Disruption of polycystin-1 function interferes with branching morphogenesis of the ureteric bud in developing mouse kidneys

Katalin Polgar, Christopher R. Burrow, Deborah P. Hyink, Hilda Fernandez, Katie Thornton, Xiaohong Li, G. Luca Gusella, Patricia D. Wilson*

Department of Medicine, Division of Nephrology, Mount Sinai School of Medicine, 1425 Madison Avenue, New York, NY 10029, USA

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Abstract

The polycystic kidney disease (PKD1) gene-encoded protein, polycystin-1, is developmentally regulated, with highest expression levels seen in normal developing kidneys, where it is distributed in a punctate pattern at the basal surface of ureteric bud epithelia. Overexpression in ureteric epithelial cell membranes of an inhibitory pMyr-GFP-PKD1 fusion protein via a retroviral (VVC) delivery system and microinjection into the ureteric bud lumen of embryonic day 11 mouse metanephric kidneys resulted in disrupted branching morphogenesis. Using confocal quantitative analysis, significant reductions were measured in the numbers of ureteric bud branch points and tips, as well as in the total ureteric bud length, volume and area, while significant increases were seen as dilations of the terminal branches, where significant increases in outer diameter and volumes were measured. Microinjection of an activating 5TM-GFP-PKD1 fusion protein had an opposite effect and showed significant increases in ureteric bud length and area. These are the first studies to experimentally manipulate polycystin-1 expression by transduction in the embryonic mouse kidney and suggest that polycystin-1 plays a critical role in the regulation of epithelial morphogenesis during renal development.

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Introduction

The mammalian kidney derives from intermediate mesoderm cells that migrate caudally to form the nephric (Wolffian) duct, giving rise to a non-functional pronephros, a transiently functional mesonephros and the permanent metanephros. At embryonic day 10.5 in mice and day 28 in man, the ureteric bud appears as an outgrowth of the posterior portion of the nephric duct that then migrates and invades the undifferentiated metanephric mesenchyme, where reciprocal interactions lead to iterative rounds of ureteric bud branching and elongation, as well as mesenchymal condensation and nephron induction at the tips. The process of branching morphogenesis is controlled by a variety of soluble growth factors and insoluble matrix proteins via interactions with their receptors. These include: glial-derived neurotrophic factor (GDNF) that signals via c-Ret and GFRα1, as well as fibroblast growth factors, such as FGF-7, that activate diverse FGF receptors. Other receptor-dependent signaling pathways that have been implicated in branching morphogenesis in the kidney include those activated by bone morphogenetic protein (BMP) 7, activin, wnts 2b, 7 and 11, pleiotrophin, midkine, laminin-5 and fibronectin (reviewed in Burrow, 2000; Davies, 2002; Piscione and Rosenblum, 2002; Vainio and Lin, 2002). Strict regulation, coordination and integration of multiple pathways are essential for the appropriate development and differentiation of the normal metanephric kidney. Several human diseases result in a cystic kidney phenotype after genetic disruption of the ureteric bud branching process including: renal coloboma syndrome, Townes–Brock syndrome, branchio-oto-renal syndrome, Beckwith–Wiedemann syndrome, Zellweger syndrome, Alagille syndrome and
Simpson–Golabi–Behmel syndrome (Piscione and Rosenblum, 2002).

Autosomal dominant polycystic kidney disease (ADPKD) is a very common progressive renal disease affecting 1:800 humans and is caused by mutations in the PKD1 gene in 85% of cases. Loss of function of the PKD1-encoded polycystin-1 protein in heterozygous human patients results in the formation of multiple tubular epithelial cysts, derived from every segment of the nephron, that enlarge progressively and eventually obliterate renal structure and function. The pathophysiological changes leading to disease result from abnormal persistent proliferation and luminal fluid secretion associated with apical membrane mispolarization of epidermal growth factor receptor and NaK-ATPase as well as alterations in matrix receptors and adhesion properties (Wilson, 1996).

Several lines of evidence suggest that ADPKD is of developmental origin. Mutant mice, with a variety of homozygous targeted disruptions in the PKD1 gene, develop enlarged cystic kidneys and die in utero or perinatally, either of renal or vascular malformations (Boulter et al., 2001; Kim et al., 2000; Lu et al., 1997). This may also be true in humans since no human patients homozygous for PKD1 mutations have been identified, suggesting embryonic lethality. In some heterozygous ADPKD patients, multiple renal cysts have been detected in utero, even though symptoms of renal insufficiency and hypertension may not develop until adulthood (MacDermot et al., 1998; Sedman et al., 1987). Furthermore, some fetal genes and proteins, including the β2 subunit of NaK-ATPase, the ErbB2 variant of EGF receptor and the transcription factors Pax-2 and WT-1, fail to be appropriately down-regulated after birth in ADPKD kidneys. Abnormalities associated with persistent expression of these fetal proteins are thought to contribute to the increased cell proliferation and fluid transport defects in ADPKD cysts (Ostrom et al., 2000; Wilson, 1996, 2004b; Wilson et al., 2000).

