Functional polycystin-1 expression is developmentally regulated during epithelial morphogenesis in vitro: downregulation and loss of membrane localization during cystogenesis

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Polycystin-1 is a protein mutated in the majority of cases of autosomal dominant polycystic kidney disease (ADPKD), but its role in the molecular pathway of tubulogenesis and cystogenesis is not understood. To define the role of polycystin-1 during dynamic changes in formation of intercellular contacts and cell polarity accompanying epithelial morphogenesis, we have utilized a 3D MDCK in vitro model of tubulogenesis and cystogenesis. Here we demonstrate that polycystin-1 is a novel component of desmosomal junctions of epithelial cells. A striking downregulation of polycystin-1 mRNA was detected in cysts as compared to tubules, leading to altered protein expression and localization. While polycystin-1 is localized to basolateral membranes of MDCK tubules, it is only detected in cytoplasmic pools in cystic cells. Furthermore, the expression of polycystin-1 is modulated during distinct stages of HGF-induced tubulogenesis from MDCK cysts. Thus, polycystin-1 is not detected in intercellular contacts at early steps of tubulogenesis, but assumes its basolateral localization at the time of cell polarization and lumen formation. An important role of polycystin-1 is further demonstrated using the pancreatic ductal epithelial cell line SU.86.86 which undergoes in vitro differentiation resulting in the formation of domes. Dome formation is thought to parallel tubular differentiation and morphogenesis in vivo. Our data reveal significant upregulation of polycystin-1 mRNA and protein levels in domes. Collectively, our results demonstrate a critical importance of controlled level of polycystin-1 expression for proper tubular differentiation and maturation. We suggest that the loss of polycystin-1 from its basolateral location in tubular epithelium may alter critical pathways controlling normal tubulogenesis leading to cystic transformation.

INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is a common genetic disorder accounting for 8–10% of all cases of end stage renal disease (1,2). At least 85% of cases of ADPKD can be attributed to the mutations in the PKD1 gene encoding polycystin-1 (3,4). The remaining 15% of ADPKD cases are caused by a mutation in the PKD2 gene encoding polycystin-2 (5). The main feature of the disease is the formation and progressive growth of fluid-filled cysts in the kidney nephrons as well as in the ductal epithelia of other organs such as the liver, pancreas and spleen. The process of cystogenesis, whether caused by the mutations in the PKD1 gene or by mutations in a number of non-allelic genes, appears to be similar and is characterized by abnormal proliferation of epithelial cells lining the cyst cavity, intracystic fluid secretion, altered cellular polarity and protein sorting and thickening of basement membrane (reviewed in refs 6 and 7). The role of polycystin-1 in the process of cystic transformation has not been defined.

Polycystin-1 is a large transmembrane protein with several motifs in the extracellular domain: two leucine-rich repeats (LRR), a C-type lectin domain, an LDL-A domain, multiple Ig-like domains (or PKD domains) and a REJ domain (8,9). This unique arrangement of adhesive motifs suggested an important role in cell–cell/matrix interactions (8). We have recently demonstrated that polycystin-1 can mediate cell–cell adhesion through the homophilic interaction of its Ig-like domains (10). This arrangement of adhesive motifs suggested an important role in cell–cell/matrix interactions (8). We have recently demonstrated that polycystin-1 can mediate cell–cell adhesion through the homophilic interaction of its Ig-like domains (10). We have also shown that its membrane localization requires functional activity of tuberin, a product of the TSC2 gene (11).
Important clues to the role of polycystin-1 in normal and cystic state came from studies of knock-out mouse models suggesting that polycystin-1 is important for tubular morphogenesis and maturation (12,13). Not only did reduction or loss of functional protein lead to cystic development, but over-expression of polycystin-1 in transgenic mice resulted in a cystic phenotype, suggesting that the level of polycystin-1 expression is critical for normal function (14).

Polycystin-1 is predominantly expressed in ductal epithelial cells. Several studies aimed at examining the role of polycystin-1 in cell–cell adhesion yielded conflicting results. Localization of polycystin-1 to tight junctions, adherence junctions or desmosomes have been reported, depending upon the reagents and experimental system used (15-17). Efforts to define the expression and localization of polycystin-1 during cystogenesis and tubulogenesis have been hampered by the lack of species-specific molecular tools. Cyst formation by Madin-Darby canine kidney cells (MDCK) in 3D matrix in vitro is an attractive system for the study of molecular mechanisms of cystogenesis (18), but the role of polycystin-1 in this model was difficult to address due to the lack of canine-specific reagents. To be able to study the role of polycystin-1 in tubulogenesis and cystogenesis using MDCK cells, we have cloned the canine Pkd1 gene (Dackowski et al., submitted). Comparative analysis of human and dog polycystin-1 demonstrated a differential degree of conservation among specific domains ranging from 97% identity for LRR repeats down to 62% identity for the LDL-A motif and allowed selection of a highly specific antibody for localization studies (Dackowski et al., submitted).

