Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2011;28:673-682

Accepted: September 15, 2011

Shear Stress Reverses Dome Formation in Confluent Renal Tubular Cells

Irene Cattaneo[1*], Lucia Condorelli[1*], Anna R. Terrinoni[2], Luca Antiga[1], Fabio Sangalli[1] and Andrea Remuzzi[1,3]

[1]Department of Biomedical Engineering, Mario Negri Institute for Pharmacological Research, Bergamo, [2]Centro COGES and [3]Department of Industrial Engineering University of Bergamo, Dalmine-Bergamo, *these authors contributed equally to this work

Key Words
MDCK • Shear stress • Cytoskeleton • ZO-1 • Cilia

Abstract
Background/Aims. It has been shown that MDCK cells, a cell line derived from canine renal tubules, develop cell domes due to fluid pumped under cell monolayer and focal detachment from the adhesion surface. *In vitro* studies have shown that primary cilia of kidney tubular epithelial cells act as mechano-sensors, increasing intracellular calcium within seconds upon changes in fluid shear stress (SS) on cell membrane. We then studied the effect of prolonged SS exposure on cell dome formation in confluent MDCK cell monolayers. Methods. A parallel plate flow chamber was used to apply laminar SS at 2 dynes/cm^2 to confluent cell monolayers for 6 hours. Control MDCK cell monolayers were maintained in static condition. The effects of Ca^{2+} blockade and cell deciliation on SS exposure were also investigated. Results. Seven days after reaching confluence, static cultures developed liquid filled domes, elevating from culture plate. Exposure to SS induced almost complete disappearance of cell domes (0.4±0.8 vs. 11.4±2.8 domes/mm^2, $p<0.01$, $n=14$). SS induced dome disappearance took place within minutes to hours, as shown by time-lapse videomicroscopy. Exposure to SS importantly affected cell cytoskeleton altering actin stress fibers expression and organization, and the distribution of tight junction protein ZO-1. Dome disappearance induced by flow was completely prevented in the presence of EGTA or after cell deciliation. Conclusions. These data indicate that kidney tubular cells are sensitive to apical flow and that these effects are mediated by primary cilia by regulation of Ca^{2+} entry in to the cell. SS induced Ca^{2+} entry provokes contraction of cortical actin ring that tenses cell-cell borders and decreases basal stress fibers. These processes may increase paracellular permeability and decrease basal adhesion making dome disappear. Elucidation of the effects of apical fluid flow on tubular cell function may open new insights on the patho-physiology of kidney diseases associated with cilia dysfunction.

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1015-8987/11/0284-0673$38.00/0

Accessible online at:
www.karger.com/cpb

Andrea Remuzzi, Eng. D.
Head, Department of Biomedical Engineering
Mario Negri Institute for Pharmacological Research
Via Stezzano, 87-24126 Bergamo (Italy)
Tel. + 39 (035) 42131, Fax+ 39 (035) 319.331, E-Mail aremuzzi@marionegri.it

Introduction

In physiological conditions epithelial cells lining the inner surface of both proximal and distal tubules in the kidney are exposed to tubular fluid flow. These cells are characterized by the presence of primary cilia, which project from the apical surface into the tubular lumen, and act as mechanosensors [1, 2]. Changes in tubular fluid volume flow are expected to result in changes in shear stress (SS) and cilia bending, with following increase in intracellular Ca^{2+} and consequent activation of nuclear transcription factors. These intracellular changes directly affect gene expression and cell cycle regulation [3-8]. Evidence suggests that in patients affected by polycystic kidney disease (PKD) this mechanosensor function of tubular epithelial cells is impaired by mutations of the transmembrane proteins associated with ciliary membrane, polycystine-1 (PC1) and-2 (PC2) [2]. It is not known at the moment how these signals are related to formation and growth of tubular dilation and ultimately to renal cysts. Tubular epithelial cell function modulation by fluid flow, or physical stimulation by a micropipette, has been studied *in vitro* using MDCK cells, a cell line derived from distal tubule/collecting duct of normal dog kidney [9]. These studies mainly investigated the acute effects of fluid flow that take place within seconds or minutes after cell stimulation. However, the effects of more prolonged exposure to fluid SS on function and structure of these cells have not been characterized yet.

As shown in details in the literature, confluent MDCK cells maintained in culture for more than seven days undergo formation of dome structures, consisting in groups of cells that elevate above the adhesion surface [10]. These structural alterations likely derive from active fluid reabsorption at luminal level and basolateral secretion, with consequent cell detachment from the substratum and reorganization of junctional contacts. These events induce and sustain focal elevation of groups of cells along the monolayer, and are associated with cell differentiation and expression of pro-apoptotic proteins [11]. Cell dome formation is also involved in tubular cell differentiation and morphogenesis [12]. In the present study we then investigated the effects of exposure of confluent monolayers of MDCK cells to fluid SS, studying morphological and functional changes of polarized MDCK cells in response to steady laminar SS using a parallel-plate flow chamber. We quantified the surface density and spatial extension of domes in cell monolayers using optical and electron microscopy, after 6 hour exposure to SS, and we documented the dynamic response of MDCK cells to flow by time-lapse videomicroscopy. Cytoscheletal organization (polimerized actin fibers), expression of tight junction (TJ) protein ZO-1 and of Na^+,K^+-ATPase protein, as well as Ca^{2+} entry into the cells were also investigated in cells exposed to flow or maintained in static condition to elucidate the cascade of events potentially triggered by flow exposure. In addition, we investigated the effect of fluid flow on the morphology of cell domes during Ca^{2+} blockade, and after deciliation, to selectively antagonize intracellular Ca^{2+} increase and the related intracellular signals induced by cilia-mediated mechanotransduction.

