Polaris, a protein disrupted in orpk mutant mice, is required for assembly of renal cillum

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Yoder, Bradley K., Albert Tousson, Leigh Milican, John H. Wu, Charles E. Bugg, Jr., James A. Schafer, and Daniel F. Balkovetz. Polaris, a protein disrupted in orpk mutant mice, is required for assembly of renal cillum. Am J Physiol Renal Physiol 282: F541–F552, 2002; 10.1152/ajprenal.00273.2001.—Cilia are organelles that play diverse roles, from fluid movement to sensory reception. Polaris, a protein associated with cystic kidney disease in Tg737°orpk mice, functions in a ciliogenic pathway. Here, we explore the role of polaris in primary cilia on Madin-Darby canine kidney cells. The results indicate that polaris localization and solubility change dramatically during cilia formation. These changes correlate with the formation of basal bodies and large protein rafts at the apical surface of the epithelia. A cortical collecting duct cell line has been derived from mice with a mutation in the Tg737 gene. These cells do not develop normal cilia, which can be corrected by reexpression of the wild-type Tg737 gene. These data suggest that the primary cilia are important for normal renal function and/or development and that the ciliary defect may be a contributing factor to the cystic disease in Tg737°orpk mice. Further characterization of these cells will be important in elucidating the physiological role of renal cilia and in determining their relationship to cystic disease.

Tg737: ciliogenesis; cell line; polycystic kidney disease; Madin-Darby canine kidney cells; Oak Ridge Polycystic Kidney

Cilia and flagella are complex organelles found throughout the animal kingdom, within protozoa, and in some plants, where they perform a broad range of functions (5, 35, 54). Cilia on epithelial cells in the lung or ependymal cells lining the ventricles of the brain are motile and function in the movement of mucus and cerebral spinal fluid, respectively. Motile cilia on the surface of the node, an important early embryonic-stage organizing center, play a critical role in specification of left-right body axis during development (34, 36, 50). In addition, there are primary nonmotile cilia in many cells in the nephron, the bile and pancreatic ducts, on retinal rods in the eye, and on olfactory neurons (3, 8, 32, 43, 53). Although the specialized cilia on the rods and olfactory neurons are involved in reception of extracellular stimuli, the role of the primary nonmotile cilia in the kidney, pancreas, and liver is unknown and has been relatively ignored. The nearly ubiquitous nature (http://www.wadsworth.org/BMS/SCBlinks/cilia1.html) of these solitary cilia has led some to propose that they are vestigial organelles of little consequence to normal tissue function (14, 53). However, recent data have suggested that aberrant formation of cilia on cells in the murine kidney can result in cystic kidney disease (37, 51).

The formation of cysts in the kidney is a pathological entity common to a number of inherited and acquired diseases (16). Of these cystic disorders, polycystic kidney disease (PKD) is the most common, most extensively studied, and one of the leading causes of end-stage renal failure in humans. Most cases of the dominant form of human PKD (ADPKD) arise from mutations in either of two genes, PKD1 and PKD2 (8a, 13a, 22a). The gene responsible for the less common, recessive form of human PKD (ARPKD) has yet to be cloned. Pathological findings in PKD include the formation of epithelia-lined cysts throughout the nephron in ADPKD and predominantly in the collecting duct in ARPKD, changes in extracellular matrix composition, improper epithelial cell differentiation, and alterations in cell polarity. In addition to the lesions in the kidney, abnormalities are often found in other tissues, including the liver and pancreas (30, 31).

The considerable morbidity and mortality resulting from cystic kidney diseases in humans have prompted intense investigative efforts to identify the molecular mechanisms involved in cystogenesis and cystic disease progression. A good deal of knowledge about human PKD has come from the availability of numerous mouse models (4, 6, 19, 33). These models exhibit similar pathology to human PKD with regard to cyst localization, epithelial polarity defects, and extrarenal involvement. Pkd1 and Pkd2 mutant mice have been engineered, and these mice are now being used to

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study the human disease and the function of their respective protein products (28, 55). In addition to the Pkd1 and Pkd2 mice, several interesting mouse models for PKD have arisen spontaneously, through chemical mutagenesis, and through transgenic insertional mutagenesis. Two of the best-studied models are the Oak Ridge Polycystic Kidney disease (orpk; Tg737<sup>orph</sup>) and the congenital polycystic kidney disease (cpk) mutants (6, 33). Both orpk and cpk mice develop renal cysts along with hepatic and pancreatic abnormalities (17, 20, 33).

There are several lines of evidence suggesting a connection between PKD and cilia. First, polaris, the protein associated with the cystic lesions in Tg737<sup>orph</sup> mice, localizes to the basal body and cilia axoneme (51). Because of the defect in polaris function, the cilia are aberrantly formed in orpk mutant mice (37, 51). Studies of the polaris homologues in Caenorhabditis elegans and Chlamydomonas indicate that the homologues function as a component of the intraflagellar transport (IFT) system (21, 37, 41). IFT is a process that describes the kinesin- and dynein-mediated movement of large protein rafts, of which polaris is a component, along the axoneme of cilia and flagella (25). An additional connection between cilia and PKD is seen in cystic lesion of the cpk mouse. A candidate gene responsible for the cpk phenotype has recently been cloned (Guay-Woodford LM, unpublished observations). Although the function of cystin, the protein encoded by the cpk gene, remains to be determined, exogenously expressed epitope-tagged cystin is detected in the ciliary axoneme. Finally, although the function and subcellular localization of mammalian polycystins remain somewhat controversial, it is intriguing that the C. elegans homologues of polycystin-1 (lov-1) and polycystin-2 (pdk2) are in cilia on a subgroup of sensory neurons that also express the polaris homologue (osm-5) (7, 21, 41). In contrast to osm-5 mutants, cilia appear normal in lov-1 and pdk-2 mutant worms. However, lov-1 and pdk-2 mutant worms do exhibit sensory defects associated with worm mating behavior that are similar to those in the osm-5 mutants, suggesting that male lov-1 and pdk-2 mutants have lost their cilium-mediated sensory function (7).

