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MiR-146a regulates osteogenic differentiation and proliferation of bone marrow stromal cells in traumatic femoral head necrosis

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Abstract. – OBJECTIVE: To investigate the regulatory mechanism of micro ribonucleic acid (miR)-146a in osteogenic differentiation and proliferation of bone marrow stromal cells (BMSCs) in traumatic femoral head necrosis.

PATIENTS AND METHODS: Femoral neck fracture patients undergoing surgery were divided into necrosis group and non-necrosis group. The expression level of miR-146a in BMSCs isolated from these patients was detected via quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The clinical corr[illegible] of miR-146a with BMSCs in traumatic f[illegible] head necrosis was explored. The regulato[illegible]f-fects of miR-146a on osteogenic differentia[illegible] and proliferation of BMSCs in traumatic fe[illegible]ral head necrosis were detecte[illegible]reover, t[illegible] cell proliferation was analyze[illegible]counting kit-8 (CCK-8) assay. The d[illegible]sition [illegible]calcium on the cell surface was d[illegible]ted via [illegible]arin red staining to evaluate the [illegible]geni[illegible]tion. The messenger [illegible]A ([illegible]xpressi[illegible]s of osteogenesis-s[illegible]ic gen[illegible]kaline phosphatase (ALP), a[illegible]teocalcin ([illegible]in BMSCs undergoing os[illegible]differentia[illegible]ere detected via qR[illegible]CR.

RESULTS: Expressio[illegible] of miR-146a in BMSCs of [illegible]osis group w[illegible]nificantly lower than th[illegible] non-necrosis gr[illegible], and the difference[illegible]s statis[illegible]lly significant ($p<0.01$). CCK-8 a[illegible]revea[illegible]that the proliferation of BMSCs was [illegible]ly en[illegible]ed in miR-146a-mimic group[illegible]pared[illegible] that in miR-NC group, but it sig[illegible]an[illegible]eclined in miR-146a-inhibitor group co[illegible]d with that in miR-NC group. The[illegible]sults of [illegible]arin red staining showed that th[illegible]tion of calcium obviously increased in [illegible]mimic group compared with that in [illegible]R-NC group, indicating that the osteogenic [illegible]erentiation ability is significantly enhanced, [illegible]it markedly decreased in miR-146a-inhibitor [illegible]oup compared with that in miR-NC group. The detection of osteogenesis-specific genes via qRT-PCR manifested that the mRNA expressions of ALP and Ocn remarkably increased in miR-146a-mi[illegible]oup compa[illegible]n those in miR-NC g[illegible], a[illegible]ere were s[illegible]istically significant di[illegible]erences[illegible]05). The mRNA expressions [illegible]P and Oc[illegible]arkably decreased in mi[illegible]hibitor grou[illegible]mpared with those i[illegible]R-NC group, and there were statistically s[illegible]ificant differ[illegible]ces ($p<0.05$), suggesting the [illegible]ited osteog[illegible]c differentiation ability.

CONCLUSIONS: We showed that miR-146a re[illegible]s the o[illegible]genic differentiation and proliferation of BMSCs in traumatic femoral head necrosis.

Keywords:
MiR-146, Bone marrow stromal cells, Traumatic femoral head necrosis.

Introduction

Femoral head necrosis is a severe hip joint disease characterized by insufficient blood supply in the femoral head and intraosseous hypertension[1]. Traumatic femoral head necrosis is an important type mostly caused by femoral neck fracture, dislocation of hip joint and other hip joint diseases. In recent years, the incidence rate of femoral head necrosis is increasing year by year, ranking first in tuberculosis of hip joint[2]. Multiple therapeutic approaches have been applied for femoral head necrosis, such as nucleus pulposus decompression, bone grafting, joint formation, and joint replacement[3]. Nowadays, the modified bone marrow stromal cells (BMSCs) via gene transfection technique have become a new therapeutic regimen for femoral head necrosis.

