubules (arrow in Fig. 2H), indicating that the loss of primary cilia and cyst formation was specific to Kif3a mutant cells.

The frequency of cyst formation in different nephron segments in the postnatal kidney (Fig. 3A) did not correlate with the efficiency of Cre/loxP recombination (Fig. 1D). To determine whether cyst formation correlated instead with cell proliferation, proliferative activity was measured in different nephron segments by staining with an Ab against Ki-67. Ab staining of control kidneys at P4 and co-staining with anti-NKCC2 Ab or DBA revealed Ki-67 expression in 39 ± 3.3% of cells in the thick ascending limbs of loops of Henle and 9.9 ± 6% of cells in the collecting ducts (Fig. 3B). These results indicated that cyst formation in the P2 Kif3a mutant mice correlated with the rates of cell proliferation rather than the efficiency of cre-mediated recombination.
Kidney-specific inactivation of Kif3a at P10 or older ages does not cause cyst formation despite the loss of cilia

To study the effects of Kif3a inactivation in adult mice, control and conditional Kif3a mutant mice were injected with tamoxifen at P35 for 5 days, and the kidneys were analyzed 4 weeks later. Both control and Kif3a mutant mice exhibited normal kidney histology and did not develop any kidney cysts within 4 weeks after tamoxifen administration (Fig. 4A and D). Conditional Kif3a mutant mice that were injected with tamoxifen at P10, P14 and P21 and followed for up to 2 months also did not develop kidney cysts (data not shown). LacZ-positive tubular epithelial cells were present in both the tamoxifen-treated mutant and control mice, which indicated that the absence of kidney cysts in the mutant mice was not due to the absence of Cre/loxP recombination. To determine whether inactivation of Kif3a at P35 affected the formation of cilia, kidney sections were stained with an Ab against acetylated tubulin. Co-staining with an Ab against β-galactosidase was used to identify the cells in which Cre/loxP recombination had been induced. Primary cilia were detected on the apical surface of most lacZ-positive cells in the tamoxifen-treated control mice (Fig. 4B and C) but were absent from most lacZ-positive cells in the Kif3a mutant mouse. Cilia were detected on 267 of 469 (57 ± 3.8%) lacZ-positive renal tubular cells in the control mice but only 69 of 479 (14 ± 1.3%) lacZ-positive cells in the Kif3a mutant mice (P < 0.0001, Student’s t-test) (Fig. 4G). Cilia were present on lacZ-negative cells in both mutant and control mice, indicating that the loss of cilia was specific to Kif3a mutant cells. To determine whether the absence of cysts in older mice was due to a decrease in cell proliferation, the proliferative activity in the postnatal kidney was measured at various ages after birth by staining mitotic cells with an Ab against phosphohistone H3. Figure 3C shows that the percentage of mitotic cells declined during postnatal kidney development from 0.99% at P1 to 0.06% at P21 (P = 0.0006, ANOVA). Taken together these results indicated that inactivation of Kif3a at P10 or an older age, when the rates of kidney cell proliferation were lower than at birth, did not produce kidney cysts. The absence of cyst formation in adult mice was observed despite the loss of primary cilia.

Acute kidney injury stimulates cyst formation in adult Kif3a mutant mice

Acute kidney injury (AKI) caused by renal ischemia or nephrotoxins produces tubular cell death, which is followed by tubular regeneration and restoration of kidney function. Recent studies have shown that tubular regeneration results primarily from proliferation of surviving tubular epithelial cells (22, 23). If cyst formation in adult Kif3a mutant mice is dependent on cell proliferation, then AKI might stimulate cell proliferation and induce cyst formation. To test this possibility, conditional mutant mice were treated with tamoxifen at 5 weeks of age to inactivate Kif3a. After 1 week, the mice were subjected to unilateral renal ischemic/reperfusion injury (IRI) by clamping the left renal pedicle followed by release of the clamp. Previous studies from our laboratory have shown that this procedure results in tubular cell death followed by increased cell proliferation in regenerating renal tubules (23). Mice were analyzed 2.5 weeks after renal IRI. Examination of the uninjured right kidney revealed normal histology and expression of lacZ in non-cystic renal tubules (Fig. 5A and B). In contrast, the injured kidney contained multiple cysts that were lined by lacZ-positive epithelial cells and were located near the cortico-medullary junction (Fig. 5C–E). Cysts were not seen in tamoxifen-treated control mice subjected to IRI (data not shown). To determine the origins of the cysts in the injured Kif3a mutant kidneys, kidney sections were stained with markers of specific nephron segments. The cysts in the injured kidneys stained positive for Lotus tetragonolobus agglutinin (LTA) and NKCC2 (data not shown), indicating that they originated from the proximal tubules and thick ascending limbs of loops of Henle, which are the nephron segments that are most susceptible to ischemic injury. To visualize the primary cilia, the kidney sections were stained with an Ab against acetylated tubulin and co-stained with an Ab against β-galactosidase. The lacZ-positive epithelial cells lining the cysts in the injured kidneys lacked primary cilia (Fig. 5G). Cilia were also absent in the non-cystic tubules in the uninjured kidneys (Fig. 5F). The absence of cilia was specific to Kif3a mutant cells, since cilia were observed in lacZ-negative cells in both the injured and uninjured kidneys. Quantification of cyst formation showed that the cyst index was 211 ± 83 in
Loss of cilia precedes cyst formation in kidney-specific Kif3α mutant mice

