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Nikki Mehran April 15, 2015

Lithium-mediated Elongation of Renal Cilia

by

Nikki A. Mehran

Dr. Mitsi A. Blount
Adviser

Biology

Dr. Mitsi A. Blount

Adviser

Dr. Victor Corces

Committee Member

Dr. Jacobus de Roode

Committee Member

2015

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By

Nikki A. Mehran

Dr. Mitsi A. Blount

Adviser

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Abstract

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Polycystic kidney disease (PKD), a ciliopathy that features multiple inner medullary collecting duct (IMCD)-derived cysts, affects 600,000 Americans and has no current cure. Concentrations of 3'5'-cyclic adenosine monophosphate (cAMP) are elevated the PKD kidney and are thought to play a role in cyst development. Cyclic-AMP is produced by adenylyl cyclases (ACs), enzymes shown to stimulate cilia growth. Prior research has shown that lithium, a known inhibitor of ACs, increases cilia length in the brain. Speculating that lithium could elongate cilia in the IMCD, we treated mIMCD3-SSTR3-GFP cells (a mouse IMCD cell line stably expressing a GFP-tagged cilia marker) with increasing concentrations of lithium and found that cilia length is increased correspondingly. To determine if this observation is replicated physiologically, Sprague-Dawley rats were treated with or without lithium for 14 days. Kidneys were then perfusion fixed and cilia were detected by immunofluorescence staining. Cilia length in the IMCD was increased in lithium-treated rats (~30 %). Protein expression of Arl13b, a GTPase whose overexpression is associated with cilia elongation, was significantly increased in the renal inner medulla of lithium-treated Sprague-Dawley rats. Urinary cAMP levels were dramatically decreased in lithium-treated Sprague-Dawley rats compared to controls suggesting that lithium action in the IMCD occurs through inhibition of cAMP synthesis. Treating mIMCD3-SSTR3-GFP cells with a specific inhibitor for AC, SQ-22536, in the absence of lithium increased cilia-length to the same extent of lithium-treatment alone; however, lithium-mediated cilia elongation was attenuated when ACs were stimulated with forskolin or a cAMP analogue, dibutyryl cAMP, was added. Incubating cells with SQ-22536 and lithium did not have

an additive effect. Collectively the data suggest that lithium-mediated cilia elongation in the IMCD occurs in a cAMP-dependent manner.

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Introduction

Polycystic Kidney Disease (PKD) affects nearly 600,000 individuals and has no current cure [1]. The hallmarks of PKD include large, fluid-filled cysts derived from the inner medullary collecting duct (IMCD) of the kidney, elevated cyclic AMP (cAMP) levels, and stunted or null cilia [2]. As the disease progresses, the kidney becomes massively enlarged and covered in cysts – a dramatic transformation in appearance. The deadliness of the disease lies within cyst formation and enlargement: because of this, rather than solely affecting kidney function, PKD may cause damage to the surrounding organs of the individual affected. As the cysts increase in size over the course of time, they displace and destroy the surrounding renal parenchyma. This destruction is why approximately 50% of PKD individuals develop end-stage renal disease, which can then only be treated with dialysis or kidney transplantation [3]. This progression to renal disease typically affects middle-aged or elderly patients [4]. PKD can also be the catalyst behind similar diseases of surrounding organ and tissue systems, such as liver cyst formation, which develops in more than 80% of PKD patients [1].

Renal Physiology

In order to understand how renal function is compromised by advancing PKD, knowledge of kidney morphology and physiology is necessary. The kidney's crucial functions include regulating water and electrolyte balance, excreting metabolic waste, regulating arterial blood pressure, regulating red blood cell production, vitamin D production, and gluconeogenesis [5]. Macroscopically, kidneys are organized into three layers, starting with the outermost and the least hypertonic cortex, then the outer medulla, and finally the innermost and the most hypertonic inner medulla (Figure 1). Looking more closely, the kidney is composed of functional units

called nephrons; each nephron consists of a spherical filtering component called the renal corpuscle or glomerulus and a tubule extending from it [5]. The renal corpuscle separates a protein-free filtrate from plasma, and the filtrate continues its journey in the tubules, which are made up of a single layer of epithelial cells resting on a basement membrane. The tubules are subdivided into proximal, intermediate, and distal segments (see color key in Figure 1) that are important for the recovery and modification of the glomerular filtrate. Filtrate leaving the glomerulus first enters the proximal tubule. The proximal tubule is responsible for reabsorbing the majority of the filtrate to prevent loss of metabolically useful solutes like glucose [6]. The intermediate and the distal segments of the tubule further fine-tune the composition of the filtrate and play an important role in regulating potassium, calcium, and magnesium homeostasis, as well as water absorption. To achieve these functions, each segment of the tubule has a distinct cellular morphology and expresses unique sets of solute transporters. Finally, the modified filtrate flows into the collecting duct, where the final regulation of electrolyte balance and water absorption occurs [7]. As shown in Figure 1, the long tubules traverse the three renal layers. Because an osmotic gradient exists between the cortex and medulla, there is a strong osmotic drive to extract water from the filtrate into the deep hypertonic inner medulla, ultimately producing concentrated urine [7].

Although the initial cellular event that stimulates cystogenesis remains uncertain, we do know that renal cysts are derived from tubular epithelial cells and PKD cysts in particular are localized to the collecting duct (Figure 2) [2]. Uncontrolled cyst growth causes focal expansions of the tubule epithelium into “blister like” structures that eventually pinch off to form isolated structures that continue to expand in size [3]. The macroscopic consequence of this is that the formation of fluid-filled cysts disrupts the normally compact arrangement of tubules in a healthy

kidney in addition to replacing functional kidney tubules. By displacing and destroying the adjacent renal parenchyma, the progressive cyst development associated with PKD can ultimately lead to end-stage renal disease [3].

Genetics and Development

PKD is a group of genetic disorders characterized by the growth of numerous cysts in the kidney. PKD can be inherited as an autosomal dominant trait (ADPKD) or an autosomal recessive trait (ARPKD). ADPKD, which affects all ethnic groups worldwide with an incidence of 1 in 500 to 1 in 1000, is the most common potentially lethal genetic disorder in humans [8]. Mutations in PKD1, the gene encoding polycystin-1, and PKD2, the gene encoding polycystin-2, have been identified as the cause of ADPKD [9]. Approximately 85% of ADPKD cases are due to defects in polycystin-1, while the remaining 15% of cases are caused by mutations in polycystin-2 [10]. Under normal conditions, the polycystins form multi-protein complexes that play important roles in renal tubular differentiation, cell proliferation, and apoptosis as well as cilia action and calcium channeling [1, 8, 10, 11]. Coordination of these acts is important for the maintenance and regulation of renal epithelial cell growth and function. In human development, PKD can manifest itself in the forms of either lost polarity due to improper tubule cell differentiation or over-proliferation of the tubule epithelium. Complications with lost polarity result in the misplacement of Na^+/K^+ channels, which, given normal embryogenesis, are located in the basal domain of tubules cells. Over-proliferation of tubule epithelium can result in blockage of the tubule lumen [12].

