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The role of miR-31-modified adipose tissue-derived stem cells in repairing rat critical-sized calvarial defects

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ABSTRACT

With the increasing application of microRNAs (miRNAs) in the treatment and monitoring of different diseases, miRNAs have become an important tool in biological and medical research. Recent studies have proven that miRNAs are involved in the osteogenic differentiation of stem cells. However, few studies have reported the use of miRNA-modified adult stem cells to repair critical-sized defects (CSDs) using tissue engineering technology. It is known that miR-31 is a pleiotropically acting miRNA that inhibits cancer metastasis and targets special AT-rich sequence-binding protein 2 (Satb2) in fibroblasts. However, it is not clear whether the function of miR-31 is to enhance adipose tissue-derived stem cell (ASC) osteogenesis, along with its association with Satb2, during osteogenic differentiation and bone regeneration. In this study, we systematically evaluated the function of miR-31 in enhancing ASC osteogenesis and the therapeutic potential of miR-31-modified ASCs in a rat CSD model with β-tricalcium phosphate (β-TCP) scaffolds. ASCs were treated with lentivirus (Lenti)-miR-31, Lenti-as-miR-31 (antisense) or Lenti-NC (negative control). These genetically modified ASCs were then combined with β-TCP scaffolds to repair CSDs in rats. The results showed that in cultured ASCs in vitro, Lenti-as-miR-31 significantly enhanced osteogenic mRNA and protein expression when compared with the Lenti-NC group. Moreover, we firstly found that a Runt-related transcription factor 2 (Runx2), Satb2 and miR-31 regulatory loop triggered by bone morphogenetic protein-2 (BMP-2) plays an important role in ASCs' osteogenic differentiation and bone regeneration. More importantly, we found that miR-31-knockdown ASCs dramatically improved the repair of CSDs, including increased bone volume, increased bone mineral density (BMD) and decreased scaffold residue *in vivo*. These data confirm the essential role of miR-31-modified ASCs in osteogenesis *in vitro* and *in vivo*.

1. Introduction

It is still difficult to repair critical-sized defects (CSDs) following trauma or tumor resection in the clinic. Autografts, which are osteoconductive and osteoinductive, set the gold standard for the clinical repair of bone defects. However, the disadvantages include infection, pain, loss of function and restricted supply due to donor shortage and morbidity [1]. With the recent development of materials and gene therapy, tissue engineering technology has become one of the most promising therapeutic approaches for bone regeneration in bone defects [2–4]. Due to their function in stimulating stem cell osteogenesis and angiogenesis, growth factors play an important role when tissue-engineered bone is used to repair a bone defect. Previous studies demonstrated that many factors, such as bone morphogenic proteins (BMPs), vascular endothelial growth factor (VEGF), hypoxia-inducible factor-1α (HIF-1α) and basic fibroblast growth factor (bFGF), can stimulate osteogenesis and angiogenesis in bone defects using a local gene-enhanced tissue-engineering method [4–6].

With the increasing application of microRNAs (miRNAs) in the treatment and monitoring of different diseases, miRNAs have become an important tool in biological and medical research. Recently, studies have proven that miRNAs are involved in the osteogenic differentiation of stem cells. More importantly, studies have shown that the use of miRNA gene-modified mesenchymal stem cells (MSCs) as seed cells is a promising approach for tissue regeneration [7,8].

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miRNAs, which are evolutionarily conserved, small noncoding RNAs (approximately 22 nucleotides long), are involved in nearly every biological process, including stem cell differentiation [9,10]. miRNAs bind to the 3′-UTR region of key transcription factors and regulate mRNA stability or protein translation. Consequently, miRNAs play an important role in regulating stem cell self-renewal and differentiation [10]. Recent microarray analysis has revealed a cohort of miRNAs (miR-204, miR-138, miR-30 and miR-23a) that function in regulating osteogenic differentiation [11–15]. Although various miRNAs regulate cell proliferation and differentiation, there is only a limited amount of information about the regulatory mechanisms of miRs in osteogenesis.