Normal polycystin-1 is a large developmentally regulated membrane protein, highly expressed in the human and mouse kidneys, where it is localized to ureteric bud and collecting tubule cell membranes. Low levels of expression are seen in medullary collecting tubules in normal adult kidneys (Geng et al., 1997; Van Adelsberg et al., 1997; Ward et al., 1996; Wilson, 1997; Wilson et al., 1999). Polycystin-1 can interact with extracellular matrix proteins via its large, multidomain, extracellular N-terminal portion, and it forms multiprotein complexes with focal adhesion proteins and cell–cell adherens junction proteins. The intracellular C-terminal portion of polycystin-1 has been shown to initiate intracellular signaling via phosphorylation and G protein activation, leading to transcriptional regulation of differentiation-related genes (Arnould et al., 1998; Bycroft et al., 1999; Geng et al., 2000; Kim et al., 1999; Li et al., 1999; Malhas et al., 2002; Parnell et al., 1998; Weston et al., 2001). Transduction and expression of C-terminal domain polycystin-1 fusion proteins in various renal cell lines also showed a membrane-associated pattern of distribution and co-localization with the focal adhesion protein, paxillin (Kaletta et al., 2003). In addition, over-expression of full-length normal polycystin-1 has been shown to induce tubulogenesis and migration in a renal cell line cultured in collagen gels (Boletta et al., 2000; Nickel et al., 2002). By contrast, human ADPKD epithelia, with PKD1 mutations, have aberrantly increased adhesiveness to extracellular collagen and show decreased motility. This suggested that inhibition of polycystin-1 function might cause increased matrix adhesion and decreased motility in ureteric bud epithelia in vivo and thus cause abnormalities in renal morphogenesis.

Focal adhesion complexes are structural anchorage sites that normally form at discrete regions of the basal plasma-membrane at points of contact between the cell and its extracellular matrix. The recruitment, via integrins, of a variety of specific kinases, phosphatases, Ras superfamily proteins and adaptor molecules provides a signaling complex that initiates intracellular signaling and provides a link between the matrix and the actin cytoskeleton. Mutations in other focal adhesion proteins, including tensin and nephrin, also lead to polycystic kidney diseases (Benzing et al., 2001; Lo et al., 1994).

Here, we identify a role for polycystin-1 in the regulation of normal renal development and morphogenesis in embryonic mouse kidneys in organ culture. This is the first study to utilize experimental manipulation and analysis of the effects of polycystin in this complex system that allows detailed quantitative analysis of renal epithelial differentiation in the context of normal 3-dimensional, multicellular interactions. High levels of endogenous polycystin-1 were detected in matrix-associated focal adhesion complexes in the basal membrane of the ureteric bud from the onset of metanephric development. Microinjection of a lentiviral construct encoding an inhibitory pMyr-GFP-C-terminal domain polycystin-1 fusion protein into the developing ureteric bud significantly decreased ureteric bud elongation and branching morphogenesis and also significantly increased tubule diameter and volume. By contrast, microinjection of an activating 5TM-GFP-polycystin-1 fusion protein increased ureteric bud elongation. Together with the demonstrated increases in matrix adherence, deficiencies in focal adhesion form and function as well as decreased migration capacities of ADPKD epithelia, these studies suggest that polycystin-1 plays a critical role in the regulation of early ureteric bud elongation, branching and tubule volume control during morphogenesis of the developing metanephric kidney.

Materials and methods

Human kidney tissues and cell lines

Human kidneys were perfused in situ and procured under sterile conditions (National Disease Research Interchange,
Philadelphia, PA, Anatomic Gift Foundation, PA) immediately prior to microdissection and tissue culture of renal tubule segments (Wilson et al., 1985); fixation in 4% fresh paraformaldehyde (EM Sciences, Hatfield, PA) in phosphate-buffered saline (PBS), pH 7.4 for immunolocalization and in situ hybridization studies or flash freezing in liquid nitrogen and storage at −86°C for protein extraction. All kidneys were checked for normal morphology by hematoxylin and eosin staining prior to use. Characterized, primary and conditionally immortalized cultures derived from microdissected normal human fetal collecting tubule (HFCT) and normal adult collecting tubule (NHCT) were generated and characterized as previously described (Racusen et al., 1995; Wilson, 2004a). HEK 293 and HeLa cell lines were obtained commercially (American Type Culture Collection).

**Antibodies, fusion proteins and expression constructs**

A previously characterized monospecific polyclonal antibody, raised in rabbits against a 31 peptide sequence of the human polycystin-1 protein intracellular terminal portion (residues 4169–4191) (Wilson et al., 1999), was used at 1:500 or 1:1000 for immunostaining of tissue sections or cell cultures and at 1:12,500 for Western immunoblots. A mouse monoclonal anti-calbindin antibody (Sigma, Clone D28K, 1:200 dilution), a rabbit anti-GFP antibody, raised in rabbits against a 31 peptide sequence (Wilson et al., 1999), was used as described previously (Li et al., 1999). For mammalian expression, a pMyr-EGFP-PKD1-CTD expression fusion protein with 5 transmembrane domains as well as the intracellular terminal domain (CTD) was used as described previously (Li et al., 1999; Kaletta et al., 2003) as well as a PKD1-5TM mammalian expression vector which encodes a pre-pro-trypsin GFP-polycystin-1 fusion protein with an N-terminal myristoylation signal sequence (the pp60−src sequence MGSSKSKPKDPSQRR), was used as described previously (Li et al., 1999; Kaletta et al., 2003) as well as a PKD1-5TM mammalian expression vector which encodes a pre-pro-trypsin GFP-polycystin-1 fusion protein with 5 transmembrane domains as well as the CTD (Kaletta et al., 2003).