To determine how inactivation of Kif3α produced kidney cysts, we studied pre-cystic tubules from Kif3α mutant mice before the onset of cyst formation. For these experiments, we used Pkd1/Cre transgenic mice that expressed high levels of Cre recombinase in renal collecting ducts under the control of the Pkd1 promoter. Pkd1/Cre mice were crossed with Kif3α\(^{flox/-}\) and Kif3α\(^{flox/-}\) mice to produce Pkd1/Cre;Kif3α\(^{flox/-}\) progeny. Pkd1/Cre;Kif3α\(^{flox/-}\) mice were used for these experiments rather than the Ksp/Cre;Kif3α\(^{flox/-}\) mice described previously (14) because Pkd1/Cre;Kif3α\(^{flox/-}\) mice developed cysts more slowly, which permitted analysis of pre-cystic tubules in newborn mice, and the cysts were restricted to the collecting ducts, which facilitated measurements of planar cell polarity (discussed in what follows). The R26R reporter gene was included in the cross to identify cells that had undergone Cre/loxP recombination. Pkd1/Cre;Kif3α\(^{flox/-}\)R26R mice developed numerous kidney cysts (Fig. 6D) compared with control littermates (Fig. 6A). Cyst formation first became evident at P14 (data not shown). X-gal staining of the mutant kidneys revealed that the cyst epithelial cells expressed lacZ, indicating that the cysts were composed of recombined cells (Fig. 6E). Co-staining with DBA and an Ab against acetylated tubulin revealed that the cysts originated from the collecting ducts and lacked primary cilia (Fig. 6F). To determine whether the loss of cilia occurred prior to the onset of cyst formation, Pkd1/Cre;Kif3α\(^{flox/-}\) mouse kidneys were analyzed at P7–P10, prior to the onset of tubular dilatation at P14. Staining of kidneys with DBA and an Ab against acetylated tubulin showed that primary cilia were present in the collecting ducts of control mice (Fig. 6G–I) but were absent in the pre-cystic collecting ducts of Pkd1/Cre;Kif3α\(^{flox/-}\) mice (Fig. 6J–L). Cilia were detected in 54 ± 4.9% of control collecting duct cells but in only 11 ± 1.3% of the Kif3α mutant collecting duct cells (P < 0.0004, Student’s t-test, n = 3). These results indicated that Cre-mediated inactivation of Kif3α in the collecting ducts led to the loss of primary cilia prior to the onset of cyst formation.

Loss of cilia produces abnormalities in planar cell polarity

Previous studies have shown that cell proliferation is increased in established cysts in kidney-specific Kif3α knockout mice (14). To determine whether cell proliferation was increased prior to the onset of cyst formation, kidneys from 7 to 10-day-old Pkd1/Cre;Kif3α\(^{flox/-}\) mice were stained with an Ab against Ki-67. Kidneys were co-stained with an Ab against β-galactosidase to identify cells that had undergone Cre/loxP recombination. Ki-67 was expressed in 14.2% of lacZ-positive cells in control kidneys and 13.9% of lacZ-positive cells in the Kif3α mutant kidneys, which was not significantly different (Fig. 7A). These results indicated that the loss of primary cilia did not stimulate cell proliferation in pre-cystic tubules.

