miRNA-15a-5p facilitates the bone marrow stem cell apoptosis of femoral head necrosis through the Wnt/β-catenin/PPARγ signaling pathway

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Abstract. Bone marrow stem cells (BMSCs) are a group of cells that function as an underlying cell source for bone tissue regeneration. However, the molecular mechanisms of how BMSCs are induced into apoptosis remains unclear. In the present study, it was demonstrated that the molecular mechanisms of BMSCs were exerted via microRNA-15a-5p (miR-15a-5p) in femoral head necrosis (FHN). Briefly, miRNA-15a-5p expression was elevated in a rat model of FHN. Overexpression of miR-15a-5p promoted the apoptosis of BMSCs and reduced cell growth through the Wnt/β-catenin/peroxisome proliferator-activated receptor γ (PPARγ) signaling pathway. Downregulation of miR-15a-5p reduced the apoptosis of BMSCs and promoted cell growth through the Wnt/β-catenin/PPARγ signaling pathway. The activation of Wnt attenuated the effects of miR-15a-5p on the apoptosis of BMSCs via the β-catenin/PPARγ signaling pathway. In conclusion, the present results indicated that miRNA-15a-5p was involved in the regulation of the apoptosis of BMSCs through regulating the Wnt/β-catenin/PPARγ signaling pathway, which may serve an important role in the regulation of FHN.

Introduction

Femoral head necrosis (FHN) is a type of osteocyte apoptosis resulting from multiple causes (1). Osteonecrosis may easily cause the morphological change of caput femoris, subsequently resulting in the collapse of caput femoris and limiting the joint motion of bone marrow (1). Blood supply disorder is a common cause, which presents ischemic necrosis caused by a femoral neck fracture (2). The mechanism underlying non-traumatic FHN remains unclear, and potential etiology includes steroid-induced, alcoholic, idiopathic and hematological system diseases, amongst others (3). Due to the unclarified mechanism, there remains a lack of specialized treatment at an early stage. At later periods, the collapsed caput femoris results in the loss of joint function of the bone marrow, therefore meaning that joint replacement of the marrow cannot be adopted (4). However, due to the early onset age of FHN and unresolved abrasion and dislocation for the joint replacement prosthetics of marrow, the clinical treatment of FHN remains difficult (4).

Bone marrow stem cells (BMSCs) are non-hematopoietic cells with multi-directional differentiation potential in bone marrow (5). Their self-renewal ability results in their ability to maintain the versatility of stem cells. BMSCs may be divided into osteoblast, chondroblast and adipocyte, which may further secrete a series of growth factors to promote tissue regeneration (6). For instance, vascular endothelial growth factor (VEGF) may recruit endothelial cells and promote the vascularization and endothelialization of damaged blood vessels in ischemic tissues (7). It has been demonstrated that the activity and quantity of near-end BMSCs in patients with steroid-induced FHN are reduced in comparison with those with non-femoral head necrosis (8). To uncover the pathogenesis, researchers have combined medullary core decompression with autologous BMSC transplant for the early treatment of FHN, which have yielded a positive clinical effect (9).

Several studies have revealed that microRNAs (miRNAs/miRs) are associated with BMSCs (10,11). miRNAs are a group of small endogenous non-coding RNA molecules, composed of 21-25 nucleic acids. miRNAs generally target one or multiple mRNAs which regulate gene expression at the translational level or by degrading the target Mrna (11). miRNAs serve a notable role in cell proliferation, differentiation, apoptosis, biological development and the occurrence and development of diseases (12). The structure of free miRNAs is very stable and is able to bear the degradation of ribonuclease. Liu et al (13) reported that miRNA-15a-5p regulates VEGFA in endometrial mesenchymal stem cells.
miRNA-15a-5p has been confirmed to be an adipogenic transcription factor, serving a critical regulatory function in adipose cell differentiation (14). It possesses a role in adipogenesis and is able to restrain osteogenesis. C/EBPα/enhancer binding protein α (C/EBPα) and PPARγ are important transcription factors in the process of adipogenic differentiation (15), with heterogeneous expression transdifferentiating myoblasts into adipocytes. PPARγ and C/EBPα are two core transcription factors in the process of adipogenesis (15). Substantial attention has been focused on their methylation situation, however, previous reports on the methylation of PPARγ and C/EBPα are only limited to the lipogenesis process of adipose tissues, mesenchymal stem cells and predipocytes (11,16). In addition, the C/EBP family and PPAR family are two critical transcription factor families involved in modulating fat cell differentiation (17). C/EBP was initially discovered as a transcription factor family that serves a vital role in fat cell differentiation, mainly including C/EBPα and C/EBPβ (18). The transcription factor family is equipped with a transcription-activated area and adjacent leucine zipper motif (18).

