Am J Transl Res 2019;11(6):3645-3650
www.ajtr.org /ISSN:1943-8141/AJTR0094425

Original Article

Inhibition of miR-9 attenuates fibroblast proliferation in human hyperplastic scar by regulating TGF-β1

Ruirui Zhang*, Dandan Liu*, Qun Xie, Qiang Hu, Qiong Wang, Chao Shan, Jing Yang

*Department of Plastic Surgery, Xiangyang Central Hospital of Hubei University of Arts and Science, Xiangyang 441021, Hubei, P. R. China. *Equal contributors.*

Received March 8, 2019; Accepted May 6, 2019; Epub June 15, 2019; Published June 30, 2019

Abstract: Healing of damaged tissue results in scar development, which can be difficult to manage. The present study was performed to determine the effects of inhibition of the microRNA (miR), miR-9, on the proliferation of fibroblasts in human hyperplastic scar (HS) formation. Samples of HS tissue and normal tissue were isolated from 20 patients, and the fibroblasts were transfected with small-interfering RNA (siRNA) for transforming growth factor beta 1 (TGF-β1), miR-9 mimic, and miR-9 inhibition. TGF-β1 protein and mRNA expression were examined in the fibroblasts and HS tissue samples by Western blotting and RT-PCR, respectively. Moreover, the effects of miR-9 inhibitor and mimic on cell proliferation and apoptosis were also examined in the HS tissue. Protein and mRNA expression levels of TGF-β1 were increased in the HS tissue compared to adjacent normal tissues. The levels of TGF-β1 mRNA and protein expression were reduced in siRNA-transfected cells. The miR-9 and TGF-β1 mRNA expression levels were reduced in the miR-9 inhibitor treatment group compared to both the negative control (NC) and control groups. Reduced levels of miR-9 and TGF-β1 mRNA expression were observed in the miR-9 inhibitor treatment group compared to the NC and control groups. Moreover, miR-9 inhibitor increased the percentage of apoptotic cells and decreased cell proliferation compared to the NC and control groups. In conclusion, this study showed that miR-9 plays an important role in the proliferation of fibroblasts by regulating TGF-β1 expression in HS tissue.

Keywords: Hyperplastic scar, fibroblast, small-interfering RNA, transforming growth factor-β1, miR-9

Introduction

Scars are areas in which the normal skin structure has been changed due to healing after tissue damage [1]. There are several types of scar tissue, including normal scars that are somewhat invisible, flat, and thin, as well as hyperplastic scars (HS), which are atypical raised scars occurring after surgery or trauma [2]. It is a challenge to manage HS by plastic surgery. HS tissue is characterised by many pathological changes, including deposition of extracellular matrix-3 and proliferation of fibroblasts [3]. The pathogenesis of scars is still not clear, but hyperplasia commonly occurs due to anomalous proliferation of fibroblasts in such tissue [4]. Over the past several decades, greater emphasis has been placed on determining the possible pathogenesis of scar formation.

The differentiation and growth of cells are regulated by transforming growth factor beta 1 (TGF-β1). Fibroblast differentiation, collagen formation, and proliferation of dermal cells were shown to be enhanced by upregulation of TGF-β1 [5]. The small non-coding RNAs known as microRNAs (miRs) have recently been shown to play major roles in cell proliferation, and several clinical and preclinical studies have suggested that miR expression is altered in proliferating dermal cells [6]. The expression of miR-9 is known to be induced by TGF-β1 in several tissues [7]. The present study was performed to examine the possible role of miR-9 in the pathogenesis of HS formation.

Materials and methods

Chemicals

miR-9 mimic and miR-9 inhibitor were purchased from Ibibio (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD, USA) was supplement-

ed with 10% foetal bovine serum (FBS; Hyclone, Logan, UT, USA). Anti-human TGF-β1 and β-actin antibodies used for Western blotting were purchased from Abcam (Cambridge, UK). TRIzol reagent was purchased from Thermo Fisher Scientific (Wilmington, DE, USA) and V-fluorescein isothiocyanate/propidium iodide (PI) stain was purchased from BD Biosciences (Franklin Lakes, NJ, USA).

Tissue sample collection and cell culture

Normal and HS skin samples were collected from 20 patients by auto-skin grafting biopsy during the period from March 2018 to October 2018 at Xiangyang Central Hospital, China. Patients were selected based on pathological and clinical diagnosis, and several inclusion criteria were applied as follows: no hormone treatment for 3 months before surgery, no prior scar treatment, and no systemic disease. Isolated tissue was stored in liquid nitrogen immediately after collection. Human HS fibroblasts (hHSFs) and human embryonic skin fibroblasts CCC-ESF-1 (ESF) were purchased from Aiyan Biotech Co., Ltd. (Shanghai, China), and cultured in medium supplemented with penicillin/streptomycin and FBS (10% each).

