Activation of mTORC1 in Collecting Ducts Causes Hyperkalemia

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ABSTRACT

Mutation of TSC (encoding tuberous sclerosis complex protein) and activation of mammalian target of rapamycin (mTOR) have been implicated in the pathogenesis of several renal diseases, such as diabetic nephropathy and polycystic kidney disease. However, the role of mTOR in renal potassium excretion and hyperkalemia is not known. We showed that mice with collecting-duct (CD)–specific ablation of TSC1 (CDTsc1KO) had greater mTOR complex 1 (mTORC1) activation in the CD and demonstrated features of pseudohypoaldosteronism, including hyperkalemia, hyperaldosteronism, and metabolic acidosis. mTORC1 activation caused endoplasmic reticulum stress, columnar cell lesions, and dedifferentiation of CD cells with loss of aquaporin-2 and epithelial-mesenchymal transition-like phenotypes. Of note, mTORC1 activation also reduced the expression of serum- and glucocorticoid-inducible kinase 1, a crucial regulator of potassium homeostasis in the kidney, and decreased the expression and/or activity of epithelial sodium channel-α, renal outer medullary potassium channel, and Na+,K+-ATPase in the CD, which probably contributed to the aldosterone resistance and hyperkalemia in these mice. Rapamycin restored these phenotypic changes. Overall, this study identifies a novel function of mTORC1 in regulating potassium homeostasis and demonstrates that loss of TSC1 and activation of mTORC1 results in dedifferentiation and dysfunction of the CD and causes hyperkalemia. The CDTsc1KO mice provide a novel model for hyperkalemia induced exclusively by dysfunction of the CD.


Hyperkalemia is a common clinical and potentially life-threatening metabolic problem in which serum potassium exceeds 5.5 mmol/L.1 The most important cause of hyperkalemia is a decrease in renal potassium excretion. Thus, knowledge of the physiologic mechanisms of potassium handling in the kidney is essential for understanding the causes of hyperkalemia and for its treatment.2–4

Potassium excretion mainly occurs in principal cells of the cortical collecting duct (CCD), which is regulated and varies according to physiologic needs.5,6 Potassium secretion in this segment is a two-step process involving (1) cellular potassium entry across the basolateral membrane of the principal cells via the Na+, K+-ATPase pump and (2) potassium exit across the apical membrane via the renal outer medullary K+ channels (ROMK) that open to allow secretion into an electronegative lumen.7,8 The two most important physiologic determinants of potassium excretion are the serum aldosterone concentration and the delivery of sodium to the distal nephron.9–13 The electronegativity of the lumen is largely due to Na+ reabsorption through the epithelial Na+ channel (ENaC). Aldosterone binds to the nuclear mineralocorticoid

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receptor (MR) within the distal tubule, and the principal cells and activates Na\(^+\), K\(^+\)-ATPase, thereby increasing Na\(^+\) reabsorption into the blood and the electronegativity of the lumen and providing a more favorable driving force for the secretion of potassium through ROMK.\(^{14,15}\) Aldosterone could also up-regulate ENaC and ROMK in the apical membrane of CCD. Therefore, maintaining homeostasis and function of CCD is critical for potassium secretion.\(^{16}\) However, the molecular mechanisms through which homeostasis and function of CCD are maintained are not well understood.\(^{4}\)

Mammalian target of rapamycin (mTOR) is a highly conserved Ser/Thr protein kinase and forms two distinct functional complexes, termed mTOR complex 1 (mTORC1) and mTORC2.\(^{17,18}\) mTORC1 is the sensitive target of rapamycin that phosphorylates downstream targets of S6 kinase 1 and eukaryotic initiation factor 4E-binding protein-1 and controls the cap-dependent protein translation.\(^{19-21}\) It integrates diverse signals, including nutrients, growth factors, energy, and stresses, to regulate cell growth, proliferation, survival, and metabolism. In response to these stimuli, mTORC1 is activated by two families of Ras-related small guanosine triphosphatases, Rheb and Rags.\(^{22}\) Guanosine triphosphate–bound (active) Rheb is suppressed by tuberous sclerosis complex 1/2 (TSC1/2), a functional complex that has guanosine triphosphatase–activating protein activity toward Rheb. TSC is an inherited benign tumor syndrome characterized by the formation of multiple hamartomas in a wide array of organs, including the kidney. Loss of TSC1/2 causes cells and tissues to display constitutive mTORC1 activity, contributing to their tumor phenotype.\(^{23,24}\)

