

Strategies to Inhibit Cyst Formation in ADPKD

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The many hundreds of cysts that grow and expand and ultimately overwhelm and destroy polycystic kidneys arise from the slow but unrelenting proliferation of tubular epithelial cells, eventually giving rise to very large, thin-walled, fluid-filled structures. The growth of these cystic bodies requires two processes: Cell proliferation and fluid secretion. Cyst epithelial cells seem to have a unique phenotype that could offer opportunities for therapeutic intervention. Current evidence has demonstrated that cAMP drives both abnormal cell proliferation, by stimulating the Ras/mitogen-activated protein kinase (MAPK) pathway, and cyst-filling fluid secretion, by activating the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel. Both of these cAMP-mediated processes should be considered in the design of strategies targeted to slow cyst growth and enlargement in autosomal dominant polycystic kidney disease.

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Cyst Formation

Each cyst that develops in autosomal dominant polycystic kidney disease (ADPKD) is thought to initiate from somatic mutation, causing biallelic loss of PKD gene function (1), or from haploinsufficiency or a threshold effect (2). In ADPKD, a deficiency of the proteins polycystin-1 and polycystin-2, the products of the *PKD1* and *PKD2* genes, causes cyst formation. Loss of the polycystins is thought to disrupt normal intracellular calcium signaling (3–10); one question begging an answer is what this calcium is normally doing. Once the cyst-initiating event has occurred, it is envisioned that the tubule undergoes a dilation process that involves abnormal cell growth and proliferation. As the cyst grows in size and pinches off from the remainder of the tubule, it then becomes an isolated, self-contained structure. The cyst continues to enlarge through a proliferative process increasing in cell number and size, and it fills as it enlarges by the secretion of fluid into the cyst lumen (11). Cyst growth occurs slowly, over decades, and eventually the cystic kidneys fail in approximately half of affected individuals by the time they reach their 50s. If cyst growth and enlargement could be slowed, then renal failure could be put off by years or decades, thereby effectively delaying or preventing ESRD.

Given this cyst-forming scenario, PKD in theory could be treated by preventing the earliest initiating event either by preventing the somatic second-hit mutations or by upregulating the expression of the unmutated PKD allele; however, with

our present state of knowledge, we have no effective way of preventing somatic mutations or controlling PKD protein levels *in vivo*. Short of that, is it possible to inhibit cyst formation early in the disease when small tubule dilations are first developing? And can we stop cyst growth once it has started?

cAMP-Dependent Fluid Secretion

In trying to answer the first of the two questions posed, we used a mutant *Pkd1* mouse model, which, as homozygous *Pkd1*-deficient mice, develop polycystic kidneys as embryos (12). To examine the process of cyst formation at the earliest stages, we made use of a metanephric organ culture system in which embryonic kidneys are removed from these mice during the midfetal stage, at embryonic day 15.5 or earlier (13). The embryonic kidneys continue to grow and develop over a 4-d period in culture and can be easily subjected to experimental treatment. When 8-Br-cAMP is included in the medium, the kidneys from wild-type mice develop only a few small tubular dilations (Figure 1A). By contrast, *Pkd1* $-/-$ kidneys, which have small cysts *in situ*, develop much larger cyst-like structures over the 4-d culture period. The *Pkd1* heterozygous kidneys have an intermediate cyst-forming capacity in the presence of cAMP.

There is a considerable body of literature providing support for the idea that cAMP-stimulated chloride transport is the driving force for the fluid secretion associated with cyst filling (14–16). It is currently believed that chloride is transported through cells from the basal to the luminal side of the growing cyst and that the role of cAMP in this process is to activate the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel on the apical membrane of cyst-lining epithelial cells, thereby allowing net chloride secretion and ultimately fluid transport into the cyst (15, 17–19).

