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LncRNA TUG1 influences osteoblast proliferation and differentiation through the Wnt/β-catenin signaling pathway

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Abstract. – OBJECTIVE: Long non-coding ribonucleic acids (lncRNAs) play a vital role in bone development, but the function of lncRNA taurine up-regulated gene 1 (TUG1) in osteoblast proliferation and differentiation is still unknown.

MATERIALS AND METHODS: The expression of TUG1 and the messenger RNA (mRNA) expressions of the Wnt/β-catenin signaling pathway markers [Runt-related transcription factor 2 (Runx2), Frizzled-2, axis inhibition protein 2 (Axin 2) and β-catenin] at 0 d, 1 d, 7 d and 14 d after in vitro culture of osteoblasts were detected, respectively, by Reverse Transcription-Polymerase Chain Reaction (RT-PCR). The effects of TUG1 on the Wnt/β-catenin signaling pathway markers and osteoblast proliferation and differentiation were studied through the silencing of TUG1 by short hairpin TUG1 (shTUG1). Furthermore, the effects of the Wnt/β-catenin signal on osteoblast proliferation and differentiation was verified by Wnt/β-catenin signal inhibitors.

RESULTS: With the continuous differentiation of osteoblasts, the level of TUG1 was significantly increased. The mRNA levels of the Wnt/β-catenin signaling pathway markers (Runx2, Frizzled-2, Axin 2 and β-catenin) also showed the same increasing trend. ShTUG1 notably reduced the activity of alkaline phosphatases (ALPs), the levels of osteocalcin and osteopontin and osteoblast proliferation activity. In addition, the silencing of TUG1 by shTUG1 resulted in significant reductions in the proteins of the Wnt/β-catenin signaling pathway markers (Runx2, Frizzled-2, Axin 2 and β-catenin), and Wnt/β-catenin inhibitors markedly reduced osteoblast proliferation and differentiation activity.

CONCLUSIONS: LncRNA TUG1 inhibition can suppress the Wnt/β-catenin signaling pathway and reduce osteoblast proliferation and differentiation.

Key Words:
Long non-coding RNA TUG1, Wnt/β-catenin signaling pathway, Osteoblast, Proliferation, Differentiation.

Introduction

Osteoporosis is a disease that easily causes brittle fracture and chronic disabling pain. It is mainly characterized by the decrease in bone mineral density and the deterioration of the bone microstructure. Besides, it is a systemic disease widely occurring in the middle-aged and elderly population[1,2]. The number of people suffering from osteoporosis worldwide is as high as 200 million, with the highest incidence rate of about 34% in postmenopausal women and about 17% in men[3]. As known, primary osteoporosis is mainly caused by the disorder of coordinated balance between osteoclast- and osteoblast-mediated bone absorption and bone formation[4].

Long non-coding ribonucleic acids (lncRNAs) can affect chromatin modification, transcription and post-transcriptional regulation[5]. In addition, lncRNAs have been proved to be involved in micro RNA (miRNA) post-transcriptional processing[6]. Taurine up-regulated gene 1 (TUG1), a new lncRNA with 6.7-kb nucleotides, is located on chromosome 22q12[7], and there is more and more evidence that TUG1 imbalance is involved in the occurrence of gastric cancer, small cell lung cancer and hepatocellular carcinoma[8,9]. It has been reported that inhibiting TUG1 can reduce atherosclerosis in mice, and TUG1 has a therapeutic effect on cardiovascular diseases. In the study of

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Katsushima et al[10] TUG1 maintains the dry characteristics of glioma cells by stimulating miR-145 in the cytoplasm. Cai et al[11] reported that TUG1 can be used as a molecular sponge of miR-299 to enhance tumor-induced angiogenesis in human glioblastoma. However, the roles of TUG1 in osteoblast proliferation and differentiation remain unclear.

Wnt stimulation can make hypophosphorylated β-catenin in cytosol accumulate, transfer to the nucleus and bind to TCF/LEF transcription factors[12]. Wnt signaling pathways can be canonical and non-canonical pathways. The canonical signal transduction leads to accumulation of cytosol β-catenin, which is then transported to the nucleus, where β-catenin acts as a co-activator of T-cell factor protein to regulate gene expression[13]. Therefore, this work aims to investigate whether lncRNA TUG1 affects osteoblast proliferation and differentiation through the Wnt/β-catenin signaling pathway.

