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TITLE: A New Therapeutic Strategy for Autosomal Dominant Polycystic Kidney Disease: Activation of AMP Kinase by Metformin

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Autosomal dominant polycystic kidney disease is a common inherited disorder. Patients are born with normal kidneys but, over the course of decades, they develop large fluid filled cysts that damage the normal kidney tissue. The damage caused by these cysts can lead ultimately to kidney failure, necessitating kidney transplantation or dialysis. There are currently no approved medications for this condition. Recent research reveals that cyst formation is due in part both to inappropriate cell growth and fluid secretion. The enzyme AMPK controls a number of cellular pathways, including those involved in cell growth and fluid secretion. Drugs that activate AMPK, therefore, may constitute an effective therapeutic option for slowing or preventing cyst growth. This research project is aimed at examining the potential of an approved, widely used, inexpensive and low-toxicity drug that can activate AMPK as a potential therapy for the treatment of polycystic kidney disease.

Autosomal Dominant Polycystic Kidney Disease; Metformin
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Introduction
Autosomal dominant polycystic kidney disease (ADPKD) is characterized by slow and continuous development of cysts derived from renal tubular epithelial cells. The cysts profoundly alter renal architecture, compressing normal parenchyma and compromising renal function. Nearly half of ADPKD patients ultimately require renal replacement therapy. ADPKD is a common genetic disorder, affecting at least 1 in 1,000 individuals (1). There are currently no effective specific clinical therapies for ADPKD. Cystic growth and expansion in ADPKD are thought to result from both fluid secretion into cyst lumens and abnormal proliferation of the cyst-lining epithelium. The rate of fluid secretion into the cyst lumen is directly proportional to the amount of the Cystic Fibrosis Transmembrane Regulator (CFTR) chloride channel in the apical membranes of cyst-lining epithelial cells (2). The cells surrounding the cysts manifest increased proliferation (3, 4). Mammalian Target of Rapamycin (mTOR) activity is elevated in models of PKD and is likely to be responsible, at least in part, for this hyperproliferative phenotype (3). mTOR is a serine/threonine kinase that regulates cell growth and proliferation, as well as transcription and protein synthesis. Interestingly, both the CFTR chloride channel and the mTOR signaling pathway are negatively regulated by the “energy sensing” molecule, Adenosine Monophosphate-activated Protein Kinase (AMPK). AMPK phosphorylates and directly inhibits CFTR, and indirectly antagonizes mTOR through phosphorylation of TSC2 and Raptor (5-8). Both of these actions are consistent with the role of AMPK as a regulator that decreases energy-consuming processes such as transport, secretion, and growth when cellular ATP levels are low (9). Thus, a drug that activates AMPK might inhibit both the secretory and the proliferative components of cyst expansion. Metformin, a drug in wide clinical use for both non-insulin dependent diabetes mellitus and Polycystic Ovary Syndrome, stimulates AMPK (10, 11). We therefore wish to examine whether metformin-induced activation of AMPK can slow cystogenesis through inhibition of mTOR-mediated cellular proliferation and inhibition of CFTR-mediated fluid secretion.
Research Accomplishments Associated with Each Task Outlined in the Approved Statement of Work

Task 1. Characterize the effects of AMPK stimulation on its downstream targets in renal epithelia in vivo (months 1-20). The studies encompassed in this task group are designed to determine whether and how AMPK stimulation impacts upon the cellular pathways that are involved in cyst development and expansion. These experiments will make use of cultured MDCK cells and in vitro assays of both AMPK activation and the functional status of AMPK’s downstream targets.

Task 1a. Establish AMPK stimulation by its pharmacologic activator, metformin in renal epithelial cells in vitro. By immunoblotting for the downstream targets of AMPK, we will confirm that AMPK stimulation has the same effects on its downstream targets in renal epithelia as has been reported in other tissues (months 1-8).

Research Accomplishments: Metformin stimulates AMPK and pACC.

We first treated Madin Darby Canine Kidney (MDCK) renal epithelial cells with metformin to evaluate AMPK activation. Activated AMPK is phosphorylated at residue Thr172 of its α subunit. We performed Western blotting using a phosphospecific antibody to measure the level of the phosphorylated AMPK, pAMPK (Fig. 1a). We found that incubation with metformin for as little as 2 hours significantly increases pAMPK levels (Fig. 1b). To determine whether this effect was correlated with increased phosphorylation of an AMPK target, we evaluated metformin’s effect on the AMPK-mediated inhibitory

Figure 1: Metformin activates AMPK in vitro and in vivo. (a) MDCK cells were incubated with 1.0 mM metformin for the number of hours stated. Cells lysates blotted for pAMPK, the activated form of AMPK. (b) Quantitation of pAMPK band density normalized to β-actin. Comparisons of the mean (± SEM) are shown for each timepoint. (c) MDCK cells treated as above, blotted with pACC, a downstream target of pAMPK. (d) Comparisons of the mean band density relative to β-actin (± SEM) are shown for each timepoint. (e) 8-week-old C57BL/6 mice were treated with intraperitoneal metformin or with vehicle for three. Western blot analysis of kidney homogenates using anti-pAMPK demonstrates increasing activation of AMPK with increasing metformin dosing. (f) Quantitation of Western blot of in vivo pAMPK levels by normalized band density to β-actin.
phosphorylation of Acetyl-CoA Carboxylase (ACC) (Fig. 1c). Incubation of MDCK cells with metformin produced a significant increase in pACC levels in six hours (Fig. 1d). In AMPK α1 knockdown cells, metformin’s effects on pAMPK and pACC levels are substantially blunted. Treatment of mice with increasing doses of metformin, administered daily for three days, results in increasing levels of pAMPK throughout the nephron (Fig. 1e and 1f).

Task 1b. Characterize the physiological consequences of inhibition of AMPK downstream target CFTR. This will be accomplished via short circuit current measurements performed on MDCK cells transfected to express CFTR and grown on permeable filter supports (months 6-16).

Research Accomplishments: Inhibition of CFTR-dependent Isc by Metformin in MDCK Cells is AMPK-dependent.

We next examined the effect of metformin treatment on the CFTR chloride channel, which is inhibited by AMPK phosphorylation (12-14). Since CFTR drives, at least part of the fluid secretion in PKD cystogenesis, we hypothesized that metformin-stimulated AMPK activity would inhibit CFTR channels in renal epithelial cells and slow the rate of cyst growth (15, 16). To test whether metformin inhibits CFTR via AMPK in a kidney-derived epithelial cell line, CFTR was expressed by adenoviral transduction in three different polarized MDCK type II cell lines stably transfected with either an empty vector, or shRNA plasmids directed against two isoforms of the catalytic α subunit of AMPK. MDCK cells endogenously express high concentrations of the α1 isoform of the AMPK catalytic α subunit and very low concentrations of the α2 isoform. Expression of the α1 shRNA construct reduced this protein’s expression by ~90%, whereas the α2 shRNA had no effect on α1 protein expression. Knockdown of α1 also reduced the level of total phospho-AMPK

Figure 2: Metformin inhibits Isc in an AMPK-dependent manner. (a) MDCK cells stably expressing either empty vector or shRNA plasmids directed against either the catalytic α1 or α2 subunits of AMPK (AMPK-α1 KD and AMPK-α2 KD cells, respectively) were blotted with antibodies against either pThr172, AMPKα2, or AMPKα1 to measure the level of AMPK expression. (b) A representative Isc trace of cells with or without 1 mM metformin pre-treatment. Mock-transduced or NH2-terminally GFP-tagged-CFTR-transduced MDCK “empty vector” control cells, AMPK-α1 KD or AMPK-α2 KD cells were treated with 1 mM metformin or vehicle for 2-4 h prior to Ussing chamber measurements of Isc. A representative Isc trace of vehicle pre-treated CFTR-control cells, AMPK-α1 KD or AMPK-α2 KD cells were treated with 1 mM metformin or vehicle for 2-4 h prior to Ussing chamber measurements of Isc. A representative Isc trace of vehicle pre-treated CFTR-expressing “empty vector” control MDCK cells treated with IBMX and forskolin and then CFTR-Inh172 at the indicated times is shown. (c) A similar representative trace of mock-transduced “empty vector” control cells shows no response to these cAMP agonists or to CFTR-Inh172. There was also no significant change in Isc following addition of 10 µM amiloride, indicating that the epithelial Na+ channel does not significantly contribute to Isc in these MDCK cells. (d) Comparisons of the normalized mean (± SEM) CFTR-dependent Isc in “empty vector” control, AMPK-α1 KD, and AMPK-α2 KD cells with (dark gray bars) or without (white bars) metformin pre-treatment.
(pAMPK) by ~90% (Fig. 2a). CFTR-dependent Isc was measured for cells grown on filters mounted in Ussing chambers for four days following adenoviral transduction, with or without exposure to 1 mM metformin for 2-4 hours prior to measurement. To initiate CFTR-mediated secretion, CFTR-expressing and mock-transduced MDCK cells were treated with the cAMP agonists IBMX and forskolin, and the experiment was concluded through application of the specific CFTR inhibitor CFTR-Inh172 (17). Typical traces of Isc changes are shown in Fig. 2b and Fig. 2c. In CFTR-expressing cells there was generally an early peak in Isc within 1-2 min following forskolin/IBMX treatment, followed by a lower plateau current within ~5 min. This remaining current was sensitive to inhibition by CFTR-Inh172. Metformin (1 mM) pretreatment of empty vector-transfected and AMPK-α2 knockdown MDCK cells significantly reduced CFTR-dependent Isc by 60-70% relative to cells pretreated with vehicle (Fig. 2d). However, there was no metformin-dependent inhibition of CFTR current in AMPK-α1 knockdown MDCK cells, suggesting that the metformin-induced inhibition of CFTR occurs specifically via an AMPK-α1-dependent mechanism.

**Task 1c. Assess AMPK-mediated mTOR inhibition.** This will be accomplished both by direct assay of the phosphorylation status of downstream mTOR targets and by determining the physiological implications of this inhibition through measurements of cell proliferation (months 10-20).

**Research Accomplishments:**

**Inhibition of mTOR by Metformin in MDCK Cells is AMPK-dependent.**

To determine whether metformin induces AMPK-mediated inhibition of mTOR activity, we tested whether mTOR activity is diminished in MDCK cells cultured in the presence of metformin by blotting for the phosphorylated form of the mTOR downstream target ribosomal S6K p70 subunit (p70 S6K) (Fig. 3a) relative to pan-S6K (Fig. 3b). This inhibition is time-dependent, with increasing exposure to metformin resulting in greater suppression of this pathway. Total S6K levels remain constant. The inhibition takes longer to achieve than inhibition of CFTR or ACC,
consistent with the fact that AMPK indirectly inhibits mTOR via TSC2/1 and Rheb (Fig. 3c). This effect is markedly less pronounced in AMPK-α1 KD cells. To evaluate whether these changes in phospho-protein levels translated into changes in proliferation, an Alamar Blue assay was used to quantitate proliferation in wild-type and AMPK-α1 knockdown MDCK cells. The y-axis depicts cell number measured at each given concentration of metformin and normalized to the control value, which was obtained for the same cell type at the same time point without metformin treatment. Wild-type MDCK cells exhibited a metformin dose-dependent decrease in proliferation, while this response was significantly diminished in the AMPK-α1 knockdown MDCK cells. (Fig. 3d) It should be noted that at the highest concentration of metformin tested (5 mM) substantial growth suppression was detected in AMPK knockdown cells. This may be due to the low level of residual AMPK that is expressed in these knockdown cells (see Fig. 2a) or to effects of high doses of metformin on yet to be identified AMPK-independent pathways. A similar suppressive effect of metformin treatment on proliferation is observed in vivo. We performed immunofluorescence analyses on kidneys from metformin treated and vehicle treated cystic Pkd1floX/-;Ksp-Cre mice using an antibody directed against Ki67, a marker of actively proliferating cells. In kidneys from vehicle-treated mice 19.7±3.8% s.e.m. of the cells exhibited Ki67 positivity (450 cells counted from each of n=6 mice) in comparison to 10.6±3.6% s.e.m. (450 cells counted from each of n=4 mice) in metformin-treated mice (p<0.0074). To assess whether the effects of metformin treatment on proliferation correlate with the level of mTOR activity in the cystic kidneys before and after metformin treatment, we performed immunohistochemistry using an antibody directed against the activated form of an mTOR target. We stained tissue from control and metformin treated cystic mice with an antibody that detects the phosphorylated form of 4E-BP1 ( ), an mTOR target whose level of phosphorylation is commonly used to report on levels of mTOR activity (18). We find that the level of p4E-BP1 is generally higher in the cyst lining epithelial cells in control animals as compared to those observed in metformin treated animals, consistent with the interpretation that metformin treatment reduced the level of mTOR activation.

