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The impact of silencing TRPC6 on the proliferation, apoptosis, and extracellular matrix secretion of epithelial cells lining the cyst wall in ADPKD

Liyin Chai, Binying Zhang, Zhengyang Liu, Jun Zeng, Xingqing Chen, Li Gong, Fang Wang*

Department of Nephrology; Chongqing Emergency Medical Center; Chongqing University Central Hospital; Chongqing, 400014, China.

Corresponding author: Fang Wang
E-mail: 16314645@qq.com

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ABSTRACT. Autosomal Dominant Polycystic Kidney Disease (ADPKD), a prevalent hereditary disorder, involves the Transient Receptor Potential Canonical 6 (TRPC6) protein, a key factor in disease progression. This study aimed to determine the effects of TRPC6 gene suppression on the proliferation and matrix production of ADPKD cyst-lining epithelial cells. The polycystic kidney wall lining inner epithelial cell line WT9-12 (WT9-12) cell line was treated with TRPC6-siRNA, and cell behavior was analyzed using the CCK-8 assay and flow cytometry. Expression levels of TRPC6 and matrix proteins (Type I Collagen (Col I), Type IV Collagen (Col IV), Procollagen III, and N-terminal Propeptide (PIIINP) Collagen) were measured by Real-Time Fluorescence Quantitative PCR and Enzyme-Linked Immunosorbent Assay. Signaling proteins related to cell proliferation and apoptosis were assessed by Western blot. TRPC6 silencing significantly decreased WT9-12 cell proliferation and matrix secretion, particularly Col I and Col IV. Apoptosis-related proteins, cleaved caspase 3 and 9, increased post-silencing, with Bax up-regulated and Bcl-2 and

p-ERK down-regulated (P<0.05). The activity of p38, p-p38, JNK, p-JNK, and ERK showed no significant change (P>0.05). These results suggest that TRPC6 gene silencing can inhibit epithelial cell proliferation and matrix production in ADPKD, and thus present a promising therapeutic strategy.

Key words: ADPKD; TRPC6; Proliferation; Apoptosis; Stromal secretion of epithelial cells

INTRODUCTION

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is common hereditary renal condition marked by the formation of cysts filled with fluid within the kidneys, which progressively enlarge, leading to a decline in renal function and potentially advancing to end-stage renal disease (ESRD) (Roediger R, 2022; Colbert GB, 2020; Nowak KL, 2020). According to epidemiologic studies, the incidence of ADPKD is approximately 1/1000 to 1/4000, and the risk of developing the disease does not differ significantly between males and females (Lanktree MB et al., 2018; Gall EC, 2019; Ma M, 2021). There is a wide age range for onset of the disease, with the majority of patients experiencing symptoms in late adulthood (30 to 50 years of age), although some patients may experience symptoms in childhood or adolescence, while others may live their entire lives without experiencing significant symptoms (Bergmann C, 2018). These symptoms may include low back and abdominal pain, hematuria, infections, renal insufficiency, and may be associated with complications such as liver cysts, intracranial aneurysms, and urinary stones (Gall EC, 2019). In terms of pathophysiology, the main features of ADPKD include loss of polarity of renal tubular epithelial cells, progressive increase in the size of cysts, fluid-filled cysts, and a gradual deterioration of kidney function (Zhou JX, 2023). Some studies suggested that the pathogenesis of ADPKD may involve the abnormal expression of polycystic proteins (PKD1 and PKD2), but these proteins normally regulate cell proliferation, differentiation, and mitochondrial function affecting oxidative stress, among other processes (Lakhia R et al., 2023). Overall, the pathogenesis of ADPKD is multifaceted and requires further in-depth study.

