LncRNA-KCNQ1OT1: a potential target in exosomes derived from adipose-derived stem cells for the treatment of osteoporosis

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Abstract

Background. Osteoporosis is a worldwide medical and socioeconomic threat characterized by systemic impairment of bone strength and microstructure. Exosomes derived from adipose-derived stem cells (ADSCs-Exos) have been confirmed to play effective roles in the repair of various tissues and organs. This study aimed to investigate the role of ADSCs-Exos and a noval long none coding RNA KCNQ1OT1 (lnc-KCNQ1OT1) played in osteoporosis as well as the mechanism. *Methods.* Primary osteoblasts were treated with different doses of TNF-α (0, 1, 2.5, 5, 10 ng/ml) and then co-cultured with ADSCs-Exos or exosomes-derived from lnc-KCNQ1OT1-modified ADSCs (KCNQ1OT1-Exos). The expression of miRNA-141-5p (miR-141-5p) and lnc-KCNQ1OT1 was evaluated by quantitative real-time polymerase chain reaction (qRT-PCR). The protein expression of cleaved-caspase-3, caspase-3 and Bax was determined by Western blot. Cell viability and apoptosis were assessed by cell counting kit 8 (CCK-8) and flow cytometry analysis, respectively. The binding sites between KCNQ1OT1 and miR-141-5p were validated by dual-luciferase reporter assay.

Results. Tumor necrosis factor-α (TNF-α) dose dependently increased miR-141-5p expression, inhibited viability and promoted apoptosis of osteoblasts. However, miR-141-5p silencing or co-culture with ADSCs-Exos attenuated these effects. In addition, KCNQ1OT1-Exos could more significantly attenuate the induced cytotoxicity and apoptosis compared to ADSCs-Exos. Moreover, miR-141-5p was confirmed as the target of lnc-KCNQ1OT1 by luciferase reporter assay. *Conclusions.* ADSCs-Exos attenuated cytotoxicity and apoptosis of TNF-α-induced primary osteoblasts. KCNQ1OT1-Exos had a more significant inhibitory effect compared to ADSCs-Exos by the function of sponging miR-141-5p, suggesting that KCNQ1OT1-Exos could be promising agents in osteoporosis treatment.

Introduction

Osteoporosis is a common skeletal disease characterized by structural disorders of bone mass caused by increased osteoclast activity and reduced osteoblast generation [1]. Clinical treatment strategies are based on the promotion of osteoblast proliferation and osteoblast apoptosis [2, 3]. In osteoporosis pathogenesis, tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine which has been revealed to contribute to osteoporosis by regulating both osteoblasts and osteoclasts [3, 4]. TNF-α can suppress bone formation and inhibit osteoblast differentiation by blocking RUNX2 [5, 6]. Therefore, exploring new pathways to arrest TNF-α-induced cytotoxicity and apoptosis in osteoporosis is necessary.

As a subset of mesenchymal stem cells (MSCs), adipose-derived stem cells (ADSCs) can be easily obtained from adipose tissues and possess the potential of multi-differentiation and self-renewal. ADSCs are found in abundant quantities and can be easily obtained by minimally invasive surgeries [7]. Tissue engineering based on MSCs is considered an attractive treatment for cartilage lesions and

osteoarthritis [8]. Thus, ADSCs might be a promising MSCs-based strategy for bone formation and structural remodeling in osteoporosis [9, 10].

Exosomes are small, membrane-bound extracellular vesicles that are enriched in selected proteins, lipids, nucleic acids, and glycoconjugates [11]. Exosomes have been proved to play a significant role in the etiology of bone metabolic diseases, especially osteoporosis [12]. Exosomes derived from ADSCs (ADSCs-Exos) contain bioactive substances of ADSCs and might play a similar role to ADSCs [13]. Recent researches have demonstrated that ADSCs-Exos had motivating effects in the repair of a variety of tissues and organs [8, 14, 15]. Even though, the effects of ADSCs-Exos on cell cytotoxicity and apoptosis in osteoporosis as well as the potential mechanisms still remain unclear.

Exosomes are important mediators between cells by transferring molecules, such as long none coding RNAs (lncRNA), microRNAs (miRNAs) and cytokines [16-19]. Evidence indicates that exosomes derived from mmu_circ_0000250-modified ADSCs are able to promote wound healing in diabetic mice [20]. Additionally, exosomes derived from miR-188-3p-modified ADSCs can protect Parkinson's disease[21]. Recently, a novel lncRNA, KCNQ1overlapping transcript 1 (KCNQ1OT1) was found to be able to promote the proliferation, metabolism and growth of tumor cells [22]. However, little is known about the role of KCNQ1OT1 in osteoporosis. The aim of this study was to investigate the role of ADSCs-Exos and lnc-KCNQ1OT1 played in osteoporosis as well as the mechanism.

Methods

2.1. Isolation and culture of ADSCs

The Institutional Animal Care and Use Committee of Southeast University approved the protocol for the use of animals in this study. Adipose tissues were collected from the inguinal fat pad from 2-3-week-old C57BL/6 mice (SLAC, Shanghai, China), and then rinsed in phosphate-buffered saline (PBS) and cut into 1×1mm pieces. The collected tissues were digested by collagenase type II (Sigma-Aldrich, USA) at 37 °C for 1 h. After digestion, tissues were centrifuged at room temperature (1000rpm, 5min) and the resultant cell pellet was re-suspended in Dulbecco's modified Eagle's medium (DMEM) at the cell density of 5×10^6/ml. Cells were then cultured at 37°C under a 5% CO_2 atmosphere. The culture medium was replaced every 2-3 days and cells after 3 passages were used in the present study.

2.2. Induced differentiation potential of isolated ADSCs

ADSCs were cultured in a 24-well plate at a density of 4×10^4 cells/well with basic culture medium (DMEM-LG) containing 10% FBS, 100 U/ml penicillin and 100mg/ml streptomycin. They were subjected

to induced differentiation by culturing them in osteogenic (Cyagen Biosciences, Rasmx-90021), adipogenic (Cyagen Biosciences, Rasmx-90031) and chondrogenic (Cyagen Biosciences, Rasmx-90041) medium, respectively. The outcomes were evaluated by Alizarin Red, Oil Red O and Alcian Blue staining, respectively.

2.3. Isolation and characterization of primary osteoblasts

The isolation and characterization of primary osteoblasts were conducted as described before [23]. Briefly, calvarium tissue was isolated from C57BL/6 mice which was born within 48 h, minced into 1mm3 tissue block and digested using 0.25% trypsin (Thermo Fisher, USA) containing 0.02% EDTA for 25 min at 37°C. Bone chips were digested in 5 ml Hanks solution containing 0.1% Collagenase I (Thermo Fisher, USA) and 0.05% trypsin for 1 h in a shaking incubator at 37°C with a shaking speed of 200 r/min. The released cells were collected by centrifugation for 10 minutes at 1500 r/min. The cells were suspended in 5 ml of a-MEM (containing 1 g/L D-Glucose and L-Glutamine, BI, USA) containing 10% FBS and then transferred to 25 cm2 plastic culture flask (polystyrene cell culture flask, Nest, China). The culture flask was incubated at 37°C in a 5% CO2 incubator. The images of cell morphology were taken using a microscope attaching camera (OLYMPUS IX51). The primary osteoblasts of passage 4 were selected for further experiments. The outcomes were evaluated by Alkaline phosphatase staining and Alizarin red S staining.