Using canine-specific molecular reagents, we show herein that the Pkd1 gene in MDCK cells is not duplicated and is transcribed into a 14 kb mRNA. Immunofluorescent staining coupled with confocal analysis demonstrated localization of polycystin-1 to desmosomal junctions in MDCK and other epithelial cell lines which is in agreement with Scheffers et al. (17). We report for the first time differential expression and localization of polycystin-1 during in vitro morphogenesis and differentiation which is regulated at the transcriptional level. A striking downregulation of PKD1 mRNA is detected in MDCK cysts as compared to tubules, resulting in a significant reduction of membrane bound polycystin-1 and mislocalization to the cytoplasmic pools. We also describe the upregulation of polycystin-1 during in vitro tubular differentiation of pancreatic epithelial cells. Taken together, our data demonstrate that localization at particular segments of membrane together with a controlled level of polycystin-1 expression define the outcome for morphogenetic pathway to either normal tubulogenesis or abnormal cystic transformation.

RESULTS

Canine Pkd1 gene is not duplicated and is transcribed into 14 kb mRNA in MDCK cells

It has been debated whether polycystin-1 is endogenously expressed in MDCK cells. We and others have demonstrated that polycystin-1 is localized to the lateral membranes of MDCK monolayers (10,17), but there were also reports on the lack of immunodetectable endogenous polycystin-1 (19). To overcome the problem of conflicting results obtained by employing human-specific reagents, we have cloned and characterized dog Pkd1 gene (Dackowski et al., submitted). First, we carried out fluorescence in situ hybridization (FISH) of dog genomic DNA to cultured MDCK cells. As shown in Figure 1A, we detected additional mRNA species (~8.5 kb) in human-derived cells (3). Thus, polycystin-1 mRNA is indeed expressed by MDCK cells and the level of expression is relatively low, which is comparable to the level of polycystin-1 mRNA expression described for other mammalian tissues and cell lines (3,11,14).

Subcellular localization of polycystin-1 in epithelial cells of different origin

Using human polycystin-1 domain-specific antibodies, we and others have previously demonstrated the lateral membrane localization of polycystin-1 in epithelial MDCK cells as well as in endothelial cells (10,17,20,21). However, conflicting results have been reported regarding subcellular localization for polycystin-1 with reports of tight junction (TJ) (15), adherence junction (AJ) (16) or desmosomal junctions (Dj) (17). To overcome the potential problem of interspecies antibody reactivity, we have performed a domain-specific comparative analysis of human polycystin-1 with our newly identified canine polycystin-1 (Dackowski et al., submitted) and selected anti-LRR antibody for subsequent subcellular analysis based on a striking 97% identity between human and dog LRR domains. We have used dual immunofluorescence staining to examine colocalization of polycystin-1 in MDCK cells with ZO-1 as a
Figure 2. Subcellular localization of polycystin-1 in epithelial cells. (A) Co-localization of polycystin-1 with junctional markers TJ and DJ in MDCK cells. Polycystin was stained with anti-LRR antibody (green). Staining of TJ (ZO-1) and DJ (desmoplakin) is in red. Colocalization patterns are shown in yellow (merged image). Discordant pattern of localization is seen for polycystin-1 and ZO-1 dual staining. In contrast, complete colocalization is detected for polycystin-1 and desmoplakin. (B) Colocalization of polycystin-1 (green) with desmoplakin (red) or ZO-1 (red) in human pancreatic ductal cell line SU.86.86. Merged images show colocalization patterns for polycystin-1 and desmoplakin, but not ZO-1. (C) Localization of polycystin-1 relative to AJ components of MDCK cells. Dual staining was performed with polycystin-1 anti-LRR antibodies (green) and antibodies against four different component of AJ (E-cadherin, β-catenin, γ-catenin and pp120; red color). Merged images with E-cadherin, β-catenin and pp120 produced not overlapping patterns of green and red. In dual staining with γ-catenin, a punctate yellow pattern can be seen suggesting partial overlapping. This partial overlapping is due to the presence of γ-catenin in both AJ and DJ.
TJ marker and with desmoplakin as a DJ marker (Fig. 2A). As shown in Figure 2A, no colocalization was detected with ZO-1 protein, but concordant staining was obtained for polycystin-1 and desmoplakin. Specifically, as shown by artificial pixel shift analysis in Figure 2A, a complete point-by-point colocalization can easily be seen for polycystin-1 and desmoplakin. These data agree with a recent report (17). To determine whether polycystin-1 localization to desmosomes is unique to MDCK cells, we have tested another epithelial cell line, SU.86.86, of human pancreatic ductal origin. As shown in Figure 2B, dual staining with polycystin-1 antibody (anti-LRR) and desmoplakin antibody produced a punctate overlapping pattern, while no colocalization was seen with the ZO-1 marker of TJ. In addition we have analyzed yet another epithelial cell line, LLC-PK1 of proximal tubular origin and obtained the same results (not shown). Thus, desmosomal localization for polycystin-1 seems to be a common feature for epithelial cells.