This potential connection between cilia and PKD is intriguing in light of our limited knowledge of the role of the primary cilium found on epithelial cells lining the nephron and collecting duct. Elucidating the function of primary cilia and their association with PKD will require detailed characterization of proteins involved in both ciliogenesis and cilia function, plus the development of reagents and assays to test their role in renal physiology. In this regard, we describe the generation of an SV40 conditionally immortalized cortical collecting duct cell line from orpk mice. Although these cells develop overtly normal basal bodies as the initiating step in ciliogenesis, they subsequently fail to assemble the cilia axoneme. This ciliary defect can be corrected by reexpressing wild-type polaris. We also describe basic biochemical properties of polaris during cilia assembly and analyze the effect of microtubule destabilization on polaris localization. Further functional characterization of these cells will be important in dissecting the signaling events mediated through renal cilia and how disruption of this organelle can result in PKD phenotype in mice.

**MATERIALS AND METHODS**

**Mice.** The generation and genotyping of the Tg737<sup>orph</sup> mutant lines have been described previously (33, 57). Heterozygous orpk female mice were bred with male mice homozygous for the ImmortalMouse transgene (H-2K<sup>-</sup>-tsA58; Charles River Laboratories, Wilmington, MA), and the resulting offspring were genotyped from tail biopsies. Mice that were orpk/ImmortalMouse compound heterozygotes were obtained and crossed to generate orpk homozygous mutants that carry the H-2K<sup>-</sup>-tsA58 transgene (ImmortalMouse). All mice were maintained at the University of Alabama School of Medicine in accordance with National Institutes of Health guidelines.

**Madin-Darby canine kidney cell culture, transfection of Madin-Darby canine kidney T23 cells, and inducible polaris expression.** Type II Madin-Darby canine kidney (MDCK) or transfected MDCK-T23 cells (tetracycline-off cell line; provided by K. Mostov and Y. Altschuler) were used between passages 3 and 15. Cells were cultured in MEM containing Earle's balanced salt solution supplemented with 5% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.25 µg/ml amphotericin B at 37°C and 5% CO<sub>2</sub>. MDCK-T23 cells were cotransfected with a hygromycin selection maker, and the T7-epitope-tagged polaris was cloned into the pBl-G vector (Clontech, Palo Alto, CA) or empty pBl-G vector using the calcium phosphate precipitation method. Drug-resistant clones were selected, and clonally derived lines were established in the presence of 300 mg/ml hygromycin. Expression from the pBl-G vector was repressed by addition of 20 ng/ml doxycycline (Sigma, St. Louis, MO) to the culture medium.

**Antibodies.** The generation of antisera against polaris (GN593 and GN594) and confirmation of their specificity were described previously (51). Other antibodies used include α-β-tubulin (Biogenex, Mu178-UC), α-E-cadherin (Transduction Laboratories, Lexington, KY), α-ZO (zonal occludin)-1 antibody (obtained from B. Stevenson, Univ. of Alberta) (48), α-SV40 large T antigen (Oncogene Sciences, Uniondale, NY), α-T7 (Novagen, Madison, WI), α-pan-cytokeratin (AE1/AE3; Chemicon International, Temecula, CA), and α-vimentin (3B4; DAKO, Carpinteria, CA). The secondary antibodies used include goat anti-mouse IgG-Oregon green (Molecular Probes, Eugene, OR), goat anti-mouse IgG-FITC (Jackson ImmunoResearch Laboratories, West Grove, PA), goat anti-rabbit-Texas red (TxR; Jackson ImmunoResearch Laboratories), goat anti-rat-TxR (Jackson ImmunoResearch Laboratories), donkey anti-rabbit IgG-tetramethylrhodamine isothiocyanate (Jackson ImmunoResearch Laboratories), goat anti-rabbit horseradish peroxidase (HRP; Bio-Rad, Hercules, CA), and goat anti-mouse HRP (Bio-Rad). In all cases, antibody controls were evaluated to ensure that the results obtained were not a consequence of the secondary antibodies used.

**Time course of polaris solubility and localization to MDCK cilia.** Type II MDCK cells were plated at confluence and grown on 6- and 24-mm Transwell filters (Costar, Cambridge, MA) for 6, 24, 48, 72, and 96 h. For immunofluorescent localization of polaris, 6-mm filters were used. For cell lystate preparation, cells on 24-mm filters were rinsed twice with Dulbecco's phosphate-buffered saline containing Mg<sup>2+</sup> and Ca<sup>2+</sup> (PBS+) at 4°C. Cells were solubilized in 50 µl of
50 mM NaCl, 10 mM PIPES, pH 6.8, 3 mM MgCl₂, 0.5% Triton X-100 (Triton-X), and 300 mM sucrose (CSK buffer) containing protease inhibitors [2 mM phenylmethylsulfonyl fluoride (PMSF), 50 µg/ml pepstatin, 50 µg/ml chymostatin and 10 µg/ml antipain] for 20 min at 4°C. The cells were scraped from the filter and sedimented at 4°C in a microcentrifuge. The soluble and pellet fractions were collected. The pellet was resuspended in 100 µl of 15 mM Tris (pH 7.5), 5 mM EDTA, 2.5 mM EGTA, and 1% SDS, heated at 95°C for 20 min, diluted to 0.5 ml with CSK buffer, and centrifuged for 10 min at 4°C at maximum speed. The resulting supernatant was considered the Triton-X-insoluble fraction. Protein concentration of the fractions was determined using a BCA kit (Pierce, Rockford, IL).

