An mTOR anti-sense oligonucleotide decreases polycystic kidney disease in mice with a targeted mutation in Pkd2

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Autosomal dominant polycystic kidney disease (ADPKD) is the most common life-threatening hereditary disease in the USA. In human ADPKD studies, sirolimus, a mammalian target of rapamycin complex 1 (mTORC1) inhibitor, had little therapeutic effect. While sirolimus robustly inhibits mTORC1, it has a minimal effect on mTOR complex 2 (mTORC2). Polycystic kidneys of Pkd2WS25/— mice, an orthologous model of human ADPKD caused by a mutation in the Pkd2 gene, had an early increase in pS6 (marker of mTORC1) and pAktSer473 (marker of mTORC2). To investigate the effect of combined mTORC1 and 2 inhibition, Pkd2WS25/— mice were treated with an mTOR anti-sense oligonucleotide (ASO) that blocks mTOR expression thus inhibiting both mTORC1 and 2. The mTOR ASO resulted in a significant decrease in mTOR protein, pS6 and pAktSer473. Pkd2WS25/— mice treated with the ASO had a normalization of kidney weights and kidney function and a marked decrease in cyst volume. The mTOR ASO resulted in a significant decrease in proliferation and apoptosis of tubular epithelial cells. To demonstrate the role of mTORC2 on cyst growth, Rictor, the functional component of mTORC2, was silenced in Madin-Darby canine kidney cell cysts grown in 3D cultures. Silencing Rictor significantly decreased cyst volume and expression of pAktSer473. The decreased cyst size in the Rictor silenced cells was reversed by introduction of a constitutively active Akt1. In vitro, combined mTORC1 and 2 inhibition reduced cyst growth more than inhibition of mTORC1 or 2 alone. In conclusion, combined mTORC1 and 2 inhibition has therapeutic potential in ADPKD.

INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is the most common life-threatening hereditary disease in the USA. It affects about 1:400 to 1:1000 people. ADPKD is caused by a mutation in either the Pkd1 (85% of cases) or Pkd2 genes (15% of cases). Approximately 50% of people with ADPKD develop chronic kidney disease (CKD) around age 50. ADPKD accounts for ~5–10% of end-stage renal failure in the USA requiring dialysis and renal transplantation (1). There is no federal drug administration (FDA)-approved treatment that reduces cyst growth in ADPKD. Human and experimental data provide strong evidence that abnormal proliferation in tubular epithelial cells plays a crucial role in cyst development and/or growth in PKD (2). Genetic manipulations that induce the proliferation of tubular epithelial cells in mice cause cysts to form in the kidney (3,4). The mTOR signaling pathway regulates cell growth and proliferation that are dysregulated in ADPKD (5).

Mammalian target of rapamycin (mTOR) exists in two distinct structural and functional complexes mTORC1 and mTORC2 (6). In mTORC1, the mTOR protein complex includes specific association with Raptor (regulatory associated protein of mTOR). In mTORC2, the mTOR protein complex includes specific association with Rictor, mSin1 and protor-1 (7–9). mTORC1 controls cell proliferation and protein synthesis via p70S6K and 4EBP1. mTORC2 phosphorylates AGC kinase family members, including pAktSer473, PKCα and SGK1 proteins that modulate cell
survival and actin cytoskeleton arrangement (10,11). mTORC2 regulates the pro-survival kinase pAktSer473 by phosphorylation of Ser473 (12). pAktSer473 is involved in survival, apoptosis, metabolism, and proliferation (13). As both mTORC1 and 2 are involved in cell proliferation and apoptosis and proliferation and apoptosis of tubular cells lining the cysts plays a crucial role in cyst growth (2), we developed the hypothesis that combined mTORC1 and 2 inhibition would slow cyst growth.

Hyperactivation of mTORC1 signaling is a feature of experimental PKD (14–16) and human PKD (17). The mTORC1 inhibitors, sirolimus or everolimus, have a therapeutic effect in the Han:SPRD rat model of ADPKD that has aberrant expression and mislocalization of a protein called SamCystin (16,18,19), the Balb/C polycystic kidney mouse, an orthologous model of autosomal recessive polycystic kidney disease (ARPKD) that has a disruption in the mouse homolog of the Drosophilia Bicaudal C gene (14,20), and Pkd1 knockout (15) and Pkd2 knockout (21) mouse models of PKD. Interestingly, sirolimus did not have a therapeutic effect in female Han:SPRD rats with ADPKD and the lack of therapeutic effect was associated with an increase in pAktSer473 in the cystic kidney (22). Also inhibition of mTORC1 in sirolimus does not attenuate PKD in pck rats (23). Clinical trials investigating rapalogues in human PKD did not find a significant therapeutic effect (24,25). mTORC2 activation, as measured by pAktSer473 phosphorylation, is upregulated in PKD (15,22,26).

In view of the negative human studies of sirolimus and everolimus in human PKD (24,25), the motivation for performing the present study was to determine the effect on PKD of a novel agent, an anti-sense oligonucleotide (ASO), that inhibits both mTORC1 and 2 and to determine the effect of additional inhibition of mTORC2 specifically pAkt on cyst growth. Specifically, drugs like sirolimus and everolimus bind to FKBP12 which binds Raptor and indirectly inhibits mTORC1 but not mTORC2. The effect of direct mTOR inhibition using an ASO in PKD is not known. The study presents a novel approach of mTOR inhibition using an ASO. In our previous study of sirolimus in Pkd2WS25/− mice, sirolimus had no effect on pAkt (21). In fact, sirolimus resulted in an increase in pAkt in female Han:SPRD rats associated with no protection against PKD (22).

