MicroRNA-142 protects MC3T3-E1 cells against high glucose-induced apoptosis by targeting β-catenin

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Abstract. Osteoporosis, characterized by decreased mineral density and bone mass, is triggered by various detrimental factors and often causes further complications, including fractures. Aberrant expression of microRNAs (miRs) has been associated with the pathogenesis of osteoporosis. Recently, miR-142 was reported to be downregulated in osteoblasts; however, the underlying mechanism of miR-142 in mediating the development of osteoporosis remains unclear. In the present study, high glucose induced the downregulation of miR-142 mRNA expression and promoted the apoptosis of MC3T3-E1 cells. miR-142-mimics significantly protected against high glucose-induced apoptosis, upregulated the expression levels of B-cell lymphoma 2 (Bcl-2) and downregulated the protein expression levels of β-catenin, Bcl-2 associated X (Bax) and caspase-3. Furthermore, β-catenin was identified as a direct target of miR-142 using luciferase reporter assays. Similar to the effects of miR-142 inhibitors, over-expression of β-catenin aggravated the apoptosis of MC3T3-E1 cells, as demonstrated by the upregulation of Bax and caspase-3, and the downregulation of Bcl-2 expression levels. In conclusion, miR-142 protects MC3T3-E1 cells against high glucose-induced apoptosis by targeting β-catenin.

Introduction

Osteoporosis, a systemic bone disease, is characterized by decreased mineral density and bone mass, and a deterioration

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in the bone architectural structure. The pathological development of osteoporosis may be caused by an imbalance between the formation and resorption in the bone. The increased brittleness and decreased strength of the bone may lead to fractures, which is the most common complication of osteoporosis (1). Osteoporotic fractures are associated with increased morbidity and mortality rates, and use of healthcare resources (2). Presently, there is no effective treatment to cure osteoporotic fractures and the underlying molecular mechanisms of osteoporosis pathogenesis continue to be investigated.

MicroRNAs (miRs), which are ~22 nucleotides in length, are endogenous non-protein coding RNA molecules that regulate the expression of specific genes by binding to the 3'-untranslated region (3'-UTR) of target post-transcriptional mRNAs. This may enable miRs to regulate various physiological and pathological cellular processes (3). Consequently, miRs exhibit a regulatory effect on various cellular activities by mediating the expression of specific genes. However, the degree of sequence complementarity between miRs and the 3'-UTR of target mRNAs is crucial for evaluating the possible activity of miRs (4). miRs have been implicated in the development of osteoporosis by regulating a series of target genes (5). miR-142 is considered a critical pleiotropic regulator of cellular activity in embryonic development (6). Aberrant expression of miR-142 has been observed in patients with osteoporosis and miR-142 is defined as a biomarker of serum miRs for osteoporosis (7).

However, the molecular mechanism of miR-142 in regulating the pathological development of osteoporosis remains unclear. Notably, miR-142 has been reported to regulate the Wnt/β-catenin signaling pathway by targeting adenomatous polyposis coli (APC), which is a negative factor of the Wnt/β-catenin signaling pathway (8). The deletion of miR-142 may induce the upregulation of APC; however, there were no changes in the Wnt/β-catenin signaling pathway (9). In the present study, β-catenin was revealed to be a possible target of miR-142 using online prediction tools and the possible roles of miR-142 in MC3T3-E1 cells by regulating β-catenin activity was investigated.

Materials and methods

Chemicals and materials. Murine pre-osteoblast MC3T3-E1 cells were purchased from American Type Culture Collection.

The transfection reagent (Lipofectamine® 3000) was obtained from Invitrogen, Thermo Fisher Scientific, Inc. Antibodies against B-cell lymphoma (Bcl-2; cat. no. 3498S; 1:1,000), Bcl-2 associated X (Bax; cat. no. 14796S; 1:1,000), caspase-3 (cat. no. 14220S; 1:1,000), β-catenin (cat. no. 8480S; 1:1,000) and GAPDH (cat. no. 5174S; 1:1,000) were obtained from Cell Signaling Technology, Inc.