Fluorescent labeling of proteins in MDCK cells. Cells on Transwell filters were fixed in 4% paraformaldehyde (PFA) in PBS for 20 min. After filters were washed three times with PBS+, the cells were quenched with 75 mM NH₄Cl and 20 mM glycine, pH 8.0, with KOH (quench solution) for 10 min at room temperature. Filters were washed once with PBS+ and permeabilized with PBS+ containing 0.025% saponin (PFS) for 15 min at 37°C. Cells were then probed with antibodies against polaris (rabbit polyclonal antibody), ZO-1 (rat monoclonal antibody), or β-tubulin (mouse monoclonal antibody) diluted in PFS at 1:250, 1:2, or 1:50, respectively, for 1 h at 37°C. Filters were washed with PFS at room temperature and then probed with FITC (polaris)- or Texas Red (ZO-1 and β-tubulin)-conjugated secondary antibodies (Jackson Immunoresearch Laboratories) diluted 1:100 in PBS for 1 h at 37°C. Filters were washed four times for 5 min each with PFS, once with PBS+, twice with PBS+ containing 0.1% TX-100, and once with PBS+. Cells were postfixed in 4% PFA for 15 min at room temperature, cut from the support with a scalpel, and mounted in Vectashield mounting medium (Burlingame, CA).

Conventional and confocal laser scanning microscopy of transfected MDCK cells. Fluorescently labeled MDCK cell samples were analyzed using either a Leica fluorescence microscope equipped with a Hamamatsu C9810 digital camera or a Leica confocal laser scanning microscope system configured with both an argon ion (5 mW, 488 nm) and a krypton ion (10 mW, 568 nm) laser. The captured photomicrographs were labeled using Adobe Photoshop.

Nocodazole treatment of cells. Stocks of nocodazole (10 mg/ml) were prepared in dimethylsulfoxide and stored in single-use aliquots at −20°C. Just before use, the nocodazole was diluted into the appropriate medium at a final concentration of 10 µg/ml (33 µM). Cells were incubated in medium supplemented with 33 µM nocodazole for 4 h at 4°C as described elsewhere (18). No effect of exposing the cells to 4°C treatment in the absence of nocodazole was evident in control samples.

Generation of orpk mutant cortical collecting duct cells. Cortical collecting duct segments were isolated from collageenase-treated slices of renal cortex of 21-day-old orpk mutant mice that were heterozygous for the Immortomouse transgene (H-2k-tsA58) using a procedure as described previously (44). Briefly, the capsule of the kidney was removed and 1-mm sections were cut using a Stadie-Riggs handheld microtome. The sections were examined to ensure they contained no trace of medullary tissues. The slice was incubated in DMEM containing 0.1 g/dl collagenase, 5 mM glycine, 50 U/ml DNase, and 50 µg/ml soybean trypsin inhibitor. Cortical collecting duct tubule segments were selected and transferred to individual wells in 24-well culture dishes. To establish the lines, cells were grown from the tubules under permissive conditions for SV40 large-T antigen expression (33°C, 10 U/ml interferon-γ) in CD media (DMEM/F12, 10% FBS, 1.3 µg/l sodium selenite, 1.5 µg/l 3,3’-triiodothyronine, 5 mg/l insulin, 5 mg/l transferrin, 2.5 mM glutamine, 5 µM dexamethasone, 100 U/ml penicillin, 100 µg/ml streptomycin, 5% CO₂). Once the cortical collecting duct cells were established, clonal lines were derived from individual cells. The data presented here were obtained with the 94D series, and similar results were obtained with other lines derived from these mice. To promote differentiation and SV40 large-T antigen inactivation, cells were cultured at nonpermissive conditions (39°C in the absence of interferon-γ for 3 days before the analysis).

Transfection of orpk mutant cortical collecting duct cells. The construction of the Tg737 expression construct (Tg737Bap) and transfection of the 94D cells were accomplished using Lipofectamine Plus according to the manufacturer’s protocol (Life Technologies Gibco BRL, Carlsbad, CA). Stable cell lines were generated by drug selection using 400 µg/ml G418, and several clonal derived lines were obtained. Expression from the Tg737Bap construct was evaluated on Western blots for orpk alone to ensure that selection in G418 did not alter the properties of the cells.

Fluorescent labeling of proteins in 94D cells. For immunofluorescence analysis in 94D cells, cells were fixed in 4% PFA, 0.1% Triton-X in PBS for 10 min and incubated in blocking buffer (1% BSA in PBS) for 30 min at room temperature. Blocked cells were probed with rabbit anti-polaris (1:200), rat monoclonal anti-ZO-1 (1:2), or mouse monoclonal anti-β-tubulin (1:200) diluted in blocking buffer for 1 h at room temperature. After being washed three times with PBS, cells were then immunoprobed with fluorescein-conjugated secondary antibodies. The secondary antibodies used in the analysis of 94D cells included goat anti-rabbit-TxR, goat anti-mouse IgG-HRP, and donkey anti-rabbit IgG-tetramethylrhodamine isothiocyanate (all 3 obtained from Jackson Immunoresearch Laboratories). Secondary antibodies were diluted 1:200 in blocking buffer. Cells were then washed three times in PBS and mounted onto slides, and the images were captured on an inverted fluorescent microscope. Nuclei were stained for 5 min using Hoechst 33528 (Sigma) diluted 1:1,000 in PBS.