Recent studies have demonstrated that mTOR has emerged as an important modulator of several forms of renal disease, including renal regeneration after AKI, CKD, diabetic nephropathy, polycystic kidney disease, and renal cell carcinoma.\(^{25-28}\) Balanced mTOR activity is critical for podocyte and renal tubule function.\(^{29-31}\) However, the roles of mTOR in CCD function, renal potassium excretion, and hyperkalemia are not known. Of note, TSC1 was strongly expressed in wild-type (WT) littermates, but not in KO mice (Supplemental Figure 1A), suggesting that Cre recombination of the floxed Tsc1 gene was completed after birth. Because TSC1 is an upstream negative regulator of mTORC1, loss of TSC1 should result in activation of mTORC1. We found that specific enhancement of phospho-S6 in CCD was observed in KO mice, whereas the levels of phosphorylation of Akt (S473), the site regulated by mTORC2, were stable in CDTsc1KO mice CCD (Figure 1, A and B). In summary, CDTsc1KO mice have CD-specific inactivation of the Tsc1 gene and overactivation of mTORC1 signaling.

Although we observed no obvious differences between CDTsc1KO mice and WT littermates in survival, gross physical appearance, or organ morphology at 7 weeks, all CDTsc1KO mice died around 8 weeks (Figure 1C). Blood and urine biochemical analysis revealed that CDTsc1KO mice began to show mild hyperkalemia (mean plasma \([K^+]\), 4.1±0.2 mmol/L in WT versus 4.4±0.3 mmol/L in CDTsc1KO; \(P=0.024\)) at the age of 4 weeks. Then, at 8 weeks, severe hyperkalemia (plasma \([K^+]\), 4.3±0.5 mmol/L in WT versus 7.3±0.9 mmol/L in CDTsc1KO; \(P<0.001\)) (Figure 1D) and increased serum aldosterone (plasma \([aldosterone]\), 135.3±50.7 pg/ml in WT versus 185.3±55.7 pg/ml in CDTsc1KO; \(P=0.018\)) were observed (Figure 1E), with significant metabolic acidosis (plasma \([pH]\), 7.33±0.06 in WT versus 7.21±0.04 in CDTsc1KO; \(P=0.043\)) (Supplemental Table 1), while plasma creatinine, BUN and Na\(^+\) remained unchanged in CDTsc1KO mice as compared with littermate mice (Supplemental Table 1). These results show that activation of mTORC1 in CCD successfully demonstrates features of hyperkalemia caused by abnormal function of CCD and decreased excretion of potassium such as pseudohypoaldosteronism.\(^{35,36}\)

### RESULTS

#### Activation of mTORC1 in CDs Causes Hyperkalemia

To explore the potential role of mTORC1 signaling in potassium secretion of CCD, we generated mice (CDTsc1-knockout [KO]) with a conditionally ablated Tsc1 gene in the CD (principal cells) using a Cre expression cassette under the control of the Agp2 promoter (Supplemental Figure 1, A and B). Conventional Tsc1-KO mice died in the embryonic stages because of cardiac and liver dysfunction,\(^{34}\) but CDTsc1KO mice were born with normal Mendelian ratios and grew normally (Supplemental Figure 1C, Supplemental Table 1). To confirm that recombination had occurred in CCD, the ducts from 4-week-old mice were microdissected and protein levels of TSC1 were determined by Western blotting. As expected, TSC1 was only seen in wild-type (WT) littermates, but not in KO mice (Supplemental Figure 1D), suggesting that Cre recombination of the floxed Tsc1 gene was completed after birth.