Recent studies suggest that PKD may arise from an abnormality in planar cell polarity (PCP) that manifests as randomization of the orientation of cell division (24). Normally, the
mitotic spindles in dividing cells are oriented along an axis that is parallel to the longitudinal axis of the tubule, so that cell division results in tubular elongation. In two murine models of PKD, the orientation of mitotic spindles was randomized, so that cell division resulted in tubular dilatation rather than elongation (24). To test whether the loss of primary cilia produces abnormalities in PCP, we measured the orientation of mitotic spindles in dividing cells in the kidney. Thick sections (30 μm-thick) of the kidneys from P7 to P10 mice were stained with an Ab against phosphohistone H3 (anti-H3pS10), and collecting duct cells in late anaphase or telophase were identified. The orientation of the mitotic spindles in the dividing cells was determined from the 3D coordinates of the separating chromosomes stained with anti-H3pS10 Ab. The longitudinal axis of the collecting duct was determined from the 3D coordinates of two points in the tubule proximal and distal to the dividing cells. The angle between the mitotic spindles and the longitudinal axis of the tubules was calculated as described in the Methods and Materials. In the control kidneys, 91% of the mitotic spindles were oriented within 20° of the longitudinal axis of the collecting ducts (Fig. 7B and C). In contrast, in the Kif3a mutant kidneys only 46% of the mitotic spindles were oriented at 20°. Twenty-four percent of the mitotic spindles were oriented at...
an angle greater than 40° from the axis of the tubule (Fig. 7C). The orientation of mitotic spindles was significantly different in mutant and control animals (P = 0.0004, Mann–Whitney U test).

**DISCUSSION**

The role of primary cilia in the neonatal and adult mouse kidney was studied by conditional inactivation of Kif3a,
which encodes a kinesin-II subunit that is required for ciliogenesis. Kidney-specific inactivation of Kif3a in newborn mice beginning at P2 resulted in the loss of primary cilia and the formation of kidney cysts. These results confirmed that inhibition of IFT and loss of primary cilia produces PKD. In contrast, kidney-specific inactivation of Kif3a in adult mice caused no histological abnormalities within 4 weeks after tamoxifen administration. The absence of rapid cyst formation in adult Kif3a mutant mice was observed despite a comparable loss of primary cilia: The percentage of lacZ-positive cells that contained cilia was reduced from 57 to 14% in the adult Kif3a mutant mice that did not develop cysts. A similar reduction in cilia formation from 54 to 11% was observed in newborn Pkhdl1/Cre;Kif3a<sup>lox/−</sup> mice that developed cysts. Although the loss of cilia in adult Kif3a mutant mice did not result in cyst formation within 4 weeks after tamoxifen administration, it is possible that these mice would have developed kidney cysts had we analyzed them at later time points. Our findings are consistent with a recent study from Davenport et al. (25) who showed that inhibition of IFT at E16.5, either by inactivation of Tg737 or Kif3a, caused very severe cystic disease soon after birth. However, inhibition of IFT in adult mice did not cause kidney cyst formation until after 6 months. Collectively, these results suggest that disruption of IFT during embryogenesis (25) or soon after birth (our results) causes kidney cyst formation. However, disruption of IFT in adult mice does not cause cyst formation in the short term (4 weeks).

To explore the mechanism for the distinct effects of Kif3a inactivation and loss of primary cilia in adult and newborn mice, we examined the role of cell proliferation. Here, we provide three lines of evidence that the formation of kidney cysts in Kif3a mutant mice is dependent on elevations in the basal rate of cell proliferation in the renal tubules. First, we showed that the absence of cysts in older mice correlated with a decrease in cell proliferation in the kidney. The proliferative activity in the postnatal kidney was measured at various ages after birth by staining mitotic cells with an Ab against phosphohistone H3. In newborn mice, in which inactivation of Kif3a produced kidney cysts, 0.99% of the cells in the kidney were undergoing mitosis. In adult mice that did not develop kidney cysts, only 0.06% of the cells were undergoing mitosis. Although a requirement for cell proliferation would explain the age-dependence of cyst formation, inactivation of Kif3a at P10 and P14, when the rates of cell proliferation were still higher than in the adult, did not result in the rapid formation of kidney cysts. These results suggested that a threshold of cell proliferation must be exceeded or that increased cell proliferation is only one of several factors responsible for the higher rate of cyst formation in newborn mice compared with older mice. Other mechanisms that may explain the differences in the phenotype observed at different ages include the transcriptional programming of kidney cells, which is markedly different in the postnatal period compared with the adult (26).

