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Original Article

Expression of miR-214 during fracture healing and its mechanism of action

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Abstract: Objective: To investigate the expression level and mechanism of action of micro ribonucleic acid-214 (miR-214) during fracture healing, and to provide new clues and theoretical basis for the clinical treatment of nonunion. Methods: The expression of miR-214 in human mesenchymal stem cells (hMSCs) at different time points was detected via quantitative reverse transcription-polymerase chain reaction. miR-214 inhibitor and mimics were artificially synthesized and transfected to verify the role of miR-214 in osteogenic differentiation and chondrogenic differentiation. Osteogenic differentiation of hMSCs was induced using osteogenic induction culture solution to observe the expression of miR-214, runt-related transcription factor 2 (Runx2), alpha-1 type I collagen (COL1A1), and osteocalcin genes during osteogenic differentiation of hMSCs. Chondrogenic differentiation was induced using chondrogenic induction culture solution to observe expression of miR-214, alpha-1 type II collagen (COL2A1), aggrecan, and SOX9. Moreover, the rat model of femoral fracture was established, miR-214 control, miR-214 inhibitor, and miR-214 mimics were injected into the fracture end, and micro-CT images were observed after 8 weeks. Results: miR-214 was poorly expressed during osteogenic differentiation of hMSCs, and its expression level was decreased with time during induced differentiation. Inhibiting miR-214 expression could promote osteogenic differentiation, and expression of Runx2, COL1A1 and osteocalcin genes were up-regulated. Overexpression of miR-214 expression could inhibit osteogenic differentiation, and expression of Runx2, COL1A1, and osteocalcin genes were inhibited. Inhibiting miR-214 expression could also promote chondrogenic differentiation, and expression of COL2A1, aggrecan, and SOX9 were up-regulated. Furthermore, injecting miR-214 inhibitor into the fracture end of rats could promote fracture healing and avoid the occurrence of nonunion. Conclusion: Inhibiting miR-214 expression can increase the fracture healing rate and reduce the incidence rate of nonunion, providing new therapeutic regimens for nonunion in clinical orthopedics.

Keywords: Micro ribonucleic acid-214, nonunion, fracture healing

Introduction

Currently, nonunion of fracture (nonunion) is a serious disease in orthopedic surgery, whose incidence rate is high, and even higher in patients with soft tissue injury and severe open fractures, and the therapeutic effect is poor [1]. There are many types of nonunion, and they are affected by a variety of factors, such as unstable internal fixation, inadequate blood supply, poor bone contact, and infection [2]. With rapid development of medical technology, clinical treatment of nonunion patients can be performed via mechanical method, biological method, or mechanical method combined with biological method, but the effects are still not satisfactory. Moreover, there is still a lack of understanding of the pathophysiological change process and molecular biological mechanism of nonunion. Therefore, it is urgent to explore an effective and harmless therapeutic regimen for nonunion.

As a kind of small non-coding gene, micro ribonucleic acid (miRNA) can regulate the gene expression at the post-transcriptional level. miRNA usually binds specifically to the 3'-untranslated region of the target gene messenger

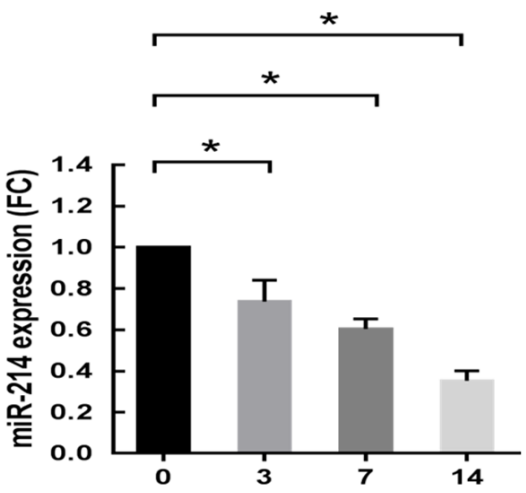

Figure 1. Detection of miR-214 expression at different time points in osteogenic differentiation of hMSCs via quantitative reverse transcription-polymerase chain reaction. miRNA was extracted at 0, 3, 7 and 14 days after osteogenic differentiation for detection, and the experiment was repeated for 3 times, *P<0.05. miR-214, micro ribonucleic acid-214; miRNA, micro ribonucleic acid; hMSCs, human mesenchymal stem cells.