Electrophoresis and Western blot analysis. Protein was isolated from cells or tissue in RIPA buffer, and the protein concentration was quantified using a DC protein assay kit (Bio-Rad, Hercules, CA) as described by the manufacturer. Equal amounts of protein were resolved by electrophoresis on SDS-PAGE gels, and the proteins were transferred to nitrocellulose (NitroBind, MSI) or Immobilon P filters (Millipore, Bedford, MA). Filters were blocked in 5% dry milk containing 0.1% Tween 20 in PBS and immunoprobed with mouse monoclonal antibody against E-cadherin (Transduction Laboratories) diluted 1:1,000, rabbit polyclonal against polaris (GN593) diluted 1:250, or mouse monoclonal anti-T7 antibody (Novagen) diluted 1:5,000 in blocking buffer. The filters were washed with PBS containing 0.1% Tween 20 and probed with goat anti-mouse IgG HRP (Bio-Rad) diluted 1:5,000 (for E-cadherin) or goat-anti-rabbit IgG HRP diluted 1:5,000 (for polaris) in block solution for 1 h. Filters were washed five times with PBS containing 0.1% Tween 20. The HRP signal was detected using a Supersignal West Femto chemiluminescence kit (Pierce) or an enhanced chemiluminescence kit (ECL; Amersham).
Northern blot analysis. Total RNA was isolated from nonciliated, nonconfluent, proliferating, or ciliated, confluent, nonproliferating MDCK cells using Trizol as described by the manufacturer (GIBCO BRL) and enriched for polyadenylated RNA by passage using oligo-dT columns. One microgram of PolyA+ RNA from MDCK cells or 2 μg of poly A+ RNA from mouse kidney were resolved by denaturing agarose gel electrophoresis, transferred to charged nitrocellulose membranes, and hybridized with mouse Tg737 cDNA or chick tubulin labeled with [α-32P]deoxyctydine 5'-triphosphate (dCTP) generated by the random hexamer method.

Fixation and scanning electron microscopic analysis. For analysis of cilia on 94D cells using scanning electron microscopy (SEM), cells were fixed in SEM grade 2.5% glutaraldehyde (Electron Microscopy Sciences, Ft. Washington, PA) in 0.1 M cacodylate buffer (pH 7.4) for 90 min, washed in cacodylate buffer, postfixed in 1% OsO4 in cacodylate, and washed twice with 0.1 M cacodylate buffer. Fixed cells were then dehydrated through a series of ethanol washes (30, 50, 70, 80, 90, 95, and 100% ethanol-cacodylate buffer) for 5 min each and then through hexamethyldisilasane (25, 33, 58, 66, 70, 80, 90, 95, and 100% ethanol-cacodylate buffer) for 5 min each. Samples were desiccated under vacuum overnight, sputter coated (50-s gold deposition), and examined using an Hitachi 7000 SEM at ×20,000.

RESULTS

Expression of polaris in MDCK cells during ciliogenesis. In mice, Tg737 encodes a 3.2-kb transcript that is expressed at low levels with a wide tissue distribution (33, 59). Data from mice and humans suggest that Tg737 is a complex gene that also encodes several low-level, alternatively spliced transcripts and protein products. The prominent 95-kDa protein encoded by the 3.2-kb Tg737 mRNA in mice is called polaris (51). In addition to this 95-kDa protein, several other protein bands are often detected. Importantly, expression of both proteins is abolished in the Tg737Δ2-3 mice, suggesting they are polaris isoforms and confirming the specificity of the affinity-purified antibodies.

To begin exploring the role of polaris in renal epithelia, we analyzed the expression of the Tg737 mRNA and its protein product in MDCK cells during ciliation. In contrast to the 3.2-kb transcript detected in the mouse, Tg737 is expressed as a 2.8-kb transcript in MDCK cells (Fig. 1A). Analysis of this transcript reveals that the difference in size is due to the presence of a larger 3'-untranslated region in the mouse gene (data not shown). Similar to the Western blot results from murine samples, we detected multiple polaris proteins in MDCK extracts, ranging in size from roughly 95 to 118 kDa (Fig. 1B). A comparison of expression levels of the Tg737 mRNA and protein in nonciliated and ciliated MDCK cells indicates that Tg737 expression is not significantly altered during ciliogenesis. The presence or absence of cilia on these cells was confirmed by immunofluorescence analysis using anti-β-tubulin.

