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AXIN2 gene silencing reduces apoptosis through regulating mitochondria-associated apoptosis signaling pathway and enhances proliferation of ESCs by modulating Wnt/β-catenin signaling pathway

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Abstract. – OBJECTIVE: Embryonic stem cells (ESCs) mainly originate from totipotent cells in early-stage of mammalian embryo and could proliferate in a manner of un-limitation. This study aimed to investigate roles of Axin2 in proliferation of ESCs and explore the associated mechanisms.

MATERIALS AND METHODS: Axis inhibition protein 2 (AXIN2) over-expression (LV5-AXIN2) and AXIN2 RNA interfere (LV3-AXIN2-RNAi) vectors were structured and transfected into H9 cells. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) was used to evaluate cell proliferative activity. Flow cytometry analysis was employed to measure apoptosis of H9 cells. AXIN2, β-catenin, transcription factor 4 (TCF4), c-myc, c-jun and Cyclin D mRNA levels and protein expressions were determined using quantitative real-time PCR (qRT-PCR) and Western blotting assay.

RESULTS: LV5-AXIN2 and LV3-AXIN2-RNAi were successfully structured with higher transfecting efficacy. AXIN2 gene silencing remarkably increased proliferative activity and AXIN2 treatment significantly induced apoptosis of H9 cells, comparing with blank vector group ($p<0.05$). AXIN2 gene silencing significantly enhanced B-cell lymphoma-2 (Bcl-2) expression and remarkably inhibited cleaved caspase-3 expression comparing to that in blank vector group ($p<0.05$). AXIN2-RNAi treatment significantly enhanced and AXIN2 over-expression significantly reduced β-catenin and TCF4 expression, comparing to that in blank vector group ($p<0.05$). AXIN2 gene silence activated down-stream molecules of Wnt/β-catenin signaling pathway, including c-jun, c-myc, and Cyclin D1 ($p<0.05$).

CONCLUSIONS: AXIN2 gene silencing reduced apoptosis by regulating mitochondria-associated apoptosis signaling pathway and enhanced proliferation by modulating molecules in Wnt/β-catenin signaling pathway. Therefore, targeting of aberrant apoptosis and AXIN2 might be a novel clinical strategy to inhibit aging and enhance self-renewal of ESCs.

Key Words:
Embryonic stem cells, AXIN2, Wnt/β-catenin signaling pathway, Proliferation, Gene silencing.

Introduction

Embryonic stem cells (ESCs) mainly origin from the totipotent cells in early-stage of mammalian embryo and could proliferate in a manner of un-limitation (also called "self-renewal") and un-differentiation (also named as "pluripotency"), undergoing appropriate conditions[1,2]. The mouse ESCs were firstly discovered in 1981[3], followed the discovery and isolation of human ESCs in 1998[1]. In recent years, ESCs have been widely studied to investigate their roles in evaluating characteristics of the induced pluripotent cells (iPCs), analyzing cancer stem cells, manipulating cell fate, and exploring therapeutic applications[4]. The ESCs usually require the appropriate culture conditions for keeping pluripotent status of cells. According to previous published studies[5-7], many effectors, including cytokines, small-molecules,

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growth factors, could affect the self-renewal of ESCs, by inhibiting or initiating the associated intra-cellular signaling pathways.

The Wnt/β-catenin signaling pathway is critical for maintaining stem cells in different tissues, controlling the branching morphogenesis, keeping functions, and developments of mammary glands[8,9]. Meanwhile, Wnt/β-catenin signaling molecules are required for the earliest periods of mammalian development, for initiating the formations of tissues differentiation[10]. Axis inhibition protein 2 (AXIN2), as a negative effector for Wnt/β-catenin signaling pathway, has been proven to participate in formation of different tissues, by modulating the Wnt/β-catenin expression[11,12]. Wnt-Axin2 also involves in the processes of stem cell self-renewal and progenitor cell differentiation and proliferation[13,14]. Wang et al[15] also proved that Wnt signaling pathway-maintained hepatic-Axin2$^+$ cells demonstrating the capability of self-renewal and playing a critical role in modulating hepatic stem cells. Therefore, the present study aimed to determine whether Wnt/β-catenin signaling pathway and Axin2 participate in the maintenance and proliferation of ESCs and explore the associated mechanisms.

Materials and Methods

Cell Culture

Human ESCs were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and were cultured based on the descriptions of previous study[1]. In brief, H9 cells were seeded on feeder-layer of mitomycin-C in-activated embryonic fibroblast in the Dulbecco's Modified Eagle's Medium (DMEM; Gibco; BRL. Co. Ltd., Grand Island, NY, USA) supplementing with 20% mTeSR (STEMCELL Technologies, Toronto, Canada) and 1% penicillin/streptomycin, at 37°C and 5% CO_2.

