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Klotho protects against LPS-induced inflammation injury by inhibiting Wnt and NF-κB pathways in HK-2 cells

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Aim: This study aimed to investigate the effect and mechanism of Klotho in lipopolysaccharide (LPS)-induced inflammation injury in HK-2 cells. *Methods:* We established LPS-induced inflammation injury model in HK-2 cells. The LPS-induced HK-2 cells were transfected with pc-Klotho, pcDNA3.1, siKlotho or siNC. Cell viability, apoptosis and reactive oxygen species (ROS) level were detected by MTT assay, Annexin V-FITC/PI Apoptosis Detection kit and 2,7-dichlorofluorescein diacetate, respectively. The levels of inflammatory factors and the expressions of proteins related to Wnt and nuclear factor-κB (NF-κB) signaling pathway were detected by RT-qPCR and western blotting, respectively. *Results:* Compared with cells transfected with pcDNA 3.1, cell viability was remarkably increased and cell apoptosis rate was decreased in LPS-induced cells with pc-Klotho ($p < 0.05$). Conversely, LPS-induced cells with siKlotho showed lower cell viability and higher cell apoptosis rate than cells with siNC ($p < 0.05$). The levels of ROS, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) was significantly reduced in LPS-induced cells with pc-Klotho compared with cells with pcDNA3.1 ($p < 0.05$). Consistently, LPS-induced cells with siKlotho showed increased levels of ROS, TNF-α and IL-6 compared with cells with siNC ($p < 0.05$). Wnt signaling pathway related protein Wnt3a and NF-κB signaling pathway related to proteins p-IκBα were significantly down-regulated in LPS-induced cells with pc-Klotho compared with cells with pcDNA3.1, while up-regulated in LPS-induced cells with pc-Klotho compared with cells with pcDNA3.1 ($p < 0.05$). *Conclusions:* Klotho may play an inhibiting role in LPS-induced inflammation injury by inhibiting NF-κB and Wnt signaling pathways in HK-2 cells.

1. Introduction

Nephritis is a common infection related immune disease, characterized by edema, hypertension and proteinuria (Anders and Mi 2011). There are various types of nephritis, including acute and chronic glomerulonephritis, pyelonephritis, purpura nephritis and lupus nephritis (Davin 2011; Lien and Lai 2011; Ortega et al. 2010). Nephritis induces inflammatory changes in the organizational structure, which may result in different degrees of renal hypofunction (Anders and Mi 2011). It is wellknown that nephritis is associated with many renal related diseases, such as renal failure (Hsu et al. 2008), diabetic nephropathy (Navarro-González and Mora-Fernández 2008), kidney calculi (Merchant, Cummins et al. 2008) and renal ischemia-reperfusion injury . Therefore, it is imperative to investigate the underlying pathogenesis and search for effective therapeutic targets for nephritis.

The *Klotho* gene was originally identified to be associated with aging and considered as an aging suppressor (Kuroo et al. 1997). It can encode a protein with single-pass transmembrane that contained a cytoplasmic domain (Tohyama et al. 2004). Klotho is found to be expressed widely, mainly in the kidney (Hu et al. 2010). In addition, because of the shedding of the ectodomain of the Klotho protein, Klotho can be secreted into the blood, urine or cerebrospinal fluid, and then act as an enzyme or hormone in an endocrine fashion (Chen et al. 2007; Hu et al. 2010). A previous study has shown that the mutation or deficiency of the *Klotho* gene may not only induce skin atrophy, muscle atrophy and shortened life span, but also be related to some diseases, such as pulmonary emphysema, osteoporosis, and arteriosclerosis (Kuroo et al. 1997). Conversely, upregulated Klotho may inhibit oxidative stress response and prolonged life span (Kurosu and Kuro-O 2005). Recently, some studies have suggested that Klotho is downregulated in the kidney of rat and human with chronic renal disease (Aizawa et al. 1998; Haruna et al. 2007). Klotho is closely associated with fibroblast growth factor 23 (FGF23) that is a key factor of calcium-phosphorus metabolism in chronic renal disease (Hu et al. 2013). Moreover, Klotho can inhibit the activation of angiotension II and oxidative stress response during the process of chronic renal disease, thereby protecting the kidney (Hu et al. 2013). Overexpressed Klotho also can ameliorate proteinuria and renal function in a chronic glomerulonephritis model (Haruna et al. 2007). However, the underlying mechanism and function of Klotho for inflammation injury of the kidney is still not fully understood.