BMSCs are adult stem cells with high self-renewal ability and multi-directional differentiation potential, existing in almost all human

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tissues. They can be isolated from the bone marrow, adipose tissue, umbilical cord, and amniotic fluid, and they expanded successfully *in vitro*[4,5]. BMSCs can not only proliferate widely, but also differentiate into different types of cells, such as adipocytes, osteoblasts, and chondrocytes. BMSCs can also secrete various cytokines to regulate immune response and promote angiogenesis through paracrine and autocrine[6]. BMSCs, as one of the important seeds of bone tissue engineering, play an important role in proliferation, osteogenic differentiation, and bone regeneration in the bone defect region. Therefore, studying the mechanism of proliferation and osteogenic differentiation of BMSCs provides an important solution for nonunion, bone cyst, bone defect, and ischemic necrosis of femoral head in the future.

Micro-ribonucleic acids (miRNAs) are a kind of small-molecular non-coding RNA with 18-24 nucleotides in length. MiRNAs alter the messenger RNA (mRNA) stability or protein translation at the post-transcriptional level through binding to the 3'-untranslated region (UTR) of their target mRNA. The complete co[illegible] mentary pairing between miRNA and it[illegible] gene can directly degrade the target gene, [illegible]e the incomplete complementary pairing betw[illegible] them leads to inhibited translation of the tar[illegible] gene[7-10]. MiRNA, as an impo[illegible]egulato[illegible] molecule, is involved in a [illegible]s c[illegible]portant pathophysiological proce[illegible], such [illegible]BMSCs proliferation, differenti[illegible] and [illegible] as well as cytokine [illegible]re[illegible] arge [illegible]-ber of researches[12-[illegible]ave dem[illegible]ated the vital role of miR[illegible]n regulati[illegible] osteogenic differenti[illegible] proliferatio[illegible] MSCs. According to [illegible] large [illegible]mber of microarray studies, t[illegible] are a larg[illegible]mber of differentially e[illegible]essed miRNAs i[illegible]MSCs, showing imp[illegible]t role[illegible] the functional maintenance an[illegible]ected [illegible]ierentiation of stem cells[17]. In this w[illegible] expre[illegible] of miR-146a in BMS[illegible] in [illegible]is gr[illegible] and non-necrosis group a[illegible]opera[illegible]emoral neck fracture was det[illegible]ed via q[illegible]PCR. The clinical correlation be[illegible]iR-146a and traumatic femoral head [illegible]osi[illegible] explored. Moreover, overexpres[illegible]on or knockdown of miR-146a expression was [illegible]ieved via lentiviral transfection in BMSCs i[illegible]rosis group. The influences of miR-146a on proliferation and osteogenic differentiation of BMSCs in traumatic femoral head necrosis was further analyzed.

Patients and Methods

Clinical Samples

All patients signed the informed conse[illegible]d this study was approved by the Ethics C[illegible] of Affiliated Hospital of Jining Me[illegible] Univer[illegible]sity. All patients with femoral ne[illegible]cture who underwent percutaneous hollow [illegible] internal fixation after traction reducti[illegible] from 2[illegible] 2017 in our hospital were selecte[illegible]mong them[illegible] tients meeting the diag[illegible]c crite[illegible] for tra[illegible]tic femoral head necr[illegible]report[illegible] the guideline[18] were enrolled into [illegible] group, [illegible]e 34 patients without [illegible]matic [illegible]ral he[illegible]ecrosis were enrolled [illegible] non-necr[illegible]r[illegible] None of the enrolled [illegible] received dr[illegible]erapy (especially ster[illegible]ori[illegible] 6 months before and after the period, and they [illegible] history of arthritis. In addi[illegible]tients with s[illegible]ic inflammatory re[illegible]s[illegible]e, autoimmune diseases, malignant tumors, [illegible] were exclude[illegible]

