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Mitochondrial TRPC3 promotes cell proliferation by regulating the mitochondrial calcium and metabolism in renal polycystin-2 knockdown cells

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Abstract

Purpose Previous studies indicate that autosomal dominant polycystic kidney disease (ADPKD) cells exhibited dysregulated calcium homeostasis and enhanced cell proliferation. TRPC3 has been shown to function in the modulation of calcium and sodium entry, but whether TRPC3 plays a role in cellular abnormalities of ADPKD cells has not been defined.

Methods Human conditionally immortalized proximal tubular epithelial cells and mouse IMCD3 cells were used with polycystin-2 (PC2, TRPP2) knockdown. Cell proliferation assay was used to detect the cell proliferations upon different treatments. QRT-PCR and western blotting were used to measure the expression profiles of TRPP2 and other proteins. High-resolution respirometry, enzymic activities and ROS levels were detected to reflect the mitochondrial functions. Calcium and sodium uptakes were measured using Fura2-AM and SBFI dyes.

Results We showed that PC2 knockdown promoted cell proliferation, ROS productions and ERK phosphorylation, compared with negative control. Meanwhile, we demonstrated that receptor-operated calcium entry (ROCE) exhibited less reductions compared with store-operated calcium entry (SOCE) upon PC2 knockdown. Inhibition of ROCE and SOCE by specific inhibitors partially reversed the enhanced cell proliferation, ROS productions and ERK phosphorylation induced by PC2 knockdown. Moreover, TRPC3 upregulation was observed upon PC2 knockdown, which acted as both SOC and ROC, promoting cation entry, cell proliferation and ERK phosphorylation. Furthermore, we showed that mitochondrial located TRPC3 was upregulated and modulating mitochondrial calcium uptake, thus promoting the ROS productions in the presence of PC2 knockdown.

Conclusions We demonstrated that TRPC3 upregulation upon PC2 knockdown aggravated the mitochondrial abnormalities and cell proliferation by modulating mitochondrial calcium uptake. Targeting TRPC3 might be a promising target for ADPKD treatment.

Keywords ADPKD · TRPP2 · TRPC3 · Mitochondria · ERK

Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is one of the genetic disorders that affects approximately 12.5 million people worldwide, which accounts for almost 10% of patients on renal-replacement therapy [1, 2]. For ADPKD, progressive expansion of multiple bilateral renal cysts leads to massive kidney enlargement and progressive renal failure. Substantially, ADPKD is caused by mutations of the genes PKD1 or PKD2, encoding the non-selective calcium ion channels polycystin 1 and polycystin 2 (PC1 and PC2), respectively, which regulate Ca^{2+} influx in primary cilia [3]. Previously, decreased PC1 and PC2 functions lead to dysregulated cellular calcium, which activated adenylyl cyclase 6 (AC6) and increased the cyclic AMP (cAMP) level, thus enhancing the protein kinase A (PKA) signaling and extracellular signaling-regulated kinase (ERK) pathway in cells derived from polycystic kidneys, promoting cell proliferation and cyst formation [4–6]. Recent studies suggested excessive

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oxidative stress was present in early ADPKD when the renal function is preserved [7, 8]. However, the source and regulation of the oxidative stress was not clear yet.

Mitochondria are responsible for the aerobic respiration of the cell and are the main source of oxidative stress. Mitochondrial dysfunction exists during ADPKD, indicating a relationship between mitochondria and renal polycystic diseases. However, the precise pathophysiological role of mitochondria and the regulation of ROS production in ADPKD remains elusive. It has been acknowledged that calcium and ROS had mutual interplayed roles, especially mitochondrial Ca^{2+}, which is vital to mitochondrial function by regulating the Ca^{2+}-sensitive enzymes in the Krebs cycle [9]. Transient receptor potential channel, subtype C, member 3 (TRPC3) is a non-selective, cation channel that modulates calcium signals in various tissues. TRPC3 has been shown to participate in the pathogenesis of several types of diseases, such as hypertension, atherosclerosis, cardiac hypertrophy, etc. [10, 11]. Recent studies indicated that TRPC3 was also located in the mitochondrial inner membrane and was increased during pathological conditions [12, 13]. There were several studies indicated that other calcium channels might complement for the decreased PC1 and PC2 functions. Miyagi et al. also indicated that PC-2 mutation-induced enhanced receptor-activated calcium entry (ROCE) was correlated with TRPC3, but the expression profiles of TRPC3 and its functions are not clearly elucidated in previous studies. Therefore, in this study, both human conditionally immortalized proximal tubular epithelial cells (ciPTEC) and the mouse collecting duct cell line IMCD3 were used and TRPP2 siRNA was stably transfected to simulate the condition of ADPKD. We showed that mitochondrial TRPC3 was significantly upregulated during TRPP2 knockdown and regulated mitochondrial ROS productions. This might become a new mechanism and a therapeutic target of TRPP2-knockdown-induced cell proliferation and cyst formation.

Methods

Cell culture and transfections

CiPTEC cells were generated as previously described [14, 15]. Briefly, primary cells were cultured through collecting mid-stream urine, then the urine sediment was resuspended in DMEM (Dulbecco's Modified Eagle Medium)–Ham's F12 medium supplemented with 10% fetal bovine serum (FBS). Meanwhile, 100 IU/ml penicillin, 100 µg/ml streptomycin, ITS (5 µg/ml insulin, 5 µg/ml transferrin and 5 ng/ml selenium), 36 ng/ml hydrocortisone, 10 ng/ml epidermal growth factor (EGF) and 40 pg/ml triiodothyronine were also added to the culture medium. After cells were immortalized, TRPP2 specific shRNA was transfected and we screened for the stably transfected cells for later experiments.

Mouse inner medullary collecting duct cell line IMCD3 was purchased from the Cell Bank of Chinese Academy of Sciences. IMCD3 cells were cultured in DMEM medium supplemented with 10% FBS (fetal bovine serum) and antibiotics added in the atmosphere of 37 °C and 5% CO_2. The cell culture reagents were purchased from Gibco (ThermoFisher Scientific, Waltham, MA, USA). The specific siRNAs for TRPP2 and TRPC3 and negative controls were synthesized and purchased from Genecopoeia (Guangzhou, China) and then stable-transfected cells were cultured and screened as described [15].