Analysis of polycystin-1 localization in adherence junctions

It has been hypothesized that the defects in cell proliferation and polarity observed in ADPKD can be mediated by E-cadherin or the catenins (22). Although polycystin-1 was precipitated in a complex containing E-cadherin and catenins, polycystin-1 and E-cadherin did not colocalize completely by immunostaining, suggesting that there were pools of polycystin-1 that did not interact with E-cadherin (22). To determine whether polycystin-1 or some pools of it can be detected in AJ, we have performed detailed colocalization studies with several components of AJ as shown in Figure 2C.
Figure 3. Differential expression of polycystin-1 in MDCK cysts and tubules. (A) Confocal localization of polycystin-1 and ZO-1 is shown in mature tubule. Single-channel images shown in black-and-white, merged images shown in dual colors. Dual staining is shown on the merged image with polycystin-1 in green and ZO-1 in red. Basolateral membrane (BM) and tubular lumen (L) are indicated. Note basolateral localization of polycystin-1. (B) Confocal analysis of dual staining of cyst with anti-polycystin-1 (green) and desmoplakin-1 (Des, red) antibodies. Locations of apical membrane (AM), basolateral membrane (BM) and cystic lumen (L) are shown. Note diffuse mostly cytosolic localization of polycystin-1 in cysts. (C) Schematic diagram of tubulogenesis in vitro drawn according to (25). (D) and (E) dynamic changes in polycystin-1 expression/distribution during early stages of tubulogenesis: formation of chains and cords respectively. Images represent the single confocal sections taken from series of confocal images capturing transformation of preformed cysts into tubules upon HGF induction. Dual staining represents the location of polycystin (green) and ZO-1 (red). The red color depicts the location of de novo forming discontinuous lumens (TL) of the tubular cords and separate lumen of the cyst (CL). Note polycystin-1 up-regulation close the regions of cell–cell contacts of forming cords contrasting with relatively weak predominantly cytoplasmic expression of polycystin-1 inside the cells comprising the cystic wall.
A punctate pattern of polycystin-1 staining differed from a rather continuous pattern for E-cadherin staining, but some regions of overlap could be observed on the merged image (Fig. 2C, top panel). Similarly, a polycystin-1 colocalization study with β-catenin and with pp120 demonstrated non-overlapping patterns (Fig. 2C). A combination of overlapping and distinct regions of localization was found for one more AJ component—γ-catenin. It is not surprising since γ-catenin (or plakoglobin) is also found in a complex with desmosomal cadherins and is involved in anchoring intermediate filaments to desmosomal plaques (23). These results were extended to other epithelial cell lines, SU.86.86 and LLC-PK1 (not shown). Taken together, our data indicate that polycystin-1 is a component of desmosomal, but not adherence or tight junctions in epithelial cells, however a transient interaction of polycystin-1 with AJ can not be excluded.

Polycystin-1 is differentially expressed during in vitro cystogenesis and tubulogenesis

To determine how the localization of polycystin-1 to desmosomes in polarized MDCK cells is affected during in vitro morphogenesis, we analysed the subcellular distribution of polycystin-1 in 3D cultures. MDCK cells form fluid-filled cysts when cultured in collagen I gels. When treated with hepatocyte growth factor (HGF), MDCK cells embedded in collagen matrix undergo morphological differentiation into tubular structures (24). It has been demonstrated that HGF-induced tubulogenesis causes differential rearrangements of cellular adhesive junctions with random surface distribution for E-cadherin and intracellular accumulation of desmoplakins (25).

We hypothesized that if polycystin-1 plays an important role in intercellular adhesion in the normal (tubular) state and is a key molecule in the cystic transformation pathway, then its expression/localization status is likely to be affected during in vitro morphogenesis. We have examined polycystin-1 localization in mature tubules formed by MDCK cells using confocal microscopy. A clear basolateral pattern of polycystin-1 localization in tubules was accompanied by weak cytoplasmic staining (Fig. 3A). The same sample was counterstained for a TJ marker (ZO-1) to delineate the luminal side of the tubule as shown in Figure 3A. The basolateral polycystin-1 localization in MDCK tubules is in agreement with normal tissue localization studies of ductal epithelia in kidney, pancreas, liver and other organs (20,26–28).

In a similar manner, we next examined subcellular localization of polycystin-1 in MDCK fully formed cysts. Surprisingly, instead of the basolateral membrane staining for polycystin-1 which is characteristic for MDCK tubules, we detected diffuse intracellular expression in cysts (Fig. 3B). A non expected lateral localization for desmoplakin was observed. The staining of polycystin-1 seems to be much brighter in tubules as compared to cysts. Therefore, we have demonstrated for the first time that the status of polycystin-1 is different in MDCK tubules (strong basolateral) versus cysts (diffuse cytoplasmic).

To assess the dynamic change in polycystin-1 expression/distribution during morphogenesis in vitro, we have addressed the status of polycystin-1 in intermediate stages of tubule development. Several stages of tubulogenesis in vitro have been described (24,25) as shown in Figure 3C. Upon HGF treatment of a preformed cyst in a 3D gel, extensions or protrusions are formed from individual cells comprising the cystic wall. Next, the linear or branching chains of cells connected to the cyst are

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Figure 4. Real time PCR analysis of polycystin-1 mRNA expression in MDCK cysts and tubules. The expression levels were normalized to β-actin. The results are shown as the mean and SD of three independent determinations.

