

# Functional Analysis of PKD1 Transgenic Lines Reveals a Direct Role for Polycystin-1 in Mediating Cell-Cell Adhesion

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**Abstract.** The *PKD1* protein, polycystin-1, is a large transmembrane protein of uncertain function and topology. To study the putative functions of polycystin-1, conditionally immortalized kidney cells transgenic for *PKD1* were generated and an interaction between transgenic polycystin-1 and endogenous polycystin-2 has been recently demonstrated in these cells. This study provides the first functional evidence that transgenic polycystin-1 directly mediates cell-cell adhesion. In non-permeabilized cells, polycystin-1 localized to the lateral cell borders with N-terminal antibodies but not with a C-terminal antibody; there was a clear difference in surface intensity between transgenic and non-transgenic cells. Compared with non-transgenic cells, transgenic cells showed a dramatic increase in resistance to the disruptive effect of a polycystin-1 antibody raised to the PKD domains of polycystin-1 (IgPKD)

in both cell adhesion and cell aggregation assays. The differential effect on cell adhesion between transgenic and non-transgenic cells could be reproduced using recombinant fusion proteins encoding non-overlapping regions of the IgPKD domains. In contrast, antibodies raised to other extracellular domains of polycystin-1 had no effect on cell adhesion. Finally, the specificity of this finding was confirmed by the lack of effect of IgPKD antibody on cell adhesion in a *PKD1* cystic cell line deficient in polycystin-1. These results demonstrate that one of the primary functions of polycystin-1 is to mediate cell-cell adhesion in renal epithelial cells, probably via homophilic or heterophilic interactions of the PKD domains. Disruption of cell-cell adhesion during tubular morphogenesis may be an early initiating event for cyst formation in ADPKD.

Autosomal dominant polycystic kidney disease (ADPKD), the most common inherited human renal disease (incidence, 1 in 1000 live births) is caused by mutations in two genes, *PKD1* (85%) and *PKD2* (15%). It is a systemic disorder characterized by the formation of fluid-filled cysts mainly in the kidney but also commonly in the liver and pancreas. ADPKD is also associated with an increased incidence of non-cystic manifestations, including hypertension, cardiac valve abnormalities, diverticular disease, and intracranial aneurysms.

Since the identification of *PKD1* and *PKD2*, investigation into the putative functions of the two ADPKD proteins, polycystin-1 and polycystin-2, has been intense, but a consensus on their likely physiologic functions has not been reached. Several possible mutational mechanisms underlying cyst formation including haploinsufficiency and a two-hit model have been proposed, but it is not clear how mutations in either gene lead

to cyst formation (1). Potentially more than one mechanism could be operative.

Polycystin-1 is a large (>460 kD) heavily glycosylated integral membrane protein (2). It is predicted to have a large N-terminal extracellular domain (approximately 2500 aa), 11 transmembrane domains, and a short C-terminal cytoplasmic tail (3). The extracellular region appears to have a modular structure suggesting the presence of potential functional motifs. These include two leucine-rich repeats (LRR), a C-type lectin, a LDL-A receptor motif and a large region (approximately 1000 aa) with strong homology to the sea urchin receptor for egg jelly (REJ) protein. The major part of the N-terminal region, however, consists of 16 novel repeats (80 to 90 aa) with low sequence homology to Ig domains. These so-called PKD domains or repeats are arranged in tandem (II–XVI) except for domain I, which is present between the LRR and lectin modules. NMR studies of a bacterial recombinant PKD domain I protein has revealed that it has a  $\beta$ -sandwich fold common to other cell surface proteins (4). The function of the various PKD domains has not been fully elucidated. It is notable, however, that synthetic peptides containing a repetitive PKD domain motif (WDFGDG) inhibit ureteric bud branching and nephron formation in organ culture (5) and recombinant PKD domain proteins display strong homophilic interactions *in vitro* (6). In the latter study, an antibody raised to epitopes within the PKD repeats (IgPKD) as well as recom-

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binant PKD domain GST fusion proteins were also found to reversibly disrupt cell adhesion in MDCK cells. In contrast, an antibody to the LRR region of polycystin-1 had no effect on cell adhesion.

A number of other studies have also implicated polycystin-1 in the regulation of cation transport, proliferation, apoptosis and tubulogenesis (7–9). In heterologous systems, polycystin-1 can interact cooperatively with polycystin-2 to form a non-selective cation channel and possibly regulate proliferation via a JAK/STAT pathway (7,10). An inhibitory effect of polycystin-2 on constitutive G protein activation by polycystin-1 has also been reported (11). However, it is uncertain whether both proteins always function together. For instance, native polycystin-2 exists both as homodimers and heterodimers (with polycystin-1) (2). The functions of polycystin-2 homodimers are likely to be distinct from those of the polycystin-1/polycystin-2 complex and could potentially account for the polycystin-1 independent polycystin-2 channel activity that has been described in some experimental systems (12–14).

Polycystin-1 is expressed ubiquitously and yet at low levels in most cell lines. This and the difficulty in generating cell lines expressing heterologous full-length polycystin-1 have led to the majority of studies concentrating on the function of the C-terminus of polycystin-1. To facilitate the study of full-length polycystin-1, we have taken the experimental approach of generating conditionally immortalized kidney cell lines from mice transgenic for *PKD1*. Using this model, we have recently described the trafficking of full-length polycystin-1 and its association with endogenous polycystin-2 to form a stable protein complex (2). In this article, we describe the likely topology of transgenic polycystin-1 and provide functional evidence that it is directly involved in mediating cell-cell adhesion.

## Methods and Materials

### *Generation of PKD1 Transgenic Lines*

The generation and characterization of a murine collecting duct *PKD1* transgenic line, M7 and a non-transgenic control M8, has been described in a recent paper (2). M7 cells stably express a transgene containing the entire human *PKD1* gene and its 5' and 3' flanking regions. M8 cells express endogenous *pkd1* alone. Both cell lines express T antigen under the control of a temperature-sensitive and  $\gamma$ -interferon inducible promoter (2). To activate T antigen expression, both lines were normally cultured at 33°C in DMEM/F12 supplemented with 5% Nuserum and recombinant mouse  $\gamma$ -interferon (Boehringer Mannheim, Germany). Pooled uncloned cells were studied at early passage (up to passage 20) and retained epithelial morphology throughout.

### *Generation of Conditionally Immortalized Normal (UCL93/3) and a PKD1 Cystic (OX161/1) Cell Lines*

Normal and cystic renal tubular cells were obtained by standard cultured techniques previously described (15). Normal renal tissue was obtained from the normal pole of an adult kidney removed because of carcinoma (UCL93). Cystic epithelial cells were isolated from ten superficial cysts of a polycystic kidney removed because of persistent infection (OX161). Primary cultured cells were transduced sequentially at early passage (P1–4) with retroviral vectors containing a temperature-sensitive T antigen (tsT) and human telomerase

(hTERT), selected for antibiotic resistance (G418 and hygromycin) and cultured at 33°C (16). Pooled uncloned cells were maintained in DMEM/F12 supplemented with 5% Nuserum and studies performed up to passage 20.

### *Specificity of Antibodies to Polycystin-1 and Polycystin-2*

The generation and specificity of polycystin-1 antibodies directed to the leucine rich repeats (LRR) and its flanking regions (7e12), the C-terminal 205 amino acids (BD3), the Ig-like domains (residues 843–2145) (anti-IgPKD) and a large N-terminal region containing the LRR motif up to the fifth PKD repeat domain (PKDNT2B) have been described in previous papers (2,6,17). The polycystin-2 antibody (p30) recognizes the C-terminal 258 amino acids of human polycystin-2 (18).