Western blotting

Western blotting was conducted according to previous reports [16]. In brief, the whole protein was extracted from cell lysates with RIPA lysis buffer supplemented with proteinase inhibitor cocktail (Roche). Afterwards, the proteins were loaded and separated with 10% SDS-PAGE gels and transferred to PVDF membrane. Then the membranes were blocked with 5% BSA solution for 2 h and incubated with primary antibodies (1:1000) diluted with 5% BSA at 4 °C overnight. Subsequently, PBST solution was used to wash the membrane and second antibodies incubated for 2 h at room temperature. ECL chemiluminescent reagent was used to visualized the bands. The primary antibodies used were as follows: anti-TRPC1, anti-TRPC3, anti-TRPC6, anti-TRPC7 (Alomone Labs, Jerusalem, Israel), anti-phosphorylated-ERK, anti-total-ERK, anti-phosphorylated-Akt, anti-total-Akt (Cell Signaling Technology, Boston, MA, USA), anti-NCX1, anti-VDAC (Abcam, MA, USA), and anti-GAPDH (Santa Cruz Biotechnology, CA, USA).

Cell proliferation assay

Cell proliferation assay was conducted using the Cell Counting Kit-8 (CCK-8, Dojindo, Japan). Briefly, ciPETCs and IMCD3 cells were seeded in triplicate in the 96-well plated for 12 h, then cells were given different treatments for indicated time and the OD450 values were measured in different treatment groups using the Fluoroskan Ascent Fluorometer (ThermoFisher, Helsinki, Finland). SOCE inhibitor 2-APB (2-Aminoethoxydiphenyl borate), ROCE inhibitor SKF-96365, mitoROS scavenger mitoTEMPO and TRPC3 inhibitors Pyr3 and Gd^{3+} were all purchased from Sigma-Aldrich (St. Louis, MO, USA).

Quantitative real-time PCR

After treatment, ciPETCs and IMCD3 cells were lysed and total RNAs were extracted with Trizol agent, and first-strand cDNA was synthesized using M-MLV transcriptase. Afterwards, quantitative real-time PCR was conducted to detect the expression levels of target genes using the Applied Biosystems 7500 PCR system. The primers used are as follows: TRPP2 (human): Forward 5′-GTCCTTGATGCAGGTGCC ATC-3′ and reverse 5′-ACTCCTTCAGCACCCCCTTC-3′; TRPP2 (mouse): Forward 5′-CAT CAC AGT CAA CGC GAC CAG-3′ and reverse 5′-TTT CTC AGG ATA CTT TGC AGG-3′. The expression levels of TRPP2 were normalized to the relative expression of GAPDH. The relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method.

Measurements of ROS levels

The ROS levels of renal tubular cells were measured using the dihydroethidium (DHE) dye for cytosolic ROS detection and the MitoSOX Red dye (ThermoFisher Scientific, Waltham, MA) for mitochondrial ROS detection. DHE and MitoSOX staining and detection were conducted as previously described [13]. In brief, cells were incubated with 5 µmol/l DHE/MitoSOX diluted with DMEM culture medium at 37 °C for 30 min, then the cells were placed on an inverted fluorescence microscope (Nikon TE2000, Tokyo, Japan) and F_{510nm}/F_{580nm} was determined using the Fluoroskan Ascent Fluorometer (ThermoFisher, Helsinki, Finland). The ROS levels are presented as a normalized value relative to the fluorescence intensity of the control group.

High-resolution respirometry

To reflect the aerobic metabolism capacities, a 2-channel titration injection respirometer (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria) equipped with 2 chambers was adopted. To evaluate the mitochondrial respiratory functions, renal tubular cells were collected, resuspended in the MiR05 solution, and transferred separately to oxygraph chambers with the final cell density of 1×10^6 cells/ml. At the beginning, routine respiration (*R* value) was assessed while respiration was stabilized, then the plasma membrane was then permeabilized with digitonin (10 µg/10^6 cells), and Complex I-dependent oxidative phosphorylation (CI_{OXPHOS}) was measured after the addition of glutamate (G), malate (M) and ADP (D). Subsequently, succinate (S) was added to induce maximal oxidative phosphorylation (OXPHOS) capacity ($CI+II_{OXPHOS}$). To better analyzing the respiratory capacity, cells were uncoupling with 2-[[4-(trifluoromethoxy) phenyl]hydrazinylidene] propanedinitrile (FCCP; injected stepwise up to 1–1.5 µmol/l) in the noncoupled state, CI + II supported noncoupled respiration ($CI+II_{ETS}$) was measured. The use of rotenone (0.5 µmol/l) inhibited the Complex I and CII_{ETS} was measured. Residual oxygen consumption rate was determined by the inhibition of Complex III with antimycin A (2.5 µmol/l). The capacity of CIV_{ETS} was measured by adding the CIV substrates ascorbate sodium salt (2 mmol/l) and TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-p-phenylenediamine dihydrochloride) (0.5 mmol/l).

Intracellular and mitochondrial calcium/sodium measurements

For the intracellular measurements of the concentrations of calcium and sodium uptake, Fura-2 AM and SBFI dyes were used and different protocols were adopted, respectively. For the calcium measurement, renal tubular cells were digested and incubated with 1 µmol/l Fura-2 AM dye in the dark at 37 °C for 30 min. The Fluorescence was measured at 510 nm emission and at excitation wavelengths of 340 nm and 380 nm, at baseline and after treatment. Both SOCE (store-operated calcium entry) and ROCE (receptor-operated calcium entry) were measured according to different protocols. The SOCE was measured by adding 1 µmol/l thapsigargin (TG, sigma) to the renal tubular cells in the calcium-free Hank's Balanced salt solution (HBSS, in mmol/l: 136 NaCl, 5.4 KCl, 0.44 KH_2PO_4, 0.34 Na_2HPO_4, 5.6 D-glucose, 10 HEPES, pH 7.4) then 1 mmol/l $CaCl_2$ was added to induced the SOCE. Meanwhile, ROCE was measured in HBSS with 1 mmol/l $CaCl_2$ and 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) was added after baseline measurements. For the detection of sodium uptake, renal tubular cells were incubated with 5 µmol/l SBFI dyes in the dark and three washes. Then Na^+ entry was measured after the agonist addition at 500 nm emission and at excitation wavelengths of 340 nm and 380 nm.