Materials and Methods

Main Test Reagents

The alkaline phosphate (ALP) assay kit was purchased from Shanghai X-Y Biotechnology Co., Ltd. (Shanghai, China), the osteocalcin test kit from Shanghai Huzhen Industrial Co., Ltd. (Shanghai, China), the osteopontin detection kit from Amyjet Scientific Co., Ltd. (Wuhan, China), and the 3-(4,5)-dimethyl thiadiazol(-z-y1)-3,5-diphenyltetrazolium bromide (MTT) cell proliferation and cytotoxicity test kits from Beyotime (Shanghai, China).

Sources of Experimental Animals

Twenty newborn Specific Pathogen Free (SPF) female Sprague Dawley (SD) rats were purchased from the Laboratory Animal Center of Jiamusi University. They were fed under the conditions of a 12/12 h light/dark cycle, 22-25°C and 65% humidity, and they had free access to food and water. This study was approved by the Animal Ethics Committee of the Jiamusi University Animal Center.

Osteoblast Isolation and Culture

Primary osteoblasts were isolated from the skull of newborn rats. In short, the skull was washed with sterile Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) containing antibiotics, cut into small pieces, digested with 5 mg/mL trypsin twice, 10 min each time, and then digested with 1 mg/mL collagenase II for 10 min the first time and 20 min the remaining 3 times. Then, the cells digested on the second to fourth times were collected and supplemented with 2-3 mL of culture medium. After the cell suspension was filtered through a 200 μm sieve to remove bone fragments, the collected cells were inoculated in a culture dish at a density of 3×10^3 cells/cm^2. When the cell density reached 70-80%, they were separated with trypsin and subcultured at 3×10^3 cells/mL.

Fluorescence Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The Revert Aid First Strand complementary deoxyribose nucleic acid (cDNA) Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to reversely transcribe mRNAs into cDNAs. Polymerase Chain Reaction (PCR) detection was performed by mixing SYBR Green PCR Master Mix with primers. Frizzled2 primers: sense: TTCTCTGAGGACGGTTAT and antisense: CTGAAGAAGTAGAGCATCA. Axis inhibition protein 2 (Axin 2): sense: GATGAACTTGAAGGATAC and antisense: TCTCTTATGTAGGTCTTG. Runt-related transcription factor 2 (Runx2) primers: sense: AATGCCTCTGCTGTTATG and antisense: TTGTGAAGACCGTTATGG. β-catenin primers: sense: GATCCGAGGACTCAATACCATTC and antisense: GTCCTCAGACATTCGGAATAGAAC. TUG1 primers: sense: TAGCAGTTCCCCAATCCTTG and antisense: CACAAATTCCCATCATTCCC. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers: sense: CGCTCTCTGCTCCTCCTGTTC and antisense: ATCCGTTGACTCCGACCTTCAC.

Enzyme-Linked Immunosorbent Assay (ELISA) Detection

On the 7th day after treatment under different conditions, the osteoblast culture was washed with sterile PBS, and the ALP activity and the content of osteocalcin and osteopontin in osteoblasts were detected using the ALP assay kit, osteocalcin assay kit and osteopontin assay kit.

Western Blotting Analysis

The cells washed with PBS were lysed with lysis buffer, and the supernatant was collected by centrifugation. Then, the protein was quantified using the NanoDrop One/OneC micro nucleic acid protein concentration analyzer, separated on

12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto a polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland). Subsequently, the protein reacted with osteocalcin, osteopontin, Runx2, Frizzled-2, Axin 2 and β-catenin antibodies at 4°C overnight, and reacted with the secondary antibody conjugated with horseradish peroxidase (HRP). Finally, the color was developed using diaminobenzidine (DAB; Solarbio, Beijing, China).

Cell Proliferation Test

Osteoblasts were paved in a 96-well plate at 1×10^5/well, and the culture medium in the well was carefully removed after incubation. Then, the osteoblasts were washed with Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA), 2-3 times and added with 200 µL MTT (5 mg/mL), and the plate was incubated in an incubator with 5% CO_2 for 6-7 h. After that, dimethyl sulfoxide (DMSO) was added to each well, thoroughly mixed using a micropipette and let stand for 45 s. The suspension was transferred to a cuvette of a spectrophotometer, the optical density (OD) value was read at 595 nm with DMSO as a blank, and cell proliferation activity was calculated.

Statistical Analysis

The data were analyzed using GraphPad Prism5 (La Jolla, CA, USA). The analysis of variance was adopted to compare the differences between groups. $p<0.05$ suggested that the difference was significant, and all the data were expressed as mean ± standard deviation.