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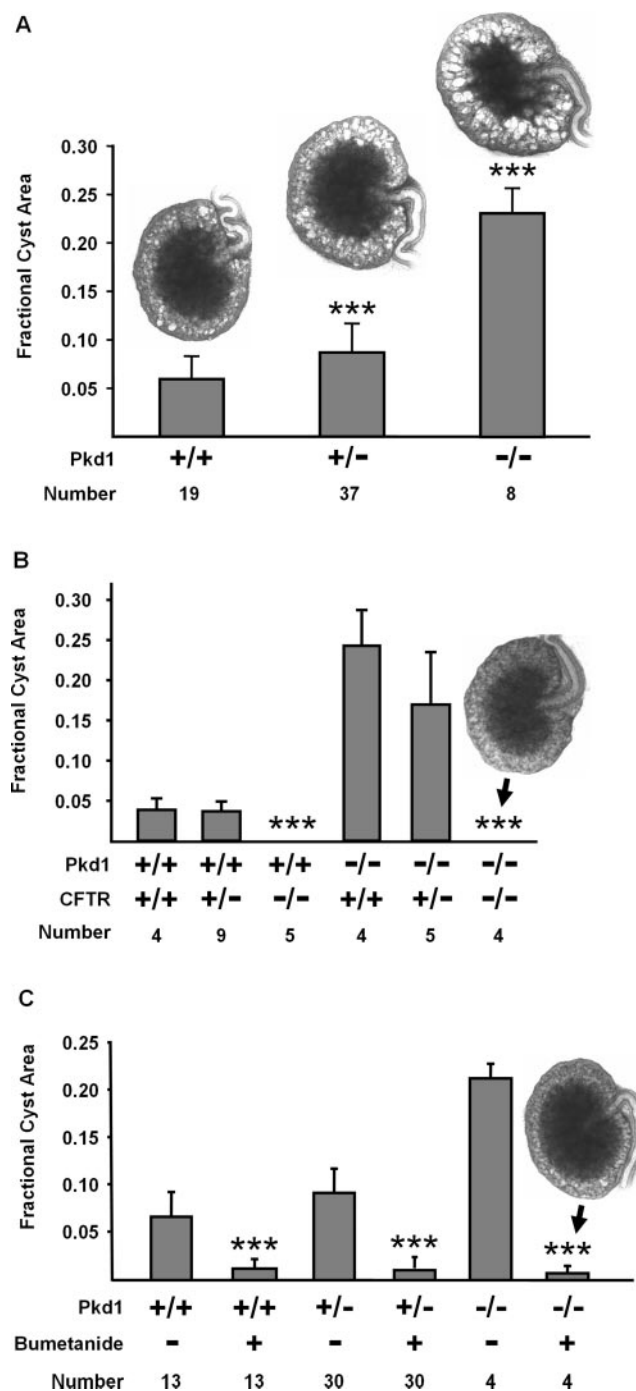


Figure 1. Roles of CFTR and NKCC1 in cyst formation in polycystic kidney disease (PKD) embryonic kidneys in metanephric culture. (A) Cyst formation in wild-type (+/+), heterozygous (+/-), and homozygous (-/-) embryonic day 15.5 (E15.5) Pkd1 kidneys that were cultured for 4 d in 100 μ M 8-Br-cAMP. (B) Effect of CFTR genotype on cAMP-dependent cyst formation in E15.5 wild-type and Pkd1 -/- kidneys that were cultured for 4 d in 100 μ M 8-Br-cAMP. (C) Effect of 100 μ M bumetanide treatment on cAMP-dependent cyst formation in E15.5 wild-type and Pkd1 +/- and -/- kidneys that were cultured for 4 d in 100 μ M 8-Br-cAMP. Bar graphs represent the average fractional cyst areas in the indicated number of animals. Images of representative kidneys are shown above the bars. *** P < 0.001 versus the control for each group. Based in part on data by Magenheimer *et al.* (13).