### *Immunofluorescence*

Cells were grown on collagen-coated glass coverslips and were fixed with ice-cold acid-ethanol for 1 h. Blocking was carried out for 1 h with 5% milk/PBS and primary antibodies were used at a dilution of 1:100 overnight at 4°C. Controls included cells stained with primary antibody omitted, an irrelevant IgG1 mAb (Serotec, Kidlington, UK) or a non-immune rabbit IgG fraction (Dako, Ely, UK). For live cell staining, cells were pre-incubated with primary antibody at 4°C for 30 min before fixation. Antibody binding was visualized using FITC-conjugated goat anti-mouse IgG and TRITC-labeled or FITC-labeled goat anti-rabbit secondary antibodies at a dilution of 1:300. Slides were viewed using a Zeiss Axioplan II equipped with 100W mercury lamp, a 63X oil immersion objective and UV, FITC, Texas Red, and Cy5 excitation/emission filter sets. Images were collected using a Digital Pixel CCD camera containing a KAF-1600 CCD chip (Digital Pixel Systems, Brighton, UK).

For dual immunofluorescence, wide field images of fluorescence samples were acquired using a  $\times 60/1.4$  NA oil immersion objective lens on a Delta Vision restoration microscope (Applied Precision, Issaquah, WA) based around an Olympus IX70 inverted microscope equipped with mercury-arc illumination, fluorescein, and rhodamine filter sets. Optical sections 0.2  $\mu\text{m}$  apart were captured with a CoolSNAP HQ CCD camera (Roper Scientific, Tucson, AZ). Digital deconvolution and image analysis was then performed on the data sets with SoftWoRx deconvolution software (Applied Precision).

### *Disruption of Cell-Cell Adhesion in Cell Monolayers*

As described previously, cells were plated in 24-well plates and cultured for 48 h to 60% confluence in normal culture media (6,19). To assess the effect of different antibodies on cell adhesion, cells were incubated for up to 4 h with normal medium containing anti-IgPKD immune serum (1:10 to 1:100), pre-immune serum (1:10 to 1:100), PKDNT2B (1:10 to 1:100), 7e12 (4 to 40  $\mu\text{g}/\text{ml}$ ) or medium alone. The effect of the antibody on cell-cell adhesion was quantified by counting the total of firmly adherent cells present in each well at the end of the incubation.

In initial experiments, we noted that M7 cells took longer to reach confluence compared with M8 cells. After 48 to 96 h of plating, the total number of M8 cells was consistently 1.5 times higher than those of M7 cells (not shown). To adjust for this difference in growth rate, the number of adherent cells per experimental well was expressed as a percentage relative to the total number present in control wells (culture medium alone). Each experiment was performed at three times in triplicate and the result expressed as mean  $\pm$  SEM ( $n = 9$ ). Statistical significance was calculated using the paired  $t$  test.

The synthesis of recombinant GST and GST-IgPKD fusion proteins (GST-Ig<sup>a</sup>, GST-Ig<sup>b</sup>, and GST-Ig<sup>c</sup>) has been described in a previous paper (6). For experiments investigating the effect of GST-IgPKD fusion proteins on cell adhesion, cells were incubated with 0.1 to 10  $\mu\text{g/ml}$  each of GST-Ig<sup>a</sup>, GST-Ig<sup>b</sup>, and GST-Ig<sup>c</sup> or control media containing 10  $\mu\text{g/ml}$  GST protein alone. The specificity of the anti-IgPKD antibody (1:30) for polycystin-1 was further assessed by pre-incubation overnight at 4°C with 10  $\mu\text{g/ml}$  each of GST-Ig<sup>a</sup>, GST-Ig<sup>b</sup>, and GST-Ig<sup>c</sup> or with 10 to 30  $\mu\text{g/ml}$  GST protein before the cell adhesion experiments.

### *Assessment of the Effect of IgPKD Antibody on Apoptosis and Cell Viability*

M8 cells were plated in 96-well plates at a density of  $1 \times 10^4$  cells and cultured for 48 h. To exclude the possibility that anti-IgPKD antibody had directly induced apoptosis leading to cell detachment, detached cells were centrifuged, washed three times with PBS, and fixed with 4% paraformaldehyde. Cytospin preparations were made and stained using a TUNEL assay (ApopTag In Situ Apoptosis Detection Kit, Intergen Company). A DAPI counterstain was used to visualize nuclear morphology. As a positive control for the TUNEL assay, cells were treated with DNaseI (1 mg/ml) for 10 min at room temperature to induce DNA strand breaks. Alternatively, to assess cell viability following antibody treatment, detached cells were counted, replated into collagen-coated wells, and cultured for a further 48 h.

### *Cell Aggregation Assay*

Cells were detached from near-confluent M7 and M8 monolayers using a 1 mM EDTA/PBS solution, washed with PBS, and resuspended in 5 ml of culture media pre-equilibrated overnight in a 5% CO<sub>2</sub> 37°C incubator. A single cell suspension was generated by gently passing the detached cells through a 21G needle and this was verified by light microscopy. An aliquot of cells (0.5 to 1 ml,  $2 \times 10^6$  cells/ml) was then incubated at 37°C for 4 h with IgPKD antibody (1:10 dilution), non-immune serum (1:10 dilution), or culture medium alone in sterile Eppendorfs on a rotator. At the end of the incubation period, an aliquot of cells was examined using a hemocytometer. The percent aggregation was estimated by recording cells in a 0.1-mm<sup>3</sup> field as either single cells or as aggregates (20). Aggregates were further graded as small (<5 cells) or large (>5 cells) as described previously (20). The remaining cells were fixed with 2% PFA for 10 min before staining with a nuclear stain (DAPI) for photography. Three independent experiments were performed, and three separate fields ( $\times 10$  objective) counted per experiment for each treatment.

### *Immunoblotting*

Total cell lysates were prepared and processed for Western blotting as described previously (2).

### *Materials*

All chemicals were purchased from Sigma (Poole, UK) unless otherwise stated. A human proximal tubular cell line (CL11) was the kind gift of Dr. L. Racusen (Baltimore, MD). A mAb to desmoplakin (clone 115F) was provided by Professor D.R. Garrod (Manchester, UK). A rat mAb to ZO-1 was obtained from the Developmental Studies Hybridoma Bank, University of Iowa. Antibodies to E-cadherin, N-cadherin, and pan-cadherin were purchased from BD Transduction Labs (Cowley, Oxford, UK) and Cambridge Bioscience (Cambs, UK) respectively.