When BMSCs are differentiated into chondrocytes, they are affected by multiple factors, including hormones and cytokines (19). Multiple signaling pathways regulate the differentiation of mesenchymal stem cells into chondrocytes, mainly including the fibroblast growth factor pathway, mitogen-activated protein kinase pathway, transforming growth factor β/bone morphogenetic protein pathway, SRY-box 9 signaling pathway and the Wnt/β-catenin signal pathway (20). Wnt/β-catenin is a newly-discovered pathway that is involved in regulating the differentiation of chondrocytes (21), which has been reported to regulate cellular morphology, function, cell-mediated immunity and stress, cellular carcinogenesis and apoptosis and participate in the development, differentiation, growth and apoptosis of cells (21). Shi et al (22) concluded that miR-15a-5p was involved in human adipocyte differentiation and obesity by regulating Wnt signaling. The present study examined the molecular mechanisms of miR-15a-5p in FHN.

**Materials and methods**

**In vivo model.** The animal study was ethically approved by the ethics committee of Hongqi Hospital Affiliated With Mudanjiang Medical University (Heilongjiang, China). Sprague-Dawley male adult rats (n=12, 160-180 g, 5-6 weeks old) were obtained from the Experimental Center of Chinese Medical Sciences University (Beijing, China) and housed in standard laboratory conditions (12 h light/dark cycle; 24–25°C; humidity, 50–55%) with ad libitum access to food and water. BMSCs were isolated from the femurs and tibias of the mice and cultured with α-MEM containing 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% foetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences). BMSCs (3x10^5 cells/ml) were trypsinized and plated into 25 cm^2 flasks in α-MEM containing 10% FBS once the cells reached 80% confluence at 37°C.

**RNA Isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was isolated from BMSCs using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to manufacturer's protocol. A total of 1-5 μl RNA was reverse transcribed using a HiFiMMLV cDNA kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's protocol. RT-qPCR was performed according to the manufacturer's protocol of the Quant SYBR Green PCR kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). PCR cycling conditions used 40 cycles (95°C for 30 sec and 60°C for 30 sec) subsequent to an initial denaturation step (94°C for 5 min). Primer sequences were as follows: miR-15a-5p forward, 5'-TAAAGGCACCCGGTTGAATGCC-3' and reverse, 5'-GCG AGCACAGATTAAATCGACTCAC-3'; U6 forward, 5'-GCT TGCCTGGCCAGCAGCATA-3' and reverse, 5'-TGCATG TCATCTTGGCTGCA-3'. Relative expression levels were calculated using the 2-ΔΔCq method (26).

**Gene expression profiling.** cDNA samples were Cy3-labeled using the SureTag DNA labeling kit (Agilent Technologies, Inc., Santa Clara, CA, USA) according to the manufacturer's protocol. Robust multi-array average background removal, quantile normalization, compensation for systematic technical differences and probe set summary were executed. The microarray data were submitted to Gene Expression Omnibus (accession number GSE80754) (27).

**Transfection.** miRNA-15a-5p (100 ng) and control negative plasmids (100 ng) were transfected into BMSCs (1x10^5 cell/ml) in 6 well plates using Lipofectamine RNAiMAX reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. β-catenin plasmid or 1 nM Aleglitazar (PPARγ agonist; Medchemexpress Co., Ltd., Shanghai, China)
were introduced into the cells by transfection alongside anti-miRNA-15a-5p or miRNA-15a-5p for 48 h at 37°C.

**Cell proliferation assay and lactate dehydrogenase (LDH) release assay.** A total of 20 µl Cell Counting Kit-8 solution (Beyotime Institute of Biotechnology, Haimen, China) were added to each well and incubated for 2 h at 37°C. The absorbance of the samples was measured at 450 nm with a spectrophotometric microplate reader (Bio-Rad 680; Bio-Rad Laboratories, Inc.). LDH activity levels were measured using LDH activity kits (C0017; Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocols. The absorbance of the samples were measured at 450 nm with a spectrophotometric microplate reader (Bio-Rad 680; Bio-Rad Laboratories, Inc.).