To determine the extent to which tubule dilation associated with cAMP-dependent cyst enlargement in metanephric organ culture requires chloride-mediated fluid secretion, we generated compound homozygous Pkd1: Cftr mutant mice (Pkd1 -/-: Cftr -/-). As seen in Figure 1B, Pkd1 mutant kidneys with one or two wild-type Cftr alleles developed numerous large cysts in the presence of cAMP, whereas kidneys from Pkd1: Cftr double-deficient mice showed absolutely no evidence of cyst formation (13). These results indicate that tubule expansion and cyst formation in metanephric culture in response to cAMP is completely dependent on CFTR and show that the CFTR chloride channel is not only present but also functional in embryonic kidneys. Parenthetically, total loss of CFTR did not increase survival of the Pkd1: Cftr double-null mice because the embryonic lethality of the Pkd1 -/- state is probably due to cardiovascular failure (20,21) or placental insufficiency (22).

These experiments suggested that cAMP-dependent fluid transport requires CFTR-mediated chloride secretion. Basolateral uptake of chloride by embryonic renal epithelial cells may involve the Na⁺, K⁺, 2Cl⁻ co-transporter, NKCC1. For testing this, embryonic kidney cultures were treated with the NKCC1 inhibitor bumetanide. As shown in Figure 1C, inhibition of NKCC1 almost completely eliminated cyst formation in all three Pkd1 genotypes, providing support for the idea that chloride transport is the driving force for fluid secretion and that the CFTR dependence is due to its role as a chloride channel.

The metanephric organ culture experiments suggest that fluid secretion can be a powerful force in driving cyst enlargement, as is demonstrated by Pkd1 -/- tubules, which impressively dilate in culture after treatment with cAMP. Of relevance to this issue is the observation that in patients who co-inherit ADPKD and cystic fibrosis, their PKD can be somewhat less severe (23). Thus, in considering future treatment strategies for ADPKD, one should think about inhibiting cyst enlargement by blocking fluid secretion, perhaps with a combination of a CFTR inhibitor and an NKCC1 inhibitor, if not at the earliest stages of cyst formation, when glomerular filtration can work to fill cysts, then perhaps later in the disease once cysts have become isolated structures and can fill only by fluid secretion. The goal would be to slow cyst enlargement to preserve the remaining renal parenchyma surrounding the cyst, thereby conserving kidney function (24), but clearly, more needs to be learned about the mechanisms of cyst-filling fluid secretion in ADPKD and the role of cAMP in this process for the eventual design and development of therapeutic approaches to target this fundamentally important aspect of cyst growth.

cAMP-Dependent Cell Proliferation

As noted, cAMP can be implicated in the cyst-forming process because of its role in chloride secretion and ultimately the fluid accumulation associated with cyst enlargement. A second role of cAMP is to stimulate the abnormal proliferation of cystic epithelial cells. As such, targeting cAMP should provide a dual benefit in slowing the progression of ADPKD, by treating both fluid secretion and cell proliferation (25,26).

It has been observed that normal renal epithelial cells and

cyst-lining epithelial cells grow at approximately the same rate in culture, unless cAMP agonists are added to the medium. In the presence of cAMP, normal cells decrease their rate of proliferation (antimitogenic response), whereas cystic cells increase their rate of proliferation (mitogenic response). We now understand that the phenotypic difference between the normal and ADPKD cell types is due to the differing and quite opposite responses of the Ras/MAPK pathway to cAMP signals (4,27–29).

The p44/p42 extracellular signal-regulated kinase 1 (ERK1)/ERK2 MAPKs are inhibited in normal cells treated with cAMP (Figure 2). This is caused by a cAMP-dependent inhibitory phosphorylation of Raf-1. In contrast, cAMP treatment of ADPKD cells stimulates ERK activity, which is caused by cAMP-dependent activation of B-Raf, not seen in normal cells (28). In further examining these pathways, we found that normal cells could be switched to a PKD-like phenotype when pretreated with a calcium channel blocker (nifedipine, verapamil, or gadolinium) or when extracellular calcium was lowered (4). These treatments resulted in decreased intracellular calcium levels, mimicking a calcium-deficient state expected with loss of polycystin function (3–5). Calcium channel blocker pretreatment caused cAMP to stimulate, rather than inhibit, ERK. Further analysis revealed that B-Raf is normally kept in a repressed state, in part by a calcium-dependent phosphatidylinositol-3-kinase/Akt pathway. As such, calcium channel blockers derepress B-Raf, allowing cAMP signals to bypass Raf-1 and to activate B-Raf, mitogen-associated/extracellular-regulated kinase (MEK), and ERK. We further analyzed this pathway by

