Rapamycin Ameliorates PKD Resulting from Conditional Inactivation of *Pkd1*

Jonathan M. Shillingford,*† Klaus B. Piontek,‡ Gregory G. Germino,‡ and Thomas Weimbs*†

*Department of Molecular, Cellular, and Developmental Biology and †Neuroscience Research Institute, University of California Santa Barbara, Santa Barbara, California; and ‡Department of Medicine, School of Medicine, Johns Hopkins University, Baltimore, Maryland

ABSTRACT

Aberrant activation of the mammalian target of rapamycin (mTOR) pathway occurs in polycystic kidney disease (PKD). mTOR inhibitors, such as rapamycin, are highly effective in several rodent models of PKD, but these models result from mutations in genes other than *Pkd1* and *Pkd2*, which are the primary genes responsible for human autosomal dominant PKD. To address this limitation, we tested the efficacy of rapamycin in a mouse model that results from conditional inactivation of *Pkd1*. Mosaic deletion of *Pkd1* resulted in PKD and replicated characteristic features of human PKD including aberrant mTOR activation, epithelial proliferation and apoptosis, and progressive fibrosis. Treatment with rapamycin was highly effective: It reduced cyst growth, preserved renal function, inhibited epithelial cell proliferation, increased apoptosis of cyst-lining cells, and inhibited fibrosis. These data provide *in vivo* evidence that rapamycin is effective in a human-orthologous mouse model of PKD.

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Autosomal dominant polycystic kidney disease (ADPKD) is characterized by the gradual replacement of normal renal parenchyma by cysts, which culminates in renal failure in approximately 50% of patients.[1] No effective drug treatment is available to slow the progression of ADPKD, which is primarily (85%) caused by mutations in the *PKD1* gene encoding polycystin-1 (PC1).[2] Our previous results suggested that PC1 may regulate the kinase mammalian target of rapamycin (mTOR) *via* its interaction with tuberin.[3] In addition, we have demonstrated that mTOR activity is low in the normal human kidney but strongly upregulated in renal cyst-lining epithelial cells in ADPKD.[3] Finally, rapamycin treatment of four nonorthologous rodent PKD models resulted in inhibition of renal cyst growth, regression of kidney size, and preservation of renal function,[3–9] which led to the proposal that mTOR inhibitors, some of which are already in clinical use as immunosuppressants, may be effective in patients with ADPKD.[10–13] Indeed, four clinical trials have been initiated to test the efficacy of mTOR inhibitors in ADPKD.[12–14] Given the immunosuppressive and other adverse effects of mTOR inhibitors, it will be important to establish a compelling rationale for their use in patients with ADPKD.

Previous studies used rodent PKD models with mutations in genes that encode proteins (polaris, bicaudal-C, samcystin, and folliculin) with poorly understood function and no known functional link to PC1.[3–9] We hypothesized that the normal function of these and other proteins involved in renal cystic diseases eventually converge on the mTOR pathway,[13] but it has remained uncertain whether mTOR inhibition would be effective in human ADPKD.

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G.G.G.'s current affiliation is National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland

Correspondence: Dr. Thomas Weimbs, Molecular, Cellular, and Developmental Biology, University of California Santa Barbara, Santa Barbara, CA 93106-9610. Phone: 805-893-4144; Fax: 805-893-4724; E-mail: weimbs@lifesci.ucsb.edu