Additional support for the role of cell proliferation was provided by the differences in the frequency of cyst formation in different nephron segments at various stages of development. Inactivation of Kif3a in postnatal mice produced cysts that originated primarily from the loops of Henle (74% from loops of Henle and 22% from collecting ducts). In contrast, inactivation of Kif3a during embryonic development produced cysts that originated primarily from collecting ducts (73% from collecting ducts and 5% from loops of Henle) (14). This difference in the origins of the cysts was not explained by differences in the efficiency of Cre/loxP recombination, since the recombination rate was actually higher in collecting ducts than in the loops of Henle in postnatal mice treated with tamoxifen. Instead, the frequency of cyst formation correlated with the relative rates of cell proliferation in different nephron segments in the postnatal kidney. The proliferative activity of cells in the postnatal loops of Henle was 4-fold higher than in the collecting ducts, which was similar to the observed ratio of cyst formation in these nephron segments. In rodents, much of the development and elongation of the loops of Henle occurs after birth, and previous studies have shown that the proliferative activity in the postnatal loops of Henle is higher than in the proximal and distal tubules (27). Moreover, the highest rate of cell proliferation in the loop of Henle is in the cells near the

**Figure 7.** Loss of renal cilia does not stimulate cell proliferation but produces aberrant planar cell polarity. (A) Quantification of Ki-67 positive cells in lacZ-positive renal tubules in control mice (shaded bars) and Pkhdl1/Cre;Kif3a<sup>lox/−</sup> mice (solid bars) at P7–P10 revealed no significant differences in the rates of cell proliferation. NS, not significant (P = 0.92, Student’s t-test, n = 3). Error bars indicate SD. (B) To determine whether the loss of cilia produced aberrant planar cell polarity, the orientation of cell division was measured in Pkhdl1/Cre;Kif3a<sup>lox/−</sup> mice at P7–P10 prior to cyst formation. Representative images of kidneys stained with Abs against phosphohistone H3 (green) and enartinin (red) showed that the orientation of cell division (lines) was parallel to the longitudinal axis of the tubule in collecting ducts from control mice (left) but not in the Pkhdl1/Cre;Kif3a<sup>lox/−</sup> mice (right). (C) Histogram showing the distribution of mitotic spindle orientations in control mice (shaded bars) and Pkhdl1/Cre;Kif3a<sup>lox/−</sup> mice (solid bars). The orientation of cell division was significantly more randomized in the Pkhdl1/Cre;Kif3a<sup>lox/−</sup> mice (P = 0.0004, Mann–Whitney U test, n = 52).
cortico-medullary junction, which may explain the abundance of cysts in this location.

A third line of evidence supporting the role of cell proliferation in inducing cyst formation in Kif3a mutant mice was provided by studies in mice with acute kidney injury. Following AKI, the kidney has a remarkable capacity to regenerate by cellular proliferation (22,23). To test whether inducing proliferation by AKI caused cyst formation, adult Kif3a mutant mice were subjected to unilateral renal IRI. We have previously shown that renal regeneration after IRI is primarily mediated by proliferation of surviving tubular epithelial cells (23). Kif3a mutant mice that were subjected to renal IRI developed cysts in the injured kidney within 2.5 weeks. No cyst formation was observed in the uninjured contralateral kidney or sham-operated Kif3a mutant mice despite the comparable loss of cilia in the recombined tubules. These results indicated that injury and/or tubular regeneration trigger cystogenesis in adult Kif3a mutant mice and supported the hypothesis that cell proliferation stimulates cyst formation. To our knowledge, these studies are the first to show that AKI stimulates cyst formation in an animal model of PKD. Further studies will be required to determine whether stimulating cell proliferation by other means also induces cyst formation in adult Kif3a mutant mice. In addition, AKI may have other effects besides increasing cell proliferation that stimulate cyst formation.