**mTORC1 Activation Causes Columnar Cell Lesions and Dedifferentiation of CDs**

We first focused on the morphologic and histologic changes of CCD and kidney after specific deletion of Tsc1 in CCD. Although no obvious differences between CDTsc1KO mice and WT littermates in terms of kidney weight (Supplemental Table 1) or global appearance (Supplemental Figure 2A) were observed in mice of all ages, CCDs began to enlarge at the age of 4 weeks in KO mice and the area of some enlarged CCDs reached 2.7 mm\(^2\) at the age of 8 weeks (Figure 2, A and B,
Supplemental Figures 2B and 3). Of note, massive columnar cell lesions, which have been previously reported in nonpalpable breast abnormalities, were observed in CCDs of 6- to 8-week-old KO mice. The lesions were characterized by columnar-shaped epithelial cells with prominent apical snouts and secretions (CAPSS) seen at the luminal aspect of the cells (Figure 2C). Moreover, detachment of columnar-shaped cells from CCDs was also seen in KO mice. Further study revealed remarkably enhanced expression of N-cadherin and vimentin in these cells, suggesting that CCD cell detachment may be due to epithelial-mesenchymal transition (EMT)-like phenotypic changes (Figure 2D). Interestingly, the expression of aquaporin-2 (AQP-2) showed a time-dependent loss and was almost abolished in 8-week-old KO mice (Figure 2A). These observations suggest that mTORC1 activation causes dedifferentiation of CCD and CD dysfunction. In contrast, we did not observe these lesions and changes in any other areas of KO mouse kidney (Supplemental Figure 4).

Activation of mTORC1 is known to increase cell growth and proliferation in various cell types. We therefore examined cell proliferation in the CCD using BrdU (5-bromo-2’-deoxyuridine) (2-hour pulse) labeling. Enhanced proliferation was observed as early as in 4-week-old KO mice (Figure 3A). Cell apoptosis was also assessed in 8-week-old mice by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining, which showed a significant increase of apoptosis in columnar and detached cells (Figure 3, B and C). It is suggested that mTORC1 activation induces abnormal cell proliferation and apoptosis in the CCD.

Rapamycin Restores the Phenotypes in CDTsc1KO Mice

We next examined whether mTORC1-dependent CCD cell injury and dysfunction could be prevented by the mTORC1 inhibitor rapamycin. To this end, we started rapamycin treatment from 4 weeks of age in CDTsc1 KO mice. Interestingly, after 4 weeks of treatment, the rapamycin-treated KO mice did not die around 8 weeks; they survived for an additional 4 weeks and died only when rapamycin administration was stopped for 1 week (Figure 4A). Furthermore, the severe hyperkalemia, increased serum aldosterone, and metabolic acidosis observed at 8 weeks were largely diminished after rapamycin treatment (Figure 4, B and C, Supplemental Table 1). Histologic analyses of CCDs revealed that enhanced expression of N-cadherin and vimentin (Figure 4D), enlargement of CCDs (Figure 4E), columnar cell lesions, and detachment of CCD cells (Figure 4F, Supplemental Figure 2B) had almost disappeared. These phenotypic changes were consistent with dephosphorylation of S6 in rapamycin-treated KO mice (Figure 4, F and G). These results indicated that mTORC1-dependent CCD pathologic phenotypes could be restored upon inactivation of mTORC1 by rapamycin.
Endoplasmic Reticulum Stress Is a Critical Factor for CCD Cell Lesions and Detachment in CDTsc1 KO Mice