ADPKD is a focal disease that involves only a small fraction of cells in the kidney even though all cells carry one copy of the mutated gene. A two-hit model of cystogenesis is the

proposed mechanism to explain the focal nature of cyst formation (Figure 3) [13]. In this model, a mutated *PKD1* (or *PKD2*) gene is inherited from the affected parent and a wild-type gene is inherited from the unaffected parent. At some point during the lifetime of the resulting offspring, the wild-type gene undergoes a somatic mutation and becomes inactivated. This results in a complete loss of *PKD1* (or *PKD2*) in the cell and initiates cyst formation. Because such somatic mutations are rare and occur in relatively few tubular epithelial cells, the formation of cysts are focal in the nephron. Thus, while the pattern of inheritance is dominant, progression of ADPKD occurs through a molecular recessive mechanism. In fact, no humans with homozygous germline mutations of either *PKD1* or *PKD2* have been observed, presumably because homozygosity is lethal to embryos [14].

Although it is established that the loss of the genes that encode for polycystin-1 and polycystin-2 leads to cyst formation, the precise mechanism remains unclear. Identification of both polycystin-1 and polycystin-2 in the primary cilia of mammalian renal epithelial cells have led to the emergence of the idea that the renal primary cilium is the central cellular compartment in the pathogenesis of cystic kidney diseases [15].

Cilia: Function

Cilia can be divided into two groups: motile cilia and immotile cilia. Immotile cilia, also known as primary cilia, share many structural similarities to motile cilia (Figure 4). Both primary cilia and motile cilia arise from a basal body consisting of a membrane-bound ciliary axoneme. The ciliary axoneme of the primary cilium is comprised of nine doublet microtubule bundles, but lacks the central microtubule pair (the '9+0' structure). Primary cilia also lack dynein arms and other affiliated structures seen in motile cilia (including the '9 + 2' structure).

Far from being a vestigial organ as they were once thought to be, the primary cilia are now credited as essential constituents of chemosensation and mechanosensation pathways – essential tools in cellular environmental interaction [16]. Their ability to detect changes in osmolality and fluid flow make it so that functional primary cilia are necessary in renal tubular cell function, particularly in the collecting duct cells [17].

The importance of primary cilia structure and function was realized by the discovery that sensory ciliary dysfunction results from several genetically heterogeneous disorders, or ciliopathies [18]. Ciliopathies present with overlapping clinical features including renal cyst development as the most frequent symptom (Table 1) [19-27]. Abnormal primary cilia, particularly stunted primary cilia, are linked to PKD, the most common of the known ciliopathies [16]. Alterations in cilia length observed in these disorders suggest that maintaining cilia length plays a major role in maintaining a healthy cellular state. Besides ciliopathies, renal cilium length is dramatically increased in response to renal injury [28]. Although this suggests that injury-induced elongation of the cilia triggers a compartmentalized signaling effect to restore a functional epithelial layer along the nephron, the mechanisms involved in cilia length and function remain largely unknown.

Primary Cilia and PKD

Renal cilia project from the apical surface of the kidney epithelium and into the lumen of the renal tubule. Their essential sensory role is conserved across a variety of species. This was shown in a study using *C. elegans* in which homologs of human polycystin-1 and -2 localized to sensory neuron cilia. Additionally, while loss of polycystin function did not alter cilia formation in *C. elegans*, there was a change in responses of cilia-regulated systems [16, 29]. Another study

found that, using *C. elegans* as a model, the root of cystogenesis in the Oak Ridge Polycystic Kindey mouse ($Tg737^{orpk}$) was due to a mutation in the IFT88 gene [30, 31]. The importance of this study lies in the fact that cilia are incapable of performing protein synthesis—thus, the transfer of proteins by intraflagellar transport (or IFT) is essential to their formation and maintenance [30]. Cilia and polycystin-1 are also responsible for regulation of the mechanistic target of rapamycin (mTOR). This finding is significant since elevated mTOR levels coupled with polycystin-1 mutations have been observed in rodent PKD models [32]. Despite focusing on other PKD pathways, the findings of these studies support the link between the polycystin genes, renal cilia function, and PKD.

Cilia: Length-Regulation Mechanism

The enzyme adenylyl cyclase (AC) regulates the elongation of primary cilia in synoviocytes through the production of the second messenger cyclic AMP (cAMP), which is localized to primary cilia [33]. Cyclic AMP is known to stimulate secretion of transepithelial fluids. Elevated cAMP production by AC is also associated with stunted or absent cilia [2].

Calcium ion levels play a role in cilia length regulation as well through the regulation of cAMP levels within renal epithelial cells (Figure 5). In normal kidney epithelial cell function, as urine flows over the primary cilia, bending of the cilia occurs. Bending of the cilia results in an increase in the calcium ion concentration within the cell, and in turn results in inhibition of cAMP levels. As a result of PKD kidney epithelial cells presenting with stunted cilia, urine is unable to bend the cilia. Thus, calcium ion concentration does not increase, and cAMP levels

rise unchecked. Together, these factors result in the renal cyst development observed in PKD in patients and in PKD animal models such as the Oak Ridge Polycystic Kidney Mouse [16].

The Use of Lithium as a Treatment for PKD

Lithium has been used clinically as an effective treatment for mental illnesses such as bipolar disorder and severe depression [34]. Interestingly, lithium has been shown to increase the length of primary cilia in the brain as observed in patients taking lithium as a psychological treatment [35]. Lithium is incredibly versatile due to its chemical structure and is able to reach many targets. As a result, lithium is able to induce changes in cellular shape and structure [33]. The link between lithium treatment and an increase in cilia length has piqued the interest of many researchers interested in cilia as a treatment for ciliopathies caused by protein mutations in primary cilia in which the cilia are either absent or stunted [18]. In the case of PKD, lithium has been documented as an inhibitor of AC by disrupting the process in which primary cilia cease to grow after reaching a certain length [16].

Lithium-Mediated Elongation: the Approach

We hypothesize that lithium will lead to cilia elongation by blunting cAMP levels (as modeled in Figure 6). Our approach is twofold. First, we will determine if lithium chloride increases cilia length in the inner medullary collecting duct (IMCD), the primary site for cyst formation in PKD. Then, we will examine if lithium treatment alters cAMP production in the IMCD.

Materials and Methods

Experimental animals and protocols

All animal protocols were approved by the Emory University Institutional Animal Care and Use Committee (IACUC). For these studies we used aged-matched (8 weeks), male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA). Sprague-Dawley rats were pair-fed standard diet (containing 23% protein) supplemented with Li_2CO_3 (40 mmol/kg; Harlan Teklad, Madison, WI) for 14 days. Sprague-Dawley rats pair-fed an un-supplemented, standard diet were used as controls for this study. All rats were given free access to tap water and a salt block to maintain sodium balance and prevent lithium intoxication. Rats were housed individually in metabolic cages for 24 hours on day 13 of lithium treatment. Urine volume, food intake and water intake were measured. Animals were sacrificed on day 14 of the study for further investigation.

Urine analysis

Urinary cAMP was measured by EIA following the manufactures' instructions (Cayman Chemical, Ann Arbor, MI).

Tissue preparation for immunofluorescence

In accordance with IACUC-approved protocols, animals were initially anesthetized with isofluorane (Hospira, Lake Forest, IL) in a chamber and then transferred to anesthesia by nose piece for the remainder of the perfusion process. The rat was opened with an abdominal incision projecting to the sternum. The diaphragm was cut to expose the abdominal aorta, and a 21-gauge needle was inserted in the aorta. The vasculature was perfused with 1× PBS solution for 1-to-2

minutes, or until the kidney changed color from red/brown to beige/white by the removal of the blood. Then, both kidneys were perfusion fixed with 4% paraformaldehyde for 7 additional minutes. The kidneys were cut transversely into thick slices (3 - 4 mm) and placed in 4% paraformaldehyde overnight at 4°C.

