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MiR-214 inhibits human mesenchymal stem cells differentiating into osteoblasts through targeting β-catenin

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Abstract. – **OBJECTIVE:** **Wnt/β-catenin signaling pathway promotes osteoblasts (OBs) differentiation through up-regulating osteoblast-specific gene runt-related transcription factor 2 (RUNX2) expression. It was showed that microRNA-214 (miR-214) was abnormally increased in bone tissue from osteoporosis patients, suggesting its role in osteogenesis. Bioinformatics analysis revealed the complementary binding site between miR-214 and 3'-UTR of β-catenin. This study investigated the effects of miR-214 in regulating β-catenin expression and bone marrow mesenchymal stem cells (BMSCs) differentiating into OB.**

MATERIALS AND METHODS: **BMSCs were induced to differentiate to OB in a specific medium. MiR-214, β-catenin, and RUNX2 expressions were detected. The regulatory relationship between miR-214 and β-catenin was confirmed by dual luciferase reporter gene assay. BMSCs were divided into five groups, including agomir-control, miR-214 agomir, pGPU6-normal control group (pGPU6-NC), pGPU6-β-catenin, and miR-214 agomir + pGPU6-β-catenin groups. β-catenin and RUNX2 levels were tested after 21 days' induction. OB differentiation degree was evaluated by alizarin red staining.**

RESULTS: **MiR-214 was down-regulated, while β-catenin and RUNX2 were enhanced in the process of BMSCs differentiating into OBs. MiR-214 agomir and/or β-catenin shRNA pGPU6-β-catenin transfection significantly reduced β-catenin expression, declined RUNX2 level, and attenuated OB differentiation in BMSCs.**

CONCLUSIONS: **Wnt/β-catenin signaling pathway was enhanced, while the miR-214 level was decreased in the process of BMSCs differentiating into OBs. Up-regulation of miR-214 inhibited the OB differentiation of BMSCs through targeted suppressing β-catenin expression and attenuating Wnt/β-catenin signaling pathway activity.**

Key Words:
miR-214, Wnt/Đ-catenin, RUNX2, Osteoblast, Differentiation.

Introduction

Mesenchymal stem cells (MSCs) are a kind of pluripotent stem cells originated from mesoderm featured as self-replication and multi-directional differentiation potential. It can differentiate into multiple connective tissue cells, including osteoblast (OB), cartilage cells, fat cells, fibroblasts, and myoblasts[1,2]. MSCs differentiating into OB is of great significance in maintaining normal bone mass, promoting bone growth and development, which is regulated by signaling pathways and proteins[3,4].

Wnt/β-catenin signaling pathway is closely associated with embryonic development, individual growth, immune response, and tumorigenesis that is highly conserved in evolution[5]. Except for transforming growth factor-β (TGF-β), bone morphogenetic protein (BMP), and mitogen-activated protein kinase (MAPK) signaling pathway, Wnt/β-catenin signaling pathway also play the critical role in OB differentiation and bone formation[6,7]. β-catenin is the key protein in canonical Wnt/β-catenin signaling pathway. It was found that down-regulation of β-catenin mediated Wnt/β-catenin signaling pathway attenuation is related to osteoporosis and fracture, indicating that β-catenin is of great significance in OB formation and skeletal development[8]. MiRNA is a type of endogenous single-stranded non-coding RNA at the length of 22-25 nt. It plays a degrading or inhibiting role on mRNA by binding with the 3'-UTR, thus participates in multiple biological processes, such as cell differentiation, tissue and embryonic development, individual growth, and senescence [9,10]. It was showed that miR-214 increased in BMSCs and bone tissue from osteoporosis mice and patients, suggesting its role in

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facilitating bone loss[11,12]. Bioinformatics analysis revealed the complementary binding site between miR-214 and 3'-UTR of β-catenin. This study investigated the effects of miR-214 in regulating β-catenin expression and OB differentiation.

