MicroRNA-141 inhibits the differentiation of bone marrow-derived mesenchymal stem cells in steroid-induced osteonecrosis via E2F3

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Abstract. Osteonecrosis of the femoral head (ONFH) affects the life of patients. MicroRNA-141 (miR-141) has been found associated with proliferation of bone marrow-derived mesenchymal stem cells (BMSCs). E2F transcription factor 3 (E2F3) has been identified as the target of miR-141 to regulate cell proliferation. The aim of the present study was to investigate whether miR-141 and E2F3 were involved in the osteogenic differentiation of BMSCs during ONFH. BMSCs from 4-week-old Sprague-Dawley rats were transduced with miR-141 mimic or inhibitor lentiviruses. Alkaline phosphatase staining was performed to confirm osteogenic differentiation. Reverse transcription-quantitative PCR, luciferase reporter assays and western blot analysis were also used to examine the interaction between E2F3 and miR-141 in BMSCs from the control and ONFH rats. The lentiviral transductions were carried out successfully. The mRNA expression levels of miR-141 in ONFH were upregulated, while those of E2F3 were downregulated compared with the control rat. The luciferase reporter assays indicated that miR-141 could target E2F3. miR-141 knockdown upregulated the mRNA expression levels of E2F3. In addition, osteogenic differentiation of BMSCs was inhibited following miR-141 overexpression, but increased following miR-141 knockdown, as evidenced by the results of the alkaline phosphatase staining and western blot analysis. In conclusion, miR-141 inhibits the osteogenic differentiation of BMSCs in ONFH by targeting E2F3. These two molecules may represent novel candidates to examine in order to investigate the mechanism underlying ONFH.

Introduction

Glucocorticoids have been widely used for the treatment of various pathological conditions, such as allergies, inflammatory conditions, malignancies and immunological diseases. Steroid-induced avascular necrosis of the femoral head can be triggered following steroid-pulse therapy and long-term use of glucocorticoids (1,2). It has been found that steroid-induced avascular necrosis of the femoral head is the most common type of non-traumatic osteonecrosis of the femoral head (3). It is estimated that 75,000-150,000 new cases of ONFH are diagnosed every year in China (4). Reduced bone mass and trabecular fracture may occur after the third stage of ONFH as a result of cell death in the femoral head (5). Femoral head collapse and osteoarthritis eventually require artificial joint replacement to improve patient quality of life, which causes a heavy economic burden for society. Therefore, early detection of ONFH and protection of the femoral head are necessary for patient quality of life. However, as the molecular mechanism underlying the cause of ONFH is not entirely clear, effective treatment options are still lacking. The mechanism may be connected with abnormal lipid distribution, which can induce the formation of microemboli in the arteries supplying the femoral head (6). Additionally, apoptosis and autophagy of osteocytes are regulated by glucocorticoids (5,7) which have multiple effects on bone formation through lipid metabolism (8). Previous studies have demonstrated that, following inhibition of the Wnt/β-catenin pathway, peroxisome proliferator-activated receptor levels are increased by sclerostin production, causing pluripotent precursor cells to differentiate into adipocytes, rather than osteoblasts (9,10). Thus, modulating lipid metabolism disorders and improving bone mass may be critical factors in the prevention of ONFH.

Bone marrow-derived mesenchymal stem cells (BMSCs) are primitive cells that have the potential to differentiate into adipocytes, chondrocytes, osteoblasts and osteocytes (11,12). Bone resorption and bone formation are balanced by
osteoclasts and osteoblasts in order to maintain bone mass homeostasis (13). Matsuya et al (14) found that autologous BMSC injection into the femoral head can slow down the progression of ONFH. Lipid metabolism and osteogenic differentiation are also strongly associated with BMSC function (15). Another study has confirmed that glucocorticoids can inhibit the osteogenic differentiation and proliferation of BMSCs (16), which can reduce BMSC numbers (16). In addition, in the long-term, BMSC metabolism and differentiation are reduced as a result of an imbalance in bone mass homeostasis, thus increasing bone fragility (17). Tian and Yu (15) have suggested that the proliferation of BMSCs is impaired in ONFH. The results of the aforementioned studies suggest that improved understanding of the mechanisms underlying BMSC proliferation and differentiation may provide insight into the pathogenesis of ONFH.