Additional Progress on Task 1 Studies:

I. Identification of Dosing Regimens that Produce AMPK Activation in Wild Type Mice

We measured the effects of varying doses of metformin on AMPK activity in mice in vivo. This analysis was performed by immunocytochemistry and by western blotting. In both cases, ischemic injury was used as a positive control to produce maximal AMPK activation. AMPK is activated in response to energy deprivation, which

![Figure 4: Immunocytochemical assessment of AMPK activation in mouse kidneys in situ. AMPK activation levels were assessed by staining sections with an antibody directed against p-AMPK. We find that treatment with 375 mg/kg produces levels of AMPK activation that are comparable to those produced by renal ischemia.](image-url)
results in the accumulation of high cytosolic levels of the enzyme’s allosteric activator AMP. Renal ischemia produces energy deprivation in renal tubules and is a powerful stimulus leading to the activation of AMPK in the kidney. The localization of activated, phosphorylated form of AMPK (p-AMPK) at baseline and after acute kidney ischemia has been characterized in the rat (19, 20). In murine kidneys, we demonstrate similar localization of p-AMPK under these conditions. Upon immunofluorescence staining using a p-AMPK-specific antibody, p-AMPK was detected in the apical membranes of distal tubules of mice at baseline. Following ischemia, there was a diffuse increase in p-AMPK fluorescence globally, including within proximal tubules and glomeruli (Fig. 4). Metformin has been demonstrated to activate AMPK in the heart, vascular tissue, and kidney homogenates (21-23). We observed that treatment with metformin delivered by IP injection results in increased fluorescence of p-AMPK in all cortical tubule segments (Fig. 4). Furthermore, this activation appears to be dose-dependent, as assessed by immunofluorescence microscopy. Western blot analysis to measure p-AMPK levels was also performed on renal tissue that was snap-frozen in situ using a specially designed forceps that had been pre-cooled in liquid nitrogen. This method also confirmed that AMPK activation occurs in response to increasing metformin doses (Figure 5). The data suggest that the metformin dose that has been used in our in vivo studies (24) produces reproducible AMPK activation in situ (Figure 6). Our efforts to assess the isoform specificity of AMPK activation in vivo will continue, as will our efforts to determine the effects of AMPK activation on CFTR distribution and mTOR activity in kidneys in vivo.

II. Further investigation of metformin’s mode of action.

Very recent published data suggests that the mechanism of action of metformin in vivo may involve pathways other than those related to AMPK (25). It is critically important to determine whether the potential therapeutic effects of metformin that we have identified in the context of Autosomal Dominant Polycystic Kidney Disease are due to its capacity to activate AMPK or are...
instead due to its effects on other targets. Recent research indicates that metformin can decrease cellular levels of cAMP (25). This is especially relevant in the setting of Autosomal Dominant Polycystic Kidney Disease because a substantial body of research has demonstrated that elevation of cAMP promotes cyst growth in vitro and in vivo (26, 27). Furthermore, drug therapies that reduce cAMP slow cyst growth both in mouse models of Autosomal Dominant Polycystic Kidney Disease (28) as well as in human Autosomal Dominant Polycystic Kidney Disease patients (29). To begin to explore this possibility, we have undertaken to assess the effects of other AMPK activators. These include AICAR, the A-769662 compound from Abbott Laboratories, and two new AMPK activating compounds provided to us by Glaxo Smith Kline on an investigational basis. We have begun to assess whether these compounds modulate cystic growth of renal epithelial cells maintained in three dimensional culture conditions. If this proves to be the case we will conclude that AMPK is indeed a pharmacological target of value in Autosomal Dominant Polycystic Kidney Disease and that the therapeutic potentials of novel compounds that activate AMPK should be explored in relevant tissue culture and animal models. We have demonstrated that these compounds activate AMPK in cells in culture. We have now undertaken dose response analyses to determine the optimal concentrations and time courses for activating AMPK in renal epithelial cells in culture (see Figure 7). Once identified, these conditions will be applied in experiments designed to assess the effects of these compounds on the cystic growth of renal epithelial cells maintained in three dimensional culture.

III. Exploration of a novel mechanism that may connect AMPK inhibition to the development of Autosomal Dominant Polycystic Kidney Disease.

A recent paper from the laboratory of Dr. Alessandra Boletta reported that cells homozygous for ADPKD-causing mutations manifest substantial perturbations in energy production (30). These cells exhibit very high levels of glycolysis and low levels of oxidative metabolism, reminiscent of the Warburg effect that is seen in tumor cells. As a result of the very high levels of glycolysis the cytoplasmic levels of ATP are very high and levels of active AMPK

![Figure 7: Dose response analysis of AMPK activation by A-769662 in cultured MDCK renal epithelial cells.](image)

![Figure 8: EGLN3 KD induces an increase in surface PC1 in polarized LLCPK cells: Left 3 panels: surface IF using Flag antibody; Right panel: fluorescence plate reader analysis measuring ratio of Flag (surface) to HA (total) signal](image)
are consequently very low. These data lend further support to the idea that small molecule AMPK-activators may have therapeutic benefit in ADPKD. Very recently, we have explored the mechanisms responsible for the “Warburg-like” excessive glycolytic activity that is observed in ADPKD cells. We have identified a key enzyme that is involved in regulating energy synthesis to be another potential drug target in ADPKD.

One of the key steps in the regulation of the tricarboxylic acid cycle and aerobic metabolism is the production of acetyl CoA from pyruvate by pyruvate dehydrogenase (PDH) (31). PDH is subjected to inhibitory phosphorylation by PDH kinase, and this inhibition is reversed by a calcium-activated PDH phosphatase. Polycystin-2 can function as an ER calcium release channel (32), and calcium release from the ER stimulates aerobic metabolism by activating PDH phosphatase and thus PDH (33). The channel activity of TRPA1, which like polycystin-2 is a member of the TRP family, is directly regulated by oxygen through the oxidation and hydroxylation of specific cysteine and proline residues, respectively (34). This prolyl hydroxylation is mediated by prolyl hydroxylase domain containing (PHD) enzymes, which are critical sensors of changes in oxygen levels (35, 36). We have found through mass spectrometric analysis that polycystin-1 and polycystin-2 are both modified by cysteine oxidation and prolyl hydroxylation at highly conserved sites. Furthermore, we have found that the polycystin 1/2 complex associates with a PHD enzyme called EGLN3, and that reducing the expression or activity of EGLN3 substantially alters polycystin 1/2 behavior.

We find that shRNA-mediated knockdown of EGLN3, which is also known as prolyl hydroxylase domain containing protein 3 (PHD3), leads to increased surface expression of PC1 (Figure 8). As noted above, the PHD proteins are cellular O2 sensors and regulate the cell’s hypoxia response pathway. In the presence of O2, PHD proteins hydroxylate proline residues on the hypoxia induced factor HIF1α and this modification targets HIF1α for degradation (35). PHD modification of TRPA1 contributes to the O2-sensitivity of TRPA1 channel gating and also modulates its cell surface expression (34). The O2-sensitivity of TRPA1 gating also involves direct non-enzymatic oxidation of TRPA1 cysteine residues. We find that knockdown of EGLN3 expression or inhibition of EGLN3 activity (using CoCl2) in LLC-PK1 cells stably expressing polycystin-1 and polycystin-2 led to a substantial increase in the quantity of polycystin-1 present in the apical plasma membrane (Fig. 8). Mass spectrometric analysis of polycystin-1 and polycystin-2 purified from transfected cells by immunoprecipitation reveals the presence of several hydroxylated proline residues and oxidized cysteine residues. The oxidized cysteines reside in close proximity to the hydroxylated prolines, and several of these residues are found in sequence motifs that resemble the PHD target site in TRPA1. EGLN3 co-immunoprecipitates with PC1, and this association is prevented by inhibiting EGLN3 with DMOG (Figure 9). Taken together, these data support the exciting hypothesis that the trafficking and channel activity of the polycystin-1/2 complex is modulated by PHD proteins in response to changes in cellular O2 levels. These findings further suggest a novel hypothesis that accounts for the metabolic perturbations that accompany the loss of polycystin 1 expression and the potential therapeutic utility of metformin. We propose that oxygen levels regulate the calcium
channel activity of the polycystin 1/2 complex, which in turn regulates oxidative metabolism by mediating the release of calcium from the ER, leading to activation of PDH-phosphatase and hence of PDH.

**Task 2.** Evaluate the *in vitro* and *in vivo* effects of metformin-induced AMPK inhibition of mTOR and CFTR in the context of *in vitro* and *in vivo* models of cystic kidney disease (months 12-36).

**Task 2a.** Determine the effect of metformin treatment on average cyst size and cyst number using *in vitro* models of cystogenesis. These experiments will be performed with renal epithelial cells that spontaneously form cysts when suspended in a collagen matrix. Cyst size and number will be determined by quantitative fluorescence microscopy techniques (months 12-24).

**Research Accomplishments:**
Metformin treatment slows cystogenesis *ex vivo.* Two-dimensional culture models do not accurately depict cell growth in the three-dimensional environment in which cysts develop. To evaluate metformin’s effects in the context of cystogenesis, we suspended MDCK cells in a three-dimensional collagen matrix and allowed them to form cysts spontaneously in the presence of forskolin and IBMX (37). Cultures co-incubated with metformin for the duration of cyst growth produced significantly smaller cysts than those similarly treated with forskolin and IBMX alone (p=0.003, unpaired t-test, n=3 gels for each experimental condition) (Fig 10a).

![Figure 10: Metformin reduces cyst size *in vitro* and *ex vivo.*](image)

(a) Representative light micrographs of MDCK cell cysts grown in collagen gels. Cysts were treated with forskolin and IBMX, to enhance apical fluid secretion, with (bottom) or without (top) 1.0 mM metformin for 20 days. Gels were melted and the cysts were allowed to precipitate to the bottom for imaging. (b) Metformin treatment reduces cyst size in an *ex vivo* model of renal cystogenesis. Embryonic kidneys were placed in culture at E12 and maintained for 5 days in the continued presence of 100 µM 8-Br-cAMP. Representative light microscopic images are shown from one mouse. Each row shows the same kidney. The contralateral kidney (bottom row) was treated with metformin for 4 days, and then switched to normal media, illustrating that the embryonic kidney remains viable and capable of cystogenesis.

We next tested the effect of metformin on *ex vivo* cystogenesis. Embryonic kidneys (E12.5) were removed from C57/B6 mice. One embryonic kidney was cultured in the presence of membrane permeable 8-Br-cAMP to stimulate fluid secretion, while the contralateral kidney was co-incubated with 8-Br-cAMP and metformin for 4 days. Culture in the presence of 8-Br-cAMP induces cyst formation in embryonic mouse kidneys (38). Metformin treatment significantly
decreased fractional cyst area (p=0.04, unpaired t-test with n=4 for each experimental condition). On day 5, metformin was removed from the treated embryonic kidney and cyst growth recommenced in the treated kidney, demonstrating that metformin treatment slowed cyst growth without affecting the viability of the tissue (Fig. 10b).

**Task 2b. Perform an in vivo trial of metformin treatment on Ksp-Cre, Pkd1\textsuperscript{flox/-} mice.** This mouse model represents a very severe model of ADPKD. These experiments will permit the potential for metformin therapy to slow disease progression to be evaluated in mice that have developed cystic disease prior to the initiation of treatment (months 12-28). These experiments will utilize 75 mice, 25 of which will be used in months 1-12 for breeding purposes, to generate a stable colony of 50 mice with the required genotypes that will be used in the experiments associated with this task.