For ureteric bud microinjections and long-term mammalian expression, a pMyr-EGFP-PKD1-CTD expression construct was generated using the VVC self-inactivating lentivirus (E. Federova and G.L Gusella, manuscript in preparation). The VVC lentivector was derived from the substitution of the U3 region of the 5′ LTR of pHmCMV vector with the human early cytomegalovirus promoter. From the resulting vector, the U3 region of the 3′-LTR was subsequently deleted to obtain the self-inactivating lentiviral vector VVC. In VVC, the expression of the viral full-length genomic RNA, required during viral production, is driven by the CMV promoter. The Xho1–BamHI fragments encoding the pMyr-EGFP-PKD1-CTD fusion protein were ligated into the VVC vector polylinker site, and the expression and titers of the PKD1-CTD constructs were tested on cultured HeLa-Tat cells. Infectious viral supernatants with a titer of >1 × 10^7 Transducing Units (TU)/ml were produced by transient transfection of 293T cells using Lipofectamine 2000 (Life Technologies). 8 µg of VVC-pMyr-EGFP-PKD1-CTD was co-transfected with 5.0 µg of the pCMVΔR8.2 plasmid producing the viral proteins and 2.5 µg of the pMDG plasmid expressing the pseudotyping VSV glycoprotein (VSVG) (Naldini et al., 1996). After 24 h, the medium was replaced with complete medium supplemented with 4 mM sodium butyrate, and supernatants were collected 48 h later. After filtration through a 0.45 µm filter, the virus was concentrated by centrifugation for 5 h at 30,000 × g, and the pellets were resuspended. The titer (TU/ml) was determined 48 h after infection of 3 × 10^5 HeLa-Tat cells/well in 24-well plates by scoring the number of EGFP expressing cells using UV microscopy.

**Immunohistochemistry and fluorescence staining**

Tissue sections of 4 h paraformaldehyde-fixed human tissue, and monolayer cultures of 10 min paraformaldehyde-fixed, 0.1% Triton X-100 permeabilized human renal epithelia, were incubated in 0.3% H_2O_2 in methanol to block endogenous peroxidase activity followed by 10% normal goat or rabbit serum in PBS, prior to 45 min incubation in primary antibody diluted 2% BSA/PBS. After 3 washes of 5 min each in PBS–Tween 20 (0.02%), sections or cells were incubated for 45 min each with biotinylated goat anti-rabbit IgG and avidin–biotin peroxidase (Vectorstain Elite, Vector Laboratories), with 3 intervening washes in PBS–Tween. After 2 washes in Tris-buffered saline (TBS), color development was carried out for 15 min using 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB) in PBS–Tween. After 2 rinses in PBS–Tween and 1 in PBS for 10 min, the secondary antibodies, donkey anti-mouse–Alexa 488 or donkey anti-rabbit–Alexa 568 (Molecular Probes), were used in 1:250 dilution, and incubation was carried out for 2 h at 37°C. After 3 washes in PBS, some kidneys were subjected to immunofluorescence after 2 h incubation at 37°C with anti-calbindin, anti-WT-1 or anti-GFP antibodies diluted 1:200, 1:100 and 1:250, respectively, in PBS–5% FBS. After 2 rinses in PBS–TWEEN and 1 in PBS for 10 min, the secondary antibodies, donkey anti-mouse–Alexa 488 or donkey anti-rabbit–Alexa 568 (Molecular Probes), were used in 1:250 dilution, and incubation was carried out for 3 h after incubation at 37°C with anti-calbindin, anti-WT-1 or anti-GFP antibodies diluted 1:200, 1:100 and 1:250, respectively, in PBS–5% FBS.
were expressed as mean transfection of the empty vector with pCRE-Luc. All values controls were carried out by co-transfection of pFC-MEKK activities were determined using a luminometer. Positive lysates were collected 24 h after transfection, and luciferase 2003) or together with the pMyr-EGFP-PKD1-CTD. Cell transfected with the PKD1-5TM alone (Kaletta et al., sample constant. In additional studies, 293 cells plasmid DNA used to keep the amount of DNA in each reporter gene (pCRE-Luc, Stratagene) and an unrelated region of the prolactin gene linked to the firefly luciferase 1996) were paired kidneys were harvested donors.

Luciferase reporter transcription assays

HEK 293 cells were co-transfected with pMyr-EGFP-PKD1-CTD and a reporter vector containing the 5'-flanking PKD1-CTD probe (the coding region for amino acids 4169–4191) was generated by PCR prior to digoxigenin substitution. Each of 5 replicate experiments was conducted on a single day and conducted under identical conditions, consisted of 25 sections comprising a developmental series of normal kidneys and age-matched ADPKD kidneys from 6 different donors.

In situ hybridization

Tissue sections of normal human fetal and adult kidneys that had been fixed in 4% fresh paraformaldehyde prepared in DEPC-treated PBS and embedded in low temperature wax were mounted on silanized Probe-On Plus slides (Fisher, Springfield, NJ), deparaffinized and subjected to digoxigenin–anti-digoxigenin in situ hybridization analysis as described previously (Wilson et al., 2000). The PKD1-CTD probe (the coding region for amino acids 4169–4191) was digoxigenin–anti-digoxigenin in situ hybridization analysis described previously (Wilson et al., 2000). The PKD1-CTD probe (the coding region for amino acids 4169–4191) was generated by PCR prior to digoxigenin substitution. Each of 5 replicate experiments was conducted on a single day and conducted under identical conditions, consisted of 25 sections comprising a developmental series of normal kidneys and age-matched ADPKD kidneys from 6 different donors.

