Ectopic expression of cadherin 8 is sufficient to cause cyst formation in a novel 3D collagen matrix renal tubule culture

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Kher R, Sha EC, Escobar MR, Andreoli EM, Wang P, Xu WM, Wandinger-Ness A, Bacallao RL. Ectopic expression of cadherin 8 is sufficient to cause cyst formation in a novel 3D collagen matrix renal tubule culture. Am J Physiol Cell Physiol 301: C99–C105, 2011. First published March 9, 2011; doi:10.1152/ajpcell.00151.2010.—While a variety of genetic mutations have been shown to be associated with renal cyst formation, mechanisms of renal cyst formation are largely unknown. In prior communications we described alterations in E-cadherin assembly in cultured cystic epithelial cells (Charron AJ, Nakamura S, Bacallao R, Wandinger-Ness A. J Cell Biol 149: 111–124, 2000). Using the same cell line we assayed cadherin expression by RT-PCR using primer pairs that anneal to highly conserved sequences of cadherin genes but flank informative regions of cadherins. Using this approach we found that autosomal dominant polycystic kidney disease (ADPKD) cells express cadherin 8, a neuronal cadherin with limited expression in the kidney. Immunohistochemistry confirmed cadherin 8 expression in cystic epithelia. To test the functional significance of cadherin 8 expression in renal epithelial cells, we adapted a three-dimensional collagen culture method in which HK-2 cells form tubule structures and microinjected adenovirus into the matrix space surrounding tubule structures. Adenovirus expressing cadherin 8 under the control of a tet promoter caused cyst structures to grow out of the tubules when coinjected with adenovirus expressing a tet transactivator. Microinjection of single adenovirus expressing either tet transactivator or cadherin 8 failed to cause cyst formation. When doxycycline was added to the culture, following coinjection of adenovirus, there was a dose-response reduction in cadherin 8 expression and cyst formation. Similarly, HK-2 cells transfected with Flag-tagged cadherin 8 form cysts in addition to tubular structures. HK-2 cells transfected with Flag-tagged N-cadherin do not form cysts. These data suggest that ectopic expression of cadherin 8 in renal epithelial cells is sufficient to cause the morphogenic pattern of cyst formation. HK-2 cells; three-dimensional collagen culture; adenovirus; cysts; autosomal dominant polycystic kidney disease

POLYCYSTIC KIDNEY DISEASE is a heterogeneous group of genetic disorders associated with a variety of phenotypes ranging from situs inversus, hereditary blindness, obesity, mental retardation, hydrocephalus, and cystic changes in kidneys, liver, and pancreas. While animal models and genomic approaches have greatly expanded our identification of the genes associated with polycystic kidney disease, our understanding of the mechanistic underpinnings of renal cyst formation is largely incomplete. Several leading hypothesis have driven our understanding of cyst formation. The two-hit hypothesis was advanced to account for the focal nature of renal cyst formation (25, 26, 34), while the cilia signaling hypothesis has linked mechano-transduction of flow to gene transcription and terminal differentiation (1, 8, 10, 12). Lastly, defects in planar cell polarity genes have been shown to be cystogenic. Particularly instructive in this regard has been the finding that FAT4 knockout, an atypical cadherin that participates in planar cell polarity, causes renal cysts (3, 5, 15, 29, 32).

In prior work, we demonstrated that cultured renal epithelial cells derived from kidney cysts fail to assemble E-cadherin in the lateral membrane. E-cadherin is made in equivalent amounts in cystic epithelial cells as compared withagematched control renal epithelial cells but fails to stably integrate into the lateral membrane (9). Since the cells form functional tight junctions, we examined what other cadherins could provide lateral membrane signaling necessary to permit tight junction assembly. We found that N-cadherin was expressed in autosomal dominant polycystic kidney disease (ADPKD) cells by immunostaining and N-cadherin served to stabilize β-catenin at the cell membrane (28). However, this approach did not provide a full evaluation of cadherin family member expression in the ADPKD cells. In this communication we demonstrate that ADPKD cells express cadherin 8, a type II cadherin expressed predominantly in neuronal cells. This expression is found in polycystic kidney, and that ectopic expression of cadherin 8 by HK-2 cells in three-dimensional (3D) culture will drive formation of cysts arising from tubules. This finding suggests that altered cadherin expression as part of a transdifferentiation event may be a final pathway for renal cyst formation.

