Mammalian Target of Rapamycin and Caspase Inhibitors in Polycystic Kidney Disease

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One of the most important abnormalities of the tubular epithelial cells lining the cysts as well as noncystic tubular epithelium is a disturbance in the balance between tubular cell proliferation and apoptosis. Activation of the mammalian target of rapamycin signaling pathway results in increased cell proliferation. Recent studies suggested abnormalities of the mammalian target of rapamycin signaling pathway in polycystic kidney disease. Mammalian target of rapamycin inhibition with sirolimus or everolimus results in attenuation of cyst formation in rat and mouse models of polycystic kidney disease. Apoptosis is a pathologic feature of most models of polycystic kidney disease, including human polycystic kidneys. Caspases, the major mediators of apoptosis, are increased in polycystic kidney disease kidneys. Both in vitro and in vivo studies suggest that caspase or apoptosis inhibition attenuates cyst formation. This review focuses on mammalian target of rapamycin and apoptosis signaling pathways in polycystic kidney disease and the role of mammalian target of rapamycin inhibitors and apoptosis inhibitors as potential therapies to reduce cyst formation.

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mTOR Signaling in PKD

Dysregulated mTOR and phosphatidylinositol-3 kinase signaling contributes to the pathophysiology of human diseases, including heart disease, diabetes, and muscular atrophy (13). Recent studies suggested abnormalities of mTOR signaling in kidney diseases: (1) Rapamycin markedly decreases disease severity in PKD, (2) rapamycin decreases uninephrectomy-induced compensatory hypertrophy in the contralateral kidney (14), and (3) rapamycin reduces renal tumors and cysts in animal models of TSC (7) and in humans with TSC and angiomylipomas (8).

IGF-1

Aberrant expression of growth factor receptors and the accumulation of growth factors in cyst fluid may modulate Akt. There is evidence for IGF-1 signaling in PKD: (1) In the pcy mouse model of PKD, mRNA levels of IGF-I in the kidney increase with disease progression (15); (2) in Han:SPRD rats, IGF-I protein levels are increased in PKD compared with control kidneys (16); (3) systemic administration of IGF-1 results in proliferation of proximal tubules in rat kidneys (17); (4) estrogens and IGF-1 markedly stimulate proliferation of an immortalized hepatic cyst epithelium cell line by acting on the IGF-1R; the proliferative effect was blocked by an IGF-1R blocking antibody (18); and (5) EGF plays an important role in cyst epithelial cell proliferation and cyst expansion in animal models and human studies. EGF receptor (EGFR) inhibition (19) (20) lessens cyst formation in animal models of PKD. EGFR-mediated mTOR/p70S6K signaling has been described in 3T3 cells. Cross-talk between EGF- and IGF-mediated pathways has been described in that EGF may regulate IGF-binding protein (21); however, the effect of EGFR tyrosine kinase inhibition on mTOR signaling in PKD is not known.

Akt

The amount of phosho-Akt (p-Akt) in cystic kidney of Pdk1−/− kidneys was more than that in wild-type kidneys (22). In 16-wk-old Han:SPRD rat kidneys, constitutive expression of Akt-1, -2, and -3 mRNA was seen in both wild-type and PKD kidneys (23); however, on immunoblot and ELISA, there was increased p-Akt (Ser473) in PKD kidneys compared with controls.

TSC1/2, mTOR, and p70S6K

In vitro studies demonstrated that the N-terminal cytoplasmic domain of polycystin 1 co-localizes with mTOR and co-localizes and interacts with tuberin (24). Phospho-mTOR and p70S6K are induced in cyst-lining epithelial cells in cysts from human kidneys (24). p70S6K (Thr389) and total S6K are increased in 12-wk-old Han:SPRD rat kidneys with PKD and

(FK506-binding protein of 12 kD-rapamycin-associated protein) (Figure 1).

