Steviol retards renal cyst growth through reduction of CFTR expression and inhibition of epithelial cell proliferation in a mouse model of polycystic kidney disease

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Cyst enlargement in autosomal dominant polycystic kidney disease (ADPKD) is associated with cAMP-activated proliferation of cyst-lining epithelial cells and transepithelial fluid secretion into the cyst lumen via cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel leading to renal failure for which no effective treatment is currently available. We previously reported that steviol retards Madin–Darby canine kidney (MDCK) cyst enlargement by inhibiting CFTR channel activity and promoting proteasomal-mediated CFTR degradation. It is imperative to examine the effect of steviol in animal models of ADPKD. Therefore, we examined the effect of steviol on renal cyst growth in an orthologous mouse model of human ADPKD (Pkd1flox/flox;Pkhd1-Cre). The results showed that daily treatment with both 200 mg/kg BW of steviol and 1000 mg/kg BW of stevioside for 14 days markedly decreased kidney weight and cystic index in these mice. However, only steviol markedly reduced blood urea nitrogen and creatinine values. Steviol also reduced cell proliferation but had no effect on cell apoptosis. In addition, steviol suppressed CFTR and mTOR/S6K expression in renal cyst-lining epithelial cells. Interestingly, steviol was found to stimulate AMP-activated protein kinase (AMPK). Our findings indicate that steviol slows cyst progression in ADPKD mouse model, in part, through the activation of AMPK which subsequently inhibits CFTR chloride channel expression and inhibits renal epithelial cell proliferation via mTOR/S6K pathway. Most importantly, steviol could markedly improve kidney function in a mouse model of ADPKD. Steviol thus has potential application for further development as a therapeutic compound for the treatment of polycystic kidney disease.

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1. Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is a progressive inherited disorder caused by mutation of either PKD1 or PKD2 gene encoding polycystin-1 and polycystin-2 protein [1], respectively. The prominent feature of ADPKD is the formation and progressive enlargement of numerous fluid-filled cysts in the kidney. These cysts replace normal renal parenchymal cell resulting in the loss of renal function and ending up with end-stage renal failure [2]. Unfortunately, no effective therapeutic treatment for ADPKD is currently available.

ADPKD progression is composed of two key processes, cell proliferation and fluid secretion, that involve several complex pathways. Cyst expansion is due to the hypersecretion of chloride and water into the cyst lumen through cystic fibrosis transmembrane conductance regulator (CFTR) channel and aquaporin (AQP) [3,4], respectively. Cyst-lining epithelial cell growth contributes to cyst formation and cell proliferation in PKD [5,6]. Several pathways were found to play an important role in cyst-lining cell proliferation. It was found that upregulation of ERK in renal
epithelial cell obtained from ADPKD patients and PKD mouse models promotes cell proliferation via Raf/MEK/ERK pathway [7,8]. Moreover, phosphorylation of ERK indirectly stimulated the mTOR signaling pathway as well as inhibited AMP-activated protein kinase (AMPK) [9]. This is in agreement with a previous study showing that PC-1 C terminus regulated mTOR signaling was upregulated in cyst-lining cells [10], whereas AMPK, a depressor of energy-consumption, was deactivated in ADPKD. Inhibition of mTOR by rapamycin decreased kidney size and slows cyst progression in rodent non-orthologous and orthologous models of PKD [11,12]. Interestingly, activation of AMPK by metformin [13] and/or glucose starvation [14] effectively retarded renal cyst growth by inhibiting CFTR chloride channel and inhibiting mTOR pathway in ADPKD mouse models. Inhibition of these two key processes in ADPKD to retard cyst growth would provide good opportunities to control the advance-ment and morbidity of the disease. In the past decade, lots of efforts have been directed towards interfering with the intracellular pathways governing cyst growth. Particularly, an inhibitory effect of CFTR function on cyst growth was widely studied. It was found that several small molecules or natural compounds shown to inhibit CFTR could retard renal cystogenesis in both cell models and mouse models of PKD [15,16]. Due to the complex pathophysiology of ADPKD, the best treatment may require a combination of therapies to inhibit the progression of the disease effectively. The novel synthetic compounds with broad capacity to inhibit cyst progression may provide effective therapeutic approaches that help ameliorate ADPKD and improve renal function.