showing that inhibition of phosphatidylinositol-3-kinase or Akt also causes derepression of B-Raf, allowing cAMP activation of ERK. We also know that the cAMP signal requires PKA, Src, and Ras and is associated with an increased level of the p90 B-Raf protein. These experiments suggest that the abnormal cell proliferation that characterizes the ADPKD phenotype is caused by impaired calcium signaling, which reprograms the Ras/MAPK pathway to allow cAMP activation of B-Raf and ERK (4).

Support for this view has come from a number of animal models of PKD, including the PCK rat, a model of human ARPKD (30), and the *jck* mouse, which has a nonorthologous form of recessive PKD (31). Cyst formation in these models was accompanied by high levels of cAMP, increased levels of the p90 B-Raf isoform relative to the p65 isoform, and increased levels of phosphorylated (activated) ERK.

A role for calcium in the cAMP stimulation of cell proliferation was also demonstrated in wild-type vascular smooth muscle cells in culture, which could be induced to proliferate by treating them with the calcium channel blocker verapamil (32). This treatment was associated with increased phosphorylated ERK levels. In this case, verapamil treatment alone caused a mitogenic response, perhaps because there was an associated increase in intracellular cAMP in cells that were treated with the calcium channel blocker. Importantly, as predicted from the previously mentioned studies, verapamil treatment of cystic Cy/+ rats significantly exacerbated their PKD (33), suggesting perhaps that the use of calcium channel blocker antihypertensive treatment be reevaluated in patients with PKD.

Further support for the key role of calcium comes from experiments with ADPKD cells themselves (34). In studies from Darren Wallace's laboratory, it was demonstrated that primary cells from various ADPKD cysts had significantly lower basal intracellular calcium, confirming that ADPKD is associated with a disruption in calcium homeostasis. These ADPKD cells responded to cAMP with increased cell proliferation through activation of the B-Raf/ERK pathway. Importantly, this response to cAMP could be normalized by re-introducing calcium to these cyst epithelial cells with an L-type calcium channel agonist, Bay-K8644, or a calcium ionophore, A23187. These treatments switched the cAMP response of these cells from mitogenic to antimitogenic, thereby rescuing them from their ADPKD state.

In our experiments, a variety of treatments to restrict calcium uptake all were able to switch cells to the PKD phenotype. These treatments included the L-type calcium channel blockers verapamil and nifedipine and the nonspecific inhibitors gadolinium and extracellular EGTA. Because of their effects, we believe that these treatments cause a tonic reduction of basal intracellular calcium and that this decrease may result secondarily in a disruption of calcium signaling. Indeed, a tonic decrease in intracellular calcium was demonstrated directly in verapamil-treated M-1 cells (4). Presumably, this decreased basal calcium would adversely affect polycystin-associated calcium signaling processes, including cilia-dependent calcium signaling events (6,35,36) and other polycystin-mediated calcium signaling events (5,37–40). Although caution should be exercised

