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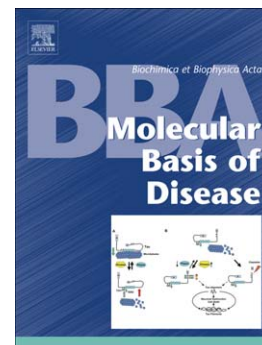
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**Protein Kinase X (PRKX) Can Rescue the Effects of Polycystic Kidney Disease-1 gene (PKD1) Deficiency.**

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**Abstract**

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is a common, genetically determined developmental disorder of the kidney that is characterized by cystic expansion of renal tubules and is caused by truncating mutations and haplo-insufficiency of the *PKD1* gene. Several defects in cAMP-mediated proliferation and ion secretion have been detected in ADPKD cyst-lining epithelia. Unlike the ubiquitous PKA, the cAMP-dependent CREB-kinase, Protein Kinase X (PRKX) is developmentally regulated, tissue restricted and induces renal epithelial cell migration, and tubulogenesis *in vitro* as well as branching morphogenesis of ureteric bud in developing kidneys. The possibility of functional interactions between *PKD1*-encoded polycystin-1 and PRKX was suggested by the renal co-distribution of PRKX and polycystin-1 and the binding and phosphorylation of the C-terminal of polycystin-1 by PRKX at S4166 *in vitro*. Early consequences of *PKD1* mutation include increased tubule epithelial cell-matrix adhesion, decreased migration, reduced ureteric bud branching and aberrant renal tubule dilation. To determine whether PRKX might counteract the adverse effects of *PKD1* mutation, human ADPKD epithelial cell lines were transfected with constitutively active PRKX and shown to rescue characteristic adhesion and migration defects. In addition, the co-injection of constitutively active PRKX with inhibitory pMyr-EGFP-PKD1 into the ureteric buds of mouse embryonic kidneys in organ culture resulted in restoration of normal branching morphogenesis without cystic tubular dilations. These results suggest that PRKX can restore normal function to *PKD1*-deficient kidneys and have implications for the development of preventative therapy for ADPKD.

## Introduction

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is a leading cause of endstage renal failure and affects 500,000 patients in USA <sup>1</sup>. At present, there is no proven therapy and 50% of individuals who inherit mutations in the *PKDI* gene will require hemodialysis or renal transplantation. *PKDI* encodes a membrane protein, polycystin-1 <sup>2</sup>, the intracellular C-terminal domain of which has specific tyrosine and serine sites for phosphorylation by c-src, focal adhesion kinase and protein kinase A (PKA) as well as an RRSSR consensus sequence for putative S4166 phosphorylation <sup>3 4 21 39 40</sup>. Polycystin-1 is highly expressed in developing kidneys, but significantly less expressed in normal adult kidneys <sup>5-7</sup>. Depending on cell differentiation state and developmental stage, polycystin-1 has been localized on basal, lateral and apical plasma membranes where it forms large multi-protein complexes with cell-matrix focal adhesions, cell-cell adhesion complexes and primary cilia, respectively <sup>6 8</sup>. Inactivation of *PKDI* results in increased adhesion of renal epithelia to collagen matrix, decreased migration in response to growth factors, stunted branching morphogenesis and cystic dilations of the terminal branches and tips of the ureteric bud <sup>9</sup>. Direct functional studies have shown that inhibition of *PKDI*-encoded polycystin-1 leads to cystic maldevelopment of mouse kidneys and suggests a critical role for normal polycystin-1 in the differentiation and controlled morphogenesis of the kidney by regulation of tubular diameters <sup>9 10</sup>. Functional decline in ADPKD patients is due to progressive renal cystic enlargement and typically does not result in endstage renal failure until the 4<sup>th</sup>-5<sup>th</sup> decade. Since cystic expansion arises early in development where it appears to be initiated in the

ureteric bud/collecting tubules, this presents a long window of opportunity to develop effective retardation and preventative therapies<sup>11 12</sup>.

Several abnormalities in response to cAMP have been documented in ADPKD, including increased cAMP-dependent proliferation, increased ATP release, and alterations in secreted ion and fluid transport<sup>13 14 15 16 17</sup>. In addition to the ubiquitously expressed PKA, cAMP-mediated responses can be mediated via Protein Kinase X (PRKX), a serine/threonine kinase gene on the X chromosome (Xp22.3)<sup>31 32</sup>. Functional differences between PRKX and PKA have been reported<sup>18</sup> including a PRKX-specific role in granulocyte/macrophage lineage differentiation<sup>33</sup>. Previously we have shown that similarly to PKA, cAMP induces translocation of PRKX to the nucleus of renal epithelial cells and activates CRE-promoter elements<sup>3</sup>. Unlike PKA, however, *PRKX* belongs to an ancient subfamily containing Dictyostelium *KAPC-DICDI*, and consists of a single catalytic and a single regulatory subunit<sup>3 34 35</sup>. PRKX activation results in the stimulation of cell migration and tubulogenesis in renal epithelial cells<sup>3</sup> and its overexpression in embryonic mouse kidneys in organ culture activates ureteric bud branching morphogenesis<sup>36</sup>, suggesting a role for PRKX in regulation of tubulogenesis during kidney development.

The current studies sought to determine whether PRKX was able to interact with polycystin-1 in renal epithelia and to evaluate its potential role in the restoration of normal renal epithelial function, morphogenesis and tubule diameter control in *PKDI*-deficient kidneys.

## Materials and Methods

### *Construction of PRKX Expression Vectors*

The generation of recombinant peGFP/PRKX, pFLAG/PRKX constructs has been described previously<sup>3</sup>. A constitutively active peGFP/PRKX.ca construct was engineered by point mutagenesis (His 93 changed to Gln and Trp 202 to Arg) as described for PKA kinase<sup>27</sup>. This construct causes activation of kinase activity and nuclear translocation in the absence of added cAMP<sup>20</sup>. For ureteric bud microinjection and long-term epithelial cell transduction, viral PRKX-expressing constructs were generated using self-inactivating lentiviral (VVC) vectors<sup>37</sup>. peGFP-PRKX and peGFP/PRKX.ca fusion proteins were excised with XhoI and BamHI and ligated with VVC vectors under the control of the CMV early promoter, to generate VVC-peGFP/PRKX and VVC-peGFP/PRKX.ca as described previously<sup>36</sup>.

### *Construction of PKD-1-CTD Expression Vectors*

Human *PKDI*-C-terminal domain (CTD) fusion protein was prepared for *E. coli* expression by PCR of a 0.6kb DNA fragment encoding amino acids 4105-4303 and subcloned into pET30 LIC (Novagen) vector which encodes in frame N-terminal His - and S-tags, as previously described<sup>4</sup>. For mammalian expression and transfection analysis, the previously described pMyrEGFP-*PKDI*-CTD expression plasmid was used,<sup>4 29</sup> and for ureteric bud microinjections and long-term expression VVC-Myr-EGFP-*PKDI*-CTD was used<sup>39</sup>.