MSCs were [illegible]ained from the bone marrow i[illegible] upper fe[illegible]l marrow cavity of patients wit[illegible]mor[illegible]moral neck fracture and isolated via [illegible] gradient separation as previously described[19]. An equal volume of Percoll separation [illegible] (Solarbio, Beijing, China) was added for [illegible]gation at 2000 rpm for 30 min. Cells were suspended in the normal growth medium [90% low-glucose Dulbecco's Modified Eagle Medium (DMEM; HyClone Laboratories Inc., Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA), penicillin (100 U/mL) and streptomycin (100 μg/mL) (Sigma-Aldrich, St. Louis, MO, USA)]. Then, the cells were inoculated in a 55 cm2 flask and cultured in an incubator with 5% CO2 at 37%. The medium was replaced once every 2-3 d.

BMSCs were identified based on cell surface antigen determination (CD29 and CD34) via flow cytometry.

QRT-PCR

MiR-146a expression in BMSCs between necrosis group and non-necrosis group was detected via qRT-PCR. BMSCs in necrosis group were divided into miR-NC group, miR-146a-mimic group, and miR-146a-inhibitor group. The expression of miR-146a in the three groups was detected via qRT-PCR. BMSCs in the three groups were cultured in the osteogenic differentiation-inducing medium, and the mRNA expressions of osteogenesis-specific genes, alkaline phosphatase (ALP), and osteocalcin (Ocn) were detected via qRT-PCR.

The total RNA was extracted using TRIzol reagent (Sangon Biotech, Shanghai, China) and reversely transcribed using the NanoDrop 2000 device (Thermo Fisher Scientific, Rockford, IL, USA). The cDNA samples were obtained using TaKaRa RNA PCR kit (TaKaRa, Dalian, China) and Oligo dT primers (Invitrogen, Shanghai, China). The miRNA and mRNA expression levels were measured via qPCR using SYBR master mixture (TaKaRa, Dalian, China) on a Light Cycler 480 device (Roche, Basel, Switzerland). Each sample was measured for 3 times. The primer design and synthesis were shown in Table I. The expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the data were analyzed using the 2-ΔΔCT method[20]. Primer sequences used in this study were as follows: ALP, F: 5'-GCAGAACAACTCCTTACTC-3', R: 5'-GCTATTGGGTGTCCGAAGGA-3'; MiR-146a, F: 5'-GACTTCTCCACAACCCTCTG-3', R: 5'-AGAGGGAAGAGTTCCCCAG-3'; Ocn, F: 5'-AGATGCACCTGTACGATCAA-3', R: 5'-CTTTCAACACGCAGGACCT-3'. U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Table I. Primer sequences.

Gene	Primer sequence
miR-146a	5'-3' TGAGAACTAAATTCCATGGCT
U6	5'-3' GCTTCGGCAGCACATAT
	3'-5' CGAATTTGCGTGTCAT
Ocn	5'-3' GCAATAAGGTAGTG AGACTCC
	3'-5' GTTTGTAGGCGGT AAGC
Alp	5'-3' ATCTTTGGTCTGGC TG
	3'-5' TTTCCCGTTC CGTCC
GAPDH	5'-3' GGTGAAGG GTGTGA
	3'-5' GACTGTG TTGAATTTG

Construction and transfection of lentiviral vector

The miR-NC, miR-1 and miR-146a-inhibitor lentivi vectors re purchased from Genechem ghai The multiplicity of infecti (M entivi s reached 50. MiR-14 expressi ter lentiviral transfection was ed via qRT

Cell Prolife on A is

Cell pr ation was ed using the cell counti -8 (CCK-8) kit (do, Kumamoto, Japa fter tr fection of BMSCs with miR-NC -146 mic or miR-146a-inhibitor lentivirus K-8 so was added at 1 d, 2 d, 4 d, d, res ively. The absorbance was red a sing a microplate reader (Infin 00, Teca ombrechtikon, Switzerland).