Moreover, for the detection of mitochondrial calcium uptake, renal tubular cells were digested and resuspended using the extracellular solution (in mmol/l: 120 NaCl, 6 KCl, 0.3 $CaCl_2$, 2 $MgCl_2$, 12 D-glucose, 12 sucrose, and 10 HEPES-free acid, pH 7.4). Cells were incubated with 5 µmol/l Rhod-2 AM and 0.025% F-127 for 30 min at 37 °C in the dark, then they were washed for three times with the extracellular solution. Additionally, cells were permeabilized with 10 µmol/l digitonin for 5 min at 37 °C in the intracellular solution (in mmol/l: 120 KCl, 10 NaCl, 1 KH_2PO_4, 20 HEPES, 2 Mg-ATP, 2 succinate and proteinase inhibitor mixtures, pH 7.2) before detections, with agonist (ATP) and Ca^{2+} added for the later treatment. The fluorescence was measured at an emission wavelength of 581 nm and an excitation wavelength of 552 nm. The statistical analysis of the calcium signal values was presented in the graphs as R_X/R_0. Among them, R is the mean emission value of several treatments induced by excitation and R_0 is the mean value of R during the pretreatment period in each experiment as baseline.

Co-immunoprecipitation assay

Renal tubular cells were cultured with confluence of 80% before treatment, with cells being lysased with the lysis buffer (Beyotime, Shanghai, China). Rabbit IgG and primary antibody were added with the ratio of 2 µg per 1 mg total protein, and then the mixtures were incubated at 4 °C for 1 h. Afterwards, protein A/G agarose (Santa Cruz, CA, USA) was added with the volume of 30 µl per sample and the mixtures were incubated at 4 °C overnight. Lysis buffer was used for washing solution for five times and the protein mixtures were denatured by western loading buffer without SDS before immunoblotting analysis.

Enzymic activities of mitochondrial Complex I and II

Another parameter is the enzymic activities of mitochondrial Complex I and II locating in the respiratory chain. The respiratory chain enzyme activities were measured using a Mitochondrial Complexes Activity Assay Kit (Millipore, Darmstadt, Germany) according to the manufacturers' instructions [17]. After treatments and cell lysis, the enzyme activities of mitochondrial Complex I in tubular cells were measured at the wavelength of 450 nm in 37 °C for 30 min, whereas Complex II activities were detected at the wavelength of 600 nm for 60 min.

Statistical analysis

The data in this study are presented as the mean ± SEM. In detail, for the comparisons between two treatment groups, unpaired Student's *t* test was used to examine the differences. For the comparisons among three or more treatment groups, ANOVA (analysis of variance) followed by Bonferroni post hoc tests were used to examine the differences. All the data analysis was performed using GraphPad Prism 6.0 (La Jolla, CA, USA) or SPSS 19.0 (IBM Analytics, NY, USA). Two-sided *P* value less than 0.05 was considered as statistically significant.

Results

Polycystin-2 knockdown cells exhibited dysregulated mitochondrial metabolism and increased proliferation

Renal tubular cells ciPETCs and IMCD3 were stably transfected with polycystin-2 (PC2, TRPP2) siRNA to simulate the model of PKD. In Fig. 1a, after transfections, ciPETCs and IMCDs cells were detected for the expression profiles of TRPP2. It showed that TRPP2 knockdown by specific shRNA/siRNA could significantly downregulate the expression levels of TRPP2, compared with negative control. Meanwhile, TRPP2 knockdown could partially increase the cell proliferation and promote the cell proliferation in both ciPETCs and IMCD3 cells (Fig. 1b). To further investigate the mechanisms of TRPP2 knockdown-induced cell proliferation, we firstly detected the levels of reactive oxygen species (ROS) in these two cells. It is shown in Fig. 1c that TRPP2 knockdown enhanced the productions of ROS, reflected by increased DHE and mitoSOX fluorescence. Furthermore, using high-resolution respirometry, it showed that TRPP2 knockdown upregulated the routine mitochondrial respiratory rate, the Complex I OXPHOS and Complex I plus Complex II OXPHOS, suggesting that the cellular metabolism was activated upon TRPP2 knockdown (Fig. 1d). These results indicated that TRPP2 knockdown enhanced the mitochondrial. Besides, we analyzed the phosphorylation of ERK and Akt kinases, which are the main pathways regulating the cell metabolism and proliferation. It is shown in Fig. 1e and siTRPP2 enhanced the phosphorylation of ERK and Akt in both ciPETC and IMCD3 cells. Therefore, these results indicated that TRPP2 knockdown enhanced the cell proliferation is correlated with the dysregulated mitochondrial metabolism.

Dysregulated calcium homeostasis is responsible for the enhanced proliferation upon polycystin-2 knockdown

Calcium is one of the most important second-messengers in the cells. Previous studies indicated that calcium signaling was dysregulated in PKD [18]. It is shown in Fig. 2a–d that TG-induced SOCE were significantly decreased, whereas OAG-induced ROCE was only slightly decreased upon TRPP2 knockdown, compared with siNC, in both ciPETC and IMCD3 cells. To further verify whether dysregulated SOCE and ROCE participated in the cell proliferation of TRPP2 knockdown, we used SOCE inhibitor 2-APB and ROCE inhibitor SKF-96365 in the cell proliferation assay. It is shown in Fig. 2e that 2-APB and SKF-96365 could significantly inhibited the cell proliferation upon TRPP2 knockdown. Moreover, 2-APB and SKF-96365 interventions could also inhibit the ROS productions and ERK phosphorylation (Fig. 2f, g), indicating that dysregulated ROCE and SOCE upon TRPP2 knockdown play important roles in the overproliferation of ADPKD tubular cells.