Cell culture and transfection. MC3T3-E1 cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) for 48 h at 37°C in a humidified incubator with 5% CO_2. The groups were divided into the following groups: i) Normal; ii) high glucose (HG); iii) miR-negative control (miR-NC); iv) miR-142-mimic; and v) miR-142-inhibitor. All groups were treated with high glucose (30 mM) (10), except for the normal group.

MC3T3-E1 cells (1×10^5 cells/well) at 60% confluence were transfected with miR-142-mimics (5'-TGTAGTGTT TCCTACTTTATGGA-3'; 50 nM; Guangzhou RiboBio Co., Ltd.) or miR-142-inhibitors (5'-mUmCmCmAmUmAmAm AmGmUmAmGmGmAmAmAmCmAmCmUmAmCmA-3'; 50 nM; Guangzhou RiboBio Co., Ltd; m, methylated.) using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The miR-NC used had the following sequence: 5'-GUGUAUGUUGUU GGCAUCA-3', which exhibited no effects on the viability of MC3T3-E1 cells. The pcDNA3.1(+)-β-catenin vector was prepared by cloning a β-catenin open reading frames into pcDNA3.1(+) vectors (Guangzhou RiboBio Co., Ltd.). Following this, MC3T3-E1 cells were transfected with pcDNA3.1(+)-β-catenin vectors, according to the manufacturer's protocol. The standard RPMI-1640 medium with 10% FBS was used for culturing the transfected MC3T3-E1 cells for an additional 48 h, following 8 h of transfection.

MTT assay. Cell viability was investigated using MTT assays. MC3T3-E1 cells were transfected with miR-142-mimics, miR-142 inhibitors or miR-NC prior to being seeded into 96-well plates at a density of 5×10^3 cells/well in RPMI-1640 supplemented with 10% FBS and cultured at 37°C in a humidified incubator with 5% CO_2 for 48 h. Subsequently, 0.5 mg/ml MTT was added to each well and incubated for 4 h at 37°C. Following this, formazan was dissolved in 150 μl dimethyl sulfoxide. The absorbance was measured at 450 nm.

Apoptosis determination. Apoptotic changes were determined using an Annexin V-FITC apoptosis assay, according to the manufacturer's protocol. The miR-142-mimics or miR-142-inhibitor-treated MC3T3-E1 cells were harvested and then incubated with Annexin V-FITC and propidium iodide, respectively. The apoptotic changes of MC3T3-E1 cells were determined by flow cytometry (FACSCalibur; BD Biosciences) and analyzed by CellQuest Pro software (version no. 5.1; BD Biosciences).

Reverse transcription-quantitative PCR (RT-qPCR). TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA from MC3T3-E1 cells, according to the manufacturer's protocol. For each sample, cDNA was synthesized using 2 μg total RNA and M-MLV reverse transcriptase (Promega Corporation), according to the standard protocols.

The expression of mature miR-142 was determined using miR-142 primers and an EzOmics SYBR qPCR kit (Biomics Biotechnologies, Inc.) using a Mastercycler® (Eppendorf). The following thermocycling conditions were used: Initial denaturation at 95°C for 6 min, followed by 40 cycles at 95°C for 40 sec, 65°C for 30 sec and 75°C for 8 min. The primer sequences for the Bcl-2, Bax, caspase-3 and β-catenin (Biomics Biotechnologies, Inc.) genes are presented in Table I and the following thermocycling conditions were used: Initial denaturation at 95°C for 6 min, followed by 40 cycles at 95°C for 40 sec, 60°C for 40 sec, 75°C for 40 sec and 75°C for 8 min. PCR products were electrophoresed using a 1.5% (wt/v) agarose gel to confirm the PCR results. Fold changes of miRs and mRNA expression were calculated and normalized to U6 and GAPDH, respectively, using the $2^{-\Delta\Delta Cq}$ method (11).