We previously identified that steviol, a derivative of natural compound stevioside, slowed cyst growth, an in vitro Madin–Darby canine kidney (MDCK) model of PKD, by directly inhibiting CFTR chloride channel activity and promoting proteasome-mediated degradation of CFTR [17]. However, this MDCK cells do not carry the mutated PKD genes or contain characteristic of genetic disorder of PKD. It is imperative to investigate the effect of steviol in an animal model that has a genetic disorder of PKD. In addition, we recently discovered that the derivative of steviol (dihydroxyosesteviol) stimulated AMPK in human colonic epithelial T84 cells [18].

In this study, we further determined the efficacy and cellular mechanisms of steviol on renal cystogenesis and renal function in Pkd1<sup>floxed</sup>:Pkhd1-Cre mouse, an in vivo model of ADPKD. Interestingly, we found that steviol retarded renal cyst growth in this mouse in part by promoting AMPK activity which further inhibited CFTR expression as well as inhibited mTOR/S6K signaling pathway. In accordance with its inhibitory effect on cyst growth or kidney volume, steviol enhanced kidney function as well. Altogether, broad inhibitory efficacy on cyst growth and defined cellular mechanism of steviol suggests its potential utility for the treatment of PKD.

### 2. Materials and methods

#### 2.1. Mouse strains and treatment protocol

The animal protocols were approved by Yale Animal Resources Center and Institutional Animal Care and Use Committee regulations. Pkd1<sup>floxed</sup> mice were crossed with Pkd1<sup>floxed</sup>:Pkhd1-Cre mice to generate Pkd1<sup>floxed/floxed</sup>:Pkhd1-Cre mice as described previously [8,19]. Pkd1<sup>floxed</sup>:Pkhd1-Cre kidneys developed a minimal cystic lesions from post-natal (P) day 10 (P10) onward and progressed to massive cystic phenotype at P24 in distal tubule and collecting duct segments. The genotyping of Pkd1<sup>floxed/floxed</sup>:Pkhd1-Cre mice was performed at P8. Pkd1<sup>floxed/floxed</sup>:Pkhd1-Cre mice were divided into two groups, vehicle treated group (DMSO treated) and experimental groups (steviode or steviol treated). Pkd1<sup>floxed</sup>:Pkhd1-Cre, Pkd1<sup>floxed/floxed</sup>:Pkhd1-Cre, or Pkd1<sup>floxed/floxed</sup>:Pkhd1-Cre mice were used as wild-type mice.

Steviol and steviolide were synthesized and purified as previously described [20,21]. The purity of all compounds was check by thin layer chromatography and nuclear magnetic resonance spectroscopy. Steviol and steviolide were dissolved in 100% DMSO and in distilled water, respectively. Experimental mice daily received steviol at doses of 40 and 200 mg/kg BW (10% of DMSO plus 90% of 0.9% normal saline) through an intraperitoneal (i.p.) injection and steviolide at doses of 500, 700, and 1000 mg/kg BW (in 100 μL of distilled water) through an oral gavages from P10 to P23 for 14 days. At P24, all mice were anesthetized by an i.p. injection with ketamine (50 mg/kg) and xylazine (10 mg/kg). Blood was collected and centrifuged for blood urea nitrogen (BUN) and creatinine measurement. Mouse's tissue was obtained by perfusion fixation with cold-PBS. All kidneys were removed and weighted. One kidney was fixed in 4% paraformaldehyde (PFA) and was cut into sagittal plane for performing cryosection and histological experiment. Another kidney was stored in liquid nitrogen for immunoblotting.