The disease tuberous sclerosis is caused by mutations in the tuberous sclerosis complex 1 (TSC1) and TSC2 genes. The major genes for tuberous sclerosis and ADPKD, TSC2 and PKD1, respectively, lie adjacent to each other at chromosome 16p13.3, suggesting a role for the PKD1 gene in the cause of renal cystic disease in tuberous sclerosis. That TSC1/2 mutations upregulate mTOR signaling suggests that rapamycin and its analogs may be useful in the treatment of TSC. In fact, the rapamycin analog CCI-779 reduces kidney tumors in TSC2−/− mice (7). A recently published study demonstrated that rapamycin reduced angiomylipoma formation in patients with TSC (8).

There may be interdependence between the mTOR signaling pathway and other pathways (e.g., the cyclin D1 cyclin-dependent kinase [CDK] pathway). The CDK inhibitor p21 is down-regulated in cystic kidneys, and there is increased expression of the cyclin D1 gene in cyst-lining epithelial cells (9). Renal expression of cyclins A2 and B1 mRNA is elevated five- and six-fold, respectively, in cystic kidneys of BALB/c-cpk/cpk mice (10). Rapamycin modulates the cyclin D1-CDK association, resulting in inhibition of G1- to S-phase cell-cycle progression (11). In addition, long-lasting arrest of murine PKD with CDK inhibitor roscovitine has been described (12). With this background, components of the mTOR signaling pathway that have been studied in PKD are reviewed.
inhibited with rapamycin treatment (25). mTOR signaling pathways that are activated in PKD are summarized in Table 1.

**mTOR Inhibition in PKD**

Rapamycin is derived from the soil bacterium *Streptomyces hygroscopicus*, which is found on Easter Island. Because Easter Island is known as Rapa Nui in the local language, the product of purification of the active compound from *Streptomyces hygroscopicus* was called rapamycin. Rapamycin is also known as sirolimus. Sirolimus specifically inhibits TOR, resulting in reduced cell growth, reduced cell-cycle progression, and decreased cellular proliferation.

Rapamycin inhibits proliferation of hematopoietic cells and is Food and Drug Administration (FDA)-approved to prevent kidney transplant rejection (3). Rapamycin inhibits the increased proliferation and migration of vascular smooth muscle cells that occurs after injury and rapamycin is FDA-approved for use on drug-eluting stents to inhibit re-stenosis. Rapamycin and its analogs CCI-779, RAD001, and AP23573 have demonstrated promising anticancer activity and show relatively mild adverse effects in phase I and II clinical studies (26).

Because increased tubular epithelial cell proliferation is a prerequisite for cyst formation and expansion in PKD (2) and sirolimus is a potent antiproliferative agent, the effect of sirolimus treatment on tubular cell proliferation, cyst formation, and renal failure was tested in the Han:SPRD rat model of PKD (27). Rats were treated with rapamycin 0.2 mg/kg per d intraperitoneally or vehicle from 4 to 8 wk of age. Rapamycin treatment (1) decreased proliferation in cystic and noncystic tubules, (2) markedly inhibited renal enlargement (65% decrease in two kidney/total body weight ratio) and cystogenesis (40% decrease in cyst volume density), and (3) prevented the loss of kidney function (59% decrease in blood urea nitrogen [BUN]) in PKD rats (27). Representative kidney sections from control, PKD, and PKD rats that were treated with rapamycin are demonstrated in Figure 2.

Whereas the proliferation index has been found to be consistently highest in cystic tubular epithelium, noncystic tubules from mice with polycystic kidneys (28) and Han:SPRD rats (29) have higher proliferation rates than tubules from age-matched controls. Rapamycin decreased tubular cell proliferation in noncystic as well as cystic tubules (27). These studies suggested that tubular cell proliferation precedes cyst formation in the Han:SPRD rat (29) and that rapamycin may decrease cyst formation, in part, by decreasing tubular cell proliferation in noncystic tubules.

Rapamycin modestly decreased the body weight in both PKD and control rats despite no apparent difference in food intake (27). Other studies in rats and mice have described weight loss as a result of rapamycin; however, long-term treatment with rapamycin has not been reported to cause weight loss in adults or children (30,31).