### *Cell Culture*

Temperature-sensitive (ts), conditionally immortalized human renal epithelial cells derived from microdissected normal human fetal collecting tubule (HFCT), normal human adult collecting tubule (NHCT) and ADPKD cyst lining epithelia (with a germline mutation truncating the polycystin-1 protein at amino acid 3078) were grown according to our standard protocols<sup>19 30</sup>. To attain full differentiation, cells were grown to 70% confluence at 33<sup>0</sup>C, and then transferred to the non-permissive temperature of 37<sup>0</sup>C for 5-10 days prior to use. JAR human choriocarcinoma cells (ATCC) were cultured in RPMI 1640 (Life Technologies, Inc.) with 10% FBS and MDCK cells were grown in Dulbecco's Modified Eagles Medium (DMEM) plus 10% FBS.

### *In Situ Hybridization*

Deparaffinized, dehydrated kidney sections were treated with proteinase K, prehybridized with triethanolamine/acetic anhydride, and hybridized overnight with digoxigenin-substituted PRKX-antisense or sense probes. After washing in 0.1 x SSC (0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0), sections were incubated in anti-digoxigenin antibody/alkaline phosphatase and color developed with Nitro Blue Tetrazolium (NBT) reagent (Roche Molecular Biochemicals).

### *Northern Blot Analysis*

Total RNA was fractionated on agarose/formaldehyde gels and the integrity of RNA monitored with ethidium bromide prior to transfer to GeneScreen membranes using 25mM sodium phosphate pH6.5. Filters were prehybridized overnight at 42<sup>0</sup>C in 50%



formamide, 0.04% PVP, 0.04% BSA, 0.04% Ficoll, 1% SDS, 0.75M NaCl, 0.075M sodium citrate and denatured salmon sperm DNA (100mg/ml). Hybridization with PRKX and 14S RNA <sup>32</sup>P-labeled cDNA probes was carried out in 0.02% PVP, 0.02% BSA, 0.02% Ficoll at 42°C for 24 hours followed by 4 washes: 2x SSC, 0.1% SDS, 15 min, 65°C; 1x SSC, 0.1% SDS, 15 min, 65°C; 0.5x SSC, 0.1% SDS, 15 min, 65°C; 0.1x SSC, 0.1% SDS, 15 min, 65°C. RNA bands were visualized by autoradiography.

### *Phosphorylation Studies*

Polycystin-1 was immunoprecipitated from HFCT cell lysates using a fully characterized mono-specific anti-human polycystin-1 C terminal antibody<sup>25</sup> and agarose beads, and then incubated for 45min at 37°C with kinase buffer (10mM HEPES, 3mM MnCl<sub>2</sub>, pH7.3) containing affinity purified pFLAG-PRKX fusion protein and 10μCi α-<sup>32</sup>P-ATP. The beads were washed 3 times with kinase buffer and 15ml TBS containing 50mM EDTA and 4ml of 5x Ramini sample buffer. After heating for 5min at 95°C, phosphorylated proteins were resolved by SDS-PAGE<sup>41</sup> followed by autoradiography. In addition, His/FLAG/PKDI-CTD or 4166/4252 truncated mutant fusion proteins made in *E. coli* and purified using a nickel column, were incubated with pFLAG-PRKX fusion protein immunoprecipitated from JAR cell lysates using anti-FLAG beads and phosphorylation determined using <sup>32</sup>γ-ATP as described above. Studies were conducted in the presence or absence of 8-Br-cAMP (100μM) or H89 inhibitor (10μM).

### *Co-immunoprecipitation of PRKX and polycystin-1*

HFCT cell lysates (800µg) were immunoprecipitated with 10µl anti-PRKX antibody (Biocompare) and 25µl A/G beads at room temperature for 2 hours. After extensive washing, aliquots were fractionated on 5% SDS/PAGE and immunoblotted with anti-PRKX (1:500) and anti-polycystin-1 (1:10,000). Lysate immunoprecipitated with beads only acted as negative control.

### *Cell Adhesion Assays*

ADPKD cells were transfected with peGFP/PRKX or peGFP/PRKX.ca using Lipofectamine 2000 (Invitrogen), washed and cultured for 24 hours prior to Fluorescence-Activated Cell Sorting (FACS) to isolate GFP positive cells. 2,000 GFP+ or control non-transfected cells were plated per well in 96 well tissue culture plates precoated with type I collagen. After 4 hours of attachment, unattached cells were removed by aspiration and adherent cells quantitated by the colorimetric aqueous MTS assay (Cell Titer 96™ Aq., Promega).

### *Modified Boyden Chamber Migration Assays*

ADPKD cells were transfected with peGFP/PRKX using Lipofectamine 2000 (Invitrogen), washed and cultured for 24 hours prior to FACS sorting. GFP+ or control untransfected cells were washed with serum-free medium and labeled with fluorescent calcein-AM (Molecular Probes). 2,000 labeled cells treated with or without 100µM 8-Br-cAMP or 10 µM H89 were plated in 0.8ml DMEM+ 1% FBS in triplicate into 24 well chambers adapted for the Fluoroblock (Falcon) apparatus. After 4 hours, 5% FBS was

added to the lower chamber medium to establish a 1-5% serum gradient and migration of cells from the upper to lower chamber quantitated at 4-48hr using a microfluorimetric plate reader (HTS 7000, Perkin Elmer Cetus).

### *Tubulogenesis Assay in 3-Dimensional Collagen Gels*

FACS-sorted MDCK cells, with or without transfection of peGFP/PRKX, peGFP/PKDI-CTD or peGFP/PKDI.tm (*PKDI* truncated mutants) were washed, grown for 24 hours, washed three times in serum-free medium and 500 cells plated per well (12-well format) in collagen type I gels: 1 vol. 10 X MEM, 1 vol sodium bicarbonate (42 mg/ml), 1/2 vol FBS, 3 vol type I collagen, 4.5 vol sterile water. After 10 days of culture in DMEM + 10% FBS, gels were fixed in ice-cold methanol and stained with rhodamine-phalloidin for 8 hours prior to examination by fluorescence microscopy.

### *Mouse Embryonic Kidney Organ Culture and Viral Vector Microinjection*

Embryonic day (E) 11-12 kidney pairs were dissected from timed pregnant CD-1 mice (Charles Rivers) using a stereomicroscope (Olympus) with a Scion imaging system. Kidneys were cultured on 24mm Transwell Clear membrane inserts for 3-5 days and media changed daily<sup>36</sup>. On day zero, 20-200nl ( $1-4 \times 10^7$  TU/ml); VVC-peGFP/PRKX.ca; VVC-pMyr-EGFP-*PKDI*-CTD; VVC-peGFP/PRKX.ca plus VVC-pMyr-EGFP-*PKDI*-CTD; VVC vector alone; or PBS (sham control) were microinjected into the ureteric bud lumen using sterile transfer tips (15 mm internal diameter), a micromanipulator and transjector (Eppendorf) and comparisons made with uninjected kidneys. After 3-5 days of organ culture, kidney explants were fixed in ice-cold methanol

for 15 min. prior to immunofluorescent staining with anti-calbindin and anti-WT-1 antibodies as described previously<sup>36</sup>.