In the present study, the effect of combined mTORC1 and 2 inhibition in vivo in Pkd2WS25/− mice, an orthologous model of human ADPKD caused by a mutation in the Pkd2 gene, was achieved through the chronic administration of a mTOR ASO that reduces mTOR expression thus inhibiting both mTORC1 and 2. To further investigate the role of Rictor and Akt in cyst growth, Rictor was knocked down in Madin-Darby canine kidney (MDCK) cells and the effect on cyst growth of addition of a constitutively active Akt was determined. Thus the aims of the study were to determine the effect on PKD of an mTOR ASO that inhibits both mTORC1 and 2, to determine the effect of sirolimus, Rictor knockdown and the combination on cyst growth in vitro and to determine the effect of Akt on cyst growth in vitro.

RESULTS

mTOR ASO penetrates the kidney and cyst epithelium

Mice were treated with the mTOR ASO or scrambled ASO (Scr ASO) from 4 to 16 weeks of age and kidney cross-sections were evaluated to determine the ASO distribution. On immunohistochemistry, the distribution of mTOR ASO accumulation throughout the kidneys demonstrated a high degree of renal ASO accumulation with a gradient of high-to-low accumulation from the renal cortex to the medulla in the cystic kidneys (Supplementary Material, Fig. S1A). Additionally, cystic epithelial cells also showed mTOR ASO accumulation (Supplementary Material, Fig. S1A).

Ps6, pAktSer473 and mTOR protein are decreased by the mTOR ASO in vivo

At an early stage of PKD, at 4 weeks of age, there was a significant increase in pS6, a marker of mTORC1 signaling and pAktSer473, a marker of mTORC2 signaling in Pkd2WS25/− mouse kidneys (Fig. 1A). The mTOR ASO resulted in a significant decrease in mTOR (Fig. 1B), pS6 (Fig. 1B), both pAktSer473 and total Akt (Fig. 1B), but not pPSGKSer422 or pPKC Ser657 (Fig. 1B) at 16 weeks of age. On immunohistochemistry, the mTOR ASO resulted in a robust decrease in mTOR protein staining in the renal cortex and medulla (Fig. 1D) compared with the Scr ASO (Fig. 1C). Reverse phase protein analysis (RPAA) showed decreased mTOR, pS6 and pAkt in mTOR ASO-treated mouse kidneys compared with scrambled ASO-treated mouse kidneys (Supplementary Material, Fig. S1B).

mTOR ASO decreases PKD and normalizes renal function in vivo

The mTOR ASO had no effect on the body weight (Table 1). The two kidney (2K) weight and the two kidney/total body weight ratio (2K/TBW) which corrects for differences in body weight, was nearly double in Pkd2WS25/− mice compared with vehicle-treated +/+ mice and normalized by the mTOR ASO compared with the Scr ASO (Table 1). The cyst volume density (CVD) was significantly increased in Pkd2WS25/− mice compared with +/+ mice and was decreased by 59.8% in the mTOR ASO compared with the Scr ASO (Table 1). The blood urea nitrogen (BUN) was significantly increased in Pkd2WS25/− mice compared with +/+ mice and normalized by the mTOR ASO compared with the Scr ASO (Table 1). Thus, the mTOR ASO resulted in a normalization of kidney weight, 2K/TBW (%) and BUN in Pkd2WS25/− mice.

Representative kidney sections of Scr ASO-treated Pkd2WS25/− mice and mTOR ASO-treated Pkd2WS25/− mice, stained with hematoxylin–eosin, at the same magnification are demonstrated in Figure 2. These representative sections show that the percentage of the kidney that is occupied by cysts is dramatically reduced in the kidney from the mTOR ASO-treated Pkd2WS25/− mice compared with Scr ASO-treated mice.

Next, differences in PKD between male and female Pkd2WS25/− mice were determined. Besides body weight, the 2K weight, 2K/TBW ratio, CVD and BUN were not significantly different between males (n = 7) and females (n = 3) in the scrambled ASO-treated group. Body weight (g) was 30 ± 0.8 in males and 24.8 ± 3 in females (P = 0.03). The 2K weight (g) was 0.65 ± 0.06 in males and 0.7 ± 0.1 in females. 2K/TBW (%) was 2.1 ± 0.2 in males and 2.9 ± 0.3 in females. CVD (%) was 27.4 ± 2.8 in males and 41.1 ± 7.6 in
females. Blood urea nitrogen (mg/dl) was 39 ± 4 in males and 47 ± 10 in females.