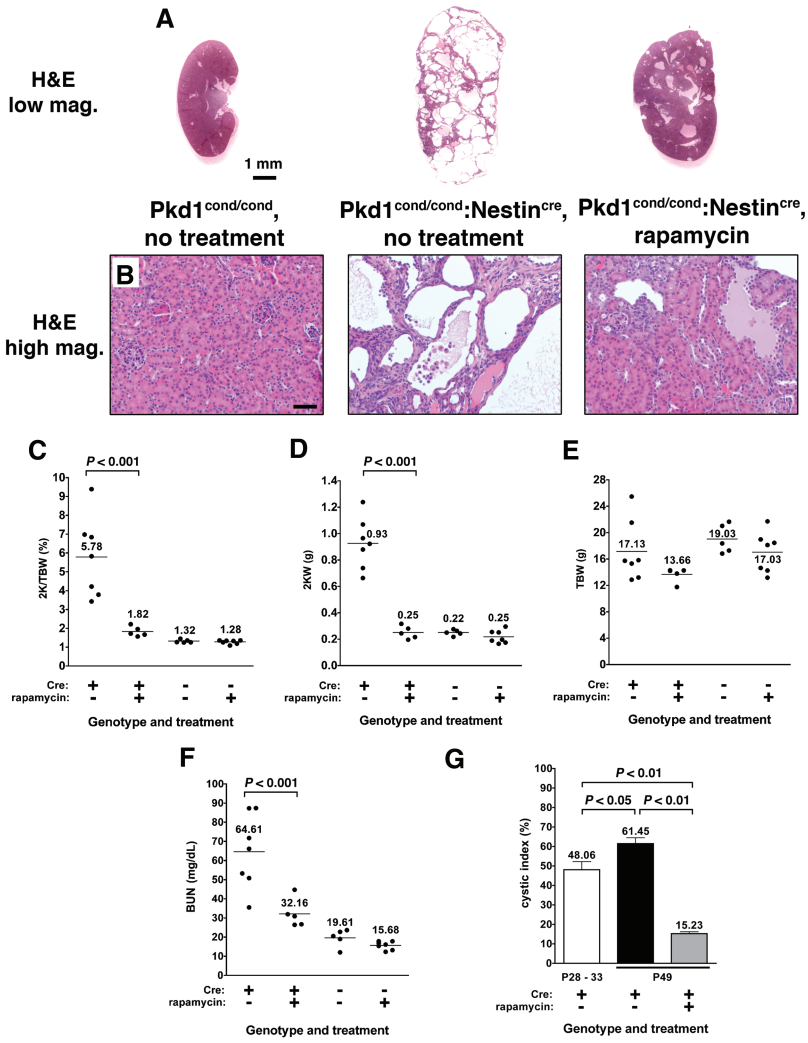

Figure 1. Rapamycin treatment of *Pkd1*$^{cond/cond}$*:Nestin*^{cre} mice significantly improves the renal cystic phenotype. All mice were treated daily with vehicle or 5 mg/kg rapamycin starting at day 28 and ending at day 49. (A) Representative low-power hematoxylin and eosin (H&E) renal sections from 49-day-old nontreated *Pkd1*$^{cond/cond}$*:Nestin*^{cre} mice (middle) showing numerous cysts compared with nontreated wild-type mice (left). Treatment of *Pkd1*$^{cond/cond}$*:Nestin*^{cre} mice with rapamycin (right) significantly improves the renal cystic phenotype. (B) Representative high-power H&E renal sections as in A. Bar = 100μm for all images. (C through F) The significant increases in the two-kidney/total body weight (2K/TBW) ratios (C), two-kidney weights (D), and BUN levels (F) observed in nontreated *Pkd1*$^{cond/cond}$*:Nestin*^{cre} mice are significantly decreased in rapamycin-treated *Pkd1*$^{cond/cond}$*:Nestin*^{cre} mice (ANOVA, Newman-Keuls posttest), whereas final body weight is not significantly different between treatment groups (E). (G) The cystic index is significantly decreased in rapamycin-treated *Pkd1*$^{cond/cond}$*:Nestin*^{cre} mice (n = 4) compared with nontreated P28 through P33 (n = 7) and P49 (n = 4) *Pkd1*$^{cond/cond}$*:Nestin*^{cre} mice (ANOVA, Newman-Keuls posttest).

To overcome this limitation, we used a mouse model in which the orthologous *Pkd1* gene is conditionally inactivated (*Pkd1*$^{cond/cond}$) by Cre-mediated recombination.[15,16] Initially, a *Pkd1*$^{cond/cond}$*:MMTV*^{cre} mouse line that resulted in infrequent renal cysts as a result of low renal Cre expression was generated[16]. We now report the development of a mouse line, *Pkd1*$^{cond/cond}$*:Nestin*^{cre}, in which the nestin promoter drives Cre expression.[17] This results in a mosaic renal expression pattern, mimicking the situation in human ADPKD whereby random somatic, second-hit mutations affect the *PKD1* locus,[1] and development of PKD with key features equivalent to the human disease. We report that rapamycin is highly effective in inhibiting all tested aspects of the disease phenotype, resulting in preservation of renal function.

RESULTS

Pkd1$^{cond/cond}$:Nestin^{cre} Animals Develop Renal Cysts and Fibrosis and Exhibit Reduced Kidney Function

Compared with 49-day-old *Pkd1*$^{cond/cond}$ animals lacking Cre (hereafter referred to as wild-type), age-matched *Pkd1*$^{cond/cond}$*:Nestin*^{cre} animals present with grossly enlarged kidneys and macroscopic cysts (Figure 1, A and B, and Supplemental Figure 1), which is reflected by the greatly increased two-kidney/body weight ratio (Figure 1C) and absolute two-kidney weight (Figure 1D). *Pkd1*$^{cond/cond}$*:Nestin*^{cre} animals exhibit highly elevated blood urea nitrogen (BUN) levels indicative of loss of kidney function (Figure 1F).

The deterioration of kidney function in ADPKD has been linked to the replacement of renal epithelium with fibrotic tissue.[18,19] To examine the extent of renal fibrosis, we assessed collagen deposition by Masson trichrome staining. As shown in Figure 2A, kidneys from *Pkd1*$^{cond/cond}$*:Nestin*^{cre} but not wild-type animals exhibited extensive deposition of collagen. Renal myofibroblasts are believed to arise from epithelial-to-mesenchymal transition in PKD and contribute to the observed collagen deposition and fibrosis.[18,19] To test whether renal myofibroblasts also may play a role in our *Pkd1*$^{cond/cond}$*:Nestin*^{cre} model, we immunostained kidney sections for smooth muscle actin (SMA). In wild-type kidneys, SMA is expressed at high levels only in the renal vascular bundles (Figure 2, B and C). In contrast, *Pkd1*$^{cond/cond}$*:Nestin*^{cre} animals showed abundant SMA-positive cells layered between cysts (Figure 2B) and normal-appearing tubules (Figure 2C) indicating the presence of myofibroblasts. Altogether, these results demonstrate that the deterioration in kidney function observed in *Pkd1*$^{cond/cond}$*:Nestin*^{cre} animals is due to