Tissue blocks from whole kidneys were dehydrated in a graded series of ethanol (2 hours each in 70, 96, and 99%, respectively) and xylene (overnight). The perfusion-fixed kidneys were subjected to paraffin embedding. Paraffin sections (5 μm thick) were cut on a Leica microtome and dried overnight at 37°C.

Immunofluorescence

Paraffin sections were placed in xylene for 24 hours and then were washed with 100% ethyl alcohol (EtOH) three times for ten minutes each, and then 95% EtOH twice for ten minutes each time. To quench with endogenous peroxides, the sections were treated with 3% H_2O_2 for 30 minutes at room temperature, then rinsed in 95% EtOH, and 75% EtOH once for five minutes. They were then rinsed in distilled water. For antigen retrieval with TEG buffer, the slides were immersed in TEG buffer and microwaved for 10 minutes at maximum power, and then microwaved again 5 minutes at 60% power and left to cool in TEG buffer for 15 minutes. The slides were then incubated in 50 mM NH_4Cl/PBS for 30 minutes at room temperature, and then in pure PBS. Next, the slides were removed, wiped, and the tissue was circled with a PAP pen. The slides were blocked with 1% BSA/PBS for 30 minutes in a humidity chamber, after which the blocking buffer was removed by suction and replaced with primary antibody Arl13b (gift of Tamara Caspary, Emory University) diluted in 1% BSA/0.1% Triton X/PBS, using 40 μL per section, and left overnight at 4°C. The slides were incubated in Alexa-conjugated secondary

antibody (anti-mouse Alexa Fluor 488) in 1% BSA/0.1% Triton X/PBS and left in a humidity chamber for 2 hours at room temperature in a dark location. An antibody against the water channel aquaporin-4 (AQP4) (Santa Cruz) in conjunction with a secondary anti-rabbit Alexa Fluor 647 antibody (Invitrogen) was used as a marker for the collecting duct. The slides were then washed with PBS three times at 5 minutes each. To visualize nuclei, sections were mounted in Prolong Gold antifade medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Molecular Probes).

Images of cilia completely contained in a single plane of focus were captured from randomly chosen high-power fields (100×, 92 × 62 μm) on a fluorescence microscope (Olympus) and measured using the Olympus cellSens™ Microscope Imaging Software. For each rat, at least 20 cilia were measured per segment.

Cell culture and cilia length measurements

To visualize cilia elongation, we used a mouse inner medullary collecting duct (mIMCD3) cell line that stably expressed somatostatin receptor 3 (SSTR3)-GFP, a ciliary membrane marker (gift of Criss Hartzell, Emory University). mIMCD3-SSTR3-GFP cells were first grown to 70-80% confluence in 2 mL of Ham's DMEM/F-12 media in 35 mm glass bottom culture dishes *(*MatTek*,* Ashland, MA). Cells were then treated with as described in the text for 24 hours. Following treatment, media was removed and cells were washed quickly with PBS. 2 mL of 25 mM HEPES in Hank's Balanced Salt Solution (HBSS) was added to the cells as a visualization media. Images of cilia were collected on an Olympus IX70 Inverted Microscope at 60X magnification. Using the CellSense software, cilia length was measured for 20 cilia randomly located per plate.

Sample preparation for Western blot analysis

Tissue: Kidneys were removed and dissected into inner medulla (IM) and outer medulla (OM) at 4°C. Fresh kidney tissues were homogenized in a glass tissue grinder in ice-cold isolation buffer (10 mM triethanolamine, 250 mM sucrose, pH 7.6, 1 μg/ml leupeptin, and 2 mg/ml PMSF), and then SDS was added to a final concentration of 1% for Western blot analysis of the total cell lysate. After centrifugation at 13,000 rpm for 20 minutes at 4°C, the protein concentration was determined by a modified Lowry method (Bio-Rad DC protein assay kit, Hercules, CA). The appropriate amount of each sample was diluted in a Tris-glycine/SDS sample buffer (125 mM Tris, pH 6.8, 4% SDS, 10% glycerol, 10% β-mercaptoethanol, 0.02% bromophenol blue) and heated at 100°C for 2 minutes.

Cell: After sufficient images were collected, the HEPES/HBSS buffer was removed and 300 μL of ice-cold RIPA solution was added to the plate. Cells were scraped, collected into individual microfuge tubes and homegoized. After centrifugation at 13,000 rpm for 20 minutes at 4°C, the protein concentration was determined by a modified Lowry method (Bio-Rad DC protein assay kit, Hercules, CA). The appropriate amount of each sample was diluted in a Tris-glycine/SDS sample buffer (125 mM Tris, pH 6.8, 4% SDS, 10% glycerol, 10% β-mercaptoethanol, 0.02% bromophenol blue) and heated at 100°C for 2 minutes.

Western blot analysis

Proteins (20 μg/lane) were size-separated by SDS-PAGE on 10% gels and then electroblotted to polyvinylidene difluoride membranes (Immobilon, Millipore, Bedford, MA). After being blocked with 5% nonfat dry milk for 1 hour, blots were incubated with the indicated

primary antibody overnight at 4°C. Blots were washed three times in tris-buffered saline with 0.5% Tween 20 and then incubated for 2 hours with Alexa Fluor 680-linked anti-rabbit IgG (Molecular Probes, Eugene, OR). Bound secondary antibody was visualized using infrared detection with the LICOR Odyssey protein analysis system (Lincoln, NE) and densitometry of the desired band was collected. Blots were then stained with Coomassie blue to determine protein loading. Bands were quantitated using Image J. The densitometry values collected from the coomassie staining were used to normalize Western blot data.

Results and Discussion

Lithium elongates cilia of the inner medullary collecting duct in vitro and in vivo.

To determine if lithium increases cilia length in renal inner medulla, we first measured the effect of increasing concentrations of lithium chloride using an mIMCD3 stable cell line expressing SSTR3-GFP, a ciliary membrane marker. Cells were grown to confluence in glass-bottomed dishes to allow for visualization of the GFP-labeled cilia with fluorescence microscopy. Lithium was added to cells at increasing concentrations 24 hours prior to collecting live images. For each plate, 3 images were collected in non-overlapping regions of the plate all at 60X. For every cilia in the captured frame, length was measured using Olympus cellSens™ Microscope Imaging Software. This experiment was repeated three times in this manner. We found that treating the cells with 5 mM lithium did not significantly increases cilia length; however, treating cells with 10, 25 and 50 mM lithium significantly increased cilia length over control levels. Treating cells with 100 mM lithium was apparently toxic to cilia growth as no cilia could be observed despite the presence of a confluent cell monolayer (Figure 7).

Next, we wanted to see if lithium elongates cilia in the collecting duct of lithium-treated animals. We collected kidneys from Sprague-Dawley rats that were fed either a standard diet or lithium diet for 14 days. To identify cilia, we probed the tissues with the known cilia marker, Arl13b. We focused on cilia of the IMCD because PKD cysts are typically derived from this region. Shown are two representative images from either control or lithium-treated rats with arrows pointing to cilia (Figure 8A). Images were collected from 3 non-overlapping areas and cilia were measured from 40X images. We looked at four rats from each group in total and found that cilia length was significantly increased with lithium treatment (Figure 8B).

Collectively our data reveal that lithium increases IMCD cell cilia length in both cells and rat models.

Lithium treatment in rats increased total cilia protein Arl13b.

