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Original Article

ORIGINAL ARTICLE

Reduction of ciliary length through pharmacologic or genetic inhibition of CDK5 attenuates polycystic kidney disease in a model of nephronophthisis

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Abstract

Polycystic kidney diseases (PKDs) comprise a subgroup of ciliopathies characterized by the formation of fluid-filled kidney cysts and progression to end-stage renal disease. A mechanistic understanding of cystogenesis is crucial for the development of viable therapeutic options. Here, we identify CDK5, a kinase active in post mitotic cells, as a new and important mediator of PKD progression. We show that long-lasting attenuation of PKD in the juvenile cystic kidneys (*jck*) mouse model of nephronophthisis by pharmacological inhibition of CDK5 using either R-roscovitine or S-CR8 is accompanied by sustained shortening of cilia and a more normal epithelial phenotype, suggesting this treatment results in a reprogramming of cellular differentiation. Also, a knock down of *Cdk5* in *jck* cells using small interfering RNA results in significant shortening of ciliary length, similar to what we observed with R-roscovitine. Finally, conditional inactivation of *Cdk5* in the *jck* mice significantly attenuates cystic disease progression and is associated with shortening of ciliary length as well as restoration of cellular

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differentiation. Our results suggest that CDK5 may regulate ciliary length by affecting tubulin dynamics via its substrate collapsin response mediator protein 2. Taken together, our data support therapeutic approaches aimed at restoration of ciliogenesis and cellular differentiation as a promising strategy for the treatment of renal cystic diseases.

Introduction

The recognition of the role of primary cilia in human disease has led to remarkable advances in our understanding of the function of this organelle in vertebrate development. Among the initial discoveries that compelled these investigations was the discovery that a number of proteins implicated in human polycystic kidney disease (PKD) localized to primary cilia. Since then, primary cilia have been characterized as crucial modulators of developmental signaling pathways including those mediated by Hedgehog, Wnt, Notch and platelet-derived growth factor alpha (1). Mutations of genes whose products localize to cilia or are required for their formation or function have been implicated in disorders now known collectively as ciliopathies, which are notable for their wide variety of symptoms, including obesity, retinal defects, skeletal defects, brain anomalies and, as noted, PKD (2–5).

Indeed, PKD is a feature of many of the ciliopathies, as well as the major cause of morbidity and mortality of these disorders. Autosomal dominant PKD (ADPKD) is one of the most common human monogenic diseases with an estimated incidence of 1:400–1:1000. ADPKD affects over 12 million people worldwide and is characterized by the development and progressive enlargement of fluid filled renal cysts leading to end-stage renal disease in 50% of affected individuals by the age of 60 years (6–9). ADPKD arises from mutations in either the *PKD1* or *PKD2* genes; their protein products PC1 and PC2 both localize to the ciliary membrane where they form receptor-channel sensory complexes (10). While ADPKD is primarily an adult disorder, the autosomal recessive PKD syndrome nephronophthisis (NPHP) is the most common genetic disease causing end-stage renal disease in children and adolescents (11,12). NPHP is a genetically heterogeneous disorder with 20 identified genes, most of which have been shown to play a role in cilia function. One of them, NPHP9, is caused by missense mutations in the kinase NEK8, which localizes to the ciliary axoneme (13). *Nek8* was originally characterized as the gene mutated in the mouse mutant *juvenile cystic kidneys* (*jck*) and the development of PKD in this model of NPHP was described previously (14,15). Interestingly, all the cystogenic *Nek8* alleles, including that of the Lewis Polycystic Kidney rat (16), are missense mutations in its RCC domain, which potentially regulates protein interactions. Null mutations of *Nek8* result in developmental defects in mice and humans (17,18) and genetic evidence suggests the *jck* missense mutation results in a gain-of-function (17). NEK8 was also identified as a critical component of the DNA damage response that links replication stress with cystic kidney disorders (19). The first ciliopathy genes described to function within the DNA damage response have been ZNF423, CEP164 and MRE11 (20). Interestingly, cyclin-dependent kinase (CDK) inhibition by R-roscovitine can cause nuclear translocation of CEP164 and inhibition of CHK1 activation (20). Of note, cilia lack the capacity for protein synthesis; therefore, all ciliary components are delivered by a specific transport pathway for protein trafficking referred to as intraflagellar transport (IFT). Defects in components of this process are also well-documented causes of PKD (21–23).

Cystogenesis is accompanied by characteristic changes in tubular epithelial cells including increased proliferation, apoptosis, acquisition of a secretory phenotype, intracellular calcium and cAMP signaling and dysregulation of the cell cycle; the last is the focus of this report. In normal cells, cilium is dynamically regulated by cell cycle transition: cilia assemble after cells exit mitosis and are resorbed during cell cycle re-entry. Reciprocally, the cilia itself is capable of influencing cell cycle as cilia resorption allows the basal body to differentiate into the centrosome, which performs important functions in mitosis as the organizer of the mitotic spindle (1,24). These data support the idea that a dysregulated cell cycle may drive cystogenesis and that therapeutic intervention targeting cell cycle dysregulation could provide therapeutic benefit for PKD. We have shown previously that pharmacological inhibition of cell cycle progression with pan CDK inhibitor, R-roscovitine, effectively attenuates cystogenesis in multiple models of PKD (25,26). Importantly, continuous daily administration of drug was not required to achieve efficacy; pulse treatment provided long-lasting arrest of PKD in *jck* model of PKD (25). While mechanistic studies demonstrated that R-roscovitine acted via cell-cycle arrest, transcriptional inhibition and blockade of apoptosis, the mechanism responsible for its long-lasting effect was not known (25). A recent efficacy study using R-roscovitine and S-CR8, a more potent second-generation analog of R-roscovitine (27,28), has confirmed its effectiveness in an orthologous mouse model with conditionally inactivated *Pkd1* genes (26). Importantly, R-roscovitine and S-CR8 share similar protein kinase selectivity profiles targeting CDK1, CDK2, CDK7, CDK9, as well as CDK5, which is an atypical member of the CDK family (27,29). Unlike mitotic CDKs, CDK5 is regulated by proteins p35 and p39, not by cyclins, and plays an important role in maintaining cellular differentiation in post-mitotic cells (30).

