A genetic screen in zebrafish identifies cilia genes as a principal cause of cystic kidney

Zhaoxia Sun[1,*], Adam Amsterdam[1], Gregory J. Pazour[2], Douglas G. Cole[3], Mark S. Miller[3] and Nancy Hopkins[1,†]

[1]MIT, Center for Cancer Research and Biology Department, Building E17 Room 340, 77 Massachusetts Avenue, Cambridge, MA 02139, USA
[2]Program in Molecular Medicine, University of Massachusetts Medical School, 373 Plantation Street, Worcester, MA 01605, USA
[3]Department of Microbiology, Molecular Biology and Biochemistry (LSS142), University of Idaho, Moscow, ID 83844, USA
*Present address: Department of Genetics, Yale University School of Medicine, 333 Cedar Street, NSB-393, P.O. Box 208005, New Haven, CT 06520, USA
†Author for correspondence (e-mail: nhopkins@mit.edu)

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Summary

Polycystic kidney disease (PKD) is a common human genetic illness. It is characterized by the formation of multiple kidney cysts that are thought to result from over-proliferation of epithelial cells. Zebrafish larvae can also develop kidney cysts. In an insertional mutagenesis screen in zebrafish, we identified 12 genes that can cause cysts in the glomerular-tubular region when mutated and we cloned 10 of these genes. Two of these genes, *vhnf1* (*tcf2*) and *pkd2*, are already associated with human cystic kidney diseases. Recently, defects in primary cilia have been linked to PKD. Strikingly, three out of the 10 genes cloned in this screen are homologues of *Chlamydomonas* genes that encode components of intraflagellar transport (IFT) particles involved in cilia formation. Mutation in a fourth blocks ciliary assembly by an unknown mechanism. These results provide compelling support for the connection between cilia and cystogenesis. Our results also suggest that lesions in genes involved in cilia formation and function are the predominant cause of cystic kidney disease, and that the genes identified here are excellent candidates for novel human PKD genes.

Supplemental data available online

Key words: PKD, Cilium, Zebrafish

Introduction

Polycystic kidney disease (PKD) is a medically important disorder that is characterized by the formation of multiple epithelium-lined, liquid-filled cysts in the kidney (Gabow and Grantham, 1997). Affecting one in 1000 live births, autosomal dominant polycystic kidney disease (ADPKD) is among the most common monogenetic disorders in humans (Gabow and Grantham, 1997). Kidney cysts are frequently connected to existing tubules or ducts in the kidney. Over-proliferation of renal epithelial cells is therefore thought to be the underlying mechanism of PKD pathogenesis and indeed is a major defect observed in PKD afflicted cells (Grantham et al., 1987; Lanoix et al., 1996; Nadasdy et al., 1995). Mutations in two genes, *PKD1* and *PKD2*, have been identified as major causes for ADPKD. These genes are responsible for most ADPKD and encode two membrane proteins, polycystin 1 and polycystin 2, respectively (The European Polycystic Kidney Disease Consortium, 1994; Mochizuki et al., 1996). Polycystin 1 is a large protein with 11 transmembrane domains, while polycystin 2 is a channel in the TRP superfamily, members of which are cation channels frequently involved in mechanosensory responses. These proteins are thought to form a complex and control the proliferation and differentiation of renal epithelial cells (Somlo and Ehrlich, 2001; Somlo and Markowitz, 2000).

In addition to ADPKD, kidney cyst formation is also a major component in a variety of genetic diseases, including autosomal recessive PKD (ARPKD), nephronophthisis (NPHP) and Bardet-Biedl syndrome (Gabow and Grantham, 1997). Multiple genes for these diseases have been cloned, including *PKHD1* for ARPKD (Onuchic et al., 2002; Ward et al., 2002); *BBS1*, *BBS2*, *BBS4*, *BBS7* and *BBS8* for Bardet-Biedl syndrome (BBS) (Ansley et al., 2003; Badano et al., 2003; Mykytyn et al., 2001; Mykytyn et al., 2002; Nishimura et al., 2001) and *NPHP1*, *NPHP2* (*INVS* – Human Gene Nomenclature Database) *NPHP3* and *NPHP4* for nephronophthisis (NPHP) (Hildebrandt et al., 1997; Olbrich et al., 2003; Otto et al., 2002; Otto et al., 2003).

Recent evidence suggests that cilia play a central role in the etiology of PKD (Ong and Wheatley, 2003). The cilium is a cell surface organelle. It is surrounded by a membrane that is contiguous with the cell membrane and it has a microtubule axoneme at its center. Eukaryotic cilia and flagella are assembled by a process called intraflagellar transport (IFT). This process was first discovered, and remains best understood, in the green algae *Chlamydomonas* (Rosenbaum and Witman, 2002). During IFT, large protein particles are carried along the ciliary microtubules by kinesin and dynein. The IFT particles are thought to be vehicles for transporting cargo needed for assembly, maintenance and function of flagella and cilia. These