Arl13b is a small GTPase and is involved many diverse of cellular processes. Because Arl13b positively regulates cilia length, Arl13b overexpression is associated with an increase in cilia elongation [36]. We performed a Western blot utilizing the antibody Arl13b on inner medullary tissues isolated from the kidneys of control Sprague-Dawley rats and Sprague-Dawley rats treated with lithium for 14 days. The Western blot showed that in the lanes where the samples for the lithium rats are placed, there is greater protein density of Arl13b (Figure 9a). We concluded that there was a statistically significant increase in the amount of cilia marker protein in the lithium treated rat inner medulla tissue (Figure 9B). This increase in Arl13b expression of lithium-treated animals suggests that the increase in this protein may play a role in the observed increase in cilia length.

Lithium treatment in rats decreases urinary cyclic AMP levels.

It has been observed that in rodent models that urinary cAMP levels closely reflect collecting duct cAMP levels [37]. Measuring urinary cAMP allowed us to determine if lithium treatment affects cAMP synthesis in the entire rodent. We collected urine for 24 hours from Sprague-Dawley rats fed a standard or lithium diet for 14 days. We then measured cAMP levels using an enzyme-linked immunoassay (EIA; Cayman Chemical, Ann Arbor, MI). We found that lithium treatment decreases urinary cAMP content, indicating that lithium does inhibit cAMP synthesis in the renal collecting duct (Figure 10).

Lithium-mediated inhibition of adenylyl cyclase activity increases cilia length.

In cells with normal cilia, adenylyl cyclase (AC) produces cAMP. This is a means by which AC can regulate cilia length depending on the amount of cAMP produced by the enzyme. To see if AC inhibition is indeed a way to increase cilia length, we treated mIMCD3 SSTR3-GFP cells with an AC inhibitor, an AC activator, and a cAMP analogue for 24 hours in the absence and presence of 25 mM lithium chloride (Figure 11). We then used fluorescent microscopy to visualize cilia and took three non-overlapping images of each plate. Cilia were measured using the Olympus cellSens software and compared to the vehicle-treated group. We found that when mIMCD cells where treated with SQ-22536, a specific inhibitor for AC, cilia length was significantly increased. Stimulating AC with forskolin (an adenylyl cyclase activator) or adding a cAMP analogue (dibutyryl-cAMP) had no effect on cilia length. Lithium-treatment alone increased cilia length significantly relative to the control group. Inhibiting AC with SQ-22536 in the presence of lithium also resulted in elongated cilia; however, the addition of the AC-specific inhibitor did not increase cilia length any more than SQ-22536- or lithium-treatment alone. This indicates that there is no synergistic response from multiple modes of AC inhibition. When AC activity is stimulated with forskolin despite the presence of lithium, cilia remain at a normal length. Furthermore, adding a cAMP-analog to mimic cAMP production in the presence of lithium does not change the normal length of cilia. Together these data show that lithium elongation of cilia involves dampening the cAMP pathway and not another contaminant pathway.

Conclusions

In conclusion, we have shown that lithium increases IMCD cilia length *in vitro* and *in vivo*. Because we observed similar findings in both model systems, when can extrapolate that flow rate does not affect cilia length in the IMCD as measurements collected in vitro were performed under static conditions. Furthermore, we found that lithium regulation of cilia length in this system is due to the dampening cAMP synthesis through lithium-mediated inhibition of native adenylyl cyclases. Although lithium has many other downstream targets, alterations of cilia length by lithium are strictly mediated though regulation of the second messenger cAMP.

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Table 1. *Common clinical features of ciliopathies.* Many similar phenotypes regularly associate with the pathology of various ciliopathies. The most commonly effected organ is the kidney followed by the pancreas and liver, all of which display cyst formation. Human biopsies, mouse models and cell culture studies have indicated that disruption of cilia length is associated with each disease.

Ciliopathy	Renal Cysts	Hepatobiliary Disease	Cilia Length
Bardet-Biedl syndrome	✓	✓	short/null [19]
Meckel syndrome	✓	✓	long [20]
Joubert syndrome	✓	✓	short/null [21]
Nephronophthisis	✓	✓	long [22]
Senior-Løken syndrome	✓	✓	null [23]
Jeune syndrome	✓	✓	short/null [24]
Oro-facial-digital syndrome type 1	✓	✓	null [25]
Alström syndrome	✓	✓	short [26]
Polycystic kidney disease	✓	✓	short/null [27]

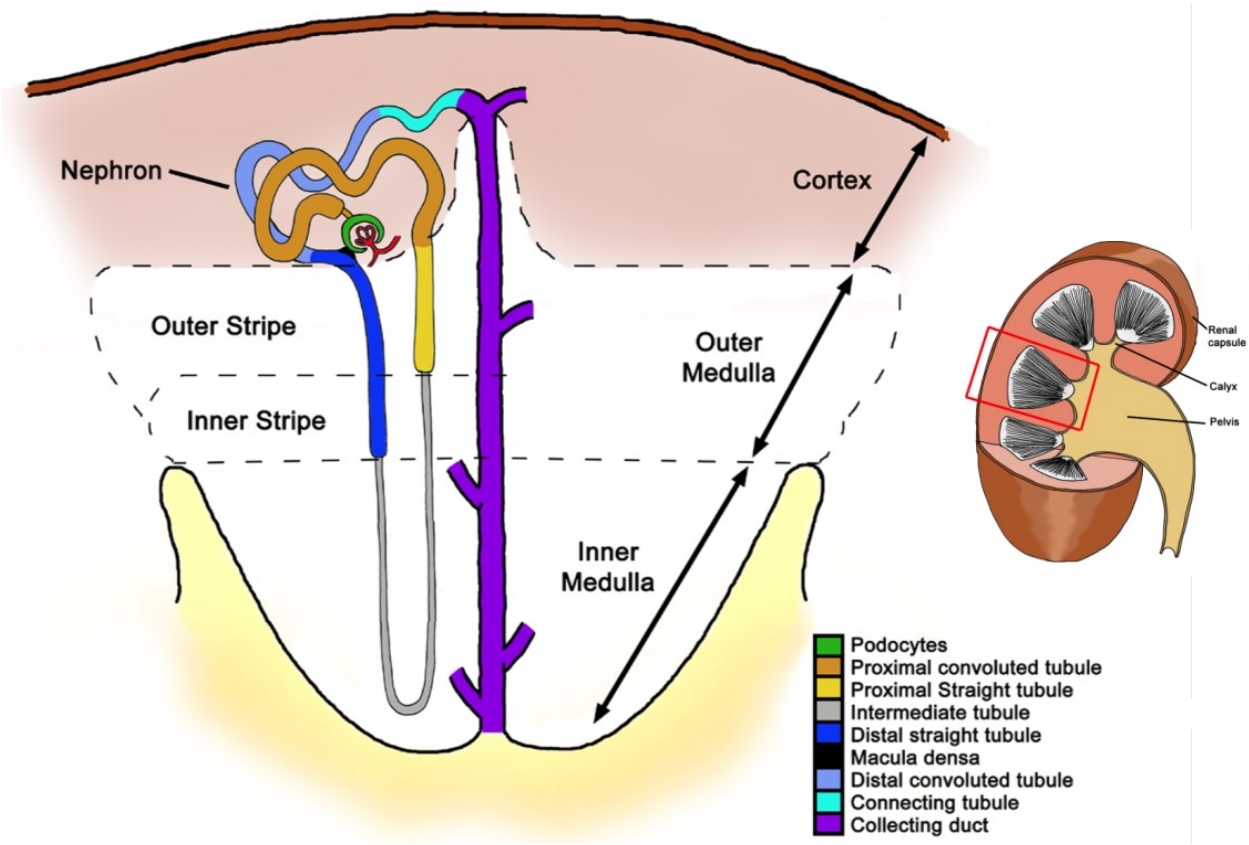

Figure 1. *Structure of the mammalian kidney.* Each kidney is comprised of a peripheral layer called the cortex, the middle layer the outer medulla, and an inner layer called the inner medulla. Nephrons are found within the cortex and medulla and have a characteristic structure that includes a glomerular blood filter containing podocytes and a tubular epithelium that loops down into the medulla. The tubule is subdivided into proximal, intermediate, and distal segments (see color key) that are important for the recovery and modification of the glomerular filtrate. Figure modified from unidentifiable source.