A separate analysis was performed of females only in the study. Despite small numbers, the 2K weight, 2K/TBW and CVD, but not BUN, was significantly decreased with mTOR ASO treatment in females. Body weight (g) was 24.8 ± 1.1 in +/+ Scr ASO (n = 6), 24.0 ± 3.0 in +/+ mTOR ASO (n = 3), 24.7 ± 3.5 in Pkd2WS25/− Scr ASO (n = 3), 24.2 ± 0.5 in Pkd2WS25/− mTOR ASO (n = 4) (not significant). 2K weight (g) was 0.3 ± 0.02 in +/+ Scr ASO (n = 5), 0.3 ± 0.02 in +/+ mTOR ASO (n = 3), 0.7 ± 0.1 in Pkd2WS25/− Scr ASO (n = 3, P < 0.001 versus +/+), 0.37 ± 0.01 in Pkd2WS25/− mTOR ASO (n = 4, P < 0.001 versus Scr ASO). 2K/TBW (%) was 1.2 ± 0.03 in +/+ Scr ASO (n = 5, P < 0.001 versus mT), 1.2 ± 0.04 in +/+ mTOR ASO (n = 3), 2.9 ± 0.3 in Pkd2WS25/− Scr ASO (n = 3, P < 0.001 versus +/+), 1.5 ± 0.03 in Pkd2WS25/− mTOR ASO (n = 4, P < 0.01 versus Scr ASO). CVD (%) was 0.4 ± 0.2 in +/+ Scr ASO (n = 5), 0.5 ± 0.4 in +/+ mTOR ASO (n = 3), 41.1 ± 7.6 in Pkd2WS25/− Scr ASO (n = 3, P < 0.01 versus +/+), 12.2 ± 3.2 in Pkd2WS25/− mTOR ASO (n = 4, P < 0.01 versus Scr ASO). Blood urea nitrogen (mg/dl) was 32 ± 2.8 in +/+ Scr ASO (n = 6), 33 ± 4.8 in +/+ mTOR ASO (n = 3), 47 ± 10.3 in Pkd2WS25/− Scr ASO (n = 3), 29 ± 1.3 in Pkd2WS25/− mTOR ASO (n = 4) (P = 0.08 versus Scr ASO).

**mTOR ASO decreases tubular cell proliferation**

The number of proliferating cell nuclear antigen (PCNA) positive cells per high power field in non-cystic tubules in the cortex was 9.0 ± 0.5 in Scr ASO-treated Pkd2WS25/− mice and 2.7 ± 0.5 in mTOR ASO-treated Pkd2WS25/− mice (P < 0.01 versus Scr ASO-treated, n = 4) (Fig. 3A). Representative pictures of PCNA-positive cells in non-cystic tubules are shown in Figure 3C and E. The number of PCNA-positive cells per cyst in the cortex was 1.9 ± 0.2 in Scr ASO-treated...
Table 1. mTOR ASO (ASO) significantly reduces PKD and improves kidney function versus a scrambled ASO (Scr ASO)

<table>
<thead>
<tr>
<th></th>
<th>+/- Scr ASO (n = 11)</th>
<th>+/- ASO (n = 6)</th>
<th>WS25-Scr ASO (n = 10)</th>
<th>WS25-mTOR ASO (n = 6)</th>
</tr>
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<tbody>
<tr>
<td>BW (g)</td>
<td>28.2 ± 1.4</td>
<td>27 ± 2.0</td>
<td>27.3 ± 1.7</td>
<td>25.7 ± 1.3</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>0.34 ± 0.02</td>
<td>0.31 ± 0.02</td>
<td>0.67 ± 0.06*</td>
<td>0.39 ± 0.03**</td>
</tr>
<tr>
<td>CVD (%)</td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.4</td>
<td>34.1 ± 4.4*</td>
<td>15.1 ± 2.9***</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>32 ± 2.8</td>
<td>33 ± 4.8</td>
<td>43.4 ± 4.1*</td>
<td>29 ± 1.3***</td>
</tr>
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*P < 0.001 versus +/-; **P < 0.05 versus +/-; ***P < 0.01 versus WS25 Scr ASO, NS versus +/-; ****P < 0.05 versus WS25 Scr ASO, NS versus +/-.

mTOR ASO decreases apoptosis

The terminal deoxynucleotidyltransferase (TdT) mediated dUTP nick-end labeling (TUNEL) method was used to detect apoptotic cells. The number of apoptotic cells per cystic tubule was 3.6 ± 0.6 in Scr ASO-treated Pkd2WS25/− mice and 1.2 ± 0.1 in mTOR ASO-treated Pkd2WS25/− mice (P < 0.01 versus Scr ASO-treated, n = 6) (Fig. 3B). Representative images of apoptosis in non-cystic tubules showed a decrease in number of PCNA-positive cells in mTOR ASO-treated Pkd2WS25/− mice compared with Scr ASO-treated mice (Fig. 4B).

mTORC2 silencing reduces cyst size in vitro

Rictor was silenced in MDCK cells by introducing a plasmid-based short hairpin RNA (shRNA) vector system. Rictor silencing was confirmed by the expression of red fluorescence protein (RFP) in the presence of doxycycline and further confirmed by western blot analysis of collagenase digested cysts. Expression of the Rictor protein was not seen in Rictor silenced cysts (Supplementary Material, Fig. S2A).

Cyst enlargement in Rictor silenced MDCK collagen cysts was determined morphologically on Day 10 by bright-field microscopy (Fig. 5A) and confocal microscopy (Supplementary Material, Movie S1A and B). Wild-type MDCK collagen cysts (no puromycin or doxycycline treatment), MDCK collagen cysts treated with puromycin and no doxycycline (Dox−) or Rictor silenced MDCK collagen cysts treated with puromycin and doxycycline (Dox+) were studied. Cyst diameter was significantly reduced on Day 10 in Rictor silenced MDCK cells compared with cells not treated with doxycycline (Fig. 5B). Rictor silencing decreased the size of the cysts independent of the number of cysts. The number of cysts per three wells on Day 10 was 196 in wild-type, 200 in Dox− and 220 in Rictor silenced Dox+ cells.