Although the pathogenesis of ADPKD is complex, processes such as inhibition of cell proliferation, imbalance of apoptosis, and matrix hypersecretion are thought to be key components of cyst formation and expansion (Hian CK, 2016). Research indicates that alterations in the TRPC6 gene could influence the development and enlargement of cysts through the modulation of these mechanisms (Englisch CN, 2022; Staruschenko A, 2023). Transient Receptor Potential Canonical 6 (TRPC6) is an ion channel protein that regulates intracellular calcium ion concentration and is closely associated with cell proliferation, apoptosis, and extracellular matrix (ECM) synthesis. Studies by Yin et al. and Fu et al. showed that aberrant expression of TRPC6 affects cyst development, and its functional inhibition or silencing may affect the balance of proliferation and apoptosis of epithelial cells within the cyst wall, thereby affecting cyst growth (Fu Y et al., 2013; Yin H, 2022). In addition, TRPC6 was involved in matrix secretion, regulates the synthesis of collagen and other components, and affects the structure and stability of the cyst wall. Gene silencing of TRPC6 slowed the progression of inflammation and fibrosis (Kim EY, 2021; Ramirez GA et al., 2018; Dryer SE, 2022). Therefore, enhanced comprehension of TRPC6's function in ADPKD could help to develop new therapies, such as gene therapy, to inhibit cyst growth and slow disease progression by silencing TRPC6.

In view of the above conclusions, this study used gene silencing technology to knock out TRPC in cyst wall-lined epithelial cells in a polycystic kidney model, aiming to explore the effects of TRPC6 deletion on cell proliferation, apoptosis, and secretion of matrix proteins such as collagen. Through the experimental design of this study, it is anticipated that the possible function of TRPC6 in the formation and dynamic regulation of ADPKD will be revealed, and a new scientific basis will be provided for early intervention and treatment strategies of the disease. At the same time, it will also help us to understand the pathophysiological process more deeply, and provide new research directions for disease prevention and control.

MATERIAL AND METHODS

Cell Source and Culture

The polycystic kidney mural lining inner epithelial cell line (WT9-12) was purchased from the American Model Strain Collection cell bank, cell number CRL-2833™. Human kidney WT9-12 cells were placed in DMEM/F12 medium supplemented with 10% FBS at a temperature of 37°C under an atmosphere of 5% CO_2. These cells exhibited a polymorphic adherent growth pattern.

Experimental grouping

The cultured cells were divided into different groups. The cell transfection section was specially treated and divided into three groups: control group, scramble group and TRPC6 siRNA group. Other experiments (e.g., cell counting Kit-8 (CCK-8) assay, cell cycle and apoptosis detection, western blotting (WB), real-time fluorescence quantitative PCR (RT-PCR), and enzyme-linked immunosorbent assay (ELISA) were simplified into control group and TRPC6 siRNA group.

Cell transfection

Lipofection was used as the transfection method in this experiment. WT9-12 cells are plated at an appropriate density in a 6-well plate, and the cells are synchronized with serum-free DMEM/F12 when they reach 50%~60% confluence. The concentration of siRNA per well was 100 pmol, added to each well containing 200 μL of DMEM/F12 medium and left to stand for 5min. Additionally, 5 μL of X-treme GENE siRNA transfection reagent (cationic lipid method) was mixed with 200 μL of DMEM/F12, thoroughly combined with the siRNA. The mixture was incubated at 15-25°C for 12-20 min, then added to 6-well plates (400 μL/well). After a 5-hour transfection, it was replaced with DMEM/F12 medium containing 2% FBS for further culture.

Cell counting Kit-assay

The CCK-8 cell counting method (CCK-8 kit, Beyotime Biotechnology Co., Ltd.) was used to estimate cultured proliferation (Zhang T, 2020) First, seed 3×10^4 cells/mL onto a 96-well plate. The cells were maintained at 37°C for incubation, and cells typically adhered to the plate within 4 h. The cells were then cultured for 24-72 h. During this period, a 10% CCK-8 medium was prepared, and the OD values were measured every 15min at 37°C after each medium change using a Bio-Tek ELX800 microplate reader (Beijing Longyue Biotechnology Development Co., Ltd.). The absorbance at 450 nm was finally determined.