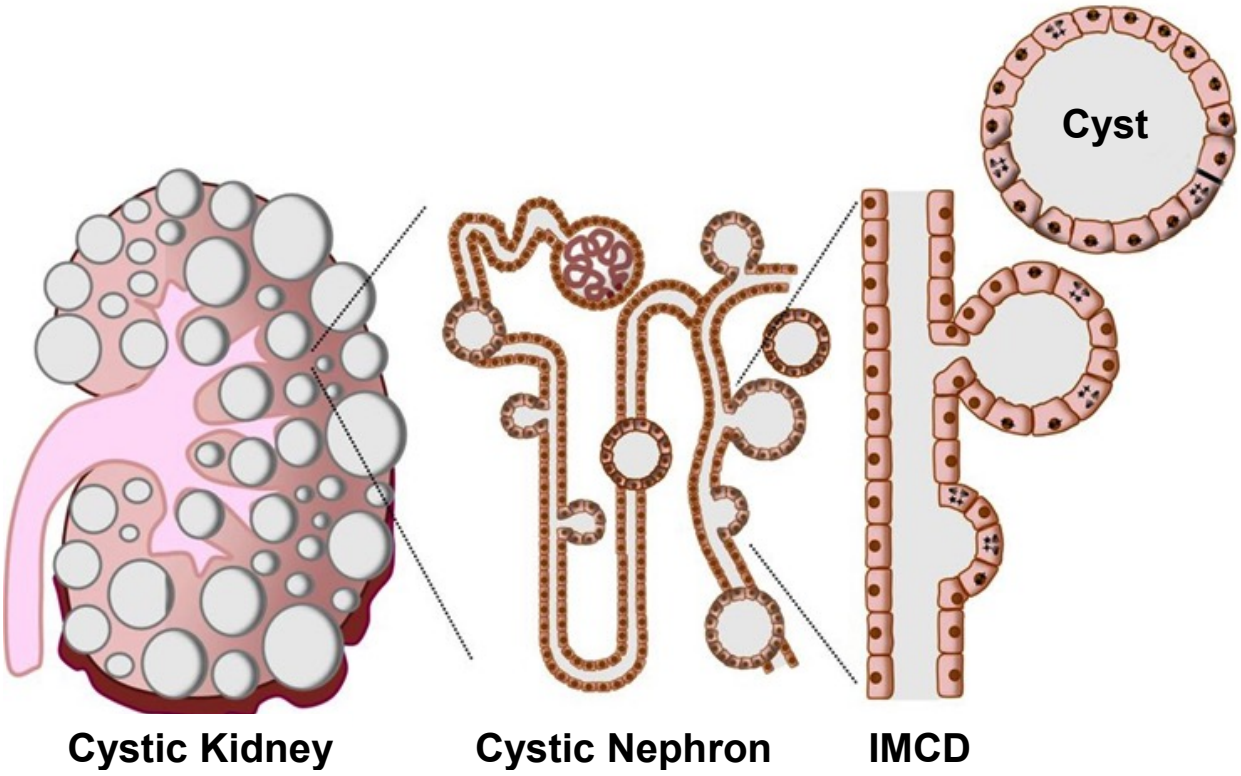

Figure 2. *Evolution of cysts from epithelial cells of the renal tubules.* Cystic kidneys (*left*), result from the growth of multiple fluid-filled cysts that originate in the renal tubules (*middle*) and continue developing through different pathophysiological mechanisms (*right*). Cysts evolve from individual cells ultimately leading to the development of blister-like structures. Once the cysts separate from the nephron that originated them, their collective effect leads to the displacement of the normal renal parenchyma and the formation of a cyst-filled kidney with reduced functional capacity. Figure modified from Blanco G, et al., 2013 [38].

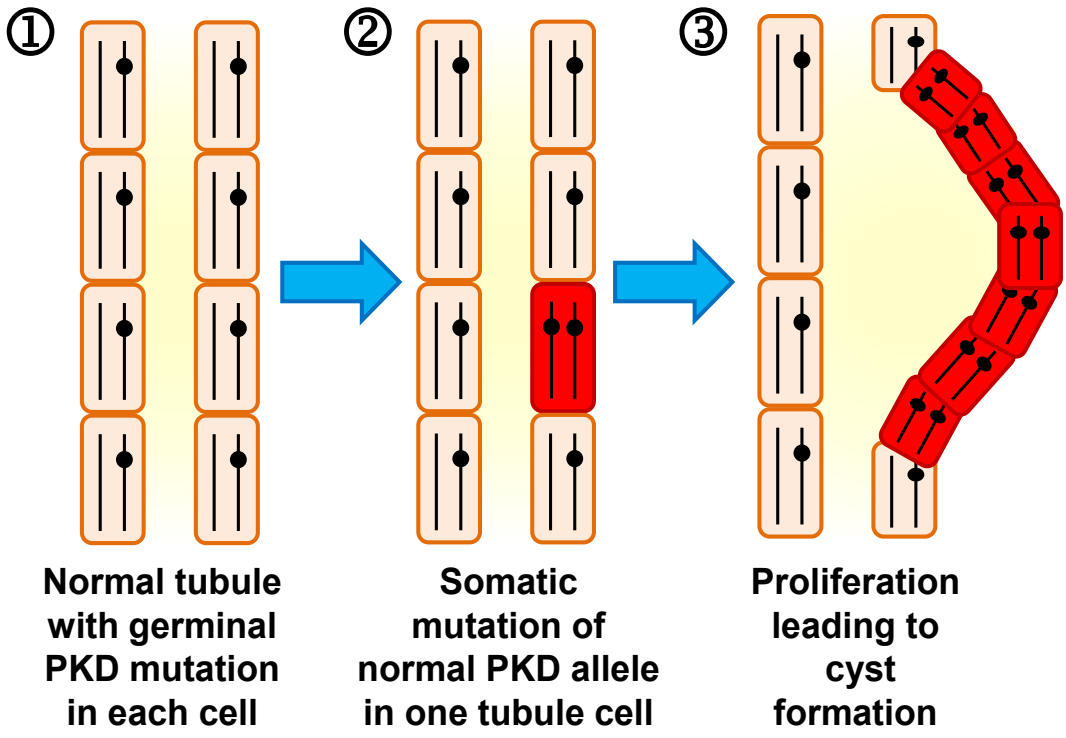

Figure 3. *The two-hit hypothesis of renal cyst formation.* (1) In non-cystic renal tubular epithelial cells, a single germline mutation in either *PKD1* or *PKD2* is present and the epithelium appears normal. (2) In cystic epithelial cells, a somatic mutation, or 'second hit' has occurred in the other *PKD* gene allele, resulting in the complete loss of normal *PKD* gene function and (3) the development of cystic epithelium.