The effect of Rictor knockdown on cyst size is shown by confocal microscopy in Supplementary Material, Figures S2B and C.

Sirolimus decreases cyst size in vitro

Cyst enlargement in MDCK collagen cysts treated with sirolimus was determined morphologically on Day 10 by bright-field microscopy (Fig. 5A) and confocal microscopy (Supplementary Material, Movie S1C). Madin-Darby canine kidney collagen cysts were treated with sirolimus (100 nM). Cyst diameter was significantly reduced on Day 10 in sirolimus-treated compared with vehicle-treated MDCK cells (Fig. 5). Sirolimus did not reduce the number of cysts. The number of cysts per three wells on Day 10 was 190 in vehicle-treated and 212 in sirolimus-treated.

Combined inhibition of mTORC1 (sirolimus treatment) and mTORC2 (Rictor silencing) further decreased the cyst size

Cyst enlargement in Rictor silenced MDCK cells treated with sirolimus was determined morphologically on Day 10 by bright-field microscopy (Fig. 5A) and confocal microscopy (Supplementary Material, Movie S1D). Madin-Darby canine kidney cell collagen cysts were treated with sirolimus (100 nM) to inhibit mTORC1 and doxycycline to knockout Rictor. Cyst diameter was significantly reduced on Day 10 in Rictor silenced MDCK cells treated with sirolimus compared with wild-type cells treated with vehicle [0.01% dimethyl sulfoxide (DMSO)] (Fig. 5B). The decrease in mean cyst diameter in Rictor silenced MDCK cells treated with sirolimus was significantly decreased compared with Rictor silencing or sirolimus alone (Fig. 5B). Cyst number was not different between groups. The number of cysts per three wells on Day 10 was 190 in vehicle-treated and 212 in Rictor silenced cells treated with sirolimus.
Rictor silencing decreases pAktSer473 in collagen cysts

On immunoblot of collagenase digested cysts, Rictor silencing resulted in decreased pAktSer473 and pPKCaSer657 but had no effect on the expression levels of pSGKSer422 (Fig. 6A).

Sirolimus decreases S6K

On immunoblot of collagenase digested cysts, the mTORC1 downstream target ribosomal pS6 kinase (S6K) was decreased relative to total S6 (Fig. 6B). The inhibition was time-dependent with maximum inhibition on Day 10 showing the role of sirolimus in suppressing the mTORC1 pathway. pAktSer473 expression levels were slightly decreased by sirolimus (Fig. 6B). It is known that high-dose long-term treatment with sirolimus can result in some inhibition of pAktSer473 (12). Sirolimus had no effect on pPKCaSer657 and pSGKSer422 (Fig. 6B).

pAktSer473 expression is absent in rictor silenced MDCK cells treated with sirolimus

On immunoblot of collagenase digested cysts, pAktSer473 expression was absent in combined Rictor silencing and sirolimus compared with wild-type cells treated with the vehicle (0.01% DMSO) (Fig. 6C). Confocal imaging shows decreased Ki67 staining in combined Rictor silencing and sirolimus treatment compared with vehicle-treated wild-type cysts (Fig. 6D).

Akt1 over-expression reverses the decrease in cyst size due to Rictor silencing

A constitutively active Akt1 was over-expressed in Rictor silenced MDCK cells. Bright-field images show a decrease in cyst size in Rictor silenced cells that is reversed in cells overexpressing Akt1 (Fig. 7A). The decrease in cyst diameter in Rictor silenced cells is reversed in cells overexpressing Akt1 (Fig. 7B). The number of cysts was not different between groups.
The number of cysts per three wells on Day 10 was 189 in wild-type, 182 in (Dox+) and 184 in (Dox+Blasticidin).

DISCUSSION

The mTOR signaling pathway regulates cell growth and proliferation that are dysregulated in ADPKD (5). Sirolimus, an mTOR inhibitor, is an FDA-approved immunosuppressive drug and is a powerful anti-proliferative drug. In view of the importance of tubular cell proliferation in cyst formation and the anti-proliferative effects of sirolimus, the effect of sirolimus on cyst formation and disease progression was previously tested in animal models of PKD.

In 2005, it was demonstrated that sirolimus decreased kidney and cyst enlargement and prevented the loss of kidney function in the male Han:SPRD rat model of ADPKD (18). The first studies of sirolimus in Pkd1 or Pkd2 knockout mouse models were reported in 2010. Sirolimus reduced cyst growth and preserved renal function in mice with PKD resulting from a conditional inactivation of Pkd1 (15). However, sirolimus reduced cyst growth, but had no effect on renal function in Pkd2WS25/− mice (21).

As a result of the positive studies in rodent models of PKD and the demonstration of mTORC1 activation in human PKD (17,27), human studies were undertaken. Three initial small studies in humans suggested that sirolimus could reduce cystic disease (14,28,29). Thus larger randomized control studies were undertaken and the results have recently been published (24,25). Both randomized clinical studies were essentially negative and associated with a high side effect and drop-out rate. There are multiple possible reasons for the unimpressive effect of the rapalogues in the human studies. One possible reason is that the rapalogues, sirolimus and everolimus, bind to FKBP12 which directly binds and inhibits mTORC1, not mTORC2. Sirolimus does not directly target mTORC2-dependent Akt-induced proliferation (30). It is possible that inhibition of both mTORC1 and 2 by the new mTOR kinase inhibitors (TORKs) or an anti-sense that inhibits mTOR directly will slow cyst growth in PKD.