Cell cycle and apoptosis detection

A certain number of WT9-12 cells (2×10^5 per well) were inoculated into a 6-well plate. Upon reaching a confluence of approximately 50% to 60%, the cells were synchronized in a serum-deprived culture medium for 24 h. Subsequently, TRPC6 siRNA was added according to the cell transfection method, with scramble siRNA as the control group. Post-treatment for durations of 24 and 48 hours, the cells were treated with 0.25% trypsin devoid of EDTA, rinsed twice using PBS, and subsequently centrifuged at 1000 rpm for 5 minutes in a centrifuge with a radius of 6 cm. followed by the addition of 70% pre-chilled ethanol, and the cells were then fixed at 4°C for 12 h. After centrifuging again and discarding the supernatant, cells were washed, 400 μL of PBS was added to each tube to suspend the cells, followed by the addition of propidium iodide (50 mg/L) 50 μL, staining in the dark for 15 min, detection using a flow cytometer, and data analysis by MultiCycle software.

Cells were synchronized and treated with siRNA once they reached 30%-40% confluence. Post-treatment, cells were digested with 0.25% trypsin (EDTA-free), collected, washed twice in PBS, and centrifuged at 1000 rpm for 5 min (radius 6 cm). The supernatant was removed, and cells were resuspended in 400 μL PBS per tube. Annexin V-FITC (5 μL) was added, followed by a 15 min incubation. Propidium iodide (10 μL) was then added for another 5 min incubation. Apoptosis was analyzed using BD FACS Calibur flow cytometry, and data were processed with MultiCycle software.

Western blotting

Total cellular protein was extracted following the BCA Kit protocol (Beijing Solarbio Science & Technology Co., Ltd.). SDS-PAGE was used to separate the proteins, which were then transferred to a PVDF membrane via wet spinning. The membrane was blocked with 5% skimmed milk powder in 1× PBST (Beijing Solarbio Science & Technology Co., Ltd.) for 120 min at room temperature, followed by overnight incubation at 4°C. The primary antibody was diluted in 1× PBST (Table 1) and applied to the membrane for a 90 min incubation at room temperature. Afterward, the membrane was incubated with HRP-conjugated goat anti-mouse IgG (1:2000, SE131, CN Solarbio) or goat anti-rabbit IgG (1:2000, ab6721, UK Abcam) for 60 min. Protein bands were visualized using ECL, and the films were scanned or photographed. The gray values of target bands and internal reference bands were analyzed by Mini-PROTEAN Tetra WB System (American Bio-Rad). Relative expression of the target protein was calculated by normalizing its gray value to that of the housekeeping protein (GAPDH).

Real-time fluorescence quantitative PCR

WT9-12 cells were cultured to yield 1×10^7 to 5×10^7 cells, which were then transferred to 1.5 mL tubes and lysed with 1 mL Trizol (American Sigma). After mixing and a 5 min room temperature incubation, total RNA was extracted using Trizol as described in [19]. For cDNA synthesis, 1 μL of RNA was reverse transcribed using a mix containing 0.4 μL each of forward and reverse primers (10 μM), 10 μL of 2× PerfecStar qPCR Super Mix, and nuclease water to a final volume of 20 μL. RT-PCR was performed on the 7700 PCR system (Applied Biosystems, Carlsbad, CA, U.S.A.) with a cycling program of 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 10 s. GAPDH and TRPC6 genes were amplified using primers listed in Table 2.

Table 1. Primary antibodies for western blot.

Primary antibody	Lot	Source	Dilution	company
Caspase 3	25128-1-AP	Mouse	1: 1000	Proteintech, USA
Caspase-9	#9505	Rabbit	1: 1000	Cell signaling, USA
Bax	ab289364	Rabbit	1: 1000	Abcam, UK
Bcl-2	ab182858	Rabbit	1: 2000	Abcam, UK
P-p38	28796-1-AP	Rabbit	1: 1000	Proteintech, USA
p38	14064-1-AP	Rabbit	1: 1000	Proteintech, USA
JNK	66210-1-Ig	Rabbit	1: 1000	Proteintech, USA
P-JNK	80024-1-RR	Rabbit	1: 1000	Proteintech, USA
TRPC6	18236-1-AP	Rabbit	1: 1000	Proteintech, USA
P-ERK1/2	28733-1-AP	Rabbit	1: 1000	Proteintech, USA
ERK1/2	11257-1-AP	Rabbit	1: 1000	Proteintech, USA
GAPDH	ab8245	Rabbit	1: 1000	Abcam, UK

Table 2. Primer sequences.

