

Chapter 4

Impaired ERK Signaling Activity in human and mouse renal cystic epithelial Cells

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Abstract

We have previously shown that polycystin-1, the protein mutated in Autosomal Dominant Polycystic Kidney Disease (ADPKD), modulates Activator Protein-1 (AP-1) activity. Moreover, AP-1 activity was aberrant in cultured ADPKD renal cystic epithelial cells and renal cystic tissue. Since AP-1 can be regulated by Mitogen Activated Protein Kinases (MAPKs), we investigated the activity of the MAPKs in human ADPKD and mouse renal cystic epithelial cells. Activity of Extra-cellular signal-Regulated Kinase (ERK) was impaired in early and end-stage renal cystic epithelial cells compared to control cells upon stimulation with Epidermal Growth Factor (EGF) and with the phorbol ester, TPA. Cell-type specific effects were detected for c-Jun N-terminal kinase (JNK), as an increase in JNK activity was observed in *Pkd1*^{-/-} mouse embryonic fibroblasts only. The involvement of polycystin-1 and polycystin-2 in the modulation of ERK and JNK activity was confirmed by overexpression of the C-terminal fragment of polycystin-1 and full-length polycystin-2. Impaired ERK reactivity upon different cellular stimuli in early- and end-stage renal cystic epithelial cells may be relevant for cyst formation and progression in ADPKD.

Introduction

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is a common inherited disorder that predominantly manifests with progressive development of fluid-filled cysts in both kidneys. Cyst formation ultimately results in chronic renal failure. In the majority of patients, the disease can be accounted for by a mutation in the *PKD1* gene (1;2). Approximately 15% of patients suffer from a mutation in the *PKD2* gene (3-5). The precise functions of the proteins encoded by the *PKD1* and *PKD2* gene, polycystin-1 and polycystin-2 respectively, remain to be elucidated. Several studies have implicated a role for polycystin-1 in signal transduction pathways governing Activator Protein-1 activity (AP-1; 6-8). Moreover, we have reported that AP-1 activity is aberrant in renal cystic cells and tissue from human and mouse ADPKD (8-9). AP-1 transcription factors regulate key cellular processes such as cell proliferation, differentiation, and survival (10-12). The activity and expression of these transcription factors can be regulated by Mitogen Activated Protein Kinases (MAPKs), including Extra-cellular signal-Regulated Kinase (ERK) and c-Jun N-terminal kinase (JNK; 13). MAPK activation itself involves phosphorylation via MAPK kinase (MAPKK), including MEK1/2 and MKKs (14;15). Depending on the cellular stimulus and cellular context, distinct MAPK signaling cascades are activated, resulting in different cellular responses. To further elucidate the molecular mechanisms disrupted in ADPKD, MAPK activity was analyzed in human and mouse renal cystic epithelial cells stimulated with well recognized activators of MAPK signaling. Our data show that EGF- and TPA(12-O-tetradecanoyl-phorbol-13-acetate)-induced signaling to ERK is significantly impaired in renal cystic epithelial cells isolated at both early- and end-stage of polycystic kidney disease. Transient overexpression of the C-terminal

fragment of polycystin-1 and full-length polycystin-2 also resulted in impaired ERK signaling. In contrast, phosphorylation of the EGF receptor (EGFR) was increased in EGF-induced human renal cystic epithelial cells. Osmotic stress-induced activity of JNK was increased in *Pkd1*^{-/-} mouse embryonic fibroblasts only.

Materials and Methods

Human control and ADPKD renal cystic epithelial cells

Human renal cystic epithelial cells were maintained in D-MEM/F12 with 100 U/ml penicillin and streptomycin, 1 mM sodium-pyruvate, 0.1 mM HEPES, 2 mM glutaMAXTM-I, and 10% heat-inactivated fetal bovine serum at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Cell culture reagents were purchased from Invitrogen B.V. (Breda, The Netherlands) and disposables from Greiner Bio-One B.V. (Alphen a/d Rijn, The Netherlands). The following human control and ADPKD renal cystic epithelial cell lines have been described previously: UCL93/3 control and OX161/1 cystic cells (16) and RCTEC control and PKD9-7WT cystic cells (17). RFH94/1 control and SKI-001 human ADPKD renal cystic epithelial cell lines were developed and characterized at the lab of Dr. A. Ong (unpublished, paper in preparation). In the following, RFH94/1, RCTEC and UCL93/3 are referred to as control cells 1, 2, and 3. SKI-001, PKD9-7WT, and OX161/1 are indicated as cystic cells 1, 2, and 3.