Materials and Methods

Main Reagents and Materials

α-minimum essential medium (α-MEM), Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin were purchased from Gibco BRL. Co. Ltd. (Grand Island, NY, USA). PRIME-XV Osteogenic Differentiation serum-free medium (SFM) was purchased from GeneTex Inc. (Irvine, CA, USA). Fibronectin was got from Corning (Corning, NY, USA). PCR primers were synthesized by Sangon Biotech. Co. Ltd. (Shanghai, China). Reverse transcription kit ReverTra Ace qPCR RT Kit and SYBR Green were purchased from Toyobo Co. Ltd. (Osaka, Japan). Mouse anti-human β-catenin, RUNX2, and β-actin antibodies were purchased from Abcam Biotech. (Cambridge, MA, USA). pGL3 luciferase reporter gene plasmid and Light Switch luciferase reporter gene detection system were obtained from Active Motif (Saranac Lake, NY, USA). MiR-214 agomir and agomir-control were designed and synthesized by Ribobio (Guangzhou, China). Transfection reagent Lipofectamine 2000 and TRIzol were purchased from Invitrogen-Life Technologies (Carlsbad, CA, USA). Alizarin red staining kit was obtained from Genmed (Shanghai, China). Ficoll-Paque PREMIUM was got from PE Gene Applied Biosystems. (Foster, CA, USA). CD73, CD90, and CD105 flow cytometry antibodies were purchased from Biolegend (San Diego, CA, USA). shRNA plasmid pGPU6-β-catenin and negative pGPU6-NC were synthesized by Genepharma (Shanghai, China).

BMSCs Isolation

The bone marrow was diluted with phosphate buffer saline (PBS) and added to the Ficoll-Paque PREMIUM. After centrifuged at 400 ×g for 30-40 min, the cells were transferred to a new centrifuge tube and added with PBS. Next, the cells were centrifuged at 400 ×g for 10-15 min and resuspended in α-MEM medium containing 10% FBS and 1% penicillin-streptomycin. Then the cells were seeded in 10 cm dish at 100,000/cm^2 and passed to identify MSCs.

MSCs Identification

P2 cells were resuspended in 100 μl loading buffer and added with 5 μl CD73, CD90, and CD105 flow cytometry antibodies. After incubated avoid of light for 30 min at 4°C, the cells were resuspended in 500 μl loading buffer and tested on flow cytometry.

OB Induction

The six-well plate was coated by Fibronectin at 2 μg/cm^2. Then, the MSCs at P2 were seeded in α-MEM complete medium. After the cell fusion reached 60%, the medium was changed to Osteogenic Differentiation SFM medium for 7, 14, and 21 days, respectively. Next, the cells were collected for detection or identification.

Dual Luciferase Reporter Gene Assay

The PCR products containing the full length of β-catenin gene 3'-UTR or mutant segment were double digested and connected to pGL3, namely pGL3-β-catenin-wt and pGL3-β-catenin-mut, respectively. Then, pGL3-β-catenin-wt (or pGL3-β-catenin-mut) was co-transfected to HEK293T cells using Superfect Transfection Reagent together with miR-214 mimic (or miR-NC). The luciferase activity was detected after cultured for 48 h using the light switch.

Cell Transfection

BMSCs were divided into five groups, including agomir-control, miR-214 agomir, pGPU6-NC, pGPU6-β-catenin, and miR-214 agomir + pGPU6-β-catenin groups. The cells were collected after 21 days' induction.

qRT-PCR

Total RNA was extracted using TRIzol and amplified using ReverTra Ace qPCR RT Kit. The primers used were as follows. β-catenin: forward, 5′-CATCTACACAGTTTGATGCTGCT-3′, reverse, 5′-GCAGTTTTGTCAGTTCAGGGA-3′; RUNX2: forward, 5′-TCAACGATCTGAGATTTGTGGG-3′, reverse, 5′-GGGGAGGATTTGTGAAGACGG-3′; β-actin: forward, 5′-GAACCCTAAGGCCAAC-3′, reverse, 5′-TGTCACGCACGATTTCC-3′. The reverse transcription system contained 0.5 μl oligdT Primer (50 μM), 0.5 μl Random 6 mers (100 μM), 0.5 μl RT Enzyme Mix, 3 μl RNA (250 ng/μl), 2 μl 5×Reaction Buffer, and 3.5 μl RNase Free dH_2O. The reverse transcription condition was 37°C for 15 min and 85°C for 5 s. The PCR system contained 4.5 μl SYBR buffer, 0.5 μl Primers (5 μM), 1 μl cDNA, and 4 μl H_2O. The PCR reaction was composed of

95°C pre-denaturation for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Real-time PCR was performed on Viia7 to test the relative expression.