Gene	Primer sequences
TRPC6	Forward primer: CTTGTGGTCCTTGCTGTTGC
	Reverse primer: CGCAATGAATGATGCTGCGA
GAPDH	Forward primer: AATGACCCCTTCATTGAC
	Reverse primer: TCCACGACGTACTCAGCGC

ELISA

The ELISA method was to detect the levels of human Col I, Col IV, and Procollagen III, N-terminal Peptide (PIIINP) collagen in the cell culture supernatant after 48 h of TRPC6 siRNA treatment. The corresponding assay kits for each protein were used as previously described. The specific experimental procedures strictly followed the manufacturer's instructions provided with the ELISA kits (Col I kit, CSB-E08082h; Col IV kit, CSB-E17116h; PIIINP kit, CSB-E11226h), all purchased from Wuhan Huamei Bioengineering Co., Ltd.

Statistical analysis

All data were tabulated using Excel. Analysis and graphical representation were conducted with GraphPad Prism version 8.3.0. Quantitative data were presented as the Mean ± Standard Deviation (SD). Statistical differences in means between pairs of groups were assessed using the Student's t-test, while multiple group comparisons were evaluated using one-way ANOVA. $P < 0.05$ was set as the threshold for statistical significance. Each experiment was conducted in triplicate to ensure reliability.

RESULTS

TRPC6 siRNA transfection efficiency

After 48 h, RNA was collected and extracted from the control group and WT9-12 cells transfected with scramble and TRPC6 siRNA. RT-PCR was conducted to evaluate the expression of TRPC6 post-transfection. findings indicated that there was no statistically significant variation in TRPC6 expression between the scrambled and control groups (P>0.05). In contrast, a significant reduction in TRPC6 expression was observed in the group treated with TRPC6 siRN (P<0.05) (Figure 1).

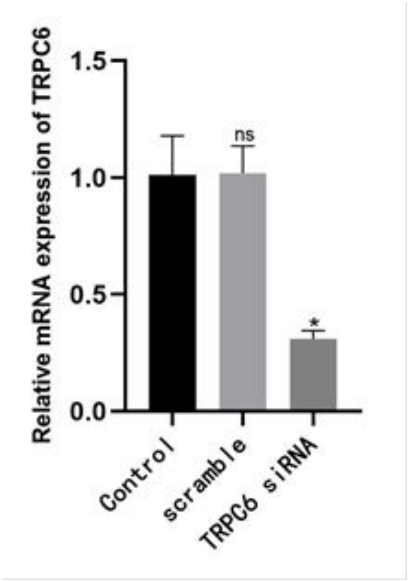

Figure 1. Transfection of WT9-12 cells with TRPC6 siRNA resulted in a change in the expression level of TRPC6. Values are means±SD (ns, not significant. *P<0.05, n=3).

Effect of interfering TRPC6 expression on the proliferation of WT9-12 cells

In WT9-12 cells, the viability of cells in the TRPC6 siRNA group was found to be reduced when compared to the control group at 12, 24, and 48 h after transfection, with a notably pronounced decrease at the 48-hour time point (P<0.05) (Figure 2).

Effect of interfering TRPC6 expression on cell cycle and apoptosis of WT9-12 cells

After 48 h of transfection with specific siRNA targeting TRPC6 in WT9-12 cells, the cell cycle progression and the rate of apoptosis were evaluated through flow cytometric analysis. The findings indicated that the rate of apoptosis in the group treated with TRPC6 siRNA was markedly elevated in comparison to the control group, accompanied by an enhanced proportion of cells in the G2/M phase (P<0.05) (Figure 3).