RNA, thereby inhibiting the translation process of protein [3]. miRNA widely exists in the body, and its specific expression can be involved in a variety of biological processes in cells, such as cell proliferation, apoptosis, and differentiation [4-7]. A study has found that miRNA inhibitors or mimics can promote bone regeneration in *in vivo* experiments [8]. However, there are few reports on the effect of injecting miRNA into fracture end on fracture healing. In this study, the effects of micro ribonucleic acid-214 (miR-214) on osteogenic differentiation and chondrogenic differentiation of human mesenchymal stem cells (hMSCs) were analyzed, and miR-214 inhibitor was injected into the fracture end, so as to explore the mechanism of miRNA in nonunion.

Materials and methods

Cell culture

hMSCs were purchased from ScienCell Company (Cat No. 7500), cultured in the high-glucose Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, inoculated into the 6 cm culture dish at a density of $8*10^6$/mL, and cultured in an incubator with 5% CO_2 at 37°C. When 80% cells were fused, they were digested with trypsin, followed by passage at a ratio of 1:2.

Main reagents

DMEM was purchased from Gibco, USA. Lipofection transfection (Lipofectamine 2000) and RNA extraction kit (RNAiso for small RNA) were purchased from Invitrogen, USA. MiRNA-214 inhibitor, miRNA-214 mimics and control were purchased from Ambion, USA.

Cell transfection

hMSCs in the logarithmic growth phase and in good condition were selected, inoculated into a 6-well plate at a density of $2*10^5$/mL and cultured in the incubator overnight. When about 50% cells were fused, transfection was performed according to instructions of the Lipofectamine 2000 transfection kit. In transfection, 100 μL miR-214 control and 5 μL Lipofectamine 2000 were added into control group, 100 μL miR-214 inhibitor (100 pmoL/L) and 100 μL miR-214 mimics (100 pmoL/L) were added into corresponding wells in the inhibition group and the expression group, respectively. After transfection for 6 h, the original medium was replaced with fresh medium. When 60%-70% cells were fused, osteogenic induction culture solution (Cat No. HUXMA-90021) was used to induce osteogenic differentiation, and cells were collected after culture for 14 d to study the role of miR-214 in osteogenic differentiation. After transfection for 6 h using the same method as above, transfected hMSCs were digested with trypsin, centrifuged at 1,000 rpm for 5 min and re-cultured in chondrogenic differentiation culture solution (Cyagen Biosciences, Cat No. HUXMA-9004) to induce chondrogenic differentiation. The original medium was replaced with the new medium once every 3 d, and cells were collected after culture for 14 d to study the role of miR-214 in chondrogenic differentiation.

Real-time polymerase chain reaction method

Detection of miR-214 expression: Induced cells were collected and washed by phosphate buffered saline (PBS) for 3 times. Total miRNA was extracted using RNAiso for small RNA, its mass was detected using agarose gel electrophoresis and its concentration was detected using a spectrophotometer. miRNA was dissolved us-