#### 2.2. Renal cyst measurement

Kidneys were fixed overnight in 4% PFA. Midline sagittal plane of kidney was embedded in paraffin and stained with hematoxylin & eosin (H&E). Kidney histology was captured by an inverted contrast phase light microscope (Nikon TE 2000-S, Nikon Corporation, Tokyo, Japan) at 20× magnifications. For cysctic measurement, cysctic index was measured using an inverted microscope (Nikon TE 2000-U) and calculated by Meta Morph software (Universal Imaging Corporation, Buckinghamshire, UK). The percent of cysctic index was shown as a ratio of cysctic area per total kidney area.

#### 2.3. Cell proliferation and apoptosis assays

Cell proliferation was assayed by immunohistochemistry. Sections were permeabilized with 1% SDS and blocked with blocking solution (0.1% BSA, 10% goat serum in TBS). Sections were incubated with 1:200 of monoclonal Ki67 antibody (Thermo scientific). In situ Cell Death Detection kit (TUNEL) was used for cell apoptosis assay (Roche, Indianapolis, IN, USA). Sections were performed according to the instruction of the manufacturer. Sections were stained with DBA (a marker of collecting duct) and DAPI (a marker of nucleus). The number of Ki67-positive cell and TUNEL-positive cell were counted using an inverted microscope (Nikon TE 2000-U) and Meta Morph software (Universal Imaging Corporation) at 20× magnifications. The percent of cell proliferation and apoptosis was calculated by multiplying the numbers of Ki67-positive cells or numbers of TUNEL-positive cells and the total numbers of cells in collecting duct. The total numbers of cells was at least 1000 cells per kidney.

#### 2.4. Immunoblotting

Kidneys were homogenized and extracted in ice-cold buffer containing 0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, and Protease inhibitorcocktail. Samples were centrifuged at 10,000 × g. Supernatant proteins (60 μg) were separated in 8% and 10% SDS-PAGE gel depending on proteins of interest and then were transferred to nitrocellulose membrane. Membranes were blocked with 5% of non fat dry milk for 1 h and were incubated overnight with mouse CFTR antibody (Abcam, Cambridge, MA, USA), rabbit phospho-ERK 1/2 and total-ERK 1/2 (Cell signaling, Beverly, MA, USA), rabbit phospho-AMPK and AMPKα
(Cell signaling), rabbit phospho-S6K and total-S6K (Cell signaling), rabbit GAPDH antibody (Cell signaling), mouse α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit Hsp90 (Santa Cruz Biotechnology). Membranes were washed by TBS-T solution and incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG antibodies. The signal was developed using enhanced chemiluminescence (ECL, Calbiochem, San Diego, CA, USA). The intensity was measured by image J and calculated using Hsp90, GAPDH, and α-tubulin as a loading control.

2.5. Statistics

All results were represented as mean ± S.E. The statistical significant of the data between wild-type group, cystic vehicle group, and cystic treatment group were analyzed using either unpaired
student’s t test or one-way ANOVA followed by Bonferroni’s post hoc test. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Steviol inhibits renal cystogenesis in $Pkd1^{flox/flox}:Pkhd1-Cre$ mice

We previously reported that steviol retards cyst growth in an in vitro MDCK cyst model by inhibiting CFTR activity and expression [17]. However, it is imperative to examine whether this inhibitory effect of steviol could be observed in the animal model of PKD, $Pkd1^{flox/flox}:Pkhd1-Cre$ mice. In this experiment, mice were daily injected with steviol at the doses of 40 and 200 mg/kg BW (steviol-treated mice) or DMSO (vehicle treated mice) via an i.p. injection at post-natal period from day 10 to day 23 ($P10–P23$) for 14 days. These doses of steviol were chosen based on the data from our previous studies in MDCK cell [17]. Kidneys were harvested at post-natal day 24 ($P24$). The kidney function, kidney weight and

![Image](image_url)