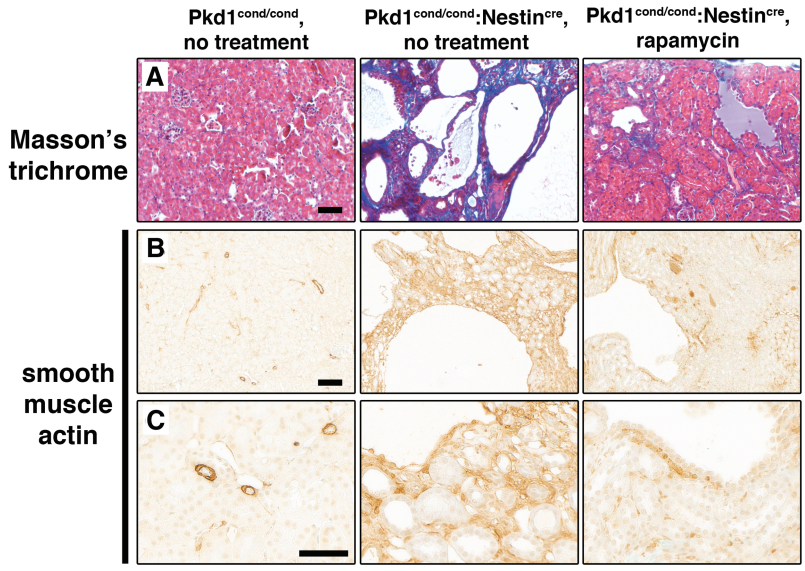

Figure 2. Renal fibrosis is reduced in rapamycin-treated *Pkd1*$^{cond/cond}$*:Nestin*^{cre} mice. All mice were treated daily with vehicle or 5 mg/kg rapamycin starting at day 28 and ending at day 49. (A) Representative Masson trichrome–stained renal sections from indicated animal genotypes. Note the extensive deposition of collagen (blue) in *Pkd1*$^{cond/cond}$*:Nestin*^{cre} mice (middle), which is significantly reduced in rapamycin-treated *Pkd1*$^{cond/cond}$*:Nestin*^{cre} mice (right). Bar = 100 μm. (B and C) Representative SMA-stained renal sections from indicated animal genotypes. Rapamycin treatment significantly reduces SMA-positive interstitial cells surrounding morphologically normal tubules (B and C, right) *versus* nontreated (B and C, middle) *Pkd1*$^{cond/cond}$*:Nestin*^{cre} mice. Bars = 100 and 50 μm in B and C, respectively.

the growth of numerous renal cysts and the pronounced accumulation of fibrotic tissue and extracellular matrix.

Renal Cysts Arise Predominantly from Collecting Duct/Distal Tubules in Pkd1$^{cond/cond}$:Nestin^{cre} Animals

A previous study of the *Nes-Cre:ROSA26* reporter line demonstrated mosaic Cre-mediated recombination in multiple renal tubule segments as early as embryonic day 15.5.[20] Figure 3 shows that the majority of renal cysts observed in postnatal day 28 (P28; Figure 3, E and F) and P49 (Figure 3, B through D) *Pkd1*$^{cond/cond}$*:Nestin*^{cre} renal tissue stain positive for the collecting duct/distal tubule marker *Dolichos biflorus* agglutinin (DBA; red) with some cysts negative for both DBA and the proximal tubule marker *Lotus tetragonolobus* lectin (LTL; green). No LTL-positive cysts were evident; therefore, renal cysts predominantly derive from collecting duct/distal tubules in *Pkd1*$^{cond/cond}$*:Nestin*^{cre} animals. Although *Nestin*^{cre} animals exhibit significant expression of Cre in the central nervous system,[17] we observed no evidence of seizures or unusual motor functions in the *Pkd1*$^{cond/cond}$*:Nestin*^{cre} animals during the course of this study.

Increased Proliferation and Apoptosis in *Pkd1*$^{cond/cond}$*:Nestin*^{cre} Animals

Increased proliferation and apoptosis of renal cystic epithelial cells are hallmarks of human ADPKD.[2] We examined proliferation and apoptosis in *Pkd1*$^{cond/cond}$*:Nestin*^{cre} animals using Ki-67 and terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) staining, respectively. As shown in Figure 4A, cyst-lining epithelial cells are highly proliferative (Figure 4D). Both morphologically normal tubules and interstitial cells in *Pkd1*$^{cond/cond}$*:Nestin*^{cre} animals were also significantly more proliferative than those in wild-type animals (Figure 4, C and D). In comparison with renal epithelial cells in wild-type animals, which exhibit very low levels of apoptosis (approximately 0.01%; Figure 5C), cyst-lining epithelial cells in *Pkd1*$^{cond/cond}$*:Nestin*^{cre} animals were highly apoptotic (Figure 5, A and D). These data demonstrate that, similar to human ADPKD, deletion of *Pkd1* results in both increased proliferation and apoptosis.

The mTOR Pathway is Activated in Pkd1$^{cond/cond}$:Nestin^{cre} Animals

To investigate mTOR pathway activity in *Pkd1*$^{cond/cond}$*:Nestin*^{cre} mice, we analyzed renal tissue lysates by immunoblotting using a P-rpS6 (S235/6) antibody, a surrogate marker of mTORC1 activation. As shown in Figure 6A, levels of P-rpS6 are greatly increased in *Pkd1*$^{cond/cond}$*:Nestin*^{cre} animals as compared with wild-type mice. Immunohistochemistry revealed high levels of P-rpS6 in cyst-lining epithelial cells and also in some morphologically normal tubules adjacent to cysts in *Pkd1*$^{cond/cond}$*:Nestin*^{cre} animals (Figure 6B). These results indicate that activation of the mTOR pathway occurs as a downstream effect of *Pkd1* deletion in this animal model, similar to the effect observed in human ADPKD.[3]

Because mTOR activity lies both upstream and downstream of Akt and previous studies showed that total Akt and P-Akt levels are upregulated in polycystic kidneys in a mouse model of nephronophthisis,[21] we determined renal levels of Akt and P-Akt (S473) in *Pkd1*$^{cond/cond}$*:Nestin*^{cre} mice (Figure 6A). In comparison with wild-type kidneys, *Pkd1*$^{cond/cond}$*:Nestin*^{cre} kidneys expressed elevated levels of total Akt and exhibited strong phosphorylation at S473. These data suggest that activation of Akt at this site, which is mediated by mTORC2,[22] may also play a role in renal cyst growth in this mouse model.