**Research Accomplishments:** Metformin treatment slows cystogenesis in an aggressive constitutive in vivo model of PKD.

We next tested whether metformin slows cyst growth in a murine model of PKD. Initially, we used the most aggressive viable murine model of PKD (Pkd1\textsuperscript{flox/-};Ksp-Cre) in which there is progression of renal cystic disease within the first week of life and death between two and three weeks of life (4). We treated these mice with daily intraperitoneal injections of metformin (300 mg/kg/day) dissolved in a 5% dextrose solution from P4 until P6. This is a dose known to activate AMPK (23). Mice were then sacrificed and kidneys harvested at P7. The vehicle treated Pkd1\textsuperscript{flox/-};Ksp-Cre kidneys (Fig. 11c) were profoundly cystic and greatly enlarged compared to the Pkd1\textsuperscript{+/+};Ksp-Cre kidneys (Fig. 11a). In contrast, cyst burden is significantly reduced in the kidneys from the metformin-treated Pkd1\textsuperscript{flox/-};Ksp-Cre mice (Fig. 11b). Because metformin can affect body weight, kidney weight: body weight ratio was not used as an endpoint (39). Instead, the effect of metformin

![Figure 11: Metformin treatment reduces cystic index in two mouse models of ADPKD.](image-url)
on renal morphology was quantitated by evaluating cystic index, which determines the fraction of a given section that corresponds to luminal area (including both tubule and cyst lumens). Untreated Pkd1flox/-;Ksp-Cre kidneys had a cystic index of 71.4±4.0% s.e.m., whereas that of metformin treated Pkd1flox/-;Ksp-Cre kidneys was 51.8±5.2% s.e.m. (p=0.029; unpaired t-test with n=4 control and n=8 metformin treated mice). In wild type kidneys, this evaluation calculates a cystic index of 10% due to tubular lumens. Notably, while the metformin-treated kidney is still cystic, it displays significantly more parenchyma than the vector-treated control. While metformin might prevent further cyst growth, it is unlikely that treatment reduces the size of pre-existing cysts.

Task 2c. Evaluate the effect of metformin in a mouse model of inducible PKD. We will use an inducible mouse model of PKD (pCAGGS-cre, tamoxifen-activatable Pkd1flox/- mice), which will allow mice to be pre-treated with metformin prior to disease induction. This will allow us to gauge the therapeutic benefit of metformin under conditions that may more accurately mimic at least certain aspects of the progression of the human disease (months 20-36). These experiments will utilize 75 mice, 25 of which will be used in months 1-12 for breeding purposes, to generate a stable colony of 50 mice with the required genotypes that will be used in the experiments associated with this task.

Research Accomplishments: Metformin treatment slows cystogenesis in an inducible in vivo model of PKD.

We established an inducible model for Pkd1 inactivation using a conditional Pkd1flox allele in combination with a tamoxifen inducible Cre recombinase (pCX-CreERTM) (4, 40, 41). Induction of Cre expression prior to P13 leads to rapidly progressive cystic disease in Pkd1flox/flox animals (42). In this system, it is possible to initiate metformin treatment prior to or during cyst development. Thus, this model might more accurately replicate the clinical scenario, in which metformin therapy could commence early in the disease process and act to prevent or slow subsequent cyst development. We initiated metformin treatment (300 mg/kg/day) at day P7 and then injected intraperitoneal tamoxifen at day P9 and P10 to initiate disease induction. We continued daily metformin injections until P18, when the animal was sacrificed and kidneys harvested for histology and cystic index evaluation. Once again, metformin treatment resulted in a smaller fractional cyst burden than vehicle-treated controls. The kidneys from vehicle-treated mice had a cystic index of 43%, whereas that of metformin treated mice was 31% (p=0.041, unpaired t-test with n=6 for vehicle treated and n=7 for metformin treated mice), a decrease of nearly one-third in the cyst burden (Fig. 11d,e).
Additional Task 2-related Research Accomplishments

I. Identification of a novel small molecule approach to correcting the perturbation in energy metabolism that is associated with Autosomal Dominant Polycystic Kidney Disease

As noted above, we hypothesize that AMPK activity may be reduced in the context of Autosomal Dominant Polycystic Kidney Disease due to the inappropriate acceleration of glycolytic activity, resulting in high levels of ATP and reduced levels of AMP. The reduced AMPK activity could contribute to the pathogenesis of Autosomal Dominant Polycystic Kidney Disease by de-repressing mTOR and CFTR activities. Thus, correcting the perturbation in energy metabolism could potentially help to correct the reduction in AMPK activity and with it the Autosomal Dominant Polycystic Kidney Disease pathological phenotype. Pyruvate dehydrogenase is inhibited via a phosphorylation event that is mediated by pyruvate dehydrogenase kinase. Dichloroacetic acid (DCA) is a very well characterized inhibitor of pyruvate dehydrogenase kinase (43). Administration of DCA stimulates the tricarboxylic acid cycle and aerobic metabolism. Furthermore, DCA has been used in human clinical trials designed to test its therapeutic potential in the setting of congenital lactic acidosis and also in the setting of cancer. Taking advantage of a 3-dimensional culture in vitro cyst growth assay that we have used extensively in our laboratory, we find that treatment with DCA dramatically inhibits cyst formation by ADPKD cells, and instead induces them to grow into tubule-like structures (Figure 12). The size and circularity of the DCA-treated versus control cysts are quantitated in Figure 13. Thus, we believe that the enzyme that we have identified constitutes another extremely interesting target for ADPKD drug development.

II. Treatment with DCA and metformin attenuates cyst development in vivo.

To assess the potential therapeutic efficacy of DCA in the setting of polycystic kidney disease, we assessed its affects using the PKD\textsuperscript{flac\textsuperscript{-}};pCX-CreER mouse model that was employed in Figure 11. DCA was delivered through the drinking water, either alone or in combination with metformin. At the end of the treatment period, mice were sacrificed and cyst development was assessed by measuring the ratio of kidney weight to body weight. As can be seen if Figure 14, treatment with DCA alone produces a trend towards reduced kidney...
weight:body weight, although this effect did not reach statistical significance in the present study. Combination therapy with DCA and metformin produced a statistically significant reduction in kidney weight:body weight. These data suggest the very exciting possibility that these two drugs produce synergistic effects, and that combination therapy employing both of them might constitute a novel and effective approach to the treatment of polycystic kidney disease.

Figure 14: Treatment with DCA, alone or in association with metformin, slows cyst growth
Key Research Accomplishments

- Metformin stimulates AMPK and pACC.
- Inhibition of CFTR-dependent I_{sc} by Metformin in MDCK Cells is AMPK-dependent.
- Inhibition of mTOR by Metformin in MDCK Cells is AMPK-dependent.
- Metformin treatment slows cystogenesis ex vivo and in vivo.
- Metformin treatment slows cystogenesis in in vivo models of PKD.
- The activity of the polycystin 1/polycystin 2 complex is regulated by cellular oxygen sensing machinery.
- Drug treatments designed to increase oxidative metabolism slow cyst growth in vitro and in vivo.
Reportable Outcomes

- Peer reviewed primary data publication:


- Invited review articles:


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**Conclusion**

AMPK activity can be pharmacologically targeted with metformin to reduce the growth of renal cysts. Metformin acts through AMPK to decrease both epithelial fluid secretion by directly inhibiting CFTR, and to decrease cellular proliferation by indirectly targeting mTOR. Metformin stimulates AMPK phosphorylation in cultured MDCK renal epithelial cells, and this phosphorylation correlates with increased AMPK activity, as evidenced by an increase in the level of the AMPK-mediated inhibitory phosphorylation of ACC. Metformin’s inhibitory action on CFTR-mediated chloride transport is AMPK-dependent. Additionally, we have shown that metformin inhibition of mTOR translates into an AMPK-dependent inhibition of cell proliferation. Using both an in vitro model of MDCK cell cystogenesis as well as embryonic kidneys ex vivo, we have demonstrated that metformin decreases cyst size, and fractional cyst area, respectively. Finally, we illustrated the potential therapeutic utility of metformin by testing it in two murine models of ADPKD, both of which are attributable to inactivation of the gene encoding polycystin-1.

In this study, only one dose known to activate AMPK in vivo was tested. When considered on a simple mg/kg basis, this dose appears considerably higher than the current maximum dose prescribed for patients with diabetes or Polycystic Ovary Syndrome. However, human equivalent dose extrapolation is more accurately calculated based on body surface area than on weight. When this calculation is performed for a 60 kg adult, the dose used in our mouse studies extrapolates to a daily dose of approximately 1500 mg, which falls well within the range in which metformin is safely used in humans. It is likely, based on the established pharmacokinetics of metformin, that single daily dosing is suboptimal, and thus we are almost certainly not observing the maximal suppressive effects that metformin could potentially exert on the severity of cyst growth.(44) Support for this contention derives from the data presented in Figure 4c, since in the embryonic kidney model, cyst growth rapidly resumes shortly after removal of metformin from the culture media. Thus, short term intermittent metformin exposure may not be adequate to optimally suppress cyst development. It is quite possible that even lower doses administered more frequently might produce beneficial effects in the setting of polycystic kidney disease. It is important to note that our efforts to assess effects of metformin treatment on renal functional parameters such as serum concentrations of BUN and creatinine were inconclusive, due in part to a large degree of inter-individual variability. Further studies, perhaps employing more slowly progressive disease models, will be required to reduce this variance and to assess the extent to which metformin treatment can protect or improve renal function in the setting of polycystic kidney disease. In addition, subsequent development of metformin for this clinical application will require pharmacokinetic and pharmacodynamic studies designed to identify an ideal dosing regimen that achieves maximal activation of renal tubular AMPK. We are now working to optimize the metformin dosing regimen to ensure that it produces the most substantial and longest lasting activation of AMPK possible. These new dosing regimens will be tested in the relevant mouse models of Autosomal Dominant Polycystic Kidney Disease.

Finally, we have made a novel discovery that may explain the recent observation that energy metabolism is perturbed in kidney cells that lack the expression of polycystin-1. We have found that the polycystins interact with and are regulated by prolylhydroxylase domain containing proteins. Thus, the polycystins may serve as components of cellular oxygen sensors. Furthermore, the calcium channel activity of the polycystins may regulate metabolism by controlling the calcium-sensitive pyruvate dehydrogenase phosphatase, which in turn activates pyruvate dehydrogenase. In
fact, stimulating pyruvate dehydrogenase activity with DCA ameliorates aspects of the cystic phenotype of renal epithelial cells grown in three dimensional culture. Thus, our data suggest a new comprehensive mechanism that accounts for the perturbations in energy metabolism that are detected in Autosomal Dominant Polycystic Kidney Disease. Furthermore, these data explain the potential therapeutic utility of AMPK activation in this context and also suggest exciting new therapeutic modalities.

**So What?**

Metformin is taken by millions of Americans each year. It is currently FDA-approved for the treatment of Non-Insulin Dependent Diabetes (Type II DM) and, intriguingly, for Polycystic Ovary Syndrome, a disease that shares a name similar to that of Polycystic Kidney Disease but whose pathogenesis is even less well understood. In fact, metformin is often considered first line therapy for the treatment of Type II DM, due to its relatively small side effect profile. Recent literature suggests that metformin’s activation of AMPK may be due to its ability to prevent AMP breakdown, although the exact mechanisms of action of metformin in Polycystic Ovary Syndrome or in Type II DM remain largely unknown. Recent reports also suggest that metformin may exert an anti-neoplastic effect. It has been reported that metformin acts in a dose-dependent manner to inhibit the proliferation of breast cancer cells and that this effect can be blocked in the presence of small interfering RNA directed against AMPK. This inhibition is also associated with a decrease in mTOR activation, suggesting that metformin’s anti-proliferative effect is directed through the activation of AMPK, and consequent inhibition of mTOR.