Materials and Methods

Cell culture and experimental design

MDCK-II cells (European Collection of Cell Cultures, Salisbury, UK), a cell line derived from distal tubule/collecting duct [9], were cultured in Eagle's minimum essential medium (MEM) with Earl's salts and $NaHCO_3$ (Sigma Aldrich, St Louis, MO) supplemented with 5% fetal bovine serum (Celbio Spa, Milano, Italy) and 2mM L-glutammine (Gibco Invitrogen Co., Paisley, Scotland). Cells were grown on Thermanox® coverslip (Nalgene Nunc Int, New York, NY) until reaching confluence. Cells were kept in culture for additional seven days to induce cell differentiation and polarization, and to allow dome formation. Cell monolayers (n=12) were then used for exposure to laminar flow or kept in static condition (n=12). In each experiment one coverslip was inserted in a parallel flow chamber and perfused with culture media in a closed circuit for 6 hours, while another coverslip was kept in the cell incubator, in static control condition, during the same time period. In additional experiments, ethylene glycol-bis-aminoethyl tetraacetic acid EGTA (Sigma) was added to the medium at 1mM concentration to block cell Ca^{2+} influx, during perfusion experiments (n=7) and in static controls (n=7). Polarized monolayers were incubated with 4mM chloral hydrate (CH, Sigma) for 24h to achieve complete deciliation [13], then monolayers were exposed to flow (n=3) or static conditions (n=3).

Perfusion chamber

The parallel plate flow chamber used to expose cell monolayers to laminar flow conditions has been previously described [14-16]. Briefly, the camber is composed of a polymetilmetacrilate plate that contains in- and out-flow tubing connections and a silicon rubber gasket (508 μm in thickness) with a rectangular opening of 15 x 32 mm. A stainless steel plate is placed on the bottom to hold the coverslip and it is joined to the upper part of the chamber by screws. The stainless steel plate has a rectangular opening to allow microscopic view. The chamber was perfused with complete medium using a roller pump (Minipuls3, Gilson, Villiers Le Bel, France) and flow rate oscillations were minimized by a compliance inserted in the afferent side of the circuit. The value of SS acting on cell surface

as a function of medium flow rate Q was calculated assuming laminar flow using the equation:

$$\tau = \frac{6\mu Q}{W \cdot h^2}$$

where μ is the medium viscosity measure at 37°C by a capillary viscometer, *W* and *h* are respectively the width and the height of the flat channel. A reservoir was used to allow O_2 and CO_2 medium exchange. At the start of the experiment, medium flow rate was gradually increased during a 10 min period, to expose the cell monolayer to a wall SS value up to 2 dynes/cm^2, then flow rate was kept constant until the end of the experiment. In additional experiments the effect of low SS exposure (0.4 dynes/cm^2) was also investigated.

Time-lapse microscopy

To investigate the dynamics of cell morphological changes during SS exposure (n=4) or in static condition (n=6), the flow chamber was placed on the stage of a phase-contrast microscope (Axiovert 40 C, Carl Zeiss Inc., Göttingen, Germany) equipped with a thermostatated chamber maintained at 37°C by an external heater (Air-Therm™ ATX, WPI Inc., Sarasota, FL). Medium pH was maintained constant by perfusion of the microscope chamber with a mixture of humidified air and 5% CO_2. To record images of cell monolayers during perfusion experiments, a digital camera was connected (PowerShot G5, Canon Inc., Tokyo, Japan) to the microscope and to a personal computer, and images acquired and stored using remote capture software. Microscopy images acquired every 30 seconds during the entire duration of the experiment (6 hrs) were then combined into digital clips at the rate of 30 frames/sec and the stream file saved in standard movie format (.mov). The effect of SS exposure in the presence of EGTA was also investigated (n=4).

Histological analysis

For histological analysis, at the end of each experiment a sample of the cell monolayer was fixed in 2% paraformaldehyde (Società Italiana Chimici, Rome, Italy) with 4% sucrose (Sigma) for 10 minutes at room temperature and then treated with 0.1M glycine (Sigma) in PBS 1x (Gibco) for 5 minutes. Cells were then permeabilised in 0.1% triton X-100 for 3 minutes and incubated in 3% BSA (Sigma) for 30 minutes at room temperature. To detect changes in stress fiber distribution, peripheral actin bands and tight junction, immunofluorescence analyses were performed. Cells were incubated with rabbit anti-ZO-1 (1:25) (Zymed Laboratories, South San Francisco, CA) over night at 4°C as primary antibody. They were then incubated with a FITC-coniugated goat anti-rabbit (1:25) antibody (Jackson Immunoresearch, West Grove, PA) for 45 minutes at 37°C followed by an incubation with rhodamine-labeled phalloidin (Invitrogen, Paisley, UK) (1:40) for 45 minutes at room temperature. For evaluation of the Na^+,K^+-ATPase protein expression, cells cultured in static condition or exposed to SS for 6 hours were fixed in a solution of 50% ethanol with 10% acetic acid diluted in PBS, permeabilised with 0.1% triton X-100 for 3 minutes and incubated in 5% BSA (Sigma). Cells were then incubated with goat anti-human Na^+,K^+-ATPase α1 antibody (Santa Cruz Biotechnology, Heidelberg, Germany) (1:40) for 2 hours at room temperature and then with mouse anti-human acetylated α-tubulin antibody (Sigma) (1:500) for one hour at room temperature. Samples were subsequently incubated with secondary antibodies, rabbit anti-goat Cy2 conjugated (Jackson Immunoresearch) (1:60) for one hour at room temperature and donkey anti-mouse Cy3 antibody (Jackson Immunoresearch) (1:100) for 45 minutes at 37°C, respectively. Counterstaining with DAPI (1 mg/ml, Sigma) for 20 minutes at 37°C was performed for cell nuclear staining. The slides were finally mounted with a fluorescent mounting medium (Dako Cytomation, Carpinteria, CA) and examined by laser confocal microscopy (LSM 510 Meta, Carl Zeiss, Jena, Germany).

Scanning electron microscopy (SEM)

For morphological analysis at SEM cell monolayers were processed with conventional methods as previously described [17]. Briefly, samples of cell monolayer in different experimental conditions, were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodilate for 1 hour, postfixed with osmium tetroxide and dehydrated through an increasing ethanol series. Cell monolayers were then drayed with pure hexamethyldisilazane (HMDS, Fluka Chemie AG, Buchs, Switzerland) [18], sputter-coated with gold and then examined on a Cross-Beam 1540EsB electron microscope (Carl Zeiss GmbH, Oberkochen, Germany). To quantify the surface density of cell domes, 3 to 6 images were acquired for each cell monolayer sample at 500x magnification and used to estimate the dimensions and mean number of dome per mm² of cell monolayers.