Following AKI, the surviving renal tubular cells transiently lose epithelial characteristics such as apical–basal polarity and differentiation markers. Similar properties are exhibited by epithelial cells from cystic kidneys. However, unlike the injured kidney where recovery is marked by re-establishment of the differentiated epithelial state, the epithelium of the cystic kidney remains persistently dedifferentiated (28). Based on these observations, it has been hypothesized that progression of cystic disease may result from failure of complete recovery after kidney injury (29). Our studies provide evidence directly linking kidney injury to cyst formation. Our findings may also explain the focal nature of cyst formation and the variability in the severity of cystic disease observed in humans affected with PKD, even among those carrying the same gene mutation. Individual differences in exposure to factors that cause subclinical kidney injury and tubular regeneration may contribute to the variability in cyst formation. Taken together, these results provide a mechanistic explanation for the differences in cyst formation at various ages after disruption of IFT. Disruption of IFT in the setting of elevated rates of cell proliferation (postnatal development or repair after AKI) causes kidney cyst formation, which suggests that baseline proliferation plays a permissive role in cyst formation.

To elucidate the mechanism by which disruption of IFT in the setting of high rates of cell proliferation leads to cyst formation, we analyzed pre-cystic kidney tubules from newborn Phkd1/Cre;Kif3a<sup>flox</sup> mice. Phkd1/Cre;Kif3a<sup>flox</sup> mice developed cysts in the renal collecting ducts only after P14, which permitted analysis of pre-cystic tubules in newborn mice at younger ages. Analysis of mutant mice at P7–P10 prior to the onset of cyst formation at P14 revealed that primary cilia were absent in lacZ-positive tubular epithelial cells. These results indicated that inactivation of Kif3a led to the loss of primary cilia prior to the onset of cyst formation. Surprisingly, the rates of cell proliferation in pre-cystic tubules in mutant mice were similar to the rates in control littermates. These results indicated that the loss of primary cilia did not stimulate cell proliferation prior to the onset of cyst formation. Rather than increased rates of cell division, we found that the loss of primary cilia produced abnormalities in the orientation of cell division as a manifestation of aberrant planar cell polarity (PCP). PCP is defined as polarity along a tissue plane that is perpendicular to the apical–basal axis (30). PCP is involved in the establishment of epithelial polarity, convergent-extension movements during embryogenesis, ciliogenesis and oriented cell division (30). In mice, PCP signaling is required for the normal development of the kidney. By orienting the direction of cell division along the longitudinal axis of the nephron, PCP signaling is thought to permit the elongation of tubules without changing their diameter (6). Fischer et al. (24) were the first to identify abnormalities of PCP in two murine models of PKD, the Pck rat and kidney-specific HNF-1β knockout mice. Measurements of the orientation of mitotic cells in pre-cystic tubules revealed that rather than dividing parallel to the longitudinal axis of the tubule, the cells in the mutant animals divided in more of a random orientation that would be predicted to cause tubular dilatation. These results suggested that PKD arises from defects in PCP that manifest as abnormalities in the orientation of cell division within the plane of the tubular epithelium. To define the role of primary cilia in PCP, we measured the orientation of cell division in pre-cystic renal tubules from Kif3a mutant mice. In control mice, cells divided along an axis that was parallel to the longitudinal axis of the tubules. However, in the Kif3a mutant mice that lacked primary cilia in the kidney, the orientation of cell division was shifted towards a more random orientation. These studies are the first to demonstrate that primary cilia are required for the maintenance of PCP in the mammalian kidney and the loss of cilia produces aberrant PCP prior to cyst formation.

The mechanism by which primary cilia regulate PCP in the kidney is not known. It has been shown that PCP signaling provides a polar bias for ciliogenesis by influencing the actin cytoskeleton and arrangement of the microtubules (31). Moreover, once the cilia are formed, fluid flow has been shown to further refine the planar polarity of the cilia within the tissue (32). It has also been reported that in response to fluid flow, the ciliary protein invasin causes upregulation of PCP signaling (33). Based on these findings it has been suggested that the primary cilium aids in the propagation of PCP signaling by (i) signaling in response to flow or an unidentified ligand(s) and (ii) providing a vectorial cue to the centrioles of the dividing cells so that mitotic spindles form in the correct orientation (34). Abnormalities of PCP in PKD were first described in rodents with mutations of the transcription factor HNF-1β or its downstream target gene, Phkd1 (24). The Phkd1 gene product, fibrocystin, is localized on primary cilia. These findings suggest that alterations in PCP in Kif3a mutant mice might arise from loss of the ciliary function or localization of fibrocystin. Consistent with this hypothesis, Kaimori et al. (35) have shown that fibrocystin undergoes proteolytic cleavage releasing an extracellular fragment from the cilium into the tubule fluid. The concentration gradient of fibrocystin...
cleavage products in the tubule fluid may be important for establishing PCP. Alternatively, abnormalities in PCP may arise from alterations in the balance between canonical and non-canonical Wnt signaling (6). In this regard, we have previously shown that kidney-specific inactivation of Kif3a leads to accumulation of nuclear β-catenin and increased expression of c-Myc, possibly reflecting activation of canonical Wnt signaling (14).