### *Microscopy and Morphometric Data Analysis*

Morphological changes were documented daily using the Olympus/Scion system. Immunofluorescence imaging was carried out by laser-scanning confocal microscopy (BioRad Radiance, MSSM core facilities) and Image J (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.

## **Results**

### *PRKX mRNA and Polycystin-1 Protein Expression in Human Kidneys*

The developmental regulation of expression and cellular localization of PRKX is very similar to that of polycystin-1 in normal fetal, normal adult and ADPKD kidneys (Figure 1). Both are highly expressed in fetal kidneys (A and D) and restricted mainly to the ureteric bud- derived collecting ducts (gestational ages 12 to 24 weeks, n=10) while little is seen in normal adult kidneys (n=10) (B and E). By contrast, PRKX and polycystin-1 expression are seen in ADPKD kidneys (n=10) where they are restricted to the cyst lining epithelia (C and F). Cell lines derived from micro-dissected normal human fetal collecting ducts (HFCT) express high levels of endogenous polycystin-1<sup>7 19</sup>.

### *Phosphorylation of Polycystin-1 by PRKX*

To determine whether polycystin-1 could be a substrate of PRKX, *in vitro* phosphorylation analysis was carried out by incubation of HFCT polycystin-1 immunoprecipitates with PRKX fusion proteins in the presence of  $\gamma^{32}$ -P-ATP<sup>4</sup> (Figure 2a). After establishing that polycystin-1 can be phosphorylated by PRKX (Figure 2a, lane 5), mutation analysis was carried out to determine the specific site at which this occurs. Immunoprecipitated pFLAG/PRKX was incubated with wild type or mutated *PKDI* fusion proteins and kinase activity determined in the presence of the activator, 8-Br-cAMP or of the inhibitor, H89. Since human polycystin-1 contains two RRSSR consensus sequences, sites S4166 and S4252 were selected for point mutation. Of interest, the S4252, but not S4166 had previously been shown to be phosphorylated by PKA<sup>4 21</sup>. The results (Figure 2b) show that PRKX phosphorylates the wildtype polycystin-1 C-terminal (lane 2), that this is enhanced by 8-Br-cAMP (lane 3) and inhibited by H89 (lane 4). While truncation of polycystin-1 and removal of the PKA site (S4252) had no effect on this activity (lanes 5-7), mutation of the S4166 significantly reduced activity (lanes 8-10) implicating polycystin S4166 as the major C-terminal target for PRKX phosphorylation.

### *Association of PRKX with Polycystin-1*

To further investigate if PRKX interacts with polycystin-1 *in vivo*, co-immunoprecipitation studies were carried out on HFCT cells that endogenously express both PRKX and polycystin-1 (Figure 3). PRKX was immunoprecipitated (lane 3) as a 68

kDa protein, the lower band being thought to be a degradation product since it was occasionally observed in Western blots using this commercial polyclonal antibody. Western immunoblot analysis of the PRKX immunoprecipitates using our C-terminal domain anti-polycystin-1 antibody<sup>25</sup> showed the presence of polycystin-1 (lane 1, arrow >430 kDa). No polycystin-1 was detected when HFCT lysates were immunoprecipitated with beads alone (lane 2). These results suggest that polycystin-1 is associated with PRKX in HFCT.

### *PRKX Reduces ADPKD Cell Adhesion and Stimulates ADPKD Cell Migration*

We next sought to determine whether PRKX could modify the aberrant functions of ADPKD epithelia *in vitro*. Human ADPKD epithelia are significantly more adherent to collagen after 4 hours of attachment than age-matched normal human collecting duct cells and are also significantly less migratory in response to a growth factor gradient<sup>9,22</sup>. Transfection of ADPKD epithelia with wild-type peGFP/PRKX or constitutively active peGFP/PRKX.ca resulted in significant decreases in the adhesion of ADPKD cells, compared with untransfected cells or ADPKD cells transfected with empty vector (Figure 4). In addition, when ADPKD epithelial cells were transfected with wild-type peGFP/PRKX migration profiles resembled those of age-matched normal (NHCT) epithelia (Figure 5A). This effect was more accentuated in the presence of 8-Br-cAMP (Figure 5B) and inhibited by H89 (Figure 5C).

*PRKX Abrogates the Effect of an Inhibitory PKD1-CTD Fusion Protein on MDCK Cell Branching Morphogenesis*

The MDCK collagen gel assay is a well established tool to study MDCK cell branching morphogenesis. In the culture gel, MDCK cells form cysts consisting of 8-10 cells by day 7 as shown in the control (Figure 6, A). We have previously demonstrated that transfection of PRKX into MDCK cells induces a tubulogenic response in 3-dimensional collagen gels, even in the absence of HGF<sup>3</sup> while transduction of the inhibitory VVC-pMyr-EGFP-*PKD1* inhibits branching morphogenesis in the embryonic mouse kidney<sup>9</sup>. Figure 6 shows that transfection of pMyr-EGFP-*PKD1* fusion protein did not induce MDCK cell tubulogenesis even in the presence of HGF (Figure 6, D), while co-transfection of pMyr-EGFP-*PKD1* plus peGFP/PRKX.ca restored tubulogenesis (Figure 6, G). Quantitative analysis showed that peGFP/PRKX.ca co-transfection with pMyr-EGFP-*PKD1* resulted in  $8.0 \pm 0.25$  (mean  $\pm$  SEM) branched epithelial structures observed per 10 epithelial structures scored (n = 4 sets of observations). Transfection of the 4166- or 4252-site mutant of pMyr-EGFP-*PKD1*, lacking the PRKX major or minor phosphorylation site, respectively, also showed inhibitory effects on MDCK tubulogenesis (Figure 6, E and F). Co-transfection of the peGFP/PRKX.ca together with the 4166- or 4252-site mutant of pMyr-EGFP-*PKD1* did not restore tubulogenesis (Figure 6, H, I). These results suggested that PRKX can overcome the deficiencies in tubulogenesis induced by inhibition of polycystin-1 only when both the PRKX major and minor phosphorylation sites in polycystin-1 are present.

*Microinjection of VVC-peGFP/PRKX.ca Rescues the Inhibitory Effects of pMyrEGFP-PKDI-CTD*

Embryonic mouse kidneys grown in organ culture, recapitulate the epithelial morphogenetic events of early kidney development<sup>23 24</sup>. Since polycystin-1 and PRKX are highly expressed in the ureteric bud, this site was chosen for the microinjection of lentiviral (VVC) expression constructs of pMyr-EGFP-*PKDI* and/or peGFP/PRKX.ca in embryonic day 11.5 mouse kidneys (Figure 7). After 3 days of subsequent organ culture, VVC-pMyr-EGFP-*PKDI* transduction alone caused reductions in ureteric bud elongation and branching as measured by numbers of branch points and branch tips by comparison to empty vector injected paired control kidneys. Glomerular numbers were also decreased, while ureteric tubular diameters were increased (Figure 7, upper panel). The opposite effects were seen after injection of VVC-peGFP/PRKX.ca alone which led to increased ureteric bud elongation and branching, increased glomerular induction and decreased tubular diameters (Figure 7, middle panel). Co-transduction by co-microinjection of both VVC-pMyr-EGFP-*PKDI* and VVC-peGFP/PRKX.ca led to the restoration of normal control levels of ureteric bud elongation and branching and glomerular induction and significantly reversed the alterations in tubule dilation (Figure 7, lower panel). These results suggest that activated PRKX can rescue the major inhibitory morphogenetic effects of inhibitory *PKDI* mutation associated with cystic initiation in developing kidneys.