Results

Expressions of LncRNA TUG1 and the Wnt/β-Catenin Signaling Pathway in Different Stages of Osteoblast Differentiation

To verify the roles of lncRNA TUG1 and the Wnt/β-catenin signaling pathway in osteoblast proliferation and differentiation, the expression of lncRNA TUG1 and the mRNA expressions of the Wnt/β-catenin signaling pathway markers (Runx2, Frizzled-2, Axin 2 and β-catenin) were detected at 0 d, 1 d, 7 d and 14 d after osteoblast culture *in vitro*, respectively. The results revealed that the level of lncRNA TUG1 was increased with the continuous differentiation of osteoblasts. In addition, mRNAs of the Wnt/β-catenin signaling pathway markers (Runx2, Frizzled-2, Axin 2 and β-catenin) showed the same increasing trend (Figure 1).

Effect of Silencing TUG1 on the Proliferation and Differentiation of Osteoblasts

The above results indicate that TUG1 has an important correlation with osteoblast differentiation. To study the effects of TUG1 on osteoblast proliferation and differentiation, TUG1 was silenced to observe whether it affects the level of osteoblast differentiation markers and its effect on osteoblast proliferation. After that, osteoblasts

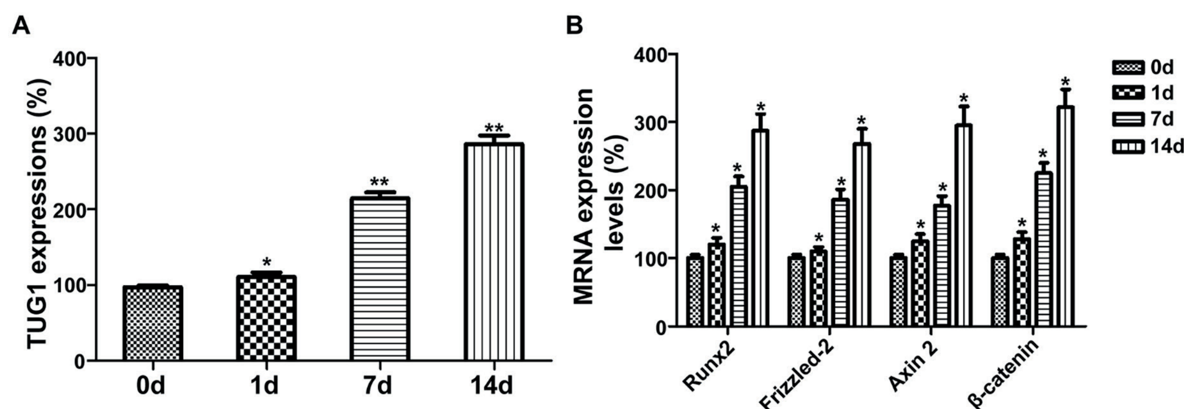

Figure 1. Levels of lncRNA TUG1 and Wnt/β-catenin signaling pathway in osteoblast differentiation. ***A,*** TUG1 expressions at 0 d, 1 d, 7 d and 14 d after the osteoblast culture (n=5). ***B,*** MRNA expression levels of the Wnt/β-catenin signaling pathway markers (Runx2, Frizzled-2, Axin 2 and β-catenin) at 0 d, 1 d, 7 d and 14 d after the osteoblast culture (n=5). $^*p<0.05$ *vs.* 0 d, and $^{**}p<0.01$ *vs.* 0 d.