In the present study, we established a cell inflammation injury model by the induction of lipopolysaccharide (LPS) in human renal cubularepithelial cell line HK-2, and then up- or down-regulated the Klotho level. Furthermore, cell growth and the levels of ROS and inflammatory factors, as well as the expressions of proteins related to Wnt and NF-κB signaling pathway were detected, aimed to investigate the effect and underlying mechanism of Klotho in LPS-induced inflammation injury in HK-2 cells.

2. Investigations and results

2.1. Effect of abnormal expression of Klotho on cell growth in LPS-induced HK-2 cells

Cell inflammation injury was induced by LPS. MTT assay showed that compared with untreated cells, 5 or 10 μg/mL LPS significantly inhibited cell viability of HK-2 cells ($p < 0.05$ or 0.01, Fig. 1A). Considering cell viability was reduced by 50% after treatment with 5 μg/mL LPS, we selected 5 μg/mL LPS in the following experiments. To confirm the effect of pc-Klotho or siKlotho plasmid transfection, we detected the expression of Klotho in LPS-induced cells. The

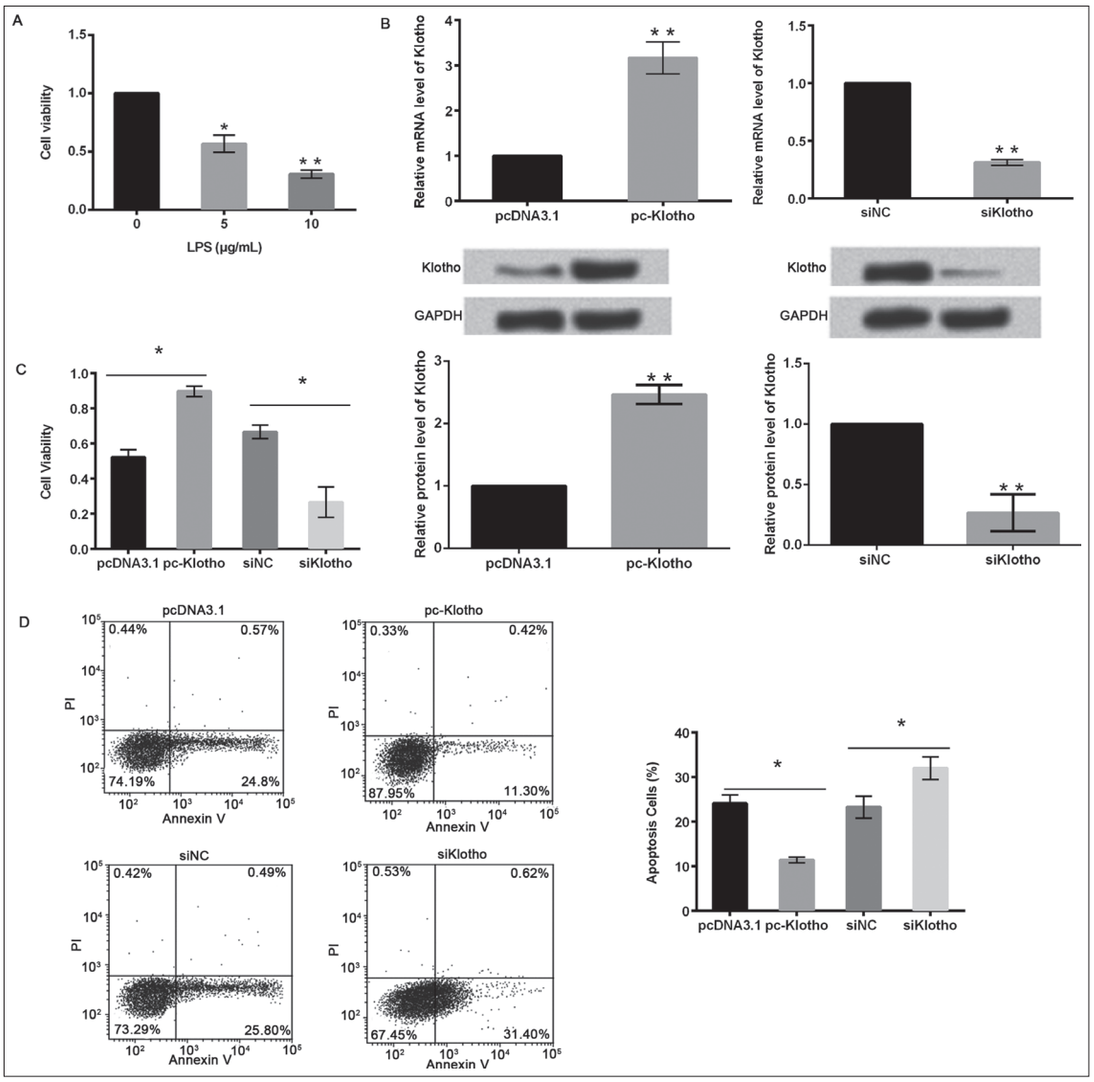

Fig. 1: Upregulated Klotho promoted cell viability and inhibited cell apoptosis in LPS-induced HK-2 cells.
A, Cell viablity in lipopolysaccharide (LPS)-induced HK-2 cells by MTT; B, Klotho level in LPS-induced cells with pc-Klotho, pcDNA3.1, siKlotho, or siNC by RT-qPCR and western blotting; C, Cell viablity in LPS-induced cells with pc-Klotho, pcDNA3.1, siKlotho, or siNC by MTT; D, Cell apoptosis rate in LPS-induced cells with pc-Klotho, pcDNA3.1, siKlotho, or siNC by Annexin V-FITC/PI Apoptosis Detection kit using flow cytometry. *$p < 0.05$ or **$p < 0.01$versus 0 μg/mL LPS (A), pcDNA3.1or siNC (B).

results revealed that the mRNA and protein expressions of Klotho were significantly increased in LPS-induced cells with pc-Klotho compared with LPS-induced cells with pcDNA3.1 ($p < 0.01$, Fig. 1B). Meanwhile, LPS-induced cells with siKlotho showed lower mRNA and protein levels of Klotho than LPS-induced cells with siNC ($p < 0.01$, Fig. 1B). Furthermore, MTT assay found that cell viability was remarkably increased when LPS-induced