## **Discussion**



In the present study, we demonstrated that PRKX is expressed in the same temporo-spatial pattern as the polycystin-1 protein in the ureteric bud epithelial of normal developing kidneys<sup>6,7</sup>. We also demonstrated that PRKX associates with polycystin-1 in human fetal kidney collecting tubule (HFCT) epithelial cells and can phosphorylate the polycystin-1 protein. These results suggest that PRKX may interact with polycystin-1 and regulate polycystin-1 functions in renal epithelial cells *in vivo*. During development of the metanephric kidney, the ureteric bud undergoes branching morphogenesis that involves epithelial cell adhesive interactions with the extracellular matrix, migration, branching, elongation and tubule diameter regulation and epithelial differentiation. During this process, polycystin-1 shows a punctate basal membrane distribution pattern and forms multiprotein complexes with integrins and component proteins of the focal adhesion complex including paxillin, talin, tensin, focal adhesion kinase, c-src and p130cas<sup>25,26</sup>. Recent studies have demonstrated a direct role for polycystin-1 in the regulation of the ordered control of epithelial cell adhesion, migration, elongation, branching morphogenesis and volume regulation necessary for normal renal development<sup>9</sup>. PRKX, a cAMP-dependent CREB kinase, also regulates similar properties, stimulating renal epithelia cell migration and branching morphogenesis<sup>3</sup>. Therefore, we reasoned that PRKX might act as a polycystin-1 kinase and modulate its functions through phosphorylation of polycystin-1 at its intracellular C terminal domain.

Sequence analysis identified 4 tyrosine residues and 2 RRSSR consensus sequences as putative target sites for phosphorylation by kinases in of the intracellular portion of polycystin-1 and functional analysis has confirmed that S4252 is a phosphorylation site for PKA, Y4127 for focal adhesion kinase (FAK) and Y4237 for c-

src<sup>3 4 21</sup>. Our current observation that PRKX phosphorylates polycystin-1 at S4166, rather than at S4252 (the only phosphorylation site for PKA) suggests that PRKX and PKA could exert distinct effects on polycystin-1. Indeed, our previous studies had demonstrated clear functional differences between PRKX and PKA with regard to renal epithelial cell function since while PRKX stimulates cell migration, and tubulogenesis PKA has no effects on those properties.<sup>3 36</sup>

Human ADPKD epithelia with mutant *PKDI* have increased adhesion and reduced migratory capacity and inhibition of *PKDI* disrupts ureteric bud branching morphogenesis leading to cystic dilation<sup>9</sup>. Since over-expression of PRKX had been shown to increase branching morphogenesis, we reasoned that it might counteract the effects of *PKDI* inhibition via modulation of polycystin-1. Using transfection strategies, PRKX was shown to significantly decrease the adhesion and increase the migration of ADPKD cells, retuning them to the levels seen in normal age-matched control cells. PRKX was also shown to stimulate MDCK cell branching morphogenesis and to abrogate the effects of an inhibitory pMyr-*PKDI*-CTD fusion protein on MDCK cell branching morphogenesis in 3-dimensional collagen gels. Most importantly, the co-injection and transduction of PRKX into the ureteric buds of E11.5 mouse embryonic kidneys in ex vivo organ culture led to an almost complete rescue of the branching morphogenesis and cystic dilation defect elicited by injection of inhibitory pMyr-EGFP-*PKDI*. Taken together these results suggest that PRKX can reverse the abnormalities in epithelial adhesion, migration and morphogenesis associated with *PKDI* inhibition and cyst formation in ADPKD. There are several potential mechanisms whereby PRKX might counteract the effects of PKD1 dysfunction. Firstly, since human ADPKD patients

are heterozygous for a single *PKD1* mutation and immunoreactive polycystin-1 protein is seen in many cyst epithelial cells in human ADPKD kidneys, PRKX may be able to counteract the deleterious effects of haplo-insufficiency by interacting with protein encoded by the normal allele. Secondly, PRKX might act in a dominant negative fashion by competing with aberrant polycystin-1, thus blocking the deleterious effects of the mutant protein. A third potential mechanism would be that PRKX interacts with other members of the polycystin-1-multiprotein complex to abrogate aberrant function. Consistent with this possibility, PRKX contains a proline-rich (WW) binding site and in vitro binding assays has been shown capable of strong interactions with PIN-1, BAG-3 and MAGI-1 shown to play a role in regulation of cell proliferation and differentiation. Further in vitro and in vivo studies in *PKD1* null homozygous and heterozygous mice are in progress to address these alternatives.

Mutation of *PKD1* in ADPKD leads to functional insufficiency associated with tubular cystic dilation. Severe inhibition of *PKD1* during renal development by injection of inactivating pMyr-EGFP-*PKD1* viral expression constructs or by *PKD1* mutation in ADPKD, disrupts and retards ureteric bud branching, reduces total glomerular induction and leads to tubular dilations<sup>9</sup>. PRKX is the first endogenous protein demonstrated to reverse the effects of *PKD1* mutation and points to the potential importance of phosphorylation of the intracellular C-terminus of polycystin-1 by this kinase in mitigating the progressive renal dysfunction seen in this monogenetic disease. 85% of ADPKD is caused by mutations in *PKD1* and 15% by mutations in *PKD2*. Recent studies suggest that the intracellular C-terminal of the *PKD2*-encoded polycystin-2 also contains putative serine target sites for phosphorylation and that inhibition of *PKD2* disrupts renal

epithelial tubulogenesis<sup>38</sup>. Polycystin-1 and polycystin-2 have been shown to interact *in vitro* and have been proposed to form functional complexes *in vivo*. Although it remains to be determined whether PRKX can phosphorylate polycystin-2, it is likely that it is capable of regulating polycystin-2 function either directly or by virtue of its interactions with polycystin-1 in the polycystin1/2 complex. Since over-expression of PRKX has been shown to be efficient at restoring normal polycystin function *in vitro* and *ex vivo*, this presents the possibility that PRKX therapy might be effective with regard to prevention and/or retardation of cystic development.

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## Legends

### Figure 1

#### **a: Northern blot analysis of PRKX mRNA expression in human kidneys.**

Human kidney total RNA (20 µg per lane) was fractionated on agarose/formaldehyde gels and transferred to a nitrocellulose membrane. The membrane was then prehybridized overnight and probed with <sup>32</sup>P-PRKX. (please see Materials and Methods for further detail)

FK: normal human fetal kidney, 16 weeks gestation .

AK: normal human adult kidney.

PKD: ADPKD kidney.

Arrow indicates the 6.4 Kb PRKX mRNA; arrowhead indicates 14S ribosomal RNA control.