particles are composed of at least 17 polypeptides (Cole et al., 1998; Piperno et al., 1998) and are highly conserved. Homologs have been found in all ciliated organisms examined, including *C. elegans*, *Drosophila* and mammals (Baker et al., 2003; Cole et al., 1998; Han et al., 2003; Haycraft et al., 2003; Huangfu et al., 2003). Mutations in genes encoding IFT particle proteins block ciliary assembly in *Chlamydomonas* (Pazour et al., 2000), *C. elegans* (Cole et al., 1998; Haycraft et al., 2001; Qin et al., 2001), *Drosophila* (Han et al., 2003) and mouse (Huangfu et al., 2003; Murcia et al., 2000; Pazour et al., 2000). In the mouse, complete null alleles block assembly of cilia on the embryonic node and result in embryonic lethality during mid-gestation (Huangfu et al., 2003; Murcia et al., 2000), while a partial loss of function allele retards cilia formation in the kidney (Pazour et al., 2000) and other organs and causes hydrocephaly, preaxial polydactyly, and cysts in the kidney, liver and pancreas (Moyer et al., 1994), indicating that cilia play important roles in vertebrate development. In support of the idea that cilia assembly defects cause PKD, targeted deletions of *KIF3A* in kidney epithelium block ciliary assembly and cause cyst formation (Lin et al., 2003). KIF3A powers IFT particle movement from the cell body to the ciliary tip (Rosenbaum and Witman, 2002). Furthermore, several PKD-associated gene products have been found on cilia, including the products of the human autosomal dominant *PKD1* and *PKD2* genes (Pazour et al., 2002b; Yoder et al., 2002), the human autosomal recessive *PKHD1* gene (Ward et al., 2003), and the human nephronophthisis *NPHP1* and *NPHP2* genes (Otto, 2003; Watanabe, 2003). These findings lead to a cilia model for PKD (Rosenbaum and Witman, 2002; Pazour and Whitman, 2003). In this model, cilia on renal epithelial cells function as antennae that detect environmental signals. Activation of sensors on the cilium, such as polycystin 2, triggers a Ca^{2+} influx into the cell and eventually regulates cell proliferation. However, the link between cilia and PKD is largely based on 'guilt by association'. Additionally, the nature of the signal sensed by cilia remains controversial and the signaling pathway that couples cilia to cell proliferation is almost completely unknown.

To further our understanding of PKD, we carried out a forward genetic screen in zebrafish to identify genes that can cause this disorder. The zebrafish pronephros is composed of two nephrons with glomeruli fused at the midline, connected to pronephric ducts that alter the blood filtrate and shunt the urine outside of the animal (Drummond et al., 1998). Zebrafish embryos can develop kidney cysts by 2 days post fertilization (Drummond et al., 1998; Sun and Hopkins, 2001). As zebrafish embryos are transparent, cyst formation is clearly visible under a stereoscope. Moreover, we have demonstrated previously that mutations in *vhnf1* (*tcf2* – Zebrafish Information Network) which is associated with human familial GCKD (glomerulocystic kidney disease) (Bingham et al., 2001), can cause kidney cysts in zebrafish (Sun and Hopkins, 2001), suggesting that kidney cyst formation in zebrafish is highly relevant to human PKD. Taken together, these characteristics of zebrafish make it feasible to perform large-scale genetic screens to search for genes involved in kidney cyst formation. From just such a screen, we have identified 12 different genes that can cause kidney cysts in zebrafish embryos when mutated. We cloned 10 of these genes, three of which encode IFT particle components. We further show that a fourth gene is also required for cilia formation through an unknown mechanism. Thus, through an unbiased genetic screen for cystic kidney mutants, we not only provide strong support for the cilial model of cystogenesis, but evidence that this may be the primary cause of human PKD.

Materials and methods

Mutant identification

We observed zebrafish embryos under a stereoscope from day 2 to day 5 post fertilization. The zebrafish nephron is located beneath somite 3-4, medial to the pectoral fins. Normally, they are invisible against a transparent background. However, cysts are clearly visible as bubbles in this region.

Molecular biology

We cloned genes responsible for the mutant phenotypes, obtained full-length cDNA and carried out RT-PCR as previously described (Amsterdam et al., 1999; Golling et al., 2002). To detect maternal expression of tested genes, we extracted RNA from embryos before the 128-cell stage with trizol reagent (Invitrogen) following the user manual. To compare gene expression in mutant and wild-type embryos, we extracted RNA from embryos at day 3-5 post-fertilization. We then reverse transcribed RNA into cDNA with the superscript II RT-PCR system (Invitrogen) and subsequently performed PCR with gene-specific primers. The primers used are as follows: *hi409*, 5′-GCCTGAAAAGAGAGAGTTTATC-3′ and 5′-CGTAATTCTTCAAGATGAGCGA-3′; *hi2211*, 5′-GATGGAGCTGCTAAAGTCACCTGT-3′ and 5′-AAATACTCCGTCGGAGACAGCA-3′; *hi3308*, 5′-TGCGGTTTTAAGGAGCGTCT-3′ and 5′-CACTCTCACATACATTGGCTGAACA-3′; *hi3417*, 5′CGGGATCCGGGATGGCGGAGGAGGAAGA-3′ and 5′-GGAATTCGTGTTTCAATAAGCCTGCGCA-3′; *hi4166*, 5′-TGGAATTCACGATGAGCTCCAGTCGCGT-3′ and 5′-GGGATACGTGCTGTGGTTCTC3′.

Histological analysis

Embryos were fixed in Bouin's fixative overnight at room temperature, washed three times with PBS, embedded in JB-4 resin from Polysciences and cut at 4 μm. Slides were then stained with Hematoxylin and Eosin.

Immunohistochemistry

Embryos were fixed in Dent's fixative as described before (Drummond et al., 1998). A monoclonal anti-acetylated tubulin antibody from Sigma was used at 1:500. α6F antibody from DSHB was used at 1:10. Alkaline phosphatase-conjugated (Vector Laboratories) anti-mouse IgG was used at 1:1000. Color was developed with NBT/BCIP as the chromogenic substrate. Embryos were then cleared with benzyl benzoate and flattened with coverslips for photography. For confocal analysis, Alexa fluor 488 conjugate anti-mouse IgG from Molecular Probes was used at 1:500.

