BJR

RESEARCH

Inhibition of microRNA-214-5p promotes cell survival and extracellular matrix formation by targeting collagen type IV alpha 1 in osteoblastic MC3T3-E1 cells

Q. S. Li,
F. Y. Meng,
Y. H. Zhao,
C. L. Jin,
J. Tian,
X. J. Yi

Eastern Medical District of Linyi People's Hospital, Linyi, China

Objectives

This study aimed to investigate the functional effects of microRNA (miR)-214-5p on osteoblastic cells, which might provide a potential role of miR-214-5p in bone fracture healing.

Methods

Blood samples were obtained from patients with hand fracture or intra-articular calcaneal fracture and from healthy controls (HCs). Expression of miR-214-5p was monitored by qRT-PCR at day 7, 14 and 21 post-surgery. Mouse osteoblastic MC3T3-E1 cells were transfected with antisense oligonucleotides (ASO)-miR-214-5p, collagen type IV alpha 1 (COL4A1) vector or their controls; thereafter, cell viability, apoptotic rate, and the expression of collagen type I alpha 1 (COL1A1), type II collagen (COL-II), and type X collagen (COL-X) were determined. Luciferase reporter assay, qRT-PCR, and Western blot were performed to ascertain whether COL4A1 was a target of miR-214-5p.

Results

Plasma miR-214-5p was highly expressed in patients with bone fracture compared with HCs after fracture ($p < 0.05$ or $p < 0.01$). Inhibition of miR-214-5p increased the viability of MC3T3-E1 cells and the expressions of COL1A1 and COL-X, but decreased the apoptotic rate and COL-II expression ($p < 0.05$ or $p < 0.01$). COL4A1 was a target of miR-214-5p, and was negatively regulated by miR-214-5p ($p < 0.05$ or $p < 0.01$). Overexpression of COL4A1 showed a similar impact on cell viability, apoptotic rate, and COL1A1, COL-II, and COL-X expressions inhibiting miR-214-5p ($p < 0.01$).

Conclusion

Inhibition of miR-214-5p promotes cell survival and extracellular matrix (ECM) formation of osteoblastic MC3T3-E1 cells by targeting COL4A1.

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Keywords: MicroRNA-214-5p, Collagen type IV alpha 1 (COL4A1), Bone fracture healing, Cell-extracellular matrix (ECM)

Article focus

- To investigate the functional role of miR-214-5p in osteoblastic cells

Key messages

- miR-214-5p is upregulated in blood samples of patients with bone fracture
- Inhibition of miR-214-5p increases viability of MC3T3-E1 cells, and expression of COL1A1 and COL-X, but decreases apoptosis and COL-II expression
- Inhibition of miR-214-5p impacts MC3T3-E1 cells by targeting COL4A1

Strengths and limitations

- Strengths: Our study may provide new insights into methods of treating a bone fracture.
- Limitations: In this study, we revealed the role of miR-214-5p in the immortalised MC3T3-E1 cells although we did not investigate whether miR-214-5p also has a similar impact on primary osteoblast, which may further improve the findings. Additionally, more in-depth investigations are needed to scrutinise the underlying mechanisms of miR-214-5p in osteoblastic cells.

■ Q. S. Li, MSc, Trauma Surgeon, Department of Traumatology,
■ Y. H. Zhao, BSc, Trauma Surgeon, Department of Traumatology,
■ C. L. Jin, BSc, Trauma Surgeon, Department of Traumatology, Eastern Medical District of Linyi People's Hospital, Linyi, China.
■ F. Y. Meng, BSc, Trauma Surgeon, Department of Traumatology, Lanling People's Hospital, Linyi, China.
■ J. Tian, BSc, Clinician, Operating Room, Linyi Cancer Hospital, Linyi, China.
■ X. J. Yi, MSc, Trauma Surgeon, Department of Traumatology, Eastern Medical District of Linyi People's Hospital, No.233, Fenghuang Street, Linyi 276000, China.