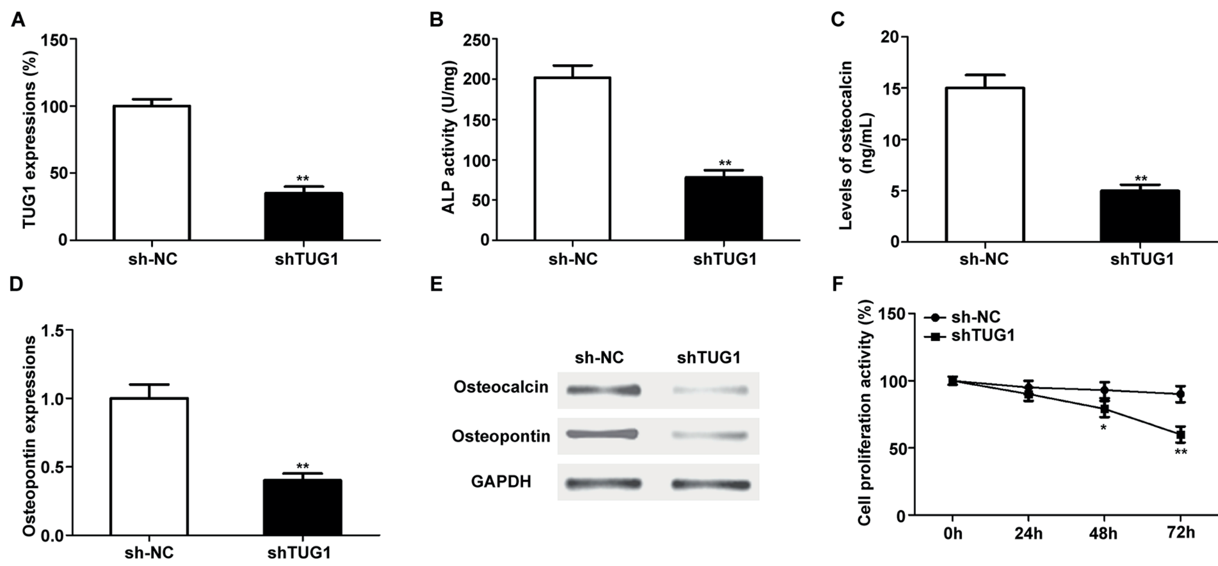

Figure 2. Effects of TUG1 silencing on osteoblast proliferation and differentiation. ***A,*** Verification of the effect of shTUG1 silencing TUG1. ***B,*** ALP activity in osteoblasts determined using the ALP assay kit. ***C-D,*** Levels of osteocalcin and osteopontin in osteoblasts detected by the ELISA kit. ***E,*** Levels of osteocalcin and osteopontin, representative osteoblast differentiation markers, determined by Western blotting. With GAPDH used for standardization, *$p<0.05$ *vs.* sh-NC, **$p<0.01$ *vs.* sh-NC.

were transfected with shTUG1 and sh-NC and then collected to detect the activity of osteoblast differentiation marker ALP and the levels of osteocalcin and osteopontin. Finally, the proliferation activity of osteoblasts was detected by MTT. According to the results, shTUG1 significantly reduced TUG1 mRNAs, ALP activity and the levels of osteocalcin and osteopontin compared with sh-NC (Figure 2A-2E). In addition, shTUG1 reduced osteoblast proliferation activity ($p<0.05$; Figure 2F).

Effect of TUG1 Silencing on the Wnt/β-Catenin Signal Transduction

To prove whether the Wnt/β-catenin expression is regulated by TUG1, the protein expressions of Runx2, Frizzled-2, Axin 2 and β-catenin were de-