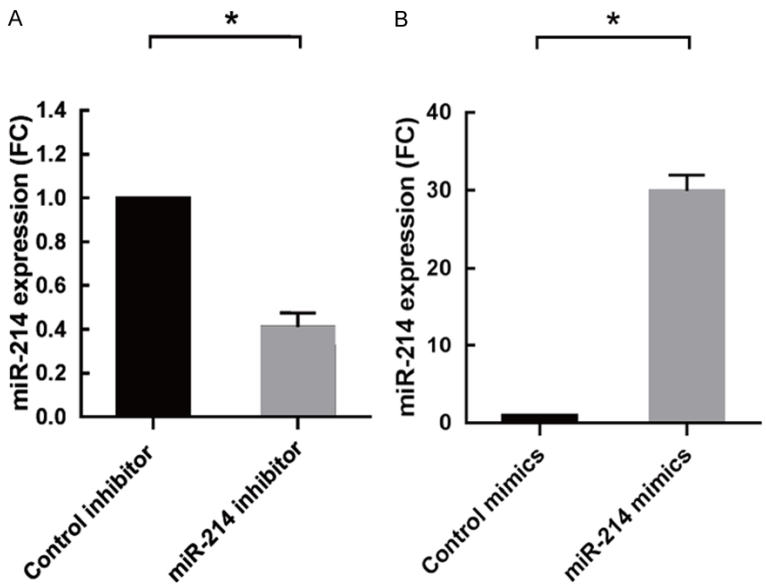

Figure 2. Expression of miR-214 in hMSCs after transfection via quantitative reverse transcription-polymerase chain reaction. A. Expression of miR-214 after transfection with miR-214 inhibitor for 24 h. The experiment was repeated for 3 times. B. Expression of miR-214 after transfection with miR-214 mimics for 24 h. The experiment was repeated for 3 times, *P<0.05. miR-214, micro ribonucleic acid-214; hMSCs, human mesenchymal stem cells.

ing 25 μL diethylpyrocarbonate (DEPC)-treated water. Complementary deoxyribonucleic acid (cDNA) was obtained via reverse transcription according to instructions of the reverse transcriptase kit, and diluted at 1:5 with DEPC-treated water. After SYBR® Premix ExTaqTM II was added according to the proportion in instructions, miR-214 expression was detected using the ABI7500 real-time quantometer (ABI, Germany) with U6 as an internal reference gene. The experiment was repeated for 3 times. miR-214: Forward primer: 5'-TGCGGACAGCAGGCACAGAC-3'; reverse primer: 5'-CCAGTGCAGGGTCCGAGGT-3'. U6: forward primer: 5'-TGCGGGTGCTCGCTTCGGCAGC-3'; reverse primer: 5'-CCAGTGCAGGGTCCGAGGT-3'.

Detection of related gene expression in osteogenic differentiation and chondrogenic differentiation

Total RNA was extracted from cells according to the operating procedure of TRIzol RNA extraction kit, and diluted with 25 μL of DEPC-treated water. The RNA content in all samples was measured using the spectrophotometer. RNA was reverse-transcribed into cDNA using the reverse transcription kit based on the RNA content. After dilution of cDNA according to the above steps, gene expression was detected with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal reference gene. The experiment was repeated for 3 times. Runt-related transcription factor 2 (Runx2): Forward primer: 5'-TGCTGCTGAAACAAACACAC-3'; reverse primer: 5'-TGCT TAGATAAATAAGCCACTTTTC-3'. Alpha-1 type I collagen (COL1A1): Forward primer: 5'-ATCAACCGGAGGAATTTCCGT-3'; reverse primer: 5'-CACC AGGACGACCAGGTTTTC-3'. Osteocalcin: forward primer: 5'-ACTTGTGCTGGGTGGTCT-3'; reverse primer: 5'-CAATACG-CAGTGGCATTAA-3'. SOX9: forward primer: 5'-TGGGAACA-ACCCGTCTAC-3'; reverse primer: 5'-ATTTCGCTGCTCCATT-TA-3'. Alpha-1 type II collagen (COL2A1): Forward primer: 5'-CCTGGCAAAGCTGGTGAG-3'; reverse primer: 5'-CTGATGG-TCCCGGTGGTCCT-3'. Aggrecan: Forward primer: 5'-GGCATCGTGTTCCATTAC-3'; reverse primer: 5'-TCTCCATAGCAGCCTTCC-3'. GAPDH: Forward primer: 5'-GCACCGTCAAGGCTGAGAAC-3'; reverse primer: 5'-TGGTGAAGACGCCAGTGGA-3'.