Localization and biochemical properties of polaris. To begin analyzing polaris properties during ciliogenesis, we plated cells at confluence on filter inserts. Triton-X-extractable and nonextractable proteins were isolated at different time points through the formation of cilia. The partitioning of polaris into either the Triton-X-soluble or -insoluble pools was analyzed on Western blots using anti-polaris antisera similar to studies conducted for E-cadherin during adherens junction formation (1). The results indicated that polaris was completely Triton-X extractable for the first 24 h after plating (Fig. 2A). However, at 48 h a small fraction of polaris protein enters the Triton-X-nonextractable pool. The amount of insoluble polaris increased through the 72-h time point. The only polaris isoform detected in the insoluble pool was the 95-kDa form of the protein. In contrast to polaris, E-cadherin was detected in the insoluble fraction within 6 h of plating (Fig. 2A). The change in E-cadherin solubility is a consequence of its association with the cytoskeleton as an adherens junction form (1, 22, 39). Similarly, we predict that the change in polaris solubility is reflective of either cytoskeletal attachment during cilia formation or its incorporation into large insoluble structures such as the IFT raft.

To correlate the changes in polaris solubility with epithelial polarization and cilia formation, we analyzed the subcellular distribution of the tight junction marker ZO-1, polaris, and β-tubulin on parallel sets of filters during the Triton-X-solubility experiment (Fig. 2B). In agreement with the early stages of polarization shown by the insolubility profile for E-cadherin, ZO-1 was detected at the lateral membrane by immunofluores-
cence within 6 h of plating. In contrast to ZO-1, polaris was detected throughout the cytoplasm as small dots for at least 24 h after plating, polaris was completely extractable for at least 24 h. The small isoform of polaris began entering the insoluble fraction at 48 h. The level of the small insoluble isoform further increased at 72 h, suggesting that it may be attaching to the cytoskeletal network. By 48 h, polaris begins to concentrate in focal spots (arrowheads) at the apical surface of the polarized epithelia. The number of cells exhibiting this focal spot of polaris increased at 72 h. By 96 h, all of the polarized cells exhibited a centrally located polaris-containing structure that extends off the apical surface of the epithelium. To confirm that these focal spots were basal bodies, cells were immunoprobed with anti-β-tubulin (red) and anti-polaris (green, 96, far right) after 96 h of culture. β-Tubulin-positive cilia (red) extend from each polaris-positive structure (green), confirming that polaris is located in or near the basal bodies at the base of individual cilia.

![Fig. 2](http://ajprenal.physiology.org/)  

Fig. 2. Polaris biochemical properties and localization change during cilia formation in MDCK cells. A: cells were plated on filters for the indicated time. The Triton X-100 (Triton-X) solubility of polaris and E-cadherin (E-Cad) were analyzed on Western blots. Although E-cadherin was found in the insoluble pool as early as 6 h after plating, polaris was completely extractable for at least 24 h. The small isoform of polaris began entering the insoluble fraction at 48 h. The level of the small insoluble isoform further increased at 72 h, suggesting that it may be attaching to the cytoskeletal network. B: to correlate the change in solubility of polaris with epithelial polarization and cilia formation, cells were plated at near confluence on filters for 6, 24, 48, and 72 h and then immunoprobed with anti-polaris (green) and anti-zonal occludin-1 (ZO-1; red) antibodies (6, 24, 48, 72, and 96 h). polaris is initially observed in punctate dots throughout the cytoplasm up until 24 h after plating. At 48 h, polaris begins to extend off the apical surface of the epithelium. To confirm that these focal spots were basal bodies, cells were immunoprobed with anti-β-tubulin (red) and anti-polaris (green, 96, far right) after 96 h of culture. β-Tubulin-positive cilia (red) extend from each polaris-positive structure (green), confirming that polaris is located in or near the basal bodies at the base of individual cilia.

![Polaris and Renal Cilia](http://ajprenal.physiology.org/)

Inducible ectopic overexpression of polaris. To further explore the role of polaris in ciliogenesis, we utilized a doxycycline-repressible expression system in T23 MDCK cells (Tet-off line, Clontech) to test whether high levels of polaris expression can saturate the IFT process and block ciliogenesis. We anticipated that these cells could then serve as an inducible model system for analyzing cilia function on the surface of a well-characterized renal epithelia cell. These cells were stably transfected with a doxycycline-responsive Tg737 construct (Tg737pBI-G, Clontech). This construct encodes a T7 epitope-tagged polaris protein that allows us to distinguish between endogenous and exogenous expression. Clonal cell lines were generated, and inducible polaris expression was analyzed on Western blots using anti-polaris and anti-T7 antibodies under repressed and nonrepressed conditions. A line was

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that do not occur with the murine forms likely arise due to alternative splicing events expressed protein suggests that the other polaris isoforms are slightly larger than 95 kDa. High levels of exogenous expression were detected as 3 bands, ranging in size from 95 to 118 kDa. High levels of exogenous polaris expression were detected in nonrepressed cells with both T7 and anti-polaris antibodies. The size of the exogenously expressed protein containing the epitope tag suggests that it represents the smallest isoform (95 kDa) of polaris. No expression from the doxycycline-regulated construct was seen on removal of doxycycline but remained repressed in its background staining in nontransfected cells.