CDK5 is a multifunctional kinase that plays an important role in regulating diverse cellular functions including differentiation, organization of focal adhesion and cytoskeleton, membrane dynamics, cellular metabolism, cell cycle arrest in post mitotic cells and cell survival, as reviewed in Ref. (30). Abnormal CDK5 activity has been actively studied in neurodegenerative diseases with hyperactivation of CDK5 with p25, the product of calpain-dependent proteolytic cleavage of p35 (31). The role of CDK5 in non-neuronal tissue is not well understood. Recent data suggest the possible function of dysregulated CDK5 in a number of kidney diseases, where it may play a pathogenic role by promoting cellular de-differentiation and apoptosis (25,32–36).

In this study, we investigated the specific role of CDK5 in promoting renal cystogenesis. We hypothesized that the long-lasting arrest of cystogenesis observed upon treatment with the pan-CDK inhibitor R-roscovitine is at least partly attributable to the inhibition of CDK5 and the restoration of cellular differentiation. Here, we report that pulse treatment of *jck* mice with the CDK inhibitors R-roscovitine and S-CR8 results in the reversal of cystic epithelial morphology to that of normal tubular epithelial cells, the normalization of the pattern of expression of markers of renal cell differentiation and the mitigation of the abnormal

lengthening of cilia seen in *jck* kidneys. To directly investigate the role of CDK5 in PKD, we generated and characterized *jck* mice with a conditionally inactivated *Cdk5* gene. We show that loss of CDK5 reduces cystogenesis, improves kidney function, reduces ciliary length and normalizes morphology of cyst-lining kidney epithelial cells. Selective inhibition of CDK5 promotes cellular differentiation and restoration of normal cellular phenotype and therefore represents a viable treatment option for PKD.

Results

Arrest of cystic disease in *jck* mice with R-roscovitine and S-CR8 is linked to a long-lasting reduction of ciliary length and restoration of renal epithelial phenotype

We and others have shown that cilia of kidney epithelial cells are lengthened in *jck* mice (8.5 ± 2.5 μm) compared to wild-type controls (2.5 ± 1.5 μm) (15,37). We examined whether the reduction in cystic disease progression conferred by treatment with

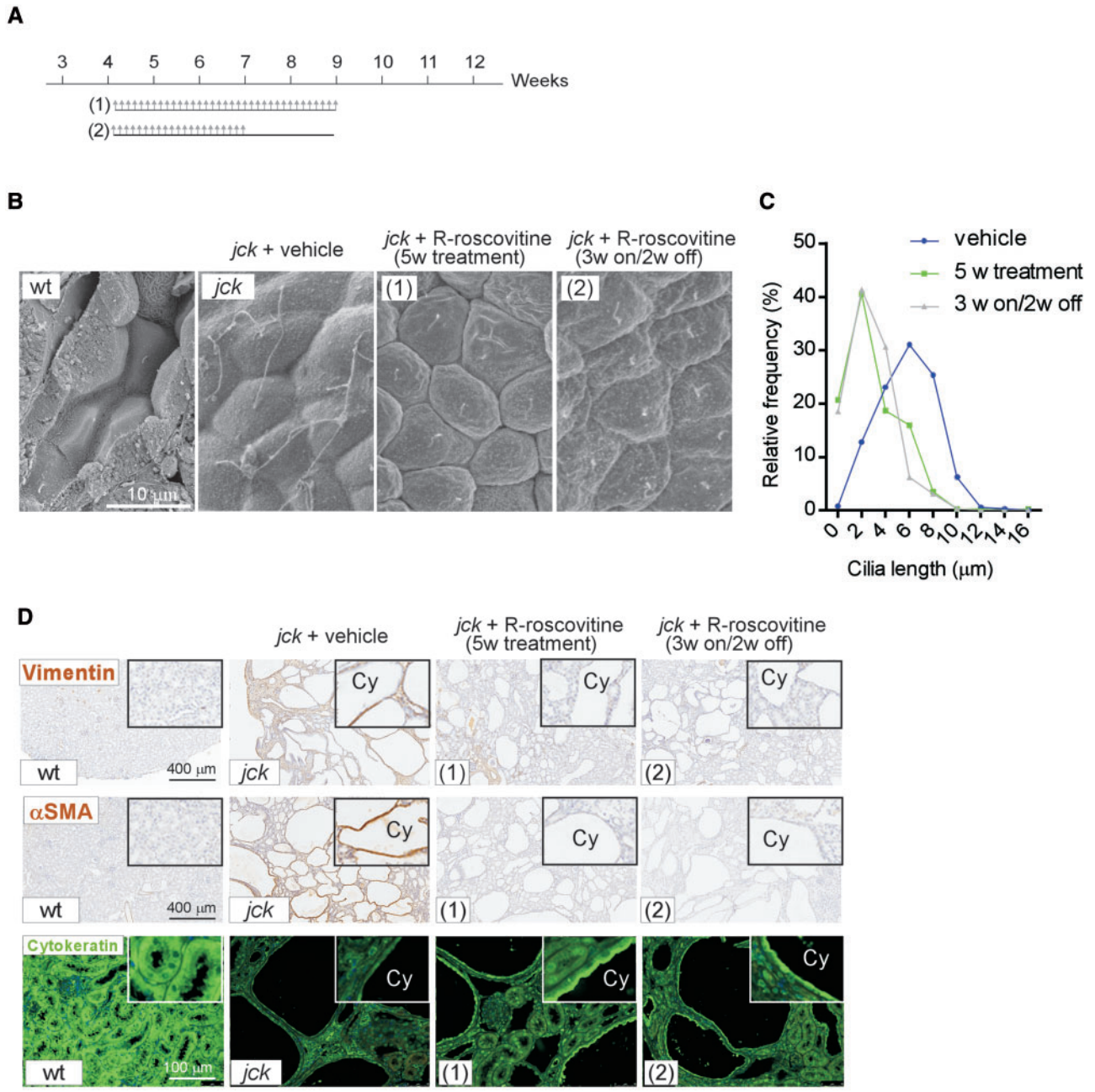

Figure 1. Sustained arrest of cystogenesis with CDK inhibitor, R-roscovitine, reduces cilia length and restores cell differentiation in *jck* kidneys. (**A**) Schematic representation of treatment regimen. *Jck* mice were treated with vehicle control or 150 mg/kg R-roscovitine by IP injections daily for 5 weeks (schedule 1), or for 3 weeks, followed by 2 weeks without treatment (schedule 2) as described (25). $N = 23$ for schedule 1 and 20 for schedule 2 . (**B**) Scanning electron micrographs of primary cilia in kidney epithelial cells from wild type (wt) and *jck* mice treated with vehicle or treated with R-roscovitine according to schedule 1 or 2 as noted. (**C**) Quantification and graphical representation of cilia length distribution of data shown in (B). $P < 0.0001$ in both schedules 1 and 2 compared to vehicle. (**D**) Markers of cellular differentiation were analyzed in kidney sections of wt, *jck* and *jck* mice treated with R-roscovitine either according to schedule 1 or 2 as noted. Panels (from top to bottom) represent, respectively, analyses of expression of vimentin, αSMA and cytokeratin 18. Cy indicates cysts.