Isolated HS tissues were washed three times with phosphate-buffered saline (PBS) (0.1 M) and skin was removed by digesting the tissue overnight at 4°C with dispase (0.25%). The tissue was then homogenised and treated with type I collagenase (0.1%) for 3 hours at 37°C. Low-glucose DMEM was mixed in an equal volume to the sample to terminate the digestion process. Fibroblasts at a density of 4×10^4/cm^2 were added to the culture plates and cultured at 100% humidity in an atmosphere containing 5% CO_2. The medium was replaced with fresh medium every day, and cells were passaged when they reached confluence.

Cells (3×10^5 cells) were incubated in 12-well plates for 24 hours at 37°C in culture medium containing FBS (10%). To silence TGF-β1, fibroblasts were transfected with small-interfering RNA (siRNA) targeting TGF-β1. As negative controls, treatment was performed with scrambled siRNA, miR-9 mimic, and miR-9 inhibitor. Lipofectamine 2000 (1 μl; Thermo Fisher Scientific) and miR-9 mimic plasmid (1.25 μl) were seeded into individual vials containing Opti MEM medium (50 μl; Thermo Fisher Scientific) when the cells reached 70% confluence. Duplicate solutions were mixed together and kept for 20 minutes. Incubation was performed for 6 hours after adding the mixture with the cells, and fibroblasts were cultured for 2 days under normal conditions.

RT-PCR

TRIzol (1 ml; Thermo Fisher Scientific) was added to 100 mg of powdered tissue sample for lysis, and total RNA was extracted by the phenol-chloroform method. A TIANScript II cDNA first-strand synthesis kit (Tiangen, Beijing, China) was used to isolate the cDNA from RNA (1 μg). PCR was performed in reaction mixtures containing cDNA (2 μl), 0.5 μl of each forward and reverse primer, and SuperReal PreMix (10 μl) in distilled water (7 μl). The expression levels of miR-9 relative to U6, and of TGF-β1 mRNA relative to β-actin mRNA, were calculated using the $2^{-\Delta\Delta Cq}$ method.

Primers	Forward	Reverse
TGF-β1	5'-GGA CAC CAA CTA TTG CTT CAG-3'	5'-TCC AGA CTC CAA ATG TAG-3'
miR-9	5'-GGTCTTTGGTTATC-TAGCTGTATGA-3'	5'-TTTCCTATG-CATATACTTCTTT-3'
U6	5'-CTC GCT TCG GCA GCA CA-3'	5'-AAC GCT TCA CGA ATT TGC GT-3'
β-actin	5'-TTC CAG CCT TCC TTC CTG G-3'	5'-TTG CGC TCA GGA GGA GGA AT-3'

Western blotting

Radioimmunoprecipitation assay lysis buffer (200 μl) was mixed with 100 mg of ground tissue sample for 30 minutes for lysis and the mixture was centrifuged for 15 minutes at 4°C, 12,000 rpm. A bicinchoninic acid (BCA) kit (Thermo Fisher Scientific) was used to estimate the protein concentration in the supernatant. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; 10%) was used to separate the proteins, which were transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with primary antibodies, i.e. rabbit anti-human TGF-β1 and β-actin, overnight at 4°C, followed by washing with PBS and incubation overnight with polyclonal goat anti-rabbit horseradish peroxidase-conjugated secondary antibody. A chemiluminescence detection kit was used to develop the enhanced images of the membranes and image analysis was performed using Image Lab software (ver. 3.0; Bio-Rad, Hercules, CA, USA).