It has been reported that hyperactivation of mTORC1 leads to endoplasmic reticulum (ER) stress and that ER stress is essential for phenotypes caused by genetically increased mTORC1 activity in hepatic cells and podocytes.31,39 We found that 78-kD glucose-regulated protein (GRP78), which is induced during ER stress, was elevated in the CCDs of CDTsc1KO mice (Figure 5A). Existence of ER stress was further confirmed by transmission electron microscopy analysis. We observed that the ER cisternae were dilated and formed large circular shapes in the principal cells of 8-week-old KO mice (Figure 5B). The accumulation of GRP78 in CCD of KO mice was reduced by rapamycin, suggesting that elevated ER stress is induced by high mTORC1 activity (Figure 5, A and C). To further explore the possible role of ER stress in CCD phenotypic changes in KO mice, we administered a chemical chaperone, 4-phenyl butyric acid (PBA), which has been previously shown to reduce ER stress in pancreatic cells and hepatic cells.39,40 Oral administration of PBA effectively reduced the accumulation of GRP78 (Figure 5, A and C) in CCD cells and prevented columnar cell lesions and detachment of CCD cells in KO mice (Figure 5D). Accordingly, reduction of ER stress with PBA treatment in KO mice significantly decreased N-cadherin and vimentin expression in CCD cells (Figure 5C). These results suggest that high mTORC1-induced ER stress is a critical factor for CCD cell lesions and detachment in CDTsc1 KO mice.
stress causes CCD columnar cell lesions and detachment in KO mice. Interestingly, despite the protective role of PBA in CCD cell damage, unlike rapamycin, it only slightly reduced the level of plasma potassium and had little effect on S6 phosphorylation (Figure 5E, Supplemental Figure 5). These data indicate that excessive activation of mTORC1-induced ER stress contributes to CCD cell lesions and detachment but does not totally explain the hyperkalemia.

mTORC1 Activation Depressed ENaCα, ROMK1, and Na+,K+-ATPase Activity in CCDs

To explore the mechanism through which mTORC1 activation induces hyperkalemia, we next investigated the effect of mTORC1 activation on ENaC and ROMK1, two important channels for Na+ reabsorption and K+ secretion in CCD. Using microdissection, we found that the protein levels of ENaCα and ROMK1, but not ENaCβ and ENaCγ, were decreased in CDTsc1KO mice (Figure 6, A and B). Consistent with the Western blot results, immunofluorescence analysis revealed that ROMK1 expression in the CCD cells was markedly reduced in KO mice (Figure 6C). Furthermore, the activity of Na+, K+-ATPase was also decreased progressively in KO mice (Figure 6D). Of note, the expression of ENaCα and ROMK1 (Figure 6, A–C), and the activity of Na+, K+-ATPase (Figure 6D) in KO mice could be restored by rapamycin, while rapamycin did not significantly affect the activity of Na+, K+-ATPase in the control mice (Figure 6E). These findings indicate that excessive mTORC1 activation reduces ENaCα, ROMK1 expression, and Na+, K+-ATPase activity in CCD.

mTORC1 Activation Reduces SGK1 Expression and Induces Aldosterone Resistance in CCDs

In CCD principal cells, binding of aldosterone to the MR rapidly induces the mRNA and protein expression of SGK1, a kinase crucial for regulating the expression and/or activity of ENaC, ROMK, and Na+, K+-ATPase in CCDs.16,41,42 We next examined the effect of excessive mTORC1 activation on SGK1 expression in CCD. We found that the protein level of SGK1 was significantly reduced in CCD of CDTsc1KO mice (Figure 7A). Importantly, rapamycin treatment reinduced the protein expression of SGK1 in CDTsc1KO mice (Figure 7A), suggesting that mTORC1 negatively regulates the expression of SGK1. In combination with the increase of plasma K+ and aldosterone in CDTsc1KO mice (Figure 2E), our findings suggest the existence of aldosterone resistance in CD-specific mTORC1 activation mice. This notion was further confirmed by measurements of fractional excretion of Na+ and K+ in WT and KO mice. Fractional excretion of Na+ increased while that of K+ decreased significantly in KO mice compared with WT mice. In addition, KO mice were insensitive to amiloride (Figure 7, B and C). Taken together, these results demonstrate that mTORC1 activation reduces SGK1 expression and induces aldosterone resistance in CCD (Figure 7D).