Establishment of AXIN2 Over-Expression and AXIN2 RNAi Plasmids and Viral Packaging

LV3-AXIN2-RNAi and LV5-AXIN2 expressing plasmids were established using pG-LV3 lentiviral vector and pG-LV5 lentiviral vector (GenePhama Co. Ltd, Shanghai, China), respectively, depending on targeting sequences, sense and anti-sense sequences showed in Figure 1A. Here, three candidate RNAi sequences were employed to obtain the optimal targeting sequence by employing PCR assay with primers (Figure 1B). The synthesis of gene fragment, preparation of DNA clones, and generation of double-chains were completed by employing GenePhama Co. Ltd (Shanghai, China). Then, LV3-AXIN2-RNAi and LV5-AXIN2 plasmids, packing-necessary elements, including PG-P2-REV, PG-p1-VSVG, PG-P3-RRE, and the RNAi-mate were mixed and cultured in 293K cells, according to manufacturer's protocol (GenePhama Co., Ltd., Shanghai, China) and previously reported study[16]. Finally, the synthesized LV3-AXIN2-RNAi and LV5-AXIN2 plasmids were identified using both enzyme digestion and sequencing approach.

3-(4,5-Dimethyl-2-Thiazolyl)-2,5-Diphenyl-2-H-Tetrazolium Bromide (MTT) Assay

H9 cells were seeded at density of 5×10^3 cells/well, and cultured on 96-well plates (Corning-Costar; Corning, NY, USA). The H9 cells were infected with LV3-AXIN2-RNAi and LV5-AXIN2 viral vectors for 24, 48, and 72 h. Then, the cell proliferative activity was measured with MTT reagent (Sigma-Aldrich, St. Louis, MO, USA) due to the former investigation reported[17]. Briefly, H9 cells were incubated using MTT at final dosage of 5 mg/ml medium at 37°C for 4 h. Then, medium of H9 cells was abandoned and the formed MTT formazan crystals were resolved using 150 µl dimethyl sulfoxide (DMSO; Amresco Inc., Solon, OH, USA) at room temperature for 10 min. Finally, absorbance of the products was measured with micro-plate reader (Mode: MK3, Thermo Fisher Scientific, Waltham, MA, USA) at 490 nm.

Flow Cytometry Analysis for Apoptosis

Apoptosis of H9 cells here was measured with flow cytometry method using commercial Apoptosis Detection Kit purchasing from BD Biosciences (Cat. No. BD 559763, Franklin Lakes, NJ, USA) basing on protocol of manufacturer. In brief, H9 cells were collected and washed using phosphate-buffered saline (PBS; Beyotime Biotech., Shanghai, China). Then, H9 cells were suspended at dosage of 5×10^3 cells/ml medium. About 100 µl cell suspensions were filled into 5-ml tubes and treating using 1 µl 7-ADD (BD Biosciences, Franklin Lakes, NJ, USA) and 5 µl Annexin V-PE (BD Biosciences, Franklin Lakes, NJ, USA) for 30 min in the dark. Eventually, the Annexin V-PE and 7-ADD stained cells were captured and analyzed with a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Quantitative Real Time-PCR Assay (qRT-PCR)

Total RNAs of H9 cells were extracted using TRIzol and associated reagents (Beyotime Biotech., Shanghai, China) due to the method reported elsewhere. Complementary DNAs (cDNAs) were synthesized with a SuperScript III cDNA Synthesis Kit (Cat. No. 18080200, Thermo Fisher Scientific, Waltham, MA, USA) due to the protocol of manufacturer. MRNA levels of AXIN2, β-catenin, transcription factor 4 (TCF4), c-myc, c-jun, Cyclin D, and β-actin were assessed with SYBR Green I PCR kit (Western Biotech., Chongqing, China) and the synthesized primers (Table I). The amplified products were captured and analyzed using Gel Scanning System (Mode: GDS8000, UVP, Sacramento, CA, USA) due to the protocol of the device. Finally, the gene expressions were analyzed using the $2^{-\Delta\Delta ct}$ approach previously reported[18].

Western Blotting Assay

The H9 cells were lysed with commercial Cell Lysis Buffer (Cat. No. P0013, Beyotime Biotech., Shanghai, China) and the protein lysates of which were separated with the 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; Beyotime Biotech., Shanghai, China). The obtained proteins were electro-transferred onto the polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories; Hercules, CA, USA) using Trans-Blot SD Semi-Dry Electrophoretic Transfer (Mode: 170-3940, Bio-Rad Laboratories; Hercules, CA, USA). PVDF membranes were treated with rabbit anti-human B-cell lymphoma-2 (Bcl-2) monoclonal antibody (1:2000, Cat. No. ab32124), rabbit anti-human cleaved caspase-3 monoclonal antibody (1:3000; Cat. No. ab32042), rabbit anti-human β-catenin polyclonal antibody (1:2000; Cat. No. 2000, ab16051), rabbit anti-human TCF4 monoclonal antibody (1:2000; Cat. No. ab217668), rabbit anti-human Cyclin D1 monoclonal antibody (1:3000; Cat. No. ab16663), rabbit anti-human c-Jun monoclonal antibody (1:3000; Cat. No. ab32137), rabbit anti-human c-myc monoclonal antibody (1:3000; Cat. No. ab68727), and rabbit anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (1:2000; Cat. No. ab128915) at 4°C overnight. Then, PVDF membranes were treated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:1000, Cat. No. ab6721) at room temperature for 2 h. All above antibodies were purchased from Abcam Biotech (Cambridge, MA, USA). PVDF membranes were incubated with a Pierce enhanced chemiluminescence (ECL) Kit (Cat. No. 32106, Thermo Fisher Scientific, Waltham, MA, USA) in the dark for 2 min. Finally, Western-blotting images were captured and analyzed by employing Labworks™ Analysis Software 4.0 (Labworks, Upland, CA, USA).