There are numerous therapies for ADPKD in development or in clinical trials, including vasopressin receptor inhibitors, calcium sensing receptor inhibitors, CFTR-inhibitors, cell cycle inhibitors, and rapamycin. Each of these strategies targets one or the other of the key processes (proliferation and secretion) thought to be involved in the pathogenesis of PKD. By acting through AMPK, metformin may offer the significant advantage of blocking both (SI 4). Moreover, metformin is already FDA approved and generally well-tolerated. The most serious, albeit rare, side effect of metformin is lactic acidosis and, since metformin is cleared by the kidney, chronic renal disease has been considered to be a potential predisposing factor for this complication. However, metformin use could ideally be initiated at an early stage in ADPKD progression, prior to the development of substantial cyst burden and compromise of renal function, thus allowing for maximal preventive benefit and minimizing the potential for renal dysfunction to limit the safe use of the drug. Given the relatively late onset and slow progression of ADPKD it is conceivable that, even if metformin were to have only modest effects in delaying or slowing cyst development, it might significantly increase the time to the development of end stage renal disease and perhaps reduce the need for renal replacement therapy.

We find that metformin stimulates AMPK, resulting in inhibition of both CFTR and mTOR, and thereby, both epithelial secretion and proliferation, respectively. Our data suggest the possible utility of metformin as a therapy for ADPKD and that AMPK is a novel potential pharmacological target for ADPKD therapy. The large body of knowledge associated with metformin administration could conceivably facilitate the translation of these findings into clinical trials to test the proposition that metformin is a safe and promising approach that exploits AMPK activity to treat this challenging disease.
References

Appendix

Reprints of:


Activating AMP-activated protein kinase (AMPK) slows renal cystogenesis

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Renal cyst development and expansion in autosomal dominant polycystic kidney disease (ADPKD) involves both fluid secretion and abnormal proliferation of cyst-lining epithelial cells. The chloride channel of the cystic fibrosis transmembrane conductance regulator (CFTR) participates in secretion of cyst fluid, and the mammalian target of rapamycin (mTOR) pathway may drive proliferation of cyst epithelial cells. CFTR and mTOR are both negatively regulated by AMP-activated protein kinase (AMPK). Metformin, a drug in wide clinical use, is a pharmacological activator of AMPK. We find that metformin stimulates AMPK, resulting in inhibition of both CFTR and the mTOR pathways. Metformin induces significant arrest of cystic growth in both in vitro and ex vivo models of renal cystogenesis. In addition, metformin administration produces a significant decrease in the cystic index in two mouse models of ADPKD. Our results suggest a possible role for AMPK activation in slowing renal cystogenesis as well as the potential for therapeutic application of metformin in the context of ADPKD.

Autosomal dominant polycystic kidney disease (ADPKD) is characterized by the slow and continuous development of cysts derived from renal tubular epithelial cells. The cysts profoundly alter renal architecture, compressing normal parenchyma and compromising renal function. Nearly half of ADPKD patients ultimately require renal replacement therapy. ADPKD is a common genetic disorder, affecting at least 1 in 1,000 individuals (1). There currently are no effective specific clinical therapies for ADPKD.

Cystic growth and expansion in ADPKD are thought to result from both fluid secretion into cyst lumens and abnormal proliferation of the cyst-lining epithelium. The rate of fluid secretion into the cyst lumen is directly proportional to the amount of the cystic fibrosis transmembrane regulator (CFTR) chloride channel in the apical membranes of cyst-lining epithelial cells (2). The evidence suggesting that CFTR acts as a significant contributor to cyst growth has inspired preclinical trials of CFTR inhibitors in cell and animal models of renal cystic disease (3, 4).

The cells surrounding the cysts manifest increased proliferation (5, 6). Mammalian target of rapamycin (mTOR) activity is elevated in models of polycystic kidney disease (PKD) and probably is responsible, at least in part, for this hyperproliferative phenotype (5). mTOR is a serine/threonine kinase that regulates cell growth and proliferation as well as transcription and protein synthesis. Rapamycin inhibits mTOR’s kinase activity (7, 8). Indeed, treatment with rapamycin has been shown to improve parameters of renal cystic expansion in several animal models of ADPKD (5, 9).

Interestingly, both the CFTR chloride channel and the mTOR signaling pathway are negatively regulated by the “energy-sensing” molecule, AMP-activated protein kinase (AMPK). AMPK phosphorylates and directly inhibits CFTR and indirectly antagonizes mTOR through phosphorylation of tuberous sclerosis protein 2 (TSC2) and Raptor (10–13). Both of these actions are consistent with the role of AMPK as a regulator that decreases energy-consuming processes such as transport, secretion, and growth when cellular ATP levels are low (14). Thus, a drug that activates AMPK might inhibit both the secretory and the proliferative components of cyst expansion. Metformin, a drug in wide clinical use for both non–insulin-dependent diabetes mellitus (type 2 DM) and polycystic ovary syndrome, stimulates AMPK (15, 16). We therefore examined whether metformin-induced activation of AMPK slows cystogenesis through inhibition of mTOR-mediated cellular proliferation and inhibition of CFTR-mediated fluid secretion.

Results

Metformin Stimulates AMPK and Phosphorylated Acetyl-CoA Carboxylase.

We first treated Madin–Darby canine kidney (MDCK) renal epithelial cells with metformin to evaluate AMPK activation. Activated AMPK is phosphorylated at residue Thr172 of its α subunit. We performed Western blotting using a phosphospecific antibody to measure the level of the phosphorylated AMPK (pAMPK) (Fig. L4). We found that incubation with metformin for as little as 2 h significantly increases pAMPK levels (Fig. 1B). To determine whether this effect was correlated with increased phosphorylation of an AMPK target, we evaluated metformin’s effect on the AMPK-mediated inhibitory phosphorylation of acetyl-CoA carboxylase (ACC) (Fig. 1C). Incubation of MDCK cells with metformin produced a significant increase in phosphorylated ACC (pACC) levels in 6 h (Fig. 1D). In AMPK-α1 knockdown (AMPK-α1–KD) cells, metformin’s effects on pAMPK and pACC levels are substantially blunted (Fig. S1). Treatment of mice with increasing doses of metformin administered daily for 3 d results in increasing levels of pAMPK throughout the nephron (Fig. 1E and F).

Inhibition of CFTR-Dependent Short-Circuit Current by Metformin in MDCK Cells Is AMPK Dependent.

We next examined the effect of metformin treatment on the CFTR chloride channel, which is inhibited by AMPK phosphorylation (17–19). Because the CFTR drives, at least in part, the fluid secretion in PKD cystogenesis, we hypothesized that metformin-stimulated AMPK activity would inhibit CFTR channels in renal epithelial cells and slow the rate of cyst growth (20, 21). To test whether metformin inhibits CFTR via AMPK in a kidney-derived epithelial cell line, CFTR was expressed by adenoviral transduction in three different polarized MDCK type II cell lines stably transfected with an empty vector or with shRNA plasmids directed against two isoforms of the catalytic α subunit of AMPK. MDCK cells endogenously express high concentrations of the α1 isoform of the AMPK catalytic α subunit.


The authors declare no conflict of interest.

* This Direct Submission article had a prearranged editor.

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and very low concentrations of the α2 isoform. Expression of the α1 shRNA construct reduced expression of this protein by ~90%, whereas the α2 shRNA had no effect on α1 protein expression. Knockdown of α1 also reduced the level of total pAMPK by ~90% (Fig. 2A). CFTR-dependent short-circuit current (Isc) was measured for cells grown on filters mounted in Ussing chambers for 4 d following adenoviral transduction, with or without exposure to 1 mM metformin for 2–4 h before measurement. To initiate CFTR-mediated secretion, CFTR-expressing and mock-transduced MDCK cells were treated with the cAMP agonists 3-isobutyl-1-methylxanthine (IBMX) and forskolin, and the experiment was concluded by the application of specific CFTR inhibitor CFTR-Inh172 (22). Typical traces of Isc changes are shown in Fig. 2B and C. CFTR-expressing cells generally showed an early peak in Isc, within 1–2 min following forskolin/IBMX treatment, followed by a lower plateau current within ~5 min. This remaining current was sensitive to inhibition by CFTR-Inh172. Metformin (1 mM) pretreatment of empty vector-transfected and AMPKα2-KD MDCK cells significantly reduced CFTR-dependent Isc by 60–70% relative to cells pretreated with vehicle (Fig. 2D). However, there was no metformin-dependent inhibition of CFTR current in AMPK-α1–KD MDCK cells, suggesting that the metformin-induced inhibition of CFTR occurs specifically via an AMPK-α1-dependent mechanism.

**Inhibition of mTOR by Metformin in MDCK Cells Is AMPK Dependent.** To determine whether metformin induces AMPK-mediated inhibition of mTOR activity, we tested whether mTOR activity is diminished in MDCK cells cultured in the presence of metformin by blotting for the phosphorylated form of the mTOR downstream target ribosomal S6 kinase S6K in 24 h (Fig. 3A). This inhibition is time dependent, with increasing exposure to metformin resulting in greater suppression of this pathway. Total S6K levels remain constant. The inhibition takes longer to achieve than inhibition of CFTR or ACC, consistent with the indirect inhibition of mTOR by AMPK via TSC2/1 and Rheb (ras homolog enriched in brain) (Fig. 3B). This effect is markedly less pronounced in AMPK−/− cells (Fig. S1). To evaluate whether these changes in phospho-protein levels translated into changes in proliferation, an Alamar Blue assay was used to quantitate proliferation in wild-type and AMPK−/− MDCK cells. In figure 3D, the y axis depicts cell number measured at each given concentration of metformin and normalized to the control value, which was obtained for the same cell type at the same point time without metformin treatment. Wild-type MDCK cells exhibited a metformin-dose-dependent decrease in proliferation, but this response was diminished significantly in the AMPK−/− MDCK cells (Fig. 3D). At the highest concentration of metformin tested (5 mM), substantial growth suppression was detected in AMPK-KD cells, perhaps because of the low level of residual AMPK that is expressed in these KD cells (Fig. 2A) or the effects of high doses of metformin on yet to be identified AMPK-independent pathways. A similar suppressive effect of metformin treatment on proliferation was observed in vivo.

We performed immunofluorescence analyses on kidneys from metformin-treated and vehicle-treated cystic Pkd1−/−;Ksp-Cre
Metformin inhibits phosphorylation of the mTOR downstream target, p70 S6K, and slows cellular proliferation in an AMPK-dependent manner. A subconfluent monolayer of MDCK cells was incubated with 1.0 mM metformin for the indicated time. Cells lysates were blotted for the downstream marker of mTOR activity, (A) p70 S6K. (B) Total S6K. (C) Quantitation of phospho-S6K Western blots band density normalized to β-actin. Comparisons of the mean (±SEM) are shown for each time point. **P = 0.0005 at 6 h, P = 0.009 at 12 h, P = 0.00009 at 24 h; one-way ANOVA with Tukey’s analysis relative to vehicle-treated control for that set of wells; n = 3 wells for each condition. (D) Effect of metformin on proliferation of control MDCK cells and MDCK cells stably transfected with shRNA against AMPK, graphed relative to control. The y axis represents cell number at each concentration of metformin, normalized to the control value measured for the same cell type at the same time point without metformin treatment. **P = 0.0008 at 0.5 mM, P = 0.009 at 1.0 mM, P = 0.004 at 5 mM; unpaired t tests between both cell lines, comparing rates of cell proliferation with n = 3 per metformin concentration).

Metformin Treatment Slows Cystogenesis ex Vivo and in Vivo. The 2D culture models do not accurately depict cell growth in the 3D environment in which cysts develop. To evaluate metformin’s effects in the context of cystogenesis, we suspended MDCK cells in a 3D collagen matrix and allowed them to form cysts spontaneously in the presence of forskolin and IBMX (24). Cultures coincubated with metformin for the duration of cyst growth produced significantly smaller cysts than those similarly treated with forskolin or IBMX alone (P = 0.003, unpaired t test; n = 3 gels for each experimental condition) (Fig. 4A).