Morpholino injection

We obtained morpholino oligos from Gene Tools and injected them into embryos at one- to four-cell stages at a concentration of 1 μM. A standard control oligo (5′-CCT CTT ACC TCA GTT ACA ATT TAT A-3′) was injected as a control. We raised morpholino injected embryos and observed for phenotypes with stereoscopes. The oligos are designed against the translational initiation sites of corresponding genes. The sequences for the morpholino oligos used are as follows: *hi459* oligo 1, 5′-TTCGCCATCAGATTGAACATTTCCC-3′; *hi459* oligo 2, 5′-TTTCCCCCCTAAATGCTTTCACTGG-3′; *hi1055B* oligo 1, 5′- TGCTTTTTACTTCTTCGATCTTCAT-3′; *hi2211* oligo 1, 5′-CATACTTCACGTTTATAATAAGACT-3′; *hi2211* oligo 2, 5′-GACTCAGGGCAGTTATAAGAACGTA-3′; *hi4166* oligo 1, 5′-AGGACGAACGCGACTGGAGCTCATC-3′.

Accession numbers

hi409/IFT81, AY618922; *hi459/scorpion*, AF506213; *hi2211/IFT172*, AY618923; *hi3417/IFT57*, AY618924; *hi3308/seahorse*, AY618925; *hi3959A/qilin*, BC045921; *hi4166/pkd2*, AY618926; *CrIFT81*, AY615519; *CrIFT172*, AY615520.

Results

An insertional mutagenesis screen in zebrafish identifies cystic kidney mutants

Using a pseudo-typed retrovirus as the mutagen, we completed an insertional mutagenesis screen in the fish to identify genes essential for the development of a 5-day-old embryo, a time when most of the major organs have formed and the embryo is turning into a free swimming larva (Amsterdam et al., 1999; Golling et al., 2002). About 550 mutants have been identified from the screen, representing mutations in roughly 400 unique essential genes or slightly more than 25% genome-wide saturation by our estimation (Amsterdam et al., 2004). Therefore, this collection of mutants represent mutations in a significant fraction of genes involved in diverse processes required in the making of a vertebrate embryo. From this mutant collection, we then specifically screened for mutants with cystic kidney phenotype by observing embryos at 2-5 days post fertilization under a stereoscope. Because of the transparency of zebrafish embryos, kidney cysts are clearly visible in live embryos under a stereoscope. We identified 15 mutant lines with this phenotype (Table 1; Fig. 1A-C). Interestingly, most of these cystic mutants also show a body curvature phenotype (example shown in Fig. 1A). A 16th mutant, showing only a body curvature phenotype but no cyst formation by day 5, turned out to be caused by an insertion in the *pkd2* gene after molecular characterization. RT-PCR revealed that *pkd2* is expressed maternally (data not shown), suggesting that maternally contributed *pkd2* might support early fish development and partially mask its functional requirement in this mutant. A morpholino directed against the translational initiation site of *pkd2* does cause cystic kidneys (Fig. 1D; Table 2). Thus, we include *pkd2* in our collection of kidney cyst mutants (Table 1)

Cloning and verification of genes mutated in cystic kidney mutants

Using the retroviral sequence as a tag to determine the site of insertion of the mutagenic proviruses, we cloned 10 genes mutated in 14 of the16 mutants described above. The two remaining mutants, *hi1392* and *hi2005*, can both complement mutations from all the other 11 complementation groups, indicating that each is disrupted in another unique gene. To verify that the genes we identified are responsible for the corresponding mutant phenotypes, RT-PCR analysis was performed using mRNA extracted from the mutant or wild-type embryos on day 3-5 post fertilization to determine if expression of the genes was altered in the corresponding mutants. At these time points, mutant embryos can be reliably distinguished from wild-type embryos based on morphology and the possibility of contamination from maternal transcripts is minimal. No wild-type mRNA corresponding to the candidate gene responsible for each mutant was detected in any of the mutants (an example is shown in Fig. 2), providing evidence that these are the genes responsible for the mutant phenotypes. It should be noted that we cannot rule out the possibility that some aberrant messages that cannot be amplified by the primers we used could be produced from the mutated genes. The identities of these genes were also verified by tight linkage analysis (Table 1). From crosses of heterozygous mutant fish, we picked embryos with wild-type and mutant morphology, extracted DNA from individual embryos and performed PCR to test whether the putative mutagenic proviral insertion is homozygous in all mutant embryos, but either absent or heterozygous in their wild-type siblings. In each case, there was tight linkage between the insertion and corresponding phenotypes in all mutants as determined by this assay (Table 1). As a further confirmation, morpholino oligos against the translational initiation sites of the four selected genes, *hi459/scorpion*, *hi1055B/pontin*,

Table 1. Cystic kidney genes identified in the screen

Genes	Mutants	Phenotypes: Kidney cyst	Phenotypes: Curved body	Linkage analysis
Group I mutants				
vhnf1	*hi548*			
	hi1843			
	hi2169	+	–	Golling et al., 2002; Sun and Hopkins, 2001
cad1/caudal	*hi2092, hi2188A hi2617*	+	–	24/24 mutant; 0/24 wild type for 2188A (Golling et al., 2002)
Group II mutants				
IFT57	*hi3417/curly*	+	+	16/16 mutant; 0/46 wild type
IFT81	*hi409/larry*	+	+	90/90 mutant; 0/36 wild type
IFT172	*hi2211/moe*	+	+	137/137 mutant; 0/55 wild type
scorpion	*hi459/scorpion*	+	+	64/64 mutant; 0/102 wild type (Golling et al., 2002)
pontin	*hi1055B*	+	+	45/45 mutant; 0/50 wild type
seahorse	*hi3308/seahorse*	+	+	43/43 mutant; 0/50 wild type
qilin	*hi3959A/qilin*	+	+	58/58 mutant; 0/87 wild type
pkd2	*hi4166*	morphant +	+	99/99 mutant; 0/93 wild type
not cloned	*hi1392/twister*	+	+	69/69 mutant; 0/90 wild type
not cloned	*hi2005/enigma*	+	+	63/63 mutant; 0/64 wild type