2.4. Isolation and characterization of exosomes

A Total Exosome Isolation kit (Invitrogen, 4478359, USA) was applied to isolate the total exosomes from the supernatant of ADSCs culture medium according to the manufacturer's protocol. The experimental procedures were conducted as described before [24]. Bicinchoninic acid assay (BCA) protein assay kit was used to measure the concentration of isolated exosomes. The protein levels of CD9, CD63, CD81 and Alix (representative markers of exosomes) were then detected.

2.5. Exosomes uptake assay

ADSCs-Exos were labeled with PKH26 (Sigma Aldrich, USA) according to the manufacturer's protocol. Isolated exosomes were resuspended in diluent C (1ml). Then 6 μl PKH26 was added into diluent C (1ml). The ADSCs-Exos and PKH26 solutions were mixed for 30 s and then centrifuged (120000×g, 2h, 4℃). Exosomes were resuspended in the complete culture medium and the PKH26-labeled ADSCs-Exos solution was added into primary osteoblasts for incubation. After 24-48h of culture, osteoblast cells were fixed with 4% formaldehyde for 10 min. DAPI was used to stain the nuclei. Cells were finally observed under a confocal microscope.

2.6. Cell Viability Assay

The viability of primary osteoblasts was determined by the Cell Counting Kit-8 (CCK-8, Dojindo, Japan) assay. Briefly, cells were seeded into 96-well plates (2×10^4cell/ml) and incubated for 24h. Then, 10 μl CCK-8 solution was added into each well for incubating 1-2 h (37 °C, 5% CO_2). Subsequently, the optical density (OD) value was measured at 450 nm using a spectrophotometer (Bio-Rad, USA).

2.7. Cell apoptosis assay

The apoptosis of primary osteoblasts was evaluated by Annexin V-FITC/PI Apoptosis Assay Kit (Keygen Biotech, China). Briefly, primary osteoblasts were collected and washed with PBS. A total of 500μl binding buffer was added to suspend cells. Firstly, 5μl annexin V-FITC was added, and then 5μl propidium iodide was added for incubation for 5-15min in the dark at room temperature. Cell apoptosis was analyzed by flow cytometry (Becton-Dickinson, FACS Calibur, USA).

2.8. Western blot analysis

The total protein of primary osteoblasts was extracted using RIPA lysis buffer (Beyotime, China) and quantified by BCA assay (Beyotime, China). Equal amounts of proteins (100μg) were separated via BeyoGel™ Plus PAGE (Beyotime, China) and then transferred to a PVDF membrane (Millipore, USA). After transferring, the membranes were blocked with 5% fat-free milk for 1h. The membranes were incubated with primary antibodies (Bax, ab32503, Caspase-3, ab32351, cleaved- Caspase-3, ab32042), which were purchased from Abcam (USA) and GAPDH, 10494-I-AP which was obtained from Proteintech (China) at 4°C overnight. The membranes were incubated with the second antibodies (Goat anti rabbit IgG HRP SE134, Goat anti mouse IgG HRP SE131, Solarbio, China) at 37°C for 1h. The ECL system (CLINX, China) was used for exposing protein bands. The intensity of the bands was analyzed using Image lab (version 3.0, Bio-Rad, USA).

2.9. Gene expression analysis using quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. For detecting miR-141-5p expression, MicroRNA cDNA Synthesis Kit (Vazyme, China) was applied for reverse transcription of cDNA, followed by qRT-PCR analysis. U6 was employed as the loading control. The primers of miR-141-5p and U6 were purchased from GeneCopoeia (Guangzhou, China). Meanwhile, the expression of lncRNA-KCNQ1OT1 was evaluated by Prime Script™ Master Mix

(Takara, Japan) and GAPDH was employed as the loading control. The data was calculated using the 2^{-DDCt} method. Primers used in this study were shown below:

lncRNA-KCNQ1OT1, F: 5'-TTGGTAGGATTTTGTTGAGG-3' and

R: 5'-CAACCTTCCCCTACTACC-3';

GAPDH, F: 5-TGGATTTGGACGCATTGGTC-3 and

R: 5-TTTGCACTGGTACGTGTTGAT-3.

2.10. Dual-luciferase reporter assay

The wild-type (WT) sequence of lncRNA-KCNQ1OT1 containing the miR-141-5p binding sites (KCNQ1OT1-WT) and the mutant sequence (KCNQ1OT1-MUT) were cloned into pMIR vectors (Promega, USA) respectively. Primary osteoblasts and HEK293 cells were co-transfected with miR-141-5p or miR-NC and KCNQ1OT1-WT or KCNQ1OT1-MUT and kept for 24 h. The luciferase activity was detected using Dual-Luciferase Reporter Assay System (Promega, USA) in the dark.

2.11. Statistical Analysis

Statistical analysis was performed with SPSS 20.0 software (IBM, Armonk, NY, U.S.A.). All quantitative data were described as mean ± SD. One-way ANOVA was used to analyze the statistical differences among three or more groups while unpaired Student's *t* test was applied to analyze the statistical differences between two groups. $P< 0.05$ was considered statistically significant.

Results

3.1. TNF-α increased miR-141-5p expression, suppressed the viability and promoted the apoptosis of primary osteoblasts in a dose-dependent manner

Primary osteoblasts were treated with increased concentrations of TNF-α (0, 1, 2.5, 5, 10 ng/ml). TNF-α dose-dependently increased miR-141-5p expression (Figure 1(a)). Meanwhile, Primary osteoblasts treated with TNF-α showed reduced cell viability and promoted apoptosis in a dose-dependent manner (Figures 1(b) and 1(c)). Furthermore, TNF-α increased the protein expression level of Bax and cleaved-Caspase-3 dose-dependently (Figure 1(d)).

3.2. Knockdown of miR-141-5p reversed the effect of TNF-α on primary osteoblasts.

Since miR-141-5p was up-regulated in TNF-α-treated primary osteoblasts, we knocked down miR-141-5p expression using anti-miR-141-5p to explore its role in TNF-α-induced cytotoxicity and apoptosis. As expected, anti-miR-141-5p reduced the expression of miR-141-5p more significantly in primary osteoblasts compared to anti-miR-NC (Figure 2(a)). CCK-8 and Annexin V-FITC/PI assays revealed that the knockdown of miR-141-5p partly reversed the inhibition of cell viability and the promotion of cell apoptosis induced by TNF-α when cells treated with PBS, TNF-α, TNF-α+anti-miR-NC, TNF-α+anti-miR-141-5p (1mg) or TNF-α+anti-miR-141-5p (2mg) respectively (Figures 2(b) and 2(c)). In line with this result, the increase of Bax and cleaved-Caspase-3 expression induced by TNF-α was inhibited by downregulating miR-141-5p (Figure 2(d)).

3.3. Characteristics of ADSCs、primary osteoblasts and ADSCs-derived exosomes

ADSCs isolated from C57/BL6 mice had a typical fibroblastic-like morphology (Figure 3(a)). Alizarin Red, Oil Red O and Alcian Blue staining was positive after the induced osteogenic, adipogenic and chondrogenic differentiation of ADSCs (Figure 3(b)). Primary osteoblasts isolated from C57/BL6 mice had shuttle, cone or cube morphology. Alkaline phosphatase staining and Alizarin red S staining were positive (Figure 3(c)). The immunoblotting showed that the ADSCs-Exos were positive for the exosomes' markers, including CD9, CD81, CD63 and Alix (Figure 3(d). Furthermore, primary osteoblasts were co-cultured with PKH26-labeled ADSCs-Exos and the red fluorescence of PKH26 label was observed in Primary osteoblasts 24 h later (Figure 3(e)), indicating primary osteoblasts could uptake ADSCs-Exos.