Rapamycin Treatment of Pkd1$^{cond/cond}$:Nestin^{cre} Animals Improves the PKD Phenotype

The aforementioned results indicate that the *Pkd1*$^{cond/cond}$*:Nestin*^{cre} mouse model faithfully replicates important characteristics of human ADPKD, including excessive proliferation, apoptosis, fibrosis, extracellular matrix deposition, and activation of the mTOR pathway. Given that this mouse model is

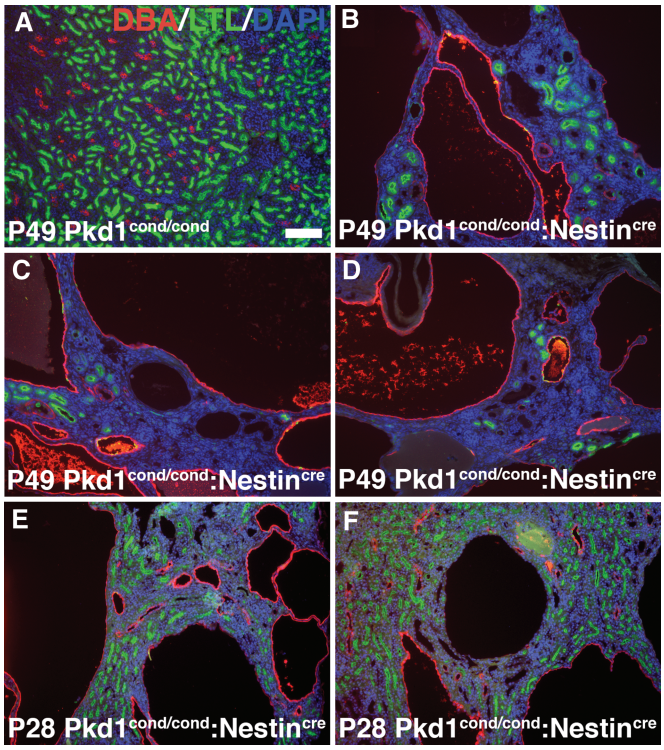

Figure 3. Renal cysts in *Pkd1*$^{cond/cond}$:*Nestin*^{cre} mice are derived from distal tubules. Renal sections derived from 49-day-old *Pkd1*$^{cond/cond}$ (A) and *Pkd1*$^{cond/cond}$:*Nestin*^{cre} (B through D) mice and 28-d-old *Pkd1*$^{cond/cond}$:*Nestin*^{cre} mice (E and F) were subjected to immunofluorescence staining with DBA (red, distal tubules), LTL (green, proximal tubules), and DAPI (blue). Note the predominantly collecting duct/distal tubule origin of cysts in *Pkd1*$^{cond/cond}$:*Nestin*^{cre} mice. Bar = 100 μm.

based on disruption of the same gene as is affected in human ADPKD, we reasoned that it can serve as a reliable model to assess the efficacy of drugs that inhibit the mTOR pathway. *Pkd1*$^{cond/cond}$:*Nestin*^{cre} mice were treated with either vehicle or rapamycin from P28 to P49. As shown in Figure 1, rapamycin treatment significantly inhibited the growth of renal cysts (Figure 1, A and B) and resulted in a greatly reduced two-kidney/total body weight ratio (Figure 1C), absolute two-kidney weight (Figure 1D), and cystic index (Figure 1G). Furthermore, the decreased plasma BUN levels in rapamycin-treated *Pkd1*$^{cond/cond}$:*Nestin*^{cre} mice (Figure 1F) demonstrated a significant improvement in overall kidney function.

To assess whether rapamycin treatment results in stasis or regression of renal cyst growth, we determined the renal cystic index in animals representing the start of rapamycin treatment. As quantified in Figure 1G and illustrated in Supplemental Figure 1, rapamycin treatment during a 3-week period resulted in a significant reduction of the cystic burden (48 *versus* 15%). The final body weight of rapamycin-treated *Pkd1*$^{cond/cond}$:*Nestin*^{cre} mice was not significantly different from that of nontreated *Pkd1*$^{cond/cond}$:*Nestin*^{cre} mice (Figure 1E).

We determined the effect of rapamycin on fibrosis by examining the extent of collagen deposition and SMA expression. Rapamycin treatment resulted in a significant reduction in renal collagen staining in *Pkd1*$^{cond/cond}$:*Nestin*^{cre} mice (Figure 2A). Some of the remaining cysts in rapamycin-treated *Pkd1*$^{cond/cond}$:*Nestin*^{cre} mice still exhibited an underlying layer of SMA-positive cells (Figure 2, B and C), similar to that observed in nontreated *Pkd1*$^{cond/cond}$:*Nestin*^{cre} animals; however, the occurrence of SMA-positive cells surrounding morphologically normal tubules was much reduced (Figure 2, B and C).

Immunoblotting revealed that rapamycin treatment of *Pkd1*$^{cond/cond}$:*Nestin*^{cre} animals greatly reduced renal P-rpS6 levels (Figure 6A). In addition, immunostaining demonstrated a loss of detectable P-rpS6 in cysts and surrounding tubules (Figure 6C). Taken together, these results demonstrate that rapamycin treatment inhibits the mTOR pathway in renal epithelial cells in this mouse model, resulting in dramatically reduced renal cyst growth and fibrosis and preservation of renal function.