Fig. 3. Exogenous overexpression of polaris does not induce or inhibit cilia assembly. A: inducible overexpression of polaris was analyzed on Western blots containing equal amounts of protein isolated from repressed (+ doxycycline (Dox)) or nonrepressed (− doxycycline) T23 MDCK cells. The cells were stably transfected with vector only (control) or with the Tg737pBI-G doxycycline-regulated construct (T7-Tg737). Endogenous and exogenous expression were detected using anti-polaris (A) and anti-T7 (B) antisera, respectively. Endogenous expression was detected as 3 bands, ranging in size from 95 to 118 kDa. High levels of exogenous polaris expression were detected in nonrepressed cells with both T7 and anti-polaris antibodies. The size of the exogenously expressed protein containing the epitope tag suggests that it represents the smallest isoform (95 kDa) of polaris. No expression from the doxycycline-regulated construct was seen under repressed conditions. B: to determine whether the high levels of polaris expression could promote ectopic cilia or saturate the intraflagellar transport (IFT) process and inhibit cilia formation, polarized cells were analyzed by immunofluorescence using antipolaris (green) and anti-ZO1 (red). A single primary cilium with normal axoneme length (2–4 μm) was detected, even on cells where polaris expression was extremely elevated (arrowhead). Immunofluorescence analysis using T7 was not feasible due to high nuclear background staining in nontransfected cells.

identified that induced high levels of polaris expression on removal of doxycycline but remained repressed in its presence (Fig. 3A). Both T7 and anti-polaris antiserum recognize the exogenously expressed protein; however, unlike endogenous canine polaris, only a single band at slightly larger than 95 kDa was detected with the T7 antibody. The slight increase in size is due to the presence of the T7 epitope tag. Thus the exogenously expressed protein of murine origin likely correlates with the lower 95-kDa form of polaris that is detected in the insoluble fraction during basal body formation. The fact that we see a single band with exogenously expressed protein suggests that the other polaris isoforms likely arise due to alternative splicing events that do not occur with the murine Tg737 cDNA cloned into the expression vector and not by posttranslational modification.

To determine whether the high-level exogenous expression of polaris had any consequence on the formation of cilia, we performed immunofluorescence analysis on well-polarized cells using the anti-polaris antibodies. The results show that high levels of polaris overexpression in MDCK cells did not have any overt inhibitory effects on ciliogenesis nor did they cause ectopic cilia formation or increased cilia length (Fig. 3B and data not shown). A single cilia axoneme, ranging in length from 2 to 4 μm, was evident on nearly all the cells, even those cells that massively overexpress polaris. The size of the cilia on these overexpressing cells was in agreement with nontransfected control line. Attempts to use the T7 antibody to detect murine polaris in MDCK cilia were not successful due to intense background staining of all the nuclei in repressed conditions as well as in nontransfected cells.

The effect of nocodazole treatment on polaris localization and cilia. IFT is a process originally identified in Chlamydomonas that describes the movement of large-protein rafts within the cilial axoneme (25). Microtubule-based motor proteins direct the movement of the IFT rafts along microtubule filaments in both anterograde and retrograde directions (24, 38, 47). Thus the microtubular network is likely to play a critical role in cilia axoneme extension, motility, and stabilization. To begin assessing the importance of intact microtubules in cilia maintenance and polaris localization, we treated polarized MDCK cells with nocodazole for up to 4 h to disrupt the microtubule filaments. After nocodazole treatment, the localization and Triton-X solubility of polaris were determined in three independent samples by immunofluorescence and Western blot analysis (Fig. 4A). Nocodazole treatment resulted in a substan-
tial reduction in the expression of the highest molecular mass (~118 kDa) polaris isoform; however, the level of the two smaller isoforms (95 and 100 kDa) remained nearly constant. In addition, the 95-kDa isoform was still detected in the Triton-X-insoluble pool. Because nocodazole did not alter the solubility of the 95-kDa isoform, we analyzed whether it had any effect on polaris localization. By immunofluorescence analysis, polaris was still detected in the ciliary axoneme and basal bodies of the nocodazole-treated cells (Fig. 4B). In contrast, β-tubulin was absent (Fig. 4B). β-Tubulin staining in cilia was not detected even after extended exposure during image capture. In non-nocodazole-treated cells, β-tubulin staining and cilia were evident on almost all cells in the monolayer.

Generation and characterization of orpk cortical collecting duct cell lines. Because we were unable to inhibit or induce excess ciliogenesis by overexpression of polaris in MDCK cells to study renal cilia function, we generated several conditionally immortalized cortical collecting duct cell lines from orpk mutants using the ImmortoMouse (see MATERIALS AND METHODS) (23). Clonal cell lines were established from single cells. Results similar to those described here for the 94D line were obtained for other cell lines derived from these mice. The 94D cells exhibit a cobblestone appearance typical of epithelial cells under both permissive and nonpermissive conditions (Fig. 5A). Western blot analysis indicates that these cells express epithelial markers such as cytokeratins, whereas fibroblast markers such as vimentin were barely detectable (Fig. 5B). These cells form adherens and tight junctions as determined by E-cadherin and ZO-1 expression detected by Western blot analysis and immunofluorescence (Fig. 5, C and D, and data not shown). Bioelectrical measurements indicate that both the mutant and the rescued cell lines form tight monolayers with transepithelial resistance measurements of 12–18 kΩ/cm² after 3 days postconfluence on Transwell filters. The temporal loss of the SV40 large T antigen under nonpermissive conditions was also evaluated by Western blot analysis. The results indicate that expression of the SV40 large T antigen was reduced within 3–4 days after the shift to the nonpermissive condition as shown for other renal cell lines derived using the ImmortoMouse (data not shown) (49).

To explore the role of polaris in the cilia of these cortical collecting duct cells, we stably transfected the 94D clonal line with a wild-type Tg737 cDNA, the expression of which is under the regulatory control of the human β-actin promoter (Tg737Bap) (56, 57). The Tg737Bap construct encodes a functional polaris pro-
tein, as evidenced in previous transgenic experiments whereby it was able to rescue the renal pathology in orpk mutants when expressed as a transgene. Expression of polaris in the rescued 94D cells was confirmed on Western blots using the α-polaris (GN593) antisera (Fig. 6) (51). Two lines (94D-Tg737Bap-1 and 94D-Tg737Bap-2) were chosen for follow-up studies because of their low-level or near-endogenous expression, respectively.