miR-31, formerly reported to be a pleiotropically acting miRNA that inhibits breast cancer metastasis [16], also targets special AT-rich sequence-binding protein 2 (Satb2) in cancer-associated fibroblasts [17]. Satb2, which is considered to be a positive regulator of multiple osteoblast-specific genes, plays an important role in osteoblast differentiation and bone development [18]. The overexpression of Satb2 can significantly promote the osteogenic differentiation of stem cells and accelerate bone regeneration, allowing the osseointegration of implants [3,19]. However, it is still not clear whether the regulation of Satb2 by miR-31 occurs during cell differentiation and how miR-31 monitors osteogenic differentiation and bone regeneration.

Due to easy isolation, relative abundance, rapid expansion and multipotency, adipose tissue-derived stem cells (ASCs) have received attention in the field of tissue engineering [1,20,21]. Previous studies have proven that an osteoinductive factor can enhance the osteogenic differentiation and bone regeneration of ASCs, such as bone morphogenetic protein-2 (BMP-2) [22,23]. Certain miRs (miR-29b and miR-2861) play important physiological roles in osteoblast differentiation and ossification [24,25], and other miRs (miR-196b, miR-17 and miR-106a) monitor the osteogenic differentiation of ASCs [26,27]. However, whether miR-31 can regulate osteogenesis in ASCs through signaling pathways of a regulatory loop and the *in vivo* role that miR-31 plays in bone formation in tissue-engineered bone are still unknown.

Runt-related transcription factor 2 (Runx2), an upstream regulator of Satb2 during osteogenic differentiation, has been identified as an early key transcription factor regulating osteogenesis [28]. The low expression of these regulatory factors in undifferentiated stem cells maintains cells' differentiation potential, and the regulation of key transcription factors, such as Runx2 or Satb2, may affect osteogenic differentiation [29,30]. Previously published data showed that the expression of osteogenesis-associated miRNAs was regulated by Runx2 [11,31], and miR-31 exhibited low expression during osteogenesis, as determined by microarray analysis [32]. Therefore, it would be interesting to confirm the role of miR-31 in enhancing ASC osteogenesis *in vitro* and *in vivo* using tissue engineering technology and to detect the mechanism by which miR-31 expression is negatively regulated during osteogenesis.

Porous β-tricalcium phosphate (β-TCP) is a synthetic and biodegradable ceramic material that has been commonly used in various applications in oral surgery [33] and has also been widely explored in the repair of bone defects [34]. Porous β-TCP is also known to be a material with good osteo-conductive capacity due to its high porosity and interconnectivity, which can facilitate intercellular communication between osteogenic cells resting in the lacunae [33,35]. Therefore, β-TCP scaffolds were used as cell carriers in our *in vivo* studies.

In this study, we systematically explored the osteogenesis of miR-31-modified stem cells *in vitro*, investigated the mechanism of miR-31's enhancement of ASCs' osteogenic differentiation and evaluated the bone-forming function of miR-31-induced ASCs in tissue-engineered bone in a rat CSD model.

2. Materials and methods

2.1. Isolation and culture of rat ASCs

F344 rats were obtained from the Shanghai Animal Experimental Center, and all procedures were approved by the Animal Research Committee of Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine. The fat pad was harvested from the inguinal area of 10-week-old male rats and minced with scissors. ASCs were isolated according to a reported protocol [36]. Briefly, the minced tissues were digested in a 0.1% collagenase solution (Sigma, St. Louis, MO, USA) and incubated at 37 °C with vigorous shaking for 1 h. The tissue was centrifuged, and the pellet was resuspended in normal medium. Next, the cells were plated in a 10 cm dish, cultured in 10 ml Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin and moved into an incubator (Thermo Fisher Scientific Inc.) at 37 °C in an atmosphere of 5% CO_2. To generate osteoinductive cells, 200 ng/ml BMP-2 (BD Biosciences, San Jose, CA, USA) was added to the culture medium, which was refreshed every 3 days. Flow cytometry was used to characterize ASCs with CD29, CD31, CD34, CD44, CD45 and CD90 staining (BD Biosciences) [21,37].