Statistical Analysis

Data was defined as mean ± standard deviation (SD) and analyzed using commercial SPSS software 20.0 (IBM, Armonk, NY, USA). The Tukey's post-hoc validated ANOVA analysis was used to compare the statistical differences among groups. All the tests or experiments were repeated at least for 6 times. The $p<0.05$ was defined as statistical difference.

Table I. Primers for the RT-PCR assay.

Genes		Primers	Length
AXIN2	Forward	AGTCGGTGATGGAGGAAAATG	155 bp
	Reverse	TTCATTCAAGGTGGGGAGATAG	
β-catenin	Forward	CGTTTGGCTGAACCATCACA	127 bp
	Reverse	CCTGGTCCTCGTCATTTAGCA	
TCF4	Forward	TAAATCCTTGCCTTTCACTTCC	198 bp
	Reverse	GGGGAGGCGAATCTAGTAAGC	
c-myc	Forward	CTCCATGAGGAGACACCGC	137 bp
	Reverse	CCAGCAGAAGGTGATCCAGAC	
c-jun	Forward	ATCGCTGCCTCCAAGTGC	132 bp
	Reverse	CTGTGCCACCTGTTCCCTG	
Cyclin D	Forward	CCTGGACGGCTCTTTACGC	160 bp
	Reverse	CGCAAGACAGAGGAAACTGGA	
β-actin	Forward	TGACGTGGACATCCGCAAAG	205 bp
	Reverse	CTGGAAGGTGGACAGCGAGG	

Results

LV3-AXIN2-RNAi and LV5-AXIN2 Viral Vectors Were Established

PCR assay showed that the RNAi sequence of AXIN2-homo-936 demonstrated the most silencing efficacy for the AXIN2 gene (Figure 1C), which was therefore selected for establishing LV3-AXIN2-RNAi in this study. According to the enzyme digestion (Figure 2A, B) and sequencing identification (Figure 2C) findings, the LV3-AXIN2-RNAi and LV5-AXIN2 viral vectors were successfully established, which would be applied for the following experiments.

LV3-AXIN2-RNAi and LV5-AXIN2 Viral Vectors Demonstrated Higher Transfecting Efficacy

The qRT-PCR assay results indicated that the expression of AXIN2 mRNA in LV3-AXIN2-RNAi transfected H9 cells was significantly lower compared to that in blank vector group (Figure 3, $p<0.05$). Meanwhile, LV5-AXIN2 transfection remarkably enhanced the AXIN2 mRNA levels compared to that of blank vector group (Figure 3, $p<0.05$).

AXIN2 Modulated H9 Cell Proliferation and Apoptosis

MTT findings showed that AXIN2-RNAi treatment remarkably increased proliferative activity of cells compared with that in blank vector group (Figure 4A, $p<0.05$), at 48 and 72 h post transfection. However, AXIN2 treatment significantly decreased the proliferative activity of cells comparing to that in blank vector group (Figure 4A, $p<0.05$). Moreover, the flow cytometry results also illustrated that AXIN2 treatment significantly induced the late apoptosis of H9 cells comparing with other groups (Figure 4B, C, $p<0.05$).