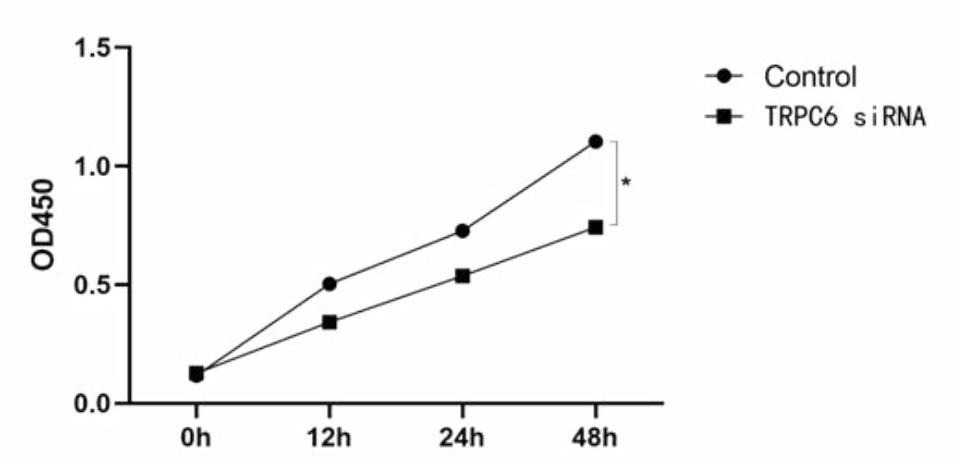

Figure 2. The effects of specific siRNA interference on TRPC6 expression on the proliferation of WT9-12 cells. Values are Means ± SD (*P<0.05, n=3).

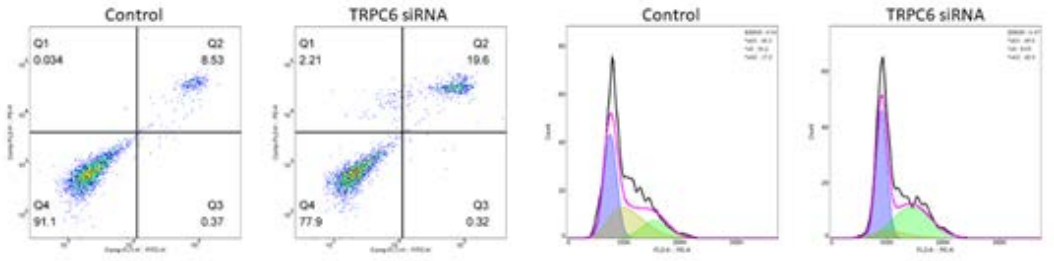

Figure 3. The effects of specific siRNA interference on TRPC6 expression on the cell cycle and apoptosis of WT9-12 cells.

Effect of interfering TRPC6 expression on protein of WT9-12 cells

Western blot analysis indicated a pronounced reduction in TRPC6 expression in the siRNA-treated group relative to the control group. There was also a notable decrease in Bcl-2 and p-ERK levels (P<0.05), while the levels of cleaved caspase-3 and cleaved caspase-9 were significantly increased (P<0.05). The expression levels of other proteins such as p38, p-p38, JNK, p-JNK, and ERK showed no significant changes (P>0.05) (Figure 4).

Effect of interfering TRPC6 expression on the secretion of WT9-12 cell

After 48 h of specific siRNA silencing of TRPC6, the secretion of Col I and Col IV was significantly reduced (P<0.05), and there was no significant effect on the secretion of PIIINP (P>0.05) (Figure 5).

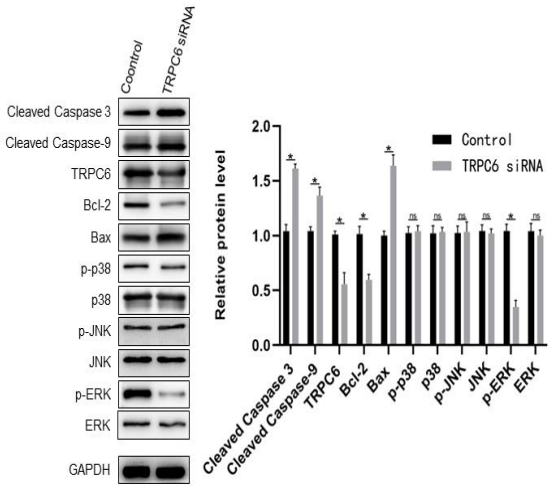

Figure 4. The effects of specific siRNA interference on TRPC6 expression on extracellular matrix secretion in WT9-12 cells. Values are Means ± SD (ns, not significant. **P*<0.05, n=3).