...uction of Osteogenic ...ifferentiation

...steogenic differentiation-inducing medium c ...ing 10% FBS, 10 mM β-glycerophosphate, 1.9×108 M dexamethasone, 50 μg/mL L-ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA), 0.01 μM 1,2,5-dihydroxyvitamin D3, 100 U/mL penicillin, and 100 μg/ in was d to induce the oste ic di ation MSCs *in vitro*. The d tion of ca cell surface was de ia alizarin ining at 14 d, and ex sion of osteogenesis-specific genes (ALP and Oc the three groups were dete qRT-PCR.

...tistical Anal...is

...atistical Pr ct and Service Solutions ( ) 19.0 soft e (IBM, Armonk, NY, USA) was for tical analysis. All quantitative data w essed as mean ± standard deviation. The comparison between the two groups e using the t-test. p-values < 0.05 were ed statistically significant.

Results

Clinical Data of Patients

A total of 19 patients meeting the diagnostic criteria for traumatic femoral head necrosis were enrolled into necrosis group, while 34 patients without traumatic femoral head necrosis were enrolled into non-necrosis group. The X-ray displayed severe ONFH in patients after operation of femoral neck fracture. The clinical image of the patient was shown in Figure 1.

Morphology of BMSCs

With the prolongation of cell culture, the spindle-shaped BMSCs began to adherence to the wall. At 14 d after continuous culture, the cell proliferation was rapid. The morphology of BMSCs was shown in Figure 2.

Identification of BMSCs

To identify the isolated BMSCs, two specific surface antigens of BMSCs were examined using a flow cytometer. The results revealed that the cells cultured showed strong positive expression

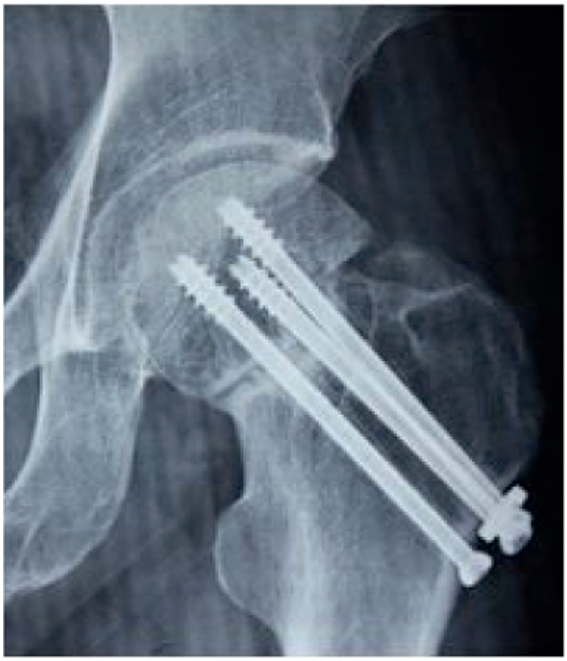


Figure 1. Clinical image of patient: Femoral head necrosis occurs at 1 year after hollow screw internal fixation.

Figure 2. Obser[illegible] of BMSC[illegible] a fl[illegible]cence microscope (400×[illegible] after continu[illegible].

sis [illegible]and the dif[illegible]e was statistically si[illegible]icant ($p<0.01$) (Figu[illegible]e 4). It is indicated [illegible] miR-146a m[illegible]y be involved in regulating [illegible]matic femor[illegible]ead necrosis, and miR-146a o[illegible]xpression [illegible]y inhibit traumatic femoral hea[illegible]osis.

of CD29 and negative expression of CD34 (F[illegible]re 3). Based on the above results, we confirmed [illegible] purity of extracted BMSCs.