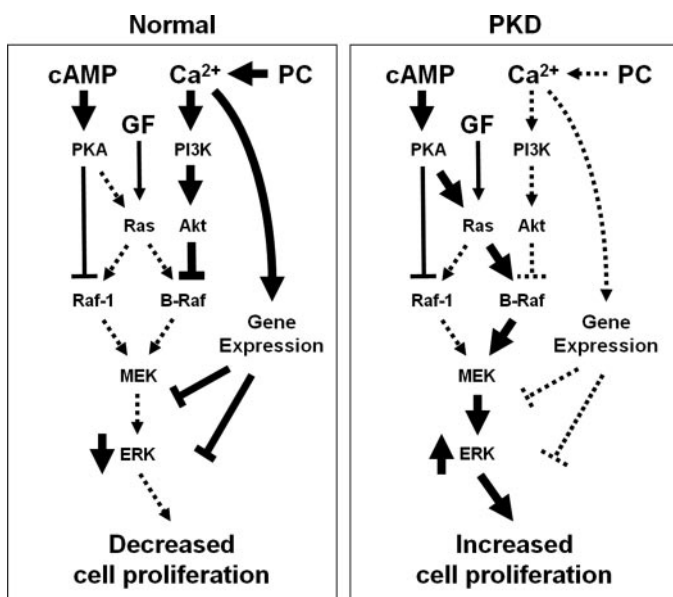


Figure 2. Pathways from cAMP and calcium to extracellular signal-regulated kinase (ERK) and cell proliferation. (Left) Normal signaling pathways. (Right) Signaling pathways in PKD. PC, polycystin; GF, growth factor; solid lines, active pathways; dotted lines, diminished pathways. Arrows by ERK indicate decreased (left) or increased (right) activity. Based on data by Yamaguchi *et al.* (4) and adapted from reference (52).

when interpreting results of experiments using chemical inhibitors, it is nevertheless interesting and perhaps instructive that the PKD state can be phenocopied by these treatments (4), that PKD can be exacerbated by calcium restriction (33), and that raising intracellular calcium can normalize ADPKD cells (34). Certainly, when thinking about potential therapies for ADPKD, one should consider methods that raise basal intracellular calcium levels (41).

It was recently suggested that loss of polycystin function *per se* may not generate a sufficient proliferative stimulus to initiate cyst growth (42,43) and that cyst formation may not require significantly increased cell proliferation (43). These ideas are based on experiments using conditional knockouts of the *Pkd1* gene in which loss of *Pkd1* early in the postnatal period, when cell proliferation is still ongoing, resulted in rapid cyst formation, whereas loss of *Pkd1* function later in adult life, when cell proliferation had ceased, resulted in significantly delayed, slow-onset cyst formation. On the basis of these experiments, it was hypothesized that renal injury followed by repair processes, including cell proliferation, may be an important permissive condition for cystogenesis (42). This has now been shown by evidence that loss of ciliary polycystin function in adult life in conjunction with injury-induced increases in cell proliferation results in rapid-onset cyst formation (44), confirming the requirement for cell proliferation in the cyst growth process. Thus, it seems that loss of polycystin function *per se* may not trigger the initiation of a proliferative process but rather predisposes cells to cyst formation by mitogenic stimuli, including cAMP agonists. This state of being “poised” for cyst formation was elegantly demonstrated in the ARPKD orthologous PCK rat model, which was bred to the AVP-deficient Brattleboro rat, giving double-deficient (*Pkhd1* $-/-$:AVP $-/-$) rats. These rats did not develop cystic kidneys until the cAMP agonist AVP was administered, effectively raising intracellular cAMP (45).

Treating cAMP-Dependent Processes

Accumulating evidence has suggested that loss of polycystin signaling brings about a transformed cellular state that permits cAMP to stimulate cell proliferation through the Ras/B-Raf/MEK/ERK pathway. A key factor in this process is that B-Raf is derepressed, allowing cAMP activation of ERK. Given this central role of cAMP, it should be possible to treat PKD by reducing intracellular cAMP. This would have a two-fold beneficial effect of inhibiting both cell proliferation and the fluid secretion associated with cyst filling. In fact, therapies designed to target the arginine vasopressin receptor and to lower intracellular cAMP in the collecting duct have proved to be highly effective in animal studies, a topic of another article in this series (46).

Given the importance of the Ras/MAPK pathway in PKD, what success has there been in targeting components of this signaling cascade to inhibit cyst formation? cAMP is known to activate B-Raf in only a few cell types, including melanocytes (47), PC12 cells (48,49), and cyst epithelial cells (4,28). In all of these cases, the cells are programmed to respond to cAMP by activating ERK through the Ras/B-Raf route rather

than through Raf-1. Thus, it would seem that targeting B-Raf might inhibit the cAMP-dependent cell proliferation. This is currently in the experimental stage in a number of laboratories.