The three IFT mutants were named *larry*, *moe* and *curly*. The mutants with mutations in novel genes were named *scorpion*, *seahorse* and *qilin* (a Chinese mythical creature similar to unicorn). The remaining two uncloned mutants were named *enigma* and *twister*. In linkage analysis, numbers show out of analyzed mutant and wild-type embryos, how many are found to be homozygous for the corresponding proviral insertion. For some mutants, the linkage data has been reported in the supplemental data of our previous publication (Golling et al., 2002; Sun and Hopkins, 2001).

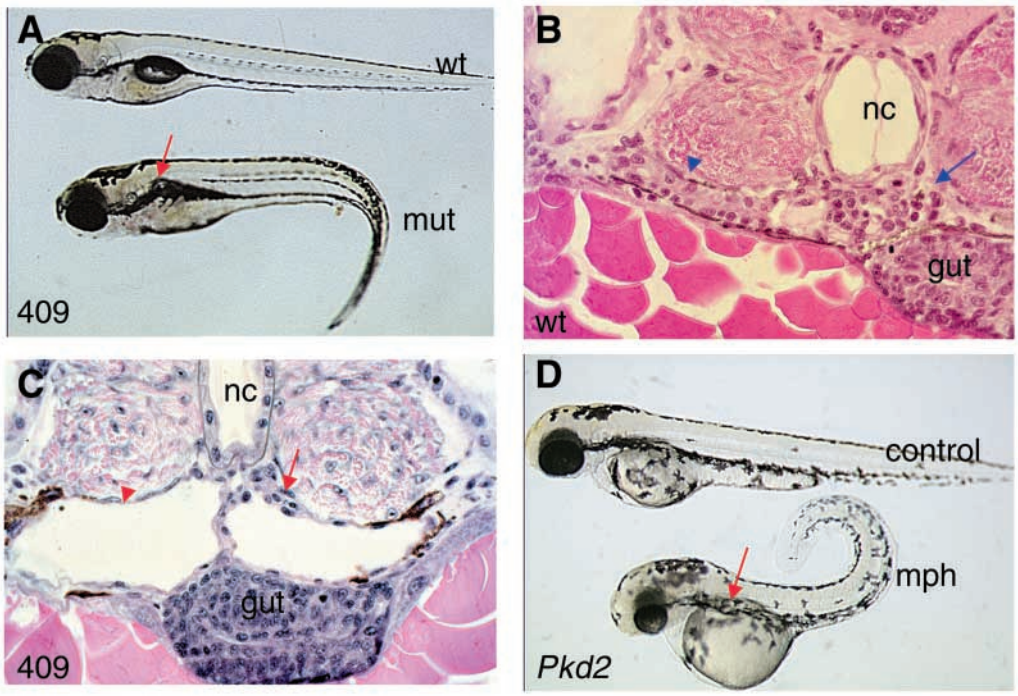

Fig. 1. Phenotypes of identified mutants. (A) A *larry*^{hi409} mutant on day 5 shows kidney cyst (arrow) and body curvature. (B,C) Cross-section of the glomerular (arrow)-tubular (arrowhead) region of wild-type (B) and *larry*^{hi409} (C) embryos at 50 hpf (hour post fertilization). (D) *pkd2* morphant at 53 hpf shows kidney cyst (arrow) and body curvature. nc, notochord; wt, wild type; mut, mutant; control, embryo injected with standard control morpholino oligo; mph, morphant.

hi2211/IFT172 and *hi4166/pkd2*, were injected into wild-type embryos. For *hi1055B/pontin* and *hi4166/pkd2*, the first oligo we attempted was able to cause phenotypes similar to that observed in the corresponding mutants (e.g. body curvature and kidney cyst formation). For *hi459/scorpion* and *hi2211/IFT172*, the first oligo we designed failed to produce significant phenotypes. However, in both cases, a second oligo was able to produce similar phenotypes. Therefore, for all genes analyzed, morpholino oligos were able to phenocopy the corresponding mutant phenotype (Table 2).

Genes identified in this screen

Table 1 summarizes the identity of the 10 genes identified to date. Importantly, two of the genes identified in this screen, *vhnf1* and *pkd2*, have previously been shown to be responsible for two different forms of human cystic kidney diseases: *VHNF1* is linked to GCKD (glomerular cystic kidney disease) and *PKD2* to ADPKD (Bingham et al., 2001; Mochizuki et al., 1996). This result strongly supports the usefulness of the zebrafish for modeling human PKDs, as well as the specificity of our cystic-kidney screen.

To facilitate the analysis of the mechanisms involved in cytogenesis, we divided these mutants into two groups based on their phenotypes, e.g. whether they show body curvature defects in addition to cystic kidney (Table 1). In Group I are the *vhnf1* and *cad1/caudal* mutants, neither of which shows body curvature defects. *vhnf1* encodes a homeobox gene. *vhnf1* mutant embryos show patterning defects in multiple organs, as we reported previously (Sun and Hopkins, 2001). *cad1* encodes a bHLH transcription factor, and its fly homolog *caudal* is involved in the anterior-posterior patterning of the body plan (Macdonald and Struhl, 1986). Reminiscent of such a role, the zebrafish *cad1* mutant also shows tail truncation in addition to the kidney cyst. Noticeably, both genes in Group I encode transcription factors.