Clinical Correlation Be[illegible]en Mi[illegible]46 and Traumatic Femo[illegible]ead N[illegible]

The expression le[illegible] o[illegible]a was [illegible]-tected via qRT-P[illegible] in ne[illegible] group and non-necrosis gr[illegible]he results s[illegible]d that the expression le[illegible]146a in ne[illegible]s group was signific[illegible]y lowe[illegible] that in non-necro-

146a Expression in the Three [illegible] (MiR-NC, MiR-146a-mimic, and MiR-146a-inhibitor) Detected Via qRT-PCR

The three lentiviruses (miR-NC, miR-146a-mimic, and miR-146a-inhibitor) were transfected into BMSCs in necrosis group to overexpress or inhibit miR-146a in BMSCs. After transfection, the miR-146a expression in the three groups (miR-NC, miR-146a-mimic, and miR-146a-inhibitor) was detected via qPCR (Figure 5). It was found that the miR-146a expres-

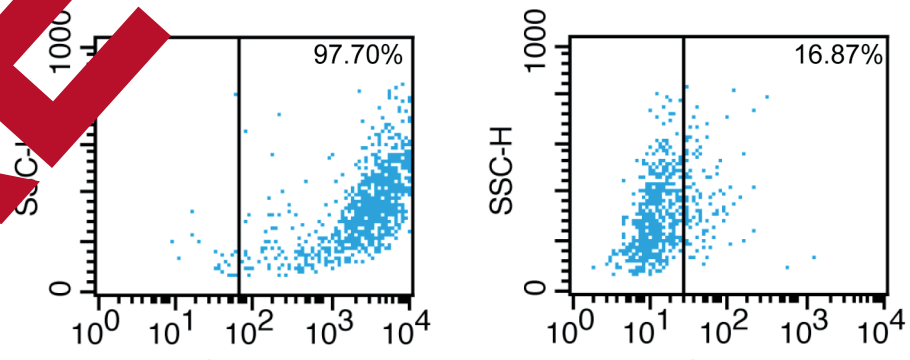


Figure 3. Identification results of flow cytometry. ***A***, Positive expression of CD29, ***B***, negative expression of CD34.

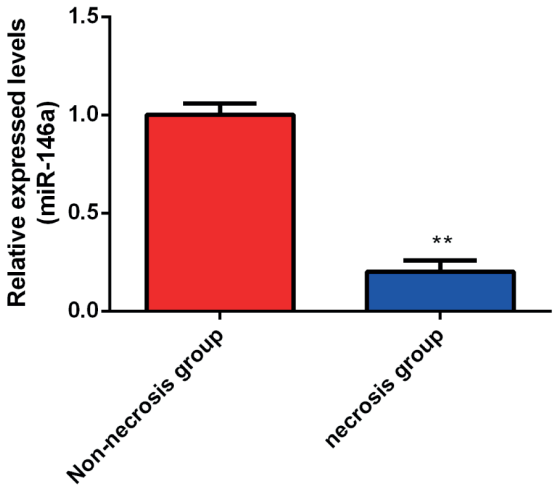


Figure 4. Expression of miR-146a in BMSCs in necrosis group and non-necrosis group detected via qRT-PCR. $^{**}p<0.01$ vs. non-necrosis group.

sion significantly increased in miR-146a-mimic group compared with that in miR-NC group, displaying a statistically significant diff ce ($p<0.01$). However, it significantly d in miR-146a-inhibitor group, also disp a statistically significant difference ($p<0$ The above results suggested that miR-146a v successfully overexpressed i -146a-m mic group and inhibited in -14 nhibitor group via lentiviral transf on.