#### **b: PRKX and PKD1 mRNA expression in human kidneys.**

*In situ* hybridization with digoxigenin-substituted antisense PRKX (A-C) or sense PRKX (G) or PKD1 (D-F) fragment riboprobes was carried out on human kidney sections and

visualized by anti-digoxigenin-alkaline phosphatase immunohistochemistry (please see Materials and Methods for full details)

A and D: Normal human fetal kidney, 12 weeks gestation.

B and E: Normal human adult kidney.

C and F: ADPKD cystic kidney.

G: PRKX sense probe on ADPKD cystic kidney.

## Figure 2

### **a: Phosphorylation of Polycystin-1 by PRKX.**

Polycystin-1 immunoprecipitated from human fetal collecting tubule (HFCT) cell lysates was incubated with pFLAG/PRKX fusion protein purified by immunoprecipitation with anti-FLAG beads. Kinase activity was measured using  $\gamma$ -<sup>32</sup>P-ATP in the presence of 8-Br-cAMP (please see Materials and Methods for details). The arrow indicates phosphorylated polycystin-1.

Lane 1: HFCT cell lysate incubated without pFLAG/PRKX

Lane 2: HFCT cell lysate incubated with pFLAG/PRKX

Lane 3: HFCT cell lysate immunoprecipitated by beads alone and incubated without pFLAG/PRKX (immunoprecipitation negative control).

Lane 4: HFCT cell lysate immunoprecipitated by polycystin-1 antibody, incubated without pFLAG/PRKX

Lane 5: HFCT cell lysate immunoprecipitated by polycystin-1 antibody, incubated with pFLAG/PRKX

### **b: Identification of PRKX phosphorylation site on polycystin-1**

Upper panel: His/FLAG/PKD1-CTD fusion protein or 4166 or 4252 site mutated fusion proteins were made in *E. coli* and purified using a nickel column. pFLAG/PRKX fusion proteins were obtained from JAR cell lysates transfected with pFLAG/PRKX by immunoprecipitation using anti-FLAG beads. In these experiments, the same amounts of His/FLAG/PKD-CTD fusion protein or of 4166-, or 4252-site mutated fusion proteins were used to incubate with equal amounts of pFLAG/PRKX fusion protein, respectively. Then equal amounts of incubation mixtures were loaded for SDS-PAGE followed by autoradiography. Kinase activity was measured using  $\gamma$ -<sup>32</sup>P-ATP in the presence or absence of 8-Br-cAMP (100 $\mu$ M) or H89 (10 $\mu$ M) inhibitor.

- 1: Control without PRKX transfection.
- 2: pFLAG/PRKX+His/FLAG/PKD-CTD
- 3: pFLAG/PRKX+His/FLAG/PKD-CTD+cAMP,
- 4: pFLAG/PRKX+His/FLAG/PKD-CTD+cAMP+H89
- 5: pFLAG/PRKX+4252 PKD mutant
- 6: pFLAG/PRKX+4252 PKD mutant +cAMP
- 7: pFLAG/PRKX+4252 PKD mutant+cAMP+H89
- 8: pFLAG/PRKX+4166 PKD mutant
- 9: pFLAG/PRKX+4166 PKD mutant+cAMP
- 10: pFLAG/PRKX+4166 PKD mutant+cAMP+H89.

Lower panel: Coomassie blue staining of the gel to show equal loading of pFLAG/PRKX fusion protein and His/FLAG/PKD-CTD fusion proteins. The molecular weight of pFLAG/PRKX is 68 kDa and the molecular weight of His/FLAG/PKD-CTD and its mutants are 32 kDa.

**Figure 3****Co-immunoprecipitation of PRKX and polycystin-1.**

800 µg HFCT cell lysate was immunoprecipitated with 25µl A/G beads alone (negative control) or 10µl anti-PRKX antibody plus 25µl A/G beads. Aliquots were fractionated by 5% SDS/PAGE and then immunoblotted with anti-PRKX (1:500) and anti-polycystin-1 (1:10,000) antibodies respectively.

Lane 1: IP with anti-PRKX plus A/G beads, Western immunoblot with anti-polycystin-1 (arrow indicates polycystin-1 band, >430 kDa)

Lane 2: IP with A/G beads alone (immunoprecipitate negative control).

Lane 3: IP with anti-PRKX plus A/G beads; Western immunoblot with anti-PRKX (arrow indicates PRKX band, 68 kDa)

**Figure 4****Effects of PRKX on ADPKD cell adhesion.**

NHCT cells and ADPKD cells were transfected without or with peGFP/PRKX or peGFP/PRKX.ca using Lipofectamine and then sorted by Fluorescence-Activated Cell Sorting (FACS) to isolate GFP-positive cells. 2,000 GFP+ or control non-transfected cells were plated per well in 96 well tissue culture plates precoated with type I collagen. Adherent cells were quantitated by the colorimetric aqueous MTS assay after 4 hours of attachment

Lane 1. NHCT cells without transfection

Lane 2. ADPKD cells without transfection.

Lane 3. ADPKD cells transfected with empty expression vector .

Lane 4. ADPKD cells transfected with peGFP/PRKX

Lane 5. ADPKD cells transfected with constitutively active peGFP/PRKX.ca.

\*  $p = < 0.01$

## Figure 5

### Effects of PRKX on ADPKD cell migration.

NHCT and ADPKD cells were transfected without and with peGFP/PRKX using Lipofectamine 2000 and sorted by FACS. 2000 fluorescent calcein-AM labeled GFP+ or control untransfected cells were placed in each well of 96 well plates and treated with or without 100  $\mu$ M 8-Br-cAMP or 10  $\mu$ M H89. Cell migration was quantitated after 4-48hr using a microfluorimetric plate reader (please see Material and Methods for details).

Values are expressed as mean  $\pm$  SEM from 2 independent experiments in triplicate. P values were calculated by T test comparisons between ADPKD+V versus

ADPKD+PRKX. \*  $= P < 0.05$

A. Untreated NHCT (blue line), ADPKD (red line), ADPKD transfected with empty vector (ADPKD+V, yellow line) and ADPKD transfected with PRKX (ADPKD+PRKX, green line).

B Treated with 100  $\mu$ M 8-Br-cAMP

C Treated with 10  $\mu$ M H89

## Figure 6

### Effects of PKD1 and PRKX on MDCK cell tubulogenesis.

MDCK cells, with or without transfection of various PRKX and PKD1 constructs were sorted by FACS and 500 GFP positive or negative cells were plated per well in collagen type I gels. After 10 days of culture in DMEM + 10% FBS, gels were fixed and stained for examination by fluorescence microscopy.

A: MDCK cells without transfection.

B: Transfected with empty expression vector pEGFP-C3.

C: Transfected with peGFP/PRKX.

D: Transfected with pMyr-EGFP-PKD1, treated with 25ng/ml HGF

E: Transfected with 4252 site mutant (M4252) of pMyr-EGFP-PKD1, treated with 25ng/ml HGF.

F: Transfected with 4166 site mutant (M4166) of pMyr-EGFP-PKD1, treated with 25ng/ml HGF.

G: Co-transfected with peGFP/PRKX plus pMyr-EGFP-PKD1, treated with 25ng/ml HGF.