The rest of the mutants display striking similarities in their phenotypes, including body curvature and kidney cysts (Fig. 1A), suggesting that they may function in the same process or even pathway. Therefore, we put them into a separate group that we designate Group II. Because of the body curvature and kidney cyst phenotype of *pkd2* morphants (morpholino knock down animals, Fig. 1D), we include the *pkd2* mutant in this group, even though it displays only the body curvature but not the cystic kidney phenotype.

In the entire insertional mutagenesis screen, novel genes account for about 20% of all the genes identified. However, among the genes mutated in the Group II kidney mutants, six out of eight are novel genes: they have human homologues, but their predicted encoded proteins had no assigned biochemical function when we first cloned them. The exceptions are *pkd2* and *hi1055B*, which encodes pontin, a component of a DNA stimulated ATPase complex. It is thought that pontin and reptin, a paralog of pontin, antagonize each other's functions (Bauer et al., 2000). A zebrafish gain-of-function mutant of reptin displays curved body and hyperplastic heart (Rottbauer

Table 2. Morpholino injection can produce similar phenotypes to those observed in the corresponding mutants

Gene	Morpholino oligo	Curly tail	Kidney cyst
hi459/scorpion	1	NA	NA
	2	15/16	15/16
hi1055B/pontin	1	21/21	14/21
hi2211/IFT172	1	NA	NA
	2	12/21	3/10
hi4166/pkd2	1	19/20	17/20

Numbers show the number of embryos that show a particular phenotype out of the embryos examined. The first oligo tested for *hi459/Scorpion* and *hi2211/IFT172* failed to produce obvious phenotype. NA, not applicable (none observed in over 40 injected embryos).

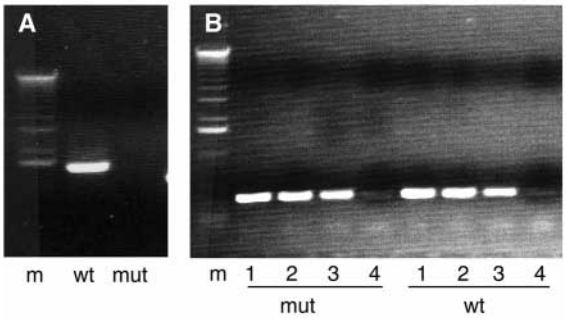

Fig. 2. mRNA is affected in mutant embryos. (A) RT-PCR on cDNA from mutant and wild-type *hi3308* embryos with a pair of *hi3308/seahorse* specific primers flanking the insertion site. (B) Control RT-PCR with a pair of actin specific primers. A serial dilution of cDNA was used to show that similar amounts of cDNA were used. m, 1 kb ladder; wt, wild type; mu, mutant; 1-4, 1:10, 1:200, 1:4,000 and 1:80,000 dilutions.

et al., 2002). The function of pontin and reptin in kidney development have not been formally studied.

Many Group II kidney mutants have defects in ciliary genes

Given the connection between cilia and PKD, we compared the sequences of the novel genes we identified to a collection of 12 IFT genes whose sequences were available to us. The IFT machinery was first identified in the green alga *Chlamydomonas* and is thought to be needed for assembly, maintenance and function of flagella and cilia (Kozminski et al., 1993; Rosenbaum and Witman, 2002). Strikingly, the sequence analyses revealed that three of our group II genes encode zebrafish homologs of IFT components. *hi409* encodes a homolog of IFT81 (D.G.C. and M.S.M., unpublished), *hi2211* encodes a homolog of IFT172 (Cole et al., 1998) and *hi3417* encodes a homolog of IFT57 (Haycraft et al., 2003) (G.J.P., G. B. Whitman, J. L. Rosenbaum and D.G.C., unpublished) (Table 1) (see Fig. S1 at http://dev.biologists.org/supplemental). We named mutants *hi409*, *hi2211* and *hi3417* *larry*, *moe* and *curly*, respectively. Intriguingly, these three IFT genes are the only known IFT genes identified in the screen among the 300 different genes cloned to date. These results provide compelling support for an intimate connection between cilia and cystogenesis.

hi409/IFT81 is expressed widely in the embryo

Despite the specific phenotype of cyst formation in PKD, a number of PKD genes have been shown to be widely expressed. To determine if this is also true for some of the genes we isolated, we performed in situ hybridization to study *hi409/larry* gene expression in embryos at the eight-somite stage, 25 hpf and 34 hpf. At all time points, *hi409/larry* transcripts can be detected in all regions of the embryos (Fig. 3). However, we also observed enriched expression in specific regions at specific time points. At the eight-somite stage, the transcript is enriched in the notochord. At 25 hpf, it is concentrated in cells surrounding the brain ventricles. At 34 hpf, enrichment can be seen in the otic vesicle and to some extent in the pronephric ducts. Interestingly, cilia are known to be present in the brain ventricle, the otic vesicle and the kidney.

Cilial defects in mutants

The results described above prompted us to examine the presence of cilia in the zebrafish pronephric duct and tubule. A monoclonal antibody against acetylated tubulin stains this region strongly (Fig. 4A). Confocal analysis verified that this signal is from cilia protruding from the apical surface of tubular and ductal cells into the lumen (Fig. 4B), suggesting that cilia are present on epithelial cells in zebrafish pronephric ducts and tubules.