cells were treated with pc-Klotho compared with cells with pcDNA 3.1 ($p < 0.05$, Fig. 1C). Also, LPS-induced cells with siKlotho showed lower cell viability than LPS-induced cells with siNC ($p < 0.05$, Fig. 1C). Consistently, flow cytometry analysis revealed that cell apoptosis rate was significantly decreased in LPS-induced cells with pc-Klotho compared with pcDNA3.1 ($p < 0.05$, Fig. 1D), and markedly increased in LPS-induced cells with siKlotho compared with siNC ($p < 0.05$, Fig. 1D).

2.2. *Effect of abnormal expression of Klotho on the levels of ROS and inflammatory factors in LPS-induced HK-2 cells*

Compared with LPS-induced cells with pcDNA3.1, ROS levels were significantly reduced in LPS-induced cells with pc-Klotho ($p < 0.05$, Fig. 2A). Also, ROS levels were remarkably elevated in LPS-induced cells with siKlotho compared with LPS-induced cells with siNC ($p < 0.05$, Fig. 2A). In addition, the mRNA levels of inflammatory factors, including TNF-α and IL-6, were obviously inhibited in LPS-induced cells with pc-Klotho compared with LPS-induced cells with pc DNA3.1 ($p < 0.05$, Fig. 2B), as well as markedly higher in LPS-induced cells with siKlotho than those in LPS-induced cells with siNC ($p < 0.05$, Fig. 2B).

2.3. *Effect of abnormal expression of Klotho on Wnt and NF-κB signaling pathway in LPS-induced HK-2 cells*

Western blotting results showed that Wnt signaling pathway related proteins, including Wnt3a, β-Catenin and Wnt5a, were significantly downregulated in LPS-induced cells with pc-Klotho compared with LPS-induced cells with pcDNA3.1 ($p < 0.05$, Fig. 3), while the LPS-induced cell with siKlotho showed obviously elevated Wnt3a expression ($p < 0.05$) but not β-Catenin and Wnt5a compared with LPS-induced cells with siNC (Fig. 3). In addition, the expressions of p-IκBα and p-p65, NF-κB signaling pathway related to proteins, were lower in LPS-induced cells with pc-Klotho than those in LPS-induced cells with pcDNA3.1 ($p < 0.05$, Fig. 3). When LPS-induced cells were treated with siKlotho, we observed elevated expression of p-IκBα ($p < 0.05$) but not p-p65 in comparison to LPS-induced cells with siNC (Fig. 3).