MATERIALS AND METHODS

Cell lines and culture. Primary cultured renal tubule epithelial cells were derived from human polycystic kidneys and normal kidneys discarded for transplantation, as previously described (9). Cell lines were maintained up to six passages at which time the cell lines go into senescence. All studies were performed in cell lines at passage 3 or less. Cells were grown in REGM media (Lonza, Basel, Switzerland) in a 5% CO₂ environment at 37°C. HK-2 cells were purchased from American Type Culture Collection and grown in DMEM media supplemented with 5% fetal calf serum. The Institutional Review Board at Indiana University reviewed and approved the acquisition of human tissue used in this study.

Antibodies and reagents. Anti-cadherin 8 was supplied by Santa Cruz Biotechnology (Santa Cruz, CA) and OriGene Technologies (Rockville, MD). Taq polymerase and reverse transcriptase were purchased from Invitrogen. TOPO cloning kits were obtained from Invitrogen (Carlsbad, CA) and used according to the manufacturer’s directions. All buffers and chemicals were purchased from Thermo Fisher Scientific.
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Fisher Scientific (Waltham, MA) and were of reagent-grade quality. Rhodamine phalloidin and Hoescht 33342 were purchased from Invitrogen. PCR primer pairs using sequences described by Suzuki et al. (33) were ordered from Eurofins MWG Operon (Huntsville, AL). All fluorescence-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). Plasmids encoding full-length Flag-tagged cadherin 2 (N-cadherin, NM_001792) and cadherin 8 (NM_001796.2) were purchased from OriGene Technologies.

RT-PCR and SSCP gel analysis. RNA was isolated from ADPKD and age-matched normal kidney cells grown to confluence in 100-mm plastic dishes using Ambion RNA isolation kits (Ambion, Austin, TX) according to the manufacturer’s directions. RNA purity was confirmed by measuring A280/260 ratios and running RNA on a 1% agarose gel. RT-PCR reactions were performed using 1 μg total RNA using conditions described by Sambrook et al. (30). PCR products were analyzed by SSCP gels using a GenoMed gel apparatus (GenoMed, St. Louis, MO). PCR products were visualized by running PCR reactions with 32P-labeled dNTPs and exposing the SSCP gels on X-ray film (XOMAT, Kodak, Rochester, NY). PCR products were cloned into TOPO plasmid and sequenced by the Molecular Biology Core facility at Indiana University.

Immunohistochemistry. ADPKD kidneys were cryoixed in isopentane cooled with dry ice. Fixed tissue was embedded in OCT (Polysciences, Warrington, PA) and sectioned in a cryomicrotome. Tissue sections were placed on ProbeON slides (Thermo Fisher Scientific) and fixed with 2% paraformaldehyde dissolved in phosphate-buffered saline (PBS) for 30 min. Chemical fixation reactions were quenched with 100 mM NH4Cl dissolved in PBS. Samples prepared for immunohistochemistry labeling were incubated in PBS with 0.3% hydrogen peroxide for 30 min to inactivate endogenous peroxidase. All samples were incubated with tissue block solution consisting of 1% bovine albumin and 0.1% Triton X-100 dissolved in PBS. Ten-micrometer sections of tissue were incubated with anti-cadherin 8 added to tissue block solution and Hoescht 33342. After incubation with primary antibody, samples were washed with PBS and then incubated with horseradish peroxidase-conjugated donkey anti-goat IgG (Vector Laboratories, Burlingame, CA). Samples were developed with Vector- Stain ABC kit according to the manufacturer’s directions and then counterstained with DAPI (Axonin, Vector Laboratory). Alternatively, after incubation with primary antibody, samples were washed with PBS and then incubated with Alexa 568-conjugated donkey anti-goat IgG (Jackson ImmunoResearch). After a second set of washings, the samples were fixed with 2% paraformaldehyde dissolved in PBS for 30 min and mounted in Mowiol.