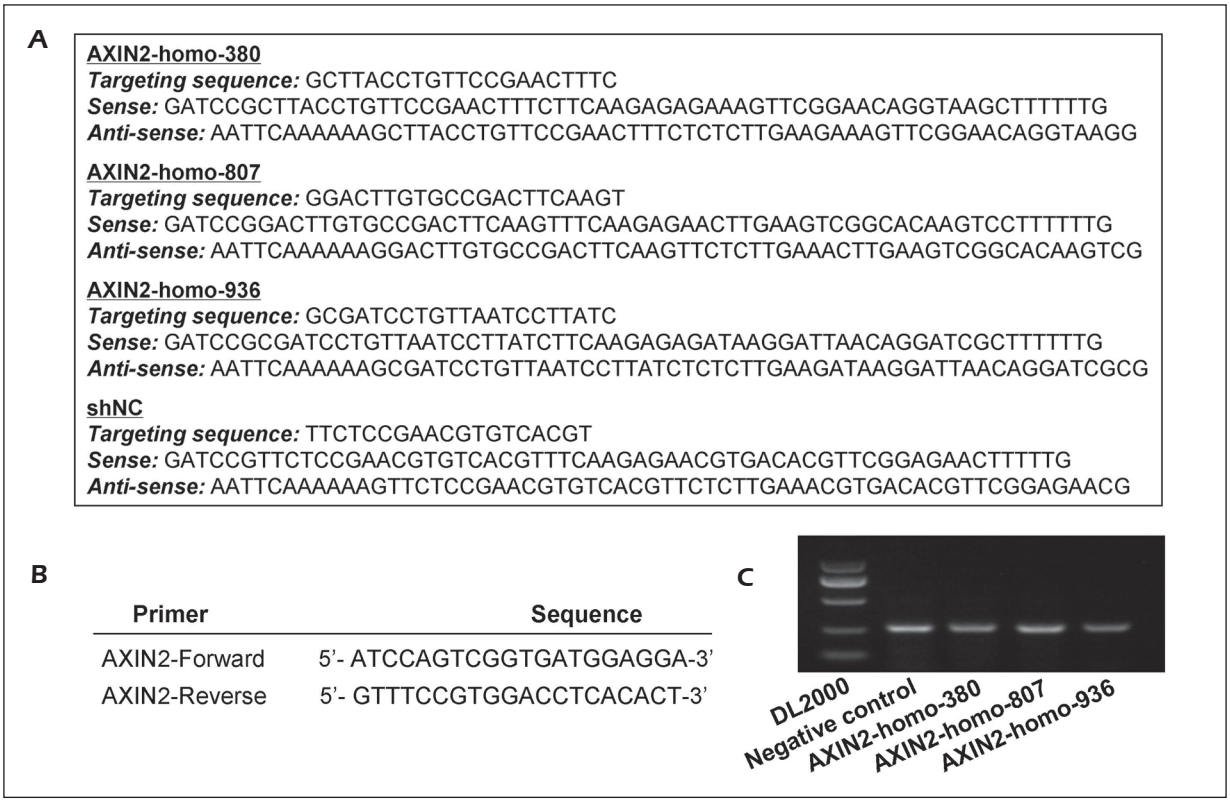

Figure 1. AXIN2-RNAi plasmids structure and identification. **A**, Targeting sequences, sense and anti-sense sequences for three AXIN2-RNAis. **B**, Primers for the amplification of AXIN2 in ESCs. **C**, PCR identification for the three AXIN2-RNAis (AXIN2-homo-380, AXIN2-homo-807, and AXIN2-homo-936).

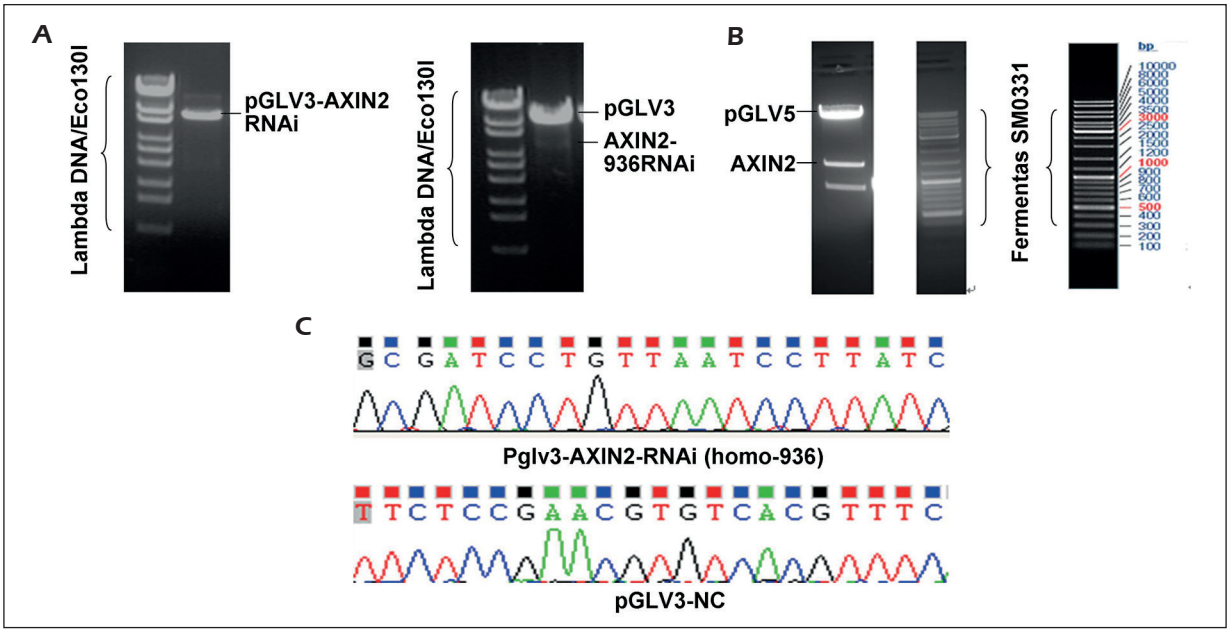

Figure 2. Enzyme digestion and gene sequencing identification for LV3-AXIN2-RNAi and LV5-AXIN2 plasmids. **A**, Enzyme digestion identification for LV3-AXIN2-RNAi. **B**, Enzyme digestion identification for LV5-AXIN2. **C**, Gene sequencing identification for LV3-AXIN2-RNAi.

AXIN2 Gene Silence Triggered Bcl-2 and Inhibited Cleaved Caspase-3 Expression

Bcl-2 and cleaved caspase-3 expressions were determined using Western blotting assay (Figure 5A). The results showed that AXIN2-RNAi treatment significantly enhanced Bcl-2 expression comparing to that in blank vector group (Figure 5B, $p<0.05$). Meanwhile, the expression of Bcl-2 was significantly reduced in LV5-AXIN2 group comparing to blank vector group (Figure 5B, $p<0.05$). However, AXIN2-RNAi administration significantly inhibited cleaved caspase-3 expression and AXIN2 viral vector treatment remarkably increased cleaved caspase-3 expression, comparing with that in blank vector group (Figure 5C, $p<0.05$).

AXIN2 Gene Silence Enhanced Expression of β-Catenin/TCF4 Complex

As the critical molecules for the Wnt/β-catenin signaling pathway, β-catenin and TCF4, were examined using qRT-PCR assay (Figure 6A, B) and Western blotting assay (Figure 7A). According to the qRT-PCR assay, AXIN2-RNAi treatment significantly enhanced and AXIN2 over-expression significantly reduced β-catenin mRNA (Figure 6A) and protein expression (Figure 7B), comparing to that in blank vector group ($p<0.05$). Moreover, the TCF4 mRNA expression (Figure 6B) and protein expression (Figure 7C) were significantly higher in LV3-AXIN2-RNAi group and significantly lower in LV5-AXIN2 group, comparing to the blank group ($p<0.05$).