3.4. ADSCs-Exos attenuated the effect of TNF-α on primary osteoblasts.

To determine the effects of ADSCs-Exos, primary osteoblasts were treated with TNF-α (5 ng/ml) and different doeses of ADSCs-Exos (PBS, TNF-α, TNF-α+Exos (25mg), TNF-α+Exos (50mg), TNF-α+Exos (100mg). ADSCs-Exos attenuated the up-regulation of miR-141-5p induced by TNF-α dose-dependently (Figure 4(a)). Similarly, ADSCs-Exos reversed the inhibition of cell viability caused by TNF-α in a does-dependent manner (Figure 4(b)). The elevated protein expression of cleaved caspase-3 and Bax induced by TNF-α was suppressed after co-culture with ADSCs-Exos (Figure 4(c)). In line with this, the results of flow cytometry indicated ADSCs-Exos does-dependently blocked cell apoptosis induced by TNF-α (Figure 4(d)).

3.5. KCNQ1OT1-Exos inhibited TNF-α-induced cytotoxicity and apoptosis of primary osteoblasts.

ADSCs were transfected with LV-KCNQ1OT1 or LV-NC for the determination of whether ADSCs transfected LV-KCNQ1OT1 into secreted exosomes. At 24 h post LV-KCNQ1OT1 transfection, the expression of KCNQ1OT1 in ADSCs or exosomes derived from the ADSCs was elevated, but the expression of miR-141-5p was down-regulated compared with that in LV-NC treated group (Figures 5(a) and 5(b)). In order to confirm whether ADSCs-Exos carrying LV-KCNQ1OT1 could deliver KCNQ1OT1 into primary osteoblasts, primary osteoblasts were co-cultured with LV-NC-Exos or LV-KCNQ1OT1-Exos. KCNQ1OT1 expression was up-regulated in primary osteoblasts treated with LV-KCNQ1OT1-Exos (Figure 5(c)). Next, to explore whether KCNQ1OT1-Exos could influence TNF-α-induced cytotoxicity and

apoptosis, primary osteoblasts were treated with TNF-α and then co-cultured with medium, ADSCs-Exos, LV-NC-Exos or LV-KCNQ1OT1-Exos. The results of CCK-8 showed the co-culture of primary osteoblasts with LV-KCNQ1OT1-Exos mitigated the negative effect of TNF-α on cell viability; ADSCs-Exos exerted a weaker stimulative effect on cell viability compared to LV-KCNQ1OT1-Exos (Figure 5(d)). Flow cytometry analysis indicated that TNF-α-induced cell apoptosis was reversed when primary osteoblasts were co-cultured with LV-KCNQ1OT1-Exos; ADSCs-Exos exerted a weaker inhibitory effect on cell apoptosis compared to LV-KCNQ1OT1-Exos (Figure 5(e)). Consistent with that, the expression of Bax and cleaved caspase-3 in primary osteoblasts was blocked after co-culture of LV-KCNQ1OT1-Exos (Figure 5(f)).

3.6. KCNQ1OT1 could sponge miR-141-5p

Given KCNQ1OT1 sequence contains potential binding sites for miR-141-5p, we predicted KCNQ1OT1 could sponge miR-141-5p (Figure 6(a)). The dual-luciferase reporter assay was conducted to confirm the combination between them. As the result showed, overexpressed miR-141-5p weakened the luciferase activity in KCNQ1OT1-WT group obviously, but the activity of luciferase reporters containing KCNQ1OT1-MUT was not changed significantly in HEK293 and primary osteoblasts (Figures 6(b) and 6(c)). Compared with ADSC-Exos and LV-NC-Exos, LV-KCNQ1OT1-Exos blocked the inhibitory effect of miR-141-5p on the luciferase activity of reporters containing KCNQ1OT1-WT (Figure 6(d)). Overexpressed KCNQ1OT1 decreased the expression of miR-141-5p, yet the down-regulation of KCNQ1OT1 increased that (Figure 6(e)). Moreover, when primary osteoblasts cultured with LV-KCNQ1OT1-Exos, the expression of miR-141-5p was inhibited (Figure 6(f)).

3.7. KCNQ1OT1-Exos inhibited the effect of TNF-α in primary osteoblasts by sponging miR-141-5p

Primary osteoblasts were transfected with miR-141-5p or miR-NC to confirm whether KCNQ1OT1-Exos attenuate TNF-α-induced cytotoxicity and apoptosis by acting as an miR-146a sponge. After the treatment of TNF-α, primary osteoblasts were transfected with miR-141-5p or miR-NC and co-cultured with ADSCs-Exos, LV-NC-Exos or LV-KCNQ1OT1-Exos. The up-regulation of miR-141-5p promoted the inhibitory effect of TNF-α on cell viability and co-culture with ADSCs-Exos or KCNQ1OT1-Exos partly reversed this phenomenon; ADSCs-Exos exerted a weaker stimulative effect on cell viability compared to LV-KCNQ1OT1-Exos (Figure 7(a)). Similarly, the up-regulation of miR-141-5p enhanced cleaved caspase-3 and Bax expression. However, these effects were attenuated following co-culture with ADSC-Exos or LV-KCNQ1OT1-Exos (Figure 7(b)). In line with this, as flow cytometry showed overexpressed miR-141-5p promoted cell apoptosis but when co-cultured with ADSC-Exos or LV-KCNQ1OT1-Exos, the cell apoptosis was inhibited; ADSCs-Exos exerted a weaker inhibitory effect on cell apoptosis compared to LV-KCNQ1OT1-Exos (Figure 7(c)).

Discussion

With the deepening understanding of osteobiology, skeletal stem cells and osteoblasts are identified as significant target in the treatment of osteoporosis by promoting bone formation and remodeling [25]. Previous studies have proven that decreasing miR-10a-3p and raising CXCL12 expression can induce the

osteogenic differentiation of BMSCs [26]. miR-26b could promote BMSCs osteogenesis through directly regulating GSK3β and activating Wnt pathway [27]. MSCs-derived exosomes are involved in multiple physiology and pathology processes, including osteogenesis, bone regeneration and osteoarthritis [28, 29]. In addition, BMSCs-derived exosomal MALAT1 was proved to be able to promote the progression of osteoblasts [30].

Similar to BMSCs, ADSCs also have the potential of multi-lineage differentiation. In 2001, Zuk et al [31] firstly isolated ADSCs from adipose tissue and found they could differentiate into adipocytes, osteoblasts and chondrocytes under different induction conditions. In addition to the commonness with BMSCs, ADSCs can be easily obtained with abundant sources. Depending on this, ADSCs might be a promising choice in the treatment of osteoporosis. However, few studies have investigated the roles of ADSCs-Exos, especially in the treatment of osteoporosis. Thus, whether ADSCs-Exos could effectively protect primary osteoblasts from the TNF-α-induced cytotoxicity and apoptosis was studied in the present study.

Our results indicated that TNF-α could increase miR-141-5p expression and inhibit the cell proliferation in primary osteoblasts. It could also promote cell apoptosis. Consistent with this, the expression of cleaved caspase-3 and Bax was also elevated. Currently, ADSCs have been widely used in tissue regeneration and bioengineering. However, with the in-depth investigations, the application of ADSCs has the potential risk of iatrogenic infection, malignant transformation, immune rejection safety issues [32-34]. Compared to ADSCs, ADSCs-Exos have no risk of malignant transformation and can hardly cause the immune rejections [35, 36]. Therefore, ADSCs-Exos can be promising regenerative agents for osteoporosis. To make clear the effects of ADSCs-Exos in osteoporosis, we attempted to culture TNF-α-treated primary osteoblasts with ADSCs-Exos. Interestingly, ADSCs-Exos promoted cell viability and impeded apoptosis, suggesting that ADSCs-Exos can be promising candidates in the treatment of OP.