There are animal studies that provide supporting evidence of mTORC2 activation in PKD. The lack of effect of sirolimus on PKD in female Han:SPRD rats was associated with an increase
in the pro-proliferative p-AktSer\textsuperscript{473}, a marker of mTORC2 activation (22). Our published data demonstrate increased p-AktSer\textsuperscript{473} and increased pPKC\textalpha Ser\textsuperscript{657}, markers of mTORC2 activation, in 8-week-old male Cy/+ rats and increased pPKC\textalpha Ser\textsuperscript{657} in 112-day-old Pkd2WS25/\textsuperscript{2} mice with PKD (21).

Increased p-AktSer\textsuperscript{473} signaling in Pkd1 knockout mice with PKD has also been demonstrated (15). With this background, the effect of combined mTORC1 and mTORC2 inhibition was tested \textit{in vivo} in Pkd2WS25/\textsuperscript{2} mice, a homologs model of human PKD caused by a mutation in the Pkd2 gene, by the chronic administration of an mTOR ASO.

The mTOR ASO resulted in a decrease of pS6, a marker of mTORC1 activation and pAktSer\textsuperscript{473}, a marker of mTORC2 activation in the kidney in Pkd2WS25/\textsuperscript{2} mice. The mTOR ASO resulted in a normalization of kidney weight, 2K/TBW ratio and kidney function. Cyst volume density was reduced by 60%. In our previous study of sirolimus in Pkd2WS25/\textsuperscript{2} mice, performed in an identical fashion to the current study, sirolimus resulted in a significant decrease in apoptosis in both non-cystic and cystic tubules. In contrast, sirolimus had no effect on apoptosis in Pkd2WS25/\textsuperscript{2} mice (21). The role of apoptosis in proliferation of tubular cells and cyst growth is controversial (31). High dose sirolimus (5 mg/kg/day) resulted in increased apoptosis in kidneys of Pkd1 knockout mice (15) whereas lower dose sirolimus (0.5 mg/kg/day) had no effect on apoptosis in kidneys of Pkd2WS25/\textsuperscript{2} mice (21) and Han:SPRD rats (22). We have demonstrated that both pharmacological (32) and genetic (33) methods of apoptosis inhibition result in slower cyst growth. Thus it is possible that part of the different effect of the mTOR ASO compared with sirolimus on cyst growth and kidney function may be related to more effective inhibition of apoptosis in cystic and non-cystic epithelium. Rapamycin is only a partial inhibitor of mTORC1 in most cell types (34). Rapamycin does not block all mTORC1 outputs (30). For example, 4E-BP1 is an important pro-proliferative protein downstream of mTORC1 that is inhibited more by mTOR kinase than by rapamycin (35). mTOR kinase inhibition more effectively targets 4E-BP1 at rapamycin sensitive and insensitive sites (30). Phosphorylation of 4E-BP1 is rapamycin resistant but is inhibited by mTOR kinase inhibition (36). Thus, the impressive effect of the mTOR ASO on PKD, despite less inhibition of pS6 than rapamycin, may be due to a greater effect on other mTORC1 outputs like 4E-BP1. Studies of the effect of rapamycin versus mTOR kinase inhibition on 4E-BP1 in PKD will be interesting.

\textbf{Figure 5.} Sirolimus treatment plus Rictor knockdown slows the growth of cysts in collagen 3D culture to a greater extent than Rictor knockdown or sirolimus treatment alone. (A) Representative bright-field micrographs of wild-type (WT) MDCK collagen cysts (no puromycin or doxycycline treatment), MDCK collagen cysts treated with puromycin and no doxycycline (Dox ), Rictor knockdown MDCK collagen cysts treated with puromycin and doxycycline (Dox+), vehicle-treated collagen cysts (Veh), sirolimus treatment (Siro) and sirolimus-treatment in Rictor knockdown cells (Dox+Siro). Scale bar 100 \textmu m. (B) Mean cyst diameter on Day 10 was decreased in (1) Dox+ versus Dox− and WT cells. \textit{P} < 0.001 versus Dox−, WT; (2) Siro versus Veh treatment. \textit{**P} < 0.001 versus Veh and (3) in Dox+ Siro compared with Veh, Dox+ or Siro. \textit{***P} < 0.001 versus Veh, Dox+ and Siro.
Figure 6. Immunoblots for pS6, pAktSer473, pPKCαSer657 and pSGKSer422. (A) Rictor silencing decreased the expression levels of pAktSer473 and pPKCαSer657 but not pSGKSer422 in (Dox+) collagen cysts as compared with (Dox−) cells. β-actin served as loading control. (B) The mTORC1 downstream target ribosomal pS6 kinase (S6K) was decreased relative to total S6 by sirolimus. pAktSer473 expression levels were slightly decreased by sirolimus. Sirolimus had no effect on pPKCα Ser657 and pPSGKSer422. β-actin served as the loading control. (C) pAktSer473 expression was decreased by combined Rictor silencing and sirolimus compared with Rictor silencing alone (Dox+). In densitometric analysis of immunoblots, data are presented as the mean of at least three separate experiments. β-actin served as loading control. (D) Proliferation was detected by immunofluorescence analysis using an antibody directed against Ki67. Representative 3D confocal images show decreased Ki67 staining (red) in sirolimus and (Dox+ and sirolimus) collagen cysts on Day 7 compared with vehicle-treated (Veh). Nuclei are stained blue with Hoechst.
It is possible that all of the benefit of the mTOR ASO in reducing PKD was due to the mTORC1 inhibition regardless of whether mTORC2 was also targeted or not. In the absence of specific mTORC2 inhibitors, the study could not be designed to test the relative effectiveness of mTORC1 versus mTORC2 inhibition in vivo. In the future, the development of specific Rictor, Pkd1 knockout mice may delineate the specific role of mTORC2 in PKD.

