Primary Cilia of inv/inv Mouse Renal Epithelial Cells Sense Physiological Fluid Flow: Bending of Primary Cilia and Ca\(^{2+}\) Influx

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ABSTRACT. Primary cilia are hypothesized to act as a mechanical sensor to detect renal tubular fluid flow. Anomalous structure of primary cilia and/or impairment of increases in intracellular Ca\(^{2+}\) concentration in response to fluid flow are thought to result in renal cyst formation in conditional kif3a knockout, Tg737 and pdk1/pkd2 mutant mice. The mutant inv/inv mouse develops multiple renal cysts like kif3a, Tg737 and pdk1/pkd2 mutants. Inv proteins have been shown to be localized in the renal primary cilia, but response of inv/inv cilia to fluid stress has not been examined. In the present study, we examined the mechanical response of primary cilia to physiological fluid flow using a video microscope, as well as intracellular Ca\(^{2+}\) increases in renal epithelial cells from normal and inv/inv mice in response to flow stress. Percentages of ciliated cells and the length of primary cilia were not significantly different between primary renal cell cultures from normal and inv/inv mutant mice. Localization of inv protein was restricted to the base of primary cilia even under flow stress. Inv/inv mutant cells had similar bending mechanics of primary cilia in response to physiological fluid flow compared to normal cells. Furthermore, no difference was found in intracellular Ca\(^{2+}\) increases in response to physiological fluid flow between normal and inv/inv mutant cells. Our present study suggests that the function of the inv protein is distinct from polaris (the Tg737 gene product), polycystins (pdk1 and pdk2 gene products).

Key words: primary cilia/kidney/Ca\(^{2+}\)/inv/flow stress/inversin

Introduction

Monocilia (primary cilia) used to be considered a vestigial or remnant structure of no functional importance. However, recent studies have shown that primary cilia are important in the establishment of body left-right asymmetry and to maintain normal renal tubular architecture. During early developmental stages, primary cilia in the node are motile and create leftward fluid flow by rapidly rotating themselves. Studies of nodal primary cilia are performed in mutants that show randomization of body situs, such as kif3a, kif3b, Tg737, iv and pdk2 mutant mice (Supp et al., 1997; Nonaka et al., 1998; Takeda et al., 1999; Murcia et al., 2000; Pennekamp et al., 2002). kif3a, kif3b and Tg737 mutants fail to produce nodal primary cilia. iv and pdk2 mutants possess primary cilia, but iv mutant cilia are immotile (Okada et al., 1999). The pdk2 mutant lacks a Ca\(^{2+}\) response in the node during development (McGrath et al., 2003). In addition to randomization of body situs, kif3a, Tg737 and pdk2 mutants develop multiple renal cysts (Moyer et al., 1994; Wu et al., 2000; Lin et al., 2003). In contrast to nodal motile cilia, primary cilia in renal epithelial cells are non-motile. Renal epithelial cells of kif3a and Tg737 mutants show a loss or shortened cilia in vivo and in vitro (Pazour et al., 2000; Yoder et al., 2002; Lin et al., 2003). Renal cells derived from the pdk1 mutant or cells treated with polycystin2 (a gene product of pdk2) antibody fail to increase intracellular Ca\(^{2+}\) concentrations in response to fluid stress (Nauli et al., 2003). Furthermore, isolated renal tubules in Tg737 mutants displayed blunted increases in intracellular Ca\(^{2+}\) concentration in response to fluid stress compared to normal tubules (Liu et al., 2005). Thus, abnormal structures in primary cilia and/or impairments in increases in intracellular Ca\(^{2+}\) concentration in response to fluid flow are thought to cause renal cyst formations.

One explanation why loss or truncation of cilia causes renal cyst formation is that non-motile primary cilia on
renal epithelial cells function as a flow sensor (Praetorius and Spring, 2003; Yokoyama, 2004). Fluid flow can bend primary cilia of rat kangaroo cells (PK1) (Schwartz et al., 1997). Bending a cilium by pipette or fluid flow induced Ca\(^{2+}\) influx in collecting tubule cells derived from normal mice, but not from pkdl null mice or renal cells treated with anti-pkd1/2 protein antibodies (Nauli et al., 2003). Taken together with a recent report concerning abundant cation-permeable channel activities in the ciliary membrane (Raychowdhury et al., 2005), it is hypothesized that the pkdl/pkd2 complex could function as a molecular sensor as well as a Ca\(^{2+}\) channel, and that a lack of flow-sensing in primary cilia could lead to renal cyst formation.