DISCUSSION

Hyperkalemia can be caused by reduced renal excretion and excessive intake or leakage of potassium from the intracellular space.1,4 Impaired elimination of potassium is a typical condition leading to hyperkalemia.2,3 This condition can be induced by decreased delivery of sodium to the distal nephron resulting from acute or chronic renal insufficiency (reduced GFR, especially when <15 ml/min per 1.73 m2 with
Figure 4. Rapamycin reverses established phenotypes in CDTsc1KO mice. (A) Rapamycin (Rapa) prolonged the life span of KO mice. Four-week-old KO mice were given long-term rapamycin treatment (from ages 4 to 12 weeks) for survival analysis. Scheme of rapamycin administration is shown. (B) Hyperkalemia in KO mice was alleviated by rapamycin (n=12 for each group). Bars indicate mean±SD. (C) Hyperaldosteronism in KO mice disappeared after rapamycin treatment (n=12 for each group). Bars indicate mean±SD. (D) Rapamycin decreased EMT transition in KO mice. Shown are representative double immunofluorescence of AQP-2 (red) and N-cadherin (green) in cortices of KO mice and KO mice with 4 weeks of rapamycin treatment. Immunoblotting of N-cadherin and vimentin from microdissection were also presented. Scale bar=20 μm. (E) CD expansion in KO mice was attenuated by rapamycin (n=12 for each group). Bars indicate mean±SD. (F) Elevated S6 phosphorylation in CDTsc1KO mice was eliminated by rapamycin. Three sequential kidney sections (2 μm) were stained with hematoxylin and eosin, AQP-2, and phospho-S6 (S235/S236) as indicated. Scale bar=50 μm. (G) Western blotting showed decreased level of phospho-S6 in KO mice after rapamycin treatment. Con, control; H&E, hematoxylin and eosin.
low urine flow); aldosterone deficiency resulting from hypoaldosteronism and congenital adrenal hyperplasia;\textsuperscript{5,6} and abnormal functioning of the CCD resulting from pseudohypoaldosteronism, a heterogeneous group of disorders of electrolyte metabolism characterized by hyperkalemia, metabolic acidosis, and normal GRF.\textsuperscript{35} These abnormalities can also result from the effects of some drugs, or from a combination of underlying diseases and drugs.\textsuperscript{15} The CDTsc1KO mice generated in this study initially presented with hyperkalemia and subsequently developed metabolic acidosis, aldosterone resistance, and severe hyperkalemia with normal plasma creatinine and BUN levels. CDTsc1KO mice successfully demonstrate features of hyperkalemia caused by drugs or disease states that interfere with the function of CCD such as pseudohypoaldosteronism.

Some tissue- or cell-specific Tsc knock-out mice have been used to study the role of mTORC1 activation in the pathogenesis of diseases and the underlying mechanisms. Tsc1 KO–induced mTORC1 activation in podocytes resulted in an EMT-like phenotypic switch in podocytes, podocyte detachment and loss, and proteinuria.\textsuperscript{29,31} Tsc1 KO in renal tubular cells increased cell proliferation and enlargement, extensive renal cyst formation, and severe polycystic kidney disease.\textsuperscript{26} Liver-specific knockout of Tsc1 resulted in sporadic hepatocellular carcinoma preceded by liver damage; inflammation; and defects in autophagy, necrosis, and regeneration.\textsuperscript{43} In the current study, CD-specific knockout of Tsc1 led to dedifferentiation of CD, including loss of AQP2, EMT-like change, detachment, and columnar cell lesions of CCD cells. Columnar cell lesions and CAPSS have been reported for a long period in the breast and have received renewed attention because of the widespread use of biopsies in nonpalpable breast abnormalities detected by screening mammography.\textsuperscript{37,38} However, the cause of these lesions is unknown. To our knowledge, our findings in CDTsc1KO mice reveal for the first time that mTORC1 activation can induce columnar cell lesions and their potential to develop carcinomas. Furthermore, AQP2 was almost abolished in 8-week-old KO mice. Accordingly, the KO mice were found to be thirsty and polyuric (Supplemental Table 1).