Western blot analysis. MC3T3-E1 cells transfected with miR-142-mimics, miR-142-inhibitors or miR-NC were harvested and lysed. Total protein was extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology) containing a protein inhibitor cocktail (Roche Diagnostics GmbH) and the concentrations were determined using the Bradford protein assay (Bio-Rad Laboratories, Inc.). A total of 30 μg of each sample was separated using 10% SDS-PAGE and proteins were transferred onto PVDF membranes. TBS containing 5% nonfat milk was used for blocking at room temperature for 1 h. Following this, membranes were incubated with primary antibodies (described previously) against Bcl-2, Bax, caspase-3, β-catenin and GAPDH at 4°C overnight. Following rinsing with PBS, membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (cat. no. A0216; 1:5,000; Beyotime Institute of Biotechnology) at room temperature for 1 h. The protein bands were visualized using an enhanced chemiluminescence detection system (Bio-Rad Laboratories, Inc.) and Quantity One software (version 4.6.2; Bio-Rad Laboratories, Inc.).

Luciferase reporter assays. According to the online prediction tool TargetScan (release 7.2; targetscan.org/vert_72), β-catenin was predicted as the possible target of miR-142. For further confirmation, luciferase reporter assays was conducted. The sequences that contained the 3'-UTR of β-catenin mRNA with wild-type and mutant binding sites for miR-142 and *Hin*dIII/SpeI were synthesized and subsequently cloned downstream of the luciferase coding region in pMiR-Report Fluc vectors (Ambion; Thermo Fisher Scientific, Inc.). MC3T3-E1 cells were cultured to 40% confluence. The reporter plasmid was co-transfected using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) with miR-142-mimics or miR-NC. Cells were collected and luciferase activity was determined using the Glomax 96 luminometer (Promega Corporation) 48 h post-transfection. Firefly luciferase reporter activity was normalized to *Renilla* luciferase activity.

Statistical analysis. All experiments were conducted in triplicate. Data are presented as mean ± standard error of the mean for each group and were analyzed using one-way ANOVA followed by Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Table I. Sequences of the forward and reverse primers used.

Primer	Primer sequence (5'-3')
Bcl-2, forward	GGCTGAGAGGAGGGCTCTTT
Bcl-2, reverse	GTGCCTGTCCTCTTAGTTCATTCTC
Bax, forward	GAAGGTATTAGAGTTGCGATT
Bax, reverse	CCTATAAACATCTCCCGATAA
Caspase-3, forward	GAGCACTGGAATGTCATCTCGCTCTG
Caspase-3, reverse	TACAGGAAGTCAGCCTCCACCGGTATC
β-catenin, forward	CGGAATTCGGGCTGACTTGATGGAGTTGGA
β-catenin, reverse	GGGGTACCCAACTGATTACTGTCACCTGG
GAPDH, forward	AAGGTCGGAGTCATCGGATT
GAPDH, reverse	CTGGAAGATGGTGATGGGATT