3D collagen culture. Cell matrix (Wako Chemicals, Richmond, VA) was mixed with growth media in equal proportions, and 1 ml was layered onto 60-mm Petri dishes with a glass coverslip mounted on the bottom (MatTek, Ashland, MA). Collagen matrix was allowed to set overnight and then HK-2 cells were plated at a density of 100,000 cells/cm² and incubated overnight. After allowing the cells to attach, excess medium was removed and the cells were overlaid with cell matrix and maintained in a 5% CO₂, 37°C culture conditions. Cultures were kept in culture for a maximum of 14 days.

Adenovirus production. Human cadherin 8 was obtained from Invitrogen and subcloned into pAdTet (11). Cadherin 8 bearing pAdTet was cotransfected into Cre8 293 cells (generously supplied by Josh Lipshutz, University of Pennsylvania, Philadelphia) with pcDNA5 Ad backbone (11). Adenovirus expressing tet trans-activator (TTA) was supplied by Josh Lipshutz. Multiplicity of infection (MOI) was determined by performing serial dilutions and assaying cadherin 8 expression by immunohistochemistry in Madin-Darby canine kidney (MDCK) TTA-expressing cells (Clonetch, Mountain View, CA).

Cyst formation assay. HK-2 cells grown in 3D collagen matrix form tubules within 24 h after collagen matrix overlay. Once tubules were formed, three different experimental conditions were evaluated. Adenovirus expressing cadherin 8, adenovirus expressing TTA, or both adenovirus 8 adenovirus and TTA adenovirus were microinjected into matrix near the tubules. Typically, 1E6 MOI virus was microinjected in a region. In addition, some experiments were performed in which cadherin 8 and TTA adenovirus were microinjected and the cells were incubated in media supplemented with either 0, 0.5, or 1 μg/ml doxycycline. After microinjection, cultures were incubated for 48 h and then fixed with 2% paraformaldehyde dissolved in PBS. After fixation, samples were labeled with rhodamine phalloidin and Hoescht 33342 (Invitrogen). In an alternative approach, HK-2 cells were transected with either plasmids bearing Flag-tagged cadherin 8 or N-cadherin using XFect (Sigma, St. Louis, MO) according to the manufacturer’s directions. After transfection, cells were grown in regular media for 24 h, passaged with trypsin-EDTA, and mixed with an equal number of untransfected HK-2 cells and plated on collagen matrix at a density of 50,000 cells/cm². Four hours after plating, the cells were overlaid with collagen and maintained in culture for 24 to 96 h before processing for light microscopy.

Fixation and staining of 3D collagen samples. Collagen matrix samples were washed three times with PBS supplemented with 0.5 mM MgCl₂ and CaCl₂. Matrix cultures were treated with 1,000 U/ml collagenase II (Worthington Biochemical, Freehold, NJ) for 5 min. After three washes with ice-cold PBS supplemented with 0.5 mM MgCl₂ and CaCl₂, samples were fixed for 30 min with 2% or 4% paraformaldehyde for 30 min. Fixation reactions were quenched with 100 mM NH₄Cl dissolved in PBS.

To stain samples, collagen matrix plugs were washed with PBS with 0.1% Triton X-100 (Thermo Fisher Scientific) supplemented with 5% normal donkey serum (Jackson ImmunoResearch). Samples were labeled with monoclonal anti-Flag (OriGene), Texas Red-conjugated phalloidin (Invitrogen), and Hoescht 33342 (Invitrogen). After labeling and washes, all samples were postfixed in paraformaldehyde and mounted with Fluorsave (EMD4Biosciences, Darmstadt, Germany).