The inv (inversion of embryonic turning) mouse mutant was discovered in a family of transgenic mice that showed situs inversus associated with multiple renal cysts (Yokoyama et al., 1993; Mochizuki et al., 1998). Mutation in the inv gene in human was later found to cause nephronophthisis type 2 (NPHP2) (Otto et al., 2003). Recently, primary cilia in the primitive node of inv/inv mutants were reported to show aberrant rotation and subsequently made turbulent nodal flow (Okada et al., 1999, 2005). Artificial leftward nodal flow rescued situs inversus in inv/inv mutants in vitro, suggesting that the turbulent nodal flow causes situs abnormalities (Watanabe et al., 2003). It is possible that a dysfunction of the primary cilia machinery is responsible for the turbulent nodal flow. Scanning electron microscopy (SEM) analysis of inv/inv kidney sections showed normal appearing primary cilia at cystic tubules (Phillips et al., 2004), but response of primary cilia in inv/inv mutants to fluid stress has yet to be clarified.

In the present study, we first examined subcellular localization of inv proteins and whether flow stimulation affects localization of inv protein. The main purpose of the present study was to examine if inv/inv mutant renal cells have any abnormalities in mechanical response of primary cilia to physiological fluid flow, or abnormalities in intracellular Ca\(^{2+}\) increase in response to fluid stress.

### Materials and Methods

#### Reagents

One-μm-diameter polystyrene beads were purchased from Polysciences, Inc. (Warrington, PA). Fura-2 AM and Pluronic F127 gel were from Molecular Probes (Eugene, OR). Fluorescein-conjugated LTA (LTA-FITC) was obtained from Vector Laboratories (Burlingame, CA). Cell culture supplements were obtained from Invitrogen (Carlsbad, CA). Unless otherwise stated, all chemicals were purchased from Sigma (St Louis, MO) or Wako Pure Chemical (Osaka, Japan).

#### Animals and primary cultures of mouse renal epithelial cells

Normal, inv/inv and inv-GFP transgenic mice (Watanabe et al., 2003) were maintained in an animal facility according to experimental procedures that were approved by the Committee for Animal Research, Kyoto Prefectural University of Medicine. Mice (postnatal day 5) were anesthetized by intraperitoneal administration of sodium pentobarbital at a dose of 50 mg/kg body weight. Kidneys were isolated and dissociated with Krebs buffer containing 10% BSA and 1 mg/ml collagenase for 30 min with gentle shaking at 37°C. Digested tissue fragments were passed through 125 μm, 105 μm and 45 μm sieves, and centrifuged at 1000 g for 10 min at room temperature. The pellet was resuspended in Dulbecco’s modified Eagle’s medium/F-12 medium containing 10% fetal bovine serum, and cells were seeded on plastic dishes or glass coverslips coated with human collagen IV (50 μg/ml). Cells were incubated at 37°C, and equilibrated with 5% CO\(_2\) or glass coverslips coated with human collagen IV (50 μg/ml). Cells were incubated at 37°C, and equilibrated with 5% CO\(_2\) in humidified air. After 24 h of incubation, culture medium was changed to D-MEM / F-12 medium containing 0.5% fetal bovine serum, 100 μM MEM non-essential amino acid solution, 5 mg/l insulin, 5 μg/l sodium selenite, 5 mg/ml transferrin, 400 μg/l dexamethasone, 10 ng/ml epidermal growth factor, 5 μg/ml 2,3,5-triiodo-l-thyronine, 10000 U/l penicillin, 100 mg/l streptomycin, and 250 μg/l Fungizone®. Medium was changed daily.

#### Imaging of primary cilia under flow stimulation

Primary renal epithelial cells were grown on type IV collagen-coated glass coverslips for at least 2 days. Cells were placed in a parallel plate-type perfusion chamber (FSC2 closed system, Biotechs, Butler, PA). The flow chamber was set on the stage of an inverted microscope (IX70, Olympus, Tokyo, Japan) equipped with a CCD camera (UC-QE, Molecular Devices Corporation, Sunnyvale, CA). One end of the chamber was connected to a reservoir filled with Hanks balanced salt solution via a silicon tube. The chamber and reservoir were maintained at 37°C by a temperature sensor and heater (FCS2 controller, Biotechs). Fluid flow was applied to cells by adjusting the height of the reservoir, and averaged volume flow (ml/s) was calculated from changes in weight of the reservoir. We captured Nomarski images of primary cilia using MetaFluor (Molecular Devices Corp., Sunnyvale, CA) for Windows every 25 msec. One μm-diameter polystyrene beads were used for determining linear fluid velocity profiles at the level of primary cilia. When averaged linear velocity in the chamber was 3.1 mm/s, linear fluid velocity at the level of the primary cilia (at 10 μm) was about 280 μm/s under our experimental conditions (Fig. 1A). Linear fluid velocity applied in the present study corresponded to an appropriate physiological range of proximal tubular flow rates (Chou and Marsh, 1987). Percentage of ciliated cells was assessed by microscope. Length of primary cilia (L, μm) was determined as follows:

\[ L = \sqrt{ac^2 + bc^2} \]  
(Fig. 1B).