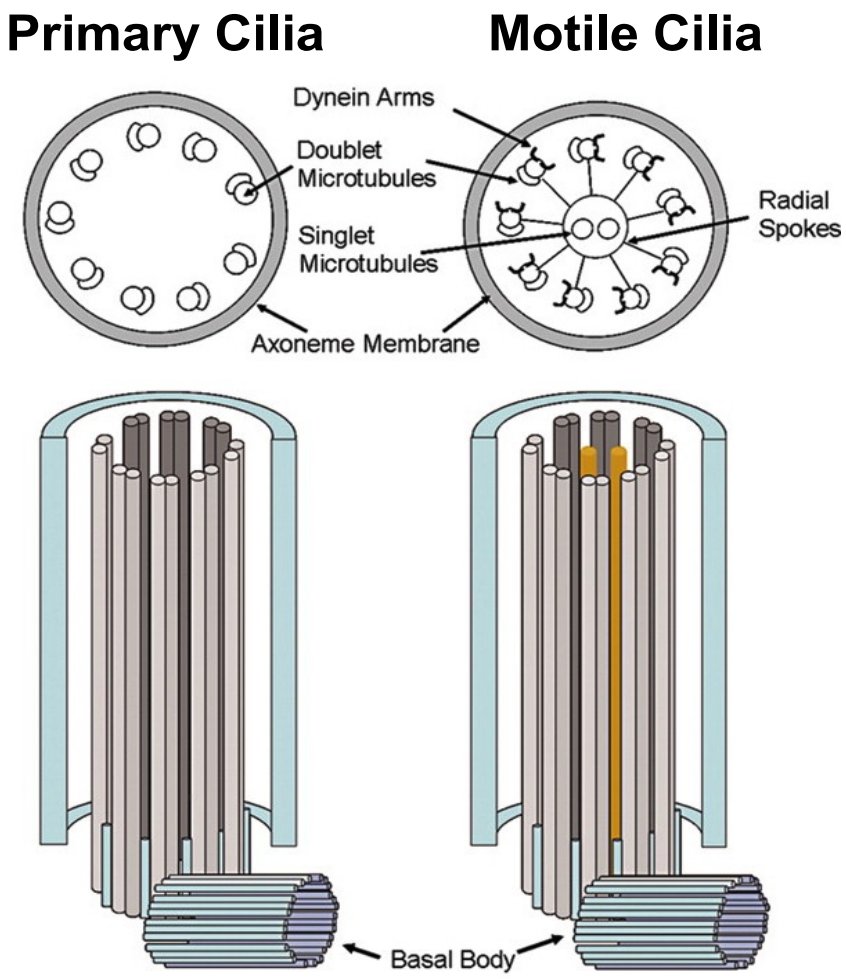

Figure 4. *Structures of primary cilia and motile cilia.* Motile cilia (right) consist of 9 doublet microtubules surrounding 2 inner singlet microtubules used to conduct force. Primary cilia (left) are lacking both singlet microtubules and dynein arms. Figure modified from Davenport JR, et al., 2005 [39].

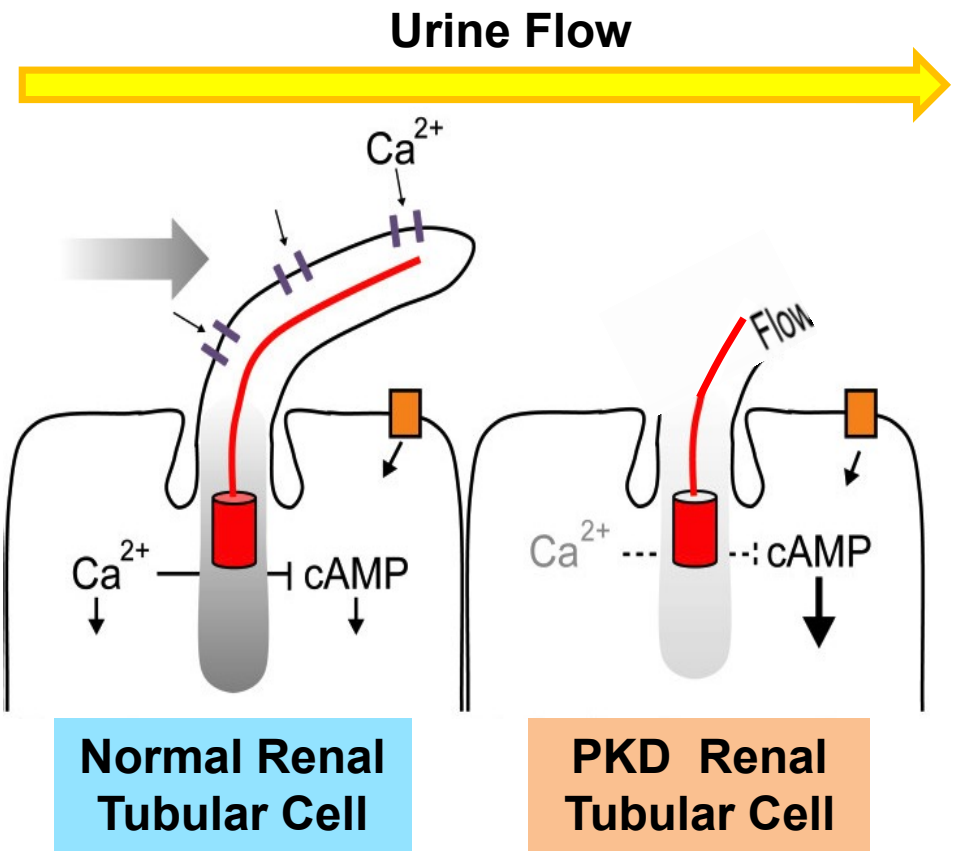

Figure 5. *Flow-regulated cilia bending controls second messenger systems in renal tubular cells.* Flow induces cilia bending, causing an increase of intracellular Ca^{2+} levels that dampens cAMP levels in a normal renal tubular epithelial cell (left). In PKD, shortened cilia fail to elevate intracellular Ca^{2+} because the mechanosensation is perturbed, leading to elevated cAMP and eventually cyst formation (right).