H: Co-transfected with peGFP/PRKX plus M4252, treated with 25ng/ml HGF.

I: Co-transfected with peGFP/PRKX plus M4252, treated with 25ng/ml HGF

In collagen gel cultures for 7 days. Rhodamine-phalloidin staining. All cells were pre-sorted for GFP positivity by FACS prior to use in tubulogenesis assays.

## Figure 7

### Upper panel:

**Inhibition of ureteric bud branching morphogenesis and glomerular induction by dominant negative PKD1.**

Kidney explants were cultured for 3 days and then labeled by anti-WT-1 and anti-Calbindin for visualization of glomeruli and ureteric bud, respectively and viewed by fluorescent confocal microscopy. Quantitative analysis using IMAGE J software was carried out to compare glomerular number, ureteric length, branch points, tips, and outer diameters at the first and second branch points (bar graphs). The values are mean  $\pm$  SEM

Paired E11.5 fetal kidneys were injected on day 0 with:

- A. Empty viral vector (VVC)
- B. VVC-pMyr-EGFP-*PKDI*

n=6 paired kidneys. \*=P<0.01.

**Middle panel:**

**Stimulation of ureteric bud branching morphogenesis and glomerular induction by PRKX.**

The kidney culture, experimental conditions and quantitative analysis are the same as described in the Upper panel. Paired E11.5 fetal kidneys were injected with

- C. Empty viral vector (VVC)
- D. VVC-peGFP/PRKX

n=6 paired kidneys. \*= P<0.01.

**Lower panel:**

**Rescue of *PKDI* dominant negative effects on ureteric bud branching morphogenesis and glomerular induction by PRKX**

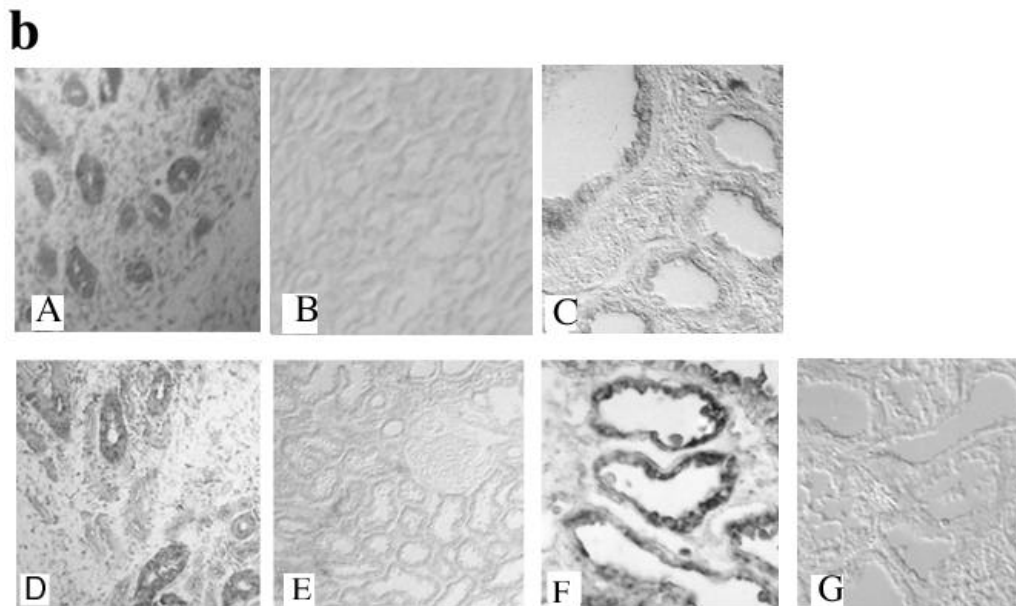
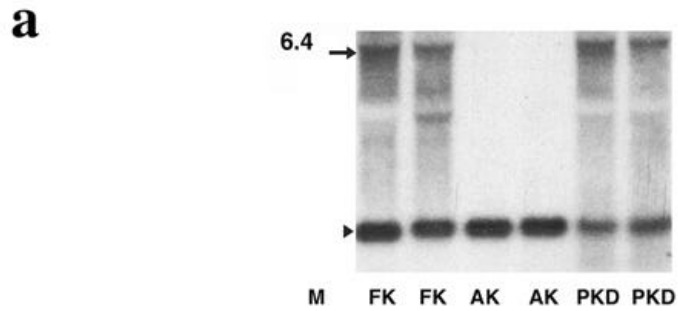
The kidney culture, experimental conditions and quantitative analysis are the same as described in the Upper panel. Paired E11.5 fetal kidneys were injected with



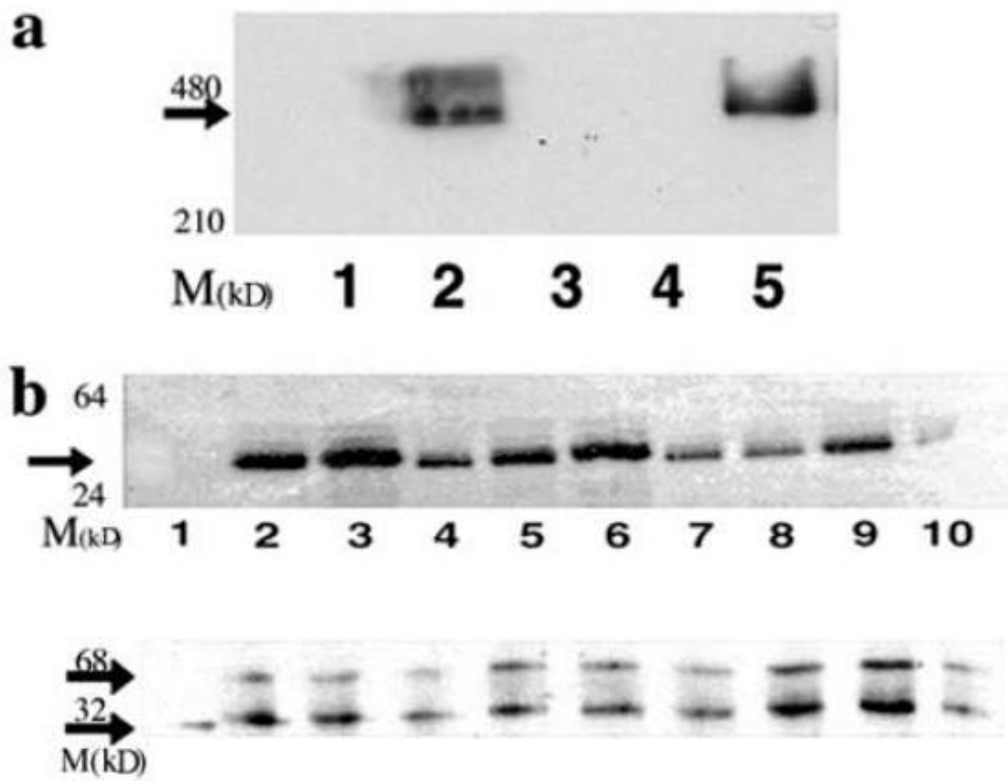
E. Empty viral vector (VVC)