R-roscovitine (25) had an effect on the ciliary length in treated kidneys. As shown in Figure 1, cilia are markedly elongated in kidneys from *jck* mutant mice and continuous R-roscovitine treatment for 5 weeks mitigates this abnormality (Fig. 1B and C). Importantly, this reduction in ciliary length is maintained when kidneys were examined 2 weeks after withdrawal of R-roscovitine treatment (Fig. 1B and C). As shown in Figure 1C, R-roscovitine restores ciliary length to levels comparable to wt controls but does not abrogate cilia formation. Of note, this is not due to persistent exposure to the drug, since pharmacokinetic analysis shows that R-roscovitine is eliminated within 24 h post-administration (38).

In addition to the reduction in cilia length, we noted that epithelial cells in the treated mice appeared to have a more ordered columnar appearance (Fig. 1B). To assess whether this was possibly due to a restoration of cellular differentiation, we assayed the cell state by immunostaining for the mesenchymal markers vimentin and alpha smooth muscle actin (αSMA) and the epithelial marker cytokeratin, which have been shown to be aberrantly expressed in PKD (39,40). We found that kidney epithelial cells are in a de-differentiated state in *jck* mice, demonstrating increased expression of vimentin and αSMA in the interstitial tissue and cystic epithelial cells and decrease expression of cytokeratin (Fig. 1D). In contrast, these markers appeared normally expressed in kidneys from *jck* mice treated with R-roscovitine, even 2 weeks after treatment withdrawal (Fig. 1D).

We similarly investigated the *in vivo* effect of S-CR8, a more potent and selective second-generation analog of R-roscovitine (27,41). Given the robust efficacy of pulse treatment with R-roscovitine in *jck* mice, we compared continuous and pulse treatment regimens of S-CR8. For this, we used several schedules of treatment: 24 mg/kg of S-CR8 was administered daily for 5 weeks (schedule 1); or for 3 weeks, followed by 2 weeks without treatment (schedule 2) or for 1 week, followed by 4 weeks without treatment (schedule 3) (Fig. 2A). Administration of S-CR8 according to schedules 1 and 2 showed similar reductions of cystogenesis compared to untreated mice, as shown by significant decreases of kidney to body weight ratio (Kidney/BW), cystic volume and blood urea nitrogen (BUN) (Supplementary Material, Table S1). The disease metrics in mice treated using schedule 3 were increased compared to those observed in schedules 1 or 2, suggesting that the therapeutic effect wanes after an extended period (4 weeks) without treatment (Supplementary Material, Table S1).

Next we tested the effect of S-CR8 treatment on cellular phenotypes. S-CR8 administered according to schedules 1 and 2 resulted in a marked reduction of cilia length (Fig. 2B and C) similar to that observed with R-roscovitine. We also observed restoration of the normal pattern of vimentin, αSMA and cytokeratin expression (Fig. 2D). This normalization of phenotype is less evident in schedule-3-treated mice (Fig. 2B–D), again consistent with a reversion to the de-differentiated state with a longer time frame of 4 weeks after treatment.

Specific targeting of CDK5 leads to cilia shortening in vitro

Since both R-roscovitine and S-CR8 inhibit multiple CDKs including CDK1, 2, 5, 7 and 9, it is not clear which CDK is responsible for long-lasting restoration of epithelial differentiation and reduction of cilia length. We asked whether CDK5 plays a pivotal role in regulating these cellular processes. First, we conducted *in vitro* experiments in a previously described immortalized *jck* kidney epithelial cell line (42) using selective inhibition of CDKs with small interfering RNA (siRNA). A shown in Figure 3, knock down of CDK5 in *jck* cells resulted in significant shortening of ciliary length, similar to what was observed with R-roscovitine. Knock down of CDK2 did not have this effect (Fig. 3).

Genetic loss of *Cdk5* inhibits cystogenesis, reduces cilia length and promotes renal epithelial cell differentiation

To further determine whether CDK5 plays a pathogenic role in PKD progression *in vivo*, we investigated the effect of genetic reduction of *Cdk5* in *jck* mice. *Cdk5* null mutant mice are embryonic lethal (43); consequently, we crossed *jck* animals with *Cdk5* conditional knock out animals and used the tamoxifen-inducible Gt(ROSA)26Sortm1 allele as a cre-driver (Supplementary Material, Fig. S1). *Cdk5* deletion was induced at 4 weeks and kidneys were examined at 9 weeks. Western analysis revealed that CDK5 protein expression was markedly, albeit not completely, reduced (Fig. 4A). Comparative analysis of *jck* mice and *jck* mice with conditionally inactivated *Cdk5* (*jck;Cdk5*^{cKO}) showed a qualitative mitigation of disease progression after *Cdk5* deletion (Fig. 4B), as well as quantitative reductions in Kidney/BW, cystic volume and BUN (Fig. 4C and Supplementary Material, Table S2).

As reported above, the mitigation of PKD progression after R-roscovitine treatment correlates with a normalization of renal epithelial cell phenotypes. We examined whether this is also true in the conditional *Cdk5* deletion model. Scanning electron microscopy (SEM) analysis of affected kidneys showed that genetic deletion of *Cdk5* in *jck* mutant mice results in decreased cilia length compared to that observed in *jck* kidneys (Fig. 5A and B). Expression of vimentin and αSMA appears less dysregulated in kidney tissue from *jck;Cdk5*^{cKO} mice (Fig. 5C). These results show that pharmacologic and genetic attenuation of *Cdk5* activity in a cystic kidney model result in similar effects on disease progression and renal cell differentiation.