**Fig. 2.** Inhibitory effect of stevioside on renal cystogenesis in $Pkd1^{flox/flox}:Pkhd1-Cre$ mice. (A) The representative image of kidney section from $Pkd1^{flox/flox}:Pkhd1-Cre$ mice at $P24$. (B) H&E staining of kidney section from wild-type, vehicle-treated cystic mice and cystic mice treated with stevioside at dose of 1000 mg/kg BW. Scale bar = 100 μm, 20× magnifications, C = cyst. The color bar represented the group of experiment (white bar; wild-type, black bar; vehicle-treated cystic mice, gray bar; cystic mice treated with stevioside). The quantitative analysis of cystic severity was represented by body weight (C), kidney weight (D), kidney weight per body weight (E), and cystic index (F). The renal function was analyzed using BUN (G) and creatinine (H) values. The results were shown as mean ± S.E., n = 5–6 mice per each group, ns; not significant, ***$P < 0.001$.}
cystic area of these mice were analyzed. It was found that steviol at the dose of 40 mg/kg BW could not inhibit cyst growth in these mice (data not shown). However, steviol at high dose (200 mg/kg BW) was able to decrease kidney size and cystic area as shown in Fig. 1A and B. Notably, steviol did not significantly alter total body weight of steviol-treated mice (Fig. 1C). Steviol decreased kidney weight (0.67 ± 0.2 g) compared with that of vehicle treated mice (1.0 ± 0.1 g), whereas kidney weight per total bodyweight ratio did not change in steviol-treated mice (Fig. 1D and E). In addition, steviol significantly reduced cystic index by 13.7 ± 4.2% compared with vehicle treated mice (Fig. 1F). The renal function following steviol treatment as determined by serum BUN and serum creatinine values was found to be improving. Steviol at dose of 200 mg/kg BW significantly decreased both serum BUN and creatinine compared with vehicle treated mice (Fig. 1G and H). These results clearly indicated that steviol could retard cyst progression and markedly improved kidney function in Pkd1<sup>ffox/ffox</sup>:Pkhd1-Cre mice.

3.2. Stevioside inhibits cyst growth in Pkd1<sup>ffox/ffox</sup>:Pkhd1-Cre mice

Steviol is the metabolite that is produced from the intestinal microflora digestion of stevioside and it is the one being absorbed into the blood and distributed to several organs including the kidney [22]. Thus, we further investigated whether stevioside (parent compound of steviol) could produce inhibitory effect on renal cystogenesis in the animal model of PKD, Pkd1<sup>ffox/ffox</sup>:Pkhd1-Cre mice, similar to that of steviol. The treatment doses of stevioside were estimated from the doses of steviol, which

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**Fig. 3.** Effect of steviol and stevioside on cell proliferation in Pkd1<sup>ffox/ffox</sup>:Pkhd1-Cre mice. (A) Cell proliferation was assessed by Ki67 staining. Kidney sections were stained with Ki67, a marker of cell proliferation (red), DBA (green), DAPI (blue). 20× magnifications, scale bar = 100 μm. (B) The percent of cell proliferation was shown as Ki67-positive nuclei in collecting duct segment in wild-type, vehicle-treated cystic, and steviol and stevioside-treated cystic kidneys. Results were expressed as mean ± S.E., n = 4 kidneys, ***P < 0.001. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
showed no toxic effect \((<1000\ mg/kg\ BW)\). Stevioside was daily orally administered at the doses of 500, 700, and 1000 mg/kg BW to the mice for 14 days. The results showed that stevioside at doses of 500 and 700 mg/kg BW had no effect on kidney weight and cystic index (data not shown), while stevioside at the high dose \((1000\ mg/kg\ BW)\) decreased kidney weight and also reduced kidney weight per total body weight ratio by \(26.8\ \pm 6.0\%\) (Fig. 2D and E). It also significantly reduced cystic index by \(11.5\ \pm 4.1\%\) (Fig. 2F). Unfortunately, stevioside ingestion has no effect on serum BUN and serum creatinine values as shown in Fig. 2G and H. These results suggested that only high dose of stevioside administration could inhibit cyst growth but could not improve renal function in \(Pkd1^{flox/flox}:Pkhd1-Cre\) mice.