a: the top of the cilium, b: the base of the cilium, c: a point where
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Localization of \textit{inv}-GFP

Primary kidney epithelial cells derived from transgenic \textit{inv/inv} mice expressing the \textit{inv}-GFP transgene were grown on type IV collagen-coated glass coverslips for at least 2 days. GFP fluorescent images were obtained with an Olympus microscope (IX70) and a CCD camera (UIC-QE). Primary cilia were bent by fluid flow to make their entire length visible.

Detection of intracellular Ca\(^{2+}\) concentrations under flow stimulation

Primary renal epithelial cells were grown on type IV collagen-coated glass coverslips for at least 2 days. Cells were incubated for 30 min with the Ca\(^{2+}\) sensitive probe Fura-2 AM (at a final concentration of 5 \(\mu\)M) and 0.01% Pluronic F127 at room temperature, in serum free medium. Cells were washed twice to remove excess Fura-2 AM, and incubated for 15 min at 37\(^\circ\)C for de-esterization. During de-esterization, cells were co-incubated with LTA-FITC (diluted 1:1000) for identification of proximal convoluted tubules (Laitinen \textit{et al.}, 1987). Subsequently, cells were placed in the chamber described above, and fluid flow was applied to the cells. Paired fluorescent images were captured using MetaFluor every 5 s at excitation wavelengths of 340 nm and 380 nm with a xenon light source. The fluorescent ratio (F340/F380) was monitored as changes in intracellular Ca\(^{2+}\) concentrations. Data were obtained from 7 regions of 2 to 5 cells.

Data analysis and statistics

Data are expressed as mean±S.E. Data obtained from the two groups were compared using a t-test. \(P\) values of less than 0.05 were considered significant.

Results

Percentage of ciliated cells and lengths of primary cilia

Primary cilia of normal mice were seen as dots at the static state under Nomarski observation, suggesting that primary cilia extended perpendicularly to the apical membrane, and became parallel to the optical axis of the microscope (indicated by arrows in Fig. 2B). When physiological fluid flow (flow profiles in Fig. 1A) was applied, primary cilia bend and easily visible as lines (indicated by dashed circles in Fig. 2C). Successive Z-axis pictures of primary cilia both static and under fluid flow are available in the Supplementary Information, Video S1. In confluent cultures, ciliated cells were 81.3±1.3\% in normal cells, and 78.2±3.7\% in \textit{inv/inv} mutant cells (Table I). Primary cilia were 11.5±0.6 \(\mu\)m in length in normal and 13.0±0.6 \(\mu\)m in \textit{inv/inv} mutant cells (Table I). The percentage of ciliated cells and lengths of

![Fig. 1.](image-url)
primary cilia were not significantly different between normal and inv/inv mutant cells (P>0.05).

**Determination of inv protein localization under physiological fluid flow stimulation**

We examined the subcellular localization of functional inv protein in primary renal epithelial cells using transgenic inv/inv mice expressing the inv-GFP transgene, which rescues the complete phenotype of inv/inv mice, including kidney cyst formation and situs inversus. Fig. 3 shows Nomarski images at the level of cells/primary cilia (Fig. 3A to E) and corresponding inv-GFP fluorescent images (Fig. 3F to J) in primary cultured renal epithelial cells. Primary cilia were not observed as dots at the level of cell nuclei (Fig. 3A and D) and we could not detect inv-GFP fluorescence in the nucleus or in the membranes between cells (Fig. 3F and I). Primary cilia were clearly seen as dots above the cell nucleus level (Fig. 3B and C). We detected strong inv-GFP fluorescence in the base of primary cilia (Fig. 3G), but not in the top (Fig. 3H). Fluid flow was applied to visualize primary cilia fully (Fig. 3E), and the corresponding inv-GFP image showed strong GFP fluorescence in the base of primary cilia (Fig. 3I). Furthermore, the inv-GFP signal in the base of cilia did not translocate or change in intensity by physiological fluid flow for 90 min or more (data not shown).