Correspondence should be sent to X. J. Yi;
e-mail: Yixijun1981@126.com

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Introduction

Fractures are common as a result of trauma.[1,2] To date, several techniques have been developed to enhance the process of bone regeneration, such as bone grafting, distraction osteogenesis, bone transport, and administration of growth factors.[3] Although most of the techniques display good results, their effectiveness and availability are limited. It has been reported that approximately 10% of fractures will not heal normally in spite of the great advances made in fracture treatment.[1] To overcome the limitations, a better understanding of the mechanisms of bone healing is necessary. It has been well demonstrated that fracture healing is a complex process that is carefully orchestrated by a variety of cellular elements and stimulating agents.[4,5] However, the precise mechanisms have not been clarified.

microRNAs (miRNAs) are short, endogenous, small, and non-coding RNAs that have the ability to modulate mRNA transcription and translation by base pairing to partially complementary sites in the untranslated region (UTR) of the mRNA.[6,7] It has been well demonstrated that miRNAs display diverse fundamental physiological and pathological processes such as cell proliferation, cell differentiation, cell apoptosis, stress response, and migration and invasion.[8-11] Recently, an increasing volume of evidence has suggested that miRNAs are involved in bone formation, development, remodelling, and regeneration. This involvement ranges from the initial response of stem cells to the metabolic response of mature tissues.[12-14] The functional characterisation of miRNAs indicates the possible important roles in fracture healing. However, very little research has focused on the functional role of miRNAs in fracture healing. A recent study has suggested that levels of plasma miR-92a are significantly higher in patients during fracture healing and that inhibition of miR-92a has therapeutic potential for fracture healing.[15]

In the present study, we aimed to investigate the functional effects of miR-214-5p on osteoblastic cells in order to reveal a potential role for miR-214-5p in fracture healing. The plasma levels of miR-214-5p were analysed in patients with intra-articular hand fractures and intra-articular calcaneal fractures. Mouse osteoblastic MC3T3-E1 cells were used to explore the possible underlying mechanism.

Materials and Methods

Preparation of blood samples. Between April 2015 and March 2016, 28 patients with a fracture who presented at our hospital were enrolled in our study (16 males and 12 females; mean age 45.16 years) (SD 17.66; 28 to 66). Among these patients, 17 were diagnosed to have an intra-articular hand fracture and 11 to have an intra-articular calcaneal fracture. Patients underwent open reduction and internal fixation (plating), closed reduction and internal fixation (pinning) or closed reduction and external fixation surgery. Surgical treatment was performed by orthopaedic hand surgeons.

Blood samples were collected from the patients on day 7, 14, and 21 post-surgery. In addition, blood samples were also collected from six healthy controls (HCs) (four males and two females, mean age 47.39 years) (SD19.58; 27 to 67). They had no evidence of inflammatory arthritis, arthralgia, heart failure, or an autoimmune disease. All the blood samples were separated, centrifuged, and stored at -80°C until analysed. Our study protocol was approved by the hospital medical ethics committees, and informed consent was obtained from all participants.

Cell culture. Mouse osteoblastic MC3T3-E1 cells were obtained from the American Type Culture Collection (ATCC) (Manassas, Virginia). Cells were maintained in α-minimum essential medium (α-MEM; Invitrogen, Carlsbad, California) and supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 100 U/mL penicillin and 100 U/mL streptomycin (Life Technologies, Carlsbad, California) at 37°C in a humidified atmosphere of 5% CO_2. The medium was changed every two to three days.

Transient transfection. Antisense oligonucleotide (ASO)-miR-214-5p and its negative control (ASO-NC), miR-214-5p mimic and its control, and pcDNA3.1 (+)-COL4A1 and its vector control, were designed and synthesised by GenePharma Corporation Ltd (Shanghai, China). Briefly, the cells were seeded in a 96-well plate and incubated for 24 hours. When the cells were at 70% to 80% confluence, they were transfected with the above plasmids or vectors using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Some 48 hours later, the cells were collected and the cell lysates were prepared.

Cell viability assay. The cell viability was measured by using 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as previously described.[16] Briefly, the cells were plated in 96-well plates at a density of 2×10^4 cells/well, and transfected with plasmids or vectors for 48 hours. After transfection at 24, 48, and 72 hours, 0.5 mg/mL MTT solution (Sigma-Aldrich, St Louis, Missouri) was added to each well and incubated for another four hours. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich) was added to all wells to dissolve formazan crystals. Absorbance at 570 nm was read by an EMax Endpoint ELISA Microplate Reader (Molecular Devices LLC, Sunnyvale, California).