It was reported previously that inhibition of mTORC1 activity may result in the suppression of a negative feedback loop that culminates in the activation of Akt *via* phosphorylation of serine 473,[23] which is mediated by mTORC2.[22] Although mTORC2 is not directly inhibited by rapamycin, it has been

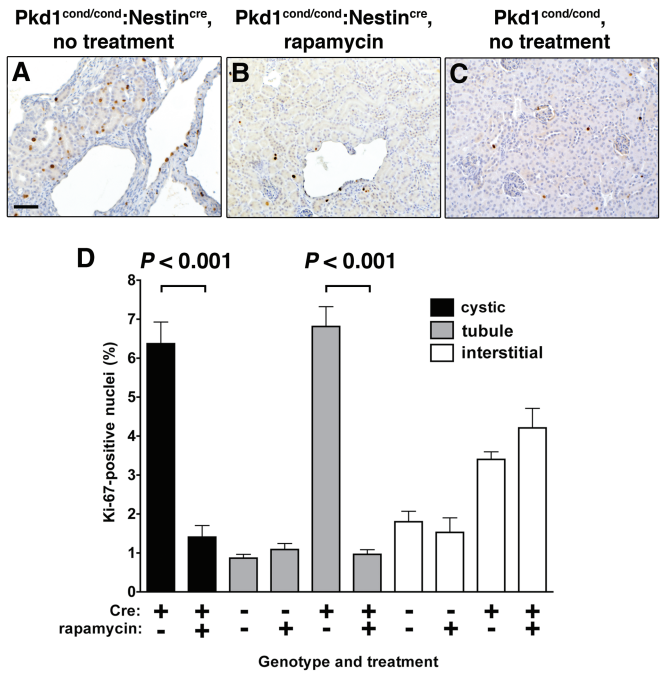

Figure 4. Rapamycin treatment of *Pkd1*$^{cond/cond}$:*Nestin*^{cre} mice decreases renal cell proliferation. All mice were treated daily with vehicle or 5 mg/kg rapamycin starting at day 28 and ending at day 49. (A through C) Representative low-power fields of renal sections from nontreated *Pkd1*$^{cond/cond}$:*Nestin*^{cre} (A), rapamycin-treated *Pkd1*$^{cond/cond}$:*Nestin*^{cre} (B) and nontreated wild-type (C) mice immunostained with a Ki-67 antibody. (D) Quantification of Ki-67 immunostaining (n = 3 for all treatment groups). Note the decrease in proliferation of cystic and morphologically normal epithelial cells in response to rapamycin treatment of *Pkd1*$^{cond/cond}$:*Nestin*^{cre} mice. Bar = 50 μm.

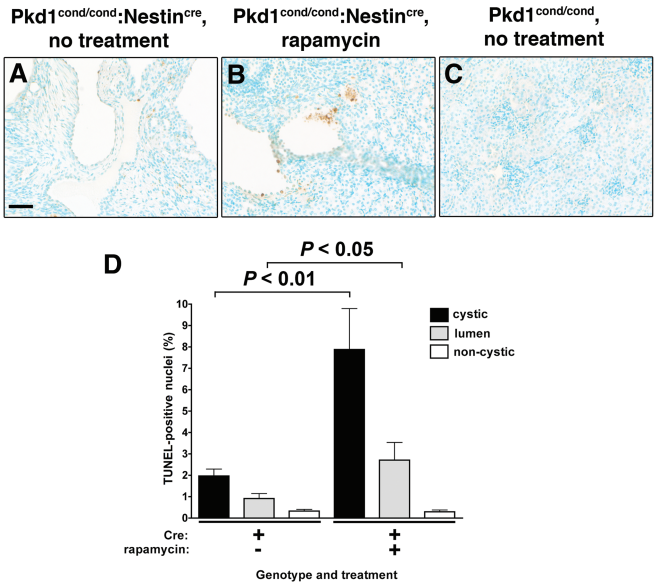

Figure 5. Rapamycin treatment of *Pkd1*$^{cond/cond}$*:Nestin*^{cre} mice increases apoptosis of cyst-lining epithelial cells. All mice were treated daily with vehicle or 5 mg/kg rapamycin starting at day 28 and ending at day 49. (A through C) Representative low-power fields of renal sections from nontreated *Pkd1*$^{cond/cond}$*:Nestin*^{cre} (A), rapamycin-treated *Pkd1*$^{cond/cond}$*:Nestin*^{cre} (B), and nontreated wild-type (C) mice processed for TUNEL staining. (D) Quantification of TUNEL-positive nuclei ($n = 3$ for all treatment groups). Note the increase in apoptosis of cystic epithelial cells in response to rapamycin treatment of *Pkd1*$^{cond/cond}$*:Nestin*^{cre} mice. Bar = 50 μm.

shown that prolonged rapamycin treatment inhibits its activity toward Akt-Ser473 in a cell-dependent manner.[24] To distinguish whether rapamycin treatment results in activation (*via* suppression of negative feedback) or inactivation (*via* inhibition of mTORC2 activity) of Akt in polycystic kidneys in this mouse model, we examined total Akt and P-Akt (S473) protein levels in renal tissue lysates. As shown in Figure 6A, rapamycin-treated *Pkd1*$^{cond/cond}$*:Nestin*^{cre} mice exhibited total Akt and P-Akt levels similar to that observed in wild-type mice. This suggests that rapamycin treatment, possibly through inhibition of mTORC2 activity, results in inhibition of Akt in *Pkd1*$^{cond/cond}$*:Nestin*^{cre} renal tissue.

