Adenylyl cyclase 5 deficiency reduces renal cyclic AMP and cyst growth in an orthologous mouse model of polycystic kidney disease

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Cyclic AMP promotes cyst growth in polycystic kidney disease (PKD) by stimulating cell proliferation and fluid secretion. Previously, we showed that the primary cilium of renal epithelial cells contains a cAMP regulatory complex comprising adenylyl cyclases 5 and 6 (AC5/6), polycystin-2, A-kinase anchoring protein 150, protein kinase A, and phosphodiesterase 4C. In *Kif3a* mutant cells that lack primary cilia, the formation of this regulatory complex is disrupted and cAMP levels are increased. Inhibition of AC5 reduces cAMP levels in *Kif3a* mutant cells, suggesting that AC5 may mediate the increase in cAMP in PKD. Here, we examined the role of AC5 in an orthologous mouse model of PKD caused by kidney-specific ablation of *Pkd2*. Knockdown of AC5 with siRNA attenuated the increase in cAMP levels in *Pkd2*-deficient renal epithelial cells. Levels of cAMP and AC5 mRNA transcripts were elevated in the kidneys of mice with collecting duct-specific ablation of *Pkd2*. Compared with *Pkd2* single mutant mice, AC5/*Pkd2* double mutant mice had less kidney enlargement, lower cyst index, reduced kidney injury, and improved kidney function. Importantly, cAMP levels and cAMP-dependent signaling were reduced in the kidneys of AC5/*Pkd2* double mutant compared to the kidneys of *Pkd2* single mutant mice. Additionally, we localized endogenous AC5 in the primary cilium of renal epithelial cells and showed that ablation of AC5 reduced ciliary elongation in the kidneys of *Pkd2* mutant mice. Thus, AC5 contributes importantly to increased renal cAMP levels and cyst growth in *Pkd2* mutant mice, and inhibition of AC5 may be beneficial in the treatment of PKD.

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Autosomal dominant polycystic kidney disease (ADPKD) is the most common renal cystic disorder, affecting approximately 1 in 500 individuals, and represents the fourth leading cause of end-stage kidney disease in adults.[1] ADPKD is caused by mutations in *PKD1* (encoding polycystin-1) or *PKD2* (encoding polycystin-2). Polycystin-1 is an integral membrane protein containing 11 transmembrane segments and a large, receptor-like extracellular N-terminal domain. Polycystin-2 (PC2) contains 6 transmembrane segments and functions as a Ca^{2+}-permeable transient receptor potential channel. Although PC2 is mainly located in the endoplasmic reticulum, polycystin-1/PC2 heterodimers are found in primary cilia in renal tubular epithelial cells.[2,3] Primary cilia are sensory organelles that are found on the surface of most cells. Primary cilia are composed of a microtubule-based axoneme that emerges from the basal body and is surrounded by the ciliary membrane. Most renal epithelial cells contain a solitary primary cilium that projects from the apical surface into the tubular lumen. Renal cilia are thought to function as mechanosensors that respond to fluid flow and regulate intracellular Ca^{2+},[4] although this latter function has recently been challenged.[5] Renal cilia also express receptors for ligands such as somatostatin and vasopressin and bind urinary exosomes.[6–8] PKD is the prototype of a ciliopathy, a group of pleiotropic genetic disorders that are characterized by abnormalities in the function or structure of the primary cilium and/or basal body. In support of this notion, we have previously shown that Kinesin Family Member 3A, a motor protein that mediates intraflagellar transport, is required for renal ciliogenesis.[9] Kidney-specific ablation of *Kif3a* results in the loss of renal cilia and is sufficient to produce kidney cysts. In addition, a cilia-dependent cyst-activating mechanism has recently been uncovered.[10]

The second messenger adenosine 3′,5′-cyclic monophosphate (cAMP) is a major driver of cystogenesis in PKD.[11] Levels of cAMP are elevated in cyst epithelial cells from humans with ADPKD and in cystic kidneys from *Pkd1* and *Pkd2* mutant mice.[12] Elevated cAMP levels contribute to cyst growth by stimulating fluid secretion through activation of the cystic fibrosis transmembrane conductance regulator chloride channel and by increasing cell proliferation through activation of the B-Raf/MEK/ERK pathway.[13] The mitogenic

response to cAMP is inhibited by treatment with Ca^{2+} ionophores, whereas treatment with Ca^{2+} channel blockers stimulates proliferation. These results suggest that the effects of elevated cAMP are dependent on a reduction in intracellular Ca^{2+} concentration. Incubation of embryonic kidneys from *Pkd1* mutant mice with 8-Br-cAMP stimulates cyst formation.[14] Conversely, drugs that reduce intracellular cAMP levels, such as tolvaptan and octreotide, inhibit cyst growth in *Pkd1* and *Pkd2* mutant mice.[15] Clinical trials have shown that tolvaptan and octreotide reduce the rate of kidney enlargement and/or slow the decline in glomerular filtration rate in humans with ADPKD. Tolvaptan is now approved for the treatment of ADPKD in some countries, although it has not been approved for this indication in the United States.[16]