## **Results**

### *Polycystin-1 Localizes to the Lateral Cell Borders of Transgenic and Non-transgenic Cells*

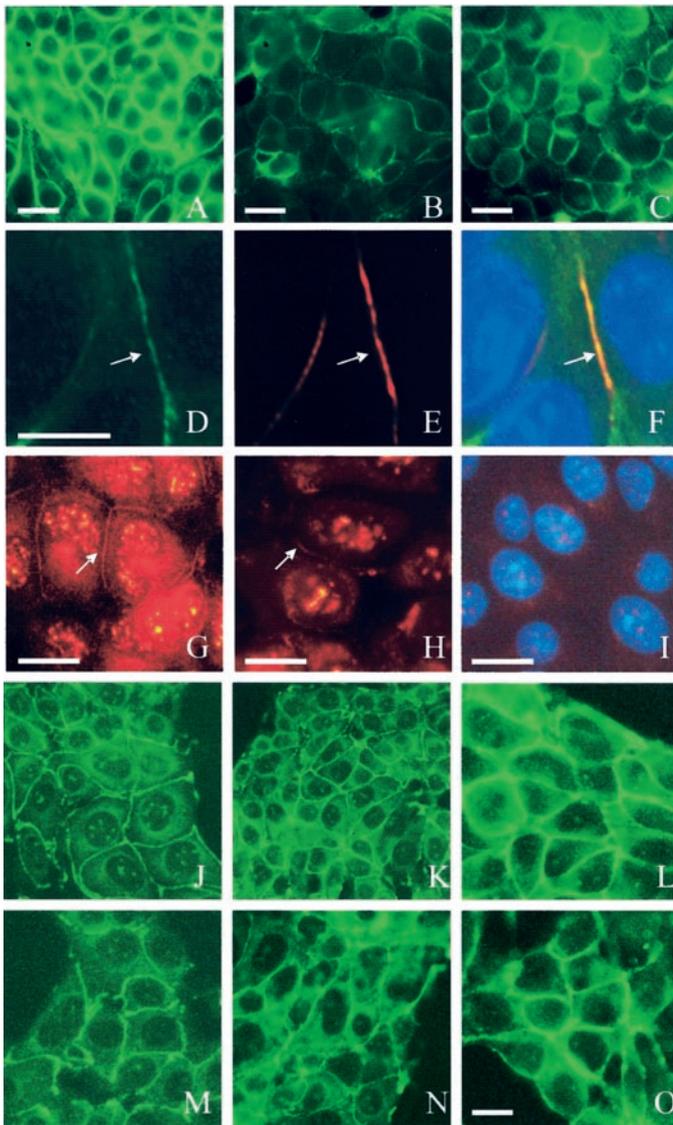
The topology of polycystin-1 expression was investigated in both non-permeabilized and permeabilized cells using three antibodies whose specificity has been demonstrated in previous studies. Immunostaining was carried out with antibodies to both extracellular (7e12, IgPKD) and intracellular (BD3) domains of polycystin-1 (Figure 1). Antibodies raised to the predicted extracellular domains of polycystin-1 showed clear membrane staining at sites of cell-cell contact in non-permeabilized cells. In general, labeling with IgPKD was weaker than that observed with 7e12 in unfixed cells. However, using dual immunofluorescent staining, we found co-localization of signals from both antibodies in the lateral cell membrane on the same optical z sections in M7 (not shown) and M8 (Figure 1, D-F) cells. No staining was seen with the secondary antibody alone, with a control mouse IgG1 monoclonal antibody or with non-immune rabbit serum (data not shown).

No staining was observed in non-permeabilized cells with BD3 (Figure 1I); however, in permeabilized cells, BD3 stains the lateral cell borders together with some intracellular cytoplasmic staining (Figure 1G,H). Cell surface expression of polycystin-1 was more intense in the M7 transgenic compared with the M8 control cells with all three antibodies. In contrast, the expression of ZO-1, E-cadherin, pan-cadherin,  $\beta$ -catenin, and plakoglobin at the lateral cell borders was identical in both lines (Figure 1, J-O). Using dual immunofluorescence, we found incomplete overlap among polycystin-1, ZO-1, E-cadherin, and pan-cadherin signals in M7 cells (data not shown). However, we were unable to detect desmoplakin at the lateral cell membranes of M7 and M8 cells using a well-characterized desmoplakin antibody that crossreacts with mouse desmoplakin (data now shown).

### *Transgenic Polycystin-1 Protects M7 Cells from the Disruptive Effect of Ig-PKD on Cell-Cell Adhesion*

In a recent study, the IgPKD antibody was shown to disrupt cell-cell adhesion in MDCK cell monolayers (6). This raised the possibility that the PKD repeats of polycystin-1 were involved in mediating cell-cell adhesion through homophilic interactions and/or through heterophilic interactions with other (as yet unidentified) cell surface molecules.

We found a similar dose-dependent and time-dependent effect of the IgPKD antibody on cell-cell adhesion in the M8 non-transgenic cells and MDCK cells (not shown): over 90% of cells had detached after a 4-h incubation with 1:10 dilution of IgPKD. Cell-cell separation could be observed within 90 min of incubation and increased with incubation time (up to 4 h) (Figure 2). In preliminary studies, we observed that the Ig-PKD antibody appeared to have much less of an effect on cell adhesion in the M7 transgenic cells. In contrast to M8 cells, no significant effect was observed in M7 cells at 1:30 dilution of IgPKD (Figure 2F). Moreover, over 50% of M7 cells remained adherent after a 4-h incubation with 1:10 dilution of the antiserum (Figure 2, E and F). In contrast, pre-



**Figure 1.** Subcellular localization of polycystin-1 and other junctional proteins in transgenic and non-transgenic cell lines. Immunofluorescence staining for polycystin-1, ZO-1, E-cadherin, and pan-cadherin was carried out on M7 (transgenic) and M8 (control) cells. Three polycystin-1 antibodies (7e12, IgPKD, BD3) were used to study the topology of polycystin-1 expression. In non-permeabilized M7 cells, polycystin-1 localized to the lateral cell membranes with the N-terminal antibodies 7e12 (A) and IgPKD (C). In non-permeabilized M8 cells, a similar pattern of much lower intensity was observed for 7e12 (B and D) and IgPKD (E): colocalization of the signals (merged color image) in the lateral membrane between two adjacent cells (arrows) at the same optical section is shown in F. Similar results were observed for M7 cells (not shown). BD3 showed no specific labeling in non-permeabilized M7 or M8 cells (I). In permeabilized cells however, BD3 detected both lateral membrane and cytoplasmic polycystin-1 expression in M7 (G) and M8 (H). Non-immune rabbit serum controls showed no staining in either non-permeabilized or permeabilized cells (not shown). Expression of other junctional markers for M7 (J–L) and M8 (M–O) showed normal localization of ZO-1 (J and M), E-cadherin (K and N), and pan-cadherin (L and O) to the lateral cell membrane. Scale bars, 10  $\mu\text{m}$ .

immune serum from the same rabbit immunized to obtain IgPKD had no effect on cell adhesion in both cell lines (Figure 2, B and F).

#### *IgPKD Antibody Does Not Induce Apoptosis*

To rule out the possibility that binding of the IgPKD antibody was directly inducing apoptosis, detached M8 cells were examined both for the presence of apoptotic nuclei using a TUNEL assay and replated after washing to assess their viability after antibody treatment (Figure 3). No induction of apoptosis was observed in detached cells (Figure 3, A and B), and replated cells proliferated normally, demonstrating that they remained viable after antibody treatment (Figure 3E). In three independent experiments, we found that detached cells (90%) replated after IgPKD treatment remained viable and continued to divide, increasing in number by nearly threefold after 48 h ( $n = 9$ ). In contrast, relatively few cells (<10%) were detached following treatment with pre-immune (PI) serum, and these did not proliferate under the same conditions probably due to the low replating density.