### 3.3. Steviol and stevioside suppress cell proliferation but have no effect on cell apoptosis in \(Pkd1^{flox/flox}:Pkhd1-Cre\) mice

The inhibitory effect of both steviol and stevioside on cyst growth could involve the suppression of cell proliferation or induction of cell apoptosis. The effect of steviol and stevioside on cell proliferation was investigated using immunohistochemistry by staining kidney sections with Ki67 antibody. The results showed that steviol at a dose of 200 mg/kg BW and stevioside at a dose of 1000 mg/kg BW strongly reduced the number of Ki67-positive cell in treated mice by \(35.7\ \pm 9.3\%\) and \(37.7\ \pm 7.7\%\), respectively, compared to that of vehicle treated mice (Fig. 3B).

Next, we examined the effect of steviol and stevioside on cell apoptosis in cystic kidneys. Using terminal deoxynucleotidyl transferase-mediated fluorescence-dUTP nick end labeling (TUNEL) assay, it was found that both steviol and stevioside did not alter the number of TUNEL-positive cells (Fig. 4). These data indicated that steviol and stevioside inhibited cyst growth in \(Pkd1^{flox/flox}:Pkhd1-Cre\) mice, in part, by suppressing cell proliferation but not by promoting cell apoptosis.

### 3.4. Steviol and stevioside reduce CFTR chloride channel expression in \(Pkd1^{flox/flox}:Pkhd1-Cre\) mice

To investigate the mechanism by which steviol retarded cyst growth in \(Pkd1^{flox/flox}:Pkhd1-Cre\) mice, whole kidney lysates from steviol-treated mice and vehicle treated mice were determined for CFTR expression by Western blot analysis. Our prior study has shown that steviol reduces MDCK cyst growth by suppression of CFTR expression [17]. We thus postulated that steviol would exert the similar effect on CFTR expression in cystic kidney of \(Pkd1^{flox/flox}:Pkhd1-Cre\) mice. Fig. 5A showed that the level of CFTR expression in collecting duct segment as observed by the expression of AQP2 in steviol-treated kidney was reduced by \(30.4\ \pm 23.0\%\) compared to that of vehicle treated kidney. In addition, stevioside \((1000\ mg/kg\ BW)\) decreased CFTR expression in renal cyst-lining epithelial cells compared to that of vehicle-treated cystic mice (Fig. 5B). In agreement with our prior data, steviol and stevioside inhibited cyst growth in the kidney of \(Pkd1^{flox/flox}:Pkhd1-Cre\) mice, in part, by reducing CFTR expression.

### 3.5. Steviol and stevioside diminish mTOR signaling pathway in \(Pkd1^{flox/flox}:Pkhd1-Cre\) mice

We further investigated whether steviol inhibited cell proliferation by targeting of ERK and mTOR signaling pathways. We found that effect of steviol did not appear to involve ERK pathway. Steviol could not suppress phosphorylation of ERK 1/2 protein in kidney of cystic treated mice (Fig. 6A). Rapamycin, the inhibitor of mTOR pathway which is upregulated in cystic kidney, has been shown to inhibit cystogenesis in PKD mouse models [11,12]. Therefore, ribosomal p70 S6 kinase (a downstream effector of mTOR pathway) was examined by Western blot analysis. An increase in phospho-S6 kinase \((p-S6K)\) content was observed in cystic kidney compared to that of wild-type kidney. Interestingly,
steviol was found to suppress p-S6K expression by 91.8 ± 24.4% compared to that of cystic vehicle treated mice as seen in Fig. 6B. Moreover, the effects of stevioside administration on p-S6K expression were determined. The result showed that stevioside tended to reduce S6K phosphorylation in renal cyst-lining epithelial cells compared to that of vehicle-treated cystic mice (Fig. 6C). The results suggested that steviol and stevioside inhibited cell proliferation in cystic kidney, in part, by counteracting mTOR/S6K signaling pathway.