Inhibition of CDK5 affects kidney expression of its specific substrate, collapsin response mediator protein 2

It has been shown previously that cilium length can be regulated through velocities of IFT machinery (44). To investigate whether such mechanism can explain the CDK5 mediated regulation of the cilium length, we analyzed IFT transport velocity by using fluorescently labeled IFT88 cells in the presence of R-roscovitine or *Cdk5* siRNA. We did not observe any effect on IFT particle velocities in the presence of CDK5 inhibitors (Supplementary Material, Fig. S2).

It is also possible that CDK5 might regulate ciliary length by affecting the dynamics of microtubules. To investigate this possibility, we set out to first identify possible downstream effectors of CDK5 in the kidneys. We compared kidney proteomes of wt, *jck* mice and *jck* mice with conditionally inactivated *Cdk5* gene (*jck;Cdk5*^{cKO}) using mass spectrometry and identified 394 proteins that specifically changed as a result of genetic deletion of *Cdk5* (Supplementary Material, Table S3). This set was cross-referenced with 40 newly identified brain-specific CDK5 substrates from CDK5$^{-/-}$ mice (45). As a result, we identified six possible kidney-specific CDK5 effectors: alpha-adducin (*Add1*) dihydropyrimidinase-like 2 (*Dpysl2*), dihydropyrimidinase-related protein 3 (*Dpysl3*), MARCKS-related protein (*Marcksl 1*), programmed cell death protein 4 (*Pdcd4*) and small nuclear

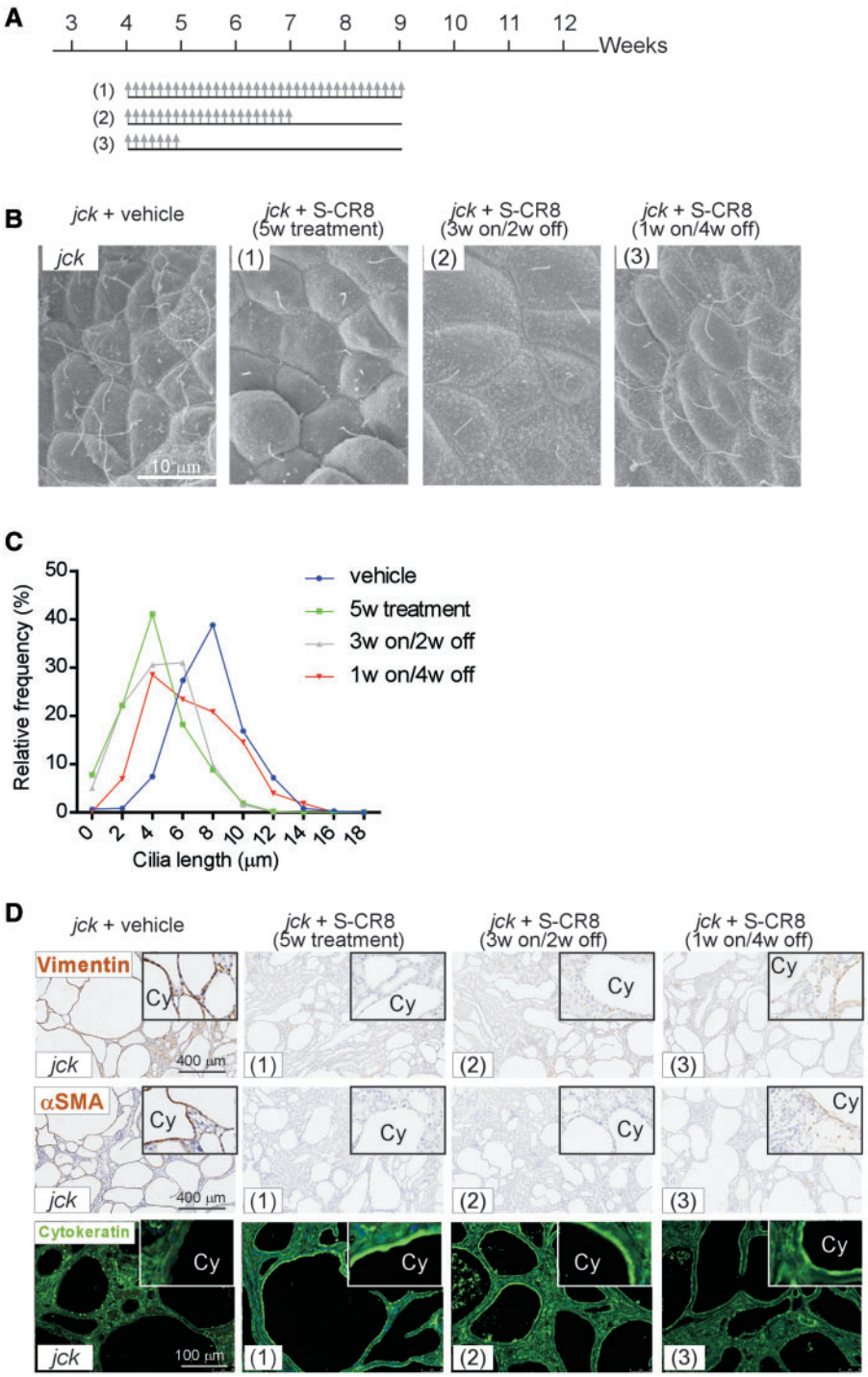

Figure 2. Long-lasting arrest of cystogenesis with CDK inhibitor, S-CR8, reduces cilia length and preserves renal epithelial cell differentiation in *jck* kidneys. (**A**) Schematic representation of treatment regimen with S-CR8. *Jck* mice were treated with vehicle control ($N = 9$) or 24 mg/kg S-CR8 daily by IP injection for 5 weeks (schedule 1, $N = 10$), for 3 weeks, followed by 2 weeks without treatment (schedule 2, $N = 8$), or for 1 week followed by 4 weeks without treatment (schedule 3, $N = 8$). (**B**) Scanning electron micrograph of primary cilia in cystic kidney epithelial cells. Representative images of vehicle control *jck* mice or treated with S-CR8 according to schedule 1, 2 or 3 as noted are shown. (**C**) Quantification and graphical representation of cilia length distribution of data shown in (B). $P < 0.0001$ in schedules 1, 2 and 3 compared to vehicle treatment. (**D**) Markers of cellular differentiation were analyzed in kidney sections of *jck* control mice and mice treated with S-CR8 according to schedule 1, 2 or 3 as noted. Panels (from top to the bottom) represent analyses of expression of vimentin, αSMA and cytokeratin 18. Cy indicates cyst.

ribonucleoprotein 70 (U1) (*Snmp70*). We have further investigated *Dpysl2*, also known as collapsin response mediator protein 2 (CRMP2), because of its known function as a regulator of microtubule dynamics in neurite outgrowth in the brain (46).