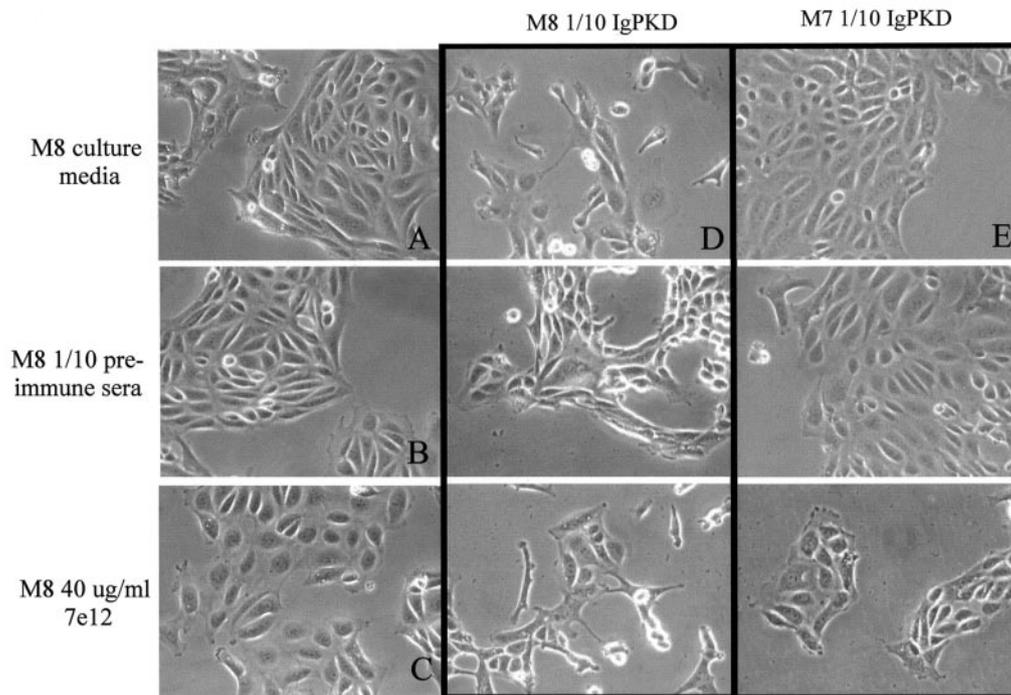
#### *Antibodies to Other Polycystin-1 Ectodomains Do Not Affect Cell Adhesion*

Similar experiments were performed with antibodies raised to other ectodomains of polycystin-1 that may be responsible for mediating cell adhesion. In particular, we examined the effect of two well-characterized N-terminal antibodies, 7e12 (Figure 2C) and NT2B, on cell adhesion in M7 and M8 cells. Unlike IgPKD, however, we found no effect of either antibody on cell adhesion, even after an extended 16-h incubation (data not shown).

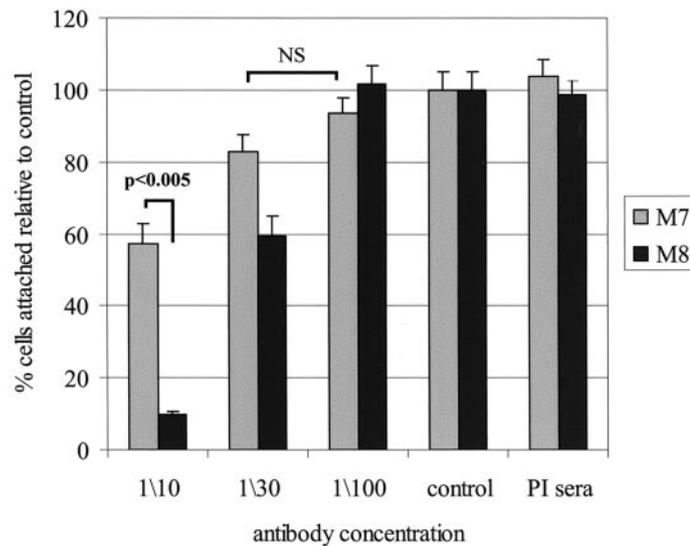
#### *IgPKD Antibody Primarily Disrupts Polycystin-1–Mediated Cell-Cell Adhesion*

To exclude the possibility that IgPKD antibody was disrupting potential cell-matrix adhesion mediated by polycystin-1 rather than cell-cell adhesion, we conducted aggregation assays with the M7 and M8 lines in the presence or absence of the antibody (Figure 4). In preliminary experiments, we observed a time-dependent increase in the number of cell aggregates for both lines; by 4 h, approximately 50% of cells had formed aggregates. Aggregates were divided approximately equally between small (<5 cells) and large (>5 cells) aggregates (20). Under control conditions, there was no difference between both lines in the number or size of aggregates found.

In the presence of the IgPKD antibody, the mean percentage of aggregating cells fell to 17% with M8 cells and to 40% in M7 cells (Figure 4B). Moreover, very few large (>5 cells) aggregates were seen in M8 cells after IgPKD treatment (<2%). Of relevance, almost all of the aggregates seen were small (<5 cells) (Figure 4A). In contrast, larger aggregates were still observed in M7 cells after IgPKD treatment, although these were fewer in number (approximately 10%) than those observed with non-immune serum treatment (approximately 25%) or culture medium alone (Figure 4A). The protective effect of transgenic polycystin-1 in this assay was



F

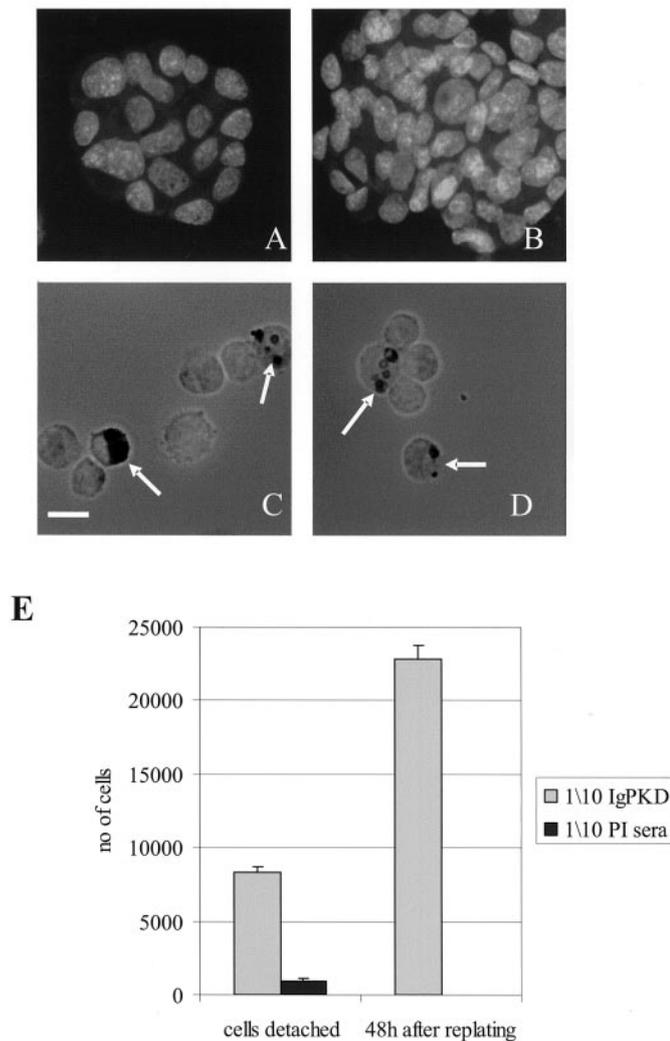


*Figure 2.* M7 cells are protected from the inhibitory effect of IgPKD antibody on cell-cell adhesion. In M8 control cells, no effect on cell-cell adhesion was observed after incubation with culture media alone (control) (A), in the presence of pre-immune (PI) rabbit serum (B), with 7e12 (C) or PKDNT2B (not shown). However, the IgPKD antibody induced a significant dose-dependent disruption of cell-cell adhesion maximal at 1:10 dilution: the severe disruption of cell-cell contacts is illustrated in three representative fields (D1–3). In contrast, overexpression of polycystin-1 appeared to confer a protective effect in M7 transgenic cells incubated with the same dilution of antibody (1:10) (E1–3). (F) M7 transgenic and M8 control cells were incubated with IgPKD antibody, control culture media or pre-immune rabbit serum for 4 h. The percentage of adherent cells relative to control culture media after a 4-h incubation period was calculated and expressed as the mean ± SEM. Each bar represents nine independent wells. Note that there is at least a threefold difference in the effect of the IgPKD antibody between M7 and M8 cells at a 1:10 dilution.

around twofold to threefold. The IgPKD antibody had no effect on the attachment or detachment of single cells (M7 or M8) plated on collagen I-coated wells (data not shown). These results confirm that the major effect of the IgPKD antibody is to disrupt polycystin-1 mediated cell-cell adhesion.