In a second study of male Han:SPRD rats, rapamycin was given orally (2 mg/kg per d) from 5 to 12 wk of age (25). There was a 39% decrease in BUN, a 34% decrease in serum creatinine, a 26% decrease in kidney size, and an 18% decrease in cyst volume density in PKD rats that were treated with rapamycin.

**Table 1. mTOR signaling in PKD**

<table>
<thead>
<tr>
<th>Signal</th>
<th>Model</th>
<th>Reference</th>
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<tr>
<td>Increased IGF-1 mRNA</td>
<td>Pcy mouse</td>
<td>(15)</td>
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<tr>
<td>Increased IGF-1 protein</td>
<td>Han:SPRD rat</td>
<td>(16)</td>
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<tr>
<td>Increased p-Akt protein</td>
<td>PKD-1/−/− mice</td>
<td>(22)</td>
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<tr>
<td>Cytoplasmic tail of polycystin-1 co-localizes with mTOR and co-localizes and interacts with tuberin</td>
<td>Han:SPRD rat</td>
<td>(23)</td>
</tr>
<tr>
<td>Phospho-mTOR and p70S6K are induced in cyst-lining epithelial cells</td>
<td>MDCK cell clones</td>
<td>(24)</td>
</tr>
<tr>
<td>Increase in p70S6K protein and total S6K is reduced by rapamycin</td>
<td>Human kidneys</td>
<td>(24)</td>
</tr>
<tr>
<td></td>
<td>Han:SPRD rats</td>
<td>(25)</td>
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*mTOR, mammalian target of rapamycin.*
In a third study of male Han:SPRD rats, everolimus (3 mg/kg per d) was given orally for 5 wk (23). Blood trough levels were 5 to 7 ng/ml. There was a 48% decrease in cyst volume density, a 30% decrease in body weight in PKD rats that were treated with everolimus. In summary, there are three independent studies that mTOR inhibition markedly slows disease progression in the male Han:SPRD rat model of PKD.

One study demonstrated that rapamycin reduced PKD in two independent mouse models of PKD (24). Orpk-rescue mutant mice and orpk-heterozygous-rescue control mice were treated with rapamycin (5 mg/kg per d) from postnatal day 150 through day 178. Rapamycin profoundly improved the cystic phenotype in the mutant mice. In the same study, bpk mice were treated with rapamycin. The bpk mouse model is characterized by an embryonic onset reminiscent of human autosomal recessive PKD (ARPKD), and the mice generally fail to live longer than 25 d. bpk mutant mice that were treated with rapamycin (5 or 1.67 mg/kg per d) from day 7 postpartum for a period of 14 d demonstrated significantly smaller cyst sizes, an improved renal cystic index, and normalization of BUN levels compared with controls.

There are no published studies of rapamycin in rats or mice with targeted mutations of human PKD orthologs; however, there is evidence that rapamycin reduces cystic disease in humans. In a retrospective study of patients who had ADPKD and received a renal transplant, Shillingford et al. (24) showed that patients who received rapamycin immunosuppression had a 24.8% decrease in size of their native polycystic kidneys on computed tomography scan compared with patients who received immunosuppression other than rapamycin and had a significantly lesser decrease in native polycystic kidney size of 8.6%. In a similar study, Qian et al. (32) retrospectively measured the volumes of polycystic livers and kidneys in patients who had ADPKD and received kidney transplants and participated in a trial that compared a sirolimus-containing immunosuppression regimen with a tacrolimus-containing regimen. Sixteen patients received computed tomography or magnetic resonance imaging scans. Treatment with the sirolimus regimen for an average of 19.4 mo was associated with an 11.9% reduction in polycystic liver volume, whereas treatment with tacrolimus for a comparable duration was associated with a 14.1% increase. A trend toward a greater reduction in native kidney volume was also noted in the sirolimus group compared with the nonsirolimus group. Hepatic cyst epithelium had markedly higher levels of phospho-AKT, phospho-ERK, phospho-mTOR, and the downstream effector phospho-S6 compared with control biliary epithelium, suggesting activation of mTOR signaling in liver cysts (32).