**Analysis of primary cilium bending speed by physiological fluid flow stimulation**

We analyzed the response of renal cilia to physiological flow stress. As shown in Fig. 2, primary cilia were observed as dots at the static state. When physiological fluid flow was applied, primary cilia of normal mice were bent. As soon as flow stimulation stopped, primary cilia quickly returned to their previous position. Fig. 4 shows consecutive pictures of a bending cilium every 25 msec when fluid flow stress was applied. It took on average 131±10 msec (n=10) from the beginning of bending to the completely bended state (Fig. 4A). When flow stress was stopped, cilia returned to the static state within an average time of 160±21 msec (n=10) (Fig. 4B). See the Supplementary Information, Video S2.

Next, we examined whether inv/inv mouse cilia showed any abnormality in bending mechanics in response to flow stress. The inv/inv mouse cilia showed the same bending mechanics in response to physiological fluid flow as that of normal mouse cilia (Fig. 5). In inv/inv renal epithelial cells, it took on average 133±11 msec (n=10) from the beginning of bending to the completely bended state (Fig. 5A). When fluid flow was stopped, cilia returned to the static state within an average time of 160±14 msec (n=10) (Fig. 5B). See the Supplementary Information, Video S3. No statis-
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A statistical difference in bending and reflecting time of primary cilia between normal and inv/inv mutant cells was observed ($P>0.05$).

**Physiological fluid flow stimulation and intracellular $\text{Ca}^{2+}$ increase**

Cells were loaded with the $\text{Ca}^{2+}$ indicator Fura-2. We selected LTA-positive proximal renal epithelial cells from both normal and inv/inv mutant mice to examine intracellular $\text{Ca}^{2+}$ response to fluid flow (Fig. 6A and B). We detected a rise in intracellular $\text{Ca}^{2+}$ concentration in response to fluid flow and this increase of intracellular $\text{Ca}^{2+}$ concentration was maintained while fluid flow was applied. In normal cells, it took on average 96.4±4.7 sec to reach peak $\text{Ca}^{2+}$ levels from the start of fluid stress. $\text{Ca}^{2+}$ levels were maintained at higher than basal levels during fluid stimulation. After the flow was stopped, intracellular $\text{Ca}^{2+}$ concentrations decreased and returned to basal levels within an average time of 153.6±15.3 sec.

Inv/inv mutant cells also showed a rise in intracellular $\text{Ca}^{2+}$ concentration in response to fluid flow and this increase of intracellular $\text{Ca}^{2+}$ concentration was maintained while fluid flow was applied. inv/inv mutant cells took on average 99.9±4.9 sec to reach peak $\text{Ca}^{2+}$ levels while fluid stress was applied. $\text{Ca}^{2+}$ levels were maintained at higher than basal levels during fluid stimulation. After the flow was stopped, intracellular $\text{Ca}^{2+}$ concentrations decreased, and returned to basal levels within an average time of 122.9±7.6 sec. There were no statistical differences between primary cilia of normal and inv/inv mutant cells in the time to reach the peak and the time to return to basal levels.

**Discussion**

The present study provides three findings about renal cells of normal and inv/inv mice in response to fluid flow. First, functional inv protein was localized at the base of primary cilia and remained there even under fluid flow stimulation. Second, primary cilia of primary inv/inv mutant mouse renal epithelial cells bend in response to physiological fluid flow in an identical manner as those of normal mouse renal epithelial cells. Third, renal cells derived from inv/inv mice increased their intracellular $\text{Ca}^{2+}$ concentration in response to physiological fluid flow.

Localization of inv protein has been reported to occur in cell membrane (Nurnberger et al., 2002; Simons et al., 2005), cytoplasm (Simons et al., 2005), nucleus (Nurnberger et al., 2002) and cilia (Morgan et al., 2002a; Otto et al., 2003; Watanabe et al., 2003). Previous reports except Watanabe et al. used antibodies against inv protein and cultured renal cell lines. Localization of inv protein using antibodies indicates the place where inv protein exists, but does not determine the place where the inv protein is functioning. The inv-GFP fusion construct rescues all the inv phenotypes.

![Fig. 3. Localization of functional inv protein to the base of primary cilia of kidney epithelial cells. inv/inv, inv-GFP mouse primary cilia of primary kidney epithelial cells. Images at the level of cell nuclei (A, D, F and I), and at the level of primary cilia (B, C, E, G, H and J) are shown. GFP fluorescence is detected at the base of primary cilia (G and J). Black arrows indicate the direction of fluid flow. Scale bars=10 μm.](image-url)
Thus, localization of the fusion protein indicates the place where the protein is functioning. In the present study, we showed that a strong GFP signal was observed at the base of primary cilia of primary cultured renal cells derived from inv-GFP mice at static state, and no translocation of the protein was observed after fluid flow stress, suggesting that the base of primary cilia is where the inv protein functions.