In summary, our results demonstrate that primary cilia maintain normal tubular diameter through PCP signaling. Loss of primary cilia does not stimulate cell proliferation per se. Rather, the loss of cilia perturbs PCP signaling causing randomization of the orientation of cell division. In settings where cell proliferation is increased, such as during kidney development or following AKI, the randomization of cell division leads to tubular dilatation and kidney cyst formation. Since human PKD is thought to involve dysfunctional cilia due to mutations in ciliary proteins, these processes may also underlie cyst formation in individuals affected with PKD.

MATERIALS AND METHODS

Transgenic mice
The plasmid Ksp/CreERT2/hGH was generated by inserting the coding sequence of pCre-ER(T2) (16) into the SfiI site of an expression plasmid (N53) containing the 1.3-kb Ksp-cadherin promoter, β-globin TATA box, and bovine growth hormone polyadenylation signal (36). The bovine growth hormone sequence was removed and replaced with a human growth hormone mini-gene from the plasmid pNuKCre (18). A DNA fragment containing the Ksp-cadherin promoter, CreERT2 coding region and human growth hormone mini-gene was excised with NotI and purified by gel electrophoresis. Transgenic mice were produced by the University of Texas Southwestern Transgenic Core Facility as described previously (36). Microinjected C57BL/6J embryos were transferred to pseudopregnant foster mothers and permitted to develop to term. Transgenic progeny were identified by PCR analysis of tail biopsies using the following primers: Cre (AGGTTCGTGCACTCATGGA and TCGACCAGTT- GACGCAG) and R26R (AAAGTCGCTCTGAGTTGTTAT, AGGGCAGACGGAAGGGTGG and TGGCAGGTCATTG- CGTGTATG). Transgenic founders were crossed with R26R-EYFP mice (provided by Dr Frank Costantini, Columbia University) (20), which express lacZ or EYFP after Cre/loxP recombination. Bitransgenic Ksp/CreERT2;R26R progeny were crossed with Kif3a<sup>fl</sup> mice (21), and the resulting progeny were intercrossed to generate Ksp/Cre-ER(T2);R26R;Kif3a<sup>fl</sup> mice (conditional Kif3a mutant). Ksp/Cre-ER(T2);R26R;Kif3a<sup>fl</sup> or Kif3a<sup></sup> litter-
mates were used as negative controls. A similar approach was used to generate Pkhd1/Cre;R26R;Kif3a<sup>fl</sup> mice (Kif3a mutant) and Pkhd1/Cre;R26R;Kif3a<sup>fl</sup> mice (control littermates). All experiments involving animals were performed under the auspices of the UT Southwestern Institutional Animal Care and Research Advisory Committee.

Tamoxifen treatment
Ksp/CreERT2 transgenic mice were administered tamoxifen to induce Cre/loxP recombination. Tamoxifen or 4-hydroxytamoxifen (Sigma), which produced equivalent results, was dissolved in 95% ethanol then diluted 10-fold in corn oil to a final concentration of 10 mg/ml. Mice that were more than 10-days-old were administered tamoxifen (4 mg/40 g body weight) daily by i.p. injection for five consecutive days. To induce recombination in newborn mice, nursing dams were injected with tamoxifen (4 mg/40 g body weight) on post-partum days 2–4. Control animals received an equal volume of vehicle alone (9.5% ethanol in corn oil). Animals were sacrificed and analyzed 4 weeks after the first day of injection. To minimize background recombination, all experiments were conducted using male mice.

Renal ischemia–reperfusion injury
Adult male Ksp/Cre-ER(T2);R26R;Kif3a<sup>fl</sup> mice were injected with tamoxifen at 5 weeks of age. At 6 weeks of age, the mice were anesthetized with isoflurane, and unilateral IRI was performed by clamping the left renal pedicle for 45 min followed by clamp release to allow reperfusion to the kidney (23). Sham-operated mice underwent the identical procedure except for clamping of the renal pedicle. Mice were sacrificed, and both kidneys were removed for analysis 2.5 weeks after IRI or sham-operation.