Cell apoptosis analysis. Cell apoptosis was determined by Annexin V-FITC/Propidium Iodide (PI) double staining (Becton Dickinson, San Jose, California) according to the manufacturer's instructions, with the use of flow cytometry analysis. The cells were seeded in 96-well plates at a density of 2×10^4 cells/well, and transfected with plasmids or vectors for 48 hours. After being washed with cold phosphate-buffered saline (PBS), the cells were suspended in a binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). The cells were then

re-suspended in Annexin V-FITC and PI for 15 minutes in the dark at room temperature. The percentage of apoptotic cells was examined by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson) and was analysed with CellQuest Software (Becton Dickinson).

Target prediction and luciferase assay. TargetScan 6.2 (Whitehead Institute for Biomedical Research, Cambridge Massachusetts) and/or microRNA. (Memorial Sloan-Kettering Cancer Center, New York, New York) were used in this study to predict the possible target gene of miR-214-5p. The wild-type (WT) or mutated (Mut) COL4A1 3'UTR sequence within the predicted target sites was polymerase chain reaction (PCR)-amplified and sub-cloned into the NheI-SalI fragment of the pmirGLO vector (Promega, Madison, Wisconsin), namely COL4A1 3'UTR WT and COL4A1 3'UTR Mut, respectively. These vectors were co-transfected with miR-214-5p mimic, ASO-miR-214-5p or its negative control using Lipofectamine 2000 (Invitrogen). After 24 hours of transfection, firefly and Renilla luciferase activities were assessed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

Quantitative real-time PCR (qRT-PCR). Total RNA, including miRNAs, was extracted from MC3T3-E1 cells and blood samples using a miRNA Isolation Kit (Applied Biosystems, Foster City, California) according to the manufacturer's protocol. Complementary DNA (cDNA) was obtained by using a NCode VILO miRNA cDNA Synthesis Kit (Invitrogen) according to the manufacturer's protocol. qRT-PCR was performed on an Applied Biosystems 7500 Thermocycler (Invitrogen) by using EXPRESS SYBR GreenER qPCR SuperMix (Invitrogen). U6 snRNA and Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH), respectively, were used as internal references of miR-214-5p and COL4A1 expression. All the primers were purchased from GenePharma. The relative expressions of miRNA and mRNA were normalised to the corresponding internal standard controls by using the comparative $2^{-\Delta\Delta CT}$ methods.

Western blot analysis. The protein level of COL4A1 was determined by using Western blot. After the cells were transfected with ASO-miR-214-5p, ASO-NC, miR-214-5p mimic and its control, and pcDNA3.1 (+)-COL4A1 and its control, for 48 hours, the cells were homogenised in radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China) and additionally, protease inhibitors. Protein concentrations were determined by a bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific Inc., Rockford, Illinois). Equivalent amounts of protein (10 µg) were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and were then transferred to nitrocellulose membranes (Sigma-Aldrich). After blocking with 5% non-fat milk, the membranes were incubated with anti-COL4A1 antibody (ab6586; 1:1000; Abcam, Cambridge, Massachusetts) at 4°C overnight. GAPDH was used as a loading control. Subsequently, the membranes were washed with Tris-buffered saline with Tween 20 (TBST) and incubated with horseradish peroxidase (HRP)-conjugated anti-goat IgG (ab181658; 1:5000; Abcam) at room temperature for two hours. An enhanced chemiluminescence (ECL) system was used to visualise the bands. Quantitative analysis was conducted by Bio-Rad Quantity One 1-D Analysis Software (Bio-Rad Laboratories, Hercules, California).

Statistical analysis. All experiments were performed in triplicate. Data are expressed as mean and SD. Statistical analyses were performed by using Student's *t* test or the one-way analysis of variance (ANOVA) test. Results were analysed using GraphPad Prism 6 (GraphPad Software, La Jolla, California). A p-value of < 0.05 was regarded as statistically significant.

Results

Plasma miR-214-5p level was upregulated after fracture. A qRT-PCR analysis was performed to assess the blood samples from both HCs and patients with a fracture on day 7, 14, and 21 post-surgery. This was to evaluate whether the expression of miR-214-5p in the blood samples of patients with fractures is changed in the fracture-healing processes. The results showed that the relative expression of miR-214-5p was significantly increased in the blood samples from both hand fracture and intra-articular calcaneal fracture patients, compared with HCs, at day 7, 14, and 21 post-surgery ($p < 0.05$ or $p < 0.01$) (Fig. 1). The results demonstrated that miR-214-5p may play a role in the processes of fracture healing.