We then examined cilia formation in all of our Group II cystic kidney mutants. Interestingly, in *scorpion*^{hi459} mutant embryos, the acetylated tubulin signal is absent (Fig. 4C,D), suggesting that the gene mutated in *hi459/scorpion* is required for cilia formation. However, in all the rest of the mutants, including the three IFT mutants, cilia were able to form (results not shown). One possibility is that IFT genes may be duplicated in the zebrafish and therefore functional redundancy may mask the function of these genes. To test this possibility, we blasted the zebrafish genome draft for all three IFT genes and found no evidence for the existence of redundant genes. Alternatively, the maternal contribution could be sufficient for the initiation of cilia formation during early development. To test this possibility, we performed a time course using RT-PCR for these IFT genes. Interestingly, all three are expressed maternally (results not shown). Therefore, the maternal

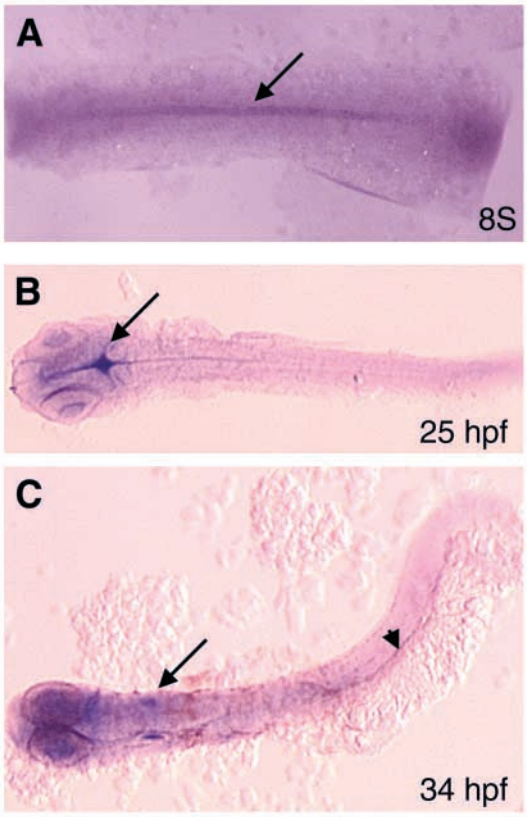

Fig. 3. *hi409/IFT81* is widely expressed. (A) In situ hybridization for *hi409/IFT81* in an embryo at the eight-somite stage (8S), *hi409/IFT81* is enriched in the notochord (arrow). (B) In embryos at 25 hpf, *hi409/IFT81* is enriched in the brain ventricle (arrow). (C) In embryos at 34 hpf, *hi409/IFT81* is enriched in the otic vesicle (arrow) and slightly in the pronephric duct (arrowhead).

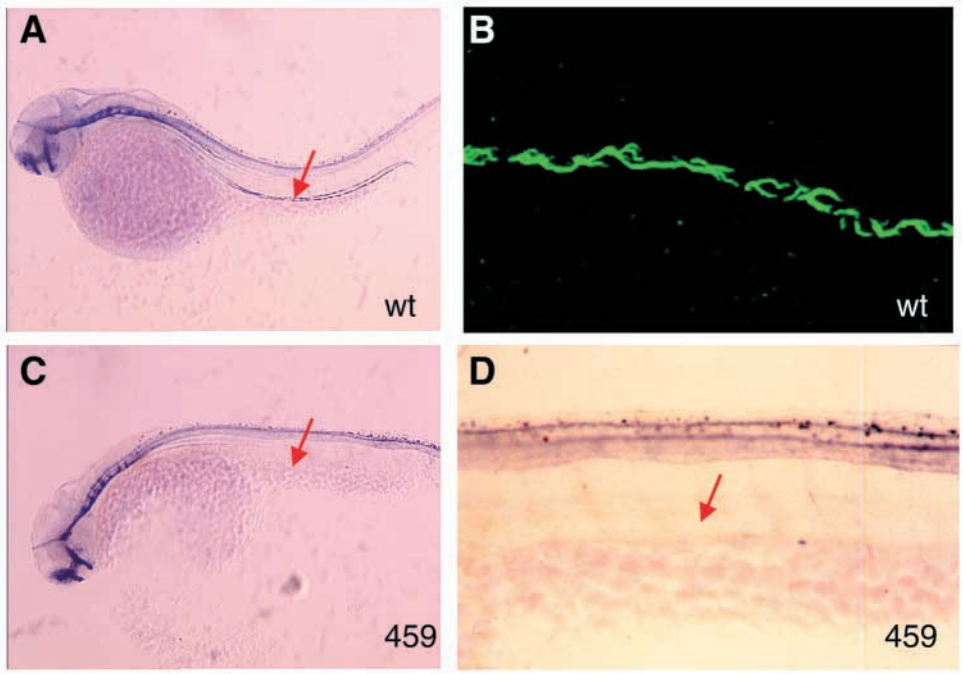

Fig. 4. Ciliary defects in *scorpion*^{hi459}. (A) The pronephric duct region (arrow) in a wild-type embryo at 50 hpf reacts strongly with an antibody against acetylated tubulin. (B) Confocal image shows cilia stained with this antibody in the pronephric duct of a wild-type embryo at 24 hpf. (D) Defect of the cilia in *scorpion*^{hi459} at 50 hpf; (D) enlarged view of C. wt, wild type; 459, *scorpion*^{hi459}.

contribution complicates the interpretation of cilia formation in these mutants. Nevertheless, based on what is known about IFT in multiple organisms, we think the function and/or maintenance of the cilia is probably affected by these mutations. Together, at least five (*hi459/scorpion*, 3 IFT genes and *pkd2*) out of the eight genes cloned for the 10 Group II mutants are involved in cilia formation or function, suggesting that lesions in cilia genes are a predominant cause of cystic kidney.