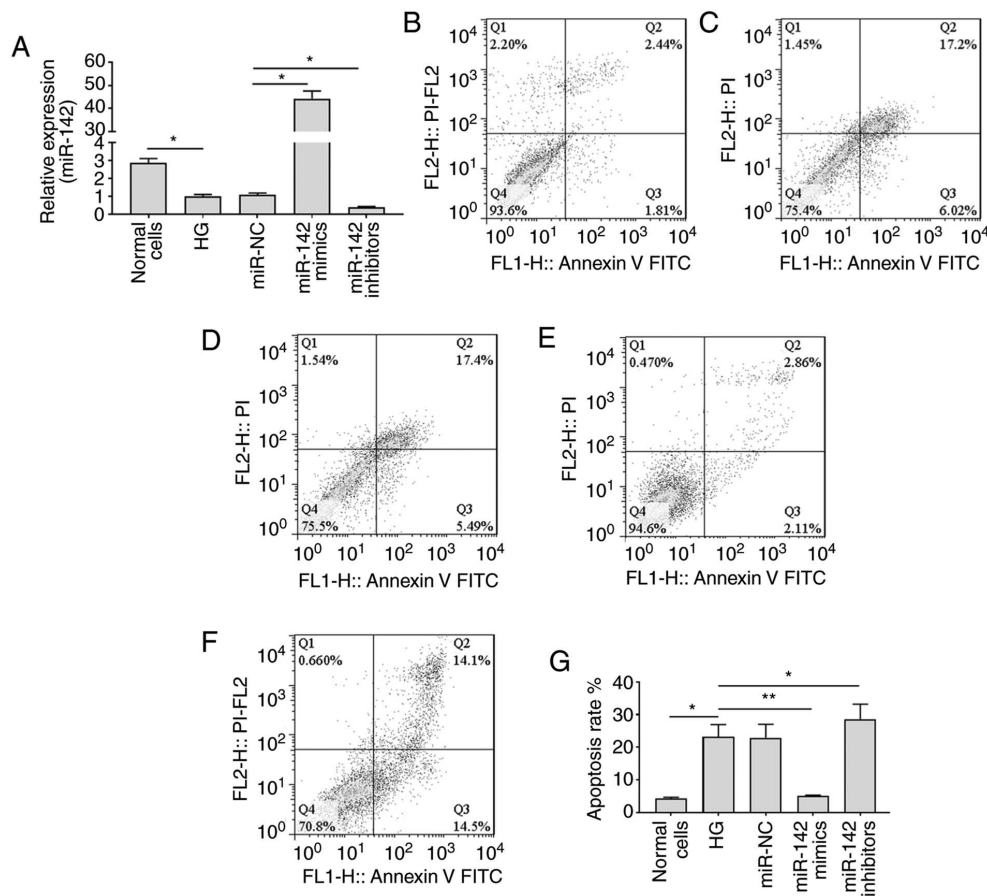Bcl-2, B-cell lymphoma 2; Bax, Bcl-2 associated X.

Figure 1. miR-142 inhibits HG-induced apoptosis in MC3T3-E1 cells. (A) The mRNA expression levels of miR-142 following transfection of miR-142-mimics or miR-142-inhibitors in MC3T3-E1 cells was determined using reverse transcription-quantitative PCR, while cell apoptosis was determined using flow cytometry in (B) the normal group, (C) the HG (30 mM) group and the HG-treated group transfected with (D) miR-NC, (E) miR-142-mimics and (F) miR-142-inhibitors. (G) Quantified apoptosis rates. Data are presented as the mean ± SD of 3 replicates. *P<0.05 and **P<0.01 vs. indicated groups. miR, microRNA; HG, high glucose; NC, negative control.