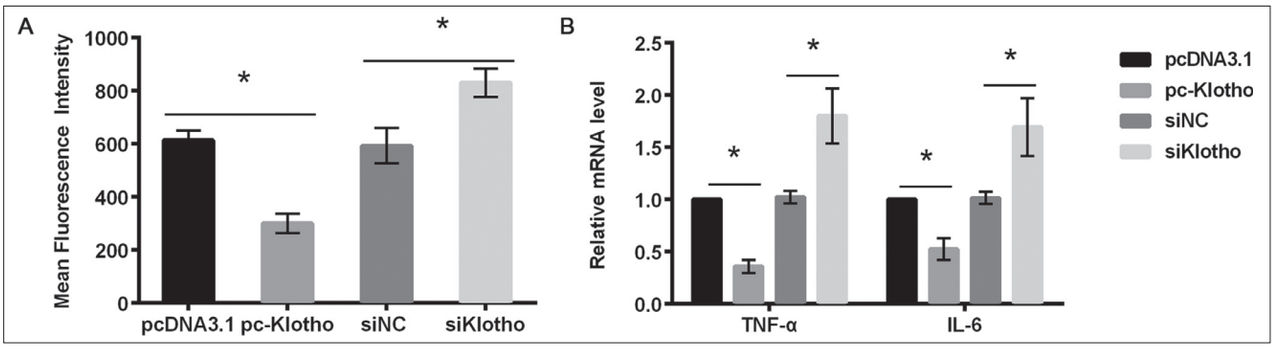

Fig. 2: Upregulated Klotho inhibited the levels of ROS and inflammatory factors in LPS-induced HK-2 cells.
A, The level of ROS in LPS-induced cells with pc-Klotho, pcDNA3.1, siKlotho, or siNC; B, The mRNA levels of TNF-α and IL-6 in LPS-induced cells with pc-Klotho, pcDNA3.1, siKlotho, or siNC by RT-qPCR.
*$p < 0.05$ LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6

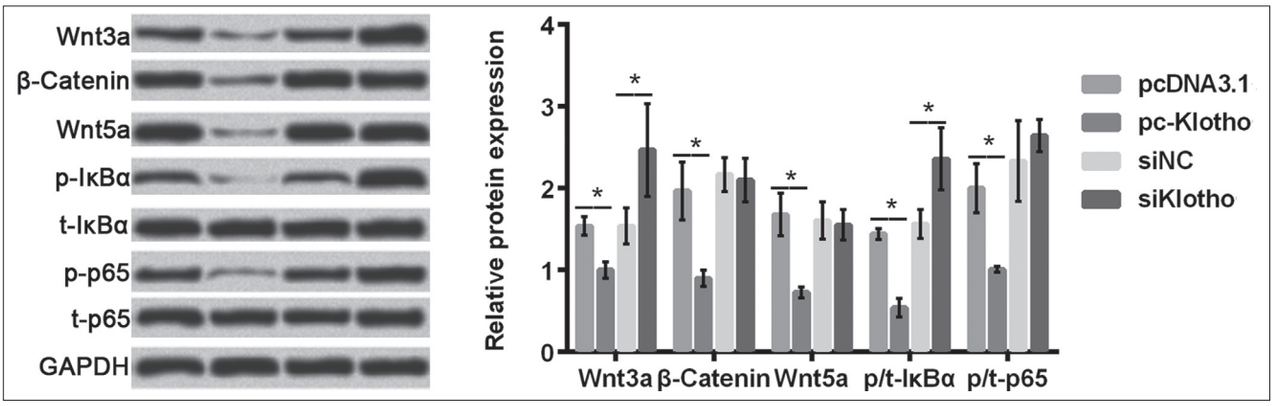

Fig. 3: Upregulated Klotho influenced Wnt and NF-κB signaling pathway in LPS-induced HK-2 cells.
The expressions of Wnt signaling pathway related proteins (Wnt3a, β-Catenin and Wnt5a) as well as NF-κB signaling pathway related to proteins (phospho-IκBα (p-IκBα), total IκBα (t-IκBα), p-p65, and t-p65) by western blotting.
*$p < 0.05$ LPS, lipopolysaccharide; NF-κB, nuclear factor-κB.