Rapamycin Treatment Decreases Proliferation and Increases Apoptosis

Rapamycin can be both antiproliferative and proapoptotic in renal cells that exhibit activated mTOR.[25] We previously showed that rapamycin treatment results in both decreased proliferation and increased apoptosis in the orpk-rescue mouse model.[3] We tested whether the observed efficacy of rapamycin in the *Pkd1*$^{cond/cond}$*:Nestin*^{cre} model is mediated through modulation of either or both of these cellular mechanisms. Quantification of Ki-67 immunostaining (Figure 4) revealed a more than four-fold reduction in proliferating cystic cells in rapamycin-treated *versus* nontreated *Pkd1*$^{cond/cond}$*:Nestin*^{cre} animals (Figure 4D). The significant increase in proliferation of morphologically normal tubules but not interstitial cells in *Pkd1*$^{cond/cond}$*:Nestin*^{cre} animals was also decreased in response to rapamycin treatment (Figure 4D). TUNEL staining (Figure 5) revealed an almost four-fold and three-fold increase in TUNEL-positive cyst-lining and luminal-shed cystic cells, respectively, in rapamycin-treated *versus* nontreated *Pkd1*$^{cond/cond}$*:Nestin*^{cre} animals (Figure 5D). In contrast, rapamycin treatment had no effect on apoptosis in morphologically normal tubules in *Pkd1*$^{cond/cond}$*:Nestin*^{cre} animals (Figure 5D, noncystic). Rapamycin treatment also had no effect in wild-type mice (data not shown), demonstrating that rapamycin alone does not induce apoptosis of normal tubule epithelial cells. Overall and consistent with our previous observations,[3] these data suggest that rapamycin exerts its beneficial effects through mTOR inhibition by a combination of reduced proliferation and increased apoptosis of cystic renal epithelial cells.

DISCUSSION

We previously demonstrated that the mTOR pathway is activated in and rapamycin effectively treats several mouse models

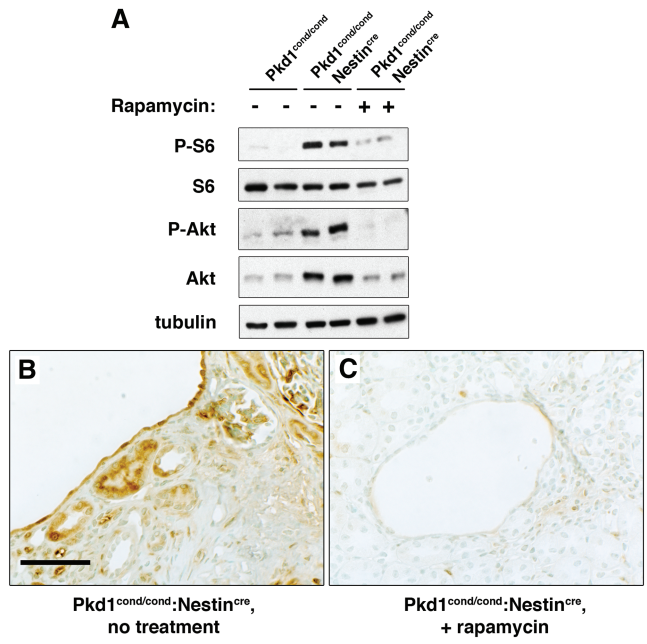

Figure 6. Rapamycin treatment of *Pkd1*$^{cond/cond}$*:Nestin*^{cre} mice reduces mTOR pathway activity. All mice were treated daily with vehicle or 5 mg/kg rapamycin starting at day 28 and ending at day 49. (A) Representative Western blots of kidney tissue lysates demonstrating that phosphorylated ribosomal protein S6 (P-rpS6) increases in *Pkd1*$^{cond/cond}$*:Nestin*^{cre} mice and is decreased by rapamycin treatment. Total levels of rpS6 and β-tubulin remain unchanged. Total levels of Akt and phosphorylated Akt (P-Akt) are increased and decreased in nontreated and rapamycin-treated cystic animals, respectively. (B and C) Immunohistochemistry showing intense P-rpS6 staining of cyst-lining and immediately adjacent epithelial cells in *Pkd1*$^{cond/cond}$*:Nestin*^{cre} mice (B) and undetectable staining in rapamycin-treated *Pkd1*$^{cond/cond}$*:Nestin*^{cre} mice (C). Bar = 50 μm.

of PKD.[3] In addition, mTOR activation and rapamycin efficacy were reported in the Han rat model[6–8] and a folliculin mouse model.[4,5] Although the genetic mutations in these rodent models are independent of *Pkd1* and *Pkd2*, the genes affected in human ADPKD, these results suggested that the mTOR pathway lies at a converging point downstream of the function of all of these gene products.[13] This study now shows that rapamycin is effective in a human-orthologous mouse model of PKD.

Previous Cre-loxP *Pkd1* models did not accurately replicate human PKD. One model resulted in a low frequency of renal *Pkd1* gene inactivation and only a few cysts,[16] whereas an inducible model resulted in *Pkd1* gene inactivation of high frequency and massive cystic transformation of renal tissue in the absence of increased proliferation.[15] Neither of these scenarios occurs in human ADPKD, which is characterized by random somatic, second-hit PKD gene mutations, leading to a progressive increase in cyst burden over time.

Herein we demonstrate that nestin Cre-mediated deletion of the *Pkd1* gene results in the development and expansion of renal cysts that, in many key aspects, resembles human ADPKD. Thus, the renal cystic progression in $Pkd1^{cond/cond}$:$Nestin^{cre}$ mice is characterized by extensive extracellular matrix deposition, myofibroblast expansion, deterioration of kidney function, increased proliferation and apoptosis of cyst-lining epithelial cells, and activation of the mTOR pathway. Interestingly, we observed mTOR activation and increased proliferation in morphologically normal tubules adjacent to cysts, suggesting that, as has been proposed for other rodent PKD models,[6,26] these tubules may receive mitogenic signals from neighboring cysts; however, among existing rodent models of PKD, $Pkd1^{cond/cond}$:$Nestin^{cre}$ mice may most closely replicate many critical aspects of human ADPKD.

Using this mouse model, we show that *Pkd1* gene inactivation leads to activation of the mTOR pathway in renal epithelium. Importantly, rapamycin treatment significantly improves all aspects of the phenotype, including cyst growth, fibrosis, proliferation, apoptosis, and overall renal function. This suggests that mTOR is necessary, directly or indirectly, for all of these mechanisms. In turn, this suggests that mTOR inhibition would be expected to lead to amelioration of the cystic phenotype in human ADPKD.