Intracellular Ca^{2+}

Calcium entry into cells exposed to static or flow condition was measured using the fluorescent Ca^{2+} indicator FluO-4 AM (Gibco), as previously described [7]. For each experiment a coverslip was esposed to a SS of 2 dynes/cm^2 for 6 hours, as previously described, in the presence of 2.5 μM FluO-4 and 5 mM probenecid (Sigma). Another cell monolayer was kept in the cell incubator in static condition during the same period with 2.5 μM FluO-4 and 5 mM probenecid. All experiments were performed using MEM medium (Sigma) without serum. Cells were then detached from culture surface by 0.5% trypsin-EDTA 1x (Gibco) for 3 minutes at 37°C, resuspended in culture medium and then centrifuged form 10 min at room temperature. Pellet was resuspended in PBS (Sigma) and used for flow cytometric analysis (FACSCanto, BD Biosciences). Fluorescence intensity was expressed relative to the baseline value obtained from cells maintained in static culture conditions without exposure to FluO-4.

Statistical analysis

Statistical analysis of dome surface density was performed using the analysis of variance and the Bonferroni test for specific comparisons. Flow cytometry data were analyzed by Student's t-test. Statistical significance $p < 0.05$ was considered statistically significant.

Results

Confluent cultures of MDCK cells showed spontaneous dome formation when grown on conventional culture dish, as shown in Fig. 1 by phase contrast and SEM. These domes consisted of cells lifted up from the solid support but remaining linked with the cell monolayer. Dome were ellipsoidal in shape with average minor and major axis of 74 and 58 μm, respectively. The density of domes in cell monolayers was 11.4 ± 2.8 domes/mm^2 (see Fig. 2). The exposure of cell monolayers to laminar fluid SS of 2 dynes/cm^2 for 6 hours induced important rearrangement of cell structure, with almost complete disappearance of cell domes (as shown in Fig. 1 and 2) with average dome surface density that dropped to 0.4 ± 0.8 domes/mm^2, ($p < 0.01$ vs. static cultures). Cell monolayers exposed for 6 hours to fluid flow showed, by the end of the observation period, slight elevation of some individual cells and formation of elongated structures that were not preferentially aligned with the main direction of the flow, as shown in Fig. 1. The same phenomenon of cell dome disappearance was observed also at a lower value of SS (0.4 dynes/cm^2) during perfusion of cell monolayers with culture media for 6 hours (n=4). MDCK cells exposed to flow showed primary cilia that were comparable in size to those present in monolayers maintained in static condition (average cilia length 83.6 ± 20.0, 82.8 ± 28.3, 83.0 ± 12.5 μm in static, flow and flow+EGTA condition, respectively). Flow-induced dome reorganization was almost completely prevented in the presence of EGTA, as shown in Fig. 1. Surface density of domes in cell monolayers exposed to SS in the presence of EGTA was comparable to that observed in static cultures (see Fig. 2). The same effect was observed in cell monolayers exposed to fluid flow after deciliation with CH (see Fig. 1 and 2).

To investigate in more details the dynamics of dome disappearance with SS exposure we used time lapse videomicroscopy technique. A representative sequence of images recorded during 6 hour exposure to flow or static conditions are reported in Fig. 3. Representative time lapse clips are available online (see: movie1 and movie2 at http://clinicalweb.marionegri.it/mdck/). Selected images of single domes recorded by videomicroscopy are also reported in Fig. 3. As shown in these time sequences, domes present at the beginning of flow exposure undergo rather fast changes, with movement of cells in the dome structure and sudden flattening of the dome, with final integration of cells in the monolayers. These rearrangements took place at different time points and

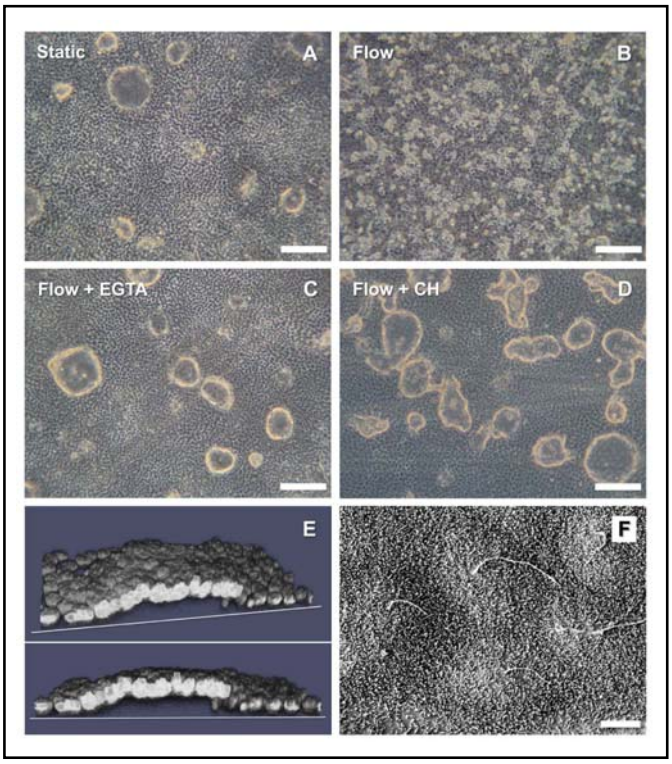

Fig. 1. Phase contrast images of MDCK cells in static condition (A-D). Static culture developed dome structures (A) while exposure to SS induced rearrangement of cell structure with complete disappearance of cell domes and reorganization of cell monolayer (B). Cell monolayers exposed to SS in the presence of EGTA (C) and after treatment with CH preserved dome formation (D) (bar = 200 μm). Three-dimensional reconstruction of cell domes by confocal microscopy (E). SEM of primary cilia in MDCK cells in static condition (F) (bar = 5 μm).

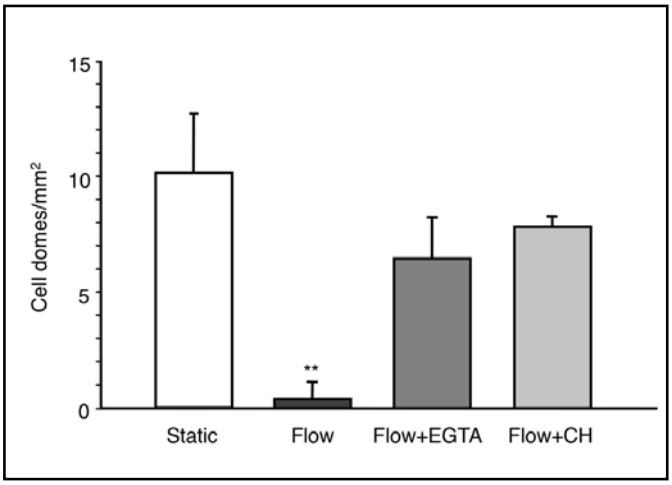

Fig. 2. Surface density of cell domes in MDCK confluent cell monolayers in static cultures conditions (n=5), after SS exposure in the absence (n=5) and in presence of EGTA (n=5), and after CH treatment (n=5). $p < 0.01$ Vs. static cultures and Vs. cells exposed to flow in presence of EGTA and after deciliation with CH.