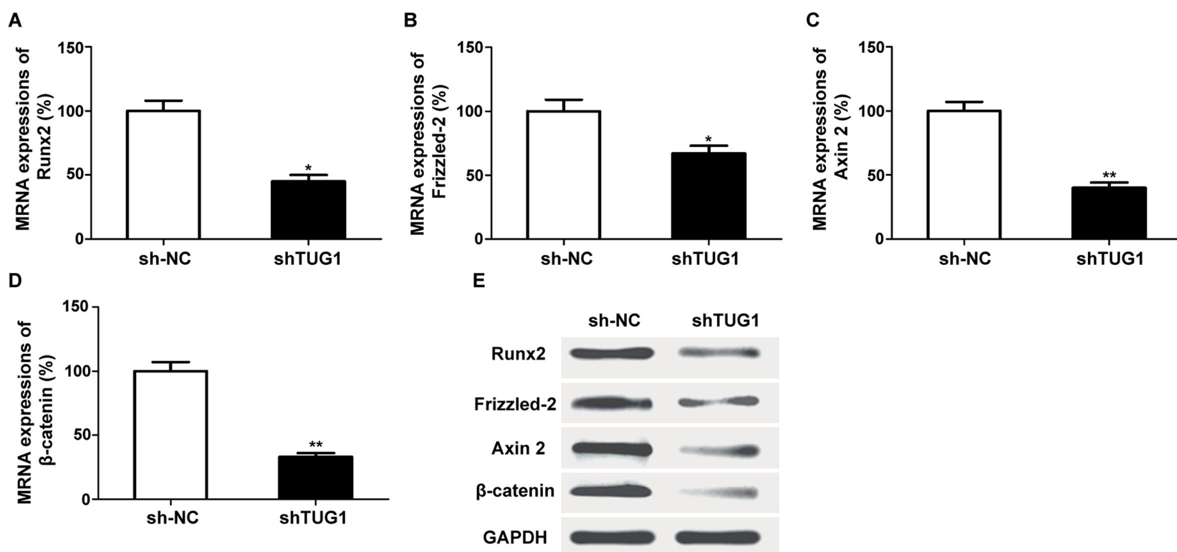

Figure 3. Effect of TUG1 silencing on the Wnt/β-catenin signal transduction. ***A-D,*** MRNA expressions of Runx2, Frizzled-2, Axin 2 and β-catenin in osteoblasts detected *via* RT-PCR. ***E,*** Protein expressions of Runx2, Frizzled-2, Axin 2 and β-catenin in osteoblasts detected *via* Western blotting. With GAPDH used for standardization, *$p<0.05$ *vs.* sh-NC, **$p<0.01$ *vs.* sh-NC.

tected after shTUG1 silenced TUG1. The silencing of TUG1 by shTUG1 resulted in significant reductions in the proteins of the Wnt/β-catenin signaling pathway markers (Runx2, Frizzled-2, Axin 2 and β-catenin; Figure 3A-3E).

Effects of Wnt/β-Catenin Inhibitors on the Proliferation and Differentiation of Osteoblasts

To further verify whether TUG1 affects the proliferation and differentiation of osteoblasts through the Wnt/β-catenin signaling pathway, osteoblasts were treated with Wnt/β-catenin inhibitors, the mRNA expressions of Runx2, Frizzled-2, Axin 2 and β-catenin were detected, respectively, ALP activity and the levels of osteocalcin and osteopontin were analyzed, and cell proliferation activity was measured. The results demonstrated that Wnt/β-catenin inhibitors remarkably reduced the mRNA expressions of Runx2, Frizzled-2, Axin 2 and β-catenin compared with control inhibitors (Figure 4A). At the same time, ALP activity, osteocalcin and osteopontin expression levels and osteoblast proliferation activity were markedly reduced (Figure 4B-4F).

Discussion

The ectopic expression of lncRNA TUG1 occurs in many cancers, but the expression and biological function of lncRNA TUG1 in osteoblasts have not been studied. This work showed that the level of TUG1 was increased with the continuous differentiation of osteoblasts. Besides, the TUG1 silencing experiment in this study revealed that TUG1 could indeed promote osteoblast differentiation, which could be proved by the increased ALP activity and expression of osteoblast differentiation markers. In addition, the silencing of TUG1 by shTUG1 resulted in significant reductions in the proteins of the Wnt/β-catenin signaling pathway markers (Runx2, Frizzled-2, Axin 2 and β-catenin). Wnt/β-catenin inhibitors markedly reduced ALP activity, osteocalcin and osteopontin levels and osteoblast proliferation activity.

It has been reported[14,15] that lncRNAs are located in the nucleus and cytoplasm, and their biological characteristics are revealed by the subcellular localization pattern of them. LncRNAs are considered to play a very crucial role in cancer development. Some studies[16-19] have found that the down-regulation of TUG1 inhibits cell proliferation in colorectal cancer, gastric cancer, bladder cancer, esophageal squamous cell carcinoma and liver cancer. TUG1 is up-regulated in squamous cell carcinoma tissues and cell lines, and the up-regulation of TUG1 in squamous cell carcinoma tissues is related to TNM staging and lymph node metastasis status[18]. *In-vitro* functional tests showed that the knockdown of TUG1 inhibits