ER stress has been previously reported to mediate high mTORC1-induced histologic and pathologic changes in several cell-specific Tsc1 KO mice.\textsuperscript{31,39} Although ER stress was also responsible for mTORC1 activation–induced CCD cell lesions and detachment in our model, reduction of ER stress with PBA only slightly reduced the level of plasma potassium, suggesting that an alternative mechanism is involved in

![Figure 5. Hyperactivation of mTORC1 induces ER stress in CDs.](image-url)
mTORC1 activation–induced hyperkalemia. We further observed that the expression of ENaC, ROMK, and Na\(^+\), K\(^+\)-ATPase in CCDs, which is essential for potassium secretion, was reduced by mTORC1 activation. SGK1 plays an essential role in renal physiology and the pathophysiology of renal disease via regulation of Na\(^+\), K\(^+\)-ATPase and ion channels, including ENaC and ROMK.\(^{44-46}\) It was recently shown that SGK1 is phosphorylated and activated by mTORC2.\(^{47,48}\) Of note, our findings suggest that mTORC1 negatively regulates expression of SGK1 because SGK1 protein level was reduced in CCD from CD Tsc1KO mice and was reinduced by rapamycin. It is suggested that high mTORC1

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**Figure 6.** Loss of ENaC, ROMK1, and dysfunction of Na\(^+\), K\(^+\)-ATPase are key events in hyperkalemia. (A) Western blotting showed decreased protein levels of ENaC\(_\alpha\) and ROMK1 in KO mice, while the level of ENaC\(_\beta\) or ENaC\(_\gamma\) was unchanged. Loss of ENaC\(_\alpha\) and ROMK1 in KO mice can be restored by both rapamycin (Rapa) and PBA. Protein lysates of indicated groups were from 8-week-old mice by microdissection. (B) Quantifications of results in A. Bars indicate mean ± SE; *P*<0.01; n.s, not significant. (C) Double immunofluorescence of AQP-2 (red) and ROMK1 (green) in CCD of indicated groups. Rapamycin ameliorated the loss of ROMK1. Renal tissues were from 8-week-old mice of indicated groups. Scale bar=20 μm. (D) Dysfunction of Na\(^+\), K\(^+\)-ATPase in KO mice was restored by rapamycin. The activities of Na\(^+\), K\(^+\)-ATPase of indicated groups were measured according to the manufacture’s instruction (n=8 for each group). Bars indicate mean±SD. (E) Rapamycin did not have significant effect on the activity of Na\(^+\), K\(^+\)-ATPase in 8-week-old control mice. Con, control.
activity may interfere with MR function as the expression of SGK1, ENaC, and ROMK and activity of Na\(^+\), K\(^+\)-ATPase can be rapidly induced by the binding of aldosterone to the MR. Although the detailed mechanisms remain to be identified, our results suggest that the dysfunction of MR signaling and development of MR-resistant hyperkalemia in CDTsc1KO mice may be the general result of dedifferentiation and dysfunction of CD, rather than a specific lesion in the MR signaling and potassium secretion pathway.

A previous clinical study reported on 13 patients with hyperkalemic metabolic acidosis,\(^{49}\) which was characterized by low fractional potassium excretion, impaired potassium excretion, and metabolic acidosis, as well as only moderately reduced GFR and no aldosterone deficiency. Importantly, these patients did not have increased fractional potassium excretion in response to mineralocorticoid, indicating the existence of aldosterone resistance. Thus, the CDTsc1KO mice highly mimicked a syndrome of hyperkalemic distal renal tubular acidosis resulting from a defect in hydrogen and potassium secretion in the distal nephron rather than from aldosterone deficiency.