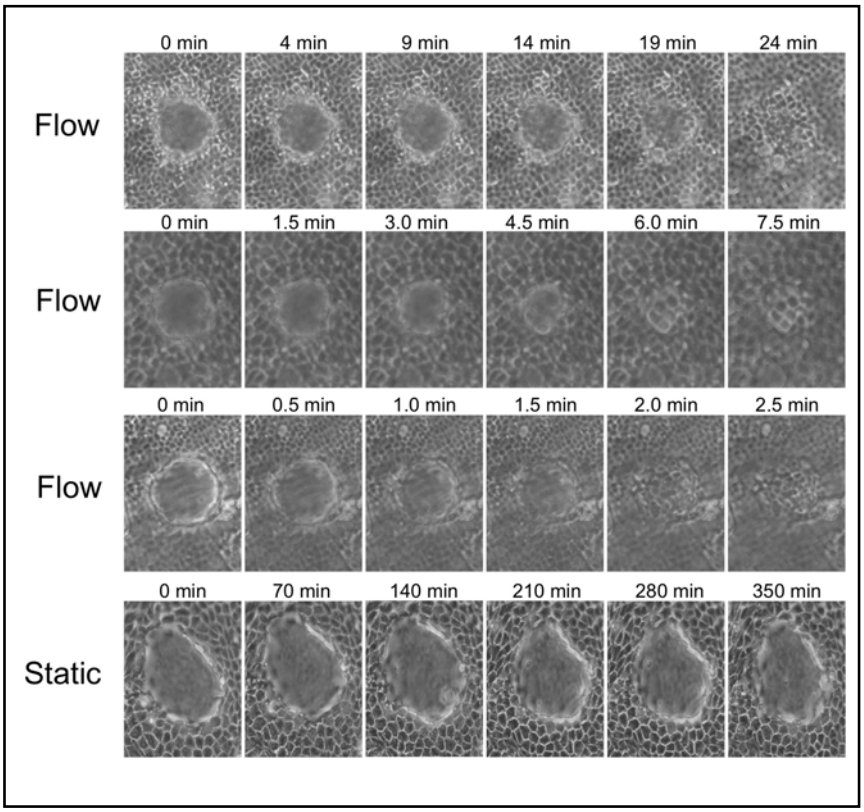

Fig. 3. Time-lapse sequences (horizontal lines) of single dome images recorded by videomicroscopy during cell exposure to flow at 2 dynes/cm^2 (first three lines) or maintained in static condition (last line).

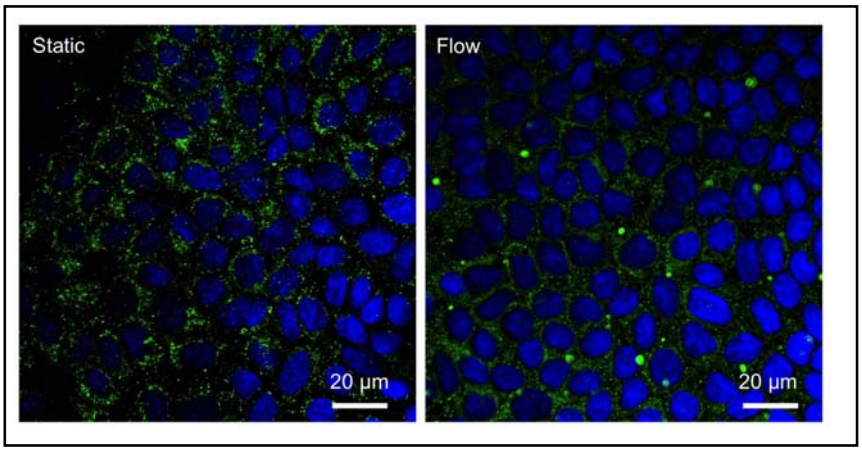

Fig. 4. Representative confocal microscopy images of cell expression of Na^+,K^+-ATPase protein (green) in cell monolayers maintained in static condition or exposed to SS. Cell nuclei are stained by DAPI in blue.

they required different time for individual domes, varying between few minutes to about 30 minutes (see movie1 online). During dome reorganization there is neither cell loss nor proliferation. By the end of the flow exposure interval (6 hours) all domes almost disappeared and the monolayer started to develop cell shape changes and reorganization, as shown in Fig. 1 and 2 (movie1 online). At variance, in static conditions most domes persisted during the entire duration of the experiment as shown in Fig. 3 (movie2 online) with cells of the domes showing some degree of movement. Time lapse videomicroscopy of cells exposed to flow in the presence of EGTA showed persistence of cell domes (see movie3 online) during the entire duration of the experiment.

Expression of Na^+,K^+-ATPase protein in representative cell monolayers maintained in static condition or exposed to SS is reported in Fig. 4. In static condition Na^+,K^+-ATPase protein was mainly expressed at the basolateral surface. After exposure to SS comparable pattern of Na^+,K^+-ATPase was observed at basolateral location. Flow exposure also induced Na^+,K^+-ATPase expression in correspondence to primary cilia, as shown by double staining images obtained with acetylated α-tubulin antibody (data not shown). Cell cytoskeleton and junctional protein distribution observed by confocal microscopy in different experimental conditions are shown in Fig. 5. As expected, in static condition rhodamine-phalloidin staining showed two