Light microscopy and image processing. Images were collected by two photon confocal microscopy using an Olympus Fluor View confocal microscope. Image processing was performed using Meta morph software (version 7.6.5; Danaher Medical Technologies, Washington, DC) running on a Dell Optiplex computer (Austin, TX). All image processing was performed using equivalent setting between control and experimental samples.

RESULTS

To determine the full profile of cadherin expression in the primary cell lines derived from renal cysts, we performed RT-PCR using primer pairs first described by Suzuki et al. (33). These primers anneal to highly conserved regions of cadherin but span highly informative regions of cadherins which permits unambiguous identification of expressed cadherins (33). To assess the results of the RT-PCR reactions, SSCP gels were run. As shown in Fig. 1, the reaction products obtained from normal kidney cells and PKD cells showed differing migration patterns, suggesting that there were differences in the PCR products. On the basis of this result we cloned the resultant PCR products and screened the plasmid inserts by sequencing. Sequence analysis identified K- and N-cadherin in the normal kidney cells. In contrast, cystic epithelia expressed K- and N-cadherin and cadherin 8 (data not shown). Since cultured cells undergo significant changes in differentiation profile during adaptation to cell culture conditions, we confirmed cadherin 8 by immunostaining for cadherin 8 in ADPKD tissue sections. Cadherin 8 was observed in epithelial cells lining the cysts in a majority of cysts observed.
We also found that, in agreement with published results, cadherin 8 is not expressed in normal adult kidneys (Fig. 2B) (6). Taken together, our data suggest that cadherin 8 is expressed in renal cystic epithelial cells both in vivo and ex vivo.

To determine the functional significance of cadherin 8 expression, we developed a novel assay for cystogenesis. This assay takes advantage of the fact that matrix overlay allows HK-2 cells to spontaneously form tubule structures within 24 h (Fig. 3A). When maintained in culture for up to 14 days, the tubule arrays extend and arborize (data not shown). When adenovirus expressing either TTA or cadherin 8 was microinjected into the matrix space surrounding the tubule sections, no changes in tubule structures were observed (Fig. 3, C–F). When the two adenoviruses were microinjected together, stalk like extensions were observed within 24 h and by 48 h independent cysts were observed (Fig. 3, G and H). Immunostaining confirmed that the cysts and stalks were cadherin 8 positive (Fig. 4, A and B). Tubule structures were N-cadherin positive (Fig. 4, C and D) in accordance with the normal cadherin profile of HK cells (2, 23). When doxycycline was added to the cell cultures following microinjection of TTA and cadherin 8 adenoviruses, there was a dose-dependent relationship between the cyst numbers observed in culture and the doxycycline dose (Table 1).

One potential interpretation of the results is that any cadherin when overexpressed can induce cyst formation. To evaluate this possibility, we transfected plasmids bearing Flag-tagged cadherin 8 or N-cadherin into HK-2 cells and evaluated the structural morphology 48 h after overlay in collagen ma-
trix. N-cadherin was chosen since HK-2 cells normally express N-cadherin (24). In Fig. 5, immunoblot analysis demonstrates that equivalent levels of cadherin 8 and N-cadherin are expressed in transfected HK-2 cells (Fig. 5A). Cells transfected with N-cadherin formed tubule arrays and chords but no cysts were observed (Fig. 5, B and C). In contrast, cadherin 8 transfected cells did form cysts (Fig. 5, D and E). This result confirms that ectopic expression of cadherin 8 is sufficient to drive cystogenic morphology and that simply overexpressing an endogenously expressed cadherin may not cause cyst formation.

DISCUSSION

Role of cadherins in tissue morphology. In vivo, renal epithelial cells express three cadherins that have been identified at various nephron segments. K-cadherin is expressed in the proximal tubule along with N-cadherin. E-cadherin is expressed more strongly in distal tubule segments (21, 22). During nephrogenesis, N-cadherin and E-cadherin predominate in expression (20). Cadherin 8 has been noted in early nephrogenesis, however, its expression is rapidly downregulated and is absent in adult nephrons (6). Cadherin 8 expression has also been noted in renal cell carcinomas but the significance of its expression is unknown (6). Normally, cadherin 8 is observed in posterior horn cells of the spine, and mouse knockouts of cadherin 8 exhibited defects in response to pain induced by heat. Neurons isolated from the posterior horn of the cadherin 8 knockout also demonstrated changes in TrpMa receptors and attenuated responses to menthol (13). No obvious kidney phenotype was observed in the animal knockout line (13).