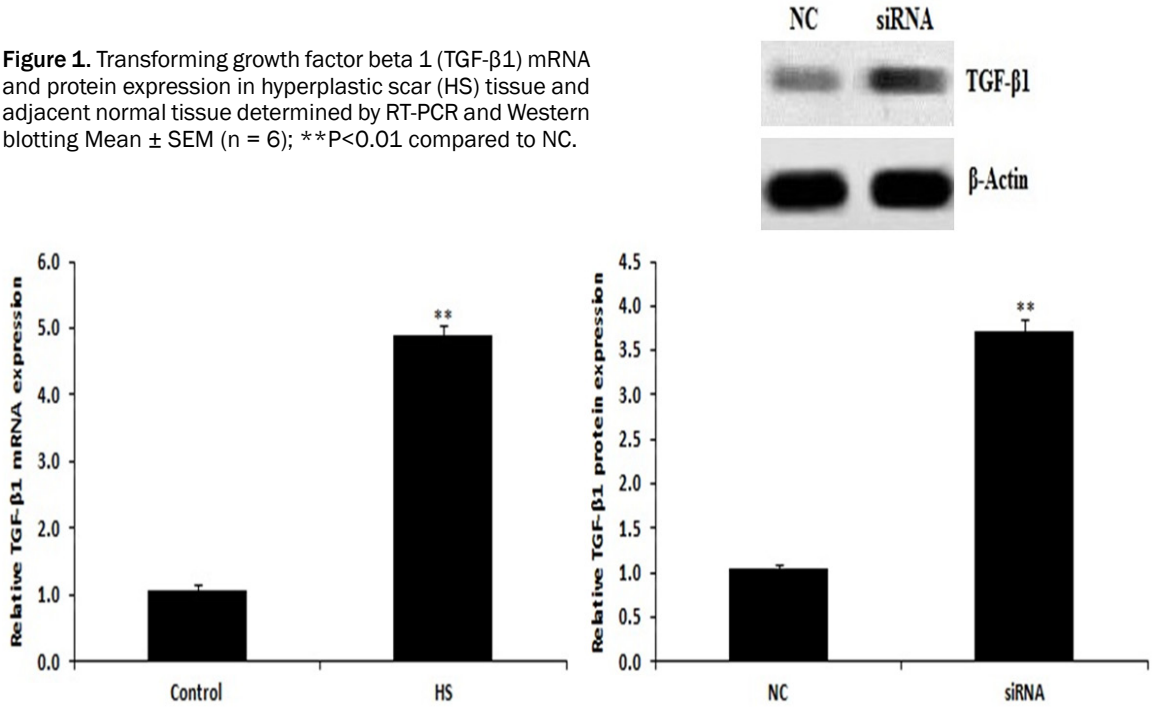

Figure 1. Transforming growth factor beta 1 (TGF-β1) mRNA and protein expression in hyperplastic scar (HS) tissue and adjacent normal tissue determined by RT-PCR and Western blotting Mean ± SEM (n = 6); **P<0.01 compared to NC.

Cell proliferation assay

Qualitative estimation of cell viability was performed by estimating the formation of water-soluble formazan dye from water-soluble tetrazolium salt using a Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). Cells were kept in 24-well plates and transfected with negative control (NC), miR-9 mimic, or miR-9 inhibitor followed 2 days later by RPMI 1640 medium (90 μl; Gibco-BRL) mixed with CCK-8 solution (10 μl). The absorbance at 450 nm (A_{450}) was determined after incubation of the plates at 37°C for 4 hours.

Apoptosis assay

The effects of miR-9 on cellular apoptosis were examined 2 days after transfection with miR-9 mimic or inhibitor, and annexin V-fluorescein isothiocyanate/PI was used to stain the cells at room temperature for 15 minutes. Apoptosis was detected by flow cytometry using WinMDI version 2.5 software (http://facs.scripps.edu) to analyse the data.

Statistical analysis

All data are expressed as the mean ± SEM (n = 6). Statistical analyses were performed using one-way analysis of variance (ANOVA). Post hoc comparison of means was carried out by Dunnett's post hoc test and Student's *t*-test using GraphPad Prism 6.1 software (GraphPad Software Inc., San Diego, CA, USA). In all analyses, $P<0.05$ was taken to indicate statistical significance.

Results

Regulation of HS by TGF-β1

RT-PCR and Western blotting analysis were performed to examine TGF-β1 mRNA and protein expression in HS tissue and adjacent normal tissue, as shown in **Figure 1**. The levels of TGF-β1 protein and mRNA expression were shown to be elevated in HS tissue compared to the adjacent normal tissue. These observations indicated that HS is regulated by TGF-β1.

Role of TGF-β1 expression in fibroblast proliferation

The roles of TGF-β1 mRNA and protein expression in the proliferation of fibroblasts were examined by transfecting cells with siRNA targeting TGF-β1 (**Figure 2**). The siRNA-transfected cells showed reduced levels of TGF-β1 mRNA and protein expression.

miR-9 expression is reduced in HS tissues with miR-9 inhibitor treatment

Figure 3 shows the results of RT-PCR analyses to determine TGF-β1 mRNA and miR-9 expression in fibroblasts prior to and after transfec-