AXIN2 Gene Silence Activated Down-Stream Molecules of Wnt/β-Catenin Signaling Pathway

The down-stream molecules of Wnt/β-catenin signaling pathway, including c-jun, c-myc, and Cyclin D1, were evaluated using both qRT-

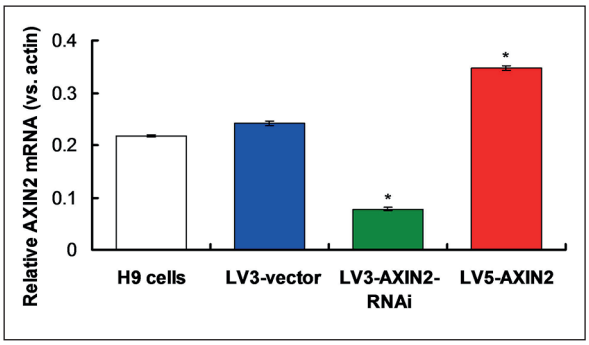

Figure 3. Determination for the AXIN2 mRNA expression in the LV3-AXIN2-RNAi and LV5-AXIN2 administrated H9 cells. *$p<0.05$ *vs.* LV3 vector group.

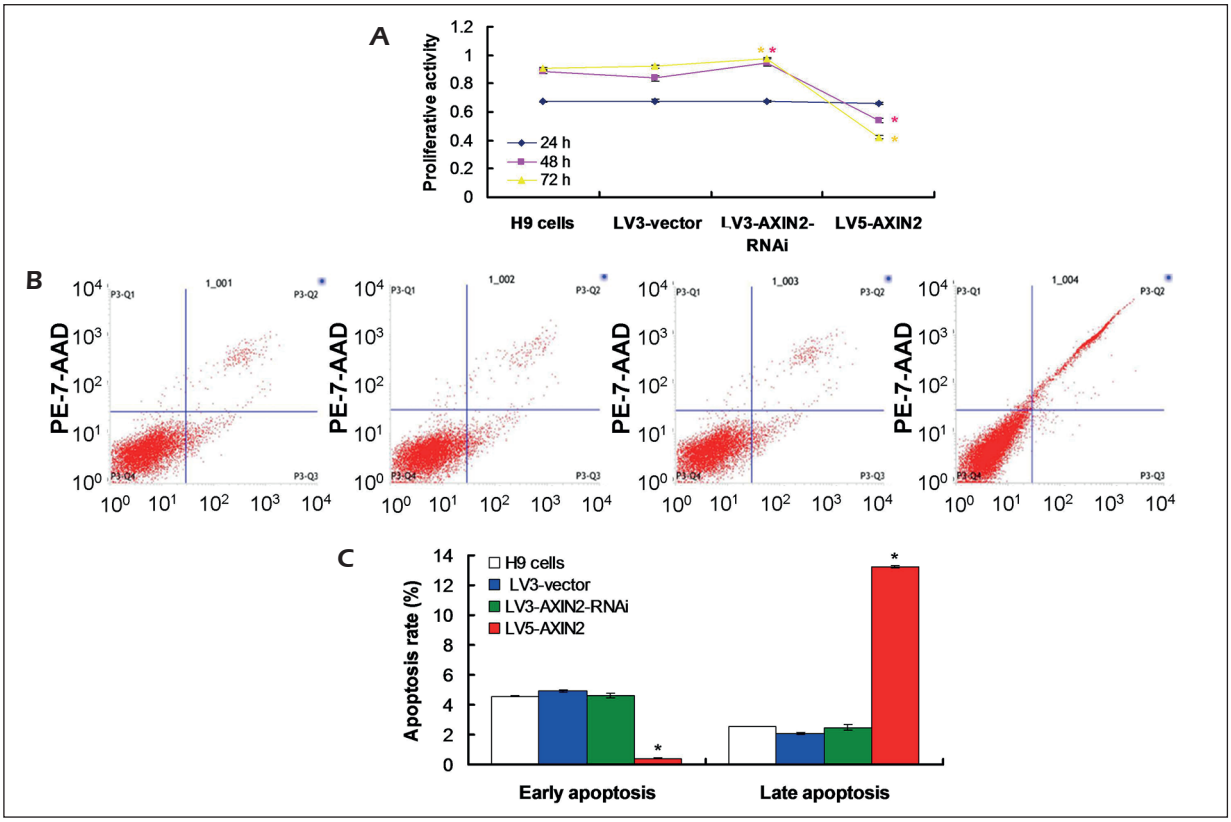

Figure 4. Measurement for the proliferative activity and apoptosis of AXIN2-RNAi and AXIN2 expressing H9 cells. **A**, Proliferative activity was measured using MTT assay. **B**, Flow cytometry assay was conducted to analyze early apoptosis and late apoptosis. **C**, Statistical analysis for the early apoptosis and late apoptosis in H9 cells of different groups. **p*<0.05 *vs.* LV3 vector group.