Results

miR-142 inhibits high glucose-induced apoptosis in MC3T3-E1 cells. To investigate the effects of miR-142 on apoptosis, miR-142-mimics or miR-142-inhibitors were transfected into MC3T3-E1 cells. The RT-qPCR results revealed that the mRNA expression level of miR-142 was markedly increased in MC3T3-E1 cells transfected with miR-142-mimics and decreased in cells transfected with miR-142-inhibitors compared with the miR-NC group (Fig. 1A). This indicated the successful transfection of miR-142-mimics or miR-142-inhibitors into MC3T3-E1 cells.
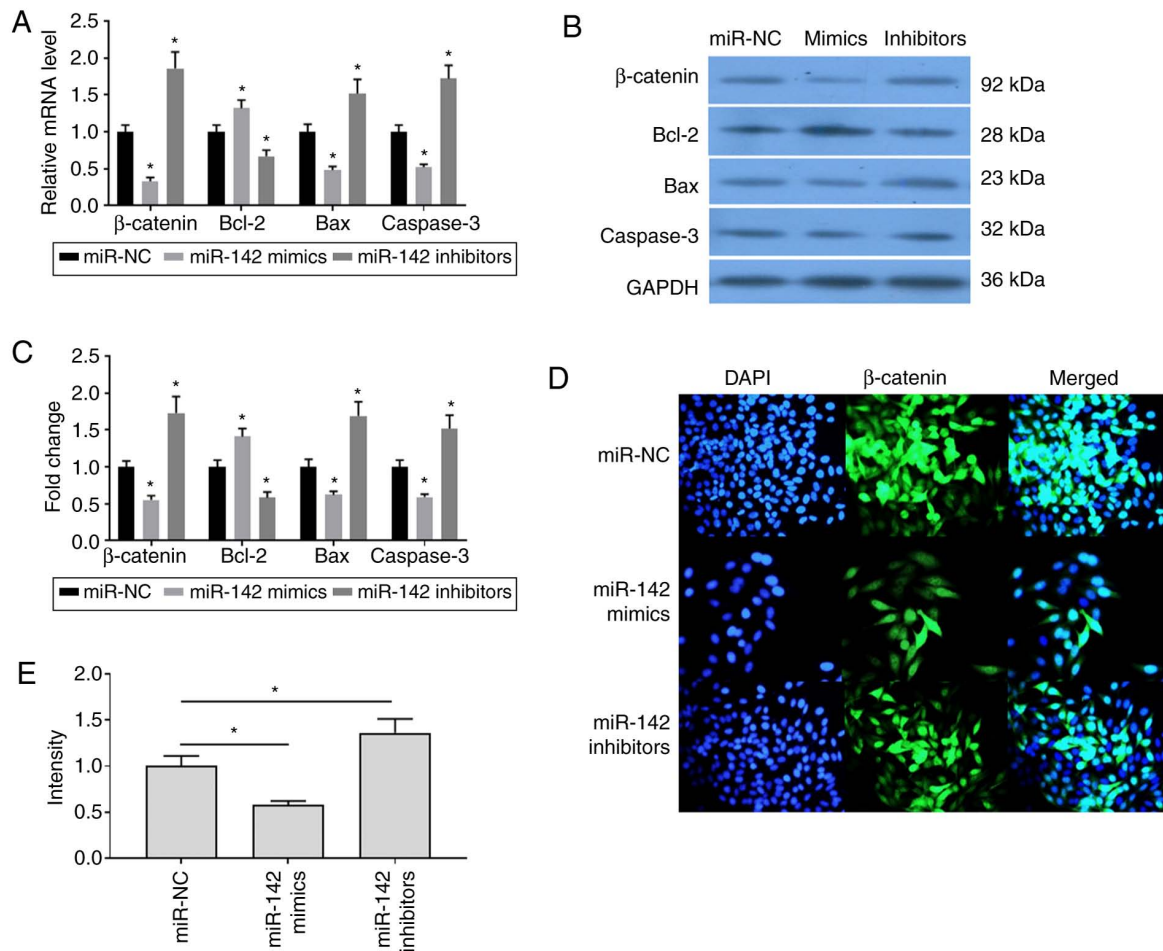
Figure 2. miR-142 downregulates the protein expression level of β-catenin and apoptosis-related proteins in MC3T3-E1 cells. (A) The mRNA and (B) protein expression levels of β-catenin, Bcl-2, Bax and caspase-3 determined using reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. (C) The summarized protein expression levels. (D) The immunofluorescence of β-catenin in MC3T3-E1 cells and (E) the summarized data. Data are presented as the mean ± SD of 3 replicates. $^*P<0.05$ vs. the miR-NC group. miR, microRNA; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2 associated X; NC, negative control.

High glucose (30 mM) induced the downregulation of miR-142 expression. Additionally, the MTT assay results revealed that there was no significant difference between the cells transfected with miR-142-mimics and miR-142-inhibitors. The effects of miR-142 on high glucose-induced apoptosis were determined using flow cytometry. As presented in Fig. 1B-G, miR-142-mimics significantly decreased high glucose-induced cell apoptosis and miR-142-inhibitors increased cell apoptosis.

miR-142 downregulates the expression of β-catenin and apoptosis-related factors. To further investigate the possible underlying mechanism of miR-142 in inhibiting the apoptosis of MC3T3-E1 cells, the expression of β-catenin and apoptosis-related factors were determined. The results revealed that miR-142-mimics decreased the expression of β-catenin, Bax and caspase-3, and increased the expression of Bcl-2 at the mRNA and protein levels (Fig. 2A-C). Furthermore, the immunofluorescence assays indicated that miR-142-mimics downregulated the protein expression level of β-catenin, while miR-142-inhibitors upregulated the protein expression level (Fig. 2D and E). Therefore, we hypothesized that β-catenin may be a direct target of miR-142.