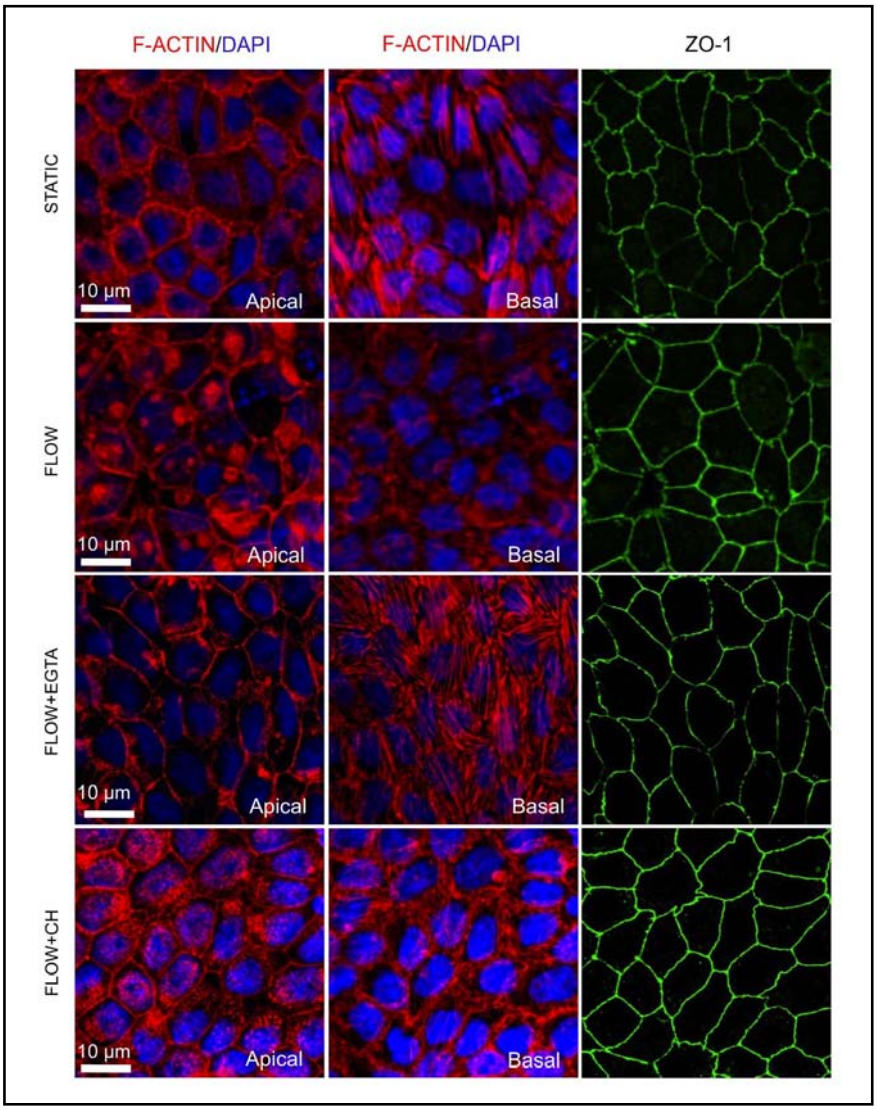

Fig. 5. Effect of flow exposure on MDCK cell cytoskeleton and junctional complexes. In static condition rhodamine-phalloidin staining (in red) of F-actin show two different patterns, in apical and in basal side of cell monolayer. SS exposure induced important rearrangement of cytoskeleton in both apical and basal cell side. ZO-1 staining (green) appear jagged and cells randomly connected in static condition, while flow exposure induced linearization of cell junctions with organized cell connections. EGTA in perfusion media prevented changes in actin fibers at basal cell side, while at apical side they appear disorganized and staining of microvillar surface is absent. On the contrary, in cell monolayer exposed to flow after deciliation by CH actin fibers in the microvillar surface is preserved, but it is lost at basal side. ZO-1 distribution is comparable to flow condition in cell exposed to flow in the presence of EGTA and in deciliated monolayers. Bar = 10 µm.

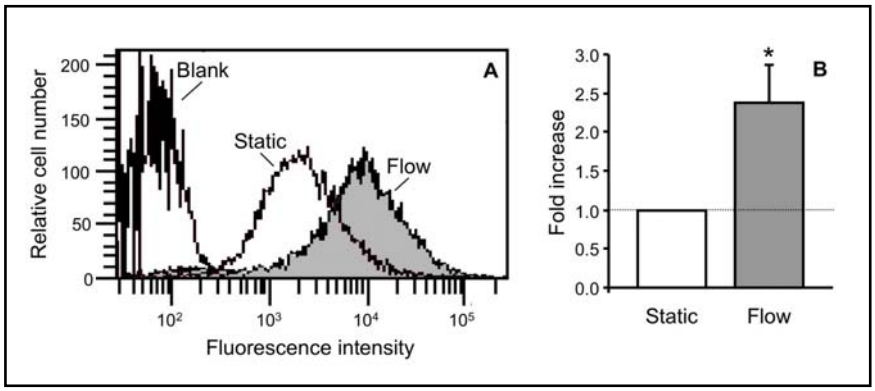

Fig. 6. Evaluation of Ca^{2+} entry into cells maintained in static condition or exposed to fluid flow for 6 hours at 2 dynes/cm^2. (A) Flow cytometric analysis of negative controls, of cells maintained in static condition and incubated with FluO-4 and of cells exposed to SS in the presence of FluO-4. (B) SS exposure induced statistically significant increase in fluorescent signal as compared to static cultures ($p < 0.05$, n=5).

distinct patterns in apical and basal cell side. In the apical side short actin fibers were distributed around cell edge and the microvillar structure. In the basal side, elongated actin stress fibers spanning the entire cross sectional area of the cells were observed. Fluid SS caused a marked rearrangement of actin fibers in the cytoskeleton. Thus, in the apical region large actin clusters were observed, with fiber distribution organized around cell edges, while in the basal side stress fibers almost completely disappeared. At variance, in cells exposed to flow in presence of EGTA, in the basal side actin fiber distribution appeared similar to that of cells in static condition, while

the apical side actin fibers were only present near cell junctions. In cells exposed to flow after deciliation by CH, stress fibers almost completely disappeared from the basal side and they were confined around cell nuclei. As for the expression of TJ associated protein ZO-1, this appeared as wavy lines in static monolayers and as straight line pattern in cells exposed to fluid SS. This flow-induced reorganization of ZO-1 pattern was not present in cells exposed to SS in presence of EGTA or in deciliated cells, and protein distribution appeared similar to that of cells in static condition.