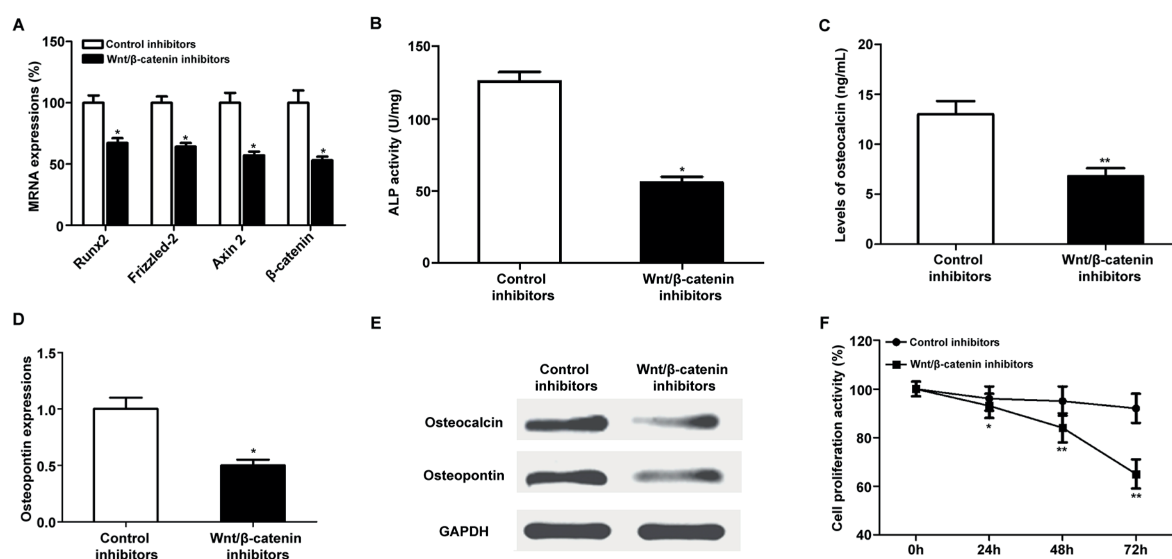

Figure 4. Effects of the Wnt/β-catenin inhibitors on osteoblast proliferation and differentiation. ***A,*** MRNA expressions of the Wnt/β-catenin signaling pathway-related markers, Runx2, Frizzled-2, Axin 2 and β-catenin detected *via* RT-PCR. ***B-E,*** Effect of the Wnt/β-catenin inhibitors on the protein expressions of osteoblast differentiation markers. ***F,*** Effect of the Wnt/β-catenin inhibitors on osteoblast proliferation activity detected *via* MTT assay. *$p<0.05$ *vs.* control inhibitors, and **$p<0.01$ *vs.* control inhibitors.

cell growth, proliferation and invasion in squamous cell carcinoma cell lines. Other research also illustrated that TUG1 knockdown induces apoptosis of squamous cell carcinoma cells and increases Caspase-3 activity. Moreover, TUG1 knockdown also affects the protein expression level of apoptosis-related mediators, including cleaved Caspase-3, cleaved Caspase-9, Bax and Bcl-2[19]. In addition, Yu et al[20] found that TUG1 is highly expressed in human aortic valve and primary valve interstitial cells. TUG1 knockdown induces the inhibition of osteoblast differentiation in calcified aortic valve diseases both *in vitro* and *in vivo*. In terms of the mechanism, TUG1 silencing increases the miR-204-5p expression and inhibits the Runx2 expression at the post-transcriptional level[20]. Furthermore, TUG1 interacts directly with miR-204-5p, positively regulates the Runx2 expression through miR-204-5p and promotes osteogenic differentiation in calcified aortic valve diseases[20]. The results of this paper are consistent with those of the above studies, which indicate that TUG1 can indeed promote osteoblast differentiation.

Runx2, Frizzled-2, Axin 2 and β-catenin are downstream factors of the Wnt/β-catenin signal transduction. Wnt/β-catenin signals enhance osteoblast proliferation and differentiation through β-catenin-dependent or β-catenin-independent pathways[21,22]. In the presence of Wnt, β-catenin is stable and can induce gene transcription, Wnt binds to its co-receptors Fz and LRP5/LRP6, and β-catenin can also induce gene expression by compounding with various transcription factors, including TCF/LEF, TBX5 and HIF-1α[23]. In this work, qRT-PCR and Western blotting analysis manifested that the expression levels of Runx2, Frizzled-2, Axin 2 and β-catenin in osteoblasts were relatively high, and their levels were significantly reduced after TUG1 silencing or Wnt/β-catenin treatment. The above results indicate that TUG1 silencing induces the inhibition of the Wnt/β-catenin signal transduction in osteoblasts. Runx2 is a transcription factor that specifically activates osteoblast differentiation and mineralization[24,25]. Osteocalcin is a downstream transcription factor of Runx2 and plays an important role in the maturation, morphology and function of bone cells[26]. It was found in this study that shTUG1 could reduce the mRNA and protein expressions of Runx2 in osteoblasts, suggesting that Runx2 participates in osteoblast differentiation. This work indicates that lncRNA TUG1 plays a vital role in osteoblast proliferation and differentiation and is closely related to the promotion of the Wnt/β-catenin signaling pathway.

Conclusions

We demonstrated that lncRNA TUG1 inhibition can suppress the Wnt/β-catenin signaling pathway and reduce osteoblast proliferation and differentiation.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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