Our observation that long-term rapamycin treatment decreases renal P-Akt (S473) levels supports previous data[24] demonstrating that prolonged rapamycin treatment can inhibit mTORC1 and mTORC2 activity. Because mTORC2-mediated Ser473 phosphorylation of Akt is required for its full activity and subsequent phosphorylation at Thr308,[22] this suggests that the suppression of a negative feedback loop (*via* S6K/IRS/PDK1)[23] is unlikely to result in significant Akt activation in rapamycin-treated animals; therefore, the therapeutic effect observed herein may be mediated through dual inhibition of mTORC1 and mTORC2. Whether P-Akt is upregulated and rapamycin sensitive in human ADPKD renal samples, as it is in polycystic liver disease samples,[27] remains to be evaluated.

Although our new mouse model closely mimics many aspects of human ADPKD, it still is an imperfect model. For example, cysts form in a period of decades in human ADPKD, which cannot be replicated adequately in mouse models. On the basis of the known onset of nestin Cre-mediated gene inactivation[20] and the observed cystic burden at P28 and P49, we estimate that some cysts begin to arise embryonically with additional cystogenesis throughout the early postnatal period in our mouse model. Another caveat is that the rapamycin dosage used in this and other rodent studies[3,6–8] significantly exceeds the dosages used in human transplant patients; however, the literature suggests that rodents may require higher dosages of rapamycin to achieve mTOR inhibition. For example, a previous study[28] found that 3 mg/kg per d rapamycin was required to inhibit mTOR activity fully in the brain of a tuberous sclerosis mouse model. Furthermore, it has been demonstrated that dosing the Han rat model of PKD with 3 mg/kg per d everolimus (a close rapamycin analogue) during a 5-week period resulted in significant improvement of the PKD phenotype and whole-blood trough levels of 5 to 7 ng/ml.[8] This level correlates well with a clinical study[29] showing that patients who maintained trough levels of rapamycin >6 ng/ml demonstrated significant mTOR inhibition and nonrejection.

Rapamycin is thought to be a highly specific drug with no known targets other than mTOR. Furthermore, even the high dosages commonly used in rodents led to little or no toxicity or any functional or structural effects on the kidneys.[30] It is therefore unlikely that the observed efficacy in our study is due to mechanisms other than mTOR inhibition. Furthermore, a recent study using the Han rat model of PKD demonstrated that prolonged low-dosage rapamycin treatment (0.2 mg/kg per d for 12 months) effectively suppressed renal cystogenesis,[9] suggesting that low dosages are sufficient to achieve effective mTOR inhibition in the kidney. These data are supported by a recent clinical trial using rapamycin in patients with tuberous sclerosis complex, which demonstrated that immunosuppressive dosage levels were sufficient to reduce the tumor burden of renal angiomyolipomas.[31] This suggests that renal drug concentrations that will inhibit mTOR can be achieved in patients. These data are also supported by our previous retrospective study in renal transplant patients with rapamycin-treated ADPKD, which exhibited a decrease in renal volume.[3] An additional retrospective study demonstrated a significant decrease in polycystic liver volumes and a trend toward renal volume decrease in transplant patients with rapamycin-treated ADPKD.[27] Interestingly, a recent report documenting the activation of mTOR in human autosomal recessive PKD (ARPKD) patients[32] suggested that rapamycin may also be effective in treating ARPKD and suggested that mTOR activation is a general feature of renal cystogenesis.[13]

Our study suggests that rapamycin treatment not only prevents further cyst growth but also results in cyst regression in $Pkd1^{cond/cond}$:Nes^{cre} mice (Figure 1G). This result is consistent with our previous observation that the renal volume in the nonorthologous orpk-rescue mouse model of PKD regresses during the course of rapamycin treatment, which we had at-

tributed to the induction of apoptosis in cyst-lining epithelial cells.[3] Because we also observed strong induction of apoptosis in the *Pkd1*$^{cond/cond}$:*Nes*^{cre} model (Figure 5), we suggest that regression of the cystic burden is due to a combination of reduced proliferation and increased apoptosis. This interpretation is also consistent with our previous observation of reduced renal volume (25%) in a retrospective study of transplant patients with ADPKD[3] and a recent report of decreased polycystic liver volumes in patients with rapamycin-treated ADPKD.[27] In addition, although only a single-patient case study, a significant reduction in kidney volume (1026 to 785 ml; 23.5% decrease) upon short-term (6 months), low-dosage rapamycin treatment of a patient with late-stage ADPKD has been reported.[33] Taken together, these data all suggest that rapamycin is capable of partially reversing renal cystic progression.

Although our study suggests that mTOR inhibitors may be effective in reducing renal cyst growth in human ADPKD, clinical trials will be needed to establish the degree of potential clinical benefit and to weigh any benefit against the expected adverse effects of treatment.

CONCISE METHODS

Animals

The generation of a mouse strain with conditionally targeted *Pkd1* alleles (exons 2 through 4; *Pkd1*$^{cond/cond}$) has been described previously.[16] This line was crossed with a mouse line expressing Cre recombinase under the control of the nestin promoter (*Pkd1*$^{cond/cond}$:*Nestin*^{cre}).[17] Animals were fed standard rodent chow and water *ad libitum* and were kept on a standard 12-hour light/dark cycle. Animals were treated according to an Institutional Animal Care and Use Committee–approved protocol.