MicroRNA (miRNA/miR) is a type of small non-coding single-stranded RNA molecule which binds to the 3' untranslated region (UTR) of target genes (18). Sedwick and Ambros were the first to propose that >30% of human genes could be regulated by miRNA (19). It has also been reported that miRNA is involved in the regulation of BMSC differentiation and proliferation (14,16). Moreover, miR-141 has been shown to inhibit proliferation and migration in several tumor cell types, such as osteosarcoma cells, SW480 colorectal cancer cells and hepatocellular carcinoma (HCC) cells (20-22). Overexpression of miR-141 inhibits the proliferation, migration and invasion of HCC cells (21). miR-141 is also associated with tissue repair and osteogenic differentiation (23). Indeed, miR-141 inhibits the proliferation of BMSCs; inhibition of miR-141 can promote the proliferation of BMSCs (24) and their osteogenic differentiation (5), possibly by targeting vitamin C transporter 2 (25). Therefore, the inhibition of miR-141 could be harnessed for the prevention and treatment of osteonecrosis caused by glucocorticoids.

E2F transcription factor 3 (E2F3) is a member of the E2F transcription factor family, which is involved in the regulation of cell proliferation (26). E2F3 has been identified as the target of miR-141 in HCC cells, and the overexpression of E2F3 can partially reverse the tumor-suppressive effects of miR-141 (27). It has been demonstrated that E2F3 transcript (E2F3-α). Sprague-Dawley rat embryos develop normally without fatal disease. However, weight loss, delayed growth and skeletal dysplasia were observed, indicating that E2F3 played a key role in muscle and skeletal development (28). However, how E2F3 functions in different tissues and environments remains unclear.

To the best of our knowledge, few studies have evaluated the role of E2F3 in osteogenic differentiation. It may be hypothesized that E2F3 could promote osteogenic differentiation of BMSCs and that miR-141 could inhibit the process by targeting this transcription factor. A previous study has indicated that E2F3 may be the target of microRNA (27). The aim of the present study was to evaluate the role of miR-141 and E2F3 in BMSC proliferation in ONFH. In addition, the role of miR-141 and E2F3 in the femoral head bone tissue changes induced by glucocorticoids was also examined.

Materials and methods

**Isolation of rat BMSCs.** After 7 days adaptive feeding (26°C, 60% humidity, 12 h of light and 12 h of darkness every day, 200 ml water per day, free access to food), animal health and behavior were monitored every day. In the present study, two 4-week-old male Sprague Dawley rats (SCXXK2017-0001) were used (29). Their weights were 70.2 and 70.3 g, respectively. The rats were anesthetized by intraperitoneal injection of pentobarbital sodium (3% solution for a dose of 40 mg/kg), then sacrificed by cervical dislocation. The rats were immersed in 75% ethanol for about 5-10 min, and dissection was carried out under sterile conditions. The bilateral femurs and tibia were removed with sterile surgical instruments, and the attached muscles were removed. Three holes were made in the femur and tibia, and a 10-ml syringe was used to extract medium inserted into the femurs and tibia. The cells in the bone marrow were repeatedly flushed into a Petri dish until the femur and tibia were pale.

The cell suspensions were collected, purified and centrifuged at 100 x g for 5 min at room temperature, then re-suspended in α-MEM (HyClone; Cytiva) and incubated at 37°C with 5% CO₂. When the cells reached 80-90% confluence, the culture medium was removed. The cells were trypsinized and washed in PBS (HyClone; Cytiva). Trypsin (HyClone; Cytiva) was used for digestion for 3 min. The cells were then sub-cultured at a 1 in 3 ratio.