TRPC3 upregulation participated in regulating calcium flux by combination with NCX1

TRPCs has been shown to play important roles in regulating calcium homeostasis and has been shown to dysfunctional upon TRPP2 knockdown [19, 20], but the concrete

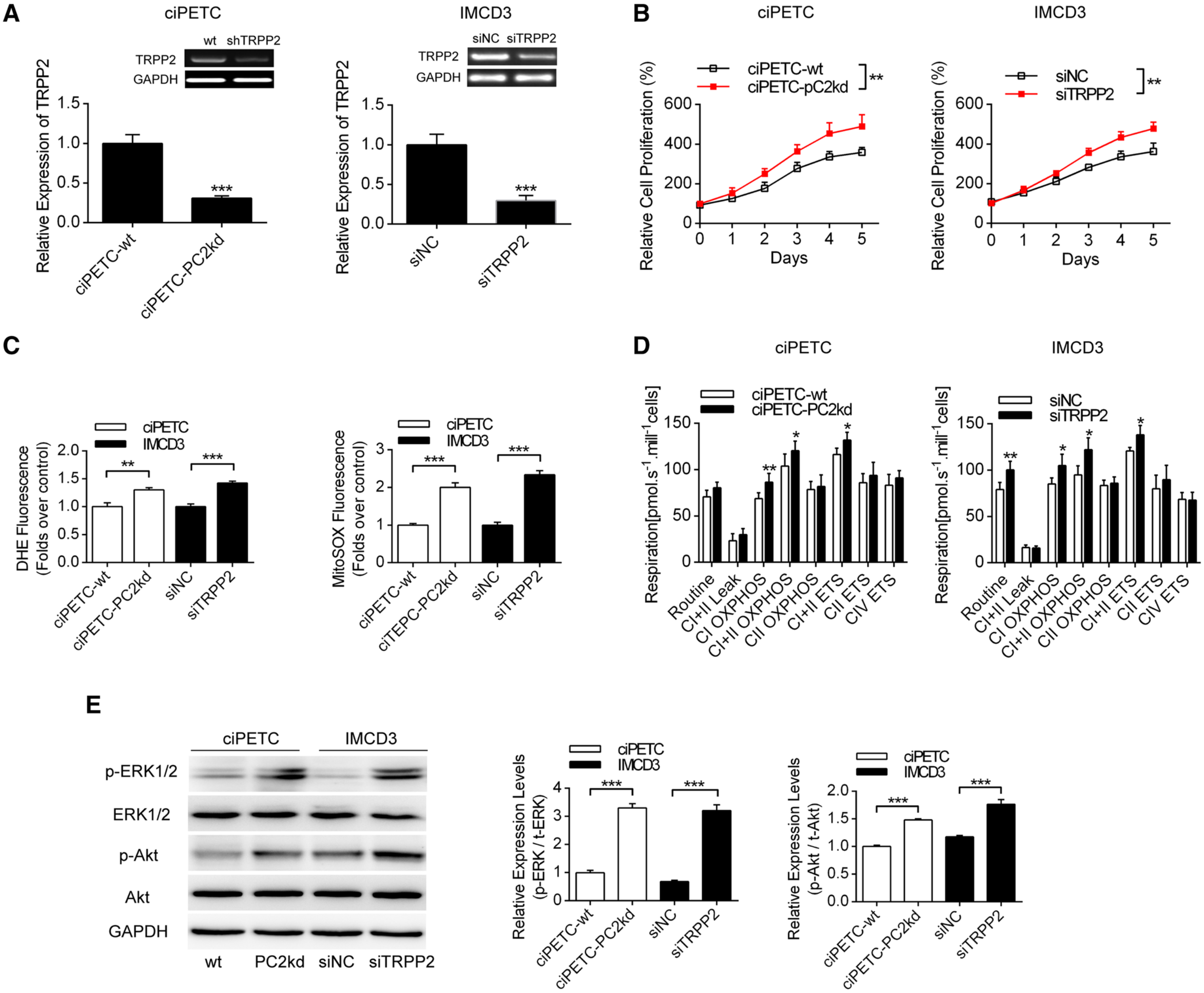


Fig. 1 The effects of TRPP2 knockdown on cell proliferations and related pathways. **a** The human renal tubular cells ciPETC and mouse collecting duct cell line IMCD3 were transfected with TRPP2 shRNA and siRNA, respectively, with negative controls, then qRT-PCR was conducted to detect the expression levels of TRPP2 mRNA. $N=3$, $P<0.0001$. **b** Cell proliferation assay was performed after TRPP2 knockdown in both ciPETC and IMCD3 cells, then the OD450 values were measured and analyzed with fold changes. $N=5$, $P<0.01$. **c** The cells were treated as **a**, then the DHE and mitoSOX fluorescence was detected. $N=6$, $**P<0.01$, $***P<0.0001$. **d** High-resolution respirometry was conducted to analyze the effects of TRPP2 knockdown on mitochondrial respiratory functions in both ciPETC and IMCD3 cells. $N=6$, $*P<0.5$, $**P<0.01$. **e** Renal tubular cells were treated as **a**, then western blot was conducted to analyzed the expression profiles of phosphorylated-ERK (p-ERK), total-ERK1/2, phosphorylated-Akt (p-Akt), total-Akt, using GAPDH as a loading control. Three western blot bands were quantified and analyzed for possible statistically significance. $N=3$, $***P<0.0001$. Data were presented as mean ± SEM

mechanisms have not been identified yet. Figure 3a detected the expression profiles of TRPC channels. It was shown that the expression levels of TRPC1, TRPC6 and TRPC7 remained unchanged while the TRPC3 expression levels are upregulated upon TRPP2 knockdown. Since TRPC3 was reported to be a non-selective cation channel that mediated both ROCE and SOCE [21]. We showed that TRPC3 knockdown could downregulate OAG-mediated calcium and sodium entry and TG-mediated calcium entry, indicating that TRPC3 participated in the SOCE and ROCE upon TRPP2 knockdown (Fig. 3b–d). Meanwhile, TRPC3 knockdown by siTRPC3 or TRPC3 inhibitor Pyr3 decreased the cell proliferations in ciPETC cells (Fig. 3e). Furthermore, we showed that TRPC3 inhibitor Pyr3 or Gd^{3+} could also suppressed the cell proliferation induced by PC2 knockdown (Fig. 3f). Additionally, TRPC3 knockdown by specific siRNA could partially attenuate the activated ERK upon TRPP2 knockdown, compared with siNC (Fig. 3g), while Fig. 3h shows the enhanced combination of TRPC3 and NCX1 upon TRPP2 knockdown while inhibition of