Luciferase reporter transcription assays

HEK 293 cells were co-transfected with pMyr-EGFP-PKD1-CTD and a reporter vector containing the 5'-flanking region of the prolactin gene linked to the firefly luciferase reporter gene (pCRE-Luc, Stratagene) and an unrelated plasmid DNA used to keep the amount of DNA in each sample constant. In additional studies, 293 cells were transfected with the PKD1-5TM alone (Kaletta et al., 2003) or together with the pMyr-EGFP-PKD1-CTD. Cell lysates were collected 24 h after transfection, and luciferase activities were determined using a luminometer. Positive controls were carried out by co-transfection of pFC-MEKK plasmid with pCRE-Luc and negative controls by co-transfection of the empty vector with pCRE-Luc. All values were expressed as mean ± SEM for 2 separate transfection experiments, each conducted in triplicate.

Adhesion assays

Equal numbers of normal CT and ADPKD cells were suspended by treatment with collagenase and plated onto type I collagen-coated plastic surfaces in multiwell cluster plates. After 4 h, unattached cells were removed by aspiration, and the numbers of attached cells remaining on the plate quantified by the colorimetric MTS assay (Cell Titer Aq, Promega) followed by microdensitometry.

Migration assays

A modified Boyden chamber assay (Fluoroblock system; Falcon) was employed followed by microfluorometric analysis on an automated plate reader. Equal numbers of NHCT and ADPKD cells were suspended and preloaded with the non-toxic fluorescent indicator calcein AM, prior to plating 5000 cells in the upper chamber of a Falcon HTS insert (8 μM filter membrane pore size) in a well of a 24-multiwell cluster plate and in the presence of a growth factor (serum) gradient of 1% (in the upper chamber) to 5% (in the lower chamber). Fluorescence was detected only in cells that migrated through the membrane into the lower medium compartment.

Mouse metanephric embryonic fetal kidney organ culture and microinjection

Embryonic day (E) 11.5–12.5 kidneys were retrieved from timed pregnant CD-1 mice (Charles Rivers Laboratories, Wilmington, MA), and paired kidneys were harvested by microdissection using a dissecting stereomicroscope equipped with a Scion computer imaging system (Olympus). Kidneys were placed on uncoated 24 mm Transwell Clear membrane inserts, 0.4 μm pore size, in a 6-well cluster (Corning Incorporated, Corning NY), and 1 ml supplemented serum-free organ culture media containing 50% DMEM, 50% Ham F12; 15 mM HEPES and L-glutamine (Cellgro, Mediatech, VA) 4.5 g/l glucose, 45 mM sodium bicarbonate, 1× insulin/transferrin/selenium (ITS, Sigma), 2 × 10⁻⁹ T3, 7 × 10⁻⁸ M prostaglandin E1 (Avner et al., 1985; Ekblom et al., 1983; Vilar et al., 1996) was added to the lower chamber of each well. Plates were placed into a tissue culture incubator (5% CO₂) for up to 5 days with daily media changes.

For the microinjections, sterile transfer tips with 15 μm internal diameter (ES, Eppendorf, Hamburg, Germany) were used. On day zero, an Eppendorf micromanipulator 5171 and transjector 5246 were used to inject 20–200 nl of 10⁷ particles per ml stock of the VVC-pMyr-EGFP-PKD1-CTD VSV pseudotyped packaged viral particles; VVC alone (empty vector); or PBS (sham) into the ureteric bud of E11.5–12.5 mouse kidneys.

Microscopy and data analysis

Morphological changes were documented daily using an Olympus dissecting microscope, equipped with Scion computer imaging system. Immunofluorescence analysis was carried out using a laser scanning confocal microscope with inverted configuration using immersion lenses (BioRad Radiance 2000, MSSM-Microscopy Shared Research Facility).

Image J analysis (NIH), an open domain Java image processing system, was used to calculate area and pixel value statistics, to measure distances and volumes and to create skeletonized images.

Statistics

Means, standard deviations and standard errors of the mean were determined in sets of paired PKD1 vector-treated versus control, empty vector-treated kidneys form
multiple individual animals \((n = 5 \text{ to } n = 10)\). The unpaired \(t\) test was used to compare each parameter measured and \(P\) values determined, \(P < 0.05\) were considered statistically significant.

**Results**

**Developmental regulation of polycystin-1 in focal complexes of human mesonephric and metanephric kidneys**

Using our previously characterized anti-human polycystin-1 C-terminal domain antibody (Wilson et al., 1999) and Figs. 1A and B, immunoblot analysis showed high levels of polycystin-1 expression in early human fetal metanephric kidneys (12–16 weeks) and low levels in adult kidneys (32–59 years) (Fig. 1C). Similarly, conditionally immortalized epithelial cell cultures derived from normal microdissected human fetal collecting tubules (HFCT) showed high levels of polycystin-1 expression, while similar cultures derived from normal adult collecting tubules (NHCT) showed lower levels of expression (Fig. 1D).