To that end, we evaluated the levels of total CRMP2 and its CDK5-specific phosphorylated form p-CRMP2 (Ser522) in kidneys from *jck* mice with conditionally inactivated *Cdk5* (Fig. 5D). Total CRMP2 and p-CRMP2 (Ser522) are elevated in *jck* kidneys

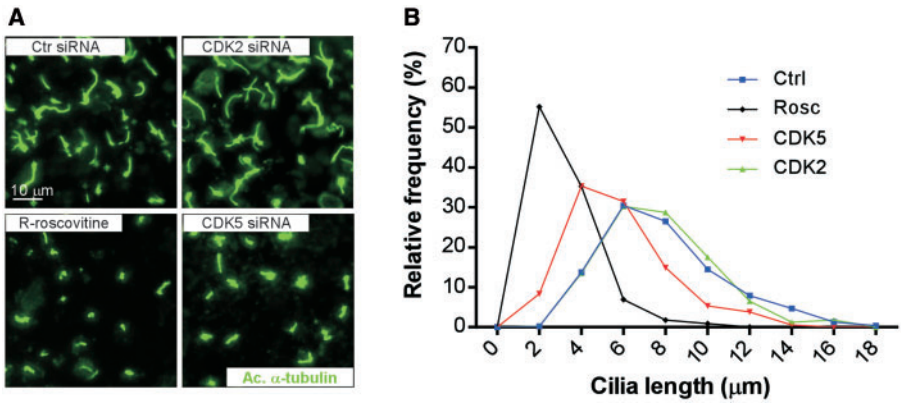

Figure 3. The effect of R-roscovitine and CDK5 siRNA on cilia length in *jck* kidney epithelial cells *in vitro*. (**A**) Immortalized *jck* kidney epithelial cells were either transiently transfected with negative control (Ctrl), CDK2 or CDK5 silencing RNA or incubated with R-roscovitine as described in the Materials and Methods section. Cells were stained with acetylated α-tubulin as primary cilium marker (green). Scale bar = 10 μm. (**B**) Quantification of the relative frequency of cilia length distribution in Ctrl, CDK2 or CDK5 siRNA-transfected or R-roscovitine-treated *jck* cells. $P < 0.001$ in R-roscovitine or CDK5 siRNA compared to Ctrl siRNA.

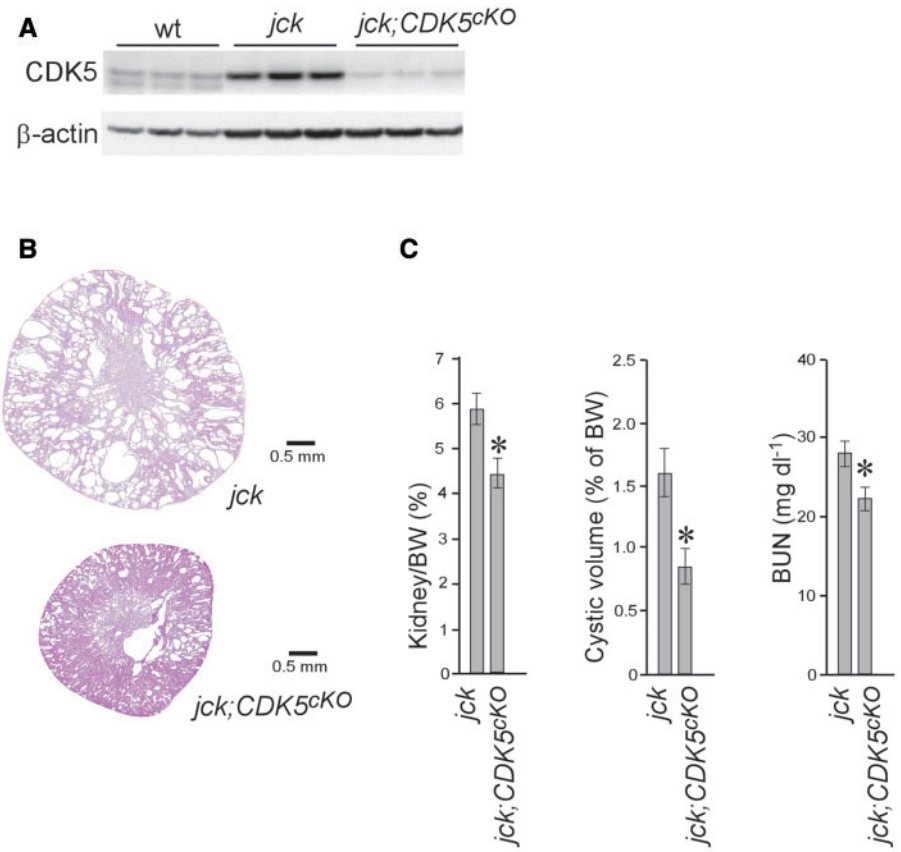

Figure 4. Conditional inactivation of CDK5 reduces cystogenesis in *jck* mice. (**A**) Protein expression of CDK5 after genetic reduction in jck kidneys. (**B**) Representative hematoxylin and eosin (H&E)-stained kidney sections from *jck* and *jck;Cdk5*^{cKO} animals. (**C**) The effect of *Cdk5* genetic reduction on PKD as measured by kidney/BW, cystic volume and BUN; *$P < 0.05$ compared with *jck* control animals. Error bars indicate SEM. *jck* $N = 14$ and *jck;Cdk5*^{cKO} $N = 8$.

compared to wild type and decreased in *jck;Cdk5*^{cKO} kidneys. Next, we examined whether specific depletion of CRMP2 in cultured *jck* cells leads to cilia shortening. As shown in Figure 5E and F, knock down of CRMP2 resulted in shortening of cilia similar to that observed previously with *Cdk5* knock down (Fig. 3).

These results suggest that CDK5 may play an important role in regulating cilia length in kidney, at least in part, via its direct target CRMP2.