β-catenin is a direct target of miR-142. Using the online prediction tool TargetScan, β-catenin was revealed to be a direct target of miR-142 (Fig. 3). The 3'-UTR of β-catenin, containing either the wild-type or mutant miR-142 binding sites, were cloned downstream of the firefly luciferase reporter gene. The results revealed that the relative luciferase activity of the vector containing wild-type β-catenin 3'-UTR was suppressed by >60%. By contrast, there was no significant difference between vectors containing mutant β-catenin and NC vectors (Fig. 3). These results confirmed our hypothesis that miR-142 decreased the expression of β-catenin by binding to the 3'-UTR of β-catenin and, therefore, that β-catenin was a direct target of miR-142.

miR-142 decreases cell apoptosis through attenuation of β-catenin activity. The Wnt/β-catenin signaling pathway has been implicated in the regulation of cell apoptosis (12). β-catenin was overexpressed in MC3T3-E1 cells following transfection of the pcDNA3.1(+)-β-catenin vector (Fig. 4A-C). The rescued expression of β-catenin impacted the effects of miR-142 on apoptosis, as revealed by the increase in protein expression of Bax and caspase-3, and the decrease in the expression of Bcl-2 (Fig. 4D and E), which is similar to the effects of miR-142-inhibitors on MC3T3-E1 cells.