Although we've found ADSCs-Exos can be beneficial in the treatment of OP, the underlying mechanism has not been revealed. Exosomes derived from MSCs contain multiple lncRNAs, which can be transported and transferred to other cells to regulate biological functions through targeting downstream genes [37, 38]. In osteoarthritis, exosomal KLF3-AS1 originated from hMSCs boosted cartilage repair and chondrocyte proliferation [39]. KCNQ1OT1, a lncRNA which is closely related to cell proliferation, migration and apoptosis, has been reported to be an oncogene in a variety of tumors[40]. Evidence has showed that KCNQ1OT1 can promote cell proliferation and migration [41]. KCNQ1OT1 was found to enhance glioma cell proliferation and invasion via regulating the miR-375/YAP pathway and thereby accelerated the progression of glioma[42]. Meanwhile, in colorectal cancer, KCNQ1OT1 can induce cell apoptosis by sponging miR-329-3p[40]. Herein, KCNQ1OT1 was targeted for further investigation in this

study. We confirmed the presence of KCNQ1OT1 in ADSCs-Exos, and further investigated the role of KCNQ1OT1 in the treatment of osteoporosis. KCNQ1OT1-Exos had a more significant inhibitory effect on TNF-α-induced cytotoxicity and apoptosis compared to ADSCs-Exos. Therefore, KCNQ1OT1-Exos are expected to be superior candidates in osteoporosis treatment.

LncRNAs can function as miRNA sponges by binding miRNAs [43]. As previously reported, lncRNA-HOTAIR can induce the apoptosis of osteoblasts via modulating the expression of miR-138 [44]. In postmenopausal osteoporosis, lncRNA LOXL1-AS1 regulates osteogenic and adipocytic differentiation of BMSCs via sponging miR-196a-5p [45]. For further mechanistic investigations, we predicted KCNQ1OT1 sequence contained miR-141-5p binding sites *via* bioinformatics analysis. As reported before, miR-141-5p promoted preeclampsia via regulating MAPK1/ERK2 signaling [46]. In chronic myeloid leukemia, microRNA-141-5p acts as a tumor suppressor by downregulating RAB32 [47]. However, the role of miR-141-5p played in osteoporosis has not been reported. In TNF-α-treated primary osteoblasts, we found increased expression of miR-141-5p. Moreover, the knock-down of miR-141-5p promoted cell viability and inhibited cell apoptosis after the treatment of TNF-α. As dual-luciferase reporter assay showed, miR-141-5p was proved to be the target gene of KCNQ1OT1. Furthermore, the rescue experiments revealed that when co-cultured with KCNQ1OT1-Exos, the effects induced by miR-141-5p on cell viability and apoptosis in TNF-α-treated primary osteoblasts were partly reversed, suggesting that KCNQ1OT1-Exos functioned by sponging miR-141-5p.

Conclusions

We demonstrated that ADSCs-Exos attenuated the effect of TNF-α on primary osteoblasts. KCNQ1OT1-Exos had a more significant inhibitory effect on TNF-α-induced cytotoxicity and apoptosis compared to ADSCs-Exos. KCNQ1OT1 exerted its role by sponging miR-141-5p, suggesting that KCNQ1OT1-Exos might provide a potential precise target for osteoporosis. Further explorations of the pleiotropic effect of KCNQ1OT1 and the crosstalk between KCNQ1OT1 and miR-141-5p will provide new insights for developing new treatments to improve the therapeutic efficacy based on ADSCs-Exos.

Declarations

Data Availability

The data used to support the findings of this study are available from the corresponding authors upon request.

Authors' contributions

SZW performed the experiments, collected data, and drafted the manuscript. JJ participated in the experimental design and experimentation, and helped collect the data. CHC took part in the study design

and revised the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Figures

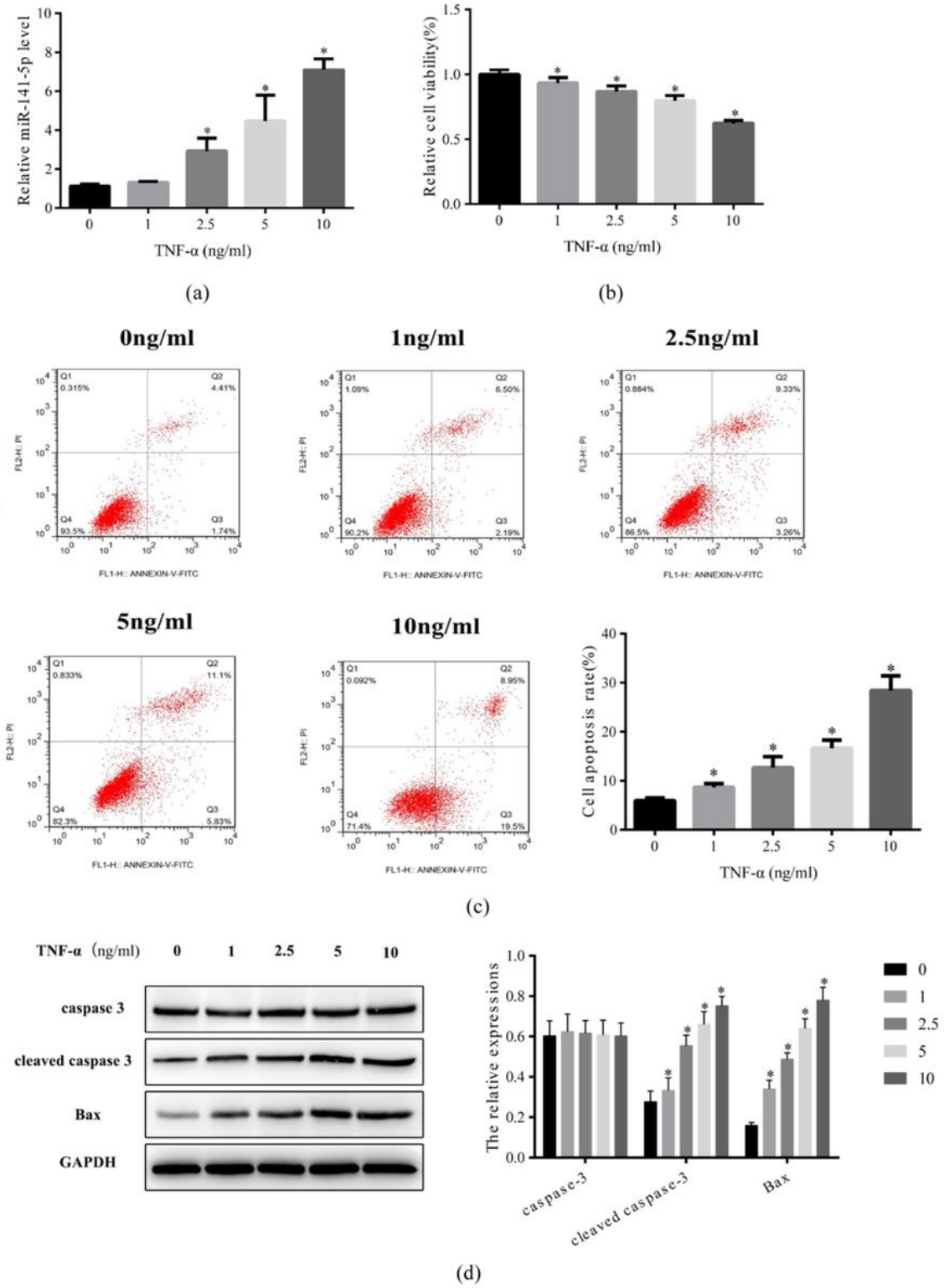

Figure 1

TNF-α increased miR-141-5p expression, suppressed the viability and promoted the apoptosis of primary osteoblasts in a dose-dependent manner. (a) TNF-α dose-dependently increased miR-141-5p expression as detected via qRT-PCR. (b, c) TNF-α reduced cell viability and promoted cell apoptosis in a dose-dependent manner as detected by CCK-8 assay and flow cytometry respectively. (d) TNF-α increased the protein expression level of Bax and cleaved-Caspase-3 dose-dependently as detected by immunoblotting. Significant differences ($P < 0.05$) are indicated by asterisks.