In summary, our study demonstrates that balanced mTORC1 activity is critical for CD function and renal potassium secretion. Loss of TSC1 and activation of mTORC1 results in dedifferentiation and dysfunction of CD and causes hyperkalemia. The CDTsc1KO mice provide a novel model for hyperkalemia induced exclusively by dysfunction of CD.

**CONCISE METHODS**

**Mice, Husbandry, and Genotyping**

Both AQP2-Cre mice and Tsc1 loxped mice were from the The Jackson Laboratory (Jax no. 006881 and 005680, respectively). Male

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**Figure 7.** mTORC1 activation reduces SGK1 and induces aldosterone resistance in cortical collecting ducts. (A) mTORC1 activation decreased the protein level of SGK1 in vivo, which was reversed by rapamycin (Rapa) and PBA. Protein lysates were from microdissection. (B and C) Fractional Na\(^+\) (B) and K\(^+\) (C) excretion in control and KO mice before and after amiloride treatment. Eight-week-old control and KO mice were injected subcutaneously with amiloride (5 mg/kg), and urine and serum were collected for biochemical analysis. Data were expressed as mean±SD; \(n=5\) for control group, \(n=7\) for amiloride group. (D) Schematic of the disruption of potassium homeostasis in principle cells of CCD in CDTsc1KO mice. Deletion of Tsc1 and hyperactivation of mTORC1 induce dedifferentiation of CCD, which further results in loss of SGK1, ENaC\(_\alpha\) and ROMK1, and dysfunction of Na\(^+\), K\(^+\)-ATPase in principle cells of CCD, and therefore disrupts potassium homeostasis and causes hyperkalemia. Con, control.
homozygous Tsc1 loxped (Tsc1<sup>loxp/loxp</sup>) mice were mated with female AQP2-Cre mice to yield mice heterozygous for loxped Tsc1 and heterozygous for AQP2-Cre. These mice were then bred with mice homozygous for loxped Tsc1 to obtain mice homozygous for loxped Tsc1 and heterozygous for AQP2-Cre. These mice carried CD-specific deletion of Tsc1 and were termed CD<sub>Tsc1KO</sub>, and the mice homozygous for loxped Tsc1 without AQP2-Cre from the same litter were used as controls (Tsc1<sup>loxp/loxp</sup>). Genotyping the mice involved the use of the following primers: AQP2-forward: 5′-CTCTGCAG-GAACCTGGTGCTGG-3′; AQP2-reverse: 5′-GGCAATCTT-CAGGTTCTGCGG-3′; Tsc1-forward: 5′-GTCAGACCGTAGGA-GAAC-3′; Tsc1-reverse: 5′-GAA TCA ACC CCA CAG AGC AT-3′. DNA extraction, PCR amplification, and agarose electrophoresis were performed according to the Jackson Laboratory’s instructions. All animal experiments were approved by the Committee on the Use and Care of Animals and were performed in accordance with the Committee’s guidelines and regulations.

Blood and Urine Physiologic Assessment and Hormone Measurement

Daily water intake was measured and urine was collected under oil for physiologic analysis.

After anesthesia, a midline dermotomy of the chest was performed, and blood was then drawn by cardiac puncture. After 15 minutes of centrifugation at 3000 g, serum was collected for routine physiologic analysis using an automatic biochemical analyzer (Olympus AU 5400), and serum aldosterone levels were measured using an ELISA kit (Nanjing Jiancheng Bioengineering). For the measurements of amiloride-sensitive Na<sup>+</sup> reabsorption, amiloride, 5 mg/kg per day, was administrated intraperitoneally to control and CD<sub>Tsc1KO</sub> mice. Urine and serum were collected for biochemical analysis.