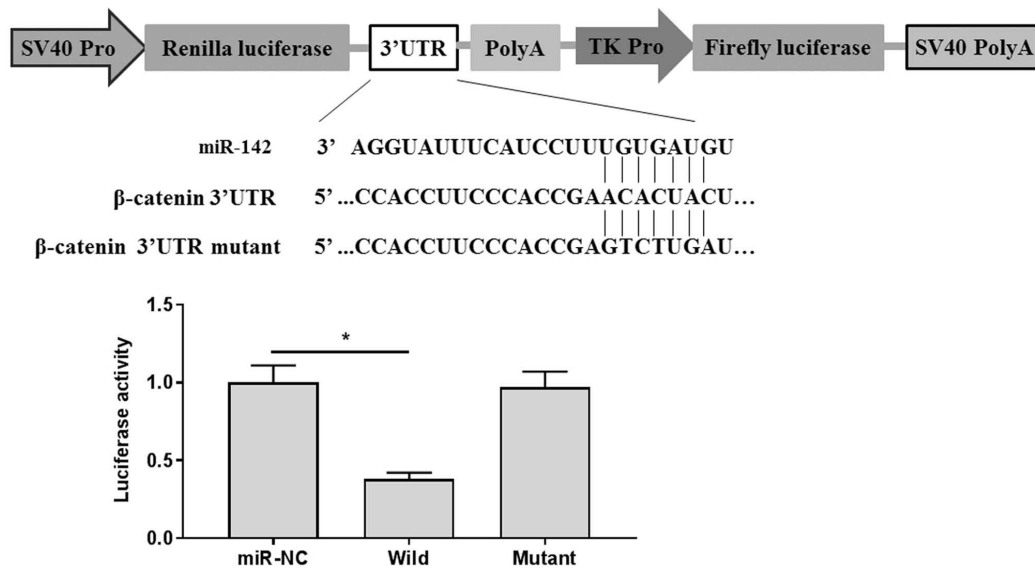
Figure 3. β-catenin was identified as a direct target of miR-142. The complementary sequence of β-catenin 3'-UTR with miR-142 was indicated. MC3T3-E1 cells were transfected with luciferase constructs and miR-142 mimics. Firefly luciferase reporter activity was normalized to Renilla luciferase activity. Data are presented using mean ± SD of 3 replicates. *P<0.05 vs. indicated groups. miR, microRNA; UTR, untranslated region. miR, microRNA; UTR, untranslated region; NC, negative control.
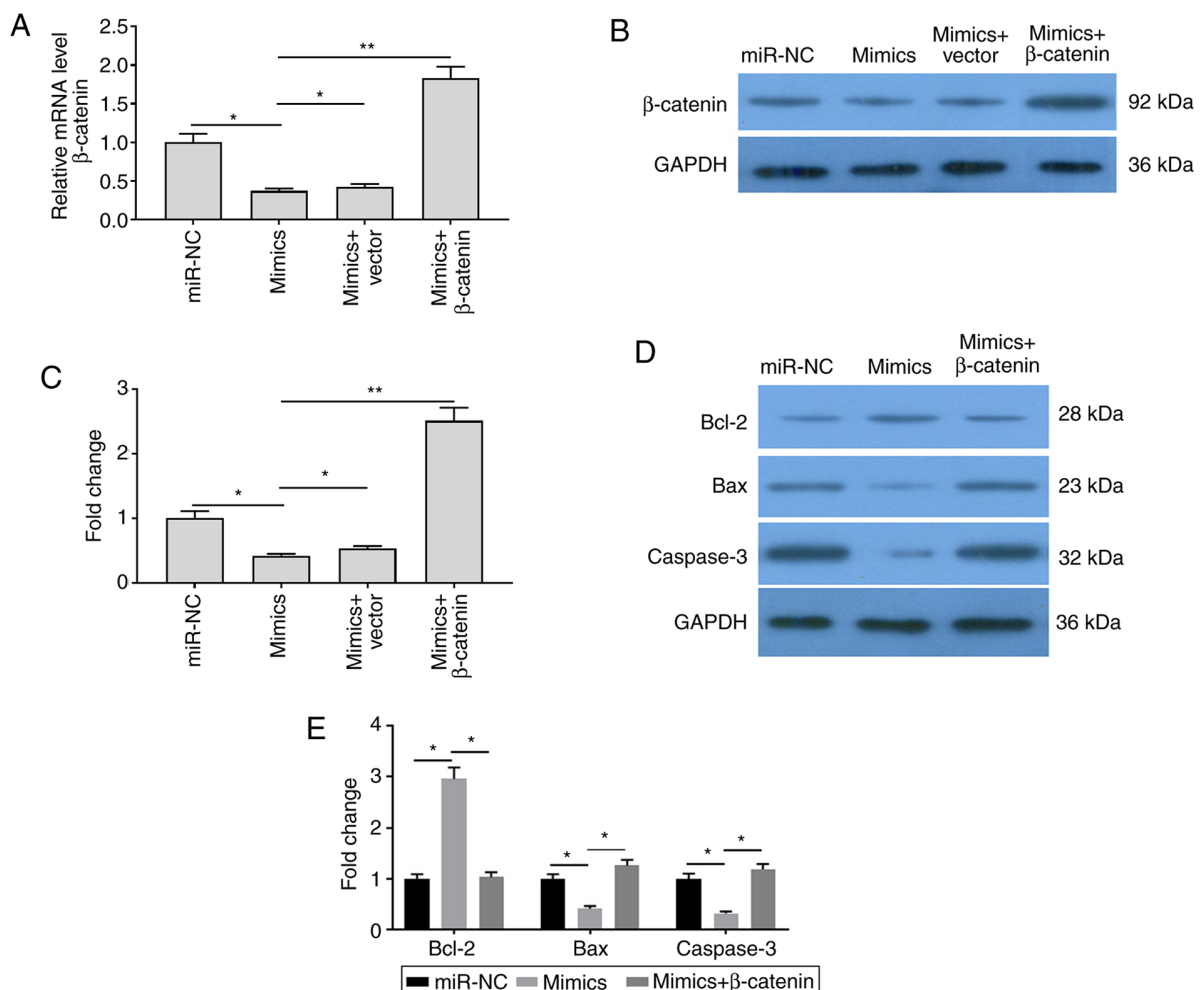

Figure 4. miR-142 regulates the expression levels of β-catenin and apoptosis-related proteins. The (A) mRNA and (B) protein expression levels of β-catenin following transfection of the pcDNA3.1(+)-β-catenin vector into MC3T3-E1 cells transfected with miR-142-mimics was determined using reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. (C) The summarized β-catenin protein expression data. (D) The protein expression levels of apoptosis-related proteins determined using western blot analysis and (E) the summarized data. Data are presented as the mean ± SD of 3 replicates. *P<0.05. **P<0.01 vs. indicated groups. miR, microRNA; NC, negative control; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2 associated X.