Animals

Pkd1^{nl/nl} mice develop polycystic kidney disease within 3–4 weeks after birth (18). *Pkd1*^{-/-} mice have been described previously (19). Animal experiments were performed with approval of the animal experimentation committee of the Leiden University Medical Center according to the animal experimentation guidelines of the Dutch government.

Isolation and culture of primary renal cells

Primary renal cells were isolated from neonate (postnatal day 4) *Pkd1*^{+/+}, *Pkd1*^{+nl}, and *Pkd1*^{nl/nl} offspring of heterozygous *Pkd1*^{+nl} crosses as described (20). Briefly, mice were sacrificed after which both kidneys were removed. Kidneys were finely sliced with sterile knives and incubated in collagenase IX (Sigma, Zwijndrecht, The Netherlands) at 37°C for 30 minutes under continuous shaking. Cells were vigorously resuspended in culture medium and centrifuged at 1200 rpm for 5 minutes at 4°C. Cells were seeded in D-MEM/F12 with 100 U/ml penicillin and streptomycin, 1 mM sodium-pyruvate, 0.1 mM HEPES, 2 mM glutaMAXTM-I, and 10% heat-inactivated fetal bovine serum at 37°C in a humidified atmosphere of 95% air and 5% CO₂ on T182 culture flasks (Greiner Bio-One B.V., Alphen a/d Rijn, The Netherlands). Experiments were performed with passage 2 cells.

Isolation and culture of primary mouse embryonic fibroblasts (MEFs)

Pkd1^{+/+}, *Pkd1*^{+/-}, and *Pkd1*^{-/-} primary mouse embryonic fibroblasts (MEFs) were isolated from a heterozygous *Pkd1*^{+/-} cross (19). Briefly, pregnant mice were sacrificed. Individual embryos were dissected to remove the head and soft tissues (liver, heart, and other viscera). Carcasses were washed twice in PBS, finely sliced with sterile knives, and incubated with trypsin-EDTA (Invitrogen Life Techn., Breda, The Netherlands) overnight at 4°C. The next day, 1:1 trypsin-EDTA was added and followed by incubation at 37°C for 30 minutes. Cells were vigorously resuspended in D-MEM with 4500 mg/l glucose, 100 U/ml penicillin and streptomycin, 1 mM sodium-pyruvate, 0.1 mM HEPES, 2 mM glutaMAX™-I, and 10% heat-inactivated fetal bovine serum, until no debris were left. Cells were seeded on T182 culture flasks (Greiner Bio-One B.V.). Experiments were performed at passage 2-3.

Genotyping

Genotyping was performed to detect *Pkd1*^{wt} and *Pkd1*^{nl} alleles in the primary cell cultures by PCR with 0.2 μM loxF (5'-CAG AGG CAT TGA TTC TGC TC-3'), loxR (5'-TCG GGA GCG GCG ATA CCG TAA CG-3'), and delF (5'-CAC TGT GGT GCG GGG TTA TC-3') primers, pim mix (45 mM Tris pH 8.3, 11 mM (NH₄)₂SO₄, 4.5 mM MgCl₂, 0.5 mM β-mercaptoethanol, 0.5 mM dNTP's, 0.11 μg/μl BSA fraction V, 5 μM EDTA), and RedTaq® polymerase (Sigma). PCR was performed as follows: denaturation at 94°C for 5 minutes, 38 cycles of denaturation at 94°C for 1 minute, annealing at 57°C for 1 minute, and elongation at 72°C for 40 seconds, with a final elongation at 72°C for 7 minutes. PCR products were visualized using agarose gel electrophoresis. To detect *Pkd1*^{wt} and *Pkd1*⁻ alleles in the MEFs, PCR was performed using 10 pmol/μl 2075 (5'-CAG AGG CAT TGA TTC TGC TC -3'), 1450 (5'-TCG GGA GCG GCG ATA CCG TAA AG -3'), and delF primers with 35 cycles of annealing at 60°C for one minute and elongation at 72°C for 90 seconds.