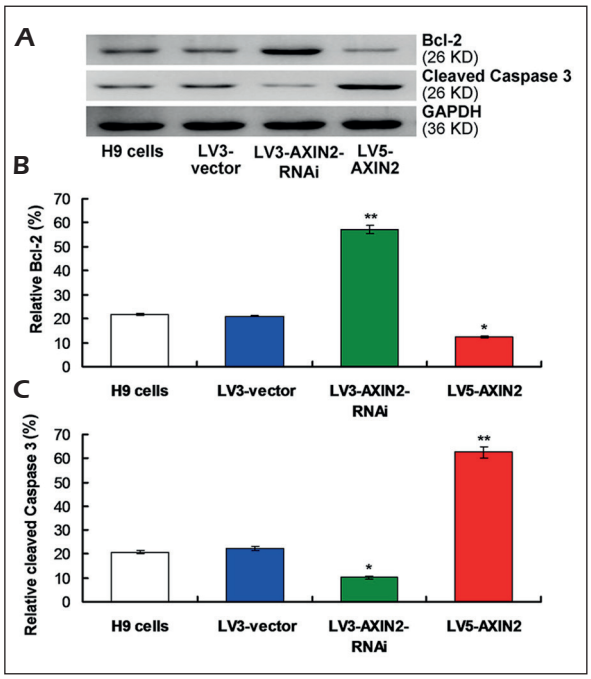

Figure 5. Effects of AXIN2-RNAi and AXIN2 expression on Bcl-2 and cleaved caspase-3 expression. **A**, Western blotting images for protein expressions. **B**, Statistical analysis for Bcl-2 expression. **C**, Statistical analysis for cleaved caspase-3 expression. * *p*<0.05, ***p*<0.01 *vs.* LV3 vector group.

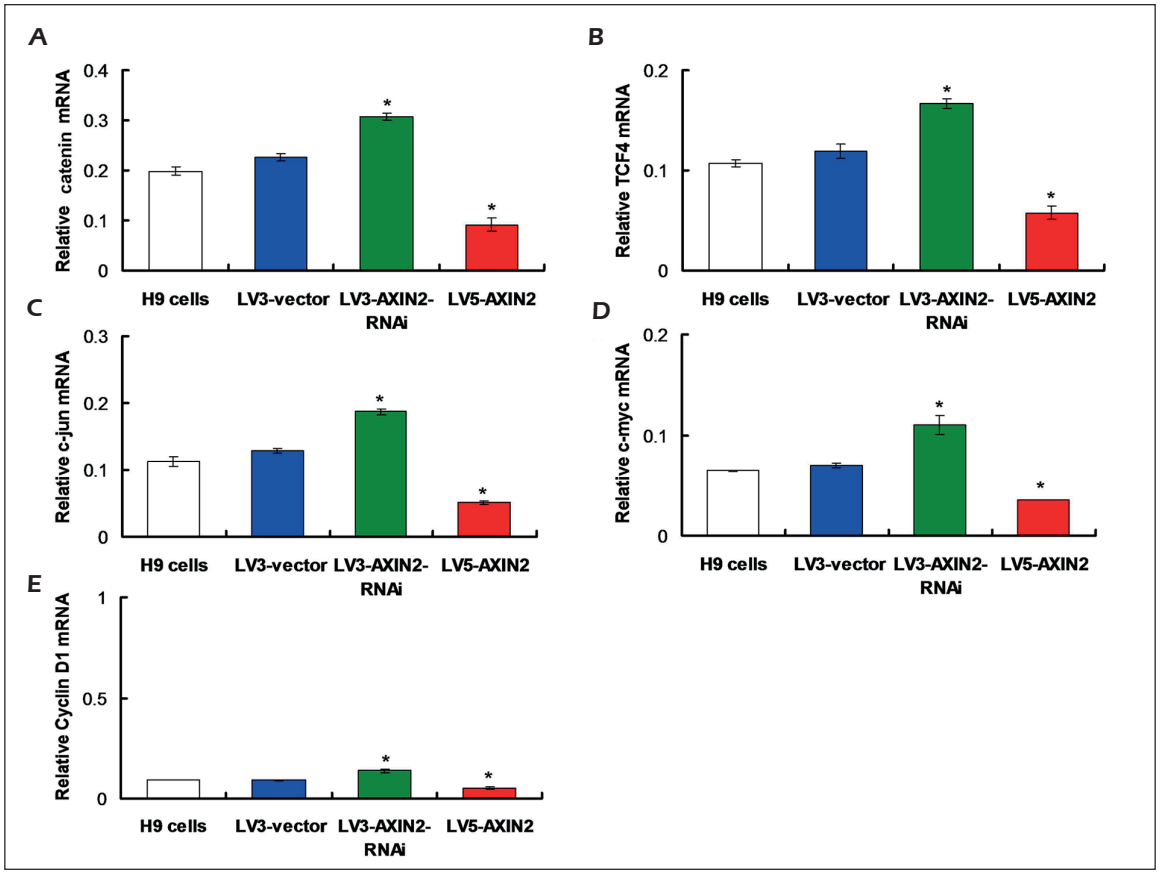

Figure 6. Evaluation for the mRNA expression of molecules in Wnt/β-catenin signaling pathway. **A**. Statistical analysis for catenin mRNA expression. **B**, Statistical analysis for TCF4 mRNA expression. **C**, Statistical analysis for c-jun mRNA expression. **D**, Statistical analysis for c-myc mRNA expression. **E**, Statistical analysis for Cyclin D1 mRNA expression. *p<0.05 *vs.* LV3 vector group.