Inhibition of miR-214-5p promoted cell viability, suppressed apoptosis, and changed collagen (COL) levels. Next, MC3T3-E1 cells were used and the expression of miR-214-5p in cells was altered by transfection with ASO-miR-214-5p. The transfection efficiency was assessed by qRT-PCR. As expected, the relative expression of miR-214-5p was significantly decreased by ASO-miR-214-5p compared with the ASO-NC group ($p < 0.01$) (Fig. 2a). After transfection, cell viability and apoptotic rate were determined. We observed that the cell viability was significantly upregulated by transfection with ASO-miR-214-5p, compared with the control group, after 24, 48 and 72 hours of transfection ($p < 0.05$ or $p < 0.01$) (Fig. 2b). As indicated in Figure 2c, the apoptotic rate was statistically reduced by transfection with ASO-miR-214-5p compared with the control group ($p < 0.01$). We then analysed the effects of the inhibition of miR-214-5p on the expression of collagen type I alpha 1 (COL1A1), type II collagen (COL-II), and type X collagen (COL-X). Our data demonstrated that the inhibition of miR-214-5p markedly increased the expression of COL1A1 and COL-X, but significantly decreased the expression of COL-II compared with the control group (all $p < 0.01$) (Fig. 2d). These results indicate that miR-214-5p inhibition promoted the

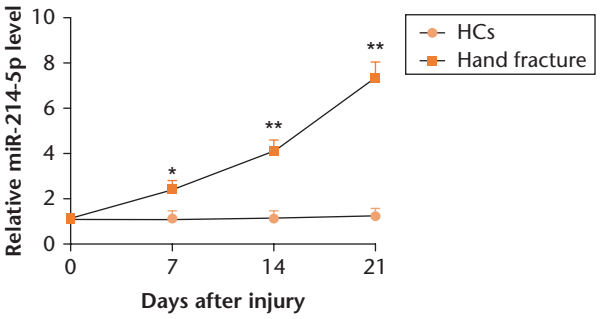


Fig. 1a

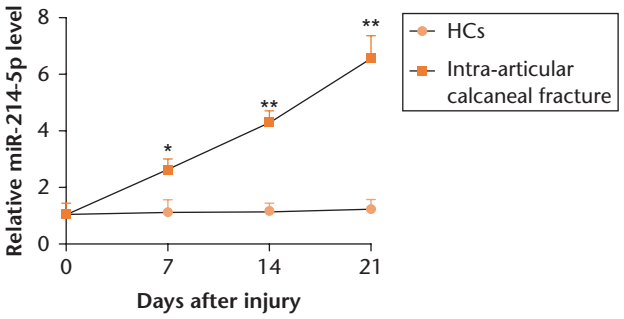


Fig. 1b

Plasma miR-214-5p level is upregulated after fracture. The expressions of miR-214-5p in patients with (a) hand fracture and (b) intra-articular calcaneal fracture at day 7, 14, and 21 post-surgery. *p < 0.05, **p < 0.01 compared with healthy controls (HCs).