*The Effect of IgPKD Antibody on Cell Adhesion Is Neutralized by Pre-Incubation with Ig-PKD Fusion Protein*

Because of the larger volumes of antibody required to conduct the aggregation assays, further experiments were con-



**Figure 3.** The IgPKD antibody does not induce apoptosis in M8 cells. No nuclear changes typical of apoptosis were observed using a TUNEL assay in cytopsin of M8 cells detached following incubation with anti-IgPKD antibody (A and B). As a positive control for the assay, M8 cells (C and D) were treated with DNaseI for 10 min according to the manufacturer’s instructions. Apoptotic bodies are marked by the white arrows. In all panels nuclei were counterstained with DAPI. Scale bars, 10  $\mu$ m. (E) Cells detached following incubation with anti-IgPKD antibody proliferated normally, and cell numbers increased by nearly threefold over a 48-h period. The small number of cells (<1000) detached after incubation with pre-immune (PI) sera meant that the replating density was very low, resulting in no observable growth after 48 h. Results shown represent the mean  $\pm$  SEM. From three independent experiments performed in triplicate ( $n = 9$ ).

ducted on adherent cells. The specificity of the effect of IgPKD on cell-cell adhesion was tested by pre-incubating IgPKD antiserum with GST-IgPKD fusion proteins. This resulted in the neutralization of the effect of IgPKD on cell-cell adhesion in M8 cells to levels seen in control wells (Figure 5). In contrast, pre-incubation of IgPKD antibody with GST protein alone did not abolish the ability of the antiserum to disrupt cell-cell adhesion.

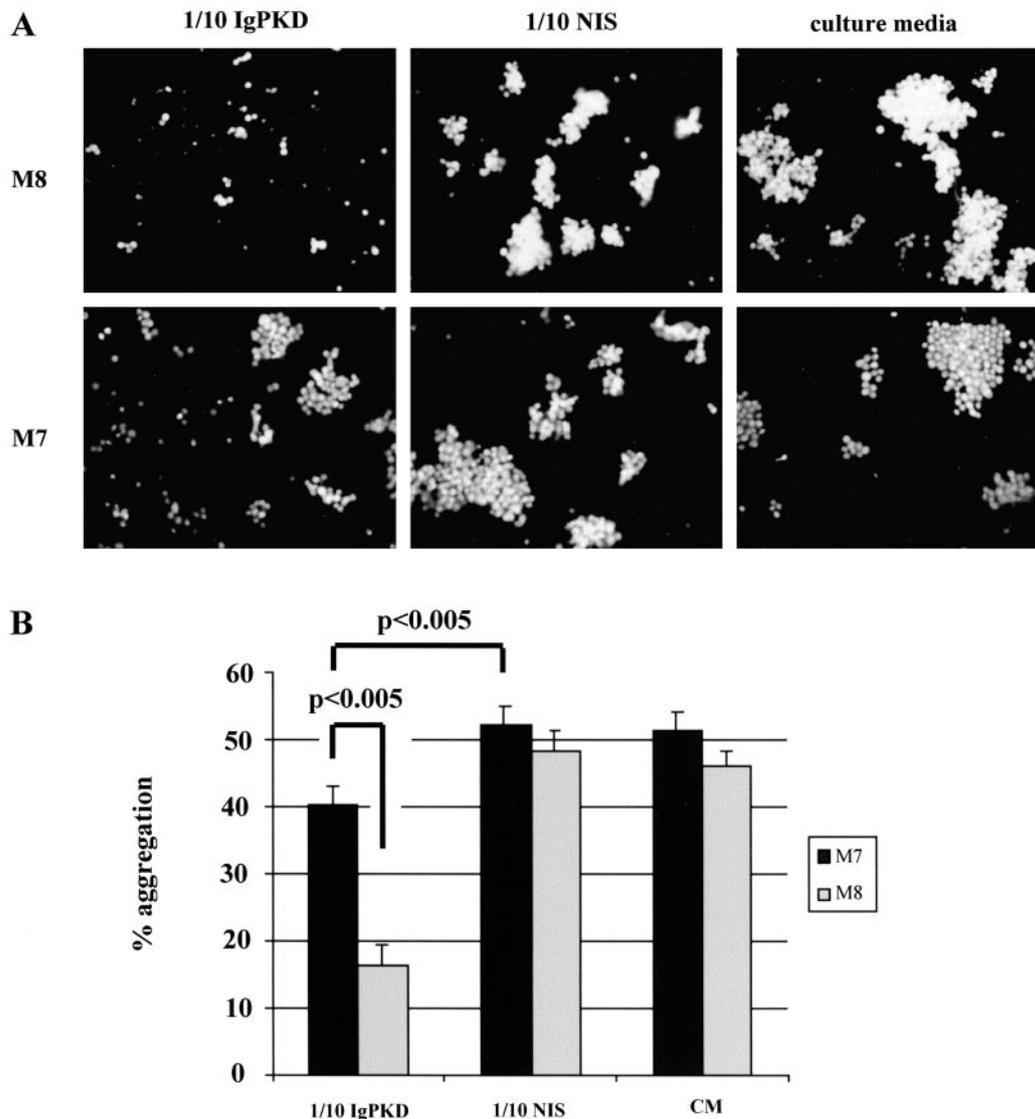
*Differential Effects of Ig-PKD Fusion Proteins on Cell-Cell Adhesion between M7 and M8 Cells*

The specificity of the disruptive effect of IgPKD and the importance of the PKD repeats in mediating cell-cell adhesion was further demonstrated by examining the effect of soluble fusion proteins containing overlapping regions of the PKD repeats of polycystin-1 (GST-Ig<sup>a</sup>, GST-Ig<sup>b</sup>, and GST-Ig<sup>c</sup>) on cell-cell adhesion in M7 and M8 cells. As shown in Figure 6, no disruption to cell-cell adhesion was observed in either cell line at the lowest concentration tested (0.1  $\mu$ g/ml). A differential effect on cell adhesion was observed at 1  $\mu$ g/ml of fusion protein: cell adhesion diminished by approximately 20% in M7 cells but by over 50% in M8 cells (Figure 6G). Increasing the concentration of the fusion proteins further to 10  $\mu$ g/ml abolished the differential effect seen at 1  $\mu$ g/ml. This indicates that the protective effect observed in M7 cells must be due to increased polycystin-1 surface expression rather than secondary to changes in another unidentified protein involved in cell-cell adhesion. Finally, GST protein alone (10  $\mu$ g/ml) had no effect on monolayer integrity demonstrating that the effects observed were specific to the PKD repeats of polycystin-1.