Next, to determine that specific inhibition of mTORC2 results in less cyst growth and to further demonstrate that combined mTORC1 and 2 inhibition results in smaller cysts than inhibition of mTORC1 or 2 alone, mTORC1 inhibition was achieved using sirolimus and combined mTORC1 and mTORC2 inhibition was achieved by treating Rictor silenced MDCK cells with sirolimus. To understand the signaling pathways involved in cyst growth in the MDCK cells that form cysts in culture, the effect of mTOR inhibition on pS6, the major marker of mTORC1 and pAktSer473, pPKCoSer657 and pPSGKSer422 was determined. The best characterized downstream mTORC2 substrates are the AGC kinases pAktSer473, pPKCoSer657 and pPSGKSer422. In a landmark paper in Science in 2005, it was shown that the Rictor-mTOR complex directly phosphorylates Akt on Ser473 and that a reduction in Rictor expression inhibited pAktSer473 (45). Knockout of mTORC2 does not affect mTORC1 suggesting that mTORC2 activates a pool of Akt that is not upstream of mTORC1 (8). mTORC2 is essential for pPKCoSer657 signaling (46). pPSGKSer422 is a recently discovered mTORC2 substrate (47) The phosphorylation of SGK1 on Ser422 and SGK1 activity are inhibited in fibroblasts from mice lacking mTORC2 but still possessing mTORC1 activity (48,49). In the present study, inhibition of mTORC2 in MDCK cells resulted in less pAktSer473 and pPKCoSer657, but had no effect on the expression levels of pPSGKSer422. In vivo, the mTOR ASO resulted in less pAktSer473, but had no effect on pPSGKSer422 and pPKCoSer657 in the kidney. Thus, the mTOR ASO in vivo or Rictor knockdown in vitro had a different effect on pPKCoSer657 and pPSGKSer422. However, both the mTOR ASO in vivo or Rictor knockdown in vitro resulted in inhibition of pAktSer473 and slower cyst growth. In addition, Akt1 over-expression reversed the decrease in cyst size due to Rictor silencing, confirming the importance of pAktSer473 in mTOR-mediated cyst growth.

Sirolimus resulted in a decrease in pS6, a marker of mTORC2 in vivo model was used to answer the question whether Rictor plays a role in cyst growth and to understand general mechanisms of tubule formation and maintenance and not to study signaling pathways as they relate to the polycystins. The model was also used to determine the effect of reintroduction of a constitutively active Akt on cyst size in Rictor knockout cells.

In the cell culture model, inhibition of mTORC2 signaling by silencing Rictor, the functional component of mTORC2, resulted in smaller cysts. To demonstrate that mTORC2-mediated production of pAktSer473 mediates cyst growth in vitro, re-introduction of a constitutively active Akt1 reversed the decrease in cyst growth mediated by Rictor inhibition.

Next, it was demonstrated in vitro that combined inhibition of mTORC1 and 2 reduces cyst growth more than inhibition of mTORC1 or 2 alone. mTORC1 inhibition was achieved using sirolimus and combined mTORC1 and mTORC2 inhibition was achieved by treating Rictor silenced MDCK cells with sirolimus. To understand the signaling pathways involved in cyst growth in the MDCK cells that form cysts in culture, the effect of mTOR inhibition on pS6, the major marker of mTORC1 and pAktSer473, pPKCoSer657 and pPSGKSer422 was determined. The best characterized downstream mTORC2 substrates are the AGC kinases pAktSer473, pPKCoSer657 and pPSGKSer422. In a landmark paper in Science in 2005, it was shown that the Rictor-mTOR complex directly phosphorylates Akt on Ser473 and that a reduction in Rictor expression inhibited pAktSer473 (45). Knockout of mTORC2 does not affect mTORC1 suggesting that mTORC2 activates a pool of Akt that is not upstream of mTORC1 (8). mTORC2 is essential for pPKCoSer657 signaling (46). pPSGKSer422 is a recently discovered mTORC2 substrate (47) The phosphorylation of SGK1 on Ser422 and SGK1 activity are inhibited in fibroblasts from mice lacking mTORC2 but still possessing mTORC1 activity (48,49).

Human Molecular Genetics, 2014, Vol. 23, No. 18

Figure 7. Akt1 overexpression reverses the decrease in cyst size due to Rictor silencing. A constitutively active Akt1 was overexpressed in Rictor silenced MDCK cells. (A) Bright-field images on Day 10 show a decrease in cyst size in Rictor silenced cells (Dox-) that is reversed in cells overexpressing Akt1 (Dox+ + Blasticidin). Scale bar, 10 μm. (B) The decrease in cyst diameter on Day 10 in (+Dox) is reversed in (Dox+ + Blasticidin) collagen cysts. *P < 0.01 versus (Dox+ + Blasticidin) and WT.