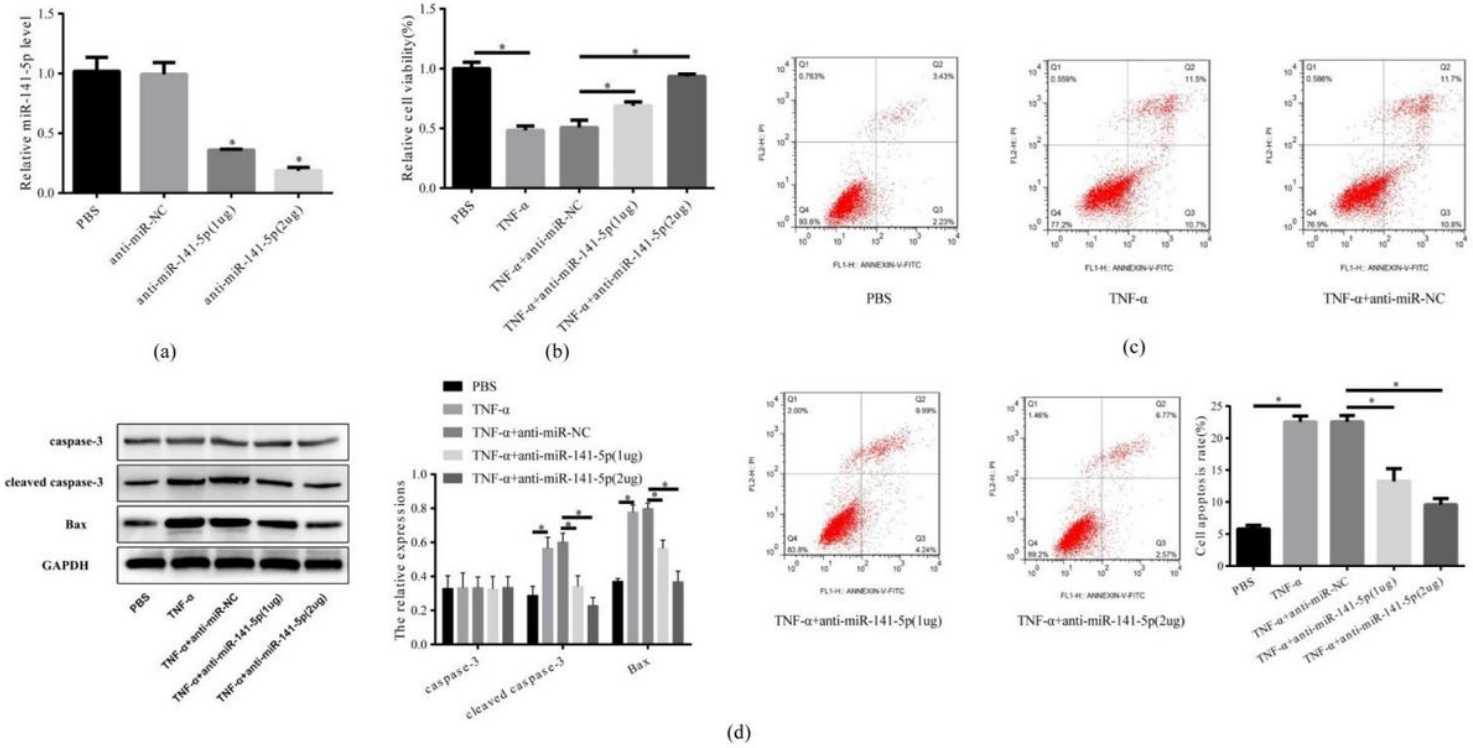

Figure 2

Knockdown of miR-141-5p reversed the effect of TNF-α on primary osteoblasts. (a) anti-miR-141-5p reduced the expression of miR-141-5p more significantly in primary osteoblasts compared to anti-miR-NC. (b, c) CCK-8 and Annexin V-FITC/PI assays revealed that the knockdown of miR-141-5p partly reversed the inhibition of cell viability and the promotion of cell apoptosis induced by TNF-α when cells treated with PBS, TNF-α, TNF-α+anti-miR-NC, TNF-α+anti-miR-141-5p (1μg) or TNF-α+anti-miR-141-5p (2μg) respectively. (d) The increase of Bax and cleaved-Caspase-3 expression induced by TNF-α was inhibited by downregulating miR-141-5p. Significant differences ($P < 0.05$) are indicated by asterisks.