Figure 5. Analysis of membrane polarity in MDCK cysts. (A) Basolateral localization of Na⁺/K⁺-ATPase. Basolateral (BM), apical membrane (AM) and lumen (L) are indicated. Arrow shows immunostaining of basolateral membrane with anti Na⁺/K⁺ antibody (red), as expected for appropriate epithelial polarity. (B) Mislocalization of EGFR in MDCK cysts. Arrow depicts strong apical localization of EGFR (red) in cysts as shown by an arrow. Images on the right of each panel show dual staining with DAPI to indicate nuclei.
initiated followed by formation of cord with discontinuous lumens. Finally, the tubule is developed when discontinuous lumens fuse. It was reported that intercellular adhesion is maintained during tubulogenesis, but TJ, AJ and DJ components are differentially regulated (25). To analyze the status of polycystin-1 during HGF induced tubulogenesis, we collected stacks of immunofluorescent images of 0.4 μm confocal sections for early stages of tubulogenesis, namely formation of chains and cords (Figs 3D and E). Representative optical sections of the dual staining with TJ (ZO-1; red) and polycystin-1 (green) are shown in Figures 3D and E. As expected at the stage of chains and cords formation, ZO-1 remained at sites of cell–cell contacts and can be clearly seen at sites of polarization and de novo formation of discontinuous lumens (Fig. 3E). As shown in Figure 3E, polycystin-1 was intracellularly distributed in the cells of the cystic wall, but appeared enhanced towards the sites of cell–cell contacts of extending cords, particularly where tubular lumens were forming. This suggests that polycystin-1 is important for intercellular adhesion during tubule formation, maturation and maintenance of tubular structures.

Downregulation of polycystin-1 in cysts occurs at transcriptional level

A dramatic difference in polycystin-1 expression in tubules versus cysts can be explained by its redistribution from basolateral location in the normal state to cytoplasmic in the cystic state. Alternatively, the lack of polycystin-1 at basolateral site of cystic cells can be due to general downregulation of its expression.

To address differential expression of polycystin-1 during in vitro morphogenesis of canine MDCK cells at the transcriptional level, we have recently cloned and characterised the dog PKD1 gene. Using canine-specific primers, we have performed real-time PCR analysis to quantitatively compare polycystin-1 mRNA expression levels in cysts versus tubules (Fig. 4). As a result, a downregulation of approximately 50% of polycystin-1 mRNA in cysts was detected. Therefore, the regulation of polycystin-1 expression during tubulogenesis and cystogenesis occurs at the transcription level.

Abnormal expression of membrane markers in MDCK cysts parallels alterations in protein sorting in ADPKD

It has been reported that ADPKD is characterized by abnormalities in epithelial protein sorting machinery that lead to mislocalization and abnormal expression of several membrane proteins including Na+/K+ ATPase and epidermal growth factor receptor (EGFR) in addition to its primary defect in polycystin-1 (29–32). Since we also determined abnormal expression of polycystin-1 in MDCK cysts, it is likely that MDCK cyst formation parallels that of ADPKD in...
Figure 7. Differential expression of cell-cell junctional markers in SU.86.86 domes. (A) Expression of ZO-1 marker of TJ in SU.86.86. domes. The top panel shows XY projections of ~40 μm stack; dotted lines indicate the position of cells forming domes. XY projections show equal level of ZO-1 expression in monolayer and domes. Z projections support this finding. The representative Z sections show the polarity of cells in domes and monolayer. The bottom panel shows enlarged views of the Z sections; basolateral membrane (BM) is underlined by a dotted line; arrowheads depict the location of TJ facing the medium. Basolateral (BM), apical membrane (AM) and dome fluid-filled cavity (C) are shown. (B) Northern blot analysis of ZO-1 mRNA expression (shown by an arrow) in human fetal kidney (K), SU.86.86. domes (D) and monolayers (M). Left panel indicates equal loadings of RNA samples stained by ethidium bromide. (C) Both polycystin-1 and E-cadherin are upregulated in domes. Images were obtained from ~40 μm of confocal stacks to show XY projections (top panel). Merged images indicate polycystin-1 staining in green and E-cadherin in red. The overexpression of E-cadherin and polycystin-1 is clearly indicated on XY projections and Z projections, however, no colocalization is observed by Z section. (D) Northern blot analysis of E-cadherin (arrow) expression confirms its mRNA overexpression in SU.86.86 domes (D) as compared to monolayers (M). RNA from human fetal kidney (K) was used as a control. Left panel indicates equal loadings of blotted RNA samples stained by ethidium bromide. (E) Expression of desmplakin in SU.86.86 domes. XY and Z projection show unchanged level of expression in SU.86.86 monolayers and domes. (F) Northern blot analysis of desmplakin mRNA (arrow) expression in human fetal kidney (K), SU.86.86 domes (D) and monolayers (M). Left panel indicates equal loadings of RNA samples stained by ethidium bromide.
other aspects as well. A mislocalization of Na\(^+\)/K\(^+\) ATPase from basolateral surface to the apical membrane of ADPKD cysts was reported (29). However, no evidence of apical localization for Na\(^+\)/K\(^+\) ATPase was found by other investigators (33). We stained for Na\(^+\)/K\(^+\)-ATPase in MDCK cysts and a clear and exclusive basolateral localization was detected (Fig. 5A). These data are in agreement with previous observations (34). In contrast to Na\(^+\)/K\(^+\) ATPase, EGFR appears to be mislocalized to the apical membrane in MDCK cysts (Fig. 5B), which parallels the finding of EGFR mislocalization and abnormal function in ADPKD cysts (32).