In three independent studies, there was no significant difference
between 2K/TBW ratio and cyst volume density in male versus female Pkd2WS25/− mice (51–53). In the present study the two kidney weight, 2K/TBW, cyst volume density and BUN was not different between scrambled ASO-treated males versus females. In a sub-analysis of females only, mTOR ASO-treated females had less PKD than Scr ASO-treated females.

In summary, our data on Pkd2WS25/− mice with PKD, demonstrate (1) an early increase in pS6 and pAktSer473 in the kidney, (2) the mTOR ASO resulted in a significant decrease in mTOR protein, pS6 and pAktSer473 in the kidney and (3) the mTOR ASO-treatment resulted in a normalization of kidney weight, 2K/TBW ratio and BUN and a 60% decrease in cyst volume density. In MDCK cells that form cysts in culture, specific inhibition of Rictor, the functional component of mTORC2, resulted in decreased cyst size that was reversed by introduction of a constitutively active Akt1 into the Rictor silenced cells.

In conclusion, combined mTORC1 and 2 inhibition has therapeutic potential in PKD and combined inhibition of mTORC1 and mTORC2 by using specific mTOR kinase inhibitors may offer a new therapeutic option in PKD. The potential future clinical use of mTOR kinase inhibition in PKD is supported by five Phase I/II clinical studies of the mTOR kinase inhibitor AZD8055 (AstraZeneca) in patients with various advanced solid tumors (see clinicaltrials.gov).

MATERIALS AND METHODS

Materials

Details of inhibitors used and antibodies are provided in the Supplementary Material, Methods.

Animals

The study was conducted in Pkd2WS25/− mice and normal littersmate control (+/+ ) mice. The Pkd2WS25/− mouse develops clinically detectable polycystic kidney disease by 16 weeks of age as evidenced by a cyst volume density of more than 30% of the kidney and renal failure compared with +/+ control mice (48,50,51). A colony of Pkd2WS25/− mice was established in our animal care facility from a litter that was obtained from Stefan Somlo at Yale University. The study protocol was approved by the University of Colorado Denver Animal Care and Use Committee. Mice had free access to tap water and standard rat chow. C57BL/6 Pkd2+/− and C57BL/6 Pkd2WS25/+ mice were used as breeding pairs to generate Pkd2WS25/− mice for the study. Mice were genotyped by Southern blotting (48,49). Pkd2WS25/− mice closely model the human condition by having one copy of Pkd2 knocked out and having a second, recombinant-sensitive allele (i.e. WS25) that undergoes high rates of recombination to yield knockouts of the second copy of the gene in somatic cells during the life span of the animals.

Experimental protocol

Litters from Pkd2+−/− mice crossed with Pkd2WS25/+ mice were weaned at 3 weeks of age and then genotyped. Mice were treated with an mTOR ASO or littermate controls were given a control mismatch scrambled ASO (Scr ASO) from 4 to 16 weeks of age. Three of the Pkd2WS25/− littermates were not treated with the Scr ASO. Both ASOs were administered once weekly via intraperitoneal (i.p.) injections at 100 mg/kg/week for the first 4 weeks, and then 50 mg/kg/week for the remaining 8 weeks of treatments. The mTOR ASO and Scr ASO were obtained from ISIS Pharmaceuticals, Carlsbad, CA. Both ASOs were 20-mer second generation MOE gapmers of the 5-10-5 design. Specifically, the phosphorothioate oligonucleotides contained 2′-O-(2-methoxyethyl)-modified ribonucleosides (2′-MOE) groups at positions 1–5 and 16–20 with 2′-deoxynucleosides at positions 6–15. The sequences of both ASOs were as follows: mTOR ASO, 5′-TCCACTTTTACACGACTGC-3′ and Scr ASO, 5′-CCCTCCTGAAGGTCCCTCC-3′. The mTOR ASO was chosen after a screen of ~150 prospective leads in primary murine hepatocytes. The top in vitro leads were then tested in C57BL/6 mice, and the lead mTOR ASO was chosen based on renal activity and tolerability. The Scr ASO does not hybridize to any known target and was used to control for any ASO class effects that could impact cystogenesis and/or renal function.

Cyst volume density in vivo

Hematoxylin–eosin stained sections were used to determine the cyst volume density. This was performed by a reviewer, blinded to the identity of the treatment modality, using point counting stereology (54). In Pkd2WS25/− mice, 80% of the cysts originate from distal nephron segments (48). At least 10 areas of the kidney were randomly selected at each of 90°, 180° and 270° from the hilum to guard against field selection variation.

PCNA staining

Immunohistochemical detection of PCNA staining was performed using an anti-PCNA antibody. The sections were incubated with alkaline-phosphatase labeled polymer (DAKO EnVision System, Cat# K4016, DAKO, Carpinteria, CA) and visualized with the substrate chromogen, fast red. Negative control sections showed no staining. The number of PCNA-positive cells per cyst was counted using a Nikon Eclipse E400 microscope equipped with a digital camera connected to Spot Advanced imaging software (Version 3.5) by an observer blinded to the treatment modality. To avoid confusion between non-cystic tubules and small cysts as well as potential changes in tubular cells lining massive cysts, PCNA-positive tubular cells were counted in ‘medium-sized cysts’ of ~50–250 μm diameter. The number of PCNA-positive cells in non-cystic tubules (defined as tubules <50 μm diameter) was expressed per high power field and counted in areas of cortex devoid of cysts. At least 10 areas of the kidney were randomly selected at each of 90°, 180° and 270° from the hilum to guard against field selection variation.