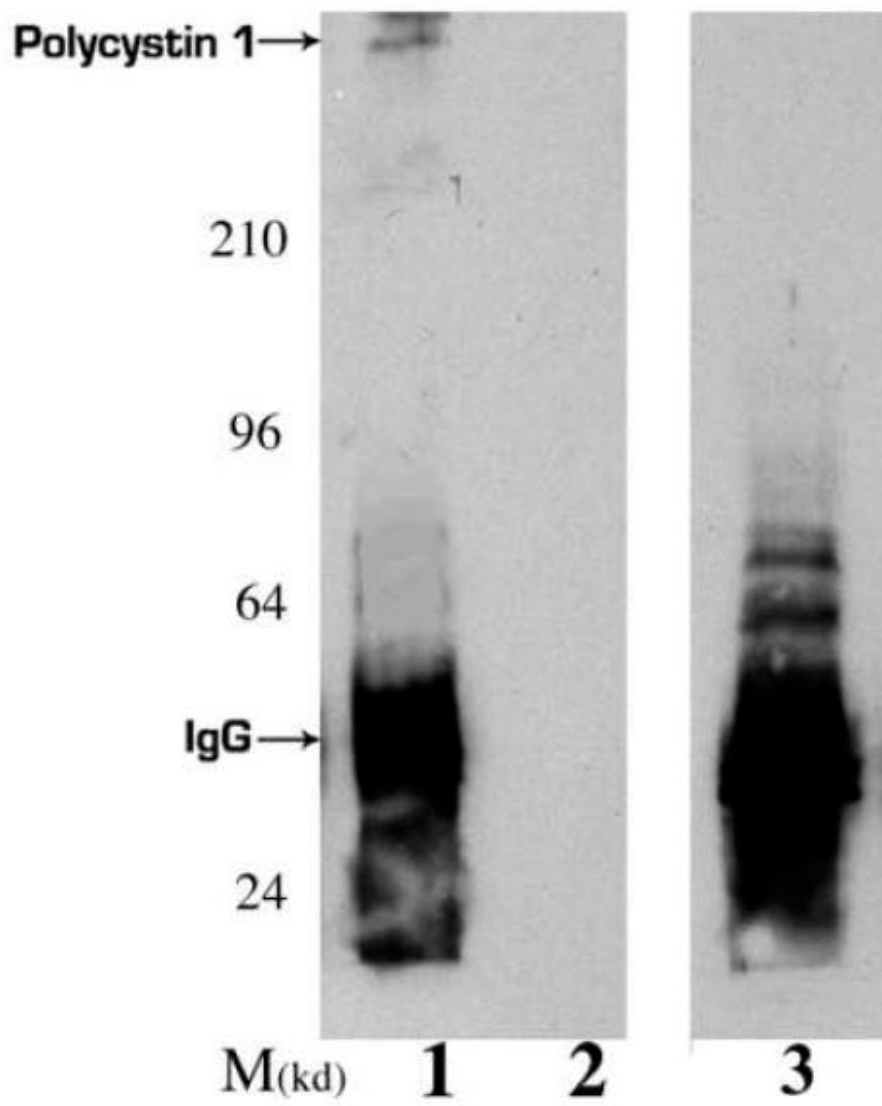
F. VVC-pMyr-EGFP-*PKDI* plus VVC-peGFP/PRKX.ca

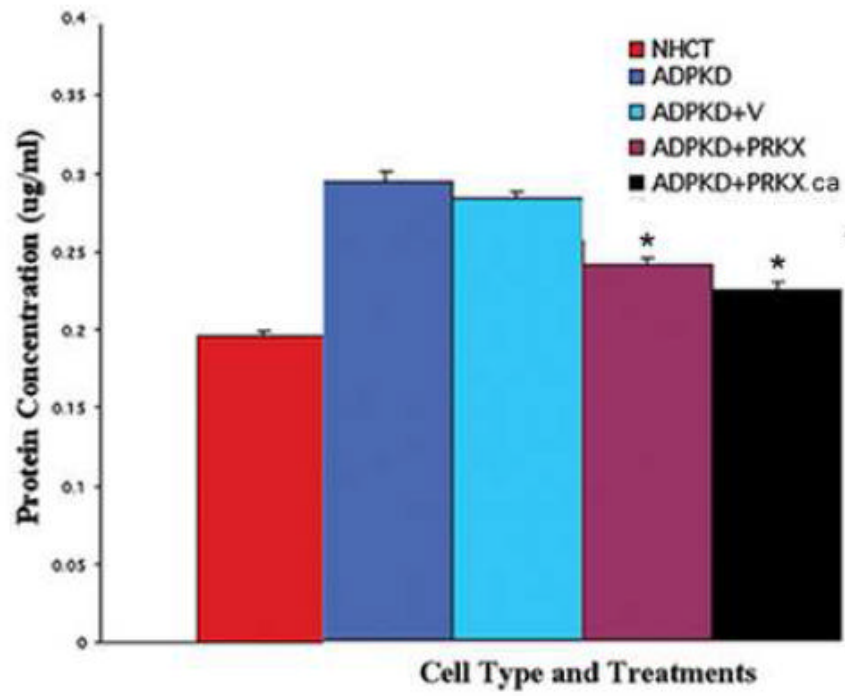
n=6 paired kidneys. \*=P<0.01, \*\*P<0.05.