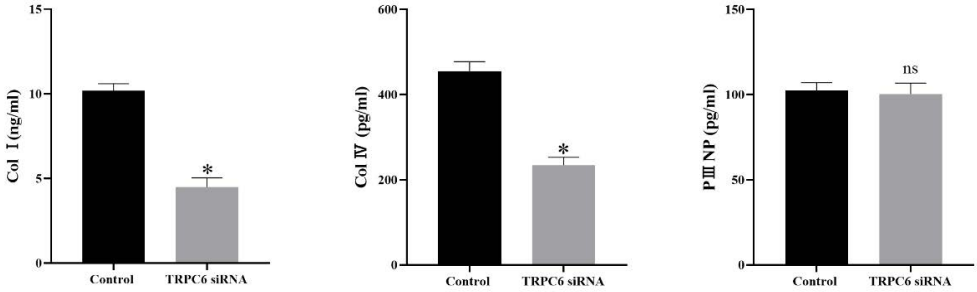

Figure 5. The effects of specific siRNA-mediated TRPC6 gene silencing on the secretion of Col I, Col IV, and PIIINP in the conditioned medium of WT9-12 cells after 48 h. Values are Means ± SD (ns, not significant. **P*<0.05, n=3).

DISCUSSION

ADPKD is characterized by multiple progressive cysts in both kidneys, ultimately leading to kidney tissue damage and renal failure, which in turn led to abnormal proliferation of WT9-12 and abnormal secretion of cyst fluid (Chandra AN, 2021). Previous investigations have identified that mutations in the TRPC6 gene correlate with the occurrence of hereditary ADPKD, which may lead to an imbalance of calcium ion homeostasis in renal tubular cells, thereby promoting the development of polycystic kidney disease (Staruschenko A, 2023). Research on the relationship between TRPC6 and polycystic kidney disease is ongoing. Some studies suggested that an elevated expression of TRPC6 could be linked to the onset and advancement of renal disorders, and that inhibition of TRPC6 expression or function might have helped slow the progression of kidney disease. In

addition, several studies explored the potential role of TRPC6 in the treatment of kidney disease, for example, TRPC6 protected against renal fibrosis in type 2 diabetic mice by inhibiting the CN-NFAT2 signaling pathway (Sun R et al., 2024). The role of the TRPC6 protein as a significant contributor to the pathogenesis of ADPKD has been established. Therefore, inhibiting the proliferation and apoptosis of cyst epithelial cells and reducing cyst fluid secretion have become the main strategies for treating ADPKD (Englisch CN, 2022). This study's findings indicate that inhibition of TRPC6 expression by RNAi technology suppressed the proliferation and stromal secretion of WT9-12 cells in ADPKD, but promoted the process of apoptosis, thus slowing down the onset and progression of ADPKD.

Given the established link between TRPC6 and ADPKD progression, our study aimed to elucidate the molecular mechanisms underlying TRPC6's role in modulating cellular behaviors pivotal to the disease's pathogenesis. Salemkour et al. showed that TRPC6 is highly expressed in a mouse model of diabetic nephropathy and when overexpressed, TRPC6 promoted podocyte injury. On the contrary, when TRPC6 expression was decreased, the injury is alleviated (Salemkour Y et al., 2023). Additionally, TRPC6 is involved in the regulation of intracellular calcium levels in podocytes, and the interaction between TRPC6 and NADPH oxidase 4 may contribute to renal injury in diabetic nephropathy (Ilatovskaya DV et al., 2018). Consequently, this research specifically targeted TRPC6 within the WT9-12 cell line and monitored its expression levels. The RT-PCR data demonstrated a significant reduction in TRPC6 expression, consistent with the anticipated outcomes. The cellular activity was then examined by CCK-8 assay, revealing that the down-regulation of TRPC6 expression significant inhibited the proliferation of WT9-12 cells. Further flow cytometry analysis indicated that down-regulation of TRPC6 expression may inhibit proliferation by affecting the cell cycle, retention of cells in the G0/G1 phase and preventing them from entering the S phase. Apoptosis studies indicated that TRPC6 affects proliferation and induces apoptosis by regulating the cell cycle. WB experiments verified that after siRNA transfection, the expression levels of apoptosis-related proteins caspase-3 and caspase-9 increased, Bax expression was up-regulated, and the expression of Bcl-2 and p-ERK decreased. Expression of p38, p-p38, JNK, p-JNK, and ERK showed no significant changes. These findings further confirmed that down-regulation of TRPC6 expression by modulated signaling pathway activity affecting the proliferation, apoptosis and stromal secretion process of WT9-12 cells. However, TRPC6 may primarily exert its effects through alternative signaling pathways rather than the MAPK signaling pathway.