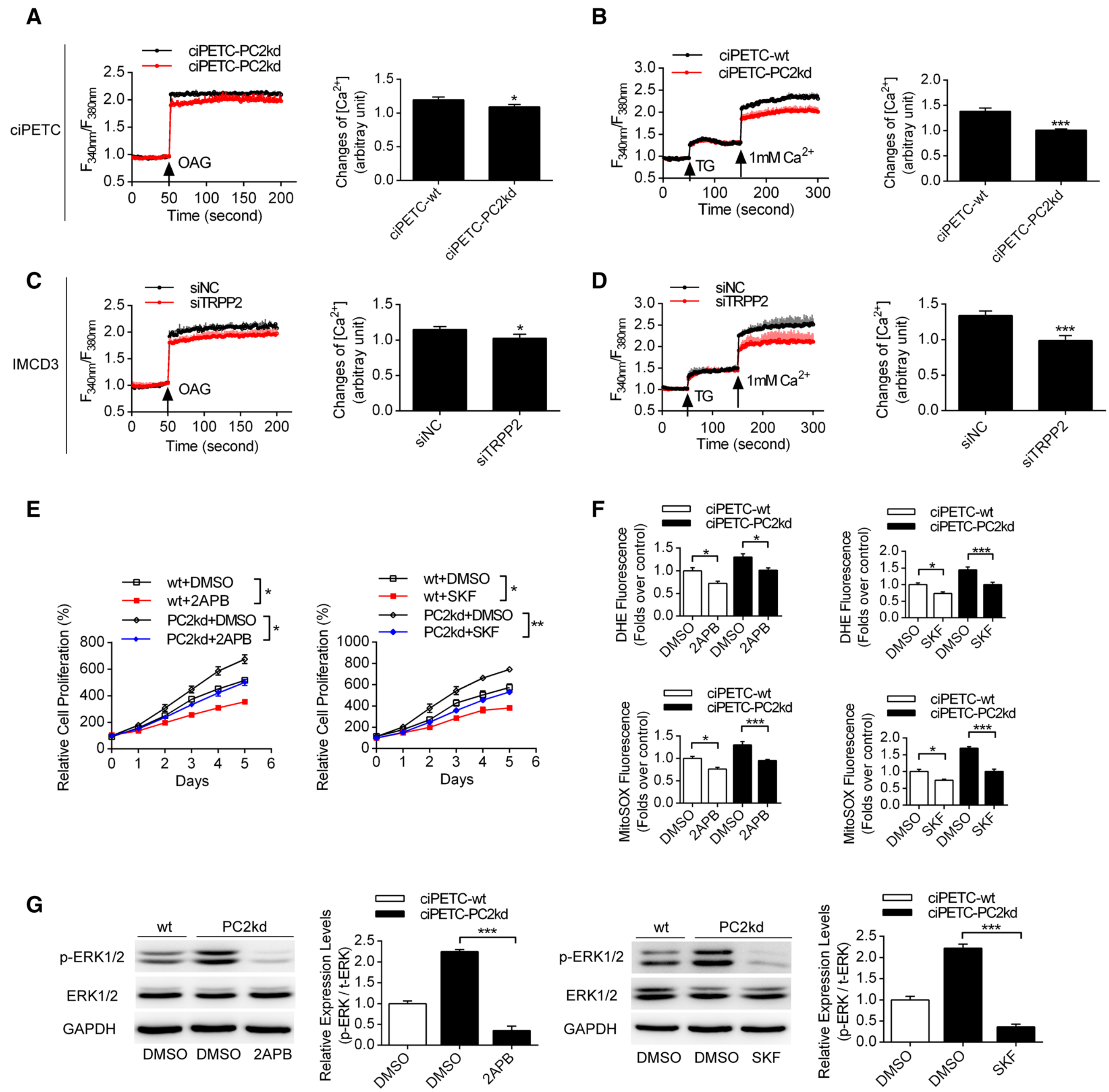


Fig. 2 The effects of enhanced SOCE and ROCE on cell proliferation and mitochondrial functions upon TRPP2 knockdown. **a–d** SOCE and ROCE were measured in both ciPETC and IMCD3 cells via different protocols. $N=3$, $^{***}P<0.0001$. **e** CiPETC cells were stably expressed with shNC or shTRPP2 plasmids, then both SOCE inhibitor 2-APB and ROCE inhibitor SKF96365 were added to examine the effects of SOCE and ROCE on cell proliferations. $N=3$, $^{*}P<0.5$, $^{**}P<0.01$. **f** After treatments with 2-APB or SKF96365 for 8 h, both DHE and mitoSOX dyes were incubated and the fluorescence was measured. $N=6$, $^{*}P<0.05$, $^{***}P<0.0001$. **g** Renal tubular cells were treated as **f**, then the expression profiles of p-ERK1/2 and t-ERK1/2 were detected by western blotting, using GAPDH as a loading control. $N=3$, $^{***}P<0.0001$

NCX1 by KB-R7943 could also inhibit the SOCE. These results indicated that TRPC3–NCX1 complex promoted the enhanced calcium entry and cell proliferation upon TRPP2 knockdown.