Formation of dome-like structures by epithelial cells SU.86.86 is accompanied by up-regulation of polycystin-1 expression

Studying polycystin-1 expression in the human pancreatic ductal cell line SU.86.86 as described above, we have noticed that this cell line can undergo differentiation in vitro. Specifically, postconfluent cultures form fluid filled domes or blisters. Domes are formed by the detachment of the cell layer from the substratum and asymmetric transcellular transport of water and ions (35). The phenomenon of dome formation has been described for several ductal epithelial cell lines and the
development of domes can be accelerated by elevated serum concentration, cAMP or its analogues, DMSO and hydrocortisone (36). The formation and morphology of SU.86.86 domes induced by elevated serum concentration is shown in Figure 6A. It has been shown that the ability of domes to accumulate fluid requires modulation and tightening of intercellular junctions (36). Since polycystin-1 may play an important role in cell–cell adhesion, we evaluated the expression of polycystin-1 in domes versus monolayers of SU.86.86 cells (Fig. 6B). A striking overexpression of polycystin-1 in domes as compared to monolayers was detected by immunofluorescence. As shown in Figure 6B, a very bright polycystin-1 staining in red readily delineates the location of dome even under low (10×) magnification. Polycystin-1 is localized to desmosomes in monolayers and its membrane staining intensifies in the cells positioned close to the edge of the dome (Fig. 6B, panel a). An intense cytoplasmic staining in addition to the membrane staining is detectable in the center of the dome (Fig. 6B, panel b). To determine whether upregulation of expression of polycystin-1 in domes occurs at the transcription level, we have performed Northern blot analysis. As shown in Figure 6C, polycystin-1 14 kb mRNA is upregulated 3-fold in domes (D) versus monolayers (M). The level of expression in human fetal kidney (K) was used as control.

Modulation of polycystin-1 expression relative to intercellular junctions and cell polarity during in vitro differentiation into dome structures

To investigate how intercellular junctions and cell polarity are modulated during formation of domes in the context of enhanced polycystin-1 expression, we performed confocal laser scan analysis of domes stained with anti-polycystin-1 antibody and counterstained with either E-cadherin, desmoplakin or ZO-1 antibodies. Recent studies demonstrated that dome formation strictly correlates with the presence of intact TJ seals (37). We investigated if the polarity of SU.86.86 cells was affected during in vitro differentiation into domes by localization studies of ZO-1 marker of TJ. The localization and level of expression of the ZO-1 marker in young and mature domes are shown in Figure 7A. When viewed in the X–Y axis (i.e. a section parallel to the plane of the support), the ZO-1 expression levels are indistinguishable between monolayer and dome structures. Further analysis of Z projection (image stacks of Z-sections) demonstrated equal distribution of ZO-1 label in domes and monolayers. Northern analysis supported this finding demonstrating approximately equal levels of ZO-1 mRNA in monolayers and domes (Fig. 7B). Importantly, the polarity of cells during in vitro differentiation is not changed as shown by the Z-section in Figure 7A. Confocal X–Z section views of the TJ marker ZO-1 indicated that the apical surface of cells comprising domes is facing the medium.

Upregulation of E-cadherin in a mammary cell line during dome formation has been reported (38). We have addressed the expression and localization of E-cadherin relative to polycystin-1 in SU.86.86 domes. Interestingly, we have also detected an increased level of E-cadherin expression in domes versus monolayers as shown in Figure 7C. X–Y projection and Z projection of dual staining for E-cadherin and polycystin-1 revealed concentration of immunostaining in dome structures, but no colocalization of these markers was observed by Z section (Fig. 7C). Northern analysis has demonstrated an increased level of E-cadherin mRNA in domes versus monolayers (Fig. 7D).
Staining for a DJ marker desmoplakin showed no increase in desmoplakin deposition in domes (Fig. 7E). These data were in agreement with mRNA expression analysis, demonstrating unchanged level of expression for desmoplakin during dome formation (Fig. 7F). Upregulation of polycystin-1 in domes results in increase of cytoplasmic distribution, but colocalization of polycystin-1 with desmoplakin at sites of cell-cell contacts remains unchanged (not shown).