In situ hybridization (Figs. 2A, B) and immunohistochemistry confirmed and extended these findings, showing high levels of ureteric bud mRNA expression and strong punctate staining for polycystin-1 at the basal membranes of the developing ureteric bud-derived collecting duct epithelium. This was particularly marked in the inner truncal portions of the ureteric bud (Figs. 2E, F, L). By contrast, in normal human adult kidneys, low levels of expression were seen only in the medulla (Fig. 2D versus C; H versus G), where light immunostaining for polycystin-1 was mostly restricted to the apical/lateral membranes of inner medullary collecting ducts (Fig. 2H, arrowheads). To determine the stage of onset of renal polycystin-1 expression, first trimester kidneys were examined (Figs. 2I–L). Strong focal staining for polycystin-1 was seen on the basal membranes of mesonephric ducts (Fig. 2I) and in the ureteric bud at the earliest stages of metanephric kidney development (Fig. 2J). By the time of its first bifurcation (Fig. 2K), a punctate pattern of staining was seen at the basal membrane regions of the ureteric bud epithelium, and this localization was maintained throughout the second trimester (Figs. 2H and L).

A similar strong focal pattern of endogenous polycystin-1 staining was demonstrated by immunocytochemical and immunofluorescent analysis of subconfluent HFCT cells after attachment to type I collagen in vitro (Fig. 3A, \(x/y\) plane; Fig. 3B, \(x/z\) plane). In confluent monolayers,
polycystin-1 showed a “chicken-wire” pattern of immuno-fluorescent staining that is consistent with basolateral membrane localization (Fig. 3C). This pattern of staining was consistent with previous studies showing polycystin-1 recruitment to multiprotein complexes within focal adhesion plaques (Wilson et al., 1999) and with the focal patterns of polycystin-1 and tensin staining in mouse embryonic kidneys (Geng et al., 1997; Lo et al., 1994). Of importance for the subsequent microinjection analysis, similar patterns of GFP fluorescence were seen after transfection of pMyr-EGFP-PKD1-CTD or 5TM-EGFP-PKD1 into renal epithelia in vitro (Figs. 3D–F).

**Defects of adhesion and migration in polycystic kidney disease epithelia**

Our previous studies had shown that primary cultures of ADPKD cyst lining epithelia were more adherent to extracellular collagen matrices than cultured cells derived from age-matched normal renal tubule epithelia (Wilson et al., 1996, 1999). Increased adhesion to type I collagen matrix was also characteristic of conditionally immortalized human ADPKD epithelia by comparison to normal adult collecting tubule (NHCT) cells in culture (Fig. 3G). In addition, as might be predicted, ADPKD epithelia showed a markedly reduced ability to migrate in response to a growth factor gradient by comparison to NHCT (Fig. 3H).

**Overexpression of a pMyr-EGFP-C-terminal domain polycystin-1 fusion protein, which inhibits polycystin-1-mediated activation of AP-1, inhibits ureteric bud branching morphogenesis**

In studies of AP-1 activation conducted in 293 cells, transfections with the PKD1-TM5 plasmid (encoding amino acid residues 3871–4303 of polycystin-1) activated AP-1 as shown by a luciferase reporter minigene assay (Fig. 4). Co-transfection of the pMyr-EGFP-PKD1-CTD construct, but
not vector alone, inhibited polycystin-1-dependent AP-1 activation produced by transfection with PKD1-TM5 (Fig. 4). We then developed a lentiviral vector encoding the same pMyr-EGFP-CTD-polycystin-1 fusion protein (see Materials and methods) which inhibited polycystin-1-dependent AP-1 activation and delivered this viral vector by microinjection (20 nl of 10^7 particles/ml) into the ureteric bud lumens of E11.5/12.5 normal mouse embryonic kidneys in organ culture (Fig. 5). Expression was monitored daily by examination of GFP fluorescence and shown throughout the structure of the developing ureteric bud (Fig. 5D), where it demonstrated a membrane pattern of staining (Fig. 5E). Phase contrast microscopy of untreated normal mouse embryonic kidney organ cultures after 1, 2, 3 and 4 days of culture showed increased organ size and complexity of morphogenesis. Injection of PBS ( sham) or VVC vector lacking the pMyr-EGFP-PKD1-CTD insert (“empty vector”) showed similar degrees of development as the untreated control over 4 days in organ culture. After microinjection of the inactivating VVC-pMyr-EGFP-PKD1-CTD into mouse kidney ureteric buds on day zero, however, a decrease in size and reduction in complexity of differentiation were observed after 4 days in culture (Fig. 6).

Comparative qualitative and quantitative analyses of ureteric bud branching were carried out on pairs of embryonic kidneys from multiple animals. Whole mount kidney rudiments were stained by immunofluorescence with antibodies against the ureteric bud constituent protein,
Fig. 4. The pMyc-PKD1-C terminal domain construct inhibits polycystin-1-mediated activation of AP-1. HEK 293 cells were co-transfected with AP-1-LUC and empty vector (negative control) or AP-1-LUC together with different PKD1 constructs. It is seen that overexpression of the PKD1-5TM construct activated AP-1 and stimulated the 7X AP-1 promoter element (bars 7 and 8), while co-expression of the pMyc-EGFP-PKD1-CTD inhibited the activation of AP-1 mediated by PKD1-5TM (bars 11 and 12).