Primary cilia of mouse primary cultured renal epithelial cells stood straight and never displayed active beating under static conditions. In response to physiological fluid flow, primary cilia were bent, hence were easily visualized. As soon as the fluid flow stopped, primary cilia were able to...
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![Image of Flow-induced Ca\textsuperscript{2+} responses in lectin-labeled, proximal convoluted tubule cells.](image)

**Fig. 6.** Flow-induced Ca\textsuperscript{2+} responses in lectin-labeled, proximal convoluted tubule cells. Epithelial cells of proximal convoluted tubule origin were detected using LTA-FITC as markers. Cells loaded with Fura2-AM were exposed to fluid flow. Flow-induced Ca\textsuperscript{2+} responses in normal (A) and inv/inv (B) cells were analyzed. Representative data are shown. Detailed procedures are described in 'Materials and Methods'.

return to their previous position without overshooting. These results correlated well with a previous report using renal cell lines of rat kangaroo (PtK1 cells) (Schwartz et al., 1997). Mutations in \textit{kif3a} and \textit{Tg737} caused structural abnormalities of renal primary cilia \textit{in vivo} and \textit{in vitro} (Pazour et al., 2000; Yoder et al., 2002; Lin et al., 2003). Recently, primary cilia in the primitive node of \textit{inv/inv} mutants were reported to show aberrant rotation and subsequently produced turbulent nodal flow, suggesting the possibility of a structural or functional alteration of primary cilia in \textit{inv} mutant mice (Okada et al., 1999, 2005). However, our study showed that the lengths of primary cilia were almost identical in both normal and \textit{inv/inv} mutant mice, and that irregularities in bending-and-return mechanics of \textit{inv/inv} primary cilia were not observed under physiological fluid flow. Furthermore, ten times faster fluid flow did not eliminate primary cilium from the cell, indicating that \textit{inv} primary cilia were also firmly anchored to the cell (data not shown). Together with a previous SEM study (Phillips et al., 2004), it is unlikely that renal primary cilia in \textit{inv/inv} mutants have structural abnormalities that cause renal cyst formation. Bending primary cilia in MDCK was reported to increase intracellular Ca\textsuperscript{2+} concentrations (Prætorius and Spring, 2001). Renal cells of \textit{pkd1} mutants or cells treated with anti-polycystin2 were unable to increase their intracellular Ca\textsuperscript{2+} concentration in response to physiological flow stress (Nauli et al., 2003). However, \textit{inv/inv} cells showed intracellular Ca\textsuperscript{2+} increases after physiological flow stress that could bend primary cilia of renal cells the same way as normal cells. Although we cannot deny that more subtle difference may exist between normal and \textit{inv} cells in the response or resting level of Ca\textsuperscript{2+}, the present results strongly suggested that \textit{inv} renal cells have the same Ca\textsuperscript{2+} response mechanism to flow stress as normal renal cells have.

\textit{Inv} protein contains calmodulin-binding motifs, and Ca\textsuperscript{2+} controls calmodulin-\textit{inv} binding (Yasuhiro et al., 2001; Morgan et al., 2002b). The polycystin complex acts as a Ca\textsuperscript{2+} channel (Hanaoka et al., 2000). Both \textit{inv} protein and polycystin are localized in cilia. Losses of \textit{inv} protein and polycystin-2 function lead not only to cyst formation, but also to \textit{situs inversus} (Yokoyama et al., 1993; Pennekamp et al., 2002). Thus, there is a possible relationship between \textit{inv} and the polycystin signaling pathway. Importantly, when mutant cells that lack \textit{inv} were exposed to fluid flow, we detected Ca\textsuperscript{2+} influx. The present results suggest that \textit{inv} protein participates in downstream signaling of Ca\textsuperscript{2+} influx. Recently, \textit{inv} protein was shown to act on the Wnt pathway (Simons et al., 2005). It would be interesting to investigate whether polycystins also modulate the Wnt signaling pathway, and share a common pathway with \textit{inv}.

In summary, \textit{inv} renal cells show no structural abnormalities of cilia, and intracellular Ca\textsuperscript{2+} increases in response to physiological fluid flow are the same as in normal renal cells. Although the \textit{inv} protein is localized in the cilia like polaris, \textit{kif3} and polycystins, the present results suggest that the \textit{inv} protein has a distinct function.

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