**DISCUSSION**

The epithelium lining ADPKD cyst has been extensively studied and major abnormalities were revealed, including increased proliferation and apoptosis, changes in cellular polarity, abnormal fluid secretion and matrix composition (reviewed in (6,39,40)). The role of polycystin-1 in molecular cystogenesis has not been defined, but several lines of evidence suggest that polycystin-1 is an important factor in epithelial cell morphogenesis. The analysis of temporal and spatial patterns of polycystin-1 expression in normal and cystic tissues suggested an important role for polycystin-1 in epithelial cell differentiation and maturation (20,41–43). The role of polycystin-1 in normal morphogenesis during elongation and maturation of tubular structures was corroborated by Pkd1 gene knock-out studies (12,15). Recently, we have demonstrated that polycystin-1 plays an important role in cell–cell adhesion through homophilic interactions of its Ig-like domains (10). Collectively, these data point to polycystin-1 as a key player in normal tubular epithelial morphogenesis where coordinate regulation of cell–cell adhesion and migration is crucial.

Here we addressed the dynamic role of polycystin-1 in the context of complex cellular rearrangements during cystogenesis and tubulogenesis in vitro. We have chosen the MDCK model which reflects many properties of polarized epithelium in vivo as it undergoes differentiation and morphogenesis. Although we and others have previously demonstrated expression of polycystin-1 at sites of cell–cell contacts of MDCK monolayers (10,42), conflicting data were reported regarding its subcellular localization. Polycystin-1 was reported to be a component of tight junction (TJ) (15), adherence junction (AJ) (22) or desmosomal junction (DJ) (17). One explanation for these discrepancies could be the use of different cell types in distinct stages of differentiation. It is also possible, that the use of human-specific antibodies on other species can be limited if homologies between particular human and canine epitopes are low. To resolve the issue of antibody interspecies crossreactivity, we have cloned the canine Pkd1 gene (Dackowski et al., submitted). Herein we show that polycystin-1 mRNA is expressed by MDCK cells. Comparative analysis between human and dog polycystin-1 allowed for delineation of the most conserved domains. A striking degree of conservation (97% identity) was determined for the LRR domain between dog and human, while the C-terminal domain demonstrated 84% identity (Dackowski et al., submitted). The lowest degree of homology of 62% was found for LDL-A motif. These data can explain controversial subcellular localization results. Therefore, we have chosen to use the anti-LRR antibody to follow the fate of polycystin-1 in MDCK cells.

Structural and functional organization of polarized epithelia is established and maintained by several junctional complexes (44–46). We have now demonstrated that polycystin-1 is localized to the desmosomal junctions of MDCK cells. No evidence of TJ or AJ location for polycystin-1 was obtained. We have also shown that DJ localization of polycystin-1 is not unique for MDCK cells, but is common for several types of epithelial cells. The adhesion receptors of the desmosomes consist of desmogleins and desmocollins of the cadherin superfamily. They are involved in intercellular adhesion, but unlike classical E-cadherin, neither molecule can confer strong homophilic adhesion in vitro (47). Expression of desmosomal cadherins in different combinations resulted in little or no aggregation of fibroblastic L-cells, while cells expressing E-cadherin exhibited strong aggregation (48). It is possible that polycystin-1 may provide strong intercellular adhesion in DJ through homophilic interactions of its Ig-like repeats (10). It is also possible that polycystin-1 localization with desmosomes is important for signaling functions as suggested by Scheffers et al. (17). The role of polycystin-1 in signal transduction has not been defined, but the C-terminal tail of polycystin-1 evidently can modulate Wnt signaling (49). The identification of desmosomal proteins that might directly interact with polycystin-1 will be the focus of future study.