Discussion

In the present study, miR-142 was found to protect MC3T3-E1 cells against high glucose-induced apoptosis by targeting β-catenin. Specifically, miR-142-mimic- and miR-142 inhibitor-transfected cells exhibited anti- and pro-apoptotic effects, respectively. Additionally, β-catenin was identified as a direct target of miR-142. Overexpression of β-catenin may alter the effects of miR-142 by aggravating the pro-apoptotic events in MC3T3-E1 cells.

Recently, increased osteoblast apoptosis has been demonstrated to serve an important role in bone development and maintenance, and osteoporosis development (13,14). Research has highlighted the roles of miRs as the critical mediators of osteogenic development. miR-765 inhibited the osteogenic differentiation of human bone marrow mesenchymal stem cells by targeting BMP6 (15). miR-214 may protect MC3T3-E1 cells against apoptosis by targeting ATF4 and ameliorating oxidative stress (16). The present study demonstrated that miR-142 exhibited protective effects against apoptosis in MC3T3-E1 cells.

An increasing number of studies have reported that the excessive administration of glucocorticoids induced oxidative injury and apoptosis in osteoblasts (17,18). Osteoblast apoptosis is modulated by a variety of signaling pathways. High glucose has been associated with the activation of oxidative stress by decreasing the expression of the NRF2/ARE signaling pathway in human retinal endothelial cells (19). miR-590 has been reported to promote MC3T3-E1 cell differentiation against the inhibitory effects of high glucose (20). In the present study, miR-142-mimics were reported to inhibit glucose-induced apoptosis in MC3T3-E1 cells.

In previous studies, H_2O_2 treatment induced oxidative stress and upregulated the expression of FOXO1 and β-catenin, and their downstream factors, leading to an increase in apoptosis of MC3T3-E1 cells (21,22). Other previous studies have also indicated that high glucose significantly induce oxidative stress (23-25). The Wnt/β-catenin signaling pathway is involved in cell apoptosis and regulates the pathological activity of osteoporosis (26,27). In addition, high glucose has been demonstrated to activate the Wnt/β-catenin signaling pathway (28). This is consistent with the results of the present study as high glucose was demonstrated to promote the expression of β-catenin and induced apoptosis in MC3T3-E1 cells. Furthermore, the protein expression levels of Bax and caspase-3 were upregulated and Bcl-2 expression was downregulated. The cleaved caspase-3 is the active form of caspase-3 and contributes toward apoptosis initiation (29). In the present study, only the protein expression level of caspase-3, not cleaved caspase-3, was detected. However, the expression of caspase-3 may provide more insightful information regarding apoptosis.

The Wnt/β-catenin signaling pathway is also regulated by miRs (12). miR-503 has been demonstrated to inhibit proliferation and promote apoptosis by targeting Wnt3a in MC3T3-E1 cells (30), while miR-375 has been reported to negatively mediate osteogenesis in MC3T3-E1 cells through directly targeting LRP5, which is a critical co-receptor of the Wnt/β-catenin signaling pathway (31). In the present study, β-catenin was identified as a direct target of miR-142 and miR-142-mimics targeted and degraded β-catenin, and inhibited apoptosis in MC3T3-E1 cells. By contrast, miR-142 inhibitors inhibited β-catenin degradation and aggravated cell apoptosis. Additionally, overexpression of β-catenin exhibited a similar effect to miR-142 inhibitors, affecting the activity of miR-142 mimics on MC3T3-E1 cells. The Wnt/β-catenin signaling pathway has been considered to serve an important role in the development of osteoporosis (32); therefore, miR-142 and β-catenin may be involved in the management of osteoporosis.

In conclusion, miR-142 protected MC3T3-E1 cells against high glucose-induced apoptosis by targeting β-catenin.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

QJ and JM designed the current study and wrote the manuscript. TZ, GJ, JC, JL and TL performed experiments and analyzed data. JC, JL, TL, QJ and JM revised and finalized the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no completing interests.

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