Cellular stimuli and plasmid constructs

Cells were treated with 20 nM EGF (Peprotech Inc, NJ, USA) or 100 ng/ml 12-O-tetradecanoyl-phorbol-13-acetate (TPA; Sigma). Osmotic shock was induced by addition of 500 μM NaCl to culture medium. Total cell lysates were prepared 15 minutes post-stimulation, unless stated otherwise. The membrane targeted mouse C-terminal polycystin-1 fusion protein construct, mPKD1HT, and its corresponding empty vector control, pcDNA1.1 was reported earlier (21;8). The full length human polycystin-2 constructs fused to enhanced green fluorescent protein, PKD2-EGFP, and the corresponding empty vector control, pEGFP-N1, has been described by Scheffers *et al.* (22).

Western blot analysis

Cells were lysed in FOSRIPA buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 1% DOC, 0.1% SDS) with complete® protease inhibitor cocktail (Roche B.V., Almere, The Netherlands), 0.5 μ M sodium-fluoride, and 0.5 μ M sodium-vanadate (Sigma). Western blotting was performed as described (23). Briefly, total cell lysates were separated by 10% SDS-PAGE and blotted on Immobilon-P membranes overnight at 150 mA and 4°C (Millipore). Protein transfer to the membrane was checked with Ponceau S staining (Sigma). Membranes were then blocked in 5% non-fat dry milk/TBST/0.5 μ M sodium-fluoride/0.5 μ M sodium-vanadate, incubated overnight at 4°C with primary antibodies, and with secondary antibodies for 4 hours at 4°C. Rabbit anti-phospho-Thr202/204-ERK1/2 were diluted 1:1000 (Cell Signaling Technology, Beverly, MA, USA), rabbit anti-phospho-Thr183/Tyr185-JNK 1:500, rabbit anti-phospho-Ser217/221-MEK1/2 1:1000, mouse-anti-phospho-Tyr1173-EGFR 1:500 (9H2, Alexis, Breda, The Netherlands), rabbit-anti-ERK1/2 1:3000 (Santa Cruz Biotechnology, Santa Cruz, Ca, USA), mouse-anti-human IgG, Fc γ fragment specific, 1:1000 (Jackson ImmunoResearch Laboratories Inc., West Grove, USA), and mouse-anti-AFP 1:5000 (BD, Alphen a/d Rijn, The Netherlands). Primary antibodies were detected using sheep-anti-mouse-HRPO conjugate, diluted 1:10000 (Amersham Biosciences Europe BmbH, Roosendaal, The Netherlands) or goat-anti-rabbit-HRPO 1:10000 (Jackson ImmunoResearch Laboratories Inc.). Proteins were visualized using enhanced chemiluminescence (Sigma) on Kodak BioMax Light Film (Kodak, Roosendaal, The Netherlands).

Results

Impaired ERK activity in human ADPKD renal cystic epithelial cells

To elucidate the molecular defects in ADPKD, MAPK signaling activity was analyzed in three human ADPKD renal cystic epithelial cell lines, SKI-001, PKD9-7WT, and OX161/1 (referred to as cystic cells 1, 2, and 3) and three normal human renal epithelial control cell lines (RFH94/1, RCTEC and UCL93/3, referred as control cells 1, 2, and 3; 16;17; Ong ACM, unpublished). These cells were stimulated with well known activators of MAPK signaling, including EGF and TPA. EGF and TPA are potent activators of ERK in these cells (Figure 1A). ERK activity (phospho-ERK) was impaired in all cystic cell lines compared to control renal epithelial cells upon stimulation with TPA and EGF (Figure 1A). To investigate this further, we analyzed the activity of the upstream MAPK kinase (MKK) of ERK, MEK1/2. Similar to the MAPKs, activity of MEK1/2 is also regulated via phosphorylation. Impaired ERK activity correlated with impaired activity of MEK1/2 (phospho-MEK1/2 level) in cells stimulated with TPA and EGF (Figure 1B, lower panel). Since EGF normally activates intracellular signaling pathways via binding and consequent activation of its receptor, EGFR, we assessed EGFR activation, i.e. phosphorylation, in these cells. As shown in Figure 1B, EGFR phosphorylation could only be detected after addition of EGF, and was enhanced