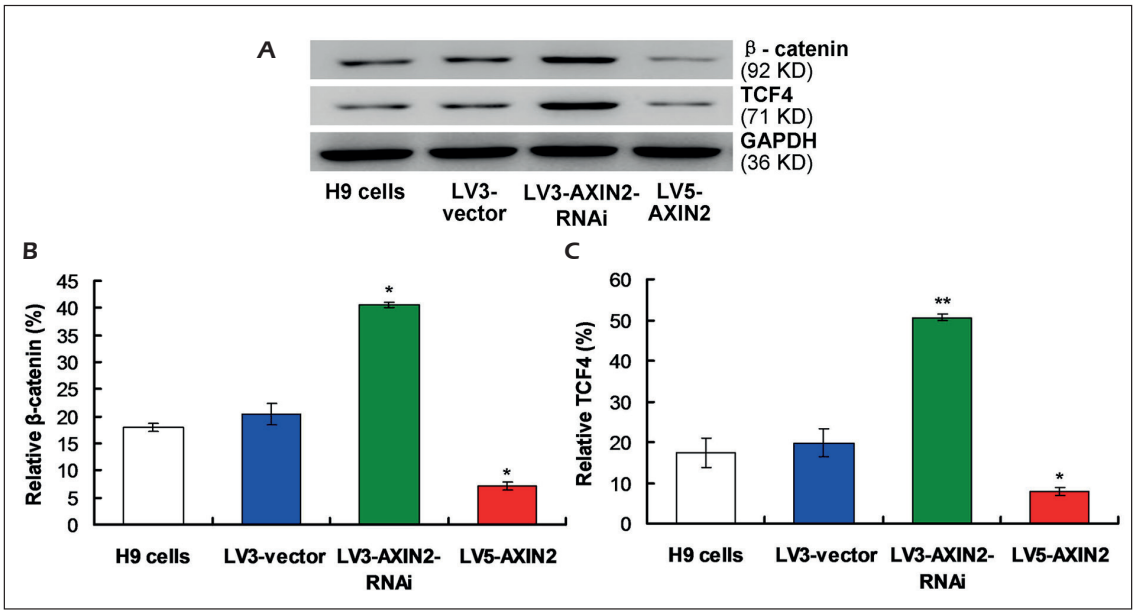

Figure 7. Effects of AXIN2-RNAi on expression of β-catenin and TCF4 using Western blotting assay. **A**, Western blotting images for β-catenin and TCF4 in H9 cells. **B**, Statistical analysis for β-catenin expression. **C**, Statistical analysis for TCF4 expression. *p<0.05, **p<0.01 *vs.* LV3 vector group.

PCR assay (Figure 6) and Western blotting assay (Figure 8A). Our results showed that AXIN2-RNAi treatment significantly up-regulated the mRNA levels of c-jun (Figure 6C), c-myc (Figure 6D), and Cyclin D1 (Figure 6E), comparing to blank group. Meanwhile, c-jun, c-myc, and Cyclin D1 mRNA expression was significantly lower in LV5-AXIN2 group compared to that in blank group (Figure 6C, D, E, $p<0.05$). Furthermore, the Western blotting assay also illustrated that AXIN2-RNAi significantly activated the Cyclin D1 (Figure 8B), c-jun (Figure 8C), and c-myc (Figure 8D) expression, which were all suppressed by administering LV5-AXIN2 ($p<0.05$).

Discussion

The present research demonstrated that AXIN2 gene silencing mediated Wnt/β-catenin signaling pathway could promote proliferation and inhibit apoptosis of human ESCs. Our results showed that β-catenin/TCF4 complex[19] involving in modulating ESCs fates might provide an explanation for the efficacy of Wnt/β-catenin signaling pathway. Of note, this study revealed a novel mechanism of canonical Wnt/β-catenin signaling pathway, which is assigned as an interaction between β-catenin and TCF4 molecule.

In this study, we found that the synthesized LV3-AXIN2-RNAi could enhance proliferation and LV5-AXIN2 could reduce proliferation by modulating the apoptosis of ESCs, which have never been clarified in ESCs according to our acknowledgement. To verify the reason for the AXIN2-associated cell apoptosis, the biomarker of mitochondria-associated apoptosis, Bcl-2, and cleaved caspase-3[20], were measured. Our data showed that AXIN2-RNAi significantly increased Bcl-2 expression and decreased cleaved caspase-3 expression, while AXIN2 over-expression remarkably decreased Bcl-2 expression and increased cleaved caspase-3 expression in ESCs. These results suggest that AXIN2 modulated the proliferation by activating the mitochondria-associated apoptosis signaling pathway.