Discussion

While treatment options for PKD and other ciliopathies are presently very limited, recent advances in characterizing the molecular pathogenesis of these disorders have suggested new therapeutic possibilities (47). The results presented here reveal the previously unrecognized role of CDK5 dysregulation in renal cystic disease, which affects primary cilia length and tubular epithelial differentiation. Mechanistic studies suggest that

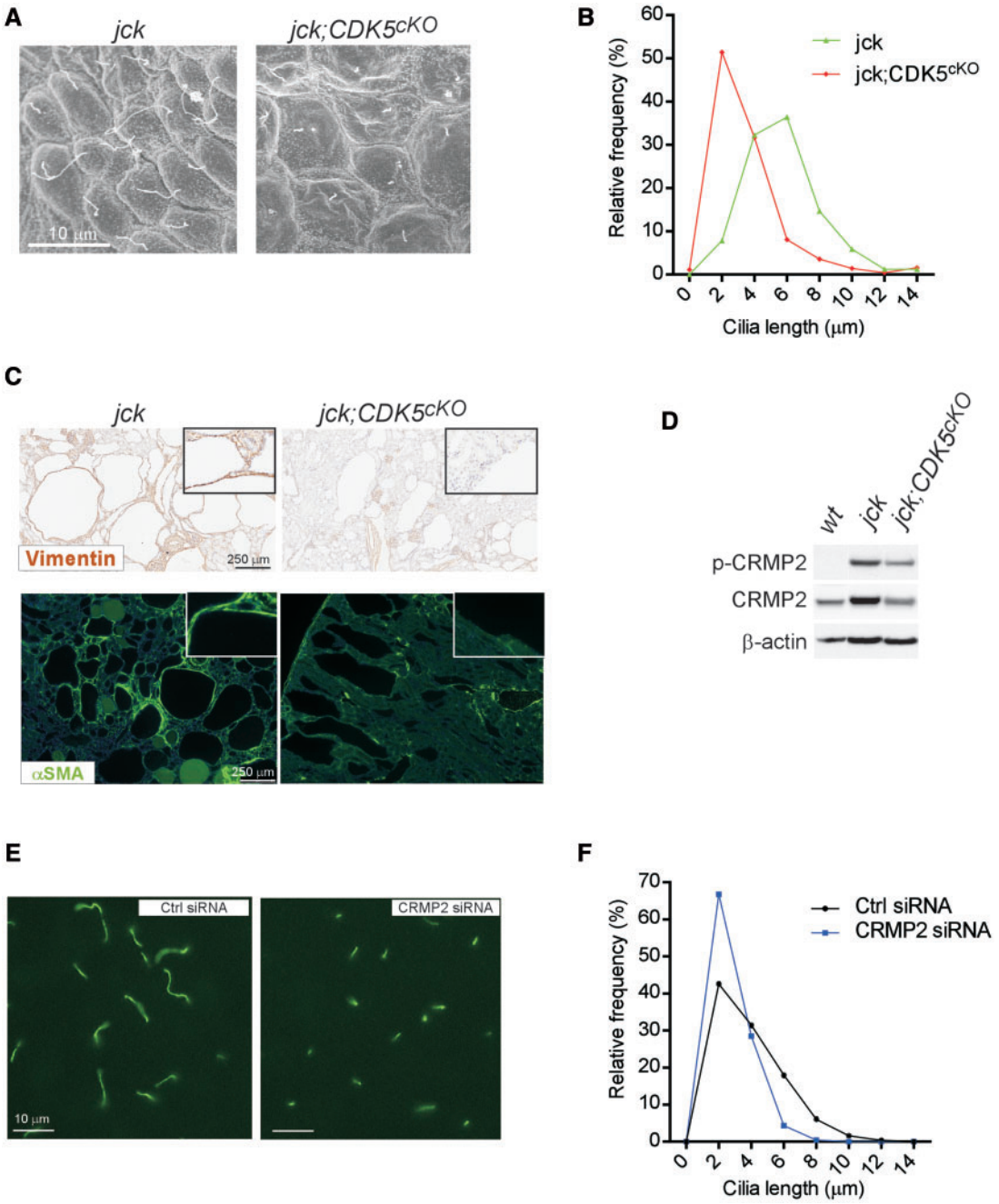

Figure 5. The effect of genetic reduction of CDK5 on cilia, cell differentiation markers and downstream target CRMP2. (**A**) Scanning electron micrographs of renal epithelial cells in *jck* and *jck* CDK5 knockout mice (*jck;CDK5*^{cKO}). (**B**) Graphical representation of cilia length distribution of data shown in (A). $P < 0.001$ in *jck;Cdk5*^{cKO} compared to *jck*. (**C**) Analysis of markers of cellular differentiation in *jck* and *jck;Cdk5*^{cKO} kidneys: Panels (from top to the bottom) represent expression of vimentin and αSMA respectively. (**D**) Immunoblot analysis of CRMP2 and pCRMP2 (Ser522) expression in wt, *jck* and *jck;Cdk5*^{cKO} kidneys. (**E**) The effect of CRMP2 knock down on ciliary length in cultured *jck* cells. (**F**) Quantitation of ciliary length distribution of data shown in (E). $P < 0.001$ in CRMP2 siRNA compared to Ctrl siRNA.

CDK5 may act through its downstream target CRMP2 by altering microtubule dynamics and thereby affecting cilia length. Moreover, our findings identify CDK5 as a new and attractive therapeutic target for the treatment of PKD because of its ability to induce sustained epithelial differentiation.

While CDK5 is ubiquitously expressed in all tissues, its role, along with the activator proteins p35 and p39, has been mainly studied in brain, where it is shown to regulate neuronal differentiation, migration and cytoskeletal dynamics (30,48). We have previously shown that R-roscovitine, an inhibitor of CDK1, 2, 5, 7 and 9, arrests cystogenesis in multiple models of PKD via blockade of the cell cycle, induction of apoptosis and transcriptional inhibition (25). We were particularly intrigued by our observation of a long-lasting therapeutic effect after withdrawal of drug. The specific mechanism of this observation is not known. We have hypothesized that CDK5 plays a role in regulating kidney tubular epithelial differentiation and that this accounts for the mechanism of sustained efficacy with pulse treatment. We have previously shown that CDK5 is, in fact, hyperactivated in cystic kidneys by the conversion of p35 to p25, similar to

neurodegenerative diseases (25,48). SEM analysis of kidneys treated with R-roscovitine or its analog S-CR8 either continuously for 5 weeks or treated for only 3 weeks followed by 2 weeks off-drug resulted in dramatic improvement of epithelial morphology. Specifically, we observe a more normal columnar epithelium, as well as reduction of cilia length, which is abnormally elongated in *jck* kidneys (15). These data suggested that both R-roscovitine and S-CR8 treatment may restore cellular differentiation. De-differentiation and perturbation of polarized epithelial phenotype represent an important aspect of PKD pathogenesis that is common to disorders caused by different genetic mutations (39,40). Indeed, we have shown restoration of epithelial differentiation by analysis of specific cellular markers. Interestingly, even a short pulse treatment with S-CR8 for only 1 week followed by 4 weeks off-drug showed effective PKD inhibition; however, examination at this time point shows a reversion of epithelial differentiation along with aberrant cilia elongation.