In situ detection of DNA fragmentation

The terminal deoxynucleotidyltransferase (TdT) mediated dUTP nick-end labeling (TUNEL) method was used to detect in situ DNA strand breaks. The Deadend™ Colorimetric TUNEL assay kit (Promega, Madison, WI) was used. Positive and negative controls for TUNEL stain were performed. All
cells with apoptotic morphology (cellular rounding and shrinkage, pyknotic nuclei or formation of apoptotic bodies) that stained positive with the TUNEL assay were counted. The number of TUNEL positive cells per tubule was counted using a Nikon Eclipse E400 microscope equipped with a digital camera connected to Spot Advanced imaging software (Version 3.5) by an observer blinded to the treatment modality, as we have previously described (18,32). Twelve areas per sample were randomly selected at 90°, 180° and 270° from the hilum of each section to guard against field selection variation. To avoid confusion between non-cystic tubules and small cysts as well as potential changes in tubular cells lining massive cysts, apoptotic cells were counted in ‘medium sized cysts’ of ~50–250 μm diameter. The number of apoptotic cells in non-cystic tubules (defined as tubules <50 μm diameter) was expressed per high power field and counted in areas of cortex devoid of cysts. At least 10 areas of the kidney were randomly selected at each of 90°, 180° and 270° from the hilum to guard against field selection variation.

**Immunohistochemistry for ASO in the kidney**

Details are given in the Supplementary Material, Methods.

**Chemistry**

Blood urea nitrogen was measured using quantitative colorimetric urea determination (QuantiChrom™ urea assay kit-DIUR-500) (Bioassay Systems, Hayward, CA).

**Cell culture**

Type I MDCK cells hereafter referred to as MDCK cells (ATCC No.CCL-34) were cultured at 37°C in a humidified 95% air/5% CO₂ atmosphere in a Minimum Essential Medium supplemented with 10% Fetal bovine serum (Fetal Plex, Gemini Bioproducts, Sacramento, CA), 10 000 U/ml penicillin, and 10 000 μg/ml streptomycin. For cysts generation, 400 individual MDCK cells were suspended in 0.8 ml of Minimum Essential Medium containing 3 mg/ml collagen (PureCol, Advanced Biomatrix, San Diego, CA) and 0.1 M NaOH. The cell suspension was plated onto 24-well plates. After gel formation, 1 ml of MDCK cell medium containing 10 μM forskolin (55,56) was added to each well and the plates were maintained at 37°C in a 5% CO₂ humidified atmosphere.

**Silencing and inhibition studies**

For mTORC2 silencing studies, a plasmid-based shRNA vector system for Rictor was employed (pTRIPZ) expressing RNAs under the Tet-On inducible promoter (Open Biosystems, Huntsville, AL). Expression of a turbo RFP as part of the Tet-On expression cassette allows cells to be monitored for expression using a Nikon Eclipse microscope (TE2000-S) with appropriate filters. Details of the transfection are given in the Supplementary Material, Methods. For testing the effect of mTORC1 inhibition, sirolimus (100 nm) was added in the culture medium. The medium containing sirolimus was changed every 24 h. 0.1% DMSO was used as the vehicle control for sirolimus. For the combination mTORC1/mTORC2 inhibition study, sirolimus (100 nm) was added to Rictor silenced MDCK cysts. All experiments were carried out in the presence of forskolin (10 μM) in the medium from Day 0 onward.

**Cyst measurements**

Individual cysts, with diameters >50 μm, were identified and counted on Day 10 using bright-field microscopy. Details of cyst measurements are given in Supplementary Material, Methods.

**Immunoblotting**

Whole kidney was homogenized in radioimmunoprecipitation assay (RIPA) buffer plus proteinase inhibitors and immunoblotted as described previously (57). Whole-kidney lysates were prepared and immunoblotted from six different animals from each of the following groups: +/- scrambled ASO-treated, WS25 scrambled ASO-treated and WS25-mTOR ASO-treated. For immunoblot analysis, actin was measured concurrently with each protein in the same sample on each blot so that each protein’s level was normalized to the level of actin in the same lane. Madin-Darby canine kidney cysts were incubated with Type 1 collagenase (catalog no. LS004194, Worthington) for 30 min at 37°C. Protein extraction of 3D cultures was performed by washing with PBS and dissolving in lysis buffer (Cell Signaling, #9803). Immunoblot was performed with monoclonal antibodies as described in Materials and Methods. Signals were detected with horseradish peroxidase-conjugated antibodies and enhanced chemiluminescence (Super-Signal West Dura Extended; Pierce, Rockford, IL, USA). Immunoblots shown in figures are representative of at least three separate experiments.

**Confocal and bright-field microscopy**

Details are given in the Supplementary Material, Methods.

**Statistical evaluation**

Values are expressed as mean ± SE. Non-normally distributed data were analyzed by the nonparametric unpaired Mann–Whitney test. Multiple group comparisons were performed by analysis of variance (ANOVA). A level of P < 0.05 was considered to be significant.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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