Rapamycin treatment

Rapamycin (LC Laboratories, Woburn, MA) was dissolved in DMSO (1 mg/ml). Twenty-eight-day-old littermate *Pkd1*$^{cond/cond}$:*Nestin*^{cre} and *Pkd1*$^{cond/cond}$ animals received daily intraperitoneal injections of either vehicle (21.5% DMSO, 21.5% EtOH, and 57% sterile saline) or 5 mg/kg rapamycin (21.5% DMSO/rapamycin, 21.5% EtOH, and 57% sterile saline) from days 28 through 49. Body weights were recorded daily, and the dosing was adjusted accordingly.

BUN Measurements

Mice were anesthetized using ketamine and xylazine and 0.9% sterile saline and weighed. Blood was drawn *via* retro-orbital bleeding using a heparinized glass capillary tube, and plasma was separated from whole blood by centrifugation using a plasma separator tube containing lithium heparin (Becton Dickinson, Franklin Lakes, NJ). The plasma urea concentration was determined using a colorimetric-based urea assay kit (DIUR-500; BioAssay Systems, Hayward, CA) and converted to BUN according to the manufacturer's instructions.

Animal and Tissue Processing

After the animals were killed, both kidneys were removed and weighed. Kidneys were bisected, and one half was fixed in 10% neutral buffered formalin and the other was frozen with liquid nitrogen and stored at −80°C. Tissue was fixed overnight and processed for paraffin embedding and sectioning.

Western Blot Analyses

Frozen kidney tissue was finely minced and boiled in SDS buffer. Tissue lysates were standardized by protein concentration, and a total of 40 μg of protein per well was resolved on SDS-PAGE gels and transferred to nitrocellulose membranes. Antibodies against S6 (1:1000), phospho-S6 (Ser235/236; 1:5000; Cell Signaling Technology, Danvers, MA), and β-tubulin (1:2000; cell line E7; Developmental Studies Hybridoma Bank) were used for immunoblotting.

Immunohistochemistry

Unstained paraffin-embedded sections were rehydrated through two changes of xylene and a graded alcohol series. Endogenous peroxidase activity was blocked by incubating sections in 3% H_2O_2 in distilled water and then rinsed in 1× PBS. Antigens were unmasked by immersing sections in a pressure cooker containing boiling 10 mM sodium citrate buffer (pH 6.0) for 10 minutes.[34] Sections were rinsed in 1× PBS and processed for immunostaining using the Vector Elite ABC Universal kit (VectorLabs, Burlingame, CA) and a DAB substrate kit (VectorLabs) according to the manufacturer's protocol. Sections were counterstained with methyl green or hematoxylin QS (VectorLabs), dehydrated through a graded alcohol series and xylene, and mounted with Permount (Fisher Scientific, Pittsburgh, PA). Primary antibodies used were phospho-S6 (Ser235/236; 1:200; Cell Signaling Technology), Ki-67 (1:200; BD Biosciences, San Jose, CA), and SMA (1:200; Sigma-Aldrich, St. Louis, MO).

Immunofluorescence

Unstained paraffin-embedded sections were rehydrated through two changes of xylene and a graded alcohol series. Sections were blocked and incubated overnight in the presence of rhodamine-conjugated DBA (1:200; VectorLabs) and biotin-conjugated LTL (1:200; VectorLabs). Autofluorescence and endogenous fluorescence were quenched by a brief Sudan Black B incubation step. After washing to remove nonspecifically bound antibody, LTL was visualized with fluorescein-conjugated streptavidin (1:200; VectorLabs).

Apoptosis Assay

TUNEL staining was performed using an ApopTag kit (Millipore, Temecula, CA) with DAB staining following the manufacturer's instructions. For the incorporation of labeled nucleotides, a 1:16 dilution of TdT enzyme was used.

Cystic Index Calculations

Six representative 100× hematoxylin and eosin images were captured from renal sections of seven nontreated P28 through P33 *Pkd1*$^{cond/cond}$:*Nestin*^{cre} mice and four nontreated or rapamycin-treated P49 *Pkd1*$^{cond/cond}$:*Nestin*^{cre} mice. Images were opened in Photoshop (Adobe Systems Inc., San Jose, CA), converted to grayscale, and resized to 800 × 598 pixels. A grid was placed over the

image to give a total of 1064 individual points. The points bisecting cyst lumens/epithelium and noncystic epithelium were counted, and the ratio of points bisecting cyst lumen/epithelium:noncystic epithelium was calculated as a measure of the cystic index.

Ki-67 and TUNEL Counts

After immunohistochemical staining of renal sections with anti-Ki-67 or TUNEL, eight representative color ×200 fields were captured from three nontreated or rapamycin-treated *Pkd1*$^{cond/cond}$:*Nestin*^{cre} and *Pkd1*$^{cond/cond}$ mice, respectively. Each field was identically contrast-enhanced and converted to grayscale. Images were opened in ImageJ[35] and scaled to 800 × 598 pixels. The number of nuclei in each field was calculated using the ITCN plug-in (Center for Bio-Image Informatics, University of California, Santa Barbara, CA) for ImageJ. The correct identification of individual nuclei was confirmed visually. The number of Ki-67– or TUNEL-positive nuclei in each field was expressed as a percentage of the total number of nuclei in a given field. TUNEL counts were expressed as either the percentage of TUNEL-positive cells per total cells in cysts and cyst lumens or morphologically normal tubule cells. A total of at least 30,000 nuclei were counted for each genotype and treatment regimen.

Statistical Analysis

Statistics were performed using Prism 4.0 software (GraphPad, San Diego, CA) using one-way ANOVA with Newman-Keuls multiple-comparison post tests or *t* test, as appropriate. $P < 0.05$ was considered significant.

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DISCLOSURES

None.

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See related editorial, "Of Mice and Men: Therapeutic mTOR Inhibition in Polycystic Kidney Disease," on pages 390–391.

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