We also investigated the effect of flow exposure on calcium entry into MDCK cells. The results of Ca^{2+} inflow during static or fluid flow incubation are reported in Fig. 6. During the 6 hours of incubation time, Ca^{2+} influx was significantly increased in cell monolayers exposed to SS as compared to static conditions, in line with the notion that mechanical stimulation of primary cilia induced cell Ca^{2+} entry.

Discussion

It has been previously reported that specialized epithelial cells develop dome formation after reaching confluence *in vitro* [19]. Cell domes that develop in focal regions of cell monolayers are characterized by fluid accumulation between cell basal membrane and the adhesion surface due trans-epithelial solute and water transport. Dome formation is sustained by tight intracellular junctions and accumulation of fluid that increases pressure between cell monolayer and adhesion surface [20]. It has been also reported that MDCK cells are characterized by selective basolateral localization of Na^+,K^+-ATPase [21, 22] and apical expression of Na^+/H^+ exchangers, that are responsible for maintenance of intracellular pH, sodium metabolism, intracellular volume and fluid homeostasis [23-25]. Previous studies demonstrated that dome formation is associated with cell Na^+ influx and cell differentiation [12, 26]. Thus, an increased expression of NHE3, a Na^+/H^+ exchanger in intestinal and renal brush border membrane that is activated by the transcription factor Stat3, was observed in MDCK dome-forming cells [24]. *In vitro* studies also showed that dome formation is associated with increased levels of intracellular cAMP [25] and that caspase-8 and p-Erk are also expressed in cells forming domes [11]. Our present results demonstrate that in MDCK cells, derived from distal tubule epithelia, exposure to flow conditions strongly affects the dynamic nature of cell dome formation, with complete disappearance of cell domes within 6 hours after beginning of monolayer perfusion. Upon exposure of cell surface to laminar fluid SS at 2 dynes/cm^2, cells forming domes reintegrated in cell monolayer without cell loss or proliferation. Our results also indicate that SS affects intracellular junctions, apical cortical acting ring along cell borders and basal stress fibers. These phenomena may be responsible for dome disappearing by increasing paracellular permeability and decreasing basal adhesion of the cell monolayer.

Literature data demonstrated that mechanical stimulation of cilia by fluid flow exposure triggers Ca^{2+} ion entry into the cell within seconds or minutes [3, 5]. We provide experimental evidence that *in vitro* flow exposure induced a significant increase in cell Ca^{2+} even during the 6 hour stimulation period, likely due to the effect of flow on primary cilia. Then to investigate whether the disappearance of cell domes during prolonged SS exposure was dependent on cilia induced elevation in intracelluar Ca^{2+}, we used EGTA to block Ca^{2+} entry into the cells during flow exposure. In another series of experiments we completely bocked cilia functiona by cell deciliation using the mitotic inhibitor CH [13] that has been shown to cause loss of cilia in sea urchin embryos [27]. In both experimental conditions we observed that prolonged SS exposure of MDCK cells prevented cell dome reorganization observed in cells exposed to SS for similar time interval. In addition the block of flow stimulated Ca^{2+} entry also prevented changes in F-actin distribution and TJ organization induced by SS.

These results suggest that mechanical stimulation is selectively mediated by Ca^{2+} entry by ciliary function that reduce fluid and solutes transport, that in static condition sustain cell dome organization. The reduction in water and solute reabsorption allows cells to adhere again to the supporting surface. Of interest we observed cell expression of Na^+,K^+-ATPase at basolateral level in static condition as well as in cells exposed to SS, while in the later condition the protein was also located near the cell cilia. Which are the precise mechanisms responsible for flow-induced changes in the function of MDCK cells within the dome cannot be derived from our present experiments, however, some considerations to this regard can be made. It is known that under flow stimulation intracellular concentration of cAMP decreases [28, 29], and it is tempting to speculate that reduction of intracellular cAMP by SS exposure is responsible for the observed changes in dome structure. In addition, it is well known that in vascular endothelial cells, that are very sensitive to SS [30, 31], exposure to laminar flow induces an anti-

apoptotic effect [32]. The same phenomena may take place in MDCK cells forming domes under flow reverting the ongoing pre-apoptotic.

At variance to the anti apoptotic effect, the effect of flow exposure on actin stress fiber seems to be different between MDCK and endothelial cells. Thus, we and others [33] observed that expression of elongated stress actin fiber in MDCK cells in static condition is almost completely lost after prolonged exposure to SS. On the contrary, endothelial cells exposed to laminar flow *in vitro* increase expression of actin fibers aligned in the flow direction [34]. Why the response to SS on cytoskeleton organization is opposite in these two types of cells is not known, but it likely depends on different cell phenotype.

Another important effect of flow stimulation on MDCK cells was the reorganization of the TJ protein ZO-1. This effect is important for cell-cell communication and for the function of epithelial monolayer itself. To this regard flow exposure of endothelial cells is known to induce expression of junctional receptors such as PECAM, VE-caderin and VEGF receptor [35]. Mechanical stimulation on these receptors is transferred to adhesion complex by the cytoskeleton and results in shape change and activation of a number of genes that are responsible for mechanotransduction. Whether similar mechanisms are involved in the response of tubular epithelial cells was beyond the scope of our study but it is worth to investigate.

Our present data, and other evidence reported in literature [2, 36], strongly suggest that fluid flow acting on apical membrane of kidney tubular cell may be important for the maintenance of physiological cell structure and function. The level of SS used in our experimental conditions is expected to occur in the proximal kidney tubule, while water reabsorption along the nephron reduces tubular flow volume and consequently wall SS. Our observation that even SS as low as 0.4 dynes/cm^2 also induced dome disappearance during 6 hour perfusion suggest that tubular fluid motion may indeed influence tubular cell structure and function also in physiological conditions along the nephron. Which cellular processes may develop *in vivo* in epithelial cells when tubular flow is stopped by tubular obstruction, or by important changes in single nephron glomerular filtration rate and/or proximal tubular fluid reabsorption, is not yet know. Our data indicate that absence of fluid motion over cell surface may importantly alter adhesion of tubular cell to basal membrane, affect cytoskeleton organization, ion and water transport, as well as cell apoptosis. Thus, beside humoral factors, like pro-inflammatory and pro-fibrotic agents, also the simple arrest of mechanical stimulation on tubular epithelial cells may be responsible for tubular cell damage. This process may then be involved in acute and/or chronic changes of kidney function in renal diseases, and may be a target for future investigations with the attempt to decrease the impact of pathological process on kidney functional degeneration.