Western Blot

Total protein was extracted by RIPA. A total of 50 µg protein was separated by 10% sodium lauryl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to membrane. Next, the membrane was blocked and incubated with primary antibody at 4°C overnight (β-catenin, RUNX2, and β-actin at 1:400, 1:200, and 1:500, respectively). Then the membrane was incubated with horseradish peroxidase (HRP) conjugated secondary antibody (1:5000) at room temperature after washed by PBS-Tween 20 solution (PBST) for three times. At last, the protein expression was detected by enhanced chemiluminescence (ECL).

Statistical Analysis

All data analyses were performed by SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). The measurement data were depicted as mean ± standard deviation and compared by the Student's *t*-test or ANOVA. Tukey's post-hoc test was used for comparing measurement data between groups. $p<0.05$ was considered statistically significant.

Results

BMSCs Isolation

Flow cytometry finding showed that MSC marker CD73, CD90, and CD105 abundantly expressed in isolated P2 cells, indicating successful isolation of BMSCs (Figure 1).

MiR-214 was Down-Regulated, while β-catenin was Increased in OB Differentiation Process of BMSCs

Alizarin red staining revealed that BMSCs staining was negative before induction, while it was slightly stained after 7 days' induction, moderately stained after 14 days, and strongly stained after 21 days, suggesting successful OB differentiation of BMSCs (Figure 2A). The qRT-PCR demonstrated that β-catenin and RUNX2 mRNA gradually elevated, while miR-214 gradually declined in OB differentiation process of BMSCs (Figure 2B). Western blot results exhibited that β-catenin and RUNX2 protein level increased in BMSCs differentiation following time extension (Figure 2C).

microRNA.org online prediction showed the targeted binding site between miR-124 and 3'-UTR of β-catenin mRNA (Figure 2D). They showed reverse relationship in the cell differentiation process, indicating their targeted regulatory relationship. Dual luciferase assay revealed that miR-214 agomir significantly declined relative luciferase activity in HEK293 cells (Figure 2E), confirming the regulatory relationship between miR-214 and β-catenin mRNA.

Up-regulation of miR-214 Restrained OB Differentiation Through Targeted Inhibiting β-catenin

MiR-214 agomir and/or β-catenin shRNA pGPU6-β-catenin transfection significantly reduced β-catenin expression and declined RUNX2 level (Figure 3A). Alizarin red staining exhibited that miR-214 agomir and/or pGPU6-β-catenin transfection weakened staining, suggesting OB differentiation attenuation (Figure 3B).

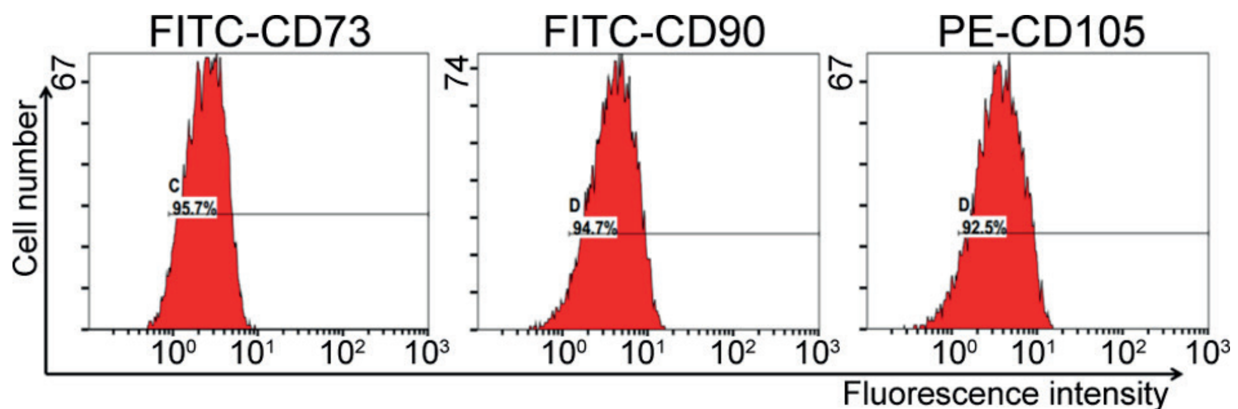

Figure 1. Flow cytometry detection of MSCs biomarker CD73, CD90, and CD105. MSCs: marrow mesenchymal stem cells.