The functions of the two other novel genes, *hi3308/seahorse* and *hi3959A/qilin*, and *pontin* in our collection of Group II mutants remain unclear. However, as vertebrates might have a wider repertoire of genes that are required for the formation and function of cilia, it is possible that some of them may also be cilia genes. Alternatively, they may be involved in connecting cilia signals to downstream events.

Further analysis of mutant phenotypes provided interesting clues for the cellular pathogenesis of kidney cysts. All the kidney cysts we observed were medial to the pectoral fins (Fig. 1A). Histological sections verified that they are located in the tubular-glomerular region (Fig. 1B,C). Interestingly, the pronephric ducts outside of the cystic regions were enlarged in all of the mutants, as shown by histological sections (an example is shown in Fig. 5A,B). We further stained whole embryos with α6F (Fig. 5C,D), an antibody that recognizes a subunit of Na^+/K^+ ATPase enriched in the basolateral surface

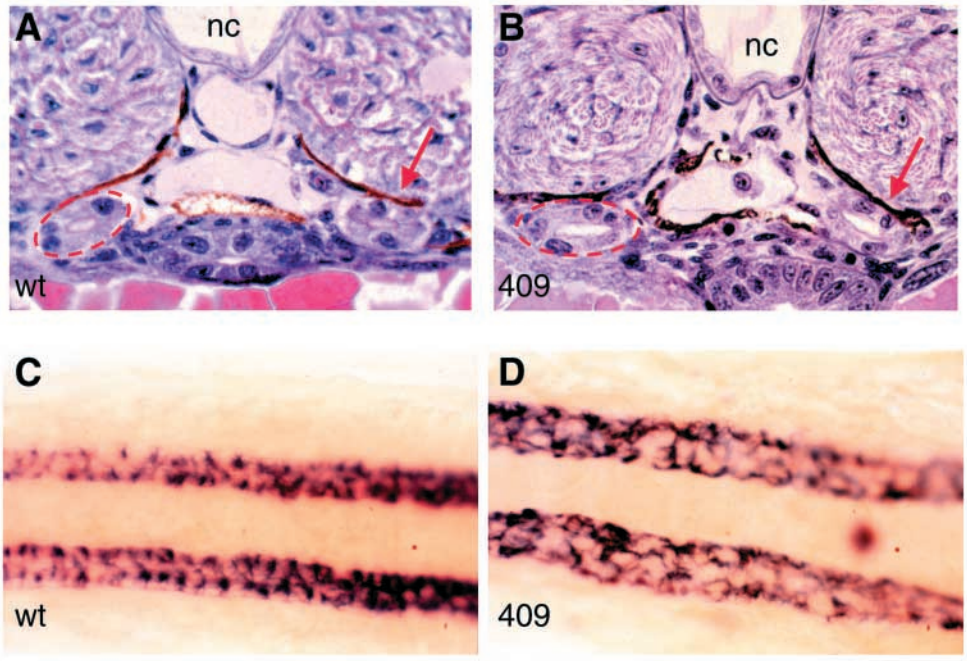

Fig. 5. Pronephric ducts. (A,B) Cross-section through pronephric ducts (arrow on the right side; encircled on the left) in wild-type (wt) and *larry*^{hi409} mutant (409) embryos. (C,D) Dorsal view of the pronephric ducts stained with α6F. nc, notochord.

of ductal and tubular cells (Drummond et al., 1998). Neither the intensity of the signal, nor the subcellular location, was significantly affected. However, the appearance of this staining also suggests that the lumen size of the pronephric ducts is increased in mutants. The size of the tubules and ducts along their lengths in these mutants also becomes variable, making it difficult to quantify the size difference.

Discussion

In an insertional mutagenesis screen that reached about 25% saturation, we identified 12 genes that can cause kidney cysts when mutated in zebrafish embryos. Two of them, *VHNF1* and *PKD2*, have been previously shown to be involved in human PKD, suggesting that cystic kidney genes isolated in zebrafish are likely to be involved in human PKD as well.

In addition to ADPKD, multiple human diseases, including ARPKD, nephronophthisis and Bardet-Biedl syndrome, show kidney cyst formation. A growing list of genes involved in these diseases have been shown to encode proteins involved in cilia formation/function (Ansley et al., 2003; Otto et al., 2003; Ward et al., 2003; Watanabe et al., 2003). A model has been proposed in which cilia are directly involved in cystogenesis (Pazour and Witman, 2003). Here, through a nonbiased forward genetic screen for cystic kidney mutants, we show that a major class of mutants involves defects in cilia formation or function. This includes three genes (*hi409*, *hi2211* and *hi3417*) whose products are part of the IFT particle that is required for cilia formation, a novel gene (*hi459/scorpion*) that is also required for ciliary assembly by an unknown mechanism, and *pkd2*, whose products are found in cilia in other organisms. This result suggests that mutations in ciliary genes are the major cause of PKD, that such genes are excellent candidate for multiple human diseases that involve kidney cyst formation, and also that the genes identified in this screen are excellent candidates for human PKD.