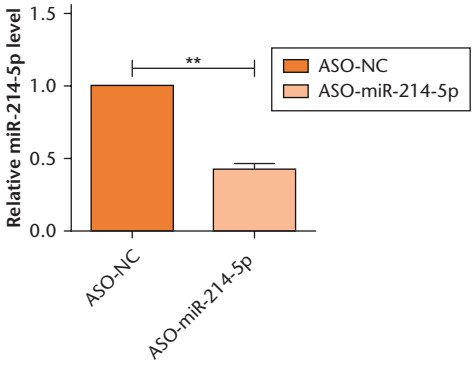


Fig. 2a

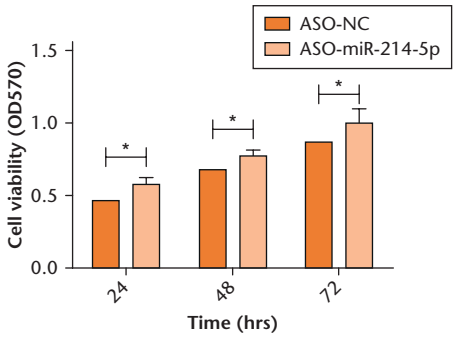
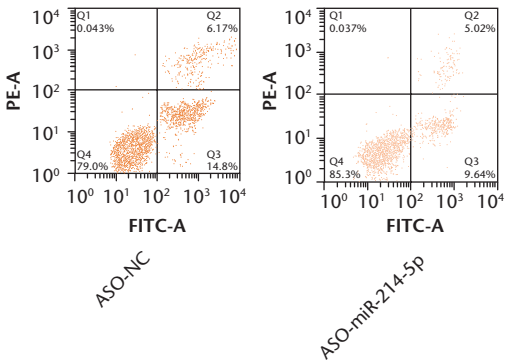


Fig. 2b



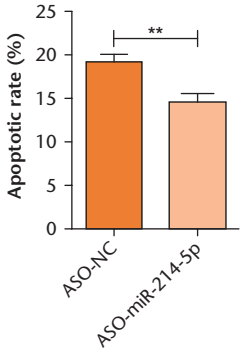


Fig. 2c

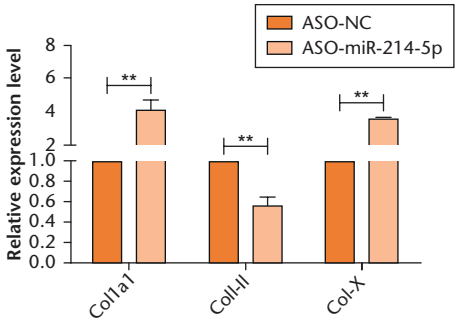


Fig. 2d

Inhibition of miR-214-5p promotes cell viability, suppresses apoptosis, and changes collagen (COL) levels. a) Relative expression of miR-214-5p after transfection with ASO-miR-214-5p; b) effects of inhibition of miR-214-5p on cell viability; c) effects of inhibition of miR-214-5p on cell apoptosis; d) effects of inhibition of miR-214-5p on the expression of COL1A1, COL-II, and COL-X. *p < 0.05, **p < 0.01 compared with ASO-NC.

viability of osteoblastic cells, suppressed apoptosis, and regulated COL levels.

COL4A1 was a direct target of miR-214-5p. To predict the target of miR-214-5p, TargetScan 6.2 and/or microRNA.org were used. As shown in Figure 3a, COL4A1 was predicted to be a target of miR-214-5p. To validate whether COL4A1 was indeed directly regulated by miR-214-5p in MC3T3-E1 cells, we produced reporter plasmids (COL4A1 3'UTR WT and COL4A1 3'UTR Mut). The results showed that the relative luciferase activity was significantly reduced by co-transfection of miR-214-5p mimic with COL4A1 3'UTR WT in MC3T3-E1 cells compared with the control mimic group, while there was no significant difference by co-transfection of miR-214-5p mimic with COL4A1 3'UTR Mut ($p < 0.01$). However, the results were reversed by co-transfection

	Predicted consequential pairing of target region (top) and miRNA (bottom)
Position 207-214 of COL4A1 3' UTR	5′ ...UUGCUGAAGGAGAAGGACAGGCA...
mmu-miR-214-5p	3′ CGUGUCGUUCACAU--CUGUCCGU
Position 1049-1055 of COL4A1 3' UTR	5′ ...CACACCUGAGCACACACAGGCAA...
mmu-miR-214-5p	3′ CGUGUCGUUCACAUCUGUCCGU