*Generation and Characterization of a PKD1 Cystic Cell Line*

We have recently generated a conditionally immortalized cystic epithelial cell line from a *PKD1* patient (OX161). The germline mutation in this patient has been described (E1537X) and would lead to truncation of polycystin-1 in the middle of the PKD repeats (17). A normal human renal cortical epithelial line (UCL93/3) was generated in identical fashion from tissue obtained from the normal pole of a kidney removed for carcinoma. Cystic and normal cells were first examined for polycystin-1 and polycystin-2 expression by immunoblotting. As shown in Figure 7A, polycystin-1 expression was markedly diminished in OX161/1 compared with two normal human kidney lines, UCL93/3 and an SV40-transformed human proximal tubular line CL11; a faint signal could however be detected in OX161/1 after prolonged exposure (not shown). In contrast, the expression of polycystin-2 and two other transmembrane proteins, E-cadherin and N-cadherin, were unaltered in all three lines. Somatic *PKD1* mutations in OX161/1 may have led to the reduction in polycystin-1 expression in these cells.

By immunofluorescence, we found that both OX161/1 and UCL93/3 expressed cytokeratin indicating their epithelial origin (Figure 7B). E-cadherin, although present in these cells, was not localized as expected to the lateral cell borders in either line but found in a cytoplasmic distribution (not shown). Desmoplakin was not detectable by immunofluorescence (not shown). To test if other cadherins might be compensating for the mislocalization of E-cadherin, we stained both lines with pan-cadherin and N-cadherin antibodies. As shown, pan-cadherin and N-cadherin antibodies both stained the lateral cell borders of both lines (Figure 7B). Polycystin-1 expression was faintly detectable at the lateral cell borders in UCL93/3 but was absent from OX161/1 (data not shown).



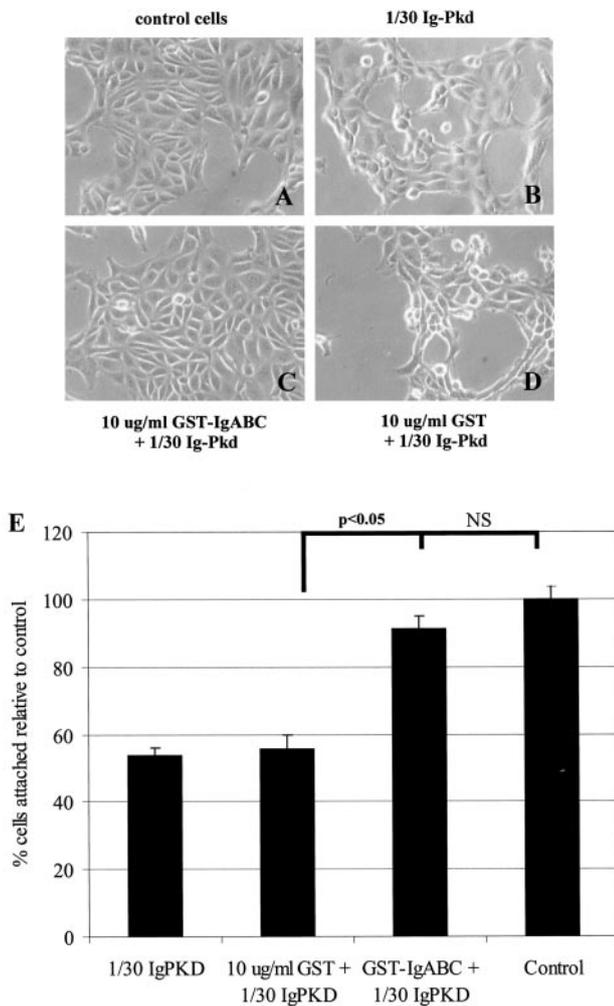
**Figure 4.** Differential disruption of polycystin-1 mediated cell aggregation in M7 and M8 cells by IgPKD antibody. The ability of IgPKD to disrupt cell aggregation in M7 and M8 cells was estimated after a 4-h incubation. The results are displayed as representative fields after DAPI staining (A) or quantitated as the percentage of cells forming aggregates *versus* single cells in three independent experiments (B). In control cells, approximately 50% of both cell lines had formed both small (<5 cells) and large (>5 cells) aggregates after a 4-h incubation. Non-immune serum (NIS) and culture medium (CM) alone had no effect on cell aggregation. In contrast, IgPKD antibody (1/10 dilution) significantly reduced cell aggregation to 40% in M7 and less than 20% in M8 cells. Note also the paucity of large aggregates in M8 cells after IgPKD treatment compared with M7.

#### *IgPKD Antibody has no Effect on Cell-Cell Adhesion in a Polycystin-1 Null Cystic Cell Line (OX161/1)*

Since OX161/1 has markedly diminished polycystin-1 expression, we predicted that the IgPKD antibody would have little or no effect on cell adhesion in these cells compared with UCL93/3. The results of these experiments are shown in Figure 7C. As with MDCK and M8 cells, a dose-dependent effect on cell-cell adhesion was found with UCL93/3. However, the IgPKD antibody had no discernable effect on cell scattering or attachment in OX161/1 cells even at the highest concentration tested (1:10).

#### **Discussion**

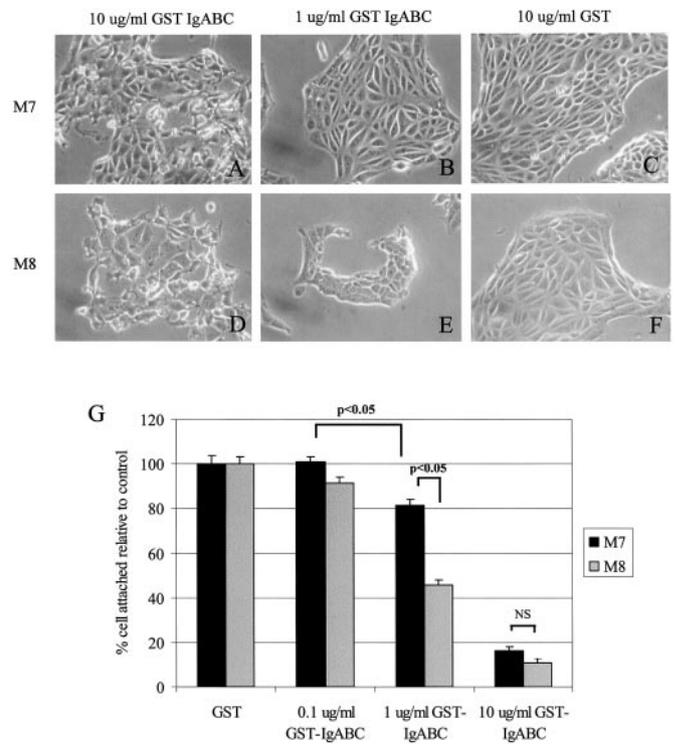
Studies of polycystin-1 function have been technically difficult because of the size, complexity, and low endogenous expression of the native protein. To overcome these difficulties, we have generated conditionally immortalized kidney cells transgenic for *PKD1* (2). Changes in the expression of *PKD1* during development mirror that of endogenous *pkd1* in *PKD1* transgenic mice. Importantly, expression of transgenic human polycystin-1 could rescue the embryonically lethal *Pkd1* knockout phenotype observed in *pkd1del34* mice (21). We reasoned therefore that cells derived from these animals



**Figure 5.** The effect of the IgPKD antibody on cell-cell adhesion can be neutralized by GST-Ig<sup>abc</sup> fusion proteins. M8 control cells were incubated in culture media alone (A) or with 1/30 anti-IgPKD antibody (B). The effect of anti-IgPKD antibody could be neutralized by pre-incubation with GST-Ig<sup>abc</sup> fusion proteins (C) but not by pre-incubation with GST alone (D). (E) Neutralization of the effect of IgPKD serum by pre-incubation with GST fusion proteins. The percentage of adherent cells relative to control culture media after a 4 h incubation period was calculated and expressed as the mean ± SEM. Each bar represents nine independent wells.