Establishment of fracture rat model and injection of miR-214

Male SD rats aged 12 weeks (purchased from SPF Biotechnology Company) were divided into 3 groups with 9 rats in each group. After rats were injected intraperitoneally with 2.5% pentobarbital at a dose of 10 mg/kg for deep anesthesia, they were fixed. A longitudinal incision was made on the right lateral knee joint, and the muscle tissue was peeled off to expose the femoral intercondylar groove. The stainless steel needle (0.45 mm) was hammered retrogradely into the right femur, the needle handle was cut off, and the wound was sutured. The rats with needle implanted were transferred onto a fracture impact table, the femur clung closely to the table, and the 350 g weight fell from 6 cm away from the ground to impact the femur. After waking up, rats took food and water, moved freely, and the body temperature was maintained. Rats in control group were injected with 30 μL sterile water at the fracture end, while those in inhibition group and expres-

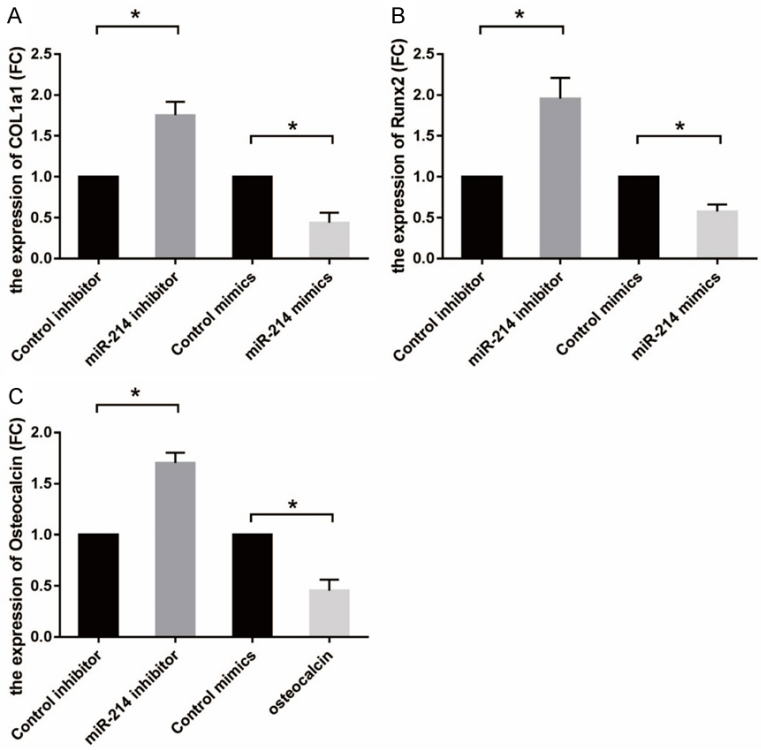

Figure 3. Detection of expression of Runx2, COL1A1, and osteocalcin genes in hMSCs with osteogenic differentiation after transfection with miR-214 inhibitor and mimics for 24 h. A. COL1A1 gene expression. The experiment is repeated for 3 times. B. Runx2 gene expression. The experiment was repeated for 3 times. C. Osteocalcin gene expression. The experiment was repeated for 3 times, *P<0.05. Runx2, Runt-related transcription factor 2; COL1A1, alpha-1 type I collagen; hMSCs, human mesenchymal stem cells; miR-214, micro ribonucleic acid-214.

sion group were injected with miR-214 inhibitor and mimics (dosage: 50 μg, 3 μL buffer, and 27 μL sterile water), respectively. Micro-CT was performed at 8 weeks, the bone surface spacing at the fracture site was calculated, and the fracture healing of rats was determined.

Statistical analysis

SPSS 20.0 statistical software was used for data analysis. One-way analysis of variance was used for the comparison among groups. Least significant difference (LSD)-t test was used for the pairwise comparison. P<0.05 suggests that the difference was statistically significant.