Our data show that pharmacological inhibition of PKD progression is accompanied by a clear effect on ciliary length. This is a particularly interesting finding in view of recent discovery demonstrating that genetic loss of cilia in mouse models of ADPKD actually inhibits cystogenesis (49). The authors demonstrated a protective effect of the loss of cilia on cystic growth in models with polycystin-1 and polycystin-2 mutations; however, the mechanism for these findings has not been explained. It is tempting to speculate that similar mechanisms can explain our observations. Of note, we and others have previously reported that the *Nek8* mutation in *jck* mice causes aberrant expression and localization of both polycystins-1 and -2 (15,37).

To prove that shortening of cilia and effect on cellular differentiation that we observed with R-roscovitine or S-CR8 treatment is mediated by inhibition of CDK5 specifically, we conducted an *in vitro* experiment in a *jck* kidney epithelial cell line with selective inhibition of CDK5 using siRNA. We found that only *Cdk5*-specific knock down reduced primary cilia length in *jck* cells. To further investigate the role of CDK5 in PKD, we generated and characterized *jck* mice with conditionally inactivated *Cdk5* gene. Analysis of these *jck;Cdk5*^{cKO} mice at 64 days of age showed significant attenuation of PKD compared to that seen in unmodified *jck* mice. Most importantly, genetic reduction of *Cdk5* in *jck* mice resulted in the restoration of kidney epithelial cell differentiation, normalized cellular morphology and cilia shortening similar to what we observed with pan-CDK inhibitors, supporting a pivotal role for CDK5 in these cellular processes.

Recent findings suggest that CDK5 is localized to the centrosome in HeLa cells where it may participate in cilia formation (50). It has also been previously proposed that cilia length can be regulated through the modulation of retrograde and anterograde cargo trafficking along the axoneme (44,51). We have shown that CDK5 inhibition with either R-roscovitine or siRNA did not affect the velocities of the cargo trafficking, suggesting another mechanism must account for shortening of primary cilia length. Because little is known about the role of CDK5 or its substrates in the kidney, we examined the significant body of data describing the role of CDK5 and its substrates in neuronal tissue. Cross referencing brain-specific CDK5 substrates and the proteome of *jck;Cdk5*^{cKO} kidney, we identified CRMP2 protein as a possible substrate for CDK5 in the kidney (45). CRMP2, the product of *Dpysl2* gene, is a multifunctional phosphorylated protein that has been widely studied in the brain where it was shown to play an important role in microtubule assembly promoting axon formation, neurite outgrowth and elongation in neuronal cells (46,52). Importantly, recent studies conducted in fibroblasts identified CRMP2 as a protein critically involved in primary cilia formation (53). Our data suggest a role of CDK5 and its substrate, CRMP2, in maintaining cilia length in the kidney. We showed that total CRMP2 and p-CRMP2 (Ser522) are increased in cystic disease and that the reduction of CDK5 leads to decreased level of both total CRMP2 and p-CRMP2. Interestingly, very recent findings by Maskey et al. described intriguing involvement of CDK5 in regulating the FBW7-NDE1 pathway and its presumptive modulation of cilia length in a cell cycle-dependent manner, at least *in vitro* (54). It is possible that CDK5 may act through multiple downstream targets and therefore, its function in kidneys needs to be further investigated in subsequent studies in multiple disease settings.

In conclusion, data presented here identify a critical role of CDK5 in controlling ciliary length and tubular epithelial differentiation. Pharmacological or genetic reduction of CDK5 leads to effective and sustained arrest of PKD. We propose that CDK5 acts on primary cilia, at least in part, by modulating microtubule dynamics. We suggest that new therapeutic approaches aimed at restoration of cellular differentiation are likely to yield effective treatments for cystic kidney diseases.

Materials and Methods

Mouse colony handling, genotyping and treatment

Jck mice (C57BL/6J-Nek8^{jck}) were kindly provided by Dr David Beier (55). *Jck* mice were bred at Biomedical Research Models "BRM" (Worcester, MA) and treated with R-roscovitine or S-CR8 at Sanofi-Genzyme R&D Center. R-roscovitine (150 mg/kg) and S-CR8 (24 mg/kg) were administered daily intraperitoneally (IP) as previously described (26).

CDK5^{cKO} mice (B6.129S4(Cg)-Cdk5$^{tm1.1Lht}$/J) and *Cre* recombinase (B6;129-Gt(ROSA)26Sor$^{tm1(cre/ERT)Nat}$/J) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained at the McLaughlin Research Institute (Great Falls, MT) (56,57). The breeding scheme is depicted in Supplementary Material, Fig. S1. Briefly we crossed *jck* heterozygous male with *Cdk5*^{cKO} homozygous females in a first set of mating and *Cdk5*^{cKO} homozygous females with *Cre* recombinase males in a second set of mating. *Jck* heterozygous/*Cdk5*^{cKO} heterozygous mice were then crossed with *Cdk5*^{cKO} heterozygous/*Cre* recombinase heterozygous mice. Finally, *Jck* heterozygous/*Cdk5*^{cKO} homozygous animals were finally crossed with *jck* heterozygous/*Cdk5*^{cKO} homozygous/*Cre* recombinase heterozygous animals to obtain the control animals: *jck* homozygous/*Cdk5*^{cKO} homozygous (herein called "*jck*") and the target animals: *jck* homozygous/*Cdk5*^{cKO} homozygous/*Cre* recombinase heterozygous (herein called "*jck;Cdk5*^{cKO}"). *Cdk5* recombination was induced upon IP injections of 250 mg/kg of body weight of Tamoxifen in 4-week-old animals. All mice were handled in accordance with McLaughlin Research Institute, BRM and Genzyme respective Institutional Animal Care and Use Committees guidelines. Genotyping was performed by Transnetyx (Cordova, TN) using a proprietary PCR-based assay. S-CR8 was synthesized as previously described (58). Treatment with S-CR8 was performed by IP injection of 24 mg/kg in 2% Tween 80, 0.3% lactic acid in M/6 sodium lactate once daily from day 26–64 after birth. All animals were euthanized by CO_2 asphyxiation. Tissues harvested for histological analysis were fixed either in Karnovsky's fixative for SEM or 4% paraformaldehyde (Electron Microscopy Science, Hatfield, PA) for histology analyses. BUN levels were measured

using a VetACE™ analyzer (Alfa Wasserman, West Coldwell, NJ).