Rescue of ciliary defects in 94D cells. In vivo studies have demonstrated that cilia are malformed on ependymal cells in the brain and on the renal collecting ducts in orpk mutant mice (37, 51). To assess whether a similar defect was occurring in vitro in our cell culture model, we plated rescued (94D-Tg737Bap-1 and 94D-Tg737Bap-2) and mutant 94D-pcDNA cells at confluence on permeable filters under nonpermissive conditions for 3 days. The effect of reexpressing wild-type polaris on cilia formation and morphology was analyzed by immunofluorescence using anti-β-tubulin antisera and by scanning electron microscopy (Fig. 7, A–C). Using either of these assays, it was evident that cilia formation was dramatically inhibited in cells lacking polaris, whereas most cells in the rescued cultures exhibited overtly normal cilia. Measurements of cilia length indicate that the cilia on rescued cells are normally ~3–4 μm long (Fig. 7B). Similar to what is seen in vivo in orpk mice, small rudimentary cilia or small extensions from the basal body structures were detected on the mutant cells in these cultures (Fig. 7, A–C) (37). The cilia on mutant cells were normally <1 μm in length, and they often exhibited bulging of the axonemal membrane (Fig. 7, B and C). Nearly identical results were obtained with either 94D-Tg737Bap-1 or 94D-Tg737Bap-2 cells; however, in general more cilia were found in the 94D-Tg737Bap-2 cultures (data not shown). This may reflect the higher levels of polaris expression in the 94D-Tg737Bap-2 line relative to the 94D-Tg737Bap-1 line.

DISCUSSION

In mice, mutations in Tg737 result in a complex series of phenotypes. The hypomorphic allele in the Tg737<sup>−/−</sup> orpk mutants results in cystic kidney disease, pancreatic and bile duct hyperplasia, hydrocephalus, and skeletal patterning defects (33, 42, 58). In contrast, the complete loss of Tg737 in the Tg737<sup>−/−</sup>-<b>Bgal</b> null mutants results in midgestation lethality (~embryonic day 9.5). Null mutants exhibit random patterning of the left-right body axis, neural tube defects, pericardial sac expansion, and enlarged limb buds (34). Polaris, the protein encoded by Tg737, is highly conserved across ciliated eukaryotes (21). Recent analysis of this protein has revealed that its function is required for normal cilia and flagella assembly. Ciliary defects have now been observed in both the hypomorphic and the null Tg737 mutant mice and in lower eukaryotes with mutations in the Tg737 homologues (21, 34, 37, 41, 51).

To begin characterizing the possible association between the cilia and the cystic kidney disease phenotype in Tg737<sup>−/−</sup> orpk mice, we initiated a study to analyze polaris expression and localization during ciliogenesis in renal epithelium using MDCK cells as a model system. The levels of Tg737 mRNA and polaris expression remained constant in nonciliated and ciliated MDCK cells, indicating that polaris expression is not altered by induction of cilia formation. Similar to what was seen in protein extracts from mice, canine polaris was detected as multiple bands on Western blots (51). It is unlikely that these additional proteins are a consequence of cross-reactivity of the affinity-purified antisera because these proteins are not detected on Western blots of protein isolated from Tg737 null mice (Tg737<sup>−/−</sup>-<b>Bgal</b>) (34, 51). They may represent differentially modified polaris isoforms; however, the fact that only a single product is seen in cells exogenously expressing murine polaris argues against this possibility. It seems more likely that these protein species are derived from alternatively spliced Tg737 transcripts. In mice, as many as five Tg737 mRNAs, ranging in size from 2.6 to >7 kb, have been observed (33, 51, 58). Similarly, longer exposure of the Northern blot of RNA from MDCK cells also reveals the presence of several less abundant transcripts; whether these transcripts are derived from the Tg737 gene remains to be determined.

We further characterized polaris by analyzing changes in its Triton-X extractability during cilia formation. The results indicate that all three of the polaris isoforms are restricted to the soluble fraction for at least 24 h after cells are plated at confluence. However, after 48 h in culture, a fraction of the 95-kDa form of polaris was detected in the insoluble pool. Comparing the solubility results with immunofluorescence data indicates that this shift in solubility is coincident with basal body formation. Similar solubility results have been obtained with β-catenin, α-catenin, and E-
cadherin as the adherens junction complex forms (1, 2, 39). The partitioning of the adherens junction proteins into the insoluble fraction is a result of their attachment to the actin-based cytoskeleton. Rather than cytoskeletal attachment, we predict that polaris insolubility is a consequence of its incorporation into the IFT rafts that assemble near the basal bodies early in ciliogenesis (9).

To evaluate whether maintenance of polaris insolubility and localization could involve microtubule filaments such as found in the cilia axoneme, we treated MDCK cells with nocodazole. Although nocodazole reduced the level of the 118-kDa-molecular-mass polaris isoform, the level of the 95- and 100-kDa isoforms remained constant. In addition, the 95-kDa protein was still detected in the insoluble fraction. Immunofluorescence results indicate that microtubule depolymerization did not alter polaris localization in the ciliary axoneme or basal bodies of treated cells. In contrast, β-tubulin could not be detected in the cilia of the nocodazole-treated cells. Thus microtubule filaments appear not to be required for maintenance of polaris in cilia once they have formed. Whether they are needed for the initial shift of polaris into the insoluble pool has not yet been evaluated. The toxicity associated with long-term culture in the presence of nocodazole to test this possibility has made this analysis problematic.