We next tested the effect of metformin on ex vivo cystogenesis. Kidneys were removed from C57/B6 mice at embryonic day 12.5 (E12.5). One embryonic kidney was cultured in the presence of membrane-permeable 8-bromo-cAMP (8-Br-cAMP) to stimulate fluid secretion, and the contralateral kidney was coincubated with 8-Br-cAMP and metformin for 4 d. Culture in the presence of 8-Br-cAMP induces cyst formation in embryonic mouse kidneys (4). Metformin treatment significantly decreased the fractional cyst area (P = 0.04, unpaired t test; n = 4 for each experimental condition). On day 5, metformin was removed from the treated embryonic kidney, and cyst growth recommenced in the treated kidney, demonstrating that metformin treatment slowed cyst growth without affecting the viability of the tissue (Fig. 4B).
Metformin Treatment Slows Cystogenesis in the in Vivo models of PKD. We next tested whether metformin slows cyst growth in a murine model of PKD. Initially, we used the most aggressive viable murine model of PKD (Pkd1fl/fl;Ksp-Cre) in which there is progression of renal cystic disease within the first week of life and death between the second and third weeks of life (6). We treated these mice with daily i.p. injections of metformin (300 mg·kg⁻¹·d⁻¹) dissolved in a 5% (mass/vol) dextrose solution from postnatal day 4 (P4) until P6. This dose is known to activate AMPK (25). Mice treated with vehicle were harvested at P7. The vehicle-treated Pkd1fl/fl;Ksp-Cre kidneys (Fig. 5C) were profoundly cystic and greatly enlarged compared with the Pkd1fl/fl;KspCre mice (Fig. 5A). In contrast, cyst burden was significantly reduced in the kidneys from the metformin-treated Pkd1fl/fl;Ksp-Cre mice (Fig. 5B). Because metformin can affect body weight, the kidney weight:body weight ratio was not used as an end point (26). Instead, the effect of metformin on renal morphology was quantitated by the fractional cyst area. Finally, we illustrate the potential therapeutic benefit of metformin treatment before or during cyst development. Thus, this model might replicate more accurately the clinical scenario in which metformin therapy could commence early in the disease process and act to prevent or slow subsequent cyst development. We initiated metformin treatment (300 mg·kg⁻¹·d⁻¹) at P7 and then injected tamoxifen i.p. at P9 or P10 to initiate disease induction. We continued daily metformin injections until P18, when the animal was killed and kidneys were harvested for histology and cystic index evaluation. Once again, metformin treatment resulted in a smaller fractional cyst burden than seen in vehicle-treated controls (31% vs. 43%; P = 0.041, unpaired t test; n = 6 vehicle-treated mice, and n = 7 for metformin-treated mice), a decrease of nearly one-third in the cyst burden (Fig. 5 D and E).

Discussion
AMPK activity can be targeted pharmacologically with metformin to reduce the growth of renal cysts. Metformin acts through AMPK to decrease epithelial fluid secretion by directly inhibiting CFTR and to decrease cellular proliferation by indirectly targeting mTOR. Metformin stimulates AMPK phosphorylation in cultured MDCK renal epithelial cells, and this phosphorylation correlates with increased AMPK activity, as evidenced by an increase in the level of the AMPK-mediated inhibitory phosphorylation of ACC. Metformin’s inhibitory action on CFTR-mediated chloride transport is AMPK dependent. Additionally, we show that metformin inhibition of mTOR translates into an AMPK-dependent inhibition of cell proliferation. Using both an in vitro model of MDCK cell cystogenesis and embryonic kidneys ex vivo, we demonstrate that metformin decreases cyst size and fractional cyst area. Finally, we illustrate the potential thera-

Fig. 4. Metformin reduces cyst size in vitro and ex vivo. (A) Representative light micrographs of MDCK cell cysts grown in collagen gels. Cysts were treated with forskolin (Forsk) and IBMX to enhance apical fluid secretion with (Lower) or without (Upper) 0.1 mM metformin for 20 d. Gels were melted, and the cysts were allowed to precipitate to the bottom for imaging. (B) Metformin treatment reduces cyst size in an ex vivo model of renal cystogenesis. Embryonic kidneys were placed in culture at E12 and maintained for 5 d in the continued presence of 100 μM 8-Br-cAMP. Representative light microscopic images from one mouse are shown. Each row shows the same kidney. The contralateral kidney (Lower) was treated with metformin for 4 d and then switched to normal medium, illustrating that the embryonic kidney remains viable and capable of cystogenesis.

Fig. 5. Metformin treatment reduces the cystic index in two mouse models of ADPKD. (A–C) Representative midsagittal sections from the kidneys of (A) a Pkd1+/+;Ksp-Cre mouse, (B) a metformin-treated Pkd1fl/fl;Ksp-Cre mouse, and (C) a vehicle-treated Pkd1fl/fl;Ksp-Cre mouse at P7. The metformin- and vehicle-treated mice were given daily weight-adjusted i.p. injections from P4 until P6. (D and E) Representative images from Pkd1fl/fl;pcX-CreER mice treated with vehicle (D) or metformin (E) from P7–P17, with Cre induction at P9 or P10.
peutic utility of metformin by testing it in two murine models of ADPKD, both of which are attributable to inactivation of the gene encoding polycystin-1.

Metformin is taken by millions of Americans each year. It currently is approved by the Food and Drug Administration for the treatment of type 2 DM and, intriguingly, for polycystic ovary syndrome, a disease that has a name similar to that of polycystic kidney disease but whose pathogenesis is even less well understood. In fact, metformin often is considered first-line therapy for the treatment of type 2 DM because of its relatively small side-effect profile. Recent literature suggests that metformin’s activation of AMPK may be the result of its ability to prevent AMP breakdown, although the exact mechanisms of action of metformin in polycystic ovary syndrome or in type 2 DM remain largely unknown (30). Recent reports also suggest that metformin may exert an antineoplastic effect. It has been reported that metformin acts in a dose-dependent manner to inhibit the proliferation of breast cancer cells, and that this effect can be blocked in the presence of siRNA directed against AMPK (31). This inhibition also is associated with a decrease in mTOR activation, suggesting that metformin’s antiproliferative effect is directed through the activation of AMPK and consequent inhibition of mTOR.

In transporting epithelial cells, AMPK not only modulates CFTR activity but also inhibits the epithelial sodium channel (ENaC) (32-34). Although in the cystic kidney this effect conceivably could lead to decreased fluid absorption and therefore perhaps to increased accumulation of cyst fluid, the role of ENaC in cyst-lining epithelial cells is uncertain (35). CFTR can inhibit ENaC channel function directly. Thus, inhibition of CFTR by AMPK could reduce such sodium channel inhibition (36, 37). Taken together, the effects of AMPK activation on ENaC function in the context of renal cystic disease are bimodal and complex. The net effect of AMPK modulation in vivo, however, is likely to reduce luminal fluid accumulation (38).

Numerous therapies for ADPKD, including vasopressin receptor inhibitors, calcium-sensing receptor inhibitors, CFTR inhibitors, cell-cycle inhibitors, and rapamycin, are in development or in clinical trials (4, 9, 39, 40). Each of these strategies targets one of the key processes (proliferation and secretion) thought to be involved in the pathogenesis of PKD. By acting through AMPK, metformin may offer the significant advantage of blocking both of these processes (Fig. S4). Moreover, metformin already is approved by the Food and Drug Administration and generally is well tolerated. The most serious, albeit rare, side effect of metformin is lactic acidosis and, because metformin is cleared by the kidney, chronic renal disease has been considered a potential predisposing factor for this complication. Ideally, however, metformin use could be initiated at an early stage in ADPKD progression, before the development of substantial cyst burden and compromise of renal function, thus allowing maximal preventive benefit and minimizing the potential for renal dysfunction to limit the safe use of the drug (41, 42). Given the relatively late onset and slow progression of ADPKD, it is conceivable that, even if metformin were to have only modest effects in delaying or slowing cyst development, it might increase significantly the time to the development of end-stage renal disease and perhaps reduce the need for renal replacement therapy.

In this study, only one dose known to activate AMPK in vivo was tested. When considered on a simple milligram per kilogram body weight basis, this dose appears considerably higher than the current maximum dose prescribed for patients with diabetes or polycystic ovary syndrome. However, human-equivalent dose extrapolation is calculated more accurately based on body surface area than on weight. When this calculation is performed for a 60-kg adult, the dose used in our mouse studies extrapolates to a daily dose of ~1,500 mg (43), well within the range in which metformin is safely used in humans. We have not tested the efficacy of lower doses or of alternative dosing regimens in these mouse models. It is likely, however, based on the established pharmacokinetics of metformin, that single daily dosing is suboptimal, and thus we almost certainly did not observe the maximal suppressive effects that metformin potentially could exert on the severity of cyst growth (44). Support for this contention derives from the data presented in Fig. 4C, because in the embryonic kidney model, cyst growth resumes rapidly shortly after removal of metformin from the culture medium. Thus, short-term intermittent exposure to metformin may not be adequate to suppress cyst development optimally. It is quite possible that even lower doses administered more frequently might produce beneficial effects in the setting of polycystic kidney disease. It is important to note that our efforts to assess effects of metformin treatment on renal functional parameters such as serum concentrations of serum urea nitrogen and creatinine were inconclusive, in part because of interindividual variability. Further studies, perhaps using more slowly progressive disease models, will be required to reduce this variance and to assess the extent to which metformin treatment can protect or improve renal function in the setting of PKD. In addition, subsequent development of metformin for this clinical application will require pharmacokinetic and pharmacodynamic studies designed to identify an ideal dosing regimen that achieves maximal activation of renal tubular AMPK.

In conclusion, we find that metformin stimulates AMPK, resulting in inhibition of both CFTR and mTOR and thereby both epithelial secretion and proliferation. Our data suggest that metformin potentially could be a safe and promising approach that exploits AMPK activity to treat this challenging disease.

Methods

Western Blotting and Proliferation Assay. Cultured MDCK cells were lysed, and protein was extracted for Western blotting using standard protocols. For experiments involving AMPK activation in vivo, kidneys were snap-frozen in situ, and homogenates were prepared according to published protocol before Western blotting (45). Details and antibodies used are given in SI Methods.

Generation of AMPK-KD Cell Lines. AMPK-KD cell lines were established by lentiviral infection. Further details and targeting sequences are given in SI Methods.

CFTR Short-Circuit Current Measurements in MDCK II Cells. MDCK cells expressing either empty vector or shRNA against one of two AMPK isotypes underwent adenoviral transduction to express GFP-tagged CFTR. I_{sc} was assessed by Ussing chamber measurement after stimulation with forskolin and 3-isobutyl-1-methylxanthine and then CFTR-inh-73 to determine the CFTR-dependent change in I_{sc}. Cells were pretreated with vector or metformin as noted. Further details are given in SI Methods.

In Vitro Cystogenesis. MDCK cells were suspended in a collagen matrix as previously described by Grantham and coworkers (24). Further details and quantitation method are given in SI Methods.

Ex Vivo Cystogenesis. Embryonic kidneys were microdissected from timed pregnant C57BL6 mice at E12.5, cultured per standard protocol with the addition of 8-Br-cAMP to promote cyst formation, and treated with either metformin or vector (4, 46). Further details are given in SI Methods.

Mouse Strains, Histology, and Cystic Index. All animal protocols were approved and conducted in accordance with Yale Animal Resources Center and Institutional Animal Care and Use Committee regulations. Pkd1<sup>flx/o</sup> and Ksp-Cre lines have been described previously (6, 47, 48). From P4 until P6, experimental mice received either metformin (300 mg/kg body weight) dissolved in 5% (mass/vol) dextrose or 5% (mass/vol) dextrose alone through daily i.p. injections. These mice were killed at P7. The pCAGGS chicken ß-actin promoter construct. Cre recombinase translocation to the nucleus was induced by...
a single dose (0.1 mg tamoxifen/kg body weight) given by i.p. injection at P9 or P10 (27, 28). Kidneys were harvested as described in Results and fixed, and the fractional cyst area was calculated via MetaMorph (Universal Imaging). Further details are given in SI Methods.