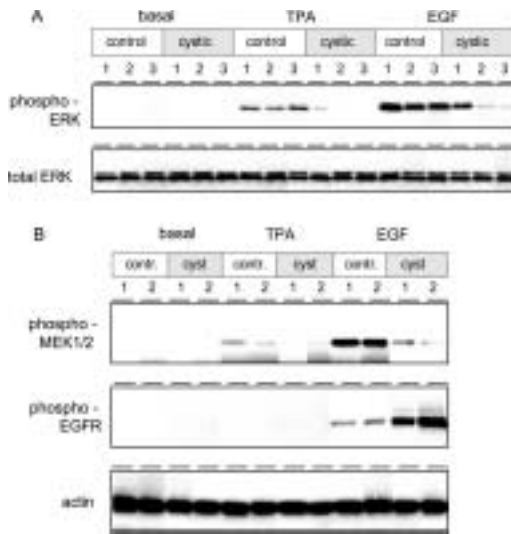


Figure 1. ERK activity is impaired in human ADPKD renal cystic epithelial cells. Three human control (RFH94/1, RCTEC and UCL93/3; referred to as controls 1, 2, and 3 respectively) and three ADPKD renal cystic epithelial cell lines (SKI-001, PKD9-7WT, and OX161/1; referred to as cystic cell lines 1, 2, and 3) were non-treated or stimulated with TPA or EGF, potent activators of MAPK signaling, as indicated. Western blot analysis was then performed using 30 μ g total cell lysate per lane. Equal loading was assessed using Ponceau S staining for total protein (*not shown*). Blots were incubated with phospho-ERK, total ERK (A), phospho-EGFR, phospho-MEK1/2 (B), and actin primary antibodies.

in cystic as compared to control cells. Thus, the impaired ERK activation by TPA and EGF in cystic cells does not seem to be due to impaired EGFR activity. Together, our data suggest that the defect in ERK activation in the human renal cystic epithelial cells tested occurs upstream of MEK and down-stream of the EGF receptor.

Impaired ERK activity in $Pkd1^{nl/nl}$ primary renal cells

Since the renal cystic epithelial cells isolated from ADPKD kidneys represent cells at end-stage renal failure, we subsequently analyzed MAPK activity in primary renal cells isolated at earlier stages of polycystic kidney disease. For this purpose, we turned to the $Pkd1^{nl/nl}$ mouse model. $Pkd1^{nl/nl}$ mice manifest with severe polycystic kidney disease at 3-4 weeks of age (18). Primary renal cells were isolated at postnatal day 4 and were therefore at relatively early stages of disease progression. Importantly, similar to the human ADPKD renal cystic epithelial cell lines, impaired ERK phosphorylation was observed in EGF stimulated $Pkd1^{nl/nl}$ renal cells (Figure 2).

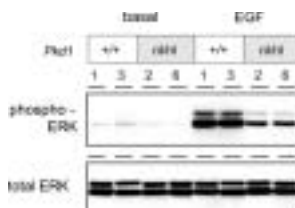


Figure 2. Impaired ERK activity in $Pkd1^{nl/nl}$ renal cells. Two control $Pkd1^{+/+}$ (1 and 3) and two $Pkd1^{nl/nl}$ renal cells (2 and 6) were non-treated or stimulated with EGF. Western blot analysis was then performed using 30 μ g total cell lysate per lane. Equal loading was assessed using Ponceau S staining for total protein (*not shown*). Blots were incubated with phospho-ERK or total ERK primary antibodies.