Results

miR-214 expression in osteogenic differentiation

In this study, quantitative reverse transcription-polymerase chain reaction was used to detect miR-214 expression at different time points in differentiation of hMSCs. The expression levels of miR-214 were decreased at 3, 7 and 14 days after osteogenic differentiation. The expression levels of miR-214 at the 3 time points had statistically significant differences compared with that at 0 d (P<0.05). See **Figure 1**. miR-214 inhibitor and miR-214 mimics were transfected into hMSCs to detect expression of miR-214. Results showed that after transfection for 24 h, miR-214 inhibitor could significantly inhibit miR-214 expression (n=3, P<0.05), and miR-214 mimics could promote expression of miR-214 in hMSCs (n=3, P<0.05). See **Figure 2**. After induction via osteogenic differentiation medium, expression levels of osteogenic differentiation markers (Runx2, COL1A1 and osteocalcin) in hMSCs after transfection were detected. Results showed that after transfection for 6 d, miR-214 inhibitor could promote the expressions of Runx2 and COL1A1, while miR-214 mimics could decrease their expressions. After transfection for 14 d, miR-214 inhibitor significantly promoted the expression of osteocalcin, while miR-214 mimics significantly inhibited expression. See **Figure 3**.

Effect of miR-214 on chondrogenic differentiation process

After chondrogenic differentiation induction medium was used for induction, the chondrogenic differentiation markers (COL2A1, aggrecan and SOX9) in hMSCs were detected at 14 d after transfection. Results revealed that inhibiting the miR-214 expression could significantly promote expression of COL2A1, aggrecan, and SOX9 genes, while promoting miR-214 expression had no effects on expression of COL2A1, aggrecan, and SOX9 genes (P>0.05). See **Figure 4**.

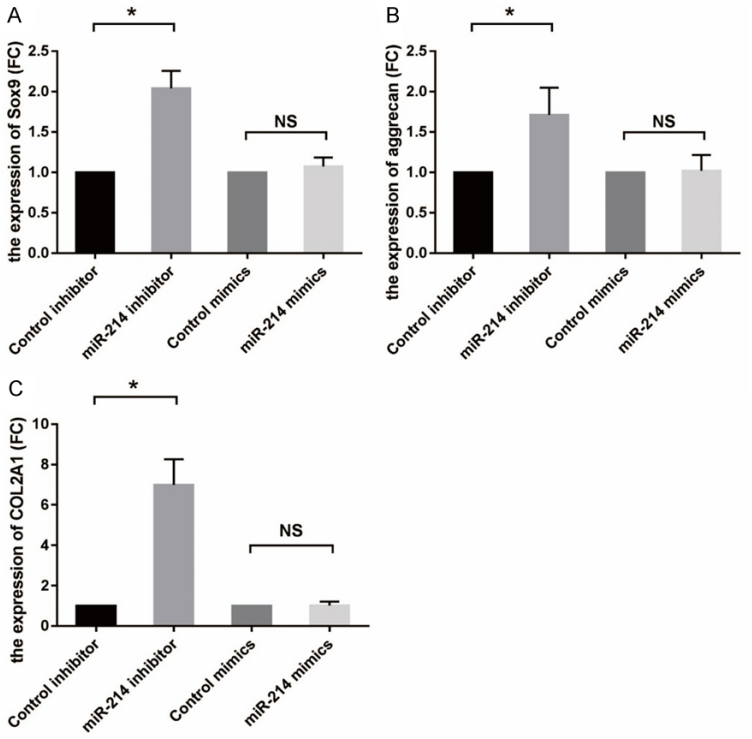

Figure 4. Detection of expression of COL2A1, aggrecan, and SOX9 genes in hMSCs with chondrogenic differentiation via quantitative reverse transcription-polymerase chain reaction after transfection with miR-214 inhibitor and mimics for 24 h. A. Expression of SOX9 gene. The experiment is repeated for 3 times. B. Expression of aggrecan gene. The experiment was repeated for 3 times. C. Expression of COL2A1 gene. The experiment was repeated for 3 times, *P<0.05. NS, not significant. COL2A1, alpha-1 type II collagen; hMSCs, human mesenchymal stem cells; miR-214, micro ribonucleic acid-214.

Effect of low expression of miR-214 on fracture healing

Using the rat model of femoral fracture established, rats were divided into the control group (n=9), the inhibition group (n=9) and the expression group (n=9).