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Fig. 5. Microinjected pMyc-EGFP-PKD1-CTD constructs transduce the whole ureteric bud of mouse embryonic kidneys in organ culture. (A) The VVC-pMyc-EGFP-PKD1-CTD self-inactivating lentiviral construct was titered on HeLa-TAT cells and microinjected (0.11 μg/ml; 5 μl) into the lumens of developing ureteric buds of E11/E12 mouse embryonic kidneys on day 0 of organ culture. (B) Schematic of injection protocol. (C) No fluorescence was seen in uninjected mouse embryonic kidneys in organ culture after 3 days. (D) Delivery and expression of the construct throughout the developing ureteric bud were demonstrated by GFP fluorescence seen here after 3 days of organ culture. (E) A high power image of the boxed portion in panel D shows expression of the pMyc-EGFP-PKD1-CTD at the basal cell membrane as indicated by the chicken-wire pattern of localization.
calbindin, after 3 days of organ culture (Fig. 7). Confocal microscopy showed distinct differences in patterns of ureteric bud branching morphogenesis in the VVC-pMyr-EGFP-PKD1-CTD-treated kidneys versus paired matched controls injected with empty vector (Fig. 7, upper panel). Kidneys microinjected with lentiviral pMyr-EGFP-PKD1-CTD showed disorganized and stunted patterns of ureteric bud branching and apparent widening of the terminal branches.

Fig. 7. Transduction of pMyr-EGFP-PKD1-CTD disrupts ureteric bud morphogenesis and glomerular induction. Whole mount immunofluorescence and confocal analysis of calbindin distribution in VVC-pMyr-EGFP-PKD1-CTD versus empty VVC (control)-injected E11.5 mouse embryonic kidneys after 3 days of subsequent organ culture (anti-calbindin antibody 1:200; upper panel). Patterns of fluorescent staining as well as Image J-derived skeletonized images show regular branching patterns in control kidneys but stunted and irregular branching patterns in VVC-pMyr-EGFP-PKD1-CTD-injected kidneys. Quantitative analysis of multiple pairs of kidneys showed that transduction of the pMyr-EGFP-PKD1-CTD construct was associated with decreases in kidney and ureteric bud volume, reduced numbers of ureteric bud branch points and branch tips and reductions in ureteric bud length and total area. Of note, however, were significant resultant increases in the outer diameters of the bud at the initial division of the ureteric bud stalk within the metanephric mesenchyme and the particularly marked increases in volumes of the terminal branches. Multiple paired sets of injected kidneys were quantified: Reduced numbers of branch points (n = 11 pairs). Reduced numbers of branch tips (n = 9 pairs). Reduced ureteric bud length (n = 8 pairs). Reduced ureteric bud area (n = 5 pairs). Increased outer diameters of the first branch points (n = 10 pairs). Increased terminal branch volume (n = 10 pairs). Transduction of pMyr-EGFP-PKD1-CTD reduces glomerular induction. FITC peanut agglutinin lectin (PNA) fluorescence analysis of glomerular development (lower panel) in VVC-pMyr-EGFP-PKD1-CTD versus empty VVC (control)-injected kidneys after 3 days of subsequent organ culture showed that significantly fewer glomeruli were induced in kidneys after transduction of pMyr-EGFP-PKD1-CTD in the ureteric bud (n = 12 pairs).
Fig. 8. Transduction of 5TM-EGFP-PKD1-CTD enhances ureteric bud morphogenesis. Whole mount phase contrast, immunofluorescence and confocal analysis of calbindin distribution in 5TM-EGFP-PKD1 versus control injected E11.5 mouse embryonic kidneys as in Fig. 7. Skeletonized imaging and quantitative analysis of paired kidneys showed increases in kidney volume and ureteric bud elongation and glomerular induction (n = 5 pairs).
Quantitative analysis confirmed a decrease in overall kidney volume and a dramatic reduction in total ureteric bud volume. Additional analyses of multiple paired sets of kidneys showed that ureteric bud transduction with VVC-pMyr-EGFP-PKD1-CTD resulted in statistically significant reductions in the numbers of ureteric bud branch points and branch tips (20.2 ± 1.88 versus 6.18 ± 0.64, P < 0.001, n = 11 pairs and 27.67 ± 3.6 versus 9.78 ± 0.85, P < 0.002, n = 9 pairs, respectively) as well as reduced ureteric bud length and ureteric bud area (5677.3 ± 1016.5 μm2 versus 1305.41 ± 152.64 μm2, P < 0.008, n = 8 pairs and 21.4 ± 1.8 mm³ versus 6.44 ± 0.38 mm³, P < 0.001, n = 5 pairs, respectively). By contrast, transduction with VVC-pMyr-EGFP-PKD1-CTD resulted in modest but statistically significant increases in the outer diameter of the ureteric bud at the initial branch point within the metanephric mesenchyme (52.35 ± 1.85 μm versus 64.71 ± 2.96 μm, P < 0.025, n = 10 pairs) and produced substantially significant increases in the volume of the ureteric bud at the terminal branch points (13.4 ± 1.73 mm³ versus 36.67 ± 8.29 mm³, P < 0.036, n = 10 pairs; Fig. 7).