Cadherins are transmembrane proteins with a large extracellular domain divided into EC1 domains and EC2 domains arranged in tandem arrays. They have a single transmembrane domain and on the cytoplasmic side there are domains that mediate binding to β- and α-catenin, scribble, and p120 (7, 16–19, 27, 36). Currently, there are 57 known cadherins in the family and they are divided into type I, type II, and atypical cadherins. Cadherin 8 is a type II cadherin with significant homology in its extracellular domain to canonical cadherins such as N- and E-cadherins (19). However, cadherin 8 lacks the...
HEV sequence found in the EC1 domain of E-cadherin (19). The cytoplasmic domain of cadherin 8 is more highly conserved to cytoplasmic domain of type I cadherins than the extra-cytoplasmic domain and has putative β-catenin and actin-binding sites (19). Since homotypic and heterotypic interactions of cadherins are closely linked to subtle differences in structural features of each family member, it was important to characterize the effect of ectopic cadherin 8 expression in renal epithelia.

To examine the effect of ectopic cadherin 8 expression, we wanted to mimic the stages of renal cyst formation first identified by Baert and Steg (4). Using microdissection techniques, Baert and Steg showed that cysts arise from fully formed tubules and can arise from any nephron segment (4). MDCK cell cysts grown in collagen culture do not form tubules, hence this model of kidney cysts may lack important aspects of cystogenesis that occur in vivo (14). In the model developed in this communication, tubular-like structures are formed prior to experimentally interventions that may lead to cyst formation. We show that ectopic expression of cadherin 8 in HK-2 cells is sufficient to cause the cystic morphogenic pattern to arise from preformed tubules. However, the nature of the cysts observed do not entirely mimic that which is seen in vivo. The lumen size is contracted and a single layer of epithelial cells lining the cyst is not seen. This suggests that this model does not completely model cyst formation and that other aspects of cystic transformation have to be added to fully recapitulate cyst formation in vivo. For example, the addition of bromo-cyclic AMP may increase the lumen size in our model. Alternatively, further derangements in lumen size control may be necessary to increase the cyst lumen. So experiments aimed at increasing activity of AMOT, Crumbs complex, or the PARS3 complex may be needed to increase cyst lumen size (31, 35). Further experiments will be needed to fully characterize this experimental system; however, this 3D culture model offers the opportunity to examine all stages of cyst formation, from initial outgrowth to sealing off the cyst, in detail.

How can the results in this communication be reconciled with current paradigms of cystogenesis? We propose that one downstream component of kidney cell differentiation, under control of integrated planar cell polarity and cilia

Table 1. Mean cyst number observed in three-dimensional cultures versus doxycycline dose

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<tr>
<th>Doxycycline Dose, μg/μl</th>
<th>Mean Cyst Number (n = 3 experiments)</th>
<th>P Value</th>
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<tr>
<td>0</td>
<td>54</td>
<td>&lt; 0.01</td>
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<tr>
<td>0.5</td>
<td>22</td>
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signaling systems, is cadherin selection. Under conditions where PKD-associated genes are mutated, cadherin selection at a genome level is altered, resulting in focal and clonal changes in cadherins expressed by the affected cells. This change is sufficient to permit changes in migratory potential and tubule morphogenic patterning of affected cells thus resulting in a kidney cyst.

In summary, our study demonstrates that cadherin 8, a type II neuronal cadherin, is expressed in cyst epithelia both in vivo and in vitro. In 3D collagen matrix culture, driving cadherin 8 expression in HK-2 cells with adenovirus is sufficient to cause a cyst-forming growth pattern arising from tubule structures.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