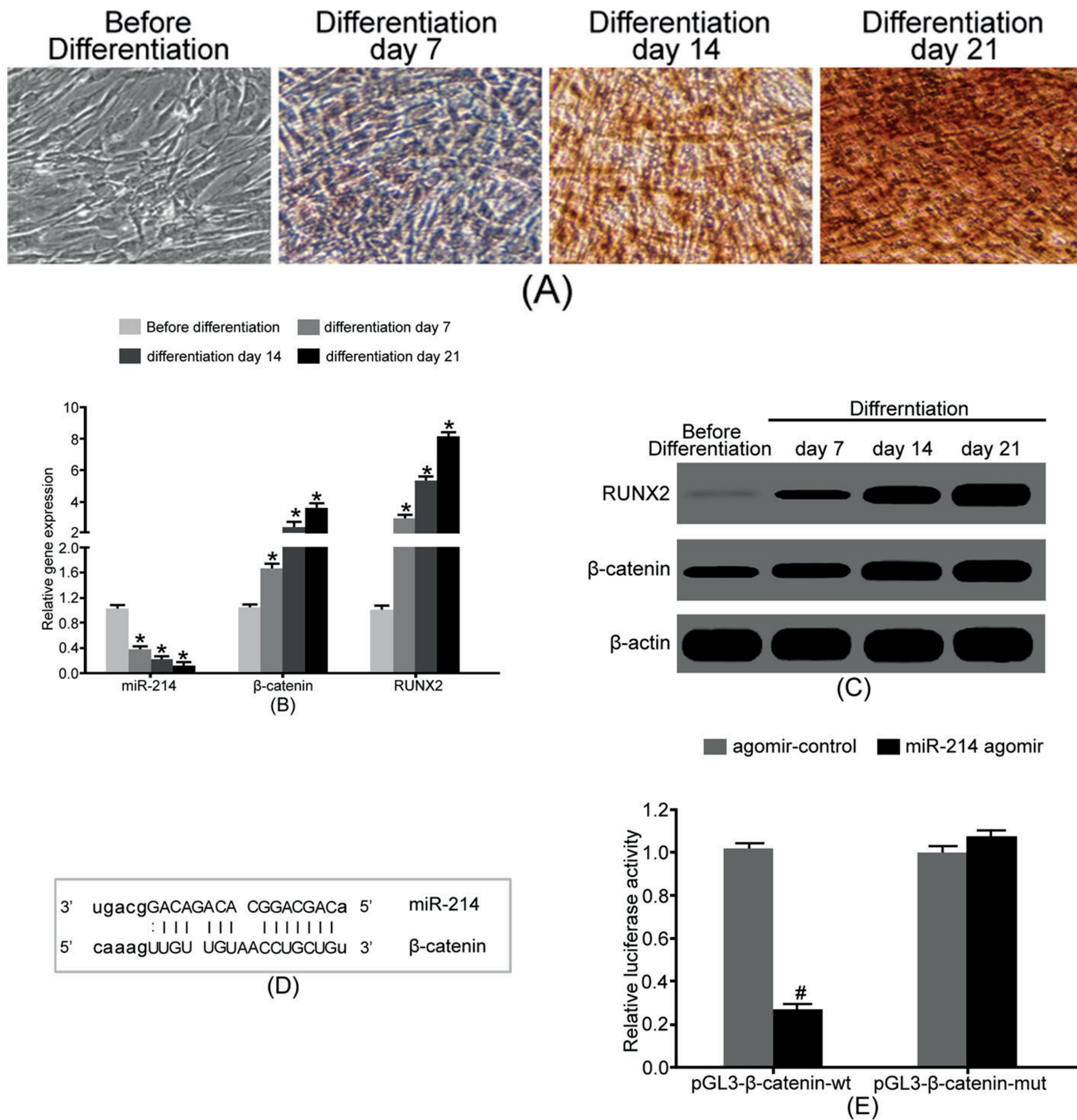

Figure 2. MiR-214 down-regulated, while β-catenin increased in the OB differentiation process of BMSCs. ***(A)*** Alizarin red staining identification of OB differentiation. ***(B)*** qRT-PCR detection of gene expression. ***(C)*** Western blot detection of protein expression. ***(D)*** The binding site between miR-124 the 3'-UTR of NFATc1 mRNA; ***(E)*** Dual luciferase reporter gene assay. $^*p<0.05$, compared with before differentiation. $^\#p<0.05$, compared with agomir-control. miR-214: microRNA-214, OB: osteoblast, qRT-PCR: quantitative RT-PCR.

Discussion

Wnt/β-catenin signaling pathway is closely associated with embryonic development, individual growth, immune response, and tumorigenesis that is highly conserved in evolution[13,14]. When lack of Wnt ligand, β-catenin binds with axin and adenomatous polyposis coli (APC), which is phosphorylated by casein kinase Iα (CKIα) and glycogen synthase kinase-3β (GSK-3β). Phosphorylated β-catenin is degraded by ubiquitin/proteasome mediated by β-transducin repeat-containing protein (β-TrCP), thus keeps in low level in cytoplasm[15]. Wnt protein can bind with frizzled (Frz) and low-density lipoprotein receptor-related protein 5 and 6 (LRP5/6), leading to dishevelled (Dvl) phosphorylation by CKIε. It obtains larger affinity to bind with FRAT and FZD, resulting in the degradation of axin in LRP5/6-Axin-FRAT complex and complex structure changes. β-catenin is dis-

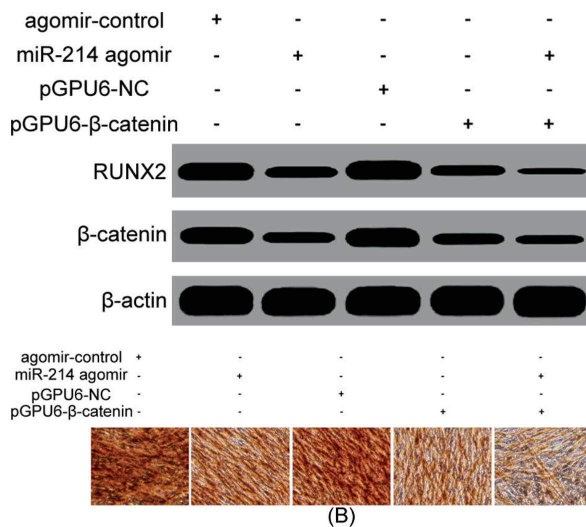