Additional targets in the Ras/MAPK pathway are also worthy of consideration. It has been observed in tumors with activating B-Raf mutations that there is unregulated, cAMP-independent cell proliferation. Of importance was a discovery that cells that contain constitutively active B-Raf mutations were hypersensitive to the MEK inhibitor CI-1040 (PD0325901) (50). This was in contrast to cells that contained activating Ras mutations, which in some cases were refractory to MEK inhibitors. The use of MEK inhibitors to treat cancer had led to disappointing results in some clinical trials, but in the case of a subset of cancer types—those with activating B-Raf mutations—there did indeed seem to be a response (50). This observation may have relevance to PKD.

In considering PKD, it was found by Omori *et al.* in 2006 (51) that the MEK inhibitor PD184352 was quite effective in slowing cyst growth. These studies made use of the *pcy* mouse model of nephronophthisis, NPHP3, a form of PKD. Their experiments showed a dramatic inhibition of kidney enlargement in *pcy/pcy* mice after 7 wk of treatment with the MEK inhibitor. There also were decreased levels of phosphorylated ERK in the treated mice and a corresponding improvement in renal function.

A possible explanation for this favorable response to MEK inhibition in the *pcy/pcy* mice may lie in the observations made in tumors that harbor B-Raf mutations. As far as is known, there are no activating B-Raf mutations in the *pcy* mouse or in any other form of PKD. Rather, B-Raf may be constitutively active, not by an activating mutation but by the mitogenic effects of cAMP (52). The key may be that if cell proliferation depends on B-Raf, then cyst growth may be sensitive to MEK inhibition. This result—the response of *pcy* mice to MEK inhibition—suggests that other forms of PKD be tested to determine whether they, too, respond. In fact, in a recent study in a *Pkd1* conditional knockout model (53), it was found that the MEK inhibitor U0126 was ineffective in slowing cyst growth and reducing kidney size, despite a decrease in phospho-ERK—reminiscent of B-Raf-independent tumors that are refractory to MEK inhibition (50). These results suggest that other signaling pathways may also be involved in cyst formation under some conditions.

Conclusions

Abnormal cell proliferation is of central importance to cyst growth in ADPKD. This abnormal cell behavior seems to result from the mitogenic actions of cAMP on an abnormally reprogrammed Ras/MAPK pathway that derepresses B-Raf, allowing it to be stimulated by cAMP signals. If so, then this constitutively active B-Raf/MEK/ERK pathway may be the Achilles heel of ADPKD if specific pharmacologic inhibitors can be developed to block B-Raf activity directly or if inhibition of its downstream effector MEK proves to be therapeutic. The loss of polycystin function in ADPKD seems to result in a cellular transformation process that leads to cAMP

sensitivity. Presumably, this reprogramming involves changes in gene expression that contribute in unknown ways to alter B-Raf/MEK/ERK signaling to allow cAMP to be mitogenic (Figure 2). As such, when these genes and their protein products are discovered, they, too, could serve as unique, cyst-specific targets; however, it should also be recognized that other, non-cAMP-stimulated signaling pathways may contribute to cyst-forming cell proliferation in some types of PKD or during certain stages of the cystogenic process (53–58). Ultimately, the answer as to which targets are the most relevant will come from studies in the human population using pathway-specific inhibitors to identify the most effective therapies.

Note Added in Proof

While this paper was in press, two articles were published that demonstrated an amelioration of PKD in rodent models by inhibition of fluid secretion [Yang B, Sonawane ND, Zhao D, Somlo S, Verkman AS. Small-Molecule CFTR Inhibitors Slow Cyst Growth in Polycystic Kidney Disease. *J Am Soc Nephrol* 2008 Apr 2; Epub ahead of print] and by inhibition of cell proliferation [Sweeney WE Jr, von Vigier RO, Frost P, Avner ED. Src Inhibition Ameliorates Polycystic Kidney Disease. *J Am Soc Nephrol* 2008 Apr 2; Epub ahead of print].

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Disclosures

None.

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