Fig. 7. Reexpression of polaris is able to rescue the ciliary defects on 94D mutant cells. A: effect of reexpressing polaris on cilia formation was analyzed in polarized 94D cells (+/- Tg737Bap construct) plated on Transwell filters at confluence and cultured under nonpermissive conditions (3 days, -IFN-γ, and 39°C) by immunofluorescence using anti-β-tubulin antibodies. β-Tubulin was detected as a small domain near the apical membrane (arrows) in mutant cells (left). In contrast, in both of the rescue lines [shown for 94D-Tg737Bap-2 (right)] β-tubulin staining extended from the surface of the epithelium (arrows). B: to better quantitate the effect of expressing polaris in mutant cells, we measured the length of the β-tubulin-positive domain. Although the β-tubulin detected in mutant 94D cells resembled that of basal body staining and was typically <1 μm in length (2 left panels), in both rescued lines β-tubulin domains were typically 2–4 μm in length (2 right panels), similar to the length in vivo in the kidney (37). C: to evaluate morphological changes in more detail, we analyzed cilia at the level of scanning electron microscopy. Similar to the data from immunofluorescence analysis, cilia on the mutant 94D cells were absent or extremely stunted (2 left panels). The few cilia that did form on the 94D cells were morphologically abnormal, with bulbous expansion of the axoneme membrane (arrowhead). In contrast to the mutant 94D cells, cilia on the 94D-Tg737Bap-1 or 94D-Tg737Bap-2 cells were consistently longer and more developed (2 right panels). All images were captured at ×20K.

The nearly ubiquitous nature of primary immotile cilia on many diverse cell types, including neurons,
epithelia, endothelium, and fibroblasts, where they have no obvious function, has led some to speculate that they are vestigial organelles of limited utility or specialization (http://www.wadsworth.org/BMS/SCBlinks/cilia1.html) (13, 29, 53). However, data from the orpk mutants and the localization of other PKD-related proteins in mice and in lower eukaryotes have raised the possibility that ciliary function may be important for normal renal function, development, or differentiation and that disruption of these organelles may result in cystic disease (7, 21, 41, 51). It is also interesting that, in addition to kidney lesions, the pkd-1, pkd-2, cpk, and orpk mutant mice exhibit extrarenal pathologies, often involving the liver and pancreas (17, 20, 27, 33, 55). In most of these cases, the pathology is associated with epithelial cells that have a primary cilium, such as those in bile and pancreatic ductule epithelium (3, 32). Thus alterations in cilia function may also be responsible for the spectrum of extrarenal phenotypes associated with cystic kidney disease.

In the kidney, cilia are present throughout much of the nephron and collecting duct (15, 53). Electron microscopic analysis in rat nephron has detected the presence of a primary cilium on cells of the parietal epithelia into the tubule lumen, where they are optimally positioned to function in a sensory capacity. Recent data have suggested that deflection of the primary cilium on MDCK cells can serve as a mechanism to evaluate fluid flow rates (40). The pathway activated in response to the cilium deflection in MDCK cells involves a stretch-activated channel located in the cilium and calcium entry through the axoneme membrane. In addition to mechanosensation, it is possible that renal cilia may function in a chemosensory capacity, such as with cilia found on olfactory neurons of mammals and in C. elegans (26, 45, 46, 52). If renal cilia do function as sensors within the tubule lumen, the cilium length may be a critical factor in signaling efficacy. Thus in orpk mutants, where cilia are severely runted, this mechanism of regulating renal function would be aberrant.

To begin elucidating the biological function of primary cilia and to determine their possible relationship with cystic kidney disease, we generated a conditionally immortalized cortical collecting duct cell line from the orpk mutants. These cells grow as tight monolayers, develop high transepithelial resistance, express markers typical of epithelia, and appear morphologically normal with the exception of their ciliary defects. The cilia that form on these mutant cells are extremely short and display bulbous extensions of the axonemal membrane. The continued presence of aberrantly formed cilia rather than complete loss of cilia on mutant cells most likely reflects the hypomorphic nature of the Tg737 orpk allele (34, 51). Similarly malformed cilia are seen in vivo in orpk mutant kidney and brain, whereas cilia are completely absent in the midgestation embryonic lethal phenotype seen in Tg737 null mutant (Tg737$^{ΔS-MGAL}$) (34, 37, 51). To evaluate the importance of polaris in renal cilia formation, we reexpressed polaris using the rescue construct that corrected the kidney defects in vivo in orpk mice (56, 57). In contrast to the mutant line, cilia on almost all of the rescued cells were morphologically normal. These data argue that polaris plays a primary role in the formation of renal cilia and that loss of cilia is a contributing factor to the renal pathology in orpk mice.

The primary cilium found on diverse cells ranging from fibroblasts to epithelial cells is a relatively unexplored organelle. The major obstacle to analyzing primary cilia is that they are near the resolution limits of conventional light microscopy, and there have not been cell lines available where cilia formation can be regulated without significantly altering the cellular differentiation state. Because the ciliary defects can be corrected in the 94D cells by reexpressing polaris, we can utilize the rescued and mutant cell lines to explore the possible function of primary renal cilia as mechanor- or chemosensors in hopes that it will give insight into the possible mechanism by which cilia loss may result in cystic kidney disease.

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