Building on our observations of TRPC6's impact on cell proliferation and apoptosis, we further investigated its influence on the renal fibrotic process, focusing on the regulation of key extracellular matrix components. Col I is a key protein in the process of renal fibrosis. In ADPKD, renal fibrosis is an important feature of disease progression, in which increased levels of Col I and Col IV are associated with the destruction of renal tissue structure and fibrosis (Fragiadaki M, 2020). Moreover, studies indicated that the WT9-12 gene in ADPKD patients promotes increased synthesis of collagen types I and IV, as well as other unidentified collagens, which contribute to the abnormal extracellular matrix and the development of cysts (Zhao P et al., 2021). TRPC6 may play a significant role in cyst growth and fluid accumulation, particularly in the formation of large cysts (Xie J et al., 2020). Sun et al., discovered that miR-182 potentially modulates the fibrotic process in the epithelial lining of polycystic kidney disease cysts by affecting Col I and Col IV through the TGF-β1/Smad3 signaling pathway (Sun L, 2020). Our research showed that upon siRNA-mediated suppression of TRPC6 expression, there was a noticeable decrease in the synthesis of Col I and Col IV within the cellular matrix, while the impact on PIIINP was minimal. Consequently, it is inferred that TRPC6 likely exerts a dominant influence on matrix secretion in the course of ADPKD.

There were some limitations in this study, such as the restricted experiments in animal models, the selection and efficiency of gene silencing techniques. The exact mechanism of the role of TRPC6 in polycystic kidney disease was not fully clarified, necessitating further studies. However, this study has provided new targets and strategies that may lead to personalized treatment approaches for polycystic kidney disease in the future. Subsequent experiments aim to examine the impact of TRPC6 on cell proliferation, apoptosis, and extracellular matrix secretion, encompassing studies on the silencing of TRPC6 in the context of ADPKD. It is hoped that the gene therapy approach will intervene in the development of polycystic kidney disease and provide clues for clinical treatment.

CONCLUSION

In summary, this study was of great value in exploring the effects of TRPC6 on the proliferation, apoptosis and matrix secretion of ADPKD endothelial cells through gene silencing. The study demonstrated that decreased TRPC6 expression inhibited the proliferation and of extracellular matrix proteins secretion in WT9-12, but promoted apoptosis, which in turn slowed the progression of polycystic kidney disease and vesicle formation. Therefore, targeting TRPC6 could potentially exert a suppressive influence on the progression of ADPKD, providing a new reference point for targeted therapy and early diagnosis of ADPKD patients.

DECLARATIONS

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIAL

The data that support the findings of this study are available on request from the corresponding author, upon reasonable request.

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DISCLOSURE

The authors declare no conflicts of interest.

AUTHORS' CONTRIBUTIONS

Conceptualization: L. Chai, F. Wan and B. Zhang; Methodology: L. Chai and F. Wang; Validation: Z. Liu, X. Chen and G. Li, J. Zhan; Formal analysis: Yang, X. Chen and G. Li, J. Zhan; Investigation: L. Chai, F. Wang, Z. Liu, X. Chen and G. Li; Data Curation: L. Chai, F. Wang, Z. Liu and B. Zhang; Visualization: J. Zeng, X. Chen, G. Li and Z. Liu; Writing - Original Draft: L. Chai, J. Zeng, X. Chen and G. Li; Writing - Review & Editing: L. Chai and B. Zhang; Supervision: L. Chai, F. Wang and B. Zhang; Project administration: L. Chai, F. Wang and B. Zhang; Funding acquisition: B. Zhang. All authors have read and agreed to the published version of the manuscript

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