Because intercellular adhesion junctions rearrange during normal organogenesis as well as in pathologic conditions, we addressed the status of polycystin-1 expression during morphogenesis in vitro. MDCK cells form cysts in 3D collagen gel matrix and differentiate into tubules in the presence of HGF, making it a convenient in vitro model of tubulogenesis and cystogenesis (24). However, the correlation between MDCK cystogenesis and the cystic transformation in ADPKD has not been addressed. Not only do mutations in the Pkd1 gene itself lead to a cystic change, but mutations in a number of non-allelic genes as well as cystogenic chemicals can lead to a development of cystic phenotype. If polycystin-1 plays a key role in cystic transformation of any etiology, its expression can be affected not only by direct mutation, but can also be altered indirectly once a cystic pathway has been triggered. Therefore, we hypothesized that polycystin-1 expression in MDCK cysts and tubules must differ. Indeed, we have found a dramatic difference in polycystin-1 expression and localization in MDCK cysts as compared to tubules. First of all, PKD1 mRNA expression in cysts was downregulated up to 50% relative to that in tubules as revealed by real-time PCR analysis. This was accompanied by aberrant expression/localization of polycystin-1 in cells comprising cysts. We have shown that in tubules polycystin-1 is expressed in the basolateral membrane, while in cysts only diffuse cytoplasmic localization was observed. A similar pattern of polycystin-1 expression was previously observed in vivo (surface expression in normal kidney versus cytoplasmic in cystic kidney) (28). Basolateral localization for polycystin-1 correlates with sites of normal tissue expression not only in ductal epithelia of the kidney, but in pancreas and liver as well (20,27,50). Polycystin-1 expression in ADPKD cysts is variable with some cysts positive and some completely negative (reviewed in ref. 43). It is possible that cells in ADPKD may be predisposed to a cystic change if protein from one allele is lost (~50% of normal expression) and cystic pathway is triggered focally by...
chance (51). Cysts can also form by a ‘second hit’ mechanism and loss of heterozygosity as was demonstrated for a proportion of ADPKD cysts (52). Our findings of upregulation of polycystin-1 expression during in vitro tubulogenesis correlate with reported induction of Pkd1 expression in maturing tubular epithelia from day E15.5 of mouse embryo suggesting a role in tubule maturation (13). This is corroborated by the data that overexpression of exogenous human Pkd1 mRNA in MDCK cells resulted in formation of tubules rather than cysts in collagen gels (19). We have tested the expression of polycystin-1 during initial stages of tubular transformation in vitro. The development of tubules from MDCK cysts can be separated into four stages: formation of cell extensions (stage 1), formation of chains of cells with a transient loss of cell polarity (stage 2), development of cords two to three cells in diameter where discontinuous lumens begin to appear (stage 3), and finally formation of a mature tubule with a continuous lumen (stage 4) (25). Interestingly, we could clearly see membrane localization of polycystin-1 rather late in morphogenesis during cord formation and tubular maturation. Polycystin-1 was not highly expressed during early stages of tubulogenesis (extension and chain formation) while strong E-cadherin expression at sites of cell–cell and cell–matrix contacts at these very stages was described (25). Therefore, we have detected a discordant pattern of E-cadherin and polycystin-1 expression/localization during tubulogenesis, which provides further evidence that these two molecules function independently. In contrast, a coordinate expression for desmoplakin and polycystin-1 in intracellular pools was apparent. As polarization of tubular cells occurs and lumens start to form polycystin-1 assumes its basolateral localization. These observations strongly suggest the role of polycystin-1 in tubular maturation.

We also report for the first time the up-regulation of polycystin-1 during epithelial differentiation in vitro resulting in dome formation in pancreatic ductal epithelial cells SU.86.86. This process occurs by the focal detachment of the cell monolayer through a transcellular transport of water and ions (35,36). The asymmetrical transport of solutes in domes is related to the ability of the cells originating from ductal epithelia to resorb ions and water from the lumen of a duct (35). We have confirmed that the polarity of cells comprising the dome is not reversed, but is colinear with the monolayer such that the apical surface is facing media. It has been shown previously that molecular mechanisms regulating formation of domes correspond with those involved in ductal epithelial differentiation. Thus, Zucchi et al. described the upregulation of several genes including cytokeratin 8 and E-cadherin in domes formed by a mammary epithelial cell line which correlated with the differentiation process of the mammary gland in vivo (38,53). The ability to accumulate fluid underneath the dome suggests the tightening of the lateral intercellular junctions that slow down paracellular fluid movement. The analysis of intercellular junctions in SU.86.86 culture has shown upregulation of both E-cadherin and polycystin-1 in domes as compared to monolayers. This suggests that similar to the role of E-cadherin in regulating AJ, polycystin-1 modulates DJ during in vitro differentiation.

Collectively, our data indicate that a combination of controlled level of polycystin-1 expression with basolateral localization is required for proper tubular differentiation and maturation. We propose that the downregulation of polycystin-1 expression and loss of protein from its basolateral location may trigger changes in cellular adhesion/polarity leading to cystic transformation.

In conclusion, we have shown dynamic changes in expression and distribution of polycystin-1 during morphogenesis in vitro. We have also demonstrated mislocalization of EGFR to the apical membranes of MDCK cysts, a hallmark of cystogenesis in vivo (31,32). Thus, our data suggest that the MDCK in vitro model of cystogenesis and tubulogenesis adequately reflects some aspects of normal and pathologic state of tubular epithelia in vivo thus providing a foundation for discovery and testing of potential therapeutics designed to block cystogenesis.

MATERIALS AND METHODS

Cell culture

MDCK and human pancreatic ductal carcinoma cell line SU.86.86. (ATCC, Rockville, Maryland) were grown in MEM/10%FBS or RPMI/10%FBS respectively. Fibroblasts Swiss 3T3 were grown in DMEM/10%FBS. MDCK cysts were grown in 3D collagen I gel as described (24). To induce tubulogenesis, HGF-containing conditioned medium was used. Briefly, MDCK cysts were grown in 3D collagen I gels for four days and treated with 3T3 fibroblasts-conditioned medium diluted 1:1 with MEM, 5% FBS every other day. Dome formation in SU.86.86 monolayer was induced in postconfluent cultures either spontaneously or facilitated in the presence of additional 5% FBS.

Antibodies

A affinity purified rabbit polyclonal antibody anti-LRR was used to detect polycystin-1. We have extensively characterized this antibody previously (10,11,15,20,27). As markers of cell–cell junctions, anti-desmoplakin (1 and 2) (Maine Biotechnology Services Inc., Portland, ME) and anti-ZO1 (Zymed, San Francisco, CA) antibodies were used. Monoclonal antibodies against E-cadherin, β-catenin, γ-catenin and pp120 were obtained from Transduction Laboratories (Lexington, KY).