Figure 3. Up-regulation of miR-214 restrained OB differentiation through targeted inhibiting β-catenin. ***(A)*** Western blot detection of protein expression. ***(B)*** Alizarin red staining identification of OB differentiation. miR-214: microRNA-214, OB: osteoblast.

sociated from LRP5/6-Axin-FRAT complex, leading to APC-Axin-GSK-3β degradation complex dysfunction. It increases the stability and accumulation of β-catenin in the cytoplasm, which enters the nucleus to promote target gene transcription and expression by binding with T-cell factor/lymphoid enhancing factor (TCF/LEF)[14,16]. Several studies showed that Wnt/β-catenin signaling pathway plays a role in promoting MSCs differentiating into OB through up-regulating RUNX2[17], Dlx5, Osterix[18], and alkaline phosphatase (ALP). It may also down-regulate CEBPα and PPARγ to suppress adipocyte differentiation of MSCs, which indirectly promotes its osteogenesis[18,19]. It was found that β-catenin reduction mediated Wnt/β-catenin signaling pathway weakening is related to osteoporosis and fracture, suggesting that β-catenin is crucial in OB formation and skeletal development[8]. It was reported that miR-214 markedly elevated in BMSCs and bone tissue from osteoporosis mice and patients, indicating its role in facilitating bone loss[11,12]. Bioinformatics analysis revealed the complementary binding site between miR-214 and 3'-UTR of β-catenin. This study investigated the influence of miR-214 in regulating β-catenin expression and bone marrow mesenchymal stem cells (BMSCs) differentiating into OB.