Fig. 3a

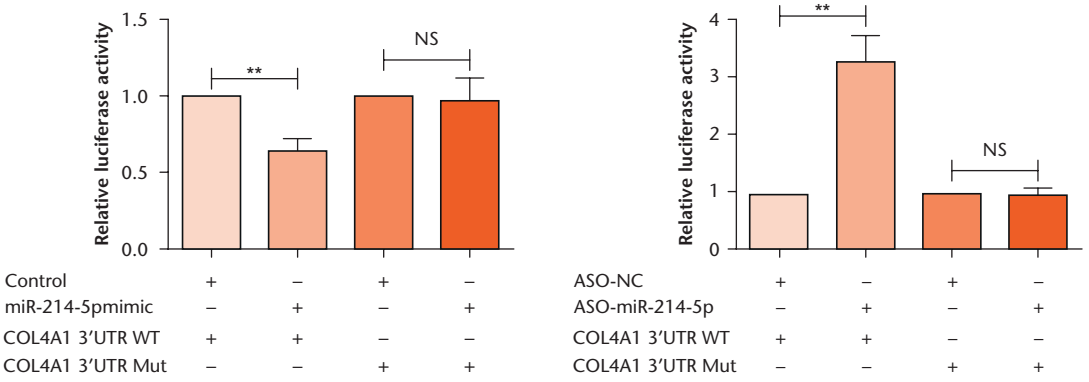


Fig. 3b

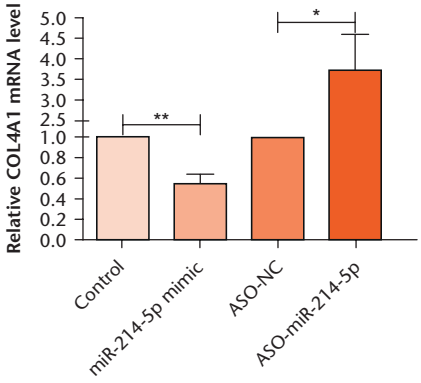
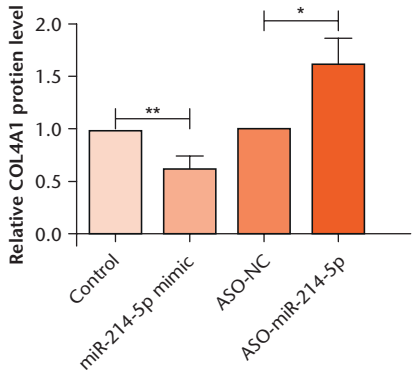


Fig. 3c



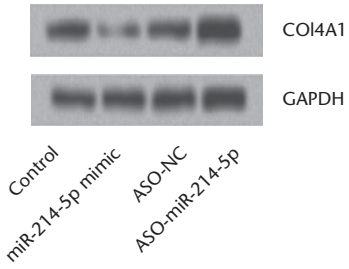


Fig. 3d

COL4A1 is a direct target of miR-214-5p. a) Software prediction of miR-214-5p as potential binding sites on COL4A1 3'UTR; b) relative luciferase activity; c) qRT-PCR for the relative expression of COL4A1 after transfection with miR-214-5p mimic or ASO-miR-214-5p; d) Western blot for the relative expression of COL4A1 after transfection with miR-214-5p mimic or ASO-miR-214-5p. *p < 0.05, **p < 0.01 compared with the control group, NS, not significant.

of ASO-miR-214-5p with COL4A1 3'UTR WT or COL4A1 3'UTR Mut (Fig. 3b). The mRNA and protein expression levels of COL4A1 were then confirmed after abnormal expression of miR-214-5p. As shown in Figures 3c and 3d, both the mRNA and protein levels of COL4A1 were statistically reduced by overexpression of miR-214-5p but significantly increased by inhibition of miR-214-5p ($p < 0.05$ or $p < 0.01$). These results suggest that COL4A1 was a direct target of miR-214-5p and was also negatively regulated by miR-214-5p.

Overexpression of COL4A1 promoted cell viability, suppressed apoptosis, and changed COL levels. To further confirm our results, the expression of COL4A1 was overexpressed and the effects of overexpression of COL4A1 on cell viability, apoptotic rate, and COL expressions were then analysed again. As expected, both the mRNA and protein levels of COL4A1 were significantly upregulated by pcDNA3.1 (+)-COL4A1 compared with those of the control group ($p < 0.01$) (Fig. 4a). Moreover, we found that overexpression of COL4A1 presented similar results to those generated by the inhibition of miR-214-5p. Overexpression of COL4A1 significantly increased cell viability, decreased the apoptotic rate, upregulated the levels of COL1A1 and COL-X, but downregulated the level of COL-II compared with the control group (all $p < 0.01$) (Fig. 4).