3.6. Steviol and stevioside enhance AMPK activation in Pkd1<sup>fl/fl</sup>:Pkhd1-Cre mice

It has been shown that the AMPK activity indirectly inhibits mTOR pathway and directly inhibits CFTR chloride channel activity resulting in retardation of renal cystogenesis in an in vivo model of PKD [13]. Moreover, our prior study has found that dihydroisosteviol, a derivative of steviol, stimulated AMPK activity in human colonic epithelial T84 cells [18]. It is therefore possible that steviol may exert its effect via AMPK pathway. Using Western blot analysis, it was found that steviol increased phosphorylation of AMPK in Pkd1<sup>fl/fl</sup>:Pkhd1-Cre kidney by 31.8 ± 40.4% and by 65.2 ± 25.1% compared to wild-type and cystic vehicle treated mice, respectively (Fig. 7A). In addition, the effects of stevioside on AMPK activation were investigated in kidneys of Pkd1<sup>fl/fl</sup>:Pkhd1-Cre mice. It was found that stevioside (1000 mg/kg BW) increased AMPK phosphorylation in renal cyst-lining epithelial cells compared to that of vehicle-treated cystic mice (Fig. 7B). However, the degree of AMPK phosphorylation induced by steviol was less that than by steviol, correlating well with their effects on improving cystic index. Taken together, our observation suggested that inhibition of cystogenesis by steviol modulated via the activation of AMPK which further inhibited CFTR channel activity and expression, and suppressed mTOR pathway in Pkd1<sup>fl/fl</sup>:Pkhd1-Cre mice.

4. Discussion

Our observation shows a novel role of steviol in retarding renal cystogenesis in Pkd1<sup>fl/fl</sup>:Pkhd1-Cre mouse, a rodent orthologous model of human ADPKD. Steviol decreased several parameters of cyst severity such as kidney weight and cystic index, and improved renal function in this mouse model. The underlying mechanism of steviol’s action appeared to involve AMPK activation, which further inhibits fluid secretion by reducing CFTR channel activity and expression, and diminishing cell proliferation by abrogating mTOR/S6K signaling pathway.

Our prior study reported that a steviol along with its derivative (dihydroisosteviol) inhibited chloride secretion by targeting CFTR [20]. Subsequently, we found that steviol could slow MDCK cyst growth by inhibiting CFTR channel activity and protein expression [17]. However, this MDCK cells do not carry the mutated PKD genes and do not recapitulate the complexity of PKD pathogenesis [23]. It is therefore imperative to investigate the effect of steviol in an animal model that closely resembles PKD. In this study, we carried on the experiment to elucidate an inhibitory effect of steviol on renal cystogenesis using an orthologous mouse model of ADPKD, Pkd1<sup>fl/fl</sup>:Pkhd1-Cre mouse [8,19]. In this mouse model, the development of cyst could be observed at early post-natal periods (P10–P24) and the pathology was more pronounced with age [24].
The low dose of steviol (40 mg/kg BW) was firstly administered to this mouse based on our results from MDCK cyst model. We found that steviol at a dose of 40 mg/kg BW had no effect on renal cyst growth, whereas steviol at higher dose (200 mg/kg BW) markedly decreased renal cyst progression with no toxic effect as observed by body weight and life span of mice. This could be due to the fact that the low dose (40 mg/kg BW) of steviol administration did not provide the therapeutic concentration of steviol in plasma as high dose (200 mg/kg BW) did. It is, therefore, revealed that steviol retards renal cyst growth not only in cell cultured model but also in mouse model of PKD.

Interestingly, when we tested whether ingestion of stevioside (a parent compound of steviol) could slow cyst progression in this mouse model, we found that only a high dose of stevioside (1000 mg/kg BW or 400 mg/kg BW steviol equivalent) was able to decrease parameters of cyst severity, with no other beneficial effects on the renal function, when compared to that of steviol. It was also noted that no effects on cyst severity or renal function were observed after treatment with 500 mg/kg BW of stevioside or 200 mg/kg BW of steviol equivalent, which is the dose that was effective in slowing cyst progression via i.p. injection. The requirement of higher dose for stevioside compared to steviol...
treated to reduce cyst severity in vivo in this study may be due to different degrees of steviol bioavailability when administered by different routes or lower numbers of gut microflora in neonatal mice. Normally, orally consumed stevioside is converted to stevioside by the intestinal microflora before being absorbed as steviol into the bloodstream [22]. In this study, the i.p. injected steviol could be completely absorbed and reached the site of renal cyst lesion, whereas oral stevioside required metabolic conversion by the intestinal flora to steviol before being absorbed into the blood circulation. Since the neonatal mice had limited amount of intestinal flora [25,26], it was possible that stevioside might not be completely converted to steviol in these neonatal mice.