TRPC3-mediated enhanced calcium influx increased ROS productions and cell proliferation

Besides regulating cellular calcium homeostasis by TRPC3, it has been reported that TRPC3 is also located in the mitochondria inner membrane and regulates mitochondrial

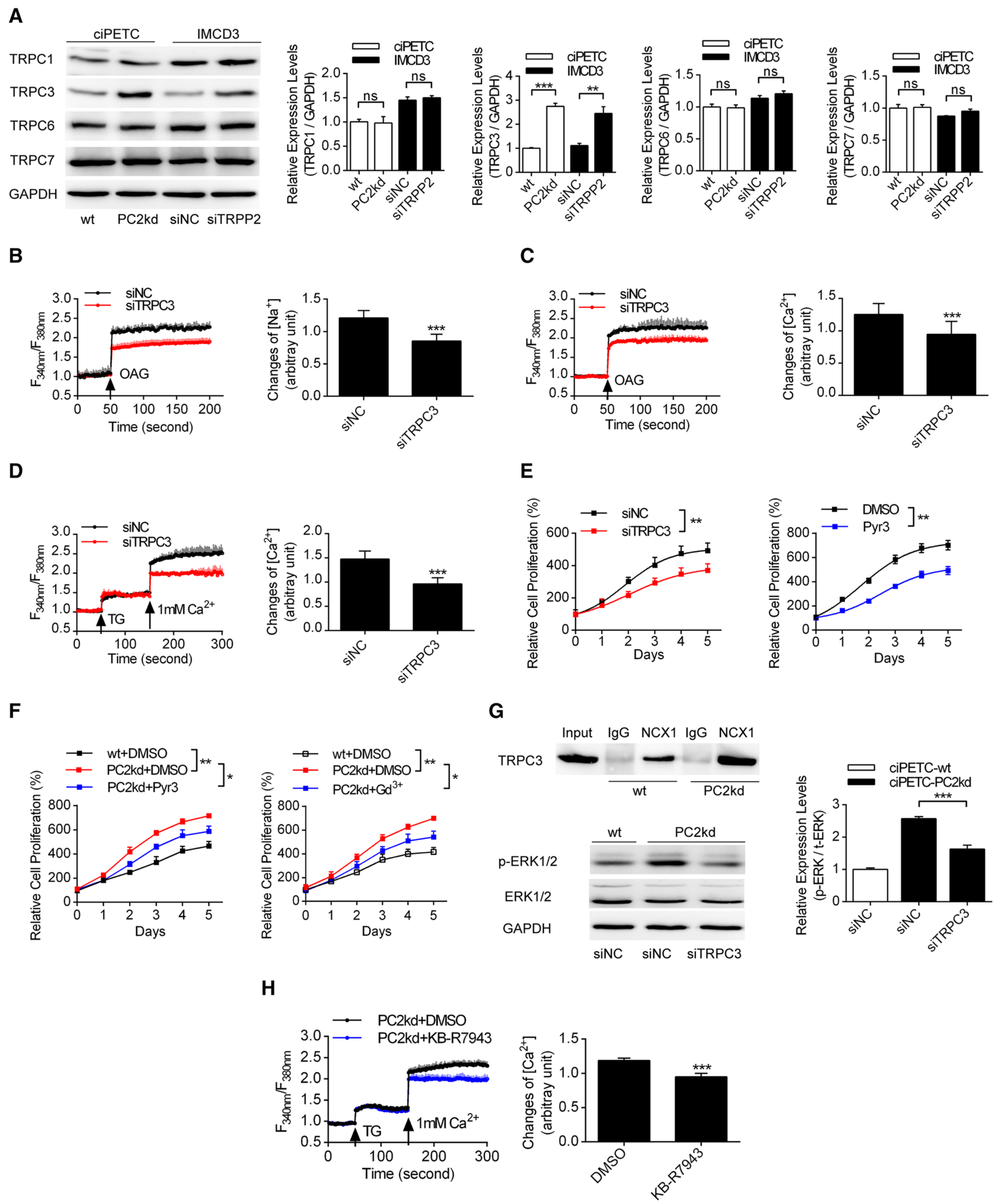
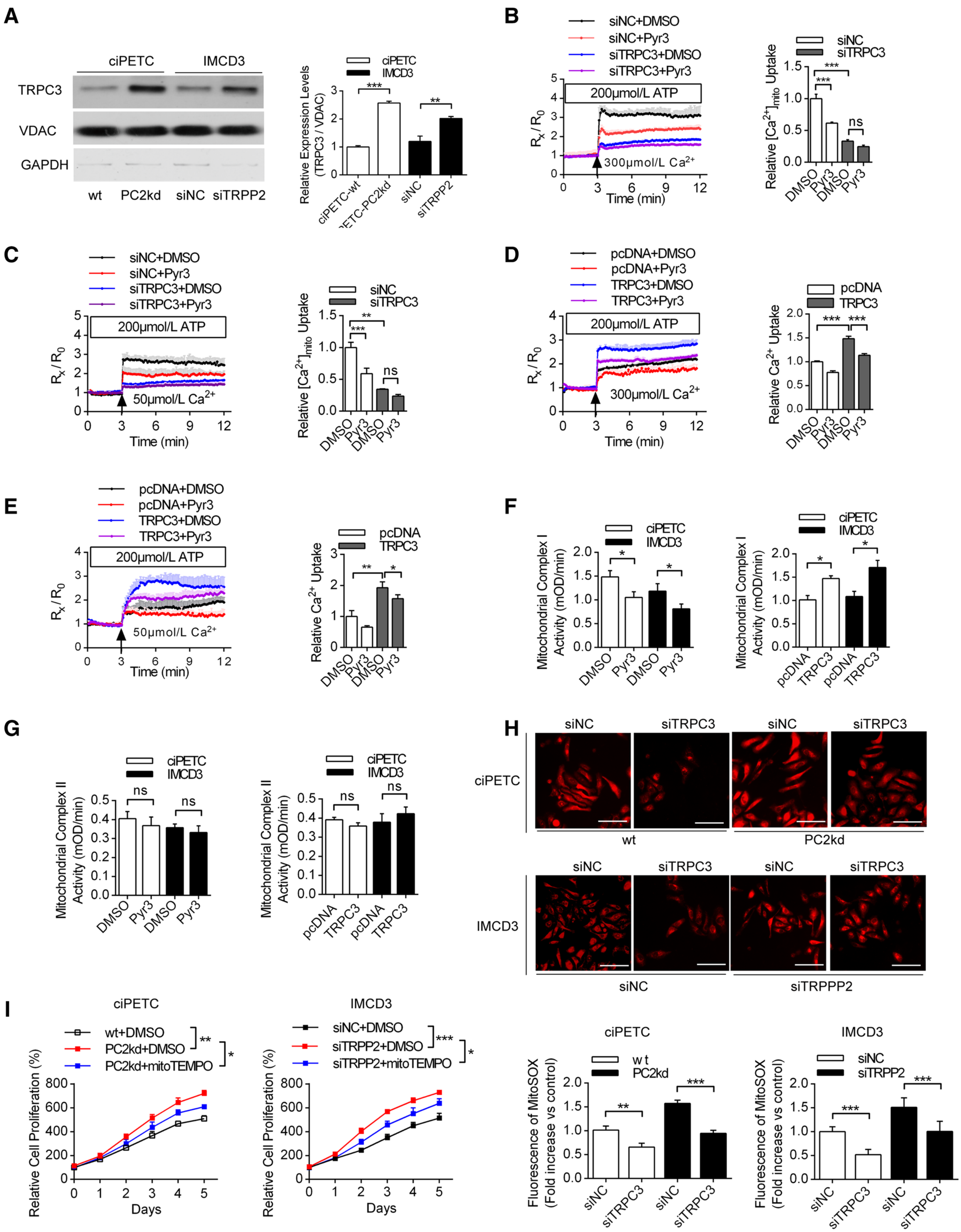