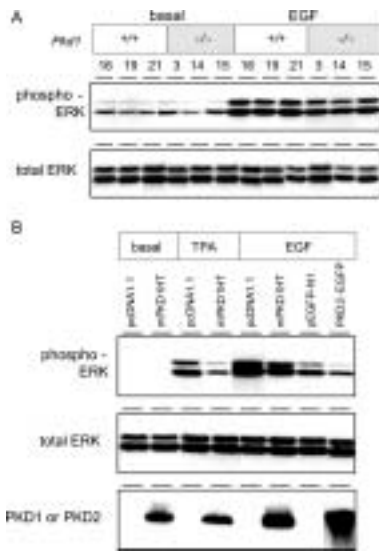


Figure 3. A) Decreased ERK activity in *Pkd1*^{-/-} MEFs. Three control *Pkd1*^{+/+} MEFs (16, 19, and 21) and three *Pkd1*^{-/-} MEFs (3, 14, and 15) were non-treated or stimulated with EGF. Western blot analysis was then performed using 30 µg total cell lysate per lane. Equal loading was assessed using Ponceau S staining for total protein (not shown). Blots were incubated with phospho-ERK and total ERK primary antibodies. B) Overexpression of polycystin-1 and polycystin-2 constructs inhibits ERK activity. HEK293 cells were transfected with a membrane targeted C-terminal 193 aa polycystin-1 construct, mPKD1HT, or a full-length human polycystin-2-EGFP fusion protein construct, PKD2-EGFP, or the corresponding empty vectors, pcDNA1.1 and pEGFP-N1 respectively. Cells were non-treated or stimulated with TPA or EGF. Western blot analysis was then performed using 30 µg total cell lysate per lane. Equal loading was assessed using Ponceau S staining for total protein (not shown). Blots were incubated with phospho-ERK or total ERK primary antibodies. mPKD1HT was detected using an anti-human-IgG(Fc) and PKD2-EGFP was detected using an anti-GFP antibody.

The C-terminal polycystin-1 region and polycystin-2 inhibit ERK activity

Since EGF induced ERK activity was significantly impaired in both early- and end-stage renal cystic cells (Figure 1 and Figure 2), we were interested whether ERK signaling was also affected in *Pkd1*^{-/-} primary mouse embryonic fibroblasts (MEFs). Interestingly, EGF stimulated ERK activity was indeed decreased in *Pkd1*^{-/-} MEFs compared to *Pkd1*^{+/+} cells, indicating that reduced polycystin-1 levels affect ERK signaling also in other cell types than renal cells (Figure 3A). We subsequently examined the effect of overexpression of polycystin-1 and polycystin-2 on ERK activity. For this, the membrane targeted C-terminal 193 amino acids of polycystin-1, mPKD1HT, and a full length polycystin-2-EGFP fusion protein construct, PKD2-EGFP, were transiently overexpressed in human embryonic kidney 293, HEK293, cells. Cells expressing these constructs showed decreased ERK phosphorylation upon TPA or EGF treatment (Figure 3B). Thus, transient overexpression of these polycystin constructs has similar effects on ERK activity as in renal cystic cells, further supporting the hypothesis that both too low and too high expression of polycystins can elicit polycystic kidney disease-associated cellular changes (24).

Increased JNK activity in *Pkd1*^{-/-} MEFs

Finally, we examined the effects of aberrant polycystin activity on JNK activation. For this assay we used osmotic shock, which was a much more potent activator than EGF in the cell types examined. In contrast to the decreased ERK activation by EGF in *Pkd1*^{-/-} MEFs, JNK phosphorylation by osmotic shock was enhanced (Figure 4A). Moreover, transient overexpression of the polycystin-1 and polycystin-2 constructs, mPKD1HT and PKD2-EGFP respectively, did increase JNK activity in osmotic shock-treated 293T cells (Figure 4B). However, the increased JNK activity detected in MEFs was not observed in human renal cystic epithelial cells or in

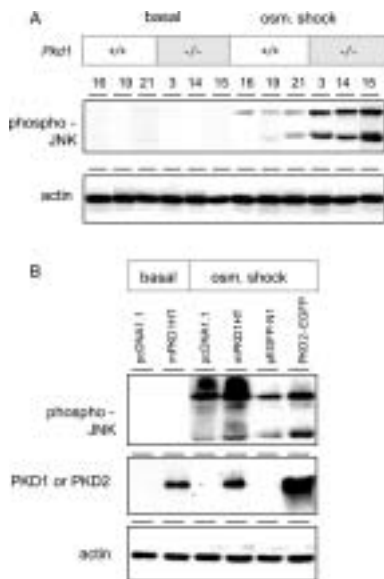


Figure 4. A) Increased JNK activity in Pkd1^{-/-} MEFs. Three control Pkd1^{+/+} MEFs (16, 19, and 21) and three Pkd1^{-/-} MEFs (3, 14, and 15) were non-treated or stimulated with osmotic shock, a potent inducer of JNK. Western blot analysis was then performed using 30 μ g total cell lysate per lane. Equal loading was assessed using Ponceau S staining for total protein (*not shown*). Blots were incubated with phospho-JNK and actin primary antibodies. B) Overexpression of polycystin-1 and polycystin-2 constructs increases JNK activity. HEK293 cells were transfected with the polycystin-1 and polycystin-2 constructs, mPKD1HT and PKD2-EGFP, or the corresponding empty vectors, pcDNA1.1 and pEGFP-N1 respectively. Cells were stimulated with osmotic shock. Western blot analysis was then performed with phospho-JNK or actin primary antibodies. mPKD1HT was detected using an anti-human-IgG(Fc) antibody and PKD2-EGFP was detected using an anti-GFP antibody.