In view of the absence of effective therapies in ADPKD and the safety of rapamycin as evidenced by long-term use in adults and children to prevent transplant rejection, four interventional studies investigating the effect of mTOR inhibition on kidney and cyst size in humans have been initiated (see http://www.clinicaltrials.gov and http://www.pkdcure.org for more details about the human studies).

Despite positive animal studies and the initiation of human studies of mTOR inhibitors in PKD, there are many unanswered questions about mTOR signaling and mTOR inhibition in PKD: (1) What are the mTOR signaling pathways and effect of mTOR inhibitors in PKD-1 and -2 gene models of PKD? (2) Does rapamycin work in female rat and mouse models? (3) What blood levels are required to achieve a therapeutic effect on cyst formation?

Caspases and Apoptosis
Apoptosis is a process of programmed cell death characterized by volume reduction, cell surface blebbing, chromatin condensation, internucleosomal cleavage of DNA, and formation of apoptotic bodies. A family of cysteine proteases, the caspases, are the major mediators of apoptosis. Caspase-3 plays a crucial and extensively studied role in the promotion of apoptotic cell death. The major pathways of caspase-mediated apoptosis are described in Figure 3.

Caspase Pathways and Apoptosis in PKD
Apoptosis is a pathologic feature of most models of PKD (33). Increased levels of apoptosis are observed in human ADPKD (34,35), the cpk mouse model of ARPKD (35), the pcy mouse model orthologous to adolescent nephronophthisis (35), the pck rat model of ADPKD (36), bpk mice (24), orpk rescue mice (24),
PKD-1–deficient mice (37), and dysplastic renal disease in rodents and humans (38). Apoptosis was detected in kidneys of humans with ADPKD regardless of renal function but not in normal kidneys (35). Increased apoptosis is a feature of the following experimental models of PKD: Transgenic mice overexpressing the proto-oncogene c-myc (SBM mice) (28), mice lacking the transcription factor AP-2β (39), c-myc transgenic mice (28), and Bcl-2–deficient mice (40).

Despite the presence of apoptosis in most PKD models, the caspase signaling pathways have only recently been described. Increased caspase activity has been detected in cystic kidneys in cpk mice, a model of ARPKD (41); however, apoptosis was localized primarily to the interstitium with little evidence of cell death in cyst epithelium or noncystic tubules.

Activation of caspase-3 and dysregulation of the balance between pro- and antiapoptotic Bcl-2 family members, specifically a downregulation of antiapoptotic Bcl-XL, correlated with increased apoptosis in the early stages of ADPKD in Han:SPRD rats (42). Bak, bax, bcl-2, and bad mRNA levels have been found to be increased in the cystic kidneys of BALB/c–cpk/cpk mice (10).

In another study, the apoptosis pathways (Figure 3) were determined in Han:SPRD rats (43). In homozygous PKD kidneys, there was an increase of (1) the pro-form of caspase-9, (2) cytochrome c release into the cytosol, and (3) caspase-2 protein and activity demonstrating involvement of the mitochondrial pathway. There was an increase in the pro-form of caspase-8 demonstrating involvement of the death receptor pathway. No differences in FasL mRNA were seen, suggesting that the death receptor pathway is independent of the death receptor ligand FasL. Survivin, an inhibitor of apoptosis protein that is increased in renal cancers, was increased in 2-wk-old homozygous Han:SPRD rat kidneys in association with activation of caspase-9 and increased apoptosis (44).