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The γ-Secretase Cleavage Product of Polycystin-1 Regulates TCF and CHOP-Mediated Transcriptional Activation through a p300-Dependent Mechanism

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SUMMARY

Mutations in Pkd1, encoding polycystin-1 (PC1), cause autosomal-dominant polycystic kidney disease (ADPKD). We show that the carboxy-terminal tail (CTT) of PC1 is released by γ-secretase-mediated cleavage and regulates the Wnt and CHOP pathways by binding the transcription factors TCF and CHOP, disrupting their interaction with the common transcriptional coactivator p300. Loss of PC1 causes increased proliferation and apoptosis, while reintroducing PC1-CTT into cultured Pkd1 null cells reestablishes normal growth rate, suppresses apoptosis, and prevents cyst formation. Inhibition of γ-secretase activity impairs the ability of PC1 to suppress growth and apoptosis and leads to cyst formation in cultured renal epithelial cells. Expression of the PC1-CTT is sufficient to rescue the dorsal body curvature phenotype in zebrafish embryos resulting from either γ-secretase inhibition or suppression of Pkd1 expression. Thus, γ-secretase-dependent release of the PC1-CTT creates a protein fragment whose expression is sufficient to suppress ADPKD-related phenotypes in vitro and in vivo.

INTRODUCTION

Autosomal-dominant polycystic kidney disease (ADPKD), a common genetic disorder, produces fluid-filled renal cysts that disrupt the normal tubular architecture and can ultimately lead to kidney failure (Wilson, 2004). Most cases (85%) result from mutations in the gene encoding polycystin-1 (Pkd1), with the remaining 15% resulting from mutations in the gene encoding polycystin-2 (Pkd2) (Harris and Torres, 2009; Rossetti et al., 2007). Polycystin-1 (PC1) has a large extracellular domain, 11 transmembrane spans, and a short carboxy-terminal cytoplasmic tail (Hughes et al., 1995). The PC1 C-terminal tail (PC1-CTT) has been implicated in the regulation of several signaling pathways, including Wnt (Kim et al., 1999; Lal et al., 2008; Zhang et al., 2007), mTOR (Shillingford et al., 2006), p21/JAK/STAT (Bhunia et al., 2002; Low et al., 2006; Talbot et al., 2011), and activator protein-1 (Arnould et al., 1998; Chauvet et al., 2004; Parnell et al., 2002). Polycystin-2 is a nonselective calcium-permeable cation channel that interacts and forms a complex with PC1 via the coiled-coil domains present in each of these proteins (Qian et al., 1997).

We show that the CTT of PC1 is released by γ-secretase-dependent cleavage and translocates to the nucleus, where it regulates transcriptional pathways involved in proliferation and apoptosis. Expression of the CTT fragment corrects several of the growth and morphogenesis-related phenotypes that characterize Pkd1 null cells grown in three-dimensional (3D) culture. Furthermore, expression of the PC1-CTT rescues the dorsal body curvature that is produced both by inhibition of PC1 expression and by inhibition of γ-secretase activity in zebrafish. Finally, we provide evidence establishing a common mechanism for PC1-CTT inhibition of proproliferative and proapoptotic signaling pathways through disruption of the relevant transcription factors’ interactions with the transcriptional coactivator p300.
RESULTS

Loss of PC1 in Mouse Renal Epithelial Cells Causes Increased Proliferation, Apoptosis, and Cyst Development in 3D Cell Culture

Clonal renal tubular epithelial cell lines derived from Pkd1^floxed/− mice were transfected with Cre recombinase to generate Pkd1^+/− cells (Joly et al., 2006; Shibazaki et al., 2008). These cell lines, which are genetically identical except for the deletion of both copies of the gene encoding PC1 in the Pkd1^+/− cells, produced strikingly different multicellular structures when grown in 3D culture. Pkd1^floxed/− cells grew into extended, tubule-like structures, whereas the Pkd1^+/− cells developed into large, spherical cysts with hollow central lumens (Figure 1A). This can be seen graphically in time-lapse videos of Pkd1^floxed/− and Pkd1^+/− cells grown in 3D culture (see Movies S1, S2, and S3 available online). The Pkd1^+/− cells acquire a hollow central lumen within the first several days of culture, whereas the Pkd1^floxed/− cells slowly form linear tubule-like structures.

Pkd1^+/− cells displayed higher levels of proliferation as compared to Pkd1^floxed/− cells, as measured by BrdU incorporation (Figure 1B). Apoptosis, as measured by staining for cleaved caspase-3, was virtually undetectable in the Pkd1^floxed/− cells, whereas apoptosis was evident in the Pkd1^+/− cells, both in cyst-lining cells (Figure 1B, arrowheads) and at the center of cell aggregates that had yet to develop a hollow central lumen (Figure 1B, inset).

To determine the effect of the isolated PC1-CTT on cystogenesis, PC1-CTT was conditionally expressed under the control of doxycycline using a TET-Off inducible expression system in a stably transfected Pkd1^+/− cell line. Pkd1^+/− cells induced to express PC1-CTT displayed decreased levels of proliferation, as measured by BrdU incorporation. In addition, expression of PC1-CTT in the Pkd1^+/− cells resulted in a dramatic change in the morphology of the structures they formed in 3D culture. Instead of large, hollow-lumen, cyst-like structures, the Pkd1^+/− cells that express the PC1-CTT developed into branched tubule-like structures lacking a hollow central lumen (Figure 1C). The average sizes of the structures formed by the Pkd1^+/− cells that express the PC1-CTT were similar to those measured for the parental Pkd1^floxed/− cells, and these structures were significantly smaller than the cystic structures formed by the Pkd1^+/− cells (Figures 1D and 1E). Immunostaining performed with an antibody directed against the HA epitope appended to the C-terminal cleavage product of PC1 (shown in red in Figure 1C and in gray scale in the lower right panel of Figure 1D) and that the PC1-CTT protein is concentrated in the nucleus (Figure S1).

The CTT Cleavage of PC1 Is Dependent upon γ-Secretase

C-terminal cleavage of PC1 was detected in HEK cells transfected with a CDNA construct encoding full-length PC1 tagged at the C terminus with the DNA-binding domain of Gal4 (Bertuccio et al., 2009). Cleavage of PC1 allows the released CTT-Gal4 to translocate to the nucleus and to stimulate luciferase production from a cotransfected UAS-Luciferase reporter plasmid. Assays are performed in the presence of clasto-lactacystin, to prevent proteasome degradation of the cleaved PC1-CTT (Bertuccio et al., 2009). The γ-secretase inhibitor DAPT was added to the media after transfection, and the cells were incubated for 24 hr (Shearman et al., 2000). PC1 cleavage, as measured by Gal4-driven luciferase expression, was inhibited in a dose-dependent manner by DAPT, indicating that PC1-CTT cleavage is dependent upon γ-secretase activity (Figure 2A).

Further evidence for γ-secretase-dependent cleavage of PC1 was obtained through DAPT treatment of LLC-PK1 cells stably expressing a full-length PC1 construct that carries a C-terminal HA tag. Lysates from cells treated with clasto-lactacystin (to prevent proteasome degradation of cleaved PC1 fragments) were fractionated to separate nuclei from cytoplasm, and the resultant fractions were analyzed by immunoblot. Bands corresponding to the cleaved PC1-CTT were detected predominantly in the nuclear fractions, and the intensity of this complex of bands was significantly decreased in cells exposed to DAPT (Figure 2B). We used siRNA to knock down expression in HEK293 cells of Presenilin-1 or Presenilin-2, each of which can serve as the catalytic subunit of the functional γ-secretase complex. Loss of Presenilin-1 did not decrease PC1-CTT cleavage as measured by the PC1-GalVP cleavage assay (Figure 2C, left panels). Presenilin-2 knockdown, however, resulted in a significant decrease in PC1-CTT cleavage (Figure 2C, right panels) and a reduction in the nuclear accumulation of PC1 cleavage products (Figure S2).

We next wished to determine whether γ-secretase-mediated cleavage of PC1 is required for the PC1 protein to exert its effects on epithelial morphogenesis. Pkd1^floxed/− cells cultured in 3D were treated with either DMSO vehicle or with DAPT for 10 days. DAPT treatment resulted in a significant change in morphology in the Pkd1^floxed/− cells. Whereas DMSO-treated cells formed linear tubule-like structures, DAPT-treated cells formed spherical cyst-like structures with hollow central lumens (Figure 2D, left panels) reminiscent of the structures formed by the Pkd1^+/− cells. DAPT treatment had no significant effect on the morphology of Pkd1^+/− cells (Figure 2D, right panels).

Expression of PC1-CTT Results in Reduced Proliferation and Apoptosis in Pkd1^+/− Cells

To quantify the effects observed in the 3D cell culture system, Pkd1^floxed/− and Pkd1^+/− cells were cultured in two dimensions on glass coverslips, and BrdU incorporation (Figure 3A) and cleaved caspase-3 staining (Figure S3) were assessed as measures of proliferation and apoptosis, respectively. Pkd1^+/− cells displayed a significantly higher level of proliferation than Pkd1^floxed/− controls. However, reintroduction of the isolated PC1-CTT significantly reduced proliferation of the Pkd1^+/− cells to levels similar to those observed in Pkd1^floxed/− cells (Figure 3B). Similarly, Pkd1^+/− cells displayed a significantly higher level of apoptosis when compared to Pkd1^floxed/− controls. When PC1-CTT expression was induced in Pkd1^+/− cells, the level of apoptosis decreased significantly. Expression of PC1-CTT in the Pkd1^+/− cells reduced apoptosis to levels similar to those seen in the Pkd1^floxed/− cells (Figure 3C; Figure S3).

PC1-CTT Directly Interacts with TCF and Inhibits Canonical Wnt Signaling

Previous data implicate canonical Wnt signaling as a driver of cyst proliferation. Recent studies show activation of Wnt target
genes in cells derived from human ADPKD cystic tissue and demonstrate an interaction between the PC1-CTT and components of the Wnt-signaling pathway (Kim et al., 1999; Lal et al., 2008; Zhang et al., 2007). The Wnt pathway regulates the size and activity of the cytosolic pool of β-catenin. At the cell membrane, β-catenin is bound by E-cadherin. In resting polarized epithelial cells, β-catenin is predominantly sequestered at the basolateral plasma membrane, where it participates in the
formation of E-cadherin-dependent adhesive junctions. Free cytoplasmic β-catenin is recognized by a “destruction complex” that mediates its phosphorylation, targeting it for proteosomal degradation. Activation of Wnt signaling prevents the destruction of free cytosolic β-catenin, which enters the nucleus to serve as a coactivator of the TCF transcription factor and thus induces proliferation (Daugherty and Gottardi, 2007). To measure endogenous Wnt-signaling activity, we employed the TopFlash assay, which utilizes a TCF-binding promoter element to drive expression of a luciferase reporter (van de Wetering et al., 1997).

Figure 2. PC1-CTT Cleavage Is Sensitive to the γ-Secretase Inhibitor DAPT
(A) HEK293 cells transfected with PC1-GalVP, UAS-Luciferase, and Renilla were treated with the γ-secretase inhibitor DAPT at the indicated concentrations in the presence of clasto-lactacystin for 24 hr prior to quantification of the luciferase signal.
(B) LLC-PK1 cells stably expressing full-length PC1 with a C-terminal 2× HA tag were exposed to DAPT for 24 hr, in the presence of clasto-lactacystin, before nuclear/cytoplasmic fractionation. Proteins were separated on a 10% SDS-polyacrylamide gel and analyzed by immunoblot. Nuclear purification was assessed using α-RNA-Pol II (nuclear fraction) and α-calnexin (nonnuclear fraction). PC1-CTT cleavage fragments were detected using α-HA.
(C) HEK293 cells were transfected with either siControl (nontargeting RNA) or siRNA directed against human Presenilin-1 or Presenilin-2. qRT-PCR was used to determine knockdown efficiency. PC1-GalVP, UAS-Luciferase, and Renilla were super-transfected into HEK cells after 48 hr of siRNA treatment to report on PC1-CTT cleavage. Data are mean ± SE of four replicates each from two independent experiments. The data in all four panels are normalized to the siControl condition.
(D) Pkd1<sup>flox/−</sup> and Pkd1<sup>flox/−</sup>/C0 cells were cultured in 3D Matrigel matrix for 10 days in media containing either DMSO (vehicle control) or 100 μM DAPT, after which they were fixed and imaged on a phase-contrast microscope.
Pkd1<sup>−/−</sup> cells demonstrated significantly higher levels of TCF activity than did the Pkd1<sup>flox/−</sup> controls. Furthermore, expression of PC1-CTT in the Pkd1<sup>−/−</sup> cell line by withdrawal of doxycycline for 48 hr prior to BrdU exposure and fixation. Proliferating cells were labeled with α-BrdU-FITC (green), PC1-CTT expression was detected with α-HA (red), and nuclei were counterstained with Hoechst 33342 (blue).