Immunostaining

Immunofluorescence staining was performed as previously described (10). Briefly, MDCK or SU.86.86 cells grown on glass coverslips in 6-well dishes were fixed in 10% acetic acid, 50% ethanol for 40 min at 4°C and washed with phosphate-buffered saline (PBS). Primary antibodies (1:50) were applied for 1 h at 37°C followed by incubation with FITC- or Cy3-labeled secondary antibody at a dilution of 1:100. Immunofluorescence staining of cysts and tubules in 3D matrix was performed as previously described (54). The specificity of staining was controlled by using secondary antibody alone as well as by blocking the signal with immunizing polypeptide.
Confocal microscopy images were acquired using a Zeiss LSM 410 Confocal System on a Zeiss Axiovert inverted microscope with a 40× 1.3NA Plan Neofluor oil immersion lens. To ensure the complete view of domes, cysts and tubules, serial focal planes were collected 0.4 μm apart for complete spanning through sample. Some fluorescence images were acquired on a Zeiss Axioplan microscope with a 40× or 100× objectives with software QED Camera Plus-In™ (version 1.3; QED Imaging, Inc., Pittsburgh, PA). Light microscopy was performed on Zeiss Axiosvert25 inverted microscope with either 4×, 10× or 20× air lenses.

Northern blot analysis
RNA extraction and hybridization were performed as previously described (11) with 20 μg of total RNA blotted per sample. To induce domes, the confluent culture of SU.86.86 cells was treated with 5% FBS for three days. When approximately 70% of monolayer was converted to the dome structures, domes were harvested by cell lifters for RNA extraction. The hybridization was performed at 42°C in the presence of 50% formamide. After hybridization the membrane was washed twice 5 min each at room temperature in 2× SSC, 20 min at 65°C in 2× SSC with 1% SDS and 15 min at room temperature in 0.1× SSC. Quantification was performed by using a PhosphorImager with ImageQuant (v. 3.2) software (Molecular Dynamics, Sunnyvale, CA). The hybridization probes were used as follows: desmoplakin: 1:1 mixture of NotI/Sall from cDNA clones (for 5′-end: Image 2984773; for 3′-end: Image 2754071); ZO-1: NotI/EcoRI fragment from cDNA clone (Image 3176987); E-cadherin: NotI/Sall fragment from cDNA clone (Image 2900672). To detect polycystin-1 in human SU.86.86 cells, a probe Xbal/NotI was derived from the 3′ end of the full-length human cDNA clone (Accession No L33243). To detect polycystin-1 in MDCK cells, a canine exon 15 specific probe was used (Accession No AF483210).

Real time PCR
For real time PCR, RNAs were extracted from single cell suspension using Trizol according to the manufacturer (Life Technologies). Genomic DNA contaminants were removed using DNA-free™ kit (Ambion, Austin, TX). Briefly, 20 μg or RNA were incubated with 1 μl of DNase-I in a final volume of 50 μl for 30 min at 37°C. DNase-I was removed using DNase-I-removing agent as described by the manufacturer. Reverse transcription of 1 μg of DNase-I treated RNA was done in RT buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl2, 500 mM dNTP, 10 mM DTT, 500 ng oligo dT, 20 U SUPERase-In™ and 200 U SuperscriptII RNase H- reverse transcriptase). The samples were incubated at 20°C for 10 min and at 42°C for 50 min, and reverse transcriptase was inactivated at 70°C for 15 min. For Taqman analysis, forward (F) and reverse (R) primers (Operon, Alameda, CA), and probes (P) (Synthegen, Houston, TX) were designed using Primer Express™ 1.5 software (Applied Biosystems, Foster City, CA). Primer sequences were as follows: F: 5′-GTGAT-CACCATGGCAACAG-3′; R: 5′-CCATACCCAGGAAGAG-AGC-3′ and P: 5′-6FAM-CCATTCGCGTCCTGACGC-TAMRA-3′ for dog β-actin; and F: 5′-TGACGGTCGCTGTCCT-CCA-3′; R: 5′-GGCTCCTCACCCTACCGA-3′ and P: 5′-6FAM-ACATCTGGGGCGCAATGACTCTG-TAMRA-3′ for dog Pkd-1. PCR reactions were carried in 25 μl reaction mixture containing 2.5 μl cDNA template or standard, 12.5 μl TaqMan Universal PCR Master Mix (Applied Biosystems), 300 nM primers and 250 nM probe. Standard curves were generated using serially diluted solutions of plasmid clones with respective inserts. The following cycling conditions were used: 50°C 2 min, 95°C 10 min and 40 cycles 95°C 15 sec and 60°C 1 min. Real time PCR assays were conducted in triplicates for each sample. The amount of target gene expression was calculated from the respective standard curves and quantitative expression of PKD-1 was normalized using dog β-actin.

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REFERENCES