Elucidation of the mechanisms responsible for tubular cell functional changes induced by the absence SS signals may open new insights into the pathophysiology of PKD, a disease that is related to genetic abnormalities of previously mentioned cilia associated proteins. This disease often progresses inexorably to end-stage renal failure and there are no pharmacological treatments effective in reversing disease progression. As mentioned before, in patients affected by PKD it has been clearly shown that intracellular signals induced in normal conditions by primary cilia of tubular cells are importantly affected by genetic alterations of cilia associated proteins such as polycystin-1 and -2 [36, 37]. It has been reported that polycystin-1 is a component of desmosomal junction of MDCK cells, and this localization is common to most epithelia [38, 39]. Cell junctions are fundamental to maintain structural and functional organization of all epithelia and previously published data demonstrate that polycystin-1 is up regulated in domes as compared to cells in monolayer in pancreatic ductal epithelial cells [12], with a diffused cytosolic expression. The same pattern was observed in cystic disease [2]. Thus, the level of polycystin-1 expression is critical for normal function while both over expression and loss or reduction of the protein lead to cyst development. It is temping to speculate that in cells defective in cilia proteins, such as in PKD patients, the flow mediated physiological state of tubular cells may be altered predisposing to cell dysfunction by initiation of a cascade of events ultimately responsible for cysts formation in renal tubule. Our results indicate that for the study of cilia dysfunction on kidney cyst formation must take into consideration the effect of prolonged exposure to shear forces that derive from tubular fluid volume flow.

In conclusion, our present investigation show that structural organization in domes of confluent MDCK cells *in vitro* is completely reversed by fluid SS acting on cell surface for 6 hours. This effect is mediated by cell ciliary function, as Ca^{2+} blockade and cell deciliation completely prevent this phenomenon. Overall our results indicate that chronic mechanical stimulation importantly affects

structure, function and differentiation of kidney tubular epithelial cells, and suggest that alteration of mechanosensing of tubular cell may be involved in pathophysiological processes responsible for renal structural and functional changes.

Additional Material

Movie1.mov: Videomicroscopy time-lapse sequence of MDCK cell monolayer during exposure to fluid SS for 6 hours. Movie2.mov: Videomicroscopy time-lapse sequence of MDCK cell monolayer during 6 hour observation period in static condition. Movie3.mov: Videomicroscopy time-lapse sequence of MDCK cell monolayer during exposure to SS for 6 hours in presence of EGTA.

Acknowledgements

This study was supported in part by Fondazione CARIPLO (project I4BIO). Irene Cattaneo was recipient of a fellowship from "Comunità Montana Valle Seriana Albino, Italy". We thank Dr. Chiara Arrigoni for technical assistance, Dr. Norberto Perico for helpful discussion and Dr. Barbara Imberti for FACS analysis.

References

1 Latta H, Maunsbach AB, Madden SC: Cilia in different segments of the rat nephron. J Biophys Biochem Cytol 1961;11:248-252.

2 Nauli SM, Alenghat FJ, Luo Y, Williams E, Vassilev P, Li X, Elia AE, Lu W, Brown EM, Quinn SJ, Ingber DE, Zhou J: Polycystins 1 and 2 mediate mechanosensation in the primary cilium of kidney cells. Nat Genet 2003;33:129-137.

3 Praetorius HA, Spring KR: Bending the MDCK cell primary cilium increases intracellular calcium. J Membr Biol 2001;184:71-79.

4 Praetorius HA, Spring KR: The renal cell primary cilium functions as a flow sensor. Curr Opin Nephrol Hypertens 2003;12:517-520.

5 Praetorius HA, Spring KR: Removal of the MDCK cell primary cilium abolishes flow sensing. J Membr Biol 2003;191:69-76.

6 Borkan SC, Wang YH, Lieberthal W, Burke PR, Schwartz JH: Heat stress ameliorates ATP depletion-induced sublethal injury in mouse proximal tubule cells. Am J Physiol 1997;272:F347-355.

7 Rydholm S, Zwartz G, Kowalewski JM, Kamali-Zare P, Frisk T, Brismar H: Mechanical Properties of Primary Cilia Regulate the Response to Fluid Flow. Am J Physiol Renal Physiol 2010; in press.

8 Siroky BJ, Ferguson WB, Fuson AL, Xie Y, Fintha A, Komlosi P, Yoder BK, Schwiebert EM, Guay-Woodford LM, Bell PD: Loss of primary cilia results in deregulated and unabated apical calcium entry in ARPKD collecting duct cells. Am J Physiol Renal Physiol 2006;290:F1320-1328.

9 Hansson GC, Simons K, van Meer G: Two strains of the Madin-Darby canine kidney (MDCK) cell line have distinct glycosphingolipid compositions. EMBO J 1986;5:483-489.

10 Lever JE: Regulation of dome formation in kidney epithelial cell cultures. Ann N Y Acad Sci 1981;372:371-383.

11 Chang YH, Lin HH, Wang YK, Chiu WT, Su HW, Tang MJ: Activation of caspase-8 and Erk-1/2 in domes regulates cell death induced by confluence in MDCK cells. J Cell Physiol 2007;211:174-182.

12 Bukanov O. N. HH, Dackowski WR, Lawrence BD, Clow PA, Roberts BL, Klinger KW, Ibraghimov-Beskrovnaya O: Functional polycystin-1 expression is developmentally regulated during epithelial morphogenesis in vitro: downregulation and loss of membrane localization during cystogenesis. Human Molecular Genetics 2002;11:923-936.

13 Overgaard KMS, Krystle S. Spiczka, Sheff DR, Alexander S, Yeaman C: Deciliation is associated with dramatic remodeling of epithelial cell junctions and surface domains. Mol Biol Cell 2009;20:102-113.

14 Kotsis F, Nitschke R, Doerken M, Walz G, Kuehn EW: Flow modulates centriole movements in tubular epithelial cells. Pflugers Arch 2008;456:1025-1035.

15 Rydholm S, Frisk T, Kowalewski JM, Andersson Svahn H, Stemme G, Brismar H: Microfluidic devices for studies of primary cilium mediated cellular response to dynamic flow conditions. Biomed Microdevices 2008;10:555-560.