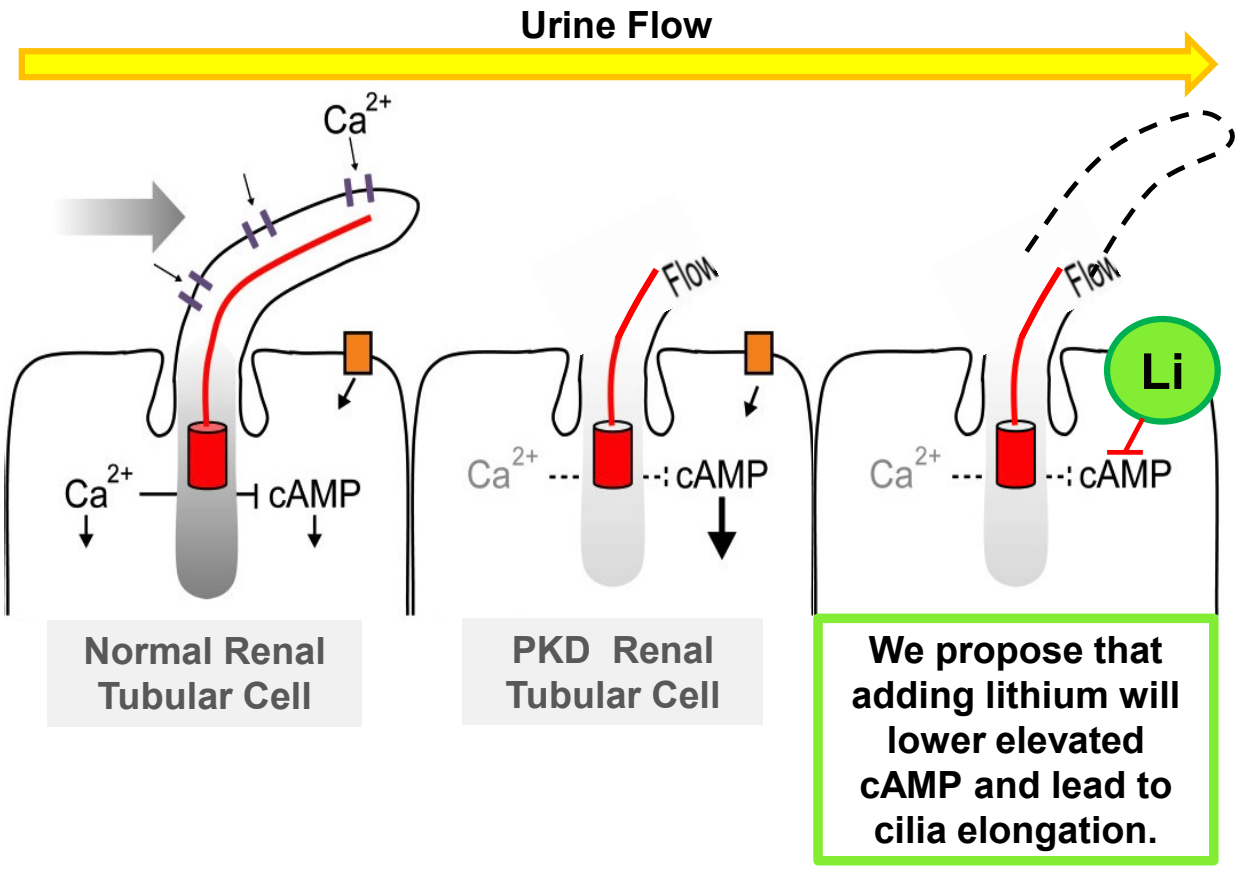

Figure 6. *Hypothetical model of proposal.* Treating renal cells with lithium will lower cAMP and lead to cilia elongation. This could ultimately alleviate advancing cyst development in PKD patients.

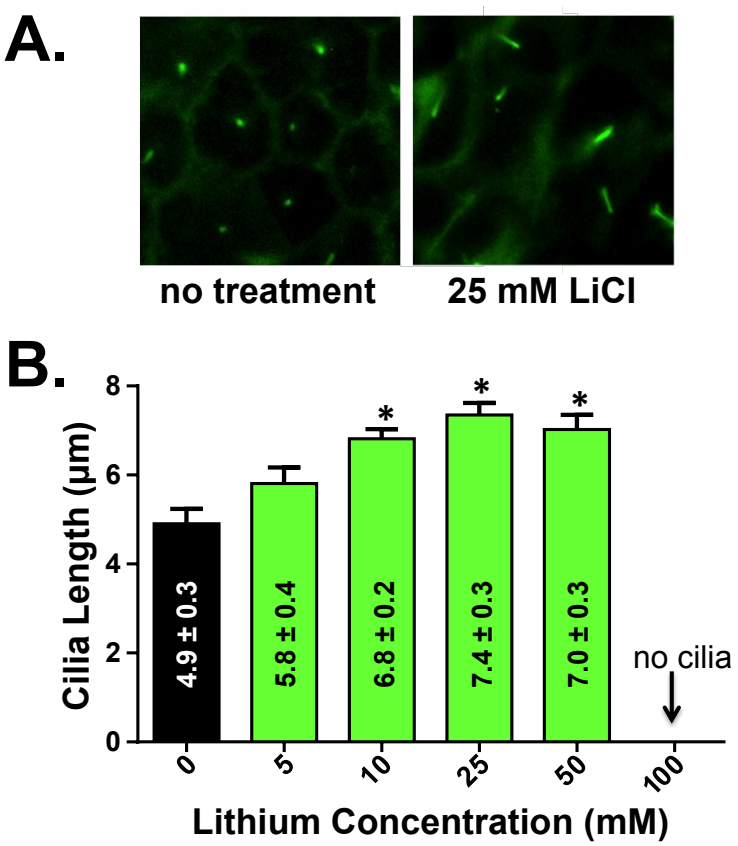

Figure 7. *Increasing lithium concentrations elongate cilia.* mIMCD3-SSTR3-GFP cells were grown to confluence in glass bottomed dishes to allow for visualization of the GFP-labeled cilia with fluorescence microscopy. Cells were then treated either with vehicle (0), 5, 10, 25, 50 or 100 mM lithium chloride (LiCl) for 24 h. A: Shown are representative pictures of cilia after no treatment and 24-h exposure to 25 mM LiCl. B: Combined analysis of all measured lengths. Data are ± SE following ANOVA where *=$p<0.05$ compared to control (n=3; 20 cilia measured per n).

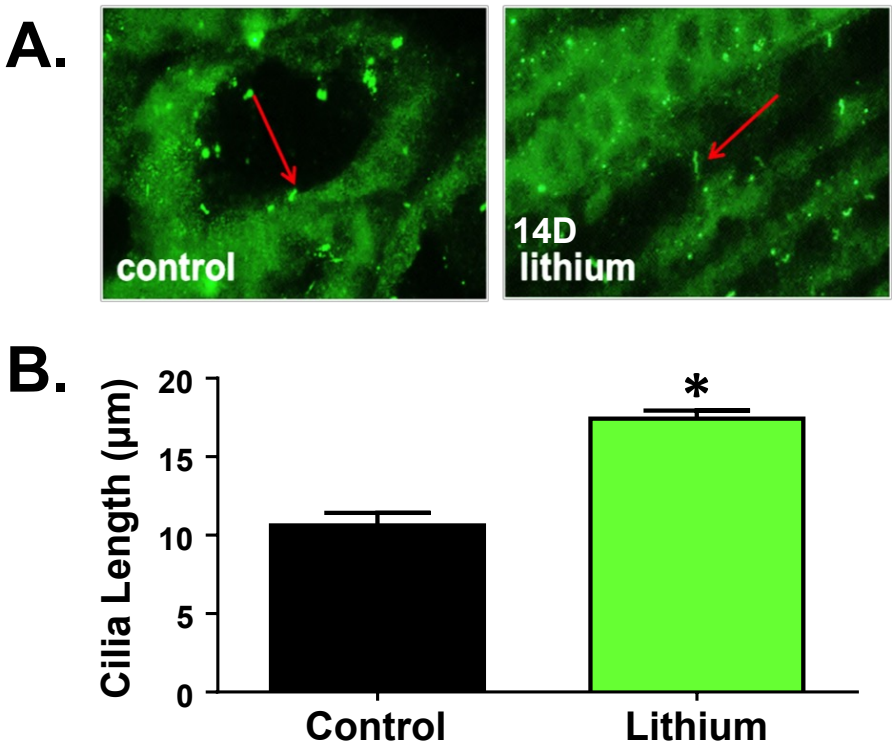

Figure 8. *Increasing lithium concentrations elongate cilia in the rat IMCD.* Male Sprague-Dawley rats (8-weeks old) were pair-fed either a standard chow diet (control) or a diet supplemented with Li_2CO_3 (40 mmol/kg) for 14 days (14D lithium). Kidneys were collected for immunohistochemistry via perfusion fixation. Paraffin embedded-sections were probed with an Arl13b antibody (a gift from Dr. Tamara Caspary, Emory University) to locate cilia in the inner medulla. A: Shown are representative pictures of Arl13b-positive cilia in the IMCD of Sprague-Dawley rats after no treatment and day 14 of lithium feeding. B: Combined analysis of all measured lengths. Data are ± SE following student t-test where *=$p<0.05$ compared to control (n=4; 20 cilia measured per n).