Fig. 3 The effects of TRPC3 upregulation on calcium homeostasis and cell proliferations. **a** Both ciPETC and IMCD3 cells were treated with TRPP2 knockdown shRNA/siRNA, then the expression levels of TRPC1, TRPC3, TRPC6 and TRPC7 were detected by western blotting, using GAPDH as a loading control. $N=3$, $^{**}P<0.01$, $^{***}P<0.0001$. **b**, **c** OAG-induced calcium and sodium entry were measured by Fura-2 AM dye in ciPETC cells with siNC or siTRPC3. $N=3$, $^{***}P<0.0001$. **d** Thapsigargin-induced calcium entry was measured by Fura-2 AM in ciPTEC cells with siNC or siTRPC3. $N=3$, $^{***}P<0.0001$. CiPETC cells were treated with siNC/siTRPC3 or DMSO/Pyr3 (**e**), DMSO/Pyr3 or DMSO/Gd^{3+} (**f**), then cell proliferation assay was conducted. $N=3$, $^{**}P<0.01$. **g** Co-immunoprecipitation was conducted in both wt and PC2kd ciPETC cells to verify the combination of TRPC3 and NCX1. Meanwhile, the expression levels of p-ERK1/2 and t-ERK1/2 were detected by western blot upon double-knockdown of TRPP2 and TRPC3. $N=3$, $P<0.0001$. **h** PC2kd ciPETC cells were treated with DMSO or NCX1 inhibitor KB-R7943, then SOCE was measured as previous described. $N=3$, $P<0.0001$


A
ciPETC
IMCD3
TRPC3
VDAC
GAPDH
wt PC2kd siNC siTRPP2
Relative Expression Levels (TRPC3 / VDAC)
ciPETC
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ciPETC-PC2kd
siNC
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siNC+Pyr3
siTRPC3+DMSO
siTRPC3+Pyr3
200μmol/L ATP
300μmol/L Ca2+
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Time (min)
siNC
siTRPC3
Relative [Ca2+]mito Uptake
DMSO
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Mitochondrial Complex II Activity (mOD/min)
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Relative Cell Proliferation (%)
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PC2kd
Fluorescence of MitoSOX (Fold increase vs control)
siNC
siTRPC3
IMCD3
siNC
siTRPP2
Fluorescence of MitoSOX (Fold increase vs control)
siNC
siTRPC3

◂**Fig. 4** The effects of TRPC3 in regulating mitochondrial calcium uptake and ROS productions. **a** CiPETC and IMCD3 cells were treated with siNC or TRPP2 knockdown siRNA, then the mitochondrial fraction was isolated and TRPC3 expression levels were detected by western blotting, using VDAC as a loading control. $N=3$, $**P<0.01$, $***P<0.0001$. **b**, **c** Mitochondrial calcium uptake was measured in cells treated with siNC/siTRPC3 or DMSO/Pyr3 in the presence of 300 or 50 µmol/l calcium, using ATP as agonist. $N=3$, $**P<0.01$, $***P<0.0001$. **d**, **e** Mitochondrial calcium uptake was measured in cells treated with pcDNA/pcDNA–TRPC3 or DMSO/Pyr3 in the presence of 300 or 50 µmol/l calcium, using ATP as agonist. $N=3$, $*P<0.05$, $**P<0.01$, $***P<0.0001$. **f**, **g** Mitochondrial enzymic activities of Complex I and Complex II were measured in both ciPETC and IMCD3 cells, treated with DMSO/Pyr3 or pcDNA/TRPC3. $N=6$, $*P<0.05$. **h** Mitochondrial ROS productions were detected by mitoSOX in both ciPETC and IMCD3 cells. $**P<0.01$, $***P<0.0001$. **i** Cell proliferation assay was conducted in both ciPETC and IMCD3 cells, treated with DMSO/10 µmol/l mitoTEMPO. $N=3$, $*P<0.05$, $**P<0.01$, $***P<0.0001$

respiratory functions by modulating mitochondrial calcium uptake. Using renal tubular cells, firstly we showed that the mitochondrial located TRPC3 was also upregulated upon TRPP2 knockdown (Fig. 4a). Meanwhile, we investigated that whether mitochondrial TRPC3 could directly regulate the mitochondrial calcium homeostasis in renal tubular cells. Figure 4b, c shows that knockdown of TRPC3 by specific siRNA or inhibition of TRPC3 by Pyr3 could significantly decrease the mitochondrial calcium uptake in permeabilized ciTEPC cells in both 300 and 50 µmol/l calcium solutions. In Fig. 4d, e, overexpression of TRPC3 by pcDNA–TRPC3 plasmid significantly upregulated mitochondrial calcium uptake in permeabilized ciPETC cells in both 300 and 50 µmol/l calcium solutions. In addition, to further verify the role of TRPC3 in modulating mitochondrial functions, we detected the key enzymic activities of tubular cells upon TRPC3 inhibition or overexpression. Figure 4f, g shows that inhibition of TRPC3 decreased the enzymic activities of mitochondrial Complex I while TRPC3 overexpression increased the enzymic activities of mitochondrial Complex I. TRPC3 did not affect the activities of mitochondrial Complex II significantly. Furthermore, we showed that TRPC3 regulated the mitochondrial ROS productions while inhibition of mitoROS by addition of mitoTEMPO could partially attenuate the enhanced cell proliferations-induced by PC2 knockdown (Fig. 4h, i). These results indicated that upregulated TRPC3 upon TRPP2 knockdown promoted the mitochondrial metabolism by promoting mitochondrial calcium uptake.