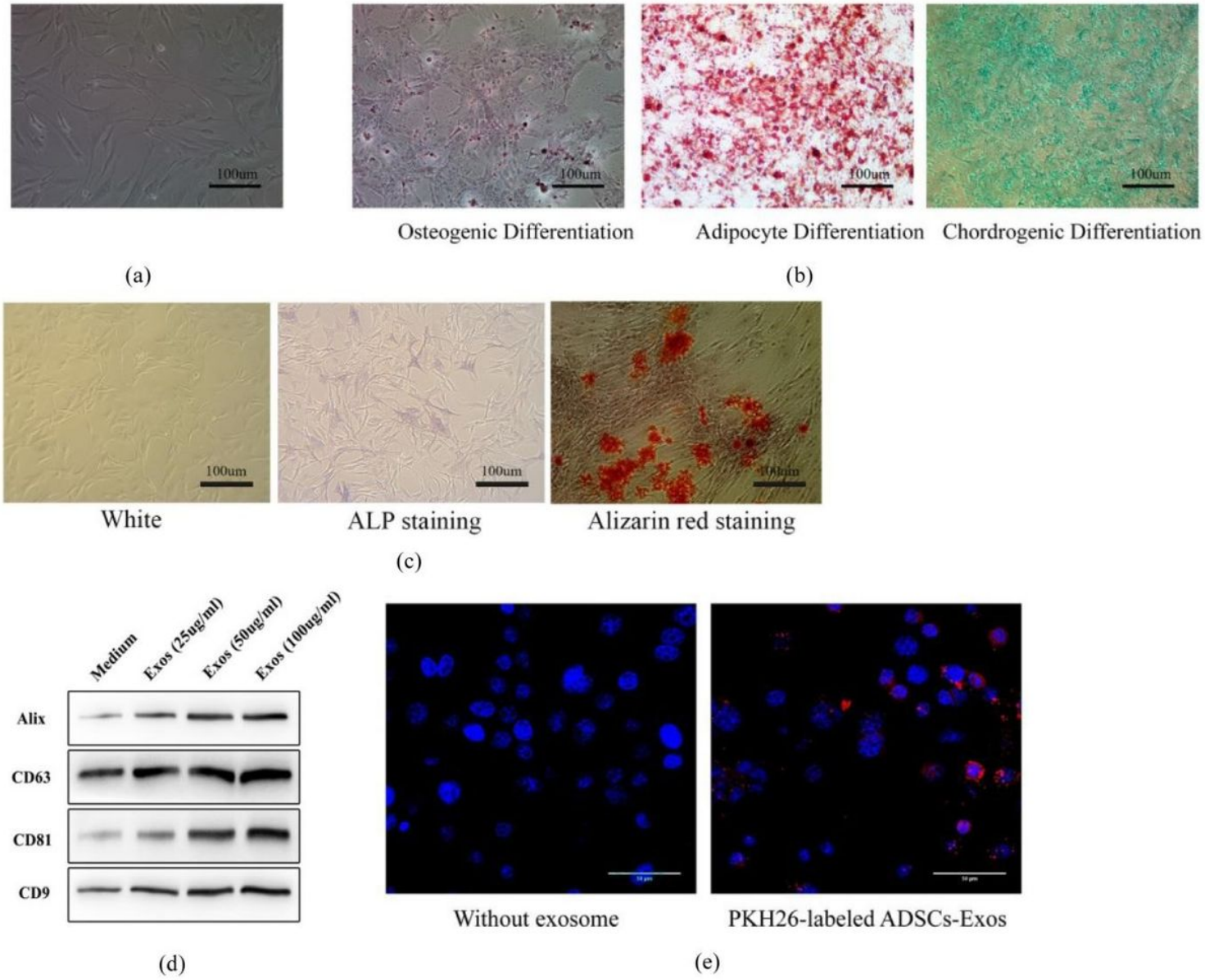

Figure 3

Characteristics of ADSCs, primary osteoblasts and ADSCs-derived exosomes. (a) The isolated ADSCs exhibited typical fibroblastic-like morphology. (b) Alizarin Red, Oil Red O and Alcian Blue staining was positive after the induced osteogenic, adipogenic and chondrogenic differentiation of ADSCs. (c) Immunoblotting showed that ADSCs-Exos were positive for Alix, CD63, CD81 and CD9. (d) Primary osteoblasts could uptake the PKH26-labeled ADSCs-Exos. (e) Primary osteoblasts exhibited shuttle, cone or cube morphology; Alkaline phosphatase staining and Alizarin red S staining were positive. Red: PKH26-labeled ADSCs-Exos. Blue: nuclei.

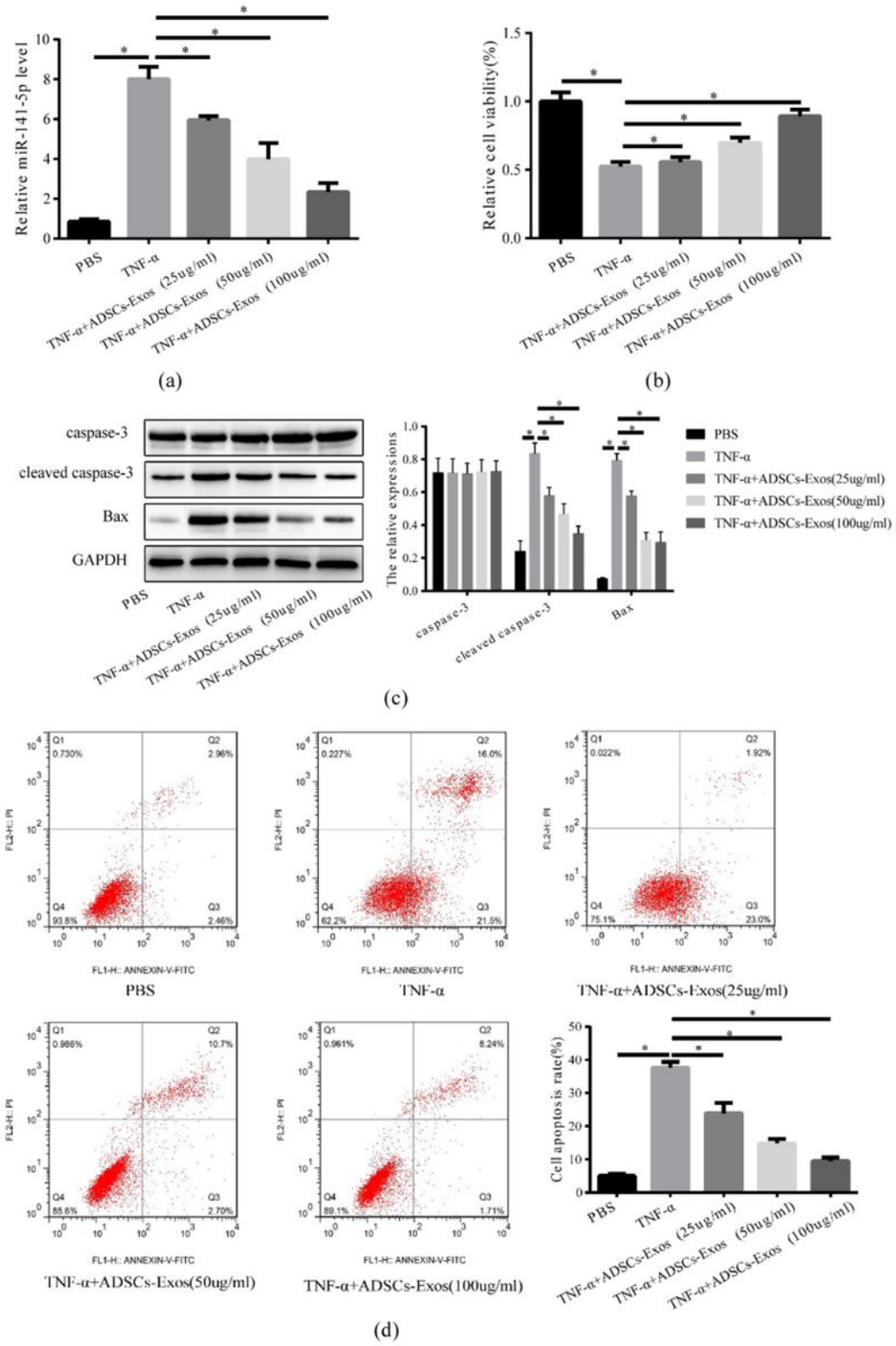

Figure 4

ADSCs-Exos attenuated the effect of TNF-α on primary osteoblasts. (a) After primary osteoblasts were treated with TNF-α (5 ng/ml) and different dose of ADSCs-Exos (PBS, TNF-α, TNF-α+Exos (25μg), TNF-α+Exos (50μg), TNF-α+Exos (100μg)), ADSCs-Exos dose-dependently decreased TNF-α induced miR-141-5p expression by qRT-PCR. (b) CCK-8 assay revealed that ADSCs-Exos mitigated the inhibition of TNF-α on cell viability in a dose-dependent manner. (c) Immunoblotting showed that ADSCs-Exos dose-

dependently decreased the promotion of TNF-α on cleaved caspase-3 and Bax expression. (d) Flow cytometry analysis showed that ADSCs-Exos dose-dependently reversed TNF-α-induced cell apoptosis. Significant differences (P < 0.05) are indicated by asterisks.