**Transfection and lentiviral transduction.** Transfection was used for luciferase assay. miR-141 mimics were purchased from Shanghai GenePharma. Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfect BMSCs according to the manufacturer’s instructions. A total of 1 µg plasmid (100 nM miRNA mimic) was diluted with 50 µl serum-free Opti-MEM. The plasmid was mixed gently and incubated at room temperature for 5 min. B. Lipo3000 was diluted with 50 µl serum-free OPtI-MEM, mix gently and incubate at room temperature for 5 min. MiR-141 mimic and lipo3000 were mixed and stood at room temperature for 20 min before transfection into cells. Subsequent luciferase assay was performed following transfection for 48 h.

The sequences used were as follows: NC, 5'-UUCGUGUCACGUTT-3' and 5'-ACGUACGUCUGAGGAATT-3'; miR-141 mimic.

**Lentiviral transduction** was used in subsequent experiments including alkaline phosphatase stain and mRNA and protein expression of E2F3. The lentivirus was packaged by Shanghai GenePharma, using a 3rd-generation lentiviral packaging system. For lentivirus production, the packaging plasmids (pGag/Pol, pRev, pVSV-G) and shuttle plasmid pLV2 were transfected into 293T cells (Genepharma, Shanghai). The supernatant of the 293T cells was collected by centrifugation at 1,000 g for 4 h at 4°C, then transferred to a syringe and filtered using a 0.45-µm filter. The plasmid was mixed with 1.5 ml serum-free DMEM medium and stood at room temperature for 20 min before transfection. Plasmid ratio 8 µg LV2 shuttle plasmid, 8 µg pGag/Pol, 4 µg pRev, 6 µg PVSV-G. 300 µl liposome was mixed in 1.2 ml serum-free medium, and left at room temperature for 5 min. Plasmid and liposome were mixed and stood at room temperature for 20 min. Then add into 293T cell culture medium. After 6 h incubation, medium was removed, and the medium containing serum was added, and the supernatant was collected after 72 h culture at 37°C. The filtrate was centrifuged at 23,000 g for 4 h at 4°C. The
plates were shaken evenly, then placed into the incubator for culture. Positive cells were screened with 0.5 µg/ml puromycin and maintained at a concentration of 2 µg/ml. After 6 h of cell culture, the medium containing the virus was replaced with fresh medium for another 72 h before subsequent experiments (cell proliferation assays and mRNA analysis).

Cell osteogenesis induction. BMSCs were inoculated at a density of 1x10^5 cells and infected with lentivirus. BMSCs were collected from the control and the ONFH rat. Cell osteogenic induction was carried out as previously reported by Yaghoobi et al (30). The BMSCs were digested with trypsin (HyClone; Cytiva) when they reached 80-90% confluence, then centrifuged at 100 x g for 5 min at room temperature. The supernatant was removed, and α-MEM (HyClone; Cytiva) was added to resuspended the BMSCs in a 24-well culture plate. The BMSCs were cultured to 70% confluence, then the culture medium was removed and replaced with osteogenic induction culture medium (Cyagen; cat. no. RASMX-90021). The medium was changed every 3 days thereafter. The osteogenesis levels were examined at day 3, 7 and 14 using alkaline phosphatase staining (31). Images were taken and saved for ALP assay.

Alkaline phosphatase staining. A total of 1x10^5 cells were inoculated in 24-well plates. The cells were cultured to 60-70% confluence, then the culture medium was replaced with osteogenic induction culture medium. The osteogenic medium was changed every 3 days, and the cells were washed with PBS at the end of the osteogenic induction process. On days 3, 7 and 14, the BMSCs were fixed with 10% formaldehyde solution at room temperature for 30 min, then washed with PBS twice. The fixed cells were then stained with 50 µl alkaline phosphatase solution at 37°C for 2 h (32).