Discussion

The molecular mechanisms of excessive cell proliferation of ADPKD cells have been studied recently. In this study, we built the model of renal tubular cells stably transfected with TRPP2 knockdown plasmid. We found that TRPP2 knockdown promoted cell proliferation, mitochondrial dysfunctions and ERK activation, which are correlated with dysregulated calcium homeostasis. Meanwhile, TRPC3 upregulation was observed upon TRPP2 knockdown, which regulating SOCE, ROCE and cell proliferation. Furthermore, mitochondrial TRPC3 upregulation was observed and modulating ROS productions through mitochondrial calcium uptakes. All together, we showed that TRPC3 upregulation promoted mitochondrial dysfunctions upon TRPP2 knockdown, which is partially responsible for the excessive cell proliferations.

Previous studies indicated that mitochondrial metabolism is closely correlated with the pathogenesis of ADPKD [22, 23]. PC1 and PC2 have been demonstrated to be required to modulate intracellular calcium signals. Since mitochondria and calcium are two mutual interplayed factors [9]. On one hand, dysregulated calcium homeostasis could affect the mitochondrial calcium concentrations and mitochondrial metabolism. A recent study suggests that PC1 affected mitochondrial functions by promoting the mitochondrial calcium uptake [24]. Meanwhile, NADH dehydrogenase (Complex I) was shown to interact with Ft, a cell adhesion protein, releasing a cleavage product imported into mitochondria and regulating Complex I activity and planar cell polarity [25, 26]. Moreover, microRNA-17 was shown to aggravate polycystic kidney disease progression through modulation of mitochondrial metabolism [27]. Our study demonstrated that TRPC3 upregulation in the mitochondrial directly regulated the mitochondrial ROS productions and thus influencing the cell proliferation. However, the mechanisms of TRPC3 upregulation have not been discussed further in our study. On the other hand, deleting liver kinase 1 (*Lkb1*), a regulator of glucose and lipid metabolism, resulted in tubulointerstitial kidney damage and altered mitochondrial morphology in mice [28]. The more direct links between mitochondrial dysfunction and cystogenesis are presented in glutaric academia type II (OMIM 231680), caused by mutations that disrupt electron transfer (ETFA, ETFB, ETFDH) [29, 30].

Studies also showed that TRPP2 could bind to PC1 and TRPC1 to induce calcium influx, while a pathogenic C-terminus-truncated PC-2 mutant enhanced ROCE via association with TRPC3 [19]. The authors also indicated that TRPC3-mediated calcium influx has been shown to play essential roles in ERK activation in multiple cell types [31, 32]. Therefore, formation of heteromeric channels comprised of mutant TRPP2 and TRPC3 and activation of ERK through calcium regulation are sufficient to enhance cell proliferation and cystogenesis. In our study, we showed that TRPC3 upregulation upon TRPP2 knockdown regulated mitochondrial functions by modulating mitochondrial calcium uptake. On the other hand, disrupted calcium homeostasis and intracellular calcium signaling have also been shown to be activated in ADPKD [6]. For example, calcium-sensing receptor (CaSR) has been shown to be activated and corrected the impaired mitochondrial functions in renal PC-1 knockdown cells. It showed that activation of CaSR rescued the impaired ATP synthesis while inhibition of IP_3R prevented the rescue of bioenergetics deficits [15]. Meanwhile, L-type calcium channel CaV1.2 was activated upon PC1 or PC2 knockdown, which contributed to the hyperproliferation and polyploidy of renal tubular cells [33].

TRPC3 has been shown to modulate the cation influx in the plasma membrane and functioned in a variety of diseases, which has been considered as an important therapeutic target recently [11]. TRPC3 function has been demonstrated to be associated with pathologies of the cardiovascular system and brain. TRPC3 channels were shown to play a role in angiotensin II- and noradrenaline-induced nuclear factor of activated T cells (NFAT) activation involved in maladaptive cardiac remodeling, arrhythmias and heart failure [34–36]. TRPC3 mediated calcium entry was directed correlated with NFAT signaling in myocytes [37]. Additionally, TRPC3 functioned as a crucial player in the proliferation and differentiation of myocardial fibroblasts and its activity was found to promote fibrosis and arrhythmias, specifically atrial fibrillation [38, 39]. Meanwhile, TRPC3 channels are also expressed in other cardiovascular cells, and TRPC3-modulated calcium homeostasis functioned in the pathogenesis of hypertension [13] and atherosclerosis [40]. In the nervous system, neuronal TRPC3 conductance was shown to lead to Ca^{2+}-dependent degradation of Purkinje cells and thus influencing motor coordination [41, 42]. In addition, TRPC3 expression in the non-excitable cells regulated the function of T cells in the immune system [43].

In the cells, TRPC3 combined with NCX1 to form a functional complex, thus regulating calcium homeostasis [34]. Additionally, TRPC3 physically and functionally interacted with Nox2 thus regulating oxidative stress productions [44]. Meanwhile, recent reports indicated that TRPC3 be located in the mitochondrial and regulated mitochondrial calcium uptakes [12, 13]. TRPC3 has been shown to play a role in caspase-dependent apoptosis and mitochondria membrane potential imbalance in glioblastoma cells [45]. TRPC3 was also correlated with α-synuclein accumulation in the brains of monkeys and mice [46]. Recent study indicated that TRPC3 enhanced the pathological cardiac hypertrophy through NCX-1-mediated mitochondrial calcium overload [47]. In our study, we showed that TRPC3 upregulation promoted calcium influx, ERK phosphorylation and cell proliferation through NCX1 combinations, while mitochondrial TRPC3 upregulation upon TRPP2 knockdown promoted mitochondrial calcium overload and ROS productions.

In conclusion, our study demonstrated that, in the cell model of TRPP2 knockdown, TRPC3 upregulation further aggravates the mitochondrial dysfunctions and cell proliferations by modulating mitochondrial calcium overload. Targeting TRPC3 might become a new promising strategy in ADPKD treatment.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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