Pkd1^{nl/nl} primary renal cells (data not shown). These data indicate that polycystins can modulate JNK activity, although the relevance of this effect for cyst formation in polycystic kidney disease is as yet unclear.

Discussion

To further elucidate the molecular defects in ADPKD, we investigated the role of MAPK signaling, a major regulator of AP-1 activity, in human and mouse renal cystic epithelial cells. For this purpose, MAPK activity was induced with well-known activators. ERK is strongly induced by TPA and EGF, whereas JNK is induced by osmotic stress in these cells. We observed increased JNK activation in Pkd1^{-/-} mouse embryonic fibroblasts, as reported recently by Nishio *et al.* (25). However, this could not be confirmed in primary Pkd1^{nl/nl} renal cells isolated at early stage of polycystic kidney disease, nor in end-stage human ADPKD renal cystic epithelial cells. Since both polycystin-1 and polycystin-2 constructs could increase JNK activity in HEK293 cells, these effects may be cell-type specific. Moreover, the strongest difference we observed was not in JNK, but in ERK activity. ERK activation was significantly impaired in both early (Figure 2) and end-stage renal cystic epithelial cells stimulated with EGF and TPA (Figure 1). Epidermal Growth Factor Receptor (EGFR) phosphorylation was detected only after stimulation with EGF and was increased in human ADPKD renal cystic epithelial cells compared to control (Figure 1B). Hyperphosphorylation of EGFR in (non-treated) renal cystic epithelial cells was reported previously (26-29). Thus, the impaired ERK activation after EGF stimulation in cystic cells does not seem to be due to impaired EGFR activity, but is most likely due to a defect in a more downstream component. Overexpression of a membrane targeted C-terminal 193 aa polycystin-1 and a full-length polycystin-2-EGFP fusion protein construct confirmed that polycystin-1 and polycystin-2 can modulate ERK and also JNK activity. Both polycystin-1 and polycystin-2 constructs inhibited ERK activity (Figure 3B). Overexpression of these polycystins thus has similar effects on ERK activity as in renal cystic cells, which is intriguing in view of the hypothesis that

both too low and too high expression of polycystins can lead to polycystic kidney disease (24).

In conclusion, our data indicate that ERK signaling is significantly impaired in early- and end-stage renal cystic cells. This impaired ERK reactivity upon different cellular stimuli may be relevant for cyst formation and progression in ADPKD. Our data are in line with a previous report by Carone *et al.* that showed that EGF induced proliferation is less efficient in 3D collagen gel culture of human ADPKD cystic cells as compared to normal kidney cells (30). Where we demonstrate significantly impaired ERK reactivity upon EGF stimulation in early- and end-stage renal cystic cells, Yamaguchi *et al.* did not report a difference in control and cystic human kidney cortex cells (HKC; 31). Our data are seemingly conflicting with other reports as well. ADPKD and *bpk* cystic cells were found to be hyper-responsive to EGF induced mitogenesis (26;32-33). These data mainly focus on the proliferative response to EGF. This proliferative response can be achieved via many intracellular signaling routes. Impaired ERK activity could also lead to increased proliferation. Indeed, Grimm *et al.* have recently reported exactly this in *Pkd2*^{-/-} cells (34). These data clearly show that further insight is required in the intracellular signaling cascades that are regulated by polycystins and that are altered in ADPKD. This is especially relevant considering the fact that inhibition of EGFR activity by pharmaceutical compounds such as EKI-785 and EKI-569, have been reported to improve disease progression in mouse models for polycystic kidney disease (35-37). Instead, our results provide a rationale for EGF supplemental treatment for polycystic kidney disease, as has been reported previously by Gattone II *et al.* and Ricker *et al.* (38-39). We report here, to our knowledge for the first time, direct molecular evidence for impaired ERK signaling activity in human and mouse renal cystic cells at early and end-stage of disease progression.

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