3. Discussion

In the current study, our results showed that in LPS-induced HK-2 cells, overexpression or knockdown of Klotho significantly increased or decreased cell growth, as well as inhibited or elevated cell apoptosis rate and the levels of ROS, TNF-α and IL-6. Furthermore, upregulated Klotho remarkably reduced the expressions of the Wnt signaling pathway related proteins Wnt3a, β-Catenin and Wnt5a, as well as NF-κB signaling pathway related proteins p-IκBα and p-p65; while downregulated Klotho only increased the expressions of Wnt3a and p-IκBα.

Several studies have shown that renal Klotho level was decreased in chronic kidney disease (Koh et al. 2001; Wang and Sun 2009), indicating a Klotho deficiency in chronic kidney disease. Klotho administration had been used as an effective treatment for kidney injury in animals, such as renal ischemia-reperfusion injury (Hu et al. 2010), renal fibrosis (Doi et al. 2011) and chronic kidney disease. Our study revealed that up-regulated Klotho significantly promoted cell growth and inhibited cell apoptosis in LPS-induced cells. Similarly, previous studies had demonstrated that up-regulated Klotho could inhibit cell proliferation and induce apoptosis in some cancers, including lung cancer (Bo et al. 2010), colon cancer (Li, et al. 2014), follicular thyroid cancer (Dai et al. 2015) and cervical cancer (Chang et al. 2012), which suggested a tumor inhibiting role of Klotho. In addition, this study found that overexpression of Klotho reduced the levels of ROS, TNF-α and IL-6 in LPS-induced cells, which suggested that upregulated Klotho might suppress cell inflammatory response and oxidative stress induced by LPS. In chronic kidney disease, the upregulation of Klotho alleviated the inflammatory response (Dounousi et al. 2016). Li et al. (2015) reported that exogenous Klotho suppressed the expression of inflammatory factors, including TNF-α and IL-6, in chronic obstructive pulmonary disease. The study of Zeng et al. (2015) revealed that downregulation of Klotho induced the production of TNF-α and IL-6 in aging-related inflammation and renal injury. In addition to inflammatory response, Oh et al. (2015) suggested that downregulated Klotho was related to enhanced oxidative stress in patients with end-stage renal disease. An *in vitro* study also reported that Klotho was involved in oxidative stress injury in mouse kidney cells (Mitobe et al. 2005). All these results suggested that overexpression of Klotho might play an inhibiting role in LPS-induced inflammation injury.

To investigate the mechanism of Klotho in LPS-induced inflammation injury, we focused on the Wnt and NF-κB signaling pathways. Wnt proteins, as highly conserved extracellular signal molecules, had been reported to be related to the formation of nephron and renal development (Schmidtott and Barasch 2008). Wnt signaling was found to play an important regulatory role in some chronic kidney disease, such as diabetic nephropathy (Zhou et al. 2012) and adriamycin nephropathy (He et al. 2011). A previous study has shown that Wnt signaling was associated with cell proliferation, apoptosis and inflammatory response (Polakis 2012). Overexpression of Klotho was reported to inhibit the activity of endogenous or exogenous Wnt (Chen et al. 2012). Similarly, our study found that ectopic expression of Klotho could influence the activity of Wnt signaling pathway. In addition, this study showed that downregulated Klotho increased the expression of the NF-κB signaling pathway related to protein p-IκBα. It has been shown that NF-κB not only is a key regulator of inflammation, but also participates in cell proliferation, apoptosis, differentiation and metastasis (Aggarwal and Sung 2011). Notably, emerging evidence

had demonstrated the synergistic effect of NF-κB and the Wnt signaling pathway (Du and Geller 2010). Therefore, we hypothesize that the anti-inflammatory role of Klotho might be mediated by both NF-κB and Wnt signaling pathway.

The present study reveals that Klotho may play an inhibiting role in LPS-induced inflammation injury in HK-2 cells, which may be associated with NF-κB and the Wnt signaling pathway.

4. Experimental

4.1. Cell culture and treatment

HK-2 cells were obtained from the Shanghai Cell Bank of Chinese Academy of Sciences and cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (3:1, Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum at 37 °C in a cell incubator with 5% CO_2 and saturation humidity. HK-2 cells were treated with different concentrations of LPS (0, 5, and 10 μg/mL) for 12 h to induce cell inflammation injury model.