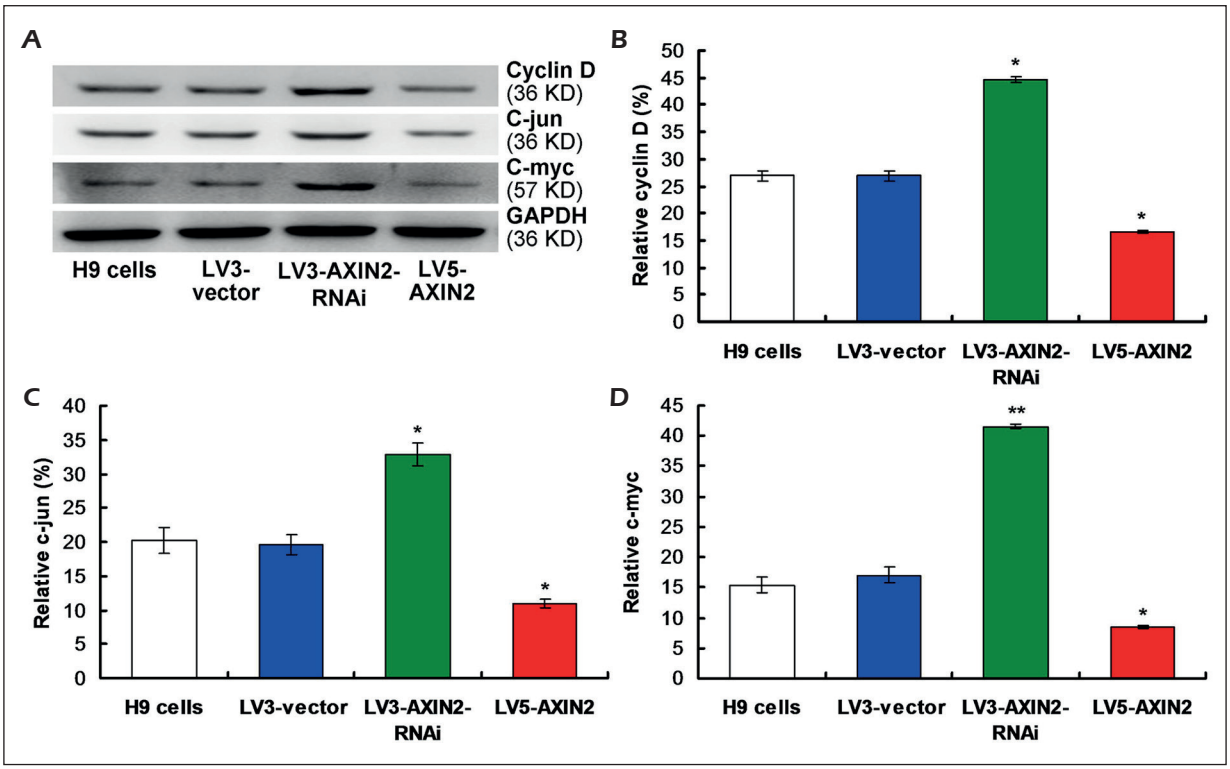

Figure 8. Evaluation for effects of AXIN2-RNAi or AXIN2 administration on expression of β-catenin and TCF4 using Western blotting assay. **A**, Western blotting images for β-catenin and TCF4 in H9 cells. **B**, Statistical analysis for Cyclin D1 expression. **C**, Statistical analysis for c-jun expression. **D**, Statistical analysis for c-myc expression. *$p<0.05$, **$p<0.01$ *vs.* LV3 vector group.

Scholars[21,22] reported that the apoptosis of cells is associated with the Wnt/β-catenin signaling pathway, whose biomarkers therefore involve in evaluating the effects of AXIN2 on proliferation and apoptosis of ESCs. The regulative role of Wnt/β-catenin signaling pathway is usually initiated by binding β-catenin molecule to TCF4 molecule[23]. In our study, the AXIN2 silence significantly activated the expression of both β-catenin and TCF4 in ESCs, which would further play multiple and critical roles in modulating cell adhesion, cellular organization, and signaling transduction in cells[24].

The molecules in the Wnt/β-catenin signaling pathway could interact with lymphoid enhancer factors and promote the transcription of the down-stream genes, including Cyclin D1, c-myc, and c-jun[25,26], all of which were also measured in ESCs. The results indicated that AXIN2 gene silence significantly activated the down-stream molecules of Wnt/β-catenin signaling pathway, including Cyclin D1, c-myc, and c-jun in ESCs. Although the specific functions are also elusive, the Wnt/β-catenin pathway and it's associated down-stream molecules appear as a requirement for keeping the proliferation and stem cell phenotype[27,28]. Therefore, the results suggest that AXIN2 gene silencing could promote the ESCs proliferation and would be enhance ESCs self-renewal by modulating Wnt/β-catenin signaling pathway and the down-stream molecules. Actually, the function of β-catenin in stem cells self-renewal is controversial and has been discussed for many years. Some studies[29,30] reported that Wnt/β-catenin signaling pathway inhibits stem cell self-renewal, but others[31] demonstrated that β-catenin is dispensable for stem self-renewal. Therefore, the Wnt/β-catenin signaling mediated effects of AXIN2 on the ESCs proliferation and self-renewal also need to be clarified in the following investigations.

Conclusions

In summary, the present findings demonstrated that the AXIN2 gene silence plays critical roles in enhancing proliferation and reducing apoptosis of ESCs, by modulating the mitochondria-associated apoptosis signaling pathway. Meanwhile, the functions of AXIN2 silence is also dependent on the regulation of Wnt/β-catenin signaling pathway, including activated β-catenin/TCF4 complex and enhanced down-stream genes, Cyclin D1, c-myc, and c-jun. Therefore, targeting of the aberrant apoptosis and AXIN2 might be a novel clinical strategy to inhibit aging and enhance self-renewal of ESCs.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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