In this study, MSC marker CD73, CD90, and CD105 abundantly expressed in isolated P2 cells, indicating successful isolation of BMSCs. Alizarin red staining revealed that BMSCs staining was negative before induction, while it gradually enhanced during induction. It exhibited strong staining after 21 days, suggesting successful OB differentiation of BMSCs. β-catenin and RUNX2 mRNA gradually elevated, while miR-214 gradually declined in OB differentiation process of BMSCs. Liu et al [20] showed that miR-214 level reduced in the OB differentiation process of hFOB 1.19 cells, which was similar to our results. It indicated that Wnt/β-catenin signaling pathway enhancement is the stimulative factor in OB differentiation, whereas miR-214 may play an inhibitory role. Li et al[11] demonstrated that miR-214 significantly increased in BMSCs from osteoporosis rat model induced by ovariectomy, indicating that miR-214 up-regulation is a negative factor in osteogenesis. Wang et al[12] found that miR-214 apparently increased in the bone tissue from fractured low bone density senile patients compared with un-fractured low bone density senile patients, revealing that miR-214 upregulation is a promoting factor in bone loss. Our study indicated that miR-214 elevation may be an obstructive factor of OB differentiation, which is similar to Li et al[11] and Wang et al[12]. Dual luciferase assay revealed that miR-214 agomir significantly declined relative luciferase activity in HEK293 cells, confirming the regulatory relationship between miR-214 and β-catenin mRNA. MiR-214 agomir and/or β-catenin shRNA pGPU6-β-catenin transfection significantly reduced β-catenin expression, declined RUNX2 level, and attenuated OB differentiation in BMSCs. It suggested that miR-214 down-regulated RUNX2 expression to attenuate OB differentiation of MSCs by targeted inhibiting β-catenin. Li et al[11] showed that inhibition of miR-214 in rat BMSCs facilitates its OB differentiation ability and enhances the bone repair ability of BMSCs transplantation in osteoporosis. Wang et al[12] reported that miR-214 *in vivo* injection significantly suppressed sclerotizing in mice. *In vitro* investigation exhibited that miR-214 suppressed OB function by targeted inhibiting ATF4 expression, while down-regulation of miR-214 markedly enhanced OB activity and promoted matrix mineralization[12]. Shi et al[21] found that miR-214 restrained OB differentiation by direct inhibiting OB specific gene Osterix expression. Sun et al[8] suggested that β-catenin significantly declined in bone tissue from patients with osteoporotic vertebral compression fracture, indicating the role of β-catenin in maintaining

normal OB differentiation and bone development. Wang et al[22] showed that miR-150 elevated under the stimulus of inflammatory factor TNF-α and alleviated OB differentiation of MSCs by targeted inhibiting β-catenin expression, revealing that down-regulation of β-catenin restained OB differentiation of MSCs. In this study, down-regulation of β-catenin was a negative factor of OB differentiation, which was in accordance with Wang et al[22]. At present, though several researches revealed that miR-214 can regulate OB differentiation, the role of miR-214 in OB differentiation through targeted suppressing β-catenin is still unclear. MiR-214 may also affect OB differentiation by targeted inhibiting other genes, such as Osterix[21] and FGFR1[23]. However, miR-214 may target inhibit β-catenin expression, regulate Wnt/β-catenin signaling pathway activity and RUNX2 expression, and affect OB differentiation of BMSCs.

Conclusions

Wnt/β-catenin signaling pathway was enhanced, while miR-214 level was decreased in the process of BMSCs differentiating into OBs. Up-regulation of miR-214 inhibited the OB differentiation of BMSCs through targeted suppressing β-catenin expression and attenuating Wnt/β-catenin signaling pathway activity.

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Conflict of interest
The authors declare no conflicts of interest.

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