X-gal staining and measurement of cyst index
Tissues were harvested from anesthetized mice and were fixed by perfusion with 4% paraformaldehyde. The tissues were then frozen in OCT after cryopreservation in 30% sucrose. Tissue sections (10-μm-thick) were incubated at 33°C overnight in the staining solution (PBS containing 20 mm Tris–Cl, pH 7.3, 1.8 mM spermidine, 2 mM MgCl2, 0.02% Nonidet P-40, 0.01% sodium deoxycholate, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide and 1 mg/ml X-gal). Sections were counterstained with eosin. To calculate the cyst index, the largest sagittal section of the kidney containing the cortex, medulla and papilla was photographed, and the cumulative area of cysts was measured using AxioVision software (Zeiss).

Immunoblot analysis
Preparation of tissue extracts and immunoblot analysis were performed as described previously (36). Immunoblots were probed with anti-GFP Ab (Molecular Probes) followed by detection by chemiluminescence. Anti-tubulin Ab (Sigma) was used as a loading control.
Ab and lectin staining

The following Abs and dilutions were used in this study: AQP3 (Chemicon International Inc., 1:400), acetylated tubulin (Sigma, 1:1000), enantactin (Chemicon, 1:1000), β-galactosidase (Molecular Probes, 1:1000), FITC-conjugated anti-GFP that cross-reacts with EYFP (Rockland, 1:200), histone H3pS10 (Sigma 1:1000), Ki-67 (Novocasta, 1:500), NCX1 (generous gift from K.D. Philipson at David Geffen School of Medicine at UCLA, 1:1000), NKCCh2 (Chemicon, 1:1000). Secondary antibodies were conjugated to Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes, 1:400). Tissue sections were incubated with primary Ab at 4°C overnight or at room temperature for 2 h after antigen retrieval with 1% SDS in PBS and blocking of non-specific binding with blocking solution (10% goat serum, 0.3% bovine serum albumin in PBS). The tissue sections were incubated with appropriate secondary antibodies for 1 h at room temperature. Lectins used in this study were Dolichos biflorus agglutinin (DBA, Vector Laboratories, 1:400) and Lotus tetragonolobus agglutinin (LTA, Vector Laboratories, 1:400). Tissue sections were stained with biotinylated lectins and detected using fluorescein-conjugated avidin D (Vector Laboratories, 1:400). The slides were mounted with Vectashield and examined using a Zeiss Axioplan 2 fluorescence microscope. Images were acquired using a digital camera and analyzed with AxioVision software (Zeiss).

Measurement of planar cell polarity

The orientation of cell division was measured as described by Fischer et al. (24). Kidney sections (30 µm-thick) were stained with anti-H3pS10 Ab to label the chromosomes of dividing cells in late anaphase and telophase. Sections were co-stained with anti-entactin Ab or DBA to label the tubular basement membranes. Z-stack images were acquired using an LSM 510 META confocal laser scanning microscope (Zeiss), and 3D images were reconstructed using Imaris software (Bitplane AG) (Supplementary Material, Video). The orientation of cell division was determined by measuring the angle between the mitotic spindles of dividing cells and the longitudinal axis of the collecting ducts. The 3D coordinates of the mitotic spindles and tubules were exported into an Excel spreadsheet, and the angle (θ) between the two axes was calculated using the formula:

$$\cos \theta = \frac{a \cdot b}{|a| |b|} = \frac{a_x b_x + a_y b_y + a_z b_z}{\sqrt{a_x^2 + a_y^2 + a_z^2} \sqrt{b_x^2 + b_y^2 + b_z^2}}$$

where a and b represent the vectors corresponding to the mitotic spindle and tubule, respectively. A total of 82 cells in late anaphase or telophase from 12 control and 15 Kif3a mutant kidneys were analyzed.

Statistical analysis

Data shown are mean ± SD. The significance of differences between the means was calculated using Student’s t-test. ANOVA was used for multiple comparisons. The Mann–Whitney U test was used to compare the distributions of mitotic spindle orientations in control and mutant mice. P < 0.05 was considered statistically significant.

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