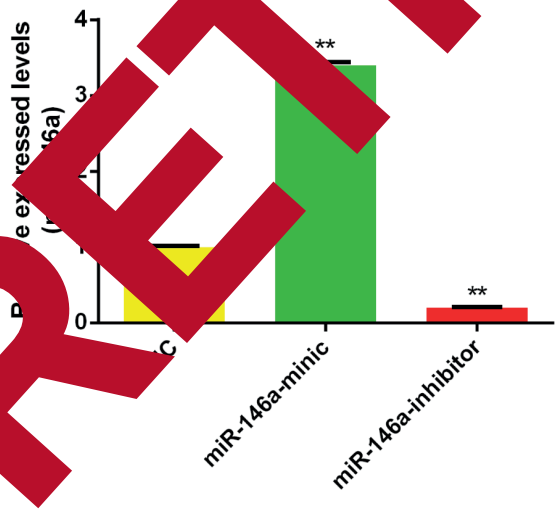


Figure 5. Expression of miR-146a in BMSCs in the three groups after lentiviral transfection. $^{**}p<0.01$ vs. miR-NC group.

CCK-8 Proliferation Assay Results

The proliferation ability of BMSCs in the three groups was detected via CCK-8 assay for consecutive five days. The results manifested that th li- feration ability of BMSCs was significa ced in miR-146a-mimic group comp with tha in miR-NC group, while it signific declined in miR-146a-inhibitor group compare that in miR-NC group. It suggested t miR- y be involved in regulating the eration of Overexpression of miR- enhan d the pr ration of BMSCs, wh R-146 ockdown re- duced the proliferat n

Culture of B s in Ost n Differenti ducing M n in the T e Gr

BMSCs transfecte h miR-NC, miR-146a-mi d miR-146a itor in the three g s were cultured in th osteogenic differen- t on-inducing dium. The deposition of cal- on the cell face was detected via alizarin re ining afte d. It was found that calcium dep m y increased in miR-146a-mimic gr pared with that in miR-NC group, indicating the elevated osteogenic differentiation On the contrary, it significantly decreased 46a-inhibitor group compared with that in miR-NC group, indicating that the osteogenic differentiation ability was significantly weakened (Figure 7). These results indicated that miR-146a may be involved in regulating the osteogenic dif-

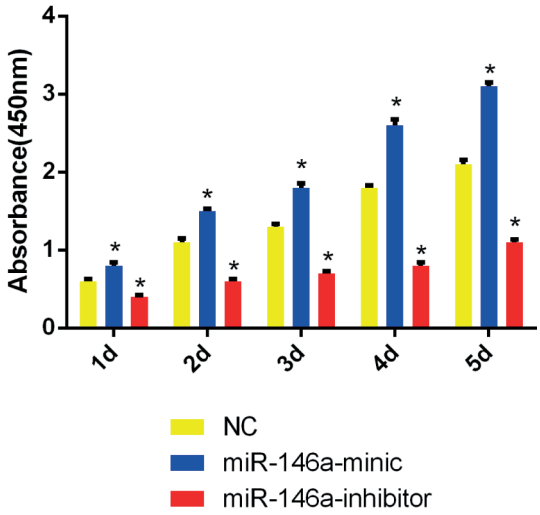


Figure 6. CCK-8 assay results. $^{*}p<0.05$ vs. NC group.