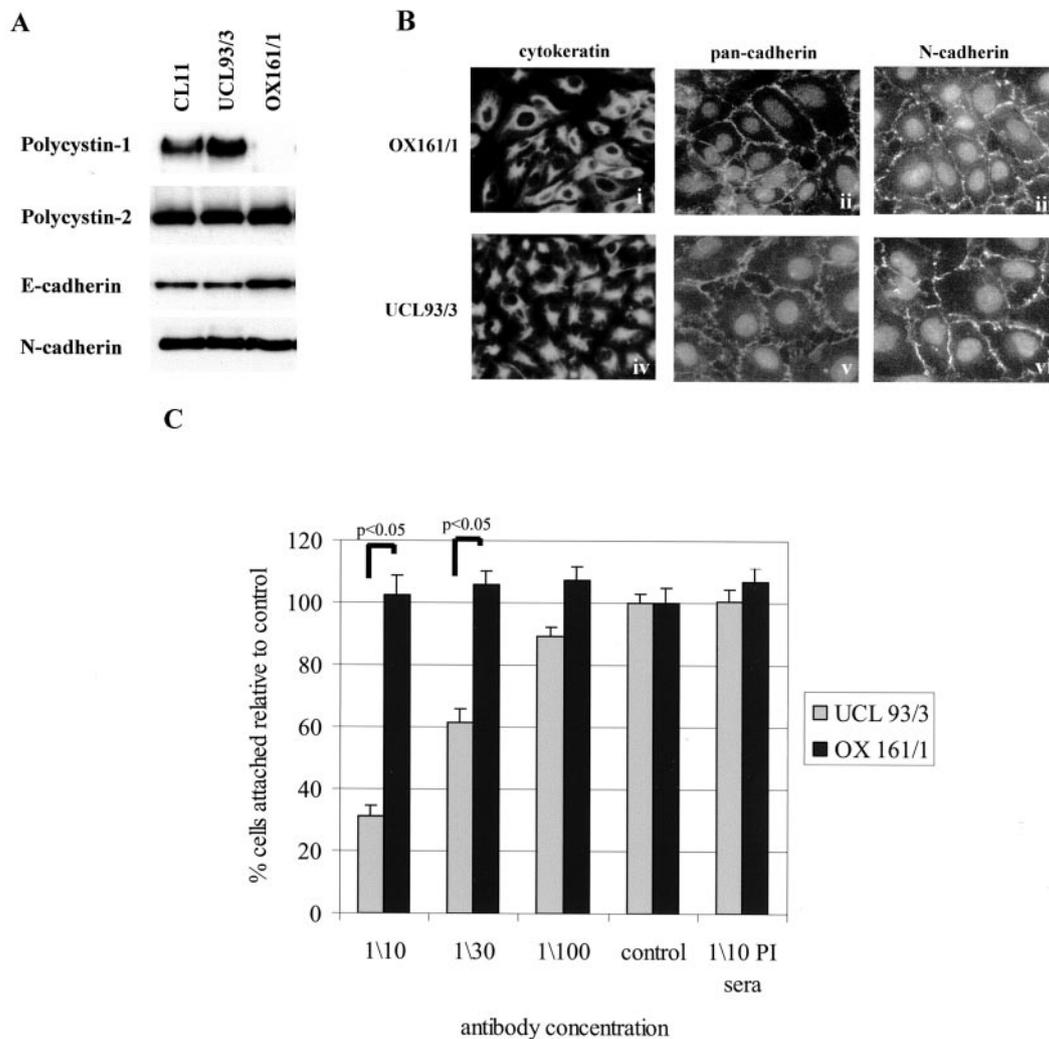
might provide good *in vitro* models to study polycystin-1 trafficking and function. Although age-dependent renal cysts were observed in some transgenic animals, these were predominantly glomerular in origin although dilatation of other tubular segments was also observed (21).

The expression of *PKD1* mRNA in transgenic cells is at least 20 times that of *pkd1* mRNA (2). We were unable to quantify the relative levels of transgenic and endogenous full-length polycystin-1 proteins, as our antibodies to human polycystin-1 crossreacted with mouse polycystin-1. However, increased amounts of an EndoH-resistant full-length polycystin-1 fraction could be co-purified with polycystin-2 from plasma membrane fractions of transgenic cells (2). In this paper, we provide the first functional evidence that this surface fraction of trans-



**Figure 6.** Differential effects of GST-Ig<sup>abc</sup> fusion proteins on cell-cell adhesion in M7 and M8 cells. The presence of 10 μg/ml GST-Ig<sup>abc</sup> disrupts cell-cell adhesion equally in M7 transgenic (A) and M8 control cells (D). M7 cells (B) were completely protected from the disruptive effect of a 4-h incubation with 1 μg/ml GST-Ig<sup>abc</sup> compared with M8 cells (E). Incubation with 10 μg/ml GST alone for 4 h had no effect on cell-cell adhesion in M7 (C) or M8 (F) cells. (G) Quantitation of the effect of GST fusion proteins on intercellular adhesion after a 4-h incubation. The percentage of adherent cells relative to control culture media was calculated and expressed as the mean ± SEM. Each bar represents nine independent wells.

genic polycystin-1 directly mediates cell-cell adhesion. First, using antibodies to specific extracellular and intracellular polycystin-1 domains, we confirmed the likely topology of the protein and also demonstrated a marked increase in lateral cell membrane expression of polycystin-1 by transgenic cells. Second, the increase in surface polycystin-1 expression was functionally significant; transgenic cells showed an up to threefold increase in resistance to the disruptive effect of polycystin-1 antibody (IgPKD) on cell-cell adhesion compared with non-transgenic cells. Importantly, the ability of polycystin-1 to mediate cell-cell adhesion was not merely due to overexpression because IgPKD also disrupted cell-cell adhesion in a dose-dependent manner in three other non-transgenic cell lines, MDCK, M8, and UCL93/3. Third, the effect of IgPKD could be neutralized by pre-incubation with GST-IgPKD polycystin-1 fusion proteins showing that an increase in polycystin-1 surface expression (rather than the expression of an unrelated protein) was directly mediating the increased resistance of the transgenic cells to IgPKD. In this context, we found no difference in the localization of several junctional markers to the lateral cell membrane (including ZO-1, E-cadherin and pan-



**Figure 7.** A PKD1 cystic cell line (OX161/1) is protected from the inhibitory effect of IgPKD on cell-cell adhesion. (A) Polycystin-1, polycystin-2, E-cadherin, and N-cadherin expression by immunoblotting in two normal human renal tubular cell lines (CL11, UCL93/3) and a PKD1 cystic cell line (OX161/1). (B) Both UCL93/3 (i-iii) and OX161/1 (iv-vi) express cytokeratin (i and iv), pan-cadherin (ii and v), and N-cadherin (iii and vi). Lateral membrane localization of N-cadherin and pan-cadherin but not E-cadherin (not shown) was observed in both lines. (C) Effect of IgPKD on cell-cell adhesion in UCL93/3 and OX161/1 after a 4-h incubation. The percentage of adherent cells relative to control culture media was calculated and expressed as the mean  $\pm$  SEM. Each bar represents nine independent wells. Note that IgPKD had no effect on cell adhesion in OX161/1 cells at the highest concentration tested (1:10).

cadherin) between transgenic and non-transgenic cells. Fourthly, a differential effect on cell-cell adhesion was observed between transgenic and non-transgenic cells using GST-IgPKD polycystin-1 fusion proteins. Of interest, polycystin-1 antibodies cognate to other extracellular domains had no effect on cell adhesion. Finally, IgPKD had no discernable effect on cell-cell adhesion in a PKD1 cystic cell line (OX161/1), which lacks polycystin-1 expression, further supporting the specificity of IgPKD for disrupting polycystin-1-mediated cell adhesion.