Hematoxylin and Eosin Staining, Immunohistochemistry, and Immunofluorescence

Kidneys and adrenal glands of mice at the indicated age were removed and weighed. The kidneys were immediately fixed in 4% paraformaldehyde and then processed using paraffin wax and standard methods. All animal experiments were approved by the Committee on the Use and Care of Animals and were performed in accordance with the Committee’s guidelines and regulations.

Immunofluorescence images were obtained using a Fluoview FV1000 confocal microscopy (Olympus).

BrdU Incorporation and Apoptosis Assay

Four- and 6-week-old control and KO mice and 6-week-old KO mice with 2 weeks rapamycin treatment were given a single intraperitoneal injection of 1 ml BrdU (Invitrogen) per 100 g body weight. Two hours later, they were euthanized by cervical dislocation; the kidneys were removed and immediately fixed in 4% paraformaldehyde, then processed using paraffin wax and standard methods. The proliferative activity of CCD was assessed on 5-μm sections using immunofluorescence as described above, and anti-BrdU primary antibody (1:1000; Sigma-Aldrich). Apoptosis of CCD cells was evaluated on 5-μm sections by TUNEL assay using a commercial kit (Promega).

Transmission Electron Microscopy

Eight-week-old control or CD<sub>Tsc1KO</sub> mice were anesthetized, and kidneys were removed and immediately fixed in 2.5% glutaraldehyde. After standard electron microscopy sample preparation, the processed samples were photographed and analyzed by an H-7650 transmission electron microscope (Hitachi).

Rapamycin and PBA Treatment

Four-week-old CD<sub>Tsc1KO</sub> mice were administered rapamycin (1 mg/kg per day) intragastrically. Some of the animals were euthanized at 8 weeks, and the serum and kidneys were collected and the kidneys were processed using paraffin wax for blood physiologic and histochemical analysis, respectively, as described above. The remaining animals were treated with rapamycin until they reached age 12 weeks and were analyzed for survival time. For PBA (500 mg/kg per day), the treatments and subsequent assays were the same as with rapamycin.

Microdissection, Na<sup>+</sup>−K<sup>+</sup>-ATPase Activity Assay, and Western Blotting

Microdissection was performed as previously described with some modification. Briefly, after anesthetization, the left kidney of mice was perfused 10 ml of solution I. Microdissection solution I was a HEPES-buffered solution containing the following (in mmol): 130 NaCl, 5 KCl, 1 Na<sub>2</sub>HPO<sub>4</sub>, 1 MgSO<sub>4</sub>, 1 Ca lactate, 2 Na acetate, 5.5 glucose, 5 l-alanine, 2 l-leucine, and 10 HEPES, with a pH of 7.4. The kidney was then perfused with solution II (solution I supplemented with 0.1% collagenase type II and 0.1% BSA). The kidney was removed and 1-mm-thick slices were incubated in solution II at 37°C for 40 minutes. After washing, the CDs were dissected on ice under a microscope. For the Na<sup>+</sup>−K<sup>+</sup>-ATPase activity assay, 30–50 ducts were analyzed using a commercial kit (Nanjing Jiancheng Bioengineering). The activity was expressed as picomoles of ATP hydrolyzed per milligram of protein per hour. For Western blotting, the ducts were boiled in SDS loading buffer and then subjected to SDS-PAGE following standard protocol.

Statistical Analyses

All experiments were carried out in duplicate. Data were expressed as mean ± SD, and differences between groups were analyzed using t test (SPSS software, version 13.0; SPSS, Inc., Chicago, IL) and one-way
ANOVA or, if the data violated a normal distribution, by non-parametric Mann–Whitney test. *p*<0.05 was considered to represent statistically significant differences. In the case of Western blot analysis, one representative set of data is shown.

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**DISCLOSURES**

None.

**REFERENCES**


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