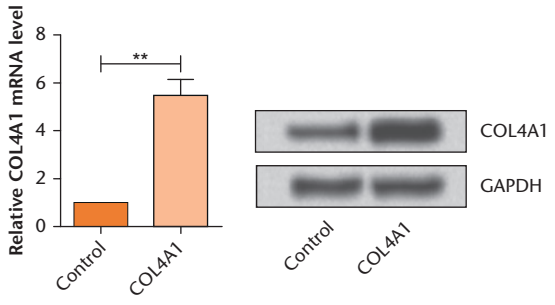


Fig. 4a

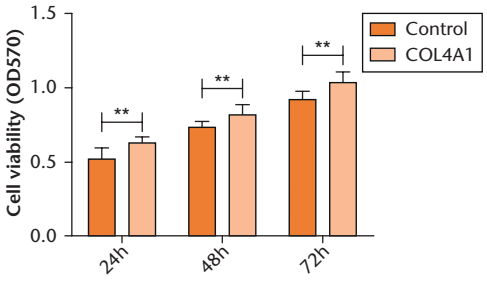


Fig. 4b

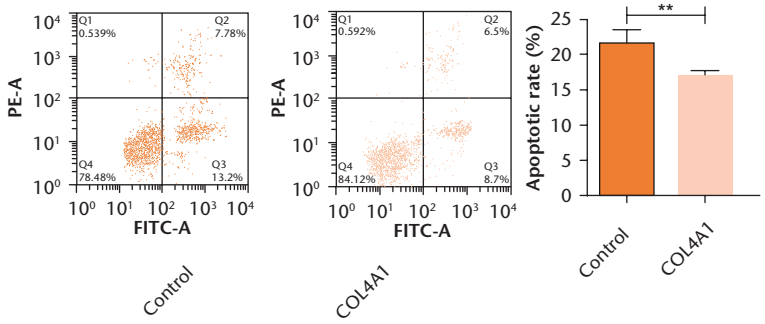


Fig. 4c

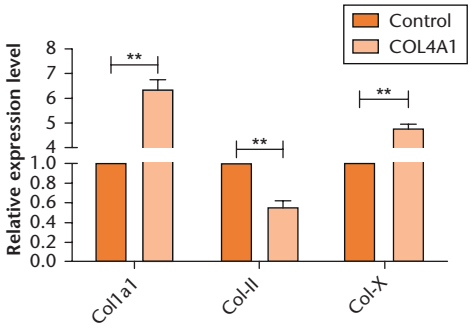


Fig. 4d

Overexpression of COL4A1 promotes cell viability, suppresses apoptosis, and changes collagen (COL) levels. a) Relative expression of COL4A1 after transfection with pcDNA3.1 (+)-COL4A1; b) effects of overexpression of COL4A1 on cell viability; c) effects of overexpression of COL4A1 on cell apoptosis; d) effects of overexpression of COL4A1 on the expression of COL1A1, COL-II, and COL-X. *$p < 0.05$, **$p < 0.01$ compared with the control group.

Discussion

In the present study, we observed that miR-214-5p was significantly increased in the plasma of patients with hand fracture and intra-articular calcaneal fracture after surgery compared with that of the healthy controls. Inhibition of miR-214-5p in MC3T3-E1 cells statistically increased the cell viability while decreasing the cell apoptosis. In addition, inhibition of miR-214-5p upregulated COL1A1 and COL-X expressions while downregulating COL-II expression. COL4A1 was a direct target of miR-214-5p and was negatively regulated by miR-214-5p. Moreover, overexpression of COL4A1 presented similar results to those produced by the inhibition of miR-214-5p.

Circulating miRNAs in the blood have been considered as non-invasive biomarkers in various diseases, including cancer[17-19] and tissue damage.[20,21] Recently, circulating miRNAs have been reported to play a significant role in bone fracture and fracture healing. For example, Murata et al[15] found that, compared with HCs, miR-92a levels in patients with a bone fracture were significantly reduced 24 hours after fracture. Seeliger et al[22] identified five miRNAs (miR-21, miR-23a, miR-24, miR-100, and miR-125b) that were associated with osteoporotic fractures. Waki et al[23] profiled miRNAs in fracture nonunion of femur in a rat model and identified five miRNAs (miR-31a-3p, miR-31a-5p, miR-146a-5p, miR-146b-5p, and miR- 223-3p) that may play an important role in the development of nonunion. Subsequently, Waki et al[24] revealed that, compared with non-healing fractures, another five miRNAs (miR-140-3p, miR-140-5p, miR-181a-5p, miR-181d-5p, and miR-451a) may play an important role in fracture healing. miR-214-5p is a product of the 110 bp miR-214 gene,[25] miR-214 has been reported to suppress bone formation and inhibition of miR-214 in osteoblasts may be a potential strategy for improving osteoporosis.[26] Furthermore, miR-214 acts as an important suppressor of osteogenic differentiation in C2C12 cells.[27] In our study, we provide the evidence that miR-214-5p was also highly expressed in the plasma of patients with bone fracture at day 7, 14, and 21 post-surgery. Therefore, we hypothesised that miR-214-5p might play a certain role during the process of fracture healing.