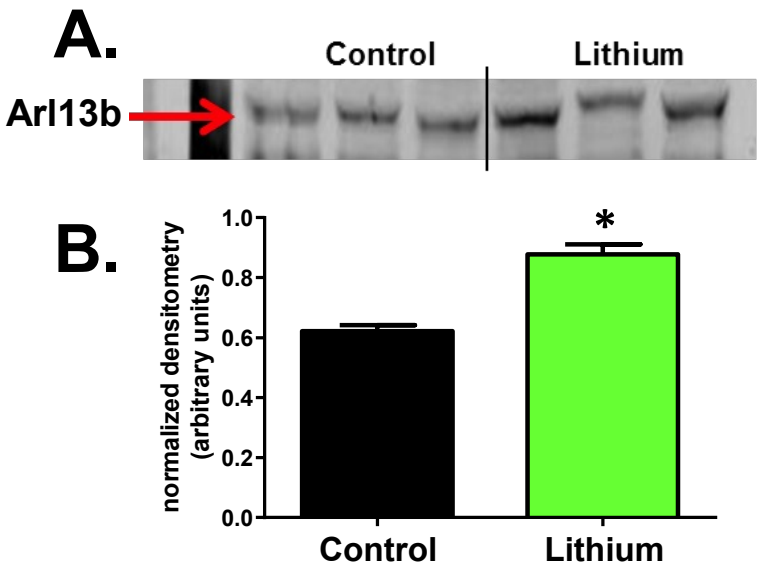

Figure 9. *Lithium treatment increases Arl13b expression in the inner medulla.* Male Sprague-Dawley rats (8-weeks old) were pair-fed either a standard chow diet (control) or a diet supplemented with Li_2CO_3 (40 mmol/kg) for 14 days (lithium). Kidneys were harvested and the inner medulla was dissected from the tissue and processed for Western blot analysis. A: Shown is a representative immunoblot for Arl13b in the isolated inner medulla from Sprague-Dawley rats after no treatment and day 14 of lithium feeding. B: Combined analysis of Arl13b abundance following normalization to comsassie staining of the membrane. Data are ± SE following student t-test where *=$p<0.05$ compared to control (n=3).

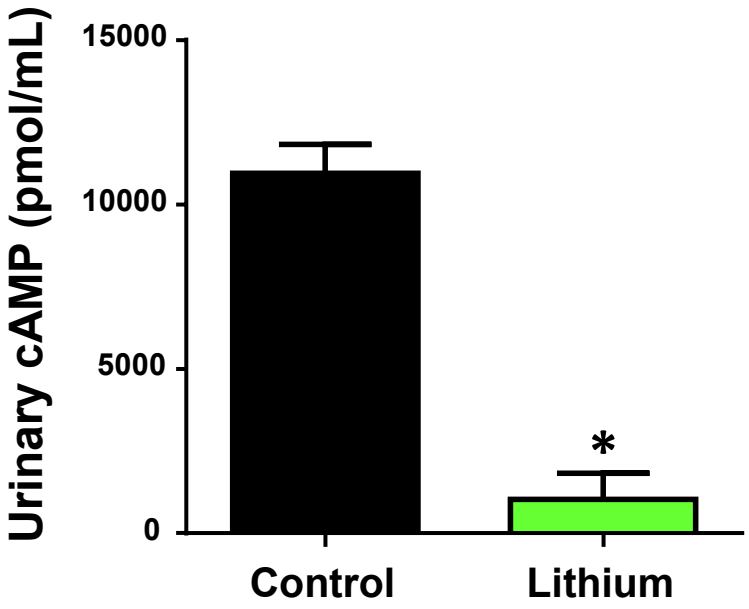

Figure 10. *Lithium treatment decreases renal cAMP synthesis.* Male Sprague-Dawley rats (8-weeks old) were pair-fed either a standard chow diet (control) or a diet supplemented with Li_2CO_3 (40 mmol/kg) for 14 days (lithium). Animals were housed individually in metabolic cages overnight on day 13 to collect 24-hour urine. cAMP was measured by EIA using collected urine. Data are ± SE following student t-test where *=$p<0.05$ compared to control (n=5).

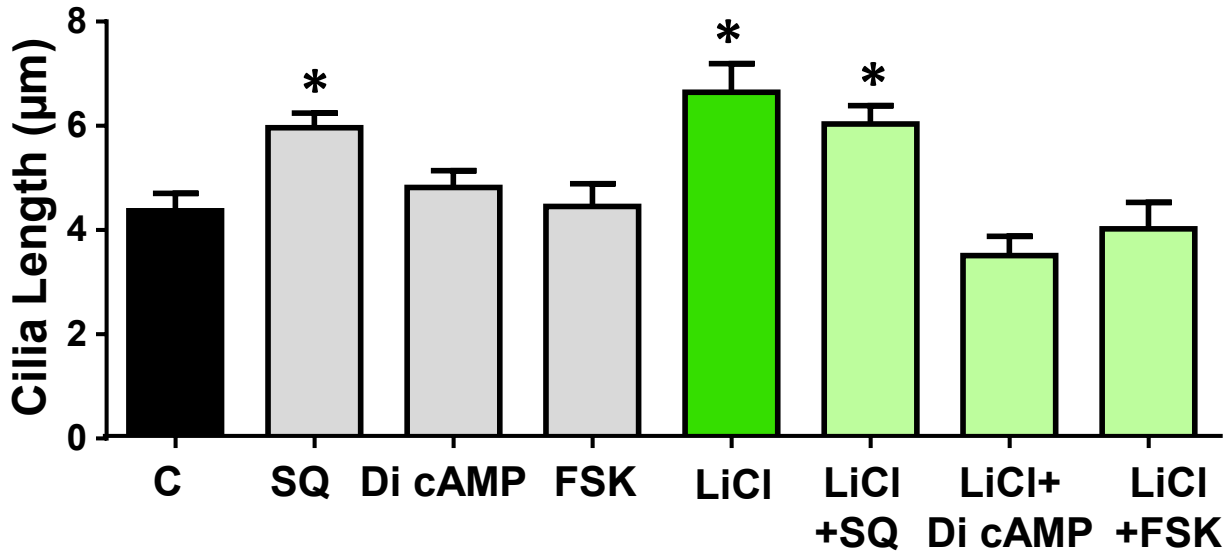

Figure 11. *Lithium elongation of cilia involves dampening the cAMP pathway.* mIMCD3-SSTR3-GFP cells were grown to confluence in glass bottomed dishes to allow for visualization of the GFP-labeled cilia with fluorescence microscopy. Cells were then treated with either vehicle (C), SQ 22536 (1 µM; SQ), Dibutyryl-cAMP (5 µM; Di cAMP), forskolin (1 µM; FSK) in the absence or presence of lithium chloride (25 mM; LiCl) for 24 hours. Graph is the analysis of all measured cilia lengths. Data are ± SE following ANOVA where *=$p<0.05$ compared to control (n=3; 20 cilia measured per n).