The mechanism whereby cAMP is elevated in PKD remains poorly understood. cAMP is synthesized from ATP by adenylyl cyclases and is catabolized by phosphodiesterases. Intracellular cAMP signaling is tightly compartmentalized by A-kinase anchoring proteins (AKAPs) that bind to adenylyl cyclases, protein kinase A, and phosphodiesterase, thereby maintaining protein kinase A in close proximity to enzymes that synthesize and degrade cAMP. Our previous studies have revealed that renal cilia contain a cAMP-regulatory complex comprising adenylyl cyclases 5 and 6 (AC5/6), A-kinase anchoring protein 150 (AKAP150), protein kinase A, and phosphodiesterase 4C (PDE4C).[17] The formation of this protein complex is disrupted in *Kif3a* mutant cells that lack primary cilia, which results in elevations in cAMP levels and activation of cAMP-dependent signaling both *in vitro* and *in vivo*. Moreover, we showed that PC2 interacts with the complex by binding to AC5/6 through its C-terminus. Ablation of PC2 increases cAMP levels, which can be corrected by re-expression of wild-type PC2 but not by mutant PC2 that lacks calcium channel activity. Because AC5/6 are Ca^{2+}-sensitive adenylyl cyclases, these findings suggest that endogenous PC2 raises the local concentration of Ca^{2+}, which suppresses AC5/6 activity. Mutation of PC2 or disruption of cilia releases the inhibition of AC5/6 and causes the cAMP elevation that drives kidney cystogenesis. Treatment of *Kif3a* mutant cells with the AC5 inhibitor NKY80 or small-interfering RNA (siRNA) knockdown of AC5 mRNA attenuates the increase in cAMP-dependent signaling. In contrast, siRNA knockdown of AC6 has no effect. These results suggest that AC5 mediates the increase in cAMP levels in *Kif3a* mutant cells and kidneys.

To test the role of AC5 in an orthologous animal model of human PKD, we generated mutant mice that were deficient in both *Pkd2* and AC5. Compared with *Pkd2* single mutant mice, concomitant ablation of AC5 reduced kidney enlargement, lowered cyst index, and improved kidney function. Importantly, cAMP levels in the kidney were increased in *Pkd2* single mutant mice and were reduced in AC5/*Pkd2* double mutant mice. These findings demonstrate that AC5 contributes to the increase in cAMP observed in PKD and provides genetic proof of concept for inhibition of AC5 as a therapeutic approach in PKD.

RESULTS

AC5 contributes to the elevation in cAMP caused by deficiency of *Pkd2* in mouse kidney cells

Our previous studies showed that inhibition of AC5 attenuates cAMP-dependent signaling in *Kif3a*-deficient renal epithelial cells that lack primary cilia.[17] To examine the role of AC5 in an orthologous model of PKD, we performed studies on *Pkd2* mutant cells and kidneys. As shown previously,[17] cAMP levels were elevated in homozygous null $Pkd2^{-/-}$ renal epithelial cells compared to $Pkd2^{+/-}$ cells (Figure 1a, left panel). Similarly, cAMP levels were elevated in kidneys from Pkhd1/Cre;$Pkd2^{F/F}$ mice,[18] in which *Pkd2* has been deleted in renal collecting ducts by Cre/loxP recombination (Figure 1a, right panel). These results confirmed that deficiency of *Pkd2* increases cAMP levels in kidney cells. Quantitative reverse-transcriptase polymerase chain reaction (PCR) showed that the levels of AC5 mRNA transcripts were 10-fold higher in kidneys from collecting duct–specific *Pkd2* knockout mice compared with their wild-type littermates (Figure 1b). Immunoblot analysis with an AC5-specific antibody (501AP) showed increased levels of AC5 protein in *Pkd2* mutant cells compared with controls (Figure 1c, upper panel). Immunoblotting with an antibody (C17) that recognizes both AC5 and AC6 also showed increased protein abundance in *Pkd2* mutant cells (Figure 1c, lower panel). We were unable to measure AC6 protein by itself because an antibody that was specific for this isoform was not available. To determine the contribution of AC5 to the elevation in cAMP levels, we first used siRNA to knockdown AC5 in $Pkd2^{-/-}$ renal epithelial cells. Compared with control cells transfected with scrambled siRNA, transfection with AC5 siRNA reduced the levels of AC5 mRNA transcripts by 50% to 60% (Figure 1d). Figure 1e shows that cAMP levels were elevated in *Pkd2* null cells, and knockdown of AC5 reduced cAMP levels by 70%. In contrast, knockdown of AC5 had no significant effect in $Pkd2^{+/-}$ cells. To address the role of AC6, we inhibited its expression in *Pkd2* null cells using siRNA. The magnitude of the inhibition of AC6 was similar to the siRNA knockdown of AC5 (Supplementary Figure 1A). The inhibition of AC6 was specific, because the expression of AC5 was unaffected by the AC6 siRNA (Supplementary Figure 1B). Similar to the effect of siRNA knockdown of AC5, knockdown of AC6 reduced cAMP levels in *Pkd2* null cells (Supplementary Figure 1C). Combined knockdown of AC5 and AC6 did not produce an additional effect (not shown). We conclude that both AC5 and AC6 contribute to the elevation of cAMP levels in *Pkd2* mutant cells.

Knockout of AC5 slows cyst progression caused by collecting duct–specific deletion of *Pkd2*

To explore the role of AC5 in the formation of kidney cysts *in vivo*, we generated *Pkd2*/AC5 double-knockout mice (DKO). For these experiments, we used AC5 null mice in which exon 2 of the AC5 gene (*Adcy5*) has been deleted by homologous recombination.[19] Homozygous null mice are globally deficient in AC5 but are healthy and have normal