Cilia project from the apical surface of renal epithelial cells into the lumen of the duct and so are ideally situated to sense the environment and regulate the proliferation and differentiation of these cells. The mechanism by which this is accomplished is unknown but may involve detection of liquid flow through the lumen, by interaction with neighboring cells or by detection of chemical or ligand signals (Lubarsky and Krasnow, 2003; Nauli et al., 2003; Praetorius et al., 2003). Activation of mechanosensory channels (i.e. polycystins) on the cilium then triggers a signaling cascade that regulates cell proliferation and/or volume (Fig. 6). Defects in this pathway can lead to uncontrolled expansion of epithelial tubes and formation of cysts in local areas.

The signaling pathway connecting cilia to cell proliferation is largely unknown. Polycystin 1 and polycystin 2 have been implicated in numerous pathways including JAK-STAT, Wnt, β-catenin, protein kinase C, cAMP, G-protein and Ca^{2+} signaling pathways (Boletta and Germino, 2003). IFT genes have been implicated in hedgehog signaling (Huangfu et al., 2003) and the lack of cilia is reported to activate β-catenin signaling (Lin et al., 2003). One gene identified in this screen, *pontin*, supports a connection to β-catenin signaling. Pontin and reptin, which are components of a DNA-stimulated ATPase (Kanemaki, 1999), have been shown to associate with and regulate the transcriptional activity of a β-catenin-containing complex (Bauer, 2000; Rottbauer et al., 2002). Pontin and reptin are thought to antagonize each other's function and a gain-of-function mutation of reptin or loss-of-function mutation of pontin leads to over-proliferation of cardiomyocytes (Rottbauer et al., 2002), consistent with the possible over proliferation defects that would explain the larger lumen size of pronephric ducts of the *pontin* mutant found in this study. We know largely from analyzing mutant phenotypes that cilia might link extracellular signals to intracellular events such as cell proliferation. An exciting new frontier in this field now is to dissect the signaling network to illustrate how cilium signaling is integrated with other pathways and how a coordinated cellular response is achieved.

In addition to their role in cystogenesis, cilia also play important roles in sensory responses. It is thought that nodal cilia are involved in both generating and sensing nodal flow, thereby breaking the bilateral symmetry of the body plan (McGrath et al., 2003; Nonaka et al., 1998). The outer segments of photoreceptor cells are specialized cilia. It has been shown that *tg737* mice, in which an IFT gene is mutated, have abnormal outer segment development and retinal degeneration (Pazour et al., 2002a). In *C. elegans*, some sensory neurons are ciliated and IFT has been observed in

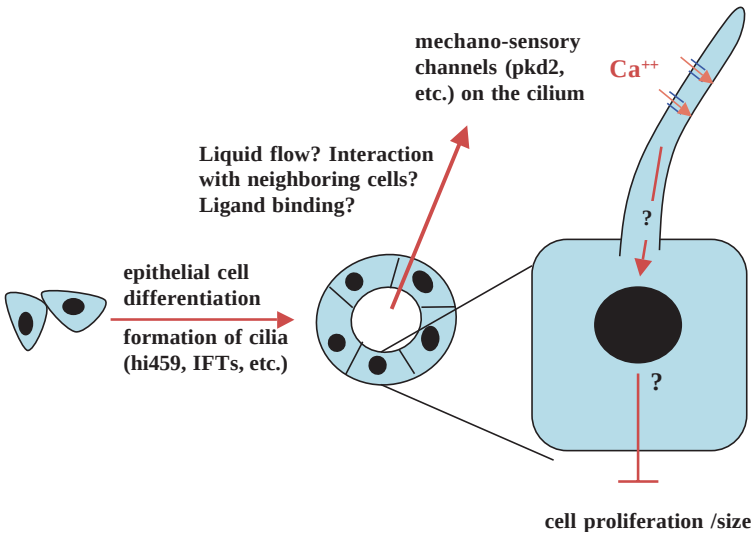

Fig. 6. A model shows the formation of cilia during the development of epithelial tubes. During epithelium differentiation, the cilium forms. The formation of the cilia requires hi459/scorpion and IFT components. The cilium protrudes into the lumen from the apical surface of epithelial cells and serves as an antenna of the cell. Mechanosensory channels (polycystins, etc.) on the cilium could be activated by liquid flow, interaction with neighboring cells or chemicals/ligands in the lumen. A resultant Ca^{2+} influx triggers a signaling cascade that regulates cell proliferation and/or size. Defects in cilia formation or function lead to uncontrolled proliferation of epithelial cells and eventually cyst formation in local areas.

chemosensory neurons (Orozco et al., 1999). In all these systems, cilia function as sensory organelles. Protruding from the cell surface, they serve as antennae of cells, albeit adapted to the diverse signals they detect. In the node, they probably sense nodal flow. In the photoreceptor cells, they sense light. In the worm, they are involved in sensing chemical signals. Given that cilia can be found on almost every type of vertebrate cell, it would not be surprising that new functions will be identified for these important but somewhat ignored organelles. As IFT genes are thought to be required for the formation or the normal functioning of cilia, the IFT mutants we identified will undoubtedly serve as powerful tools in unraveling novel functions of cilia in vertebrates.

In summary, we have identified 12 cystic kidney genes in a non-biased forward genetic screen that reached about 25% saturation, suggesting that at least 50 genes could be found by this approach. At least five out of the 10 genes we cloned are required either for cilia formation or function. Because of the similarity between cyst formation in zebrafish and human, we predict that a majority of human PKD genes are involved in cilia formation and/or function and that novel genes identified here are excellent candidates for human diseases that involve kidney cyst formation.

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Note added in proof

We have since cloned *hi1392/twister*. It encodes a novel protein. The Accession number is AY618927.

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