**Flow cytometry.** The Annexin V-fluorescein isothiocyanate (FITC) early apoptosis detection kit (Cell Signaling Technology, Inc., Danvers, MA, USA) according to the manufacturer's protocol was used to estimate apoptosis. BMSCs (1x10^5 cell/ml) were fixed with 4% paraformaldehyde for 15 min at room temperature. The cells were then stained with 10 μl Annexin V-FITC and 5 μl propidium iodide for 30 min on ice in the dark at room temperature. Apoptosis was measured using flow cytometry and analyzed using FlowJo 7.6.1 (FlowJo LLC, Ashland, OR, USA).

**Caspase-3/9 activities.** BMSCs were washed with ice-cold phosphate buffered saline and treated with RIPA buffer (Beyotime Institute of Biotechnology) for 30 min at 4°C. Supernatants were centrifuged at 12,000 x g at 4°C for 10 min and collected to measure the supernatant proteins using the BCA method (Beyotime Institute of Biotechnology). Total protein (10 μg) was used to measure caspase-3/9 activity levels using caspase-3/9 activity kits (C1116 or C1158, Beyotime Institute of Biotechnology). The absorbance of the samples were measured at 405 nm with a spectrophotometric microplate reader (Bio-Rad 680; Bio-Rad Laboratories, Inc.).

**Western blot analysis.** BMSCs were washed with ice-cold phosphate buffered saline and treated with RIPA buffer (Beyotime Institute of Biotechnology) for 30 min at 4°C. Supernatants were centrifuged at 12,000 x g at 4°C for 10 min and collected to measure the supernatant proteins using the BCA method (Beyotime Institute of Biotechnology). Total protein (50-100 μg) was subjected to electrophoresis on a 12.5% SDS-PAGE gel and then transferred onto polyvinylidene fluoride membranes (Bio-Rad Laboratories, Inc.). The membrane was blocked with tris-buffered saline with 0.1% Tween-20 containing 5% fat-free dried milk at 37°C for 1 h and incubated with anti-PPARY (cat no. sc-1981; 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), Wnt-3 (cat no. sc-74537; 1:500; Santa Cruz Biotechnology, Inc.) anti-β-Catenin (cat no. sc-31001; 1:500; Santa Cruz Biotechnology, Inc.) and anti-GAPDH (cat no. sc-48166; 1:500; Santa Cruz Biotechnology, Inc.) at 4°C overnight, followed by incubation with a goat anti-rabbit IgG secondary antibody (BA1070; 1:5,000; Wuhan Boster Biological Technology, Ltd., Wuhan, China). Immunodetection was conducted using enhanced chemiluminescence (Applygen Technologies, Inc., Beijing, China) and analyzed using Image Lab version 3.0 (Bio-Rad Laboratories, Inc.).

**Luciferase reporter assay.** miRNA-15a-5p and Wnt3a-3'UTR-phRL-TK (Promega Corporation, Madison, WI, USA) were cloned by Sangon Biotech Co., Ltd. (Shanghai, China). For luciferase activity analysis, HEK-293T cells (2x10^5 cells/well) were co-transfected with luciferase reporter constructs using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Following incubation for 48 h, the luciferase assay was performed using a dual-luciferase reporter assay system (Promega Corporation). Data were normalized to Renilla luciferase activity.

**Statistical analysis.** Data were expressed as the mean ± standard deviation using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). A Student's t-test was used for comparisons between two groups. One-way analysis of variance followed by Tukey's post-hoc test were used for multiple group comparisons. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**miR-15a-5p expression in rat models of FHN.** To investigate the mechanism of miRNAs in the regulation of the bone cell apoptosis of FHN, the gene expression of a number of miRNAs were assessed. As presented in Fig. 1A, bone cell apoptosis was observed in the FHN model group, compared with the sham rat group which did not present any apoptosis. In addition, caspase-3/9 activities and Bax protein expression levels were significantly increased in the FHN model group in comparison with the sham rat group (P<0.01; Fig. 1B-E). Gene expression profiling and RT-qPCR were further employed to analyze the changes in the expression levels of miRNAs, which revealed that miR-15a-5p expression was significantly increased in the FHN model group compared with the sham group (P<0.01; Fig. 1F-G).

**Overexpression of miR-15a-5p promoted BMSC apoptosis.** Next, the present study investigated whether miRNA-15a-5p regulated the apoptosis of BMSCs in FHN. miRNA-15a-5p mimics successfully significantly increase the expression of miRNA-15a-5p in an in vitro model of FHN compared with negative control group (P<0.01; Fig. 2A). Consequently, the overexpression of miRNA-15a-5p significantly reduced cell proliferation at 48 and 72 h post-transfection, significantly increased LDH activity and the apoptosis rate, and significantly promoted the caspase-3/9 activity levels of BMSCs compared with the negative control group (P<0.01; Fig. 2B-G).

**Downregulation of miR-15a-5p reduced the apoptosis of BMSCs.** Next, the function of miR-15a-5p on the apoptosis of BMSCs in FHN was analyzed using anti-miR-15a-5p, which successfully significantly reduced the expression levels of miR-15a-5p in an in vitro model of FHN in comparison with the negative control group (P<0.01; Fig. 3A). Furthermore, the downregulation of miR-15a-5p significantly promoted cell proliferation, significantly decreased LDH activity and apoptosis and significantly reduced the caspase-3/9 activity levels.
Figure 1. miRNA-15a-5p expression in a rat model of FHN. In order to assess apoptosis in FHN rat models, (A) haemotoxylin and eosin staining, quantified (B) caspase-3 and (C) caspase-9 activity levels and (D) Bax protein expression levels, in addition to (E) Bax protein expression assessed by western blot analysis, were used. (F) A heat map was used to determine the expression of a number of miRNAs, which identified miR-15a-5p as substantially increased in the FHN group; therefore (G) the expression levels of miRNA-15a were further assessed using a reverse transcription-quantitative polymerase chain reaction. **P<0.01 vs. the sham control group. FHN, femoral head necrosis; mir/mirna, microRNA.

Figure 2. Overexpression of miRNA-15a-5p promoted the apoptosis of bone marrow stem cells. The present study assessed the (A) miRNA-15a-5p expression levels, (B) cell proliferation, (C) LDH activity levels, (D) apoptosis rate which was also (E) quantified and (F) caspase-3 and (G) caspase-9 activity levels. **P<0.01 vs. the control group. mir/mirna, microRNA; LDH, lactate dehydrogenase.
of BMSCs compared with the negative control group (P<0.01; Fig. 3B-G).

**miR-15a-5p regulated the Wnt/β-catenin/PPARγ signaling pathway.** To further investigate the mechanism of miR-15a-5p in regulating the apoptosis of BMSCs in FHN, western blot analysis was used to measure the protein expression of the Wnt/β-catenin/PPARγ signaling pathway. As a result, the over-expression of miR-15a-5p significantly suppressed the protein expression of Wnt-3 and β-catenin, and significantly induced that of PPARγ in an in vitro model of FHn, in comparison with the negative control group (P<0.01; Fig. 4A-D). Then, a luciferase reporter assay was performed to analyze a target of miR-15a-5p, which demonstrated that PPARγ was a direct target of miR-15a-5p; and luciferase reporter activity levels were significantly increased in an in vitro model of FHn, compared with the negative control group (P<0.01; Fig. 4E-F). In addition, the downregulation of miR-15a-5p significantly induced the protein expression of Wnt-3 and β-catenin, and suppressed that of PPARγ in an in vitro model of FHN compared with the negative control group (P<0.01; Fig. 4G-J). These results revealed that the regulation of the β-catenin/PPARγ signaling pathway by Wnt may be a mechanism of miR-15a-5p in FHN.

**Activation of Wnt attenuated the effects of miR-15a-5p on the apoptosis of BMSCs via the β-catenin/PPARγ signaling pathway.** Considering the above results, in order to examine the mechanism of miR-15a-5p in regulating the apoptosis of BMSCs, a Wnt plasmid was used, and significantly induced the protein expression levels of Wnt-3 and β-catenin and significantly suppressed that of PPARγ in an in vitro model of BMSCs following miR-15a-5p, compared with the over-expression of the miR-15a-5p group (P<0.01; Fig. 5A-D). Subsequently, the activation of Wnt significantly attenuated the effects of miR-15a-5p on the inhibition of cell proliferation, the promotion of LDH activity and apoptosis and the activation of caspase-3/9 activity levels in BMSCs in comparison with the overexpression of miR-15a-5p group (P<0.01; Fig. 5E-J).

**Discussion**

BMSCs are multipotential stem cells existing in bone marrow and are equipped with the characteristics of adherence growth. Under specific conditions, they are able to be differentiated into osteoblasts, chondrocytes and adipocytes (28). Therefore, the acquisition, cultivation and expansion of BMSCs are critical steps of stem cell transplant. In order to verify the functions of certain miRNAs, it is necessary to introduce the target miRNAs into BMSCs using adenovirus transfection, lentiviral transfection and lipofection transfection (12). Suitable investigative conditions of transfection and the improvement of acquired efficiency to express miRNAs are preconditions.

**Figure 3. Downregulation of miRNA-15a-5p reduced the apoptosis of BMSCs.** The present study assessed the (A) miRNA-15a-5p expression levels, (B) cell proliferation, (C) LDH activity levels, (D) apoptosis rate which was also (E) quantified and (F) caspase-3 and (G) caspase-9 activity levels. **P<0.01 vs. the control group. miR/miRNA, microRNA; LDH, lactate dehydrogenase.**
ZHANG et al.: miRNA-15a-5p FACILITATES BONE MARROW STEM CELL APOPTOSIS

For in vivo experiments (29). To the best of our knowledge, the present study is the first to demonstrate that miR-15a-5p expression is increased in a FHN model group compared with sham group. Liu et al. (13) had previously reported that miR-15a-5p regulated VEGFA in endometrial mesenchymal stem cells.

It has been reported that the majority of osteocalcin composed by BMSCs would be secreted into a nutrient solution (29). Secretion of osteocalcin is parallel with osteogenesis (30), which is a characteristic of differentiating osteoblasts. An early characteristic of the osteogenic differentiation of BMSCs is increased cellular alkaline phosphatase (ALP) activity (31). Therefore, ALP activity in BMSCs and osteocalcin contained in a nutrient solution is considered as an important indicator used to test for osteoblast differentiation in vivo (32). In the present study it was revealed that the overexpression of miR-15a-5p reduced cell proliferation, increased LDH activity and apoptosis and promoted the caspase-3/9 activity levels of BMSCs. Krejcik et al. (33) demonstrated that miR-15a-5p/b-5p was associated with myelodysplastic syndromes and acute myeloid leukemia.

PPARγ, mainly present in adipose tissues, is a adipogenesis transcription factor closely associated with the generation of adipocytes, with a notable function in adipogenesis (18). It is able to induce preadipocytes into adipocytes, affects the storage of fatty acids in adipose tissues and serves a critical role in adipogenesis and fat cell differentiation, which is induced by numerous adipocyte genes prior to transcriptional
activation (18). Activated PPARγ in adipose tissues is able to promote the release of adiponectin and adipocyte factors, translate accumulated non-esterified fatty acids into adipose tissues and is eliminated from the liver and skeletal muscle, in order to protect these organs from loading excessive lipid matters and protect its normal functions (34). Meanwhile, PPARγ is able to regulate the expression of relevant genes involved in the release, transfer and storage of fatty acids, and also exert an important effect on regulating lipid metabolism (35). In the present study, the overexpression of mir-15a-5p suppressed the protein expression of Wnt-3 and β-catenin, and induced the expression of PPARγ in an in vitro model of FHn. Shi et al. (22) concluded that miR-15a-5p was involved in the adipocyte differentiation of human adipocytes and obesity by regulating Wnt signaling.

The Wnt/β-catenin signal pathway has been demonstrated to be closely associated with the catabolism of the cartilage matrix, the dedifferentiation of joint chondrocytes and the restraint of chondrocyte apoptosis (19). The activation of the Wnt/β-catenin pathway may protect MSCs from differentiating into chondrocytes, however the maturity of chondrocytes requires the continuous activation of β-catenin (36). In the present study it was revealed that the overexpression of miR-15a-5p significantly suppressed the protein expression of β-catenin, and the downexpression of miR-15a-5p significantly induced β-catenin protein expression in BMSCs compared with their respective control groups (P<0.01). These results suggest that the activation of Wnt attenuates the effects of miR-15a-5p on the apoptosis of BMSCs via the β-catenin/PPARγ signaling pathway. Wang et al. (37) revealed that miR-15a-5p suppressed endometrial cancer cell growth via the Wnt/β-catenin signaling pathway. In the present study, only Wnt activation was used to analyze the function of Wnt in the effects of miR-15a-5p.
on the apoptosis of BMSCs. In the future, Wnt inhibition would also be employed to further analyze the function of Wnt in the effects of miR-15a-5p on the apoptosis of BMSCs.

In conclusion, it was revealed that miR-15a-5p facilitated the adipogenesis or osteogenic differentiation of BMSCs through regulating the PPARγ/β-catenin signaling pathway. These results have provided insight into the underlying mechanism of the osteogenic differentiation of BMSCs.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SL designed the experiments; WZ, CC and XM performed the experiments; SL and WZ analyzed the data; and SL wrote the manuscript.

Ethics approval and consent to participate

The animal study was ethically approved by the ethics committee of Hongqi Hospital Affiliated with Mudanjiang Medical University (Heilongjiang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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