Although the PC1-CTT-ΔNLS construct does not alter TopFlash activity, it is worth noting that this construct is able to produce a significant signal in a reporter assay that measures the activity of the STAT6 pathway (Figure 4C). These data, which are consistent with previous observations indicating that portions of the PC1-CTT can activate STAT6 signaling (Low et al., 2006), demonstrate that loss of the NLS selectively blocks some but not all of the functional activities of the PC1-CTT.
Treatment of \( \textit{Pkd1}^{\text{floxed/}} \) cells with DAPT abolished the inhibitory effect of PC1 expression on TopFlash activity, consistent with the hypothesis that PC1-CTT cleavage and nuclear translocation of the released CTT fragment are necessary for its inhibitory effect on TCF. DAPT treatment of \( \textit{Pkd1}^{\text{--}} \) cells did not stimulate any further increase in TopFlash activity, indicating that the increase in Wnt activity obtained through inhibition of \( \gamma \)-secretase is dependent on the presence of PC1 (Figure 4D).

To dissect further the elements of the canonical Wnt-signaling pathway that interact directly with PC1-CTT, a bacterial coexpression system was employed to drive simultaneous expression of a His-tagged PC1-CTT and of GST-tagged polypeptides incorporating the sequences of \( \beta \)-catenin, the E-cadherin cytoplasmic domain, or TCF. When bacterial lysates were subjected to glutathione-Sepharose pull-down and the recovered proteins were blotted with anti-His antibody, PC1-CTT exhibited little direct interaction with \( \beta \)-catenin or with...
E-cadherin but showed a strong direct physical interaction with TCF (Figure 4C).

**PC1-CTT Interacts with CHOP and Inhibits Its Activity**

Data suggesting that the PC1-CTT may regulate apoptosis (Figures 1 and 3; Figure S3) led us to search for novel regulatory targets that could mediate this influence. To identify transcription factors regulated by PC1-CTT, we employed a “coactivator trap” screen, in which over 800 transcription factors are fused to the DNA-binding domain of Gal4 (Amelio et al., 2007). After cotransfection of each transcription factor-Gal4 construct and a Gal4-driven luciferase reporter vector into HEK293 cells, luciferase assays established baseline activities for each transcription factor. PC1-CTT was then cotransfected, and any effect of PC1-CTT on each transcription factor’s activity was measured as a change in luciferase production as compared to its baseline level. Several transcription factors were found to be significantly regulated in the presence of PC1-CTT. One of the most profoundly affected was CHOP-10/GADD153, which induces apoptosis in response to ER stress as part of the unfolded protein response (UPR) (Oyadomari and Mori, 2004) (Table S1).

To measure the effects of full-length PC1 expression on CHOP activation, Pkd1flo×/− and Pkd1−/− cells were transfected with the CHOP-Gal4 and the Gal4-luciferase reporter constructs. Pkd1−/− cells displayed significantly higher levels of CHOP activity when compared to the Pkd1flo×/− cells. Expression of the soluble PC1-CTT in the Pkd1−/− cells resulted in a significant inhibition of CHOP-Gal4 activity (Figure 5A). In addition, treatment of Pkd1flo×/− cells with DAPT abolished the inhibitory effect that PC1 expression exerts on CHOP-Gal4 activity. DAPT treatment of Pkd1−/− cells did not stimulate a further elevation in CHOP activity, indicating that the increase in CHOP activity obtained through inhibition of γ-secretase-dependent protein cleavage is dependent on the presence of PC1 (Figure 5B). Thus, the presence of the PC1 protein acts, via its released C-terminal NLS, to negatively regulate CHOP activity. Once again, this inhibitory influence on CHOP activity (as assessed by TopFlash assay) in the context of native p300 levels of p300 protein expression (Lal et al., 2008). Overexpression of p300 eliminated this inhibitory effect of PC1-CTT, suggesting that this inhibitory effect is achieved through competition between the PC1-CTT and p300 for binding to TCF (Figure 6A).

To test this possibility directly, TCF and CHOP were precipitated from HEK cells transfected with p300 and PC1-CTT (Figures 6B and 6D). As expected, in the absence of PC1-CTT, TCF and CHOP each coprecipitated with p300 (Hecht and Stemmler, 2003; Ohoka et al., 2007). These interactions were significantly disrupted in cells that express PC1-CTT, suggesting that the CTT of PC1 exerts its inhibitory effect on the activities of TCF and CHOP by interfering with their interactions with p300 (Figures 6B–6E).

**PC1-CTT Inhibits TCF and CHOP Activities by Disrupting Their Interactions with p300**

Although TCF and CHOP activate discrete transcriptional pathways, they both utilize and depend upon the common transcriptional coactivator, p300/CBP (Li et al., 2007; Ohoka et al., 2007). To determine the potential importance of p300 in the PC1-CTT-mediated regulation of TCF and CHOP, HEK cells were transfected with PC1-CTT alone, or in the presence of overexpressed p300. As shown previously, PC1-CTT expression inhibits TCF activity (as assessed by TopFlash assay) in the context of native levels of p300 protein expression (Lal et al., 2008). Overexpression of p300 eliminated this inhibitory effect of PC1-CTT, suggesting that this inhibitory effect is achieved through competition between the PC1-CTT and p300 for binding to TCF (Figure 6A).

**PC1-CTT Rescues Morphant Phenotypes in Pkd1-Null Zebrafish Embryos**

Morpholino-induced knockdown of the two zebrafish Pkd1 genes, Pkd1a and Pkd1b, produces dorsal body axis curvature, kidney cysts, hydrocephalus, and skeletal abnormalities (Mangos et al., 2010). Of these findings, the dorsal body curvature was considered to be the most reliable marker of Pkd1 knockdown, due to the substantially higher penetrance of this phenotype. Interestingly, treatment of zebrafish embryos with the γ-secretase inhibitor DAPT produces a similar phenotype, characterized by mild and moderate dorsal axis curvature (Arslanova et al., 2010). To determine the capacity of the PC1-CTT to rescue the phenotype associated with impaired Pkd1 gene expression in vivo, zebrafish embryos were injected with Pkd1a/b morpholinos alone, or with mRNA encoding the PC1-CTT. Knockdown of Pkd1a/b results in dorsal axis curvature, whereas concurrent injection of the PC1-CTT significantly decreases the severity of the body curvature at 3 dpf (Figures 7A and 7B). Injection of mRNA encoding the PC1-CTT-NLS construct did not rescue the body curvature phenotype (Figures S4A and S4B). A subset of the signaling pathways influenced by the PC1-CTT requires the NLS (Wnt and CHOP), whereas others appear to not require the presence of this motif (e.g., STAT-6) (Figures 4B, 4C, and 5C). Thus, these data suggest that the capacity of the PC1-CTT to ameliorate the severity of the body curvature phenotype involves one or more of the NLS-dependent
signaling pathways that are modulated by the PC1-CTT. Finally, injection of mRNA encoding the PC1-CTT, but not mRNA encoding control GFP, partially rescued the body curvature phenotype induced by DAPT treatment, producing a significant increase in the percentage of fish with straight bodies and a decrease in the percentage of moderately curved fish (Figure 7C).

**DISCUSSION**

Our data confirm the role of PC1 as an inhibitor of renal epithelial cell proliferation and apoptosis, and provide evidence for the mechanism responsible for this regulation, mediated by cleavage and nuclear translocation of the PC1-CTT.

Figure 5. CHOP-Gal4 Activity Is Elevated in Pkd1+/− Cells and Inhibited by PC1-CTT Expression

A) Pkd1floxed and Pkd1−/− cells stably expressing HA-PC1-CTT in a TET-Off inducible vector were transfected with CHOP-Gal4, UAS-Luciferase, and Renilla luciferase reporter constructs in the presence or absence of doxycycline, and luciferase activity was measured 24 hr later.

B) Pkd1floxed and Pkd1−/− cells were transfected with CHOP-Gal4, UAS-Luciferase, and Renilla luciferase reporter constructs and exposed to the γ-secretase inhibitor DAPT for 24 hr prior to quantification of luciferase signal. Data are mean ± SE of four replicates each from three independent experiments.

C) CHOP-Gal4 activity is significantly inhibited by expression of the PC1-CTT in HEK293 cells, whereas it is not inhibited in the presence of the PC1-CTTΔNLS.

D) Pkd1floxed and Pkd1−/− cells were reverse transfected with either noncoding siRNA (siControl), or siRNA corresponding to CHOP (siCHOP), and apoptosis levels were measured 48 hr later by cleaved caspase-3 staining.

E) HEK293 cells were cotransfected with CHOP-Gal4 and HA-PC1-CTT. Cell lysates were subjected to immunoprecipitation using α-HA Sepharose, and the immunoprecipitates were then blotted with the indicated antibodies.

F) LLC-PK1 cells stably expressing full-length PC1 with a C-terminal 2xHA tag were transfected with FLAG-CHOP, and subjected to nuclear/cytoplasmic fractionation. Endogenously cleaved PC1-CTT was immunoprecipitated from the nuclear fraction using α-HA Sepharose, and the resulting complexes were separated on a 10% polyacrylamide gel and blotted with the indicated antibodies.
Reintroduction of the PC1-CTT into Pkd1 knockout cells is sufficient to normalize their excessive proliferative and apoptotic activities, and the PC1-CTT is sufficient to rescue the dorsal tail curvature phenotype produced by morpholino-mediated disruption of Pkd1a/b expression in zebrafish. We show that PC1 cleavage is dependent upon γ-secretase activity, and that the released PC1-CTT inhibits TCF and CHOP, thereby regulating proliferation and apoptosis, respectively. Furthermore, injection of mRNA encoding the PC1-CTT is capable of partially rescuing the dorsal tail curvature phenotype produced by exposure of zebrafish embryos to the γ-secretase inhibitor DAPT. The similarity of the phenotypes produced by Pkd1a/b disruption and DAPT treatment is intriguing, and the ability of the PC1-CTT to partially rescue both suggests that at least some of the critical biological activities of the PC1 protein are dependent upon its γ-secretase-dependent PC1-CTT cleavage. Finally, we demonstrate that PC1-CTT inhibits TCF and CHOP by disrupting their interaction with the transcriptional coactivator p300, illustrating a common mechanism through which PC1-CTT is capable of regulating two distinct transcriptional pathways.
Hyperproliferation and increased apoptosis are characteristic of ADPKD (Lanoix et al., 1996; Starremans et al., 2008). We found that loss of Pkd1 in otherwise genetically identical cell lines resulted in a significant increase in both proliferation and apoptosis. These experiments were performed in vitro, thus eliminating any potential effects of the cyst microenvironment on the proliferative or apoptotic potential of the cyst-lining cells that might complicate the situation in vivo. Thus, our data establish that the loss of expression of the Pkd1 gene product is primarily responsible for the proliferative and apoptotic changes seen in ADPKD.

Pkd1−/− cells were unaffected by DAPT treatment in both the morphogenesis and the TCF and CHOP assays, however, we conclude that γ-secretase-mediated cleavage of PC1 plays an obligate role in at least a subset of this protein’s physiological functions.

The shedding of the extracellular domain of PC1 and the cleavage and nuclear translocation of its cytoplasmic domain together mark PC1 as a member of a growing collection of plasma membrane proteins that is cleaved by γ-secretase and participates in direct signaling to the nucleus (Lal and Caplan, 2011). This behavior is exemplified by the Notch (Fortini, 2009).
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EpCAM (Maetzel et al., 2009), and DCC pathways (Taniguchi et al., 2003). The precise site at which γ-secretase cleaves PC1-CTT has not yet been determined. It is worth noting, however, that γ-secretase appears to exhibit substantial promiscuity in the sequence compositions of its substrate cleavage sites (Beel and Sanders, 2008; Struhl and Adachi, 2000). This promiscuity may account, at least in part, for the number of discrete PC1-CTT cleavage products that can be detected in nuclear fractions (Figure 2B). The precise signals that stimulate γ-secretase-mediated cleavage of PC1 have yet to be discovered.