Sterile water, miR-214 inhibitor and miR-214 mimics were injected into the fracture end of rats, respectively. After 8 weeks, the fracture end was radiated via CT and observed. Results showed that compared with the control group, the fracture healing of rats in the inhibition group was significant, and there was obvious nonunion in rats in the expression group. See **Figure 5A**. Furthermore, the fracture surface spacing was analyzed and compared among the three groups of rats. Results showed that the fracture surface spacing in the inhibition group was smaller than those in the expression group and the control group, and differences were statistically significant (P<0.05). See **Figure 5B**.

Discussion

Nonunion is a common complication in orthopedic surgery, seriously affecting the life quality of fracture patient. However, its exact occurrence mechanism remains unclear. In recent years, more and more studies have found that a large number of miRNAs are involved in the regulation of bone metabolism and play important roles in the proliferation and differentiation of MSCs, osteoclasts, and osteoblasts. At the same time, they are helpful to maintain the bone metabolic balance [9]. The study has shown that miR-214 can affect the fracture healing process through a variety of ways [10]. Li et al. found that the expression of miR-214 was abnormally high in BMSCs of ovariectomized rats, and inhibiting miR-214 could promote the osteogenic differentiation and reduce the production of osteoclasts, indicating that inhibiting miR-214 expression can promote the bone formation process [11]. Yang et al. found that overexpression of miR-214 in vitro could inhibit the osteogenic differentiation of BMSCs, whereas inhibiting the miR-214 expression could promote the osteogenic differentiation process, up-regulated the expressions of osteoblastic factors (Runx2, osteocalcin, etc.), and benefited the fracture recovery [12]. Moreover, Li et al. found, through inhibiting and overexpressing miR-214, miR-214 had a targeted regulation on the expressions of alkaline phosphatase, fibroblast growth factor receptor 1, and phosphatase and tensin homolog, thus inhibiting the osteogenic differentiation process [13]. In this study, it was found that the expression level of miR-214 constantly decreased with time in the induced osteogenic differentiation of hMSCs, suggesting that miR-214 may be involved in osteogenic differentiation.