4.2. Plasmids and siRNAs transfection

The Klotho overexpression vector was constructed and named as pc-Klotho. Klotho gene was amplified from HK-2 cells by PCR and then cloned into pcDNA3.1(+). Positive pc-Klotho plasmid was identified by sequencing (GenePharma Co., Ltd, Shanghai, China). The target siRNA for Klotho gene (siKlotho) and scrambled siRNA (siNC) were obtained from GenePharma Co., Ltd. The cell inflammation injury models induced by LPS were transiently transfected with pc-Klotho plasmid, empty pcDNA3.1(+) plasmid, siKlotho or siNC by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

4.3. MTT assay

Totally, 1×10^4 cells were cultured in each well of 96-well plate for 24 h. After treatment with above LPS induction or transfection, cells were treated with 10 μL of 5 mg/mL MTT (Sigma, St. Louis, MO, USA) and then incubated for 4 h. To solubilize the formazan crystals, 100 μL dimethyl sulfoxide (DMSO, Sigma) was later added and maintained at room temperature. A microplate reader (Molecular Devices, USA) was used to measure the absorbance at 570 nm.

4.4. Apoptosis assay

Annexin V-FITC/PI Apoptosis Detection kit (Beijing Biosea Biotechnology, Beijing, China) was used to detect the cell apoptosis. The treated cells were harvested by centrifugation (1,500 rpm, 6 min), and then slightly resuspended with 1 × binding buffer. In turn, FITC-Annexin V and PI were added into resuspended cells at 25 °C in the dark for 15 min. Lastly, cells were detected using flow cytometer (BD, San Diego, CA, USA).

4.5. Reactive oxygen species (ROS) assay

ROS level was detected using 2,7-dichlorofluorescein diacetate (DCFH-DA, Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). After treatment with above transfections, the cells were washed with phosphate buffered saline (PBS) for two times and then incubated with serum-free DMEM supplemented with 10 μM DCFH-DA at 37 °C in the dark for 20 min. Next, the cells were collected using a trypsin digestion method and resuspended in PBS. A flow cytometer was used to measure the fluorescent intensities.

4.6. Real-time quantitative PCR (RT-qPCR)

Total RNA from cells was extracted using Trizol reagent (Invitrogen) and reversely transcribed into complementary DNA using the Multiscribe RT kit (Applied Biosystems, Foster City, CA, USA). Then, the levels of Klotho, TNF-α and IL-6 were detected using SYBR® Premix Ex Taq™ (Takara Biotechnology Co., Ltd., Dalian, China). Klotho sense primer was 5'-ACTTGGCCTTTATTAGCCGGGTCT-3' and antisense primer 5'-AGATGGCCTCTTCCCTGTGTTCAA-3'. Tumor necrosis factor-α (TNF-α) sense primer was 5'-CGAGTGACAAGCCTGTAGC-3' and antisense primer 5'-GGTGTGGGTGAGGAGCACAT-3'. Interleukin-6 (IL-6) sense primer was 5'-AAATGCCAGCCTGCTGACGAAC-3' and antisense primer 5'-AACAACAATCTGAGGTGCCCATGCTAC-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense primer was 5'-GCACCGTCAAGGCTGAGAAC-3' and antisense primer 5'-TGGTGAAGACGCCAGTGGA-3'. The relative quantification was carried out using comparative threshold (*C*t) cycle method ($2^{-\Delta\Delta}Ct$).

4.7. Western blotting

RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) was used to extract protein from the treated cells. The measurement of protein concentration was carried out by BCA Protein Quantitative Assay (Pierce, Appleton, WI, USA). A Bio-Rad system was used to perform western blotting. Briefly, protein sample was separated on SDS-PAGE gel, and then blotted onto nitrocellulose filter membranes. After blocked in 5% nonfat milk, the membranes were probed with mouse anti-Wnt 3a, β-Catenin, Wnt5a, phospho-IκBα (p-IκBα), total IκBα (t-IκBα), p-p65, and t-p65 polyclonal antibodies (1:500, Santa Cruz, Santa Cruz, CA, USA) and mouse anti-GAPDH monoclonal antibody (1:1000, Santa Cruz) overnight at 4°C, respectively, followed by washed with PBS and incubated with second antibody (1:5000, Santa Cruz) at room temperature for 2 h. Ultimately, the proteins were detected with Enhanced chemiluminescence (Millipore, Bedford, MA, USA).

4.8. Statistical analysis

All experiments were performed in triplicate. Statistical analyses were carried out using statistical analysis software (SPSS 19.0, SPSS Inc., Chicago, IL, USA). The results of all experiments were expressed as the mean±SD and analyzed by one-way analysis of variance. A statistically significant difference was defined as a $p < 0.05$.

Conflict of interest: Authors declare that there is no conflict of interests.

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