In the clinical setting, clearance of BUN and creatinine or their plasma levels were widely used to estimate the renal function. We found that only steviol (200 mg/kg BW), but not stevioside (1000 mg/kg BW), had an ability to improve renal function despite their similar effects in slowing cyst severity. The basis for the differences in the renal functional responses to stevioside and steviol treatments in PKD mouse remains unclear; however, it could be due to the differences in the amounts and time courses of steviol delivery to the kidney as mentioned above. We thought that intraperitoneal injection of steviol would yield an effective therapeutic concentration of steviol in plasma at an earlier time point and at a higher level than oral administration of stevioside, which requires a lag time for bacterial conversion to steviol, or for maturation of intestinal microflora in neonatal mice used in this study. Thus, it is conceivable that steviol has more benefit for renal function recovery compared to the stevioside. Supporting this statement was the finding of higher levels of AMPK phosphorylation and greater suppression of S6K phosphorylation in steviol-treated mice than in stevioside-treated mice. Previous studies have shown that stevioside and steviol decreased blood pressure in hypertensive rats by producing vasodilatation via a reduction in the total peripheral resistance (TPR) [27]. The increases in renal blood flow and glomerular filtration rate (GFR) [28] would help to lower serum levels of BUN and creatinine. These changes would be more pronounced following steviol treatment compared to stevioside due to the amount of steviol delivered to the kidney. Therefore, steviol treatment is more effective in preserving renal function than stevioside.

This study also demonstrated the mechanisms of steviol administration in delaying PKD cyst growth in vivo. Cyst expansion in PKD results from enhancement of chloride secretion through CFTR [4,29] and stimulation of cell proliferation [7,30]. Using Western blot analysis, we revealed that steviol inhibited CFTR channel expression in renal collecting duct of Pkd1flox/flox.Pkd1-Cre mouse. In addition, our results from staining with Ki67, a marker of proliferating cell, showed that steviol decreased cell proliferation via a downstream effector of mTOR pathway, S6K. In fact, several studies have shown that mTOR inhibitors slow down cyst progression and improve renal function in mouse models of PKD [11,31]. Apart from CFTR and mTOR, AMP-activated kinase (AMPK), a sensor protein which regulates the energy-consumption through prevention of ATP utilization inside the cells, was recently proposed to be a drug target for PKD [13]. Interestingly, AMPK activators such as metformin [13] and 2DG (an inducer of glucose starvation) [14] were able to inhibit renal cyst progression. AMPK has been suggested to suppress cell proliferation via inhibition of mTOR activity and decreased fluid secretion by inhibition of CFTR [13]. It is interesting that steviol in our study could promote AMPK activation in Pkd1flox/flox.Pkd1-Cre mouse. This finding is
correlated well with our previous reported that dihydrososteviol, a derivative of steviol, inhibited chloride secretion by activating AMPK activity in human colonic epithelial cell (TB4) [18].

In conclusion, we found a novel mechanism by which steviol retards PKD renal cyst growth, i.e. via stimulation of AMPK activity. In addition, steviol inhibited CFTR expression and mTOR/S6K signaling resulting in retarding renal cyst growth in Pkd1flo×flo;Pkd1Cre mouse. These findings indicate that steviol could be used for further development as a drug therapy for polycystic kidney disease. Importantly, due to the high concentration of both stevioside and steviol used to observe the beneficial effect for the treatment of PKD in our study, further study in modifying the structure of steviol to minimize its effective dose is needed.

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References