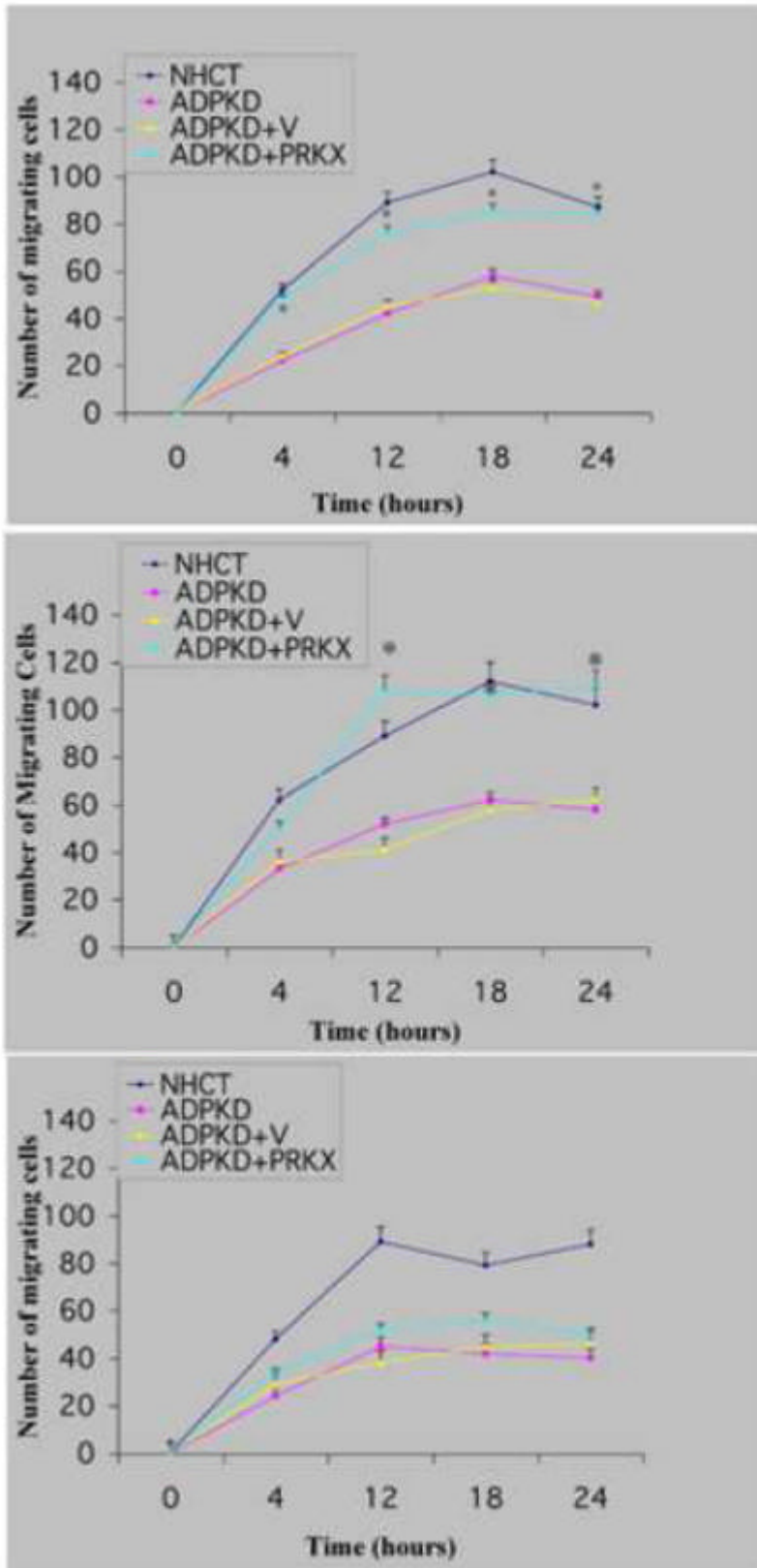
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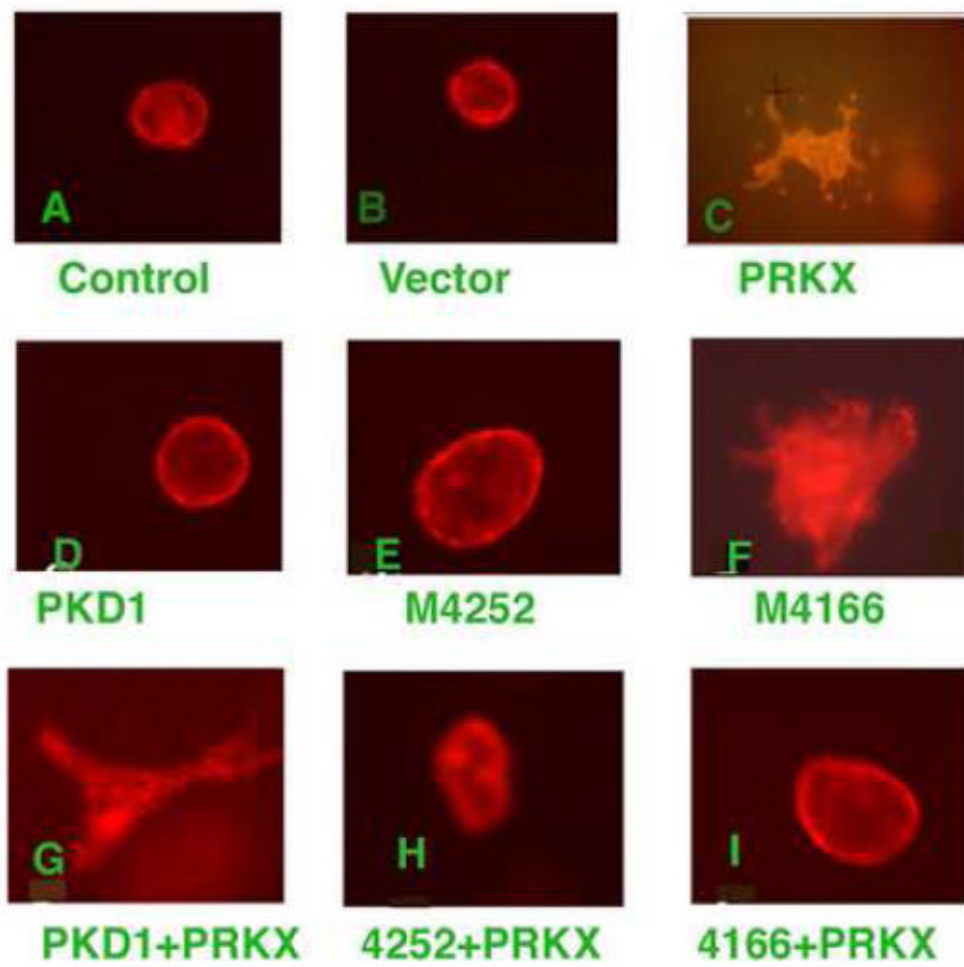




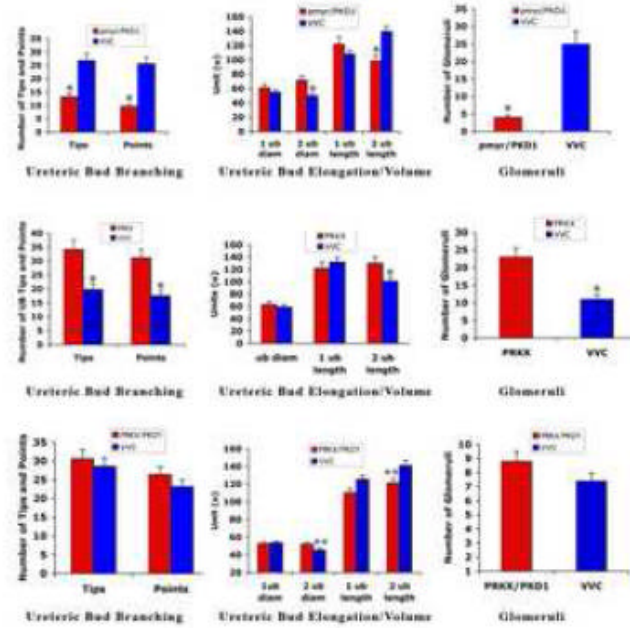
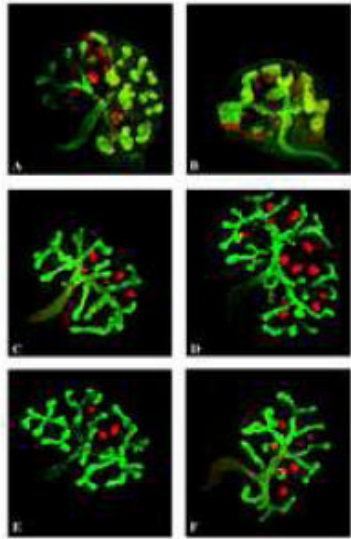


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