16 Morigi M, Zoja C, Figliuzzi M, Foppolo M, Micheletti G, Bontempelli M, Saronni M, Remuzzi G, Remuzzi A: Fluid shear stress modulates surface expression of adhesion molecules by endothelial cells. Blood 1995;85:1696-1703.

17 Morigi M, Galbusera M, Binda E, Imberti B, Gastoldi S, Remuzzi A, Zoja C, Remuzzi G: Verotoxin-1-induced up-regulation of adhesive molecules renders microvascular endothelial cells thrombogenic at high shear stress. Blood 2001;98:1828-1835.

18 Slizova D, Krs O, Pospisilova B: Alternative method of rapid drying vascular specimens for scanning electron microscopy. J Endovasc Ther 2003;10:285-287.
19 Zucchi I, Montagna C, Susani L, Vezzoni P, Dulbecco R: The rat gene homologous to the human gene 9-27 is involved in the development of the mammary gland. Proc Natl Acad Sci USA 1998;95:1079-1084.
20 Cereijido M, Ehrenfeld J, Fernandez-Castelo S, Meza I: Fluxes, junctions, and blisters in cultured monolayers of epithelioid cells (MDCK). Ann NY Acad Sci 1981;372:422-441.
21 Laughery MD, Clifford RJ, Chi Y, Kaplan JH: Selective basolateral localization of overexpressed Na-K-ATPase beta1- and beta2- subunits is disrupted by butryate treatment of MDCK cells. Am J Physiol Renal Physiol 2007;292:F1718-1725.
22 Wilson PD, Devuyst O, Li X, Gatti L, Falkenstein D, Robinson S, Fambrough D, Burrow CR: Apical plasma membrane mispolarization of NaK-ATPase in polycystic kidney disease epithelia is associated with aberrant expression of the beta2 isoform. Am J Pathol 2000;156:253-268.
23 Chakraborty M, Chatterjee D, Gorelick FS, Baron R: Cell cycle-dependent and kinase-specific regulation of the apical Na/H exchanger and the Na,K-ATPase in the kidney cell line LLC-PK1 by calcitonin. Proc Natl Acad Sci USA 1994;91:2115-2119.
24 Su HW, Yeh HH, Wang SW, Shen MR, Chen TL, Kiela PR, Ghishan FK, Tang MJ: Cell confluence-induced activation of signal transducer and activator of transcription-3 (Stat3) triggers epithelial dome formation via augmentation of sodium hydrogen exchanger-3 (NHE3) expression. J Biol Chem 2007;282:9883-9894.
25 Kennedy BG, Lever JE: Regulation of Na^+,K^+-ATPase activity in MDCK kidney epithelial cell cultures: role of growth state, cyclic AMP, and chemical inducers of dome formation and differentiation. J Cell Physiol 1984;121:51-63.
26 Zucchi I, Montagna C, Susani L, Montesano R, Affer M, Zanotti S, Redolfi E, Vezzoni P, Dulbecco R: Genetic dissection of dome formation in a mammary cell line: identification of two genes with opposing action. Proc Natl Acad Sci USA 1999;96:13766-13770.
27 Chakrabarti A, Schatten H, Mitchell KD, Crosser M, Taylor M: Chloral hydrate alters the organization of the ciliary basal apparatus and cell organelles in sea urchin embryos. Cell Tissue Res 1998;293:453-462.
28 Masyuk AI, Masyuk TV, Splinter PL, Huang BQ, Stroope AJ, LaRusso NF: Cholangiocyte cilia detect changes in luminal fluid flow and transmit them into intracellular Ca^{2+} and cAMP signaling. Gastroenterology 2006;131:911-920.
29 Thomas SR, Schultz SG, Lever JE: Stimulation of dome formation in MDCK kidney epithelial cultures by inducers of differentiation: dissociation from effects on transepithelial resistance and cyclic AMP levels. J Cell Physiol 1982;113:427-432.
30 Hahn C, Schwartz MA: Mechano-transduction in vascular physiology and atherogenesis. Nat Rev Mol Cell Biol 2009;10:53-62.
31 Davies PF: Hemodynamic shear stress and the endothelium in cardiovascular pathophysiology. Nat Clin Pract Cardiovasc Med 2009;6:16-26.
32 Wu CC, Li YS, Haga JH, Kaunas R, Chiu JJ, Su FC, Usami S, Chien S: Directional shear flow and Rho activation prevent the endothelial cell apoptosis induced by micropatterned anisotropic geometry. Proc Natl Acad Sci USA 2007;104:1254-1259.
33 Duan Y, Gotoh N, Yan Q, Du Z, Weinstein AM, Wang T, Weinbaum S: Shear-induced reorganization of renal proximal tubule cell actin cytoskeleton and apical junctional complexes. Proc Natl Acad Sci USA 2008;105:11418-11423.
34 Girard PR, Nerem RM: Shear stress modulates endothelial cell morphology and F-actin organization through the regulation of focal adhesion-associated proteins. J Cell Physiol 1995;163:179-193.
35 Tzima E, Irani-Tehrani M, Kiosses WB, Dejana E, Schultz DA, Engelhardt B, Cao G, DeLisser H, Schwartz MA: A mechanosensory complex that mediates the endothelial cell response to fluid shear stress. Nature 2005;437:426-431.
36 Nauli SM, Rossetti S, Kolb RJ, Alenghat FJ, Consugar MB, Harris PC, Ingber DE, Loghman-Adham M, Zhou J: Loss of polycystin-1 in human cyst-lining epithelia leads to ciliary dysfunction. J Am Soc Nephrol 2006;17:1015-1025.
37 Weimbs T: Polycystic kidney disease and renal injury repair: common pathways, fluid flow, and the function of polycystin-1. Am J Physiol Renal Physiol 2007;293:F1423-1432.
38 Scheffers MS, van der Bent P, Prins F, Spruit L, Breuning MH, Litvinov SV, de Heer E, Peters DJ: Polycystin-1, the product of the polycystic kidney disease 1 gene, co-localizes with desmosomes in MDCK cells. Hum Mol Genet 2000;9:2743-2750.
39 Russo RJ, Husson H, Joly D, Bukanov NO, Patey N, Knebelmann B, Ibraghimov-Beskrovnaya O: Impaired formation of desmosomal junctions in ADPKD epithelia. Histochem Cell Biol 2005;124:487-497.