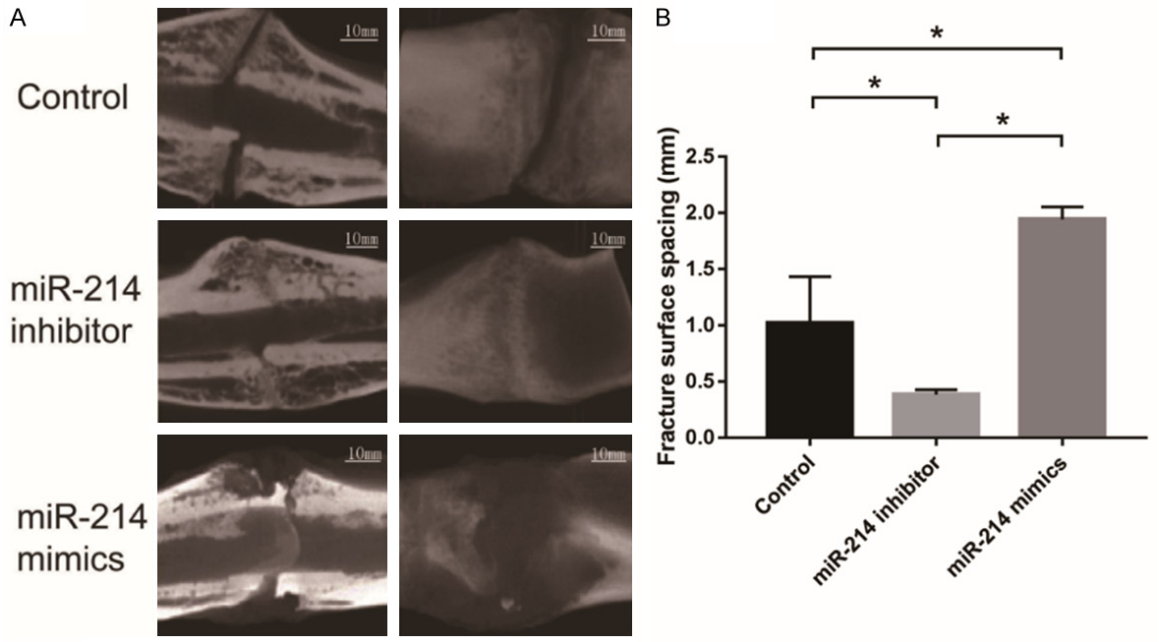

Figure 5. Effect of miR-214 on fracture healing. A. After the rat model of fracture was treated with miR-214 inhibitor and mimics for 8 weeks, CT plain (bar: 10 mm) was performed for the front (A left column) and back (A right column) of the fracture end. B. Fracture surface spacing in rats in each group was measured (n=9). Least significant difference (LSD)-t test was used, *P<0.05. miR-214, micro ribonucleic acid-214.

Osteogenic differentiation plays an important role in bone repair and regeneration, but its main mechanism of action remains unclear. Runx2 is a member of the RUNT gene family, and plays an important role in bone formation. Experiments have found that after MSCs are transfected with Runx2, osteogenesis genes, such as osteocalcin and osteopontin, are significantly up-regulated, indicating that Runx2 is able to promote osteogenic differentiation [14]. COL1A1 is one of the markers of osteogenic differentiation, and studies have found that its mutation can lead to diseases, such as achondroplasia and osteogenesis imperfecta [15]. Osteocalcin is synthesized by osteoblasts, which is one of the indexes reflecting bone turnover [16]. In this study, it was found that inhibiting expression of miR-214 in hMSCs could promote expression of Runx2, COL1A1, and osteocalcin genes, whereas promoting expression of miR-214 could inhibit gene expression in hMSCs. The above results suggest that miR-214 can negatively regulate osteogenic differentiation-related genes. Chondrogenesis is an important influencing factor in the fracture healing process, and SOX9, COL2A1, and aggrecan are also important markers in chondrogenesis. Bell et al. found that SOX9 could bind to the chondrocyte enhancer of COL2A1, and directly regulate its expression [17]. At the same time, SOX9 could regulate the COL2A1 expression in chondrogenesis. Lim et al. studied and found that aggrecan could promote chondrogenic differentiation [18]. In this study, the effect of miR-214 on chondrogenesis process was investigated in hMSCs, and it was found that inhibiting the miR-214 expression could promote the expressions of COL2A1, SOX9, and aggrecan. Therefore, it is believed that the low expression of miR-214 can accelerate the fracture healing in chondrogenic differentiation.

At present, it has been found that miRNAs are involved in the process of bone regeneration and repair [19]. Lee et al. found that injecting miR-29b-3p into the fracture end at 2 weeks after fracture of mice could significantly reduce the callus interval and promote the fracture healing [20]. Murata et al. found that injecting miR-92a inhibitors into mice could promote the fracture recovery [21]. Therefore, it has been speculated that injecting miR-214 into the fracture end of the rat model of fracture can affect the nonunion. In this study, the rat model of femoral fracture was established, and miR-214

inhibitor, miR-214 mimics, and PBS were injected into the fracture end. Results revealed that fracture healing in the miR-214 inhibition group was better without obvious nonunion. The fracture surface spacing was also compared, and results showed that the fracture healing in the miR-214 inhibition group was better. Therefore, injecting miR-214 into the fracture end can effectively prevent the nonunion and accelerate the fracture healing.

In conclusion, the role and relevant mechanism of miR-214 in the fracture healing process were analyzed in this study, and results showed that inhibiting miR-214 could promote osteogenic differentiation and chondrogenic differentiation, and injecting miR-214 inhibitors could prevent the occurrence of nonunion. However, there are still some shortcomings in this study. TargetScan showed that there are hundreds of target genes of miR-214, and whether miR-214 is involved in the regulation process through target genes is not verified yet, so further experiments are needed to clarify this point.

Acknowledgements

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Disclosure of conflict of interest

None.

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