To confirm our hypothesis, mouse osteoblastic MC3T3-E1 cells were used in the present study. After inhibition of miR-214-5p, the cell viability and cell apoptosis were determined. The results showed that inhibition of miR-214-5p significantly increased the cell viability at 24, 48, and 72 hours, while statistically decreasing the apoptotic rate. We then analysed the effects of inhibition

of miR-214-5p on the expressions of COL1A1, COL-II and COL-X. COLs are the most abundant proteins in the ECM of most animals.[28] COL1A1 is an early osteoblast marker gene,[29] which is highly expressed in most cells of the osteoblast lineage,[30] and has promoting roles in osteoblast proliferation and differentiation.[31] COL-II is a major component of hyaline cartilage and is a marker for chondrocytes, which is responsible for the promotion of chondrogenesis.[32] COL-X is a short chain collagen that is synthesised by terminally differentiating chondrocytes during skeletal development and bone growth.[33] COL-X has been reported to facilitate and regulate endochondral ossification of articular cartilage.[34] During fracture healing, endochondral ossification is a fundamental step[35] Endochondral ossification is a highly developmental complex process requiring coordination among cell-cell, cell-matrix, growth factors, hormones, and extracellular matrix proteins that leads to the generation of mineralised bone from a cartilage template.[36] In the present study, the data showed that the expression levels of COL1A1 and COL-X were significantly increased but the level of COL-II was markedly decreased by inhibition of miR-214-5p. Our findings imply that inhibition of miR-214-5p may promote chondrocyte differentiation and inhibit osteoblast differentiation, and thus may enhance bone fracture healing.

We further investigated the possible underlying mechanism of which miR-214-5p promoting ECM formation in osteoblast. COL4A1 encodes type IV collagen α-chain 1 and is a major component of almost all basement membranes.[37] Recently, it has been reported that the interaction of bone morphogenetic proteins (BMPs) with COL4A1 might partly contribute to bone development, including fracture healing, by its role in vasculogenesis and angiogenesis.[38] Given the aforementioned functions of COL4A1, we speculated that it might be a targeting gene of miR-214-5p and may have played a critical role in fracture healing. As indicated in the results, we observed that COL4A1 was a direct target of miR-214-5p by luciferase reporter assay, and was negatively regulated by miR-214-5p. To confirm whether miR-214-5p promoted ECM formation by regulating the expression of COL4A1, we measured the effects of overexpression of COL4A1 on cell viability, cell apoptosis, and COL expressions. The data demonstrated that overexpression of COL4A1 produced similar results to those generated by the inhibition of miR-214-5p, indicating that the inhibition of miR-214-5p promotes ECM formation possibly by targeting COL4A1.

In conclusion, our results suggest that inhibition of miR-214-5p promotes cell survival and ECM formation of osteoblastic MC3T3-E1 cells by targeting COL4A1. Together with our finding that the level of miR-214-5p increases in patients following a fracture, these findings provide evidence that miR-214-5p may make context-dependent contributions to fracture healing that are dependent on the stage of healing.

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Author Contribution

- Q. S. Li: Study design, Data collection and analysis, Manuscript preparation.
- F. Y. Meng: Study design, Data collection and analysis, Manuscript preparation.
- Y. H. Zhao: Data collection and analysis, Manuscript preparation.
- C. L. Jin: Data collection and analysis, Manuscript preparation.
- J. Tian: Performed surgeries, Data analysis.
- X. J. Yi: Study design, Data collection, Manuscript preparation, Final approval of paper.

Conflicts of Interest Statement

- None