**Overexpression of pMyr-EGFP-PKD-1-CTD inhibits glomerular induction**

Whole mount staining with peanut agglutinin lectin (PNA) or anti-WT-1 C19 antibody was used to detect glomeruli in paired kidneys injected with VVC-pMyr-EGFP-PKD1-CTD versus empty VVC alone (Fig. 7, lower panel, PNA). Statistically significant reductions in glomerular induction were detected after ureteric bud transduction with VVC-pMyr-EGFP-PKD1-CTD (26.5 ± 2.28 versus 5.17 ± 0.97, P = 0.001, n = 12; Fig. 7).

**Overexpression of a 5TM-polycystin-1 fusion protein, which increases polycystin-1 activation of AP-1, increases ureteric bud elongation**

An identical set of studies was carried out to evaluate the effects of microinjection of an activating 5TM-EGFP-PKD1 fusion protein into E11.5 fetal mouse kidneys. Fig. 8 shows the effects after 4 days in culture. Phase microscopy suggested that transduction with 5TM-EGFP-PKD1 caused a slight increase in size, and this was confirmed by quantitative analysis as a trend but did not reach statistical significance. Calbindin staining and confocal analysis of ureteric bud development as well as glomerular induction showed trends towards increases in most parameters and reached statistical significance with regard to increases in ureteric bud length and ureteric bud area. By contrast, the outer diameters of the first branch points tended to decrease.

**Discussion**

Several lines of evidence had led to the suggestion that PKD1-encoded protein, polycystin-1, might play an essential developmental role in the morphogenesis of the mammalian kidney. PKD1 knockout mice are embryonic or perinatal lethal with cystic kidney development, and many fetal genes are aberrantly expressed in the kidneys of mice and humans with PKD. Initial antibody studies showed high levels of polycystin-1 expression in fetal mouse and human kidneys predominantly localized to the basal plasma-membranes of the ureteric bud-derived collecting duct epithelia, in punctate focal deposits, consistent with focal adhesions (Geng et al., 1997; Ward et al., 1996; Wilson, 1997). In addition, transfection of full-length PKD1 into MDCK cells or activating PKD1 constructs into mouse inner medullary collecting duct (IMCD) cells resulted in altered cell morphology and tubulogenesis when grown in 3-dimensional collagen gels (Boletta et al., 2000; Nickel et al., 2002).

Subsequent biochemical, cellular, molecular and genetic analysis supported the conclusion that polycystin-1 acts as a mechanosensor in contact with the extracellular environment at three sites in mammalian renal tubule epithelial cells: at the basal membrane in focal adhesion complexes at the cell–matrix interface (Wilson, 1997; Wilson et al., 1999), in the apico-lateral cell adherens junctions at the cell–cell interface (Huan and van Adelsberg, 1999) and at the apical central primary cilium membrane at the cell–lumen interface (Yoder et al., 2002). Signals from the environment received by the long, extracellular, multi-domain N-terminal of polycystin-1 are coordinated by means of multiprotein complex formation and local cation fluxes at the membrane and then transduced via phosphorylation and protein–protein interactions at the intracellular C-terminal portion of polycystin-1 resulting in initiation of intracellular signaling cascades and regulation of gene transcription (Wilson, 2004b).

The current study is the first to utilize the intact mouse embryonic kidney in organ culture to analyze directly the effects of inhibition of PKD1 gene transduction. Similar to the mouse, normal polycystin-1 expression is maximal during metanephric kidney development and is also predominantly localized to the basal membrane focal adhesions of the developing ureteric bud in a punctate pattern consistent with in vivo focal adhesions. Interestingly, this localization is seen as early as in the mesonephric duct as well as after the first bifurcation of the metanephric ureteric bud. This localization persists through mid-gestation and contrasts with the lower levels of expression and predominantly apical–lateral localization of polycystin-1 in normal post-natal and adult kidney collecting tubule epithelia. Polycystin-1 also rapidly localizes to basal membrane focal adhesion structures of subconfluent cultures of human fetal collecting tubule epithelia after attachment to exogenous collagen, where it has previously been shown to co-localize, co-sediment and co-immunoprecipitate with several focal adhesion complex proteins including paxillin, vinculin, talin, α-actinin, focal adhesion kinase, c-src and p130cas (Geng et al., 2000; Wilson et al., 1999). In addition,
the punctate basal membrane distribution, as well as the co-localization with the focal adhesion index protein, paxillin, indicates focal adhesion targeting of polycystin-1 in renal epithelia transfected with either the AP-1 activating, 5TM-PKD1 or the pMyr-EGFP-PKD1-CTD expression constructs which block polycystin-1-dependent AP-1 activation (Kaletta et al., 2003). The ability of polycystin-1 to modify its preponderance of distribution in response to different cellular conditions has also been suggested by its apical–lateral pattern of distribution in confluent normal renal tubule cultures and co-localization, co-sedimentation and co-immunoprecipitation with cell–cell adherens junction proteins including E-cadherin β-, α- and γ-catenins (Charron et al., 2000; Geng et al., 2000). Ciliary staining has also been detected in fully differentiated confluent renal epithelial cultures, but not in subconfluent cultures (Nauli et al., 2003, and R. Rohatgi personal communication).

The early onset of expression of polycystin-1 in the metanephric kidney and its concentration in the focal adhesion points of contact between the basal cell membrane and the extracellular matrix in the deeper stalk portions of the trunk of the ureteric bud suggested a role for polycystin-1 in the migration and elongation of the ureteric bud during branching morphogenesis. This hypothesis was further suggested by evidence that ADPKD epithelia have marked defects in cell–matrix interactions including a significantly decreased migratory capacity and increased adherence to collagen matrix proteins. Cell–matrix abnormalities have long been known to be characteristic of ADPKD epithelia in vitro, where they contribute to the abnormal epithelial cell proliferation rates and altered apical–basal polarization of ADPKD epithelia (Carone and Kanwar, 1993; Roitbak et al., 2004; Wilson et al., 1991, 1992).