Numerous studies demonstrate that increased tubular cell proliferation is accompanied by increased tubular apoptosis in PKD: (1) Kidneys from patients with ADPKD have high levels of apoptosis as well as cellular proliferation (34). Although the compression of normal renal tissue by cysts may contribute to renal failure, only 1 to 2% of nephrons become cystic in PKD. Apoptosis is detected in normal noncystic tubules in preuremic human PKD, suggesting that apoptotic loss of noncystic nephrons may contribute to renal failure in PKD (35). (2) In human ADPKD, a 15-fold increase in c-myc expression is associated with both tubular cell proliferation and apoptosis (34). In SBM mice that overexpress c-myc, there is a 10- to 100-fold increase in both apoptosis and proliferation that occurs early in the course of the disease and precedes cystogenesis (28) (45). (3) Mice deficient in the proapoptotic Bcl-2 gene have hyperproliferation as well as apoptosis that accompanies renal cysts (40,46). (4) In Han:SPRD rats fed soy protein, the improved renal function and decreased cyst formation are accompanied by decreases in both tubular cell proliferation and apoptosis (47). Soy protein also retards cyst development in the pcy mouse model of PKD (48–50). (5) The heightened cellular proliferation and apoptosis observed in SBM mice and human ADPKD resemble the process that occurs during renal organogenesis (51). It has been suggested that epithelial cell apoptosis and proliferation are directly related and are dysregulated in ADPKD and may represent a general mechanism for cyst growth and tissue remodeling (2,34).

Caspase and/or Apoptosis Inhibition in PKD

Does apoptosis directly result in cyst formation? There are numerous in vitro and in vivo studies in which direct perturbations in caspases and/or apoptosis have resulted in changes in cyst formation.

Apoptosis is essential for Madin-Darby canine kidney (MDCK) cell cyst cavitation in collagen type I matrix. Cystogenesis in this system is inhibited by overexpression of the antiapoptotic gene Bcl-2 (52). Expression of human PKD1 in MDCK cells slows their growth and protects them from apoptosis (53). MDCK cells that express PKD1 also spontaneously form branching tubules, whereas control cells form simple cysts. Thus, PKD1 may function to regulate both apoptosis and proliferation pathways, allowing cells to enter a differentiation pathway that results in tubule formation. This study also links polycystin-1 to apoptosis.

Fibrocystin, the gene product of PKHD1 that is responsible for ARPKD, was inhibited by short hairpin RNA inhibition in IMCD cells. Inhibition of fibrocystin disrupted normal tubulomorphogenesis and resulted in increased apoptosis and proliferation (54).

Bcl-2 is an antiapoptotic protein (Figure 3). Bcl-2–deficient mice have increased apoptosis in all organs of the body, including the kidney. Renal failure results from severe PKD characterized by dilated increased proliferation of epithelium and interstitium.

Long-lasting arrest of murine PKD and preservation of kidney function with the CDK inhibitor roscovitine has been described (12). The mechanism of action of roscovitine in PKD was shown to be cell-cycle arrest, transcriptional inhibition, and attenuation of apoptosis.

We determined the effect of caspase inhibition on tubular cell apoptosis and proliferation, cyst formation, and renal failure in the Han:SPRD rat model of PKD. Using an Alzet minipump, rats were treated with the pan-caspase inhibitor IDN-8050 (10 mg/kg per d) from 4 to 8 wk of age. The pan-caspase inhibitor reduced the kidney enlargement by 44%, reduced the cyst volume density by 29%, and normalized the increase in BUN. The active form of caspase-3 and the number of proliferating cell nuclear antigen–positive tubular cells and apoptotic tubular cells in noncystic and cystic tubules was significantly reduced by the pan-caspase inhibitor. Increased apoptosis was demonstrated at an early stage of cyst formation (i.e., at birth) in homozygous PKD rats and at 2 wk of age in heterozygous PKD rats, suggesting that it may play a causative role in cyst formation rather than being a late marker of epithelial cell dysfunction.