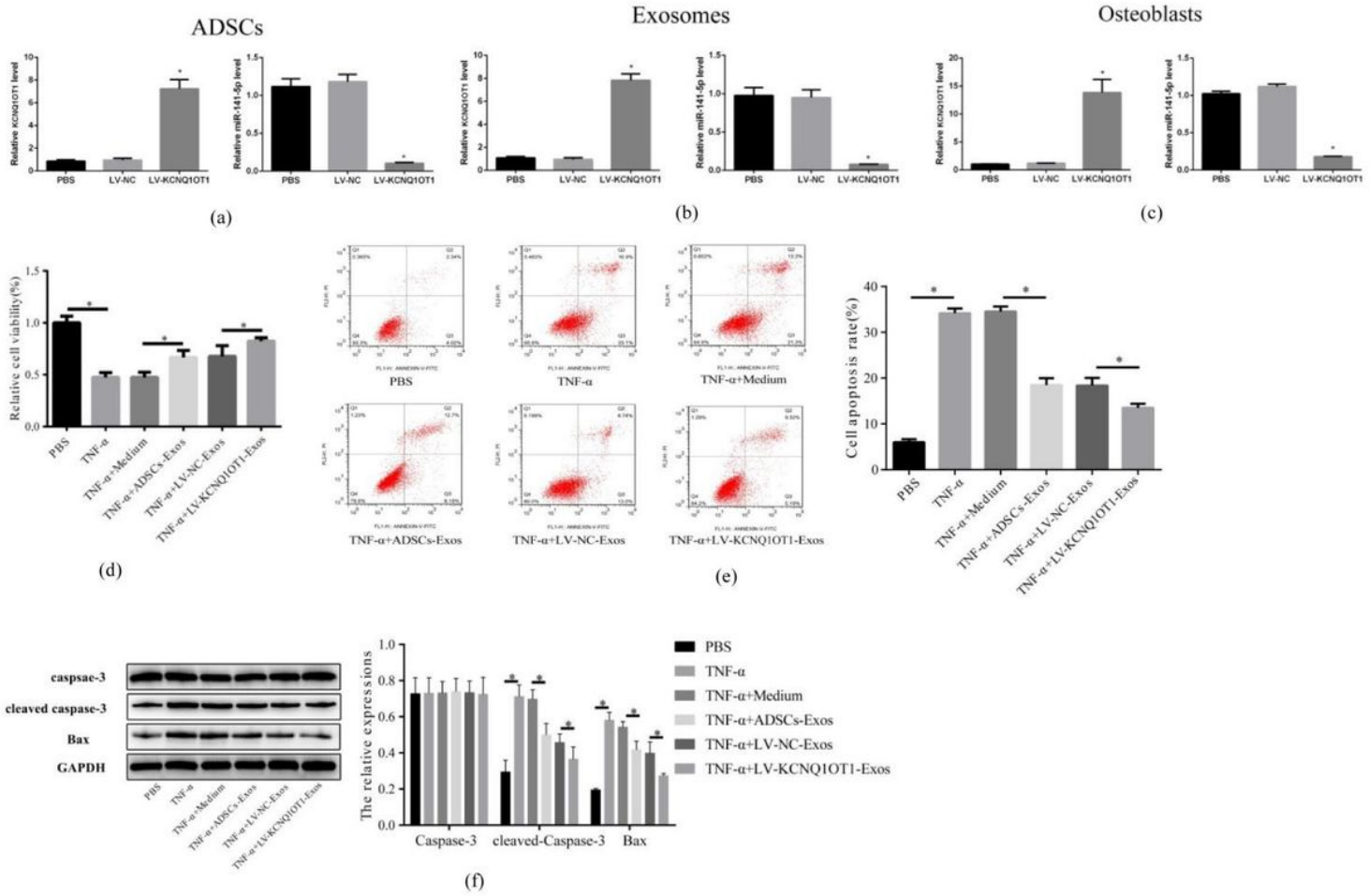

Figure 5

KCNQ1OT1-Exos inhibited TNF-α-induced cytotoxicity and apoptosis of primary osteoblasts. (a, b) The expression of KCNQ1OT1 in ADSCs or exosomes derived from the ADSCs was elevated, but the expression of miR-141-5p was down-regulated compared with that in LV-NC treated group after the transfection of LV-KCNQ1OT1. (c) KCNQ1OT1 expression was up-regulated in primary osteoblasts treated with LV-KCNQ1OT1-Exos compared to NC-Exos. (d) In primary osteoblasts, LV-KCNQ1OT1-Exos mitigated the negative effect of TNF-α on cell viability, while ADSCs-Exos exerted a weaker stimulative effect on cell viability compared to LV-KCNQ1OT1-Exos. (e) When primary osteoblasts were co-cultured with LV-KCNQ1OT1-Exos, the TNF-α-induced cell apoptosis was reversed and ADSCs-Exos exerted a weaker inhibitory effect on cell apoptosis compared to LV-KCNQ1OT1-Exos. (f) The expression of Bax and cleaved caspase-3 in primary osteoblasts was blocked after co-culture of LV-KCNQ1OT1-Exos . Significant differences (P < 0.05) are indicated by asterisks.