Our results also confirm the importance of the PKD domains in mediating cell-cell adhesion. We do not however know the critical ligand binding site(s) disrupted by the IgPKD antibody nor whether certain PKD domains are more essential than others in mediating intercellular adhesion. Unlike the IgPKD antibody, we found that antibodies generated to the LRR

domain (7e12) or to multiple N-terminal domains (NT2B) had no effect on cell adhesion. The latter result was surprising as the large recombinant protein used to generate NT2B extends from the LRR to PKD domain V (2). There are several possible explanations for this finding. First, although there are likely to be multiple epitopes recognized by this polyclonal antibody, these sequences may not be critical for either homophilic or heterophilic polycystin-1 interactions unlike those recognized by IgPKD. Second, PKD domains (II-V) have the lowest affinity homophilic interactions *in vitro* and so they may be less critical for mediating intercellular adhesion (6). Third, it is possible that NT2B could preferentially recognize epitopes in its denatured rather than native state. Against this, however, we have shown that it is able to immunoprecipitate a native polycystin-1/polycystin-2 complex (2). Taken as a whole, these

results suggest that the N-terminal domains distal to the PKD repeats are not critical to polycystin-1 mediated intercellular adhesion. A recent study showed that a recombinant polycystin-1 protein containing the LRR domain could bind to several matrix proteins including collagen I, fibronectin, and laminin (22). The LRR domain may therefore have a more critical role in mediating cell-matrix rather than cell-cell adhesion. Similarly, the C-type lectin of polycystin-1 has been shown to bind carbohydrate residues of extracellular matrix proteins *in vitro* (23).

The subcellular localization of polycystin-1 has been a highly controversial issue (reviewed in reference 24), and there are data to suggest that polycystin-1 may be expressed at tight junctions (25), adherens junctions (26), desmosomes (27), focal adhesions (28), in cytoplasmic vesicles (29), or primary cilia (30). Using dual immunofluorescence staining, we found that polycystin-1 localized to the lateral cell membrane but did not overlap completely with ZO-1, E-cadherin, or pan-cadherin expression in M7 cells. Similar data has been recently reported for MDCK cells (31). However the desmoplakin antibody we used did not label the lateral cell borders of both M7 and M8 cells; we were therefore unable to assess whether polycystin-1 localized exclusively to desmosomes in these cells. The lack of desmoplakin localization to the lateral cell membranes is difficult to explain apart from having resulted from transformation with SV40T because it affected both cell lines. In previous studies, cellular transformation using SV40T has been associated both with the retention as well as a reduction in desmosomes (32–34).

Several groups have localized polycystin-1 to desmosomes in MDCK cell monolayers (27,31,35). The functional significance of this finding is not yet clear, but it has been suggested that polycystin-1 could modulate cell adhesion indirectly, either by mediating desmosomal signaling (27) or by stabilizing desmosomal connections to intermediate filaments (35). Although polycystin-1 may indeed function in this manner, our data clearly demonstrate a more direct role for polycystin-1 in mediating cell adhesion. It is, however, noteworthy that polycystin-1 expression is dynamic and can redistribute into intracellular compartments in MDCK cysts or more diffusely over the basal surface of MDCK tubules (when induced by HGF) (31). Thus polycystin-1 may be located in different membrane domains depending on the state of cellular differentiation. Although a role for polycystin-1 in mediating cell-matrix adhesion cannot be excluded, our data indicate a primary role in mediating cell-cell adhesion.

The insensitivity of the *PKDI*-deficient cell line OX161/1 to the disruptive effects of the IgPKD antibody, yet its ability to maintain overall intercellular adhesion suggests the existence of a compensatory mechanism for the acquired deficit in polycystin-1. Homophilic interactions of the PKD repeats of polycystin-1 have been demonstrated *in vitro*, and heterophilic interactions with E-cadherin have been described (26,36). These data suggest that both homotypic/homophilic and heterotypic/heterophilic polycystin-1 interactions could mediate intercellular adhesion. Nevertheless the relative contribution of each to overall cell adhesion is not known. The

existence of both homodimeric and heterodimeric polycystin-1 complexes adds further complexity, as each is likely to possess distinct functional properties. In this regard, it should be noted that OX161/1 cells were not polycystin-1–null, as low levels of polycystin-1 were detectable by immunoblotting after prolonged exposure. Therefore, it remains possible that heterodimeric polycystin-1 complexes or heterophilic polycystin-1 interactions could be contributing to basal cell adhesion in these cells. Such complexes might be insensitive to the effect of the IgPKD antibody, which may preferentially recognize homotypic or homophilic interactions. An alternative possibility is that another adhesion molecule (an alternative cadherin or a polycystin-1 homologue) is able to compensate for the reduction in polycystin-1 expression to maintain basal intercellular adhesion. Such a mechanism would be analogous to that proposed for the ability of alternative cadherins to compensate for the known defect in E-cadherin targeting in cystic epithelial cells (36). The presence of alternative cadherins (including N-cadherin) which we found to be expressed at the lateral cell borders of OX161/1 could be compensating both for the deficit in polycystin-1 and defect in E-cadherin targeting (36,37). Such a mechanism would enable cystic epithelial cells to remain partially polarized and maintain overall, albeit dysregulated, intercellular adhesion. These changes are however clearly insufficient to abrogate the cystic phenotype.

In this study, we were unable to assess whether the intercellular adhesive function demonstrated for polycystin-1 was also dependent on its interaction with polycystin-2. Both M7 and M8 cells express identical levels of murine polycystin-2 (2). OX161/1 and UCL93/3 also express similar levels of polycystin-2 showing that a reduction in polycystin-1 expression does not significantly destabilize or upregulate polycystin-2 expression. To test whether polycystin-2 binding influences polycystin-1-mediated cell adhesion, it would be enlightening to test the effect of IgPKD antibody on cell adhesion in a polycystin-2–null line.

Given its highly modular structure, especially of the extracellular region, it is likely that polycystin-1 mediates and regulates multiple cellular functions. Changes in polycystin-1-mediated cell adhesion could account for some of the altered cellular properties found in cystic cells such as changes in proliferation, polarity, and differentiation analogous to that described for E-cadherin (38,39). Recent findings also point to the likelihood of a flow-sensitive polycystin-1/polycystin-2-mediated mechanosensory signal transduction pathway located in primary cilia, which regulates intracellular calcium concentrations (40). Polycystin-2 may operate as an ER-located calcium channel at least in some systems (14). An important next step will be to uncover which is the primary functional abnormality in ADPKD that leads to the transformation of a normal tubular cell into a cystic cell (41).

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