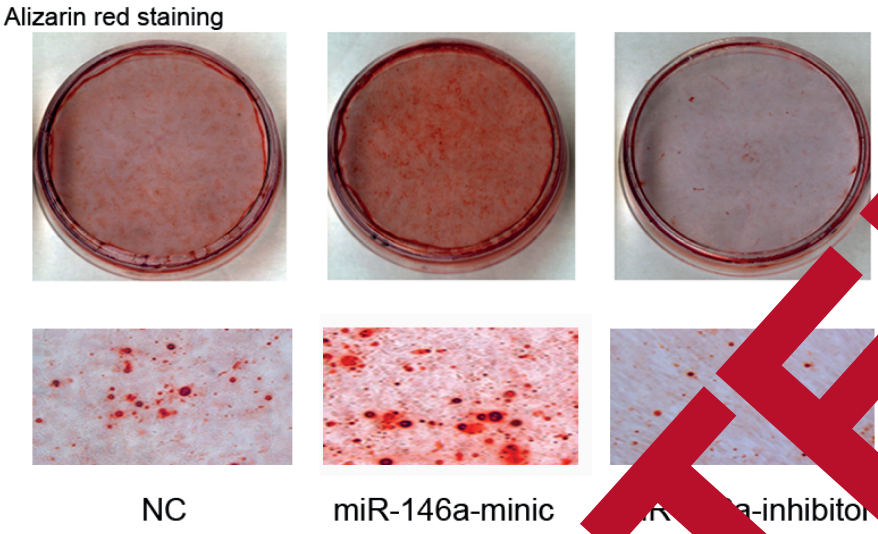


Figure 7. Alizarin red staining result[illegible]ification: 10×).

ferentiation of BMSCs, and miR-146a promoted osteogenic differentiation of BMSCs.

MRNA Expressions of Osteogenesis-Specific Genes in BMSCs in Osteog[illegible] Differentiation-Inducing Medium Detected Via qRT-PCR

BMSCs transfected with miR-NC, miR-14[illegible] mimic, and miR-146a-inhibit[illegible] the thr[illegible] groups were cultured in the [illegible]ge[illegible] differentiation-inducing medium[illegible] mRN[illegible] expressions of osteogenesis-sp[illegible] gene[illegible] Ocn) in BMSCs in the[illegible] ee [illegible] re det[illegible] via qRT-PCR. The [illegible]ts show[illegible] t the mRNA expressions of A[illegible] nd Ocn re[illegible]bly increased in miR-1[illegible] group co[illegible]ed with those in miR-[illegible] grou[illegible] there were statistically sign[illegible]nt differen[illegible]<0.05). It is suggested [illegible] the osteogenic d[illegible]entiation ability was [illegible]ificantl[illegible]nhanced. The mRNA expressio[illegible] ALP[illegible] Ocn [illegible]emarkably decreased in miR-[illegible]itor gr[illegible] compared with those in [illegible]-NC [illegible] and[illegible]re were statistically signi[illegible] differ[illegible]0.05). It is believed that the ost[illegible]enic dif[illegible]tiation ability was inhibited by mi[illegible] knockdown (Figures 8 and 9).

Discussion

Traumatic femoral head necrosis is caused by the interruption of blood supply to the femoral head, component changes in bone marrow and [illegible]ocyte death a[illegible] traumatic fracture due to the s[illegible] anatomi[illegible]ocation of the femoral neck. Tra[illegible] fe[illegible] head necrosis is the severest compli[illegible] a femoral neck fracture. Prevention and treatment of femoral head necrosis have [illegible] a hot spot recently. In this study, miR-[illegible]pression in necrosis group and non-necrosis group was detected via qRT-PCR. It is found that the expression of miR-146a in BMSCs in necrosis group was significantly lower than that in non-necrosis group. Studies have demonstrated that miRNA is involved in regulating BMSCs in traumatic femoral head necrosis. Ying et al[21] found that miR-93-5p inhibits osteogenic differentiation of BMSCs in traumatic femoral head necrosis through targeting BMP-2. MiR-146a may be involved in regulating traumatic femoral head necrosis, and miR-146a overexpression may inhibit traumatic femoral head necrosis.

Proliferation ability of BMSCs in the three groups was detected via CCK-8 assay for consecutive five days. The results manifested that the proliferation ability of BMSCs was significantly enhanced in miR-146a-mimic group compared with that in miR-NC group, while it significantly declined in miR-146a-inhibitor group compared with that in miR-NC group. We may conclude that the overexpression of miR-146a enhanced the proliferation of BMSCs, while miR-146a knockdown inhibited the proliferation of BMSCs. Some studies[22,23] have revealed that the number of local BMSCs in patients with femoral head necrosis declines, and the osteogenic differentiation ability