Since polycystin-1 is highly expressed in the ureteric bud of fetal kidneys and AP-1 activation has been implicated as an important polycystin-1 downstream signaling pathway (Kim et al., 1999), we sought to determine whether its inactivation might influence the regulation of renal development. To this end, we engineered a pMyr-EGFP-PKD1-CTD fusion protein that interferes with the activation of AP-1 by PKD1-TM5 and used a lentiviral delivery vector to determine whether inactivation of polycystin-1 signaling had a direct effect on ureteric bud morphogenesis in the embryonic mouse metanephric kidney in organ culture. The presence of a myristoylation signal sequence ensured insertion of the transduced polycystin-1 protein into the basal cell membrane of the ureteric bud, and injection at early stages of ureteric bud proliferation ensured expression throughout the developing tree. The “chicken-wire” appearance of GFP staining confirmed that the transduced protein was targeted to the basal membrane of the ureteric bud, where it would be in the same location as endogenous polycystin-1 and thus able to exert its inactivating (possibly dominant negative) effect. We speculate that this effect might be brought about through competition with protein–protein interactions and/or by inactivating polycystin-1-induced intracellular signaling cascades including those that act through JNK kinase/MAP kinase/AP-1 pathways as implicated by Kim et al. (1999) and known to often be involved with cell proliferation, differentiation and morphogenesis (Davis, 1999; Reed et al., 2001). Although sham injections or injection of empty lentiviral vector alone had no effect on the progressive elaboration of ureteric bud branching and nephron induction in vitro, the injection of dominant negative pMyr-EGFP-PKD1-CTD dramatically reduced but did not eliminate ureteric bud branching and glomerular induction. Detailed quantitative analysis also showed significantly expanded diameters of the ureteric bud at the initial T-shaped bifurcations within the metanephric mesenchyme as well as at the tips. Since our construct targets the basal membrane focal adhesions, this suggests that inactivation of polycystin-1 at this site has effects on ureteric bud elongation, branching, volume and diameter control. By contrast, the injection of activating 5TM-EGFP-PKD1 caused opposite effects, leading to statistically significant increases in ureteric bud elongation, consistent with the findings of Boletta et al. (2000) showing that polycystin-1 induced tubulogenesis in MDCK cells in collagen gels.

Although these findings do not rule out important functional roles for apical and lateral polycystin-1, they do suggest a primary role for basal polycystin-1 in early metanephric developmental branching morphogenesis. The predominant localization at cell–cell junctions in normal adult kidney epithelia as well defects in polycystin-1–cadherin-complex formation and polarity abnormalities in ADPKD epithelia suggest a critical role for apical–lateral polycystin-1, seen predominantly in adult kidneys, in the maintenance of the differentiated state (Charron et al., 2000; Roitbak et al., 2004). By contrast, recent studies are consistent with a role for apical ciliary polycystin-1 (possibly in concert with the autosomal recessive polycystic kidney disease (ARPKD) gene product fibrocystin) in the response to luminal ion concentration and fluid flow, suggesting a possible role in tubule maturation which predominates in the perinatal/pediatric periods (Nauli et al., 2003; Rohatgi et al., 2003).

The formation and dynamic turnover of focal adhesion complexes are essential for normal cell migration involving matrix attachment at the apical pole and detachment at the posterior pole (Laukaitis et al., 2001). Recent work has shown that paxillin, a marker component of the polycystin-1-containing focal adhesion complex, is also characteristic of dynamic apical adhesions essential for FAK-dependent migration (Webb et al., 2002). This is consistent with mutations in PKD1 leading to defective recruitment and phosphorylation of FAK into ADPKD focal adhesions (Wilson et al., 1999). Not surprisingly, mutations in many genes whose products are involved in focal adhesion assembly and function have also been implicated to play important roles in the control of migration: including FAK, p130cas, c-src and protein tyrosine phosphatases (Angers-
Loustau et al., 1999; Cary et al., 1998; Ilic et al., 1995; Verbeek et al., 1999) and to have impact on cell polarity (Timpson et al., 2001). Taken together, this suggests that the over-adherent, sluggishly motile phenotype of ADPKD epithelia is a consequence of functionally defective cell–matrix interactions via the focal adhesions and leads to stunted ureteric bud elongation and branching. Maintenance of the integrity of the focal adhesion complex during development of the ureteric bud is critical, as suggested by evidence that mutations in other genes, such as tensin and NPH1, whose protein products are integral components of the focal adhesion that interact with actin and p130cas, also cause cystic kidney disease (Benzing et al., 2001; Geng et al., 1997).

The essential features of normal branching morphogenesis require coordination of epithelia cell proliferation and migration to bring about appropriate elongation of the bud, the precise temporal and spatial regulation of branching, as well as strict control of the bud volume and internal and external diameters. We conclude that interference with polycystin-1 signaling pathways may interfere with regulation of the early stages of branching morphogenesis of the metanephric kidney. This is apparently a common genetic target for mutations frequently associated with renal cyst formation in human diseases.

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