The cpk mutation is a well-characterized model of ARPKD. We crossed cpk/+ mice with caspase-3–/– mice and generated cpk/cpk caspase-3+/– and cpk/cpk caspase-3–/– double-knockout mice. cpk mice died of PKD and renal failure at a mean age of 32 d. cpk caspase-3+/– mice died at a mean age...
of 56 d. Two of the cpk caspase-3+/− mice survived 113 and 105 d. The cpk caspase-3−/− mice lived approximately four times longer than littermate control cpk mice (mean age 117 versus 32 d; *P < 0.01). The 117-d-old cpk caspase-3−/− mice had a significantly lower kidney size than 32-d-old cpk and 56-d-old cpk caspase-3+/− (*P < 0.05); however, despite deletion of caspase-3, there was still an apoptotic and cystic response, likely as a result of an upregulation of caspase-7 (55).

One study suggested that increased apoptosis in tubular cells may be associated with decreased cyst formation (56). Pax2-deficient mice, which have increased apoptosis, were backcrossed into cpk mice, which have kidney cysts with cilia expression. The resultant mice had increased renal apoptosis yet less cystic disease. The results of this study support the hypothesis that cysts consist of dedifferentiated epithelial cells that require embryonic factors, such as Pax2, for continued growth and expansion.

In summary, there is much evidence that apoptosis plays a central role in cyst formation: (1) Tubular epithelial cell apoptosis occurs in most animal models of PKD and in kidneys from humans with ADPKD, (2) induction of apoptosis in tubular cells in culture results in cyst formation, (3) both apoptosis and proliferation occur in noncystic as well as cystic epithelial cells early in the course of PKD, and (4) caspase inhibition by both pharmacologic and genetic techniques results in attenuation of cyst formation. However, the precise pathways that link apoptosis and proliferation in PKD are not known.

**Link between mTOR Signaling and Caspase Signaling in PKD**

There are links between the mTOR and caspase signaling pathways. Although S6 protein is the best characterized substrate of p70S6K, p70S6K is also known to inactivate the pro-apoptotic protein BAD by preventing phosphorylation of Ser136 on BAD and blocking cell survival induced by IGF-I (57). Moreover, IGF-I–induced phosphorylation of BAD was abolished in p70S6K-deficient cells.

There is much evidence that rapamycin is proapoptotic especially in cancers, resulting in apoptotic death of the cancer cells (57); however, mTOR may have a pleiotropic function in the regulation of cell death depending on the cell type and activation state as well as downstream targets such as p53 and Bcl-2 proteins (58). There is evidence that rapamycin can be antiapoptotic (59,60). For example, rapamycin inhibits death of syncytioid via inhibition of proapoptotic Bax and inhibition of the mitochondrial cell death pathway (61,62). In this regard, we have demonstrated a role of the mitochondrial death pathway in ADPKD (45).

Studies of the effect of rapamycin on apoptosis in PKD are conflicting: Rapamycin-treated orpk-rescue mutant mice exhibit increased numbers of terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling–positive cyst-lining epithelial cells and the presence of luminal terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling–positive cells as compared with non-treated orpk-rescue mutant mice (24); however, in male Han: SPRD rats, the active form of caspase-3 (20 kD) and the number of apoptotic tubular cells in both cystic and noncystic tubules were decreased by rapamycin (63).

**Conclusions**

A disturbance in the balance between proliferation and apoptosis is one of the most important abnormalities of the tubular epithelial cells lining the cysts. Activation of the mTOR signaling pathway, which leads to increased proliferation, has been found in rat and mouse models of PKD as well as human PKD kidneys. Studies of mTOR inhibition in rodent models of PKD are ongoing, and studies of mTOR inhibition in patients with ADPKD have been initiated. Apoptosis is a pathologic feature of most models of PKD. Activation of caspase signaling pathways and dysregulation of pro- and antiapoptotic Bcl-2 proteins have been described in PKD. Although caspase inhibitors are being tested in clinical studies to prevent organ preservation injury, their use in humans with ADPKD is preliminary. It would be significant if rapamycin, which is FDA approved for human use, could be used as both an antiproliferative and an antiapoptotic agent in patients with PKD.

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**Disclosures**

None.

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