2.2. Construction and transfection of expression vectors

The total RNA extracted from MC3T3-E1 cells was used to generate cDNA by reverse transcription polymerase chain reaction PCR using a PrimeScript™ RT Reagent Kit (TaKaRa, Dalian, China). The cDNA of the PCR products was confirmed by DNA sequencing. After digestion with BglII and NotI (Promega, Madison, WI, USA) and agarose gel purification, the PCR products were subcloned into pCMV-myc. The Runx2 primer sequences and vector construction procedure have been published previously [13]. Satb2 overexpression vectors (p-Satb2-wt) were obtained from Origene Technologies (Beijing, China). The Runx2 plasmid DNA (p-Runx2-wt) and p-Satb2-wt were transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

2.3. Transfection of miRNA mimics and small interfering RNAs

miR-31 oligonucleotides, consisting of mimics (miR-31), antisense (as-miR-31) and a negative control (miR-NC), and small interfering RNAs (siRunx2, siSatb2 and si-NC) were synthesized by GenePharma Co., Ltd (Shanghai, China). For transient transfections, 50 nm of miR-31, as-miR-31, miR-NC or siRNA was mixed with 5 μl of Lipofectamine 2000 in 200 μl of Opti-MEM (Invitrogen), and the mixture was directly added to cells in six-well plates at a density of 3×10^5 cells/well. The cells were harvested at 0, 16, 32 and 48 h after transfection. For long-term detection, miR mimics or inhibitors were repeatedly transfected every 3 days. The oligonucleotide sequences of siRunx2 and siSatb2 were 5′-CACGCUAUUAAAUCCAAAU-3′ and 5′-GAUCCAAUUACAAAGUUGU-3′, respectively.

2.4. Lentiviral vector construction and transduction

GFP-labeled plasmid vectors containing miR-31, as-miR-31 or miR-NC were obtained from OriGene Technologies, and 293T cells were maintained in DMEM supplemented with 10% FBS. Lentiviruses were produced by transfecting 293T cells with 10 μg of plasmid encoding miR-31 mimics or antisense or a negative control, 5 μg of Pax plasmid and 5 μg of VSVG plasmid using Lipofectamine 2000. The culture media was then changed the next day, and the supernatant was harvested after 48 h. The lentiviruses were filtered and concentrated by ultrafiltration, and aliquots were stored at −80 °C. For transduction, cells were incubated with the virus and 8 mg/ml polybrene for 24 h. Lentiviruses (Lenti-NC, Lenti-miR-31 or Lenti-as-miR-31) were added to reach an MOI of 50 for 24 h. The transfection efficiency of the lentiviral vectors was reflected by the GFP-positive proportion of ASCs detected using flow cytometry. The expression of miR-31 in the transduced cells was determined by qPCR analysis at days 0, 1, 4, 7, 14 and 21.

2.5. Quantitative real-time PCR analysis

The total RNA was extracted from the cultured cells with Trizol (Invitrogen), the concentration and purity of the total RNA were determined spectrophotometrically at OD260 nm and OD280 nm. The samples with OD260/280 nm ratios between 1.9 and 2.1 were used for cDNA synthesis. According to the manufacturer's recommendations, reverse transcription was performed with 1 μg of total RNA in a final volume of 20 μl using a PrimeScript™ RT Reagent Kit (TaKaRa). qPCR was performed as previously reported [38] using Power SYBR Green PCR Master Mix (Applied Biosystems) in combination with a 7500 Real-Time PCR Detection System. The primer sequences are shown in Supporting Information Table S1. The mature miR-31 expression levels were examined using an miRcute miRNA qPCR detection kit (Tiangen Biotech, Beijing, China). The relative mRNA or miRNA levels were expressed as the fold change relative to the untreated controls after normalization to the expression of GAPDH or U6, respectively.

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