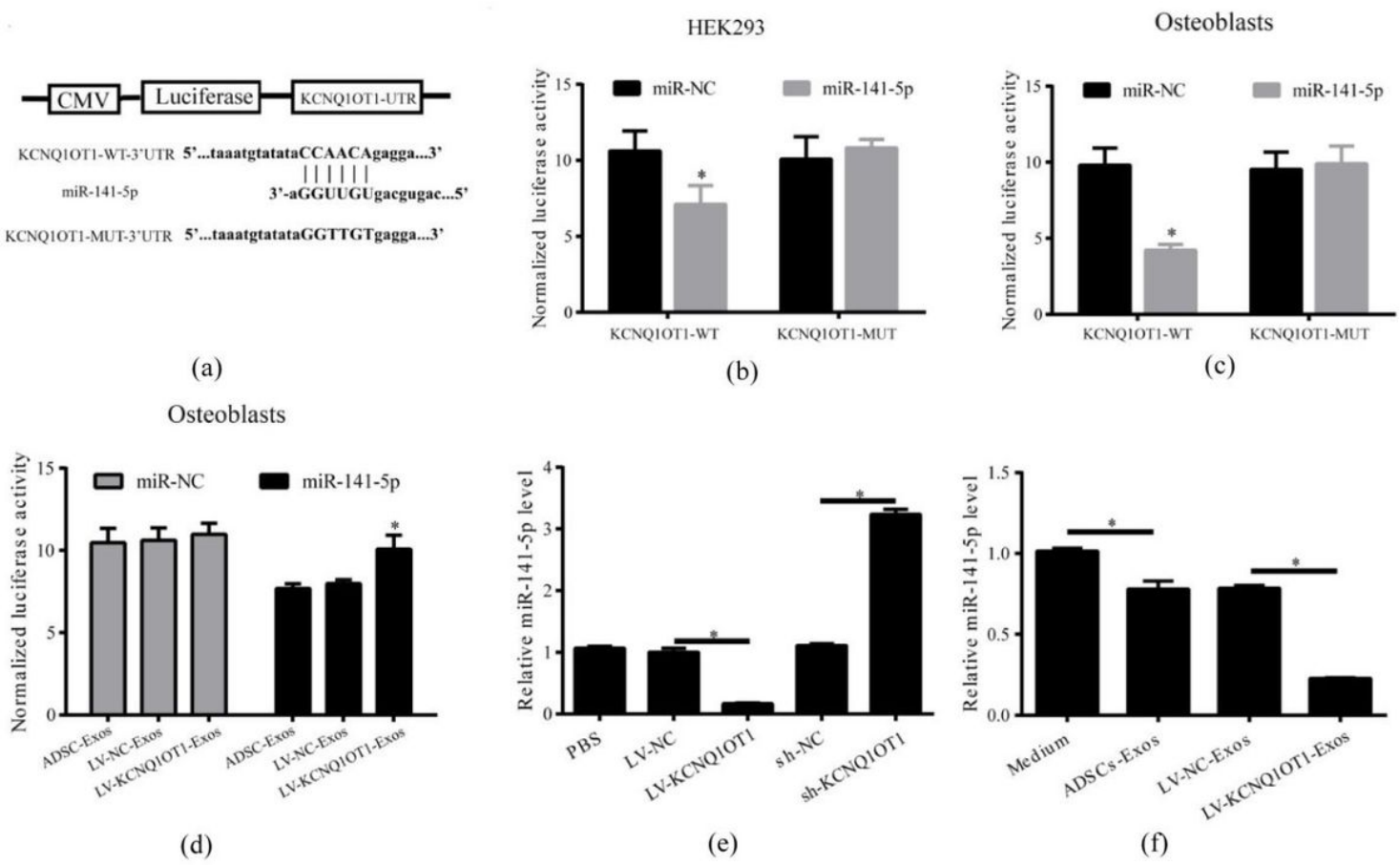

Figure 6

KCNQ1OT1 could sponge miR-141-5p. (a) KCNQ1OT1 and miR-141-5p have potential binding sites. (b,c) As dual-luciferase reporter assay showed, miR-141-5p weakened the luciferase activity in KCNQ1OT1-WT group obviously, but the activity of luciferase reporters containing KCNQ1OT1-MUT was not changed significantly in both HEK293 and primary osteoblasts. (d) LV-KCNQ1OT1-Exos blocked the inhibitory effect of miR-141-5p on the luciferase activity of reporters containing KCNQ1OT1-WT compared with ADSC-Exos and LV-NC-Exos. (e) KCNQ1OT1 suppressed the expression of miR-141-5p, but the sh-KCNQ1OT1 increased that. (f) The expression of miR-141-5p was down-regualted when primary osteoblasts co-cultured with LV-KCNQ1OT1-Exos. Significant differences ($P < 0.05$) are indicated by asterisks.

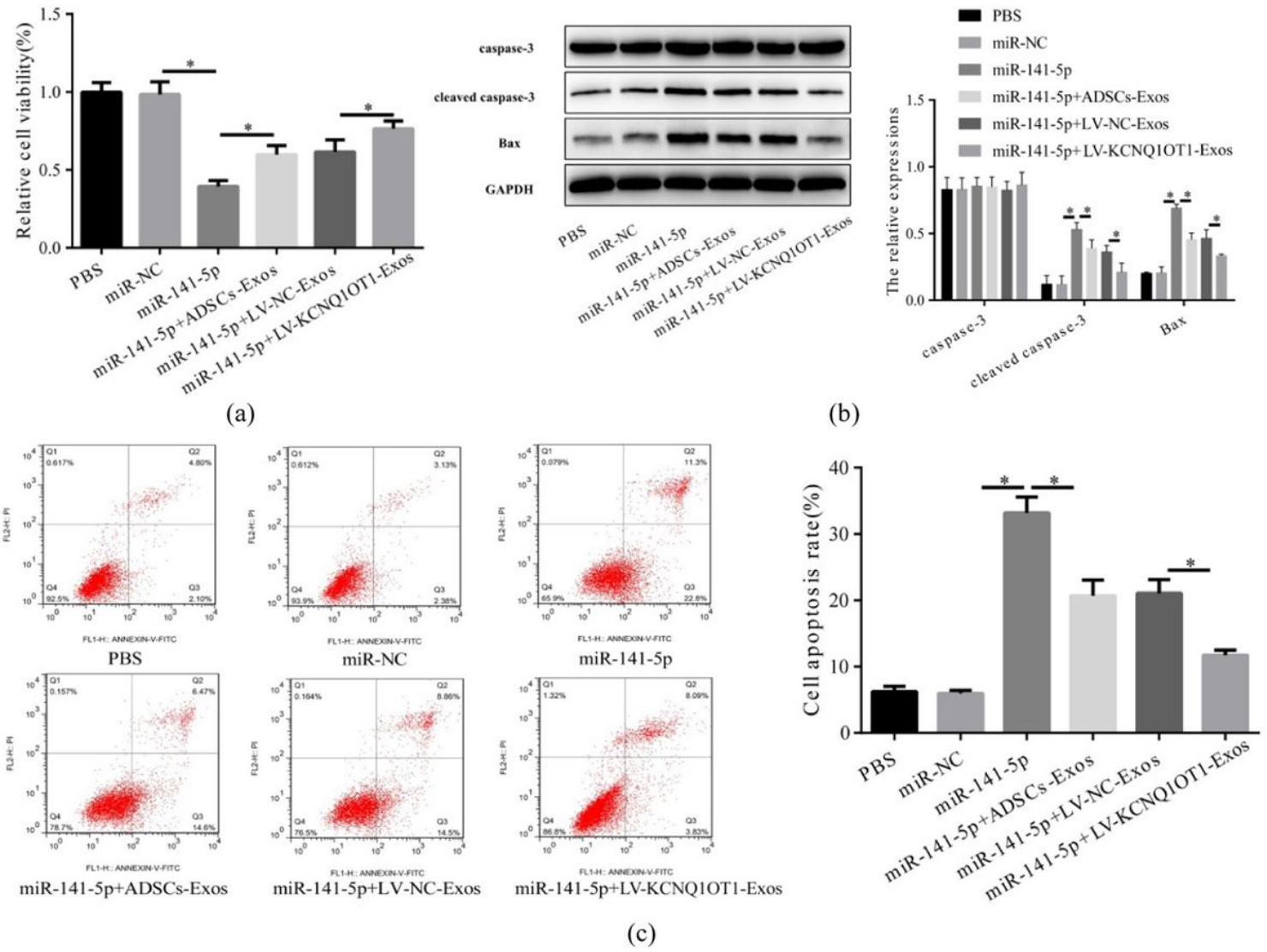

Figure 7

KCNQ1OT1-Exos inhibited the effect of TNF-α in primary osteoblasts by sponging miR-141-5p. (a) Primary osteoblasts were treated with TNF-α and then treated with miR-NC, miR-141-5p, miR-141-5p+ADSCs-Exos, miR-141-5p+LV-NC-Exos or miR-141-5p+LV-lncRNA-Exos respectively. (b) The inhibitory effect of TNF-α on cell viability was blocked by miR-141-5p and the treatment of ADSCs-Exos or KCNQ1OT1-Exos partly reversed this phenomenon; ADSCs-Exos exerted a weaker stimulative effect on cell viability compared to LV-KCNQ1OT1-Exos. MiR-141-5p enhanced the expression of cleaved caspase-3 and Bax but was attenuated following co-culture with ADSC-Exos or LV-KCNQ1OT1-Exos. (c) As flow cytometry showed, miR-141-5p promoted cell apoptosis while the treatment of ADSC-Exos or LV-KCNQ1OT1-Exos inhibited that; ADSCs-Exos exerted a weaker inhibitory effect on cell apoptosis compared to LV-KCNQ1OT1-Exos. Significant differences ($P < 0.05$) are indicated by asterisks. Exos: exosomes, ADSCs-Exos: exosomes derived from adipose-derived stem cells

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