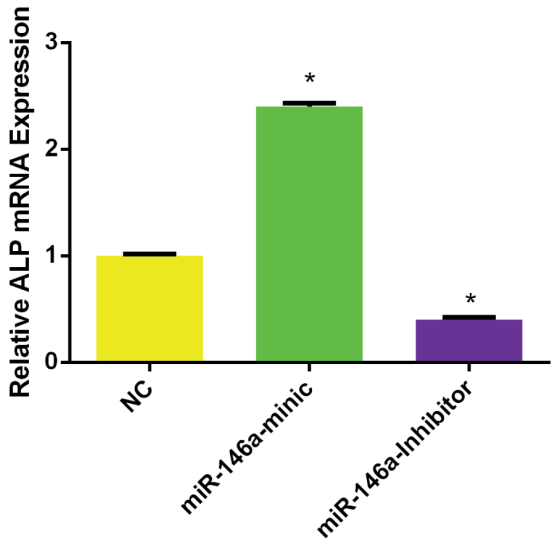
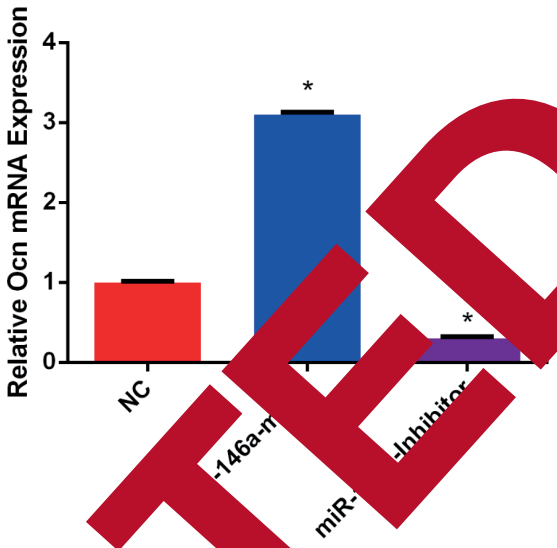


Figure 8. ALP mRNA expression in the three groups detected via qRT-PCR. *p<0.05 vs. NC group.



Figur mRNA ex in the three groups detec -PCR. *p<0.05 v group.

of BMSCs is weakened, which are consistent with our findings. In surgically-treated patients with traumatic femoral head necrosis developing femoral neck fracture, the expression of mi in BMSCs significantly reduced.

In addition, Chen et al[24] found that the oste nic differentiation of BMSCs declines in femo head necrosis. It is reported th steogen differentiation and proliferat Cs play important roles in femoral necro . In this study, miR-146a was als lved the osteogenic differ ati SCs p- tients with traumat moral ecrosis. The deposition of ca on the cel ce in the osteogenic di n-inducing dium in the three grou was de d via alizarin red staining. The lts demonst that the deposition of calc markedly increas n miR-146a-mimic p com d with that in miR-NC group, ind g th osteogenic differentiation ability is tly en ed. Calcium deposition ease iR-1 nhibitor group compared hat i group, indicating that the ost nic dif tiation ability was significantly d. Moreover, the mRNA expressions s-specific genes (ALP and Ocn) in MSCs in the three groups were detected via qRT- . The results demonstrated that the mRNA e sions of ALP and Ocn remarkably increased in miR-146a-mimic group compared with those in miR-NC group, suggesting the enhanced osteogenic differentiation ability. On the contrary, m A expressi of ALP and Ocn remarkably dec in 46a-inhibitor group compared with th iR-NC group, suggesting that the steogenic differentiation ability was inhibited. ed with that in surgically-treated patients th oral neck fracture without traumatic femoral head necrosis, the expression of miR-146a significantly declined in surgically-treated patients with traumatic femoral head necrosis developing from a femoral neck fracture.

Conclusions

We showed that miR-146 regulated the proliferation and osteogenic differentiation of BMSCs in traumatic femoral head necrosis. Further experiments are needed in the future to investigate the downstream signaling pathway of miR-146 in regulating the proliferation and osteogenic differentiation of BMSCs in traumatic femoral head necrosis and its specific mechanism.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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