SEM, histology and quantitative analyzes of cystogenesis

For SEM the cilia were imaged at the Schepens Eye Research Institute (MEEI, Boston, MA). Mouse kidneys were fixed for 48 h in 1/2 strength Karnovsky's fixative (2% paraformaldehyde + 2.5% glutaraldehyde in phosphate buffer, pH 7.4). Tissue samples were then rinsed three times in PBS, dehydrated in a graded series of Ethanol baths (35%, 50%, 70%, 95% and 2X 100% for 30 min each) and critical point dried in a Tousimis Samdri-795 Critical Point Dryer (Tousimis, Rockville, MD). Finally, kidney samples were mounted on aluminum stubs and coated with a 150A Chromium layer in a GATAN Ion Beam Coater 610. Cilia were viewed on a JEOL Field Emission Scanning Electron Microscope FESEM 7401F (JEOL, Peabody, MA). Cilia length was quantified with MetaMorph Imaging Series® software (Molecular Devices Corp, Dowington, PA). Cilia length distribution was calculated by measuring cilia in $\geq$20 random fields per sample. Total number of cilia measured in each group were $\geq$400. Statistical analysis was performed using GraphPad prism software (GraphPad Software, San Diego, CA). To determine whether these values differed significantly between experimental groups, *P* values were obtained using an unpaired parametric *t*-test.

For histological analysis, kidneys were cut 4 μm thick from both sagital and transversal sections and stained with hematoxylin and eosin using a Tissue Tek® 2000 processor (Sakura-Finetek, Torrance, CA). Slides were digitized with a Nanozoomer digital slide scanner (Hamamatsu, Bridgewater, NJ) and processed with the Metamorph Software (Molecular Devices Corp). Cystic volume was measured as percentage of cystic area to a total section area normalized by body weight.

Immunohistochemistry and immunofluorescence

Paraformaldehyde-fixed paraffin-embedded sections were deparaffinized and subjected to antigen retrieval as previously described (59). Staining for immunohistochemistry was performed using a Leica Vision Biosystems BondMax Immunostainer apparatus using Bond Polymer Refine Detection Kit (Leica Microsystems, Buffalo Grove, IL). Anti-vimentin (Epitomics, Burlingame, CA) or αSMA (Abcam, Cambridge, MA) antibodies were diluted in an antibody dilution reagent (Dako Corporation). Slides were counterstained with hematoxylin prior to mounting and digitized with a Nanozoomer digital slide scanner (Hamamatsu, Bridgewater, NJ).

Staining for immunofluorescence (IF) was performed as described previously using anti cytokeratin 18 antibodies (Sigma-Aldrich) (59). IF images were acquired on a Leica DM5500B microscope fitted with x40 objectives using Leica Application Suite Advance Fluorescence software (Leica Microsystems).

Cell culture, siRNA transfection and primary cilium staining

Wild type, *jck* and *jck;Cdk5*^{cKO} cell lines were established and maintained as described previously (42,60). *Jck* cells were cultured on collagen I coated glass slides (BD biosciences, SanJose, CA) and transiently transfected with negative control siRNAs, CDK2, CDK5, CDK7 or CRMP2 siRNA (Life Technologies, Carslbad, CA) using lipofectamine RNAiMAX as recommended by the manufacturer (Life Technologies). After transfection, cells were cultured for 48 h in complete culture medium and for an additional 48 h in serum-free medium. To study the role of R-roscovitine, cells were cultured for 48 h on collagen I-coated glass slides in complete culture medium followed by an additional 48 h in serum-free medium containing 20 μM R-roscovitine. Cells were then fixed with 4% paraformaldehyde followed by IF with anti-acetylated α-tubulin antibodies as previous described (15). Cilia length measurements and analyses were performed as described earlier.

Sodium dodecylsulfate poly-acrylamide gel electrophoresis and immunoblotting

Kidneys from mice were homogenized with 2.8 mm zirconia beads (Mo Bio Laboratories, Carlsbad, CA) using a Omni Bead Ruptor bead beater (Omni International, Kennesaw, GA) in radio immunoprecipitation assay buffer (Boston Bioproducts, Ashland, MA) complemented with complete protease inhibitors, 240 μg/ml Pefablock (Roche Diagnostics Corp, Indianapolis, IN), 1 mM $NaVO_4$ and 2 mM NaF (Sigma-Aldrich). Cell debris was pelleted by 10 min centrifugation at 20 000 g at 4°C, supernatants were harvested and protein concentrations were determined with Pierce BCA protein assay (Pierce/Thermoscientific, Rosckford, IL). Equal protein amounts were resolved by 4–12% gradient sodium dodecylsulfate poly-acrylamide gel electrophoresis and transferred onto nitrocellulose membranes using iBlot device (Life Technologies). Membranes were probed with antibodies against CDK5 (Cell Signaling, Danvers, MA), CRMP2 (Abcam, Cambridge, MA), p-CRMP2 (Ser522) (ECM Biosciences, Versailles, KY) or β-actin (Abcam, Cambridge, MA) overnight at 4°C as previously described (15). Membranes were washed in 1x Tris Buffered Saline 0.05% Tween 20 and incubated with anti-mouse or anti-rabbit IgG HRP-conjugated secondary antibodies (Promega, Madison, WI). Immunoreactive proteins were detected using enhanced chemi-luminescence (Amersham/GE, Little Chalfont, Buckinghamshire, England).

Statistics

Data are expressed as means ± SEM. Comparisons were made by two-tailed *t*-test and significance was accepted at the 0.05 level of probability ($P < 0.05$).

Supplementary material

Supplementary Material is available at *HMG* online.

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