We report a direct physical interaction between the PC1-CTT and TCF. Lal et al. have suggested that the PC1-CTT inhibits canonical Wnt signaling through an interaction with β-catenin (Lal et al., 2008). Because these experiments assessed the coimmunoprecipitation of epitope-tagged proteins coexpressed in human cell lines, the recovered protein complexes may have contained additional members of the signaling pathway, such as TCF, that were not detected in immunoblots that assessed only the presence of the tagged proteins. Thus, it seems likely that the coprecipitation of the PC1-CTT and β-catenin observed by Lal et al. (Lal et al., 2008) could be attributable to a mutual interaction of both of these proteins with TCF to form an inactive tertiary complex. The bacterial coexpression system utilized in the present study allowed us to further dissect the canonical Wnt pathway and to determine that TCF is a direct binding partner of PC1-CTT. It should be noted that, while activation of the Wnt-signaling pathway is sufficient to produce renal cystic disease (Qian et al., 2005; Saadi-Kheddouci et al., 2001), and markers of Wnt signaling appear to be elevated in the context of ADPKD-related phenotypes in vitro and in vivo.

The activities of both TCF and CHOP depend upon the common transcriptional coactivator p300 (Li et al., 2007; Ohoka et al., 2007). Our data suggest that PC1-CTT binds directly to the transcription factors TCF and CHOP, and are consistent with the hypothesis that PC1-CTT acts by blocking the p300 binding sites on both TCF and CHOP. Therefore, the p300 protein constitutes a promising convergence point that appears to be utilized by PC1-CTT to regulate two distinct transcription factors. This regulation of TCF and CHOP through interactions with the released PC1-CTT provides a simple and compelling explanation for the dysregulation of proliferation and apoptosis seen in ADPKD.

**Experimental Procedures**

**Antibodies, Plasmids, and Cell Lines**

The following antibodies and labeling reagents were used: α-HA antibody, Rat (Roche), FITC α-BrdU Kit (BD Bioscience), α-cleaved caspase-3 (Cell Signaling Technology), α-RNA Pol II (Santa Cruz Biotechnology), α-calnexin (Stratagene), α-His (QIAGEN), α-GST (Amersham), and α-FLAG and α-c-Myc (Sigma-Aldrich). For laser-scanning fluorescence microscopy, dye-coupled Alexa antibodies (Alexa 488, 594; Molecular Probes) were used as secondary reagents.

The sequence encoding the final 200 amino acids of human PC1 (4102–4302), containing a 2x HA tag at the N terminus, was cloned into the pNRt1s-21 vector (Tenev et al., 2000). The sequence for human PC1-CTT (residues 4102–4302 of Pkd1) was modified by deleting residues 4134–4154, corresponding to the putative NLS to generate the PC1-CTT-NLS (Chauvet et al., 2004). Stable cell lines were generated by transfection using Lipofectamine 2000 (Invitrogen) and selection with 350 μg ml⁻¹ Zeocin (Invitrogen). Expression was inhibited with 100 ng ml⁻¹ doxycycline. Full-length human PC1 was cloned into pcDNA3.1 neo (Invitrogen) with 2x HA tag or Gal4VP16 appended to the C terminus as described (Bertuccio et al., 2009). Stable cell clones were selected with 2 mg ml⁻¹ Geneticin (GIBCO). GL4.31[luc2P/GAL4UAS] (Promega, Madison, WI, USA) was used as a Gal4 promoter-driven firefly luciferase reporter construct. The TopFlash plasmid was purchased from Upstate Biotechnology. pRL-TK, a vector constitutively expressing Renilla luciferase, was included as an internal control to normalize for transfection differences. The sequence encoding the PC1-CTT was cloned into the pETDuet vector with an N-terminal 6xHis tag, along with either the GST-E-cadherin cytoplasmic domain, GST-β-catenin, or GST-TCF-β-catenin binding region (Gottardi and Gumbiner, 2004). The CHOP-Gal4 construct was provided by Dr. John Hogenesch (Department of Pharmacology, University of Pennsylvania). The sequence encoding full-length CHOP was cloned into the pCMV-3Tag-1A vector (Stratagene) to generate 3xFLAG-CHOP. The sequence encoding human p300 (full-length or amino acid residues 1–664) was cloned into the pCMV-Tag 3B vector (Stratagene) to generate Myc-p300.

**3D Cell Culture, α-BrdU Staining, and Cell Counting**

Pkd1fl/+, Pkd1K14fl/+, and Pkd1C14fl/−/− temperature-sensitive SV40 large T antigen renal proximal tubule cells (Joly et al., 2006; Shibazaki et al., 2008) were maintained as described.

**Cell Fractionation**

Preparation of nuclear and cytoplasmic fractions was performed as previously described (Chauvet et al., 2004). Cells grown in 10 cm dishes in the presence of 25 μM clasto-lactacystin were harvested in cold PBS, centrifuged for 5 min at 500 × g, and resuspended in hypotonic buffer (10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, and protease inhibitors). Cells were homogenized in a tissue-tight Dounce homogenizer, chilled on ice for 10 min, and then rotated for 15 min. Nonidet P-40 was added to a final concentration of 1%.
and the preparations were rotated for an additional 15 min. The lysates were centrifuged at 1,500 \( \times g \) for 5 min. The resulting supernatant formed the nonnuclear fractions. The pellets (nuclear fractions) were washed in hypotonic buffer for 10 min, resuspended in lysis buffer (50 mM HEPS [pH 7.4], 200 mM NaCl, 0.5% NP-40, 1 mM EDTA, and protease inhibitors), and rotated for 10 min. Pelleted nuclei were lysed by sonication in lysis buffer and prepared for immunoblot analysis. The protein concentration of each sample was determined using a Bio-Rad colorimetric protein concentration assay.

**Coactivator Trap Screen**

The coactivator trap screen was performed as previously described (Amelio et al., 2007). A pcDNA3.1 construct expressing HA-PC1-CTT was cotransfected with each of 837 transcription factor-Gal4 fusion proteins and the GAL4 luciferase and Renilla reporter plasmids into HEK293T/Cells in a 384-well plate. Cells were cultured for 24 hr in a humidified incubator at 37 \(^\circ\)C. The transfected cells were harvested with PBS and lysed with 100 ml of passive lysis buffer (Promega). Luciferase levels were assayed using the Dual Luciferase assay kit (Promega). Luciferase signals were determined in a GloMax 20/20 luminometer (Promega).

**Transient Transfection and Luciferase Assay**

Pkd1<sup>+/−</sup> cells, Pkd1<sup>−/−</sup> cells, and Pkd1<sup>+/−</sup> cells stably expressing pNRTIs HA-PC1-CTT were plated in 24-well tissue culture plates and transfected using Lipofectamine 2000 (Invitrogen) at 80%–100% confluency. Luciferase reporter constructs Topflash or GL4.31[luc2P/GAL4UAS] (0.2 \( \mu \)g) and PRL-TK (Renilla), were mixed with 2 \( \mu \)l Lipofectamine. HEK293 cells were transfected with STAT6-Luciferase (kind gift of Dr. S.J. Hacque, Cleveland Clinic Foundation) or with TopFlash-luciferase, or with CHOP-Gal4/Gal4-luciferase (0.2 \( \mu \)g), as well as with either control plasmid, STAT6-V5, HA-PC1-CTT or HA-PC1-CTT-NLS, and with pRL-TK (Renilla). Transfection mixtures were added drop wise to cell culture media (containing 80 \( \mu \)M DAPT [Sigma-Aldrich] when indicated) and incubated at 37 \(^\circ\)C for 24 hr. The amount of DNA in each well was equalized through the addition of a control plasmid, pcDNA3.1, which was also used for mock transfection. Transfected cells were harvested with PBS and lysed with 100 ml of passive lysis buffer (Promega). Luciferase levels were assayed using the Dual Luciferase Assay Reagent kit (Promega). Luciferase signals were determined in a GloMax 20/20 luminometer (Promega).

**siRNA Treatment**

HEK293 cells were transfected with 100 nm target-specific siRNA or control siRNA using Lipofectamine 2000. As a control siRNA, we used Silencer Negative Control siRNA#1 (#AM4611; Ambion). To knock down PSEN1 and PSEN2, we used validated siRNAs for PSEN1, SI02662688 from Qiagen; for PSEN2, AM51331 from Ambion. Knockdown of CHOP was accomplished according a published protocol (Shikawa et al., 2009).

**Immunoprecipitation, Immunoblot, and GST Pull-Down**

Cells were lysed by sonication in 50 mM HEPS (pH 7.4), 150 mM NaCl, 0.5% NP-40, 1 mM EDTA with protease inhibitors (Roche). Precleared lysates (18,000 \( \times g \), 30 min) were incubated at 4 \(^\circ\)C overnight with either monoclonal-anti-HA agarose, anti-FLAG-M2 agarose (Sigma-Aldrich), or glutathione-Sepharose 4B beads (Amersham) prebound with indicated GST fusion protein constructs harvested from BL21 bacteria by standard procedures (Stratagene). Beads were collected by centrifugation, and the pellets were washed in lysis buffer three times for 10 min with rotation at 4 \(^\circ\)C. Immunoprecipitates were eluted in SDS-PAGE loading buffer (25 mM Tris-\( \mathrm{HCl} \) [pH 6.7], 10% glycerol, 1% SDS, 50 mM DTT, bromophenol blue).

Proteins were separated on a 10% SDS-polyacrylamide gel and then electrophoretically transferred to a nitrocellulose membrane (Bio-Rad), incubated in blocking buffer (150 mM NaCl, 20 mM Tris, 5% [w/v] powdered milk, 0.1% Tween) for 60 min, and then incubated with one of the following primary antibodies at 4 \(^\circ\)C overnight: monoclonal \( \alpha \)-HA (rat antibody) (1:5,000; Roche); polyclonal \( \alpha \)-FLAG (1:5,000; Sigma-Aldrich); polyclonal \( \alpha \)-C-Myc (1:5,000; Sigma-Aldrich); polyclonal \( \alpha \)-GST (goat) (1:1,000; Amersham); and \( \alpha \)-His (1:5,000; Qiagen). Subsequently, primary antibody binding was detected with horseradish peroxidase-conjugated anti-rat, anti-rabbit, or anti-goat secondary antibodies (1:5,000–10,000; Jackson Labs), and proteins were visualized with an enhanced chemiluminescence detection kit (ECL; Amersham Biosciences).

**Zebrafish Experiments: Morpholino Antisense Oligonucleotide and mRNA Injections and Drug Treatment**

Morpholino-induced knockdown of Pkd1a and Pkd1b expression was performed as previously reported (Mangos et al., 2010). Wild-type embryos at the one- to two-cell stage were microinjected with 4.6 nl of a 0.15 mM antisense morpholino oligonucleotide solution (Gene Tools LLC) with 0.1% phenol red using a nanoject2000 microinjector (World Precision Instruments). The sequences of the morpholinos targeting Pkd1a and Pkd1b were identical to those that have been previously described (Mangos et al., 2010); briefly, the splice donor-blocking oligonucleotide sequences were: Pkd1a MO ex8, 5\'-GACCTGAGGACTCACTGTGTGATTT-3\'; and Pkd1b MO ex45, 5\'-ACATGATTTTGACCTCTTTTGTT-3\'. Gene Tools standard negative control morpholino was used as an injection control and demonstrated no effect on development. For drug treatment, after mRNA injection at the one- to two-cell stage, embryos were placed in a 10 cm dish with 30 ml embryo media containing 25 \( \mu \)M DAPT dissolved in DMSO and imaged 3 dpf as described (Arslanova et al., 2010).

**Statistical Analysis**

Results are expressed as means ± SE. Differences between means were evaluated using Student’s t test or analysis of variance as appropriate. Values of \( p < 0.05 \) were considered to be significant.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes four figures, one table, and three movies and can be found with this article online at doi:10.1016/j.devcel.2011.10.028.

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