Luciferase assays. For luciferase assays, the pGL6-mir reporter plasmid (Beyotime Institute of Biotechnology; cat. no. D2106) and the Renilla plasmid (Promega; cat. no. E2241) were transfected using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.; cat. no. L30000015). The 3'-UTR of E2F3 was amplified by PCR as previously described (33), then cloned into the pGL6-mir reporter vector (Beyotime Institute of Biotechnology; cat. no. D2106). The BMSCs were co-transfected with the wild-type or mutant pGL6-mir reporter vector (100 ng/well), and either miR-141 mimic or negative control (100 ng/well), and either miR-141 mimic or negative control (100 ng/well) in 24-well plates. The cells were cultured to 60-70% confluence, then the medium was replaced with osteogenic induction culture medium. Osteogenic medium was added to resuspended the BMSCs in a 24-well culture plate. The BMSCs were cultured to 70% confluence, then the culture medium was replaced with osteogenic induction culture medium (Cyagen; cat. no. RASMX-90021). The medium was changed every 3 days thereafter. The osteogenesis levels were examined at day 3, 7 and 14 using alkaline phosphatase staining (31). Images were taken and saved for ALP assay.

Western blot analysis of E2F3 protein expression. At day 3, 7 and 14, after discarding the RASMX-90021 medium (Cyagen; cat. no. RASMX-90021) and α-MEM (HyClone; Cytiva; SH30265) the cells were lysed in RIPA lysis buffer at 4°C overnight. The total protein was harvested via centrifugation at 13,600 g and the protein concentration was determined using the BCA method (Beyotime Institute of Biotechnology; cat. no. P0010). RIPA buffer included 50 mmol/l Tris-HCl, pH8.0, 150 mmol/L NaCl, 1% TritonX-100, 0.10 µg/ml PMSF: A total of 30 µg protein per lane was separated using SDS-PAGE on 8% gels, then transferred to a PVDF membrane (MilliporeSigma; cat. no. ISEQ00010). The membrane was then blocked with 5% skim milk in 0.1% Tween-20 Tris-buffered saline at room temperature for 1 h. The membranes were incubated with anti-E2F3 (ProteinTech Group, Inc.; cat. no. 12344-1-AP; diluted 1:1,000) or rabbit anti-GAPDH (Abbkine Scientific Co., Ltd.; cat. no. A01020; diluted 1:5,000) antibodies at 4°C overnight. This was followed by incubation with HRP-conjugated anti-mouse (Beyotime Institute of Biotechnology; cat. no. A0216; diluted 1:5,000) or anti-rabbit (Beyotime Institute of Biotechnology; cat. no. A0208; diluted 1:5,000) antibodies at room temperature for 2 h. The bands were visualized using ECL reagent (Tanon Science and Technology Co., Ltd.; cat. no. 180-5001) (39). GAPDH was used as loading control. Chemiluminescence instrument (Tanon, 5200) was used for densitometry. The software for densitometry was GelCap 1.0 (Tanon).

Statistical analysis. Each experiment was performed three times, and the results are shown as the mean ± standard deviation. SPSS 23.0 software (SPSS, Inc.) was used for statistical analysis. The differences between two groups were compared by unpaired Student's t test. The differences between three or more groups were compared using one-way analysis of variance followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of BMSCs and osteogenic induction. The morphology of BMSCs at day 0 under an optical microscope is shown in Fig. 1. BMSCs were round in shape, with strong refractivity, and varied in size. In addition, osteogenesis was examined at days 3, 7 and 14. Osteogenesis levels were higher in the control than in the ONFH group, as evidenced by cell density and osteogenic capability (Fig. 1).

Alkaline phosphatase staining. Alkaline phosphatase staining is shown in Fig. 2. The results suggested that the control...
group was markedly darker than the ONFH group at days 3, 7 and 14 (Fig. 2A). BMSCs from the normal rat were then transduced with miR-141 mimic lentivirus (miR-141), whereas those of the ONFH rat were transduced with miR-141 inhibitor lentivirus. Following transduction with the miR-141-3p mimic lentivirus, alkaline phosphatase staining in BMSCs from the normal was reduced compared with the NC. Following transduction with the miR-141 inhibitor lentivirus, alkaline phosphatase staining in BMSCs from the ONFH rat was increased compared with the inhibitor NC. These results suggest that miR-141 expression is associated with osteogenesis.

Association between E2F3 and miR-141. In order to examine the regulatory relationship between E2F3 and miR-141, luciferase reporter assays were used to determine whether miR-141 could target E2F3 expression directly through its UTR. In the wild-type group, the average E2F3 luciferase activity was significantly lower than that of the NC group (P<0.005; Fig. 3). However, average E2F3 luciferase activity in the mutant remained unchanged following transduction the miR-141-3p overexpression lentivirus.

Successful miR-141 mimic transduction in normal BMSCs and miR-141 inhibitor transduction in BMSCs from the
ONFH rat are shown in Fig. 4A (left; all P<0.01). Furthermore, RT-qPCR demonstrated that the expression levels of miR-141 in the ONFH group were significantly higher than those of the control group at days 3, 7 and 14 (all P<0.01; Fig. 4A, right). The mRNA expression of E2F3 was significantly lower in the ONFH group than in the control group at all time points (all P<0.01; Fig. 4B). This suggests that the expression of E2F3 is inversely associated with that of miR-141.

Protein expression levels of E2F3. The protein expression levels of E2F3 were determined using western blotting. E2F3 expression was significantly reduced in BMSCs from the ONFH rat than those from the control rat (Fig. 5A). The protein expression levels of E2F3 were significantly downregulated following transduction with the miR-141 overexpression lentivirus, compared with BMSCs transduced with the NC lentivirus. However, E2F3 was upregulated in BMSCs from the ONFH rat transduced with the miR-141 inhibitor lentivirus, compared with miR-141 inhibitor Nc lentivirus (Fig. 5B). This confirmed that miR-141 significantly downregulated the protein expression levels of E2F3.

Discussion

ONFH is a progressive and painful hip joint disorder affecting individuals in the 30-50 year age range (40). Previous studies have suggested that glucocorticoids are associated with lipid metabolism. Glucocorticoids facilitate decomposition of lipid into fatty acids and free fatty acids via direct action. Glucocorticoids inhibit phosphorylase and convert to terminal AMP. Glucocorticoids increase fatty acids by upregulating the activity of phenethylamylmethyltransferase, and adipose tissue. (41). Lipid metabolism and osteogenic differentiation are associated with BMSCs since BMSCs undergo adipose differentiation and osteogenic differentiation. Differentiation of BMSCs is regulated by miRNA (15,42). Lipid metabolism could lead to the collapse of the trabecular bone, causing empty lacunae and microfractures in ONFH (43). Osteocytes, which differentiate from BMSCs, are associated with ONFH; death of osteocytes lead to ONFH. A previous study has demonstrated that the osteogenic differentiation of BMSCs in cells from human ONFH tissue is downregulated, whereas adipocyte differentiation is upregulated (44). The autophagy and apoptosis rates of osteocytes were also increased in ONFH (45). It has been observed that the number of osteoblasts, which are cells that differentiate from BMSCs, are reduced in ONFH, whereas lipid differentiation is increased (44). Thus, BMSCs play an important role in the pathological changes associated with ONFH, and it is important to investigate the factors that may affect BMSCs in this disease.

miR-141 has been implicated in tissue repair and osteogenic differentiation (23). A previous study has indicated that miRNA plays an crucial role in the osteogenic differentiation of BMSCs, and abnormal expression of miRNA molecules may affect this process (46). Our previous study revealed that miR-141 can inhibit proliferation in BMSCs (24). Nevertheless, the role of this miRNA in osteogenic differentiation is not clear. The inhibition of miR-141 may promote the osteogenic differentiation of BMSCs (5) by targeting vitamin C transporter 2 (25). In acute kidney injury, it has been reported that miR-141 expression in mesenchymal stromal cells promotes tissue repair (47). miR-141 overexpression can also occur as a result of epigenetic regulation during senescence, which may lead to a declines in physiological function and tissue regeneration (48). miR-141 is a member of the miR-200c/141 cluster and expression of the miR-200c/141 cluster is regulated by DNA methylation, suggesting epigenetic regulation of this miRNA locus in aggressive breast cancer cell lines as well as untransformed mammary epithelial cells. (49). Increasing evidence suggests that several miRNA molecules participate in the development and progression of ONFH, acting either as stimulators or as suppressors (50). In the present study, miR-141 was overexpressed in BMSCs. The results demonstrated that miR-141 suppressed BMSC proliferation and osteogenic differentiation.

E2F3 participates in the regulation of cellular metastasis (26). Numerous miRNA molecules have been found to be able to modulate E2F3 expression (51), including miR-141. Indeed, E2F3 has been identified as the target of miR-141 in HCC cells, and the overexpression of E2F3 could partially reverse the tumor-suppressive effects of miR-141 (27). E2F3 expression is altered in several tumor types, and this transcription factor plays an important role in tumor development (52,53). Therefore, it was hypothesized in the present study that E2F3 and miR-141 could interact to modulate BMSC differentiation in ONFH and play a crucial role in the pathogenesis of this disease. The results suggested that miR-141 and E2F3 are potentially relevant factors of BMSC. The overexpression of miR-141 reduced the expression levels of E2F3 and inhibited osteogenic differentiation. Following miR-141 inhibition using lentiviral transduction, E2F3 expression and osteogenic differentiation significantly increased in ONFH group cells. Increased E2F3 mRNA levels may partially reverse the suppressive effects of ONFH on osteogenic differentiation. Altogether, these observations suggest that miR-141 suppresses osteogenic differentiation.
Figure 4. mRNA expression of miR-141 and E2F3. (A) Expression levels of miR-141 in group of control and ONFH at 3d, 7d and 14d. (B) mRNA expression levels of E2F3 in group of NC, miR-141, ONFH+ inhibitor NC and ONFH+ miR-141 inhibitor (left part); mRNA expression levels of E2F3 in group of control and ONFH at 3d, 7d and 14d (right part). *P<0.05, **P<0.01 vs. control. miR, microRNA; E2F3, E2F transcription factor 3; NC negative control; ONFH, osteonecrosis of the femoral head.

Figure 5. Protein expression levels of E2F3. (A) Protein expression of E2F3 in BMSCs from the control or ONFH rat at day 3, 7 and 14. (B) Protein expression of E2F3 in BMSCs from the normal rat transduced with nc or miR-141 mimic lentivirus and BMSCs from the ONFH rat transduced with the inhibitor nc or miR-141 inhibitor lentivirus. *P<0.05, **P<0.01 vs. control. miR, microRNA; E2F3, E2F transcription factor 3; NC negative control; ONFH, osteonecrosis of the femoral head; BMSCs, bone marrow-derived mesenchymal stem cells.
In conclusion, ONFH is a complex biological process involving diverse mechanisms. The present study demonstrated that miR-141 could suppress the osteogenic differentiation of BMSCs by reducing E2F3 mRNA expression levels.

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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
FX, JW, WF, TH, YL and WW participated in the design of the study and contributed to drafting and revising the manuscript. WF, YL and TH collected the data and performed the statistical analyses. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved. FX and WBW confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate
All animal experiments were approved by The Inner Mongolia Medical University Animal Ethics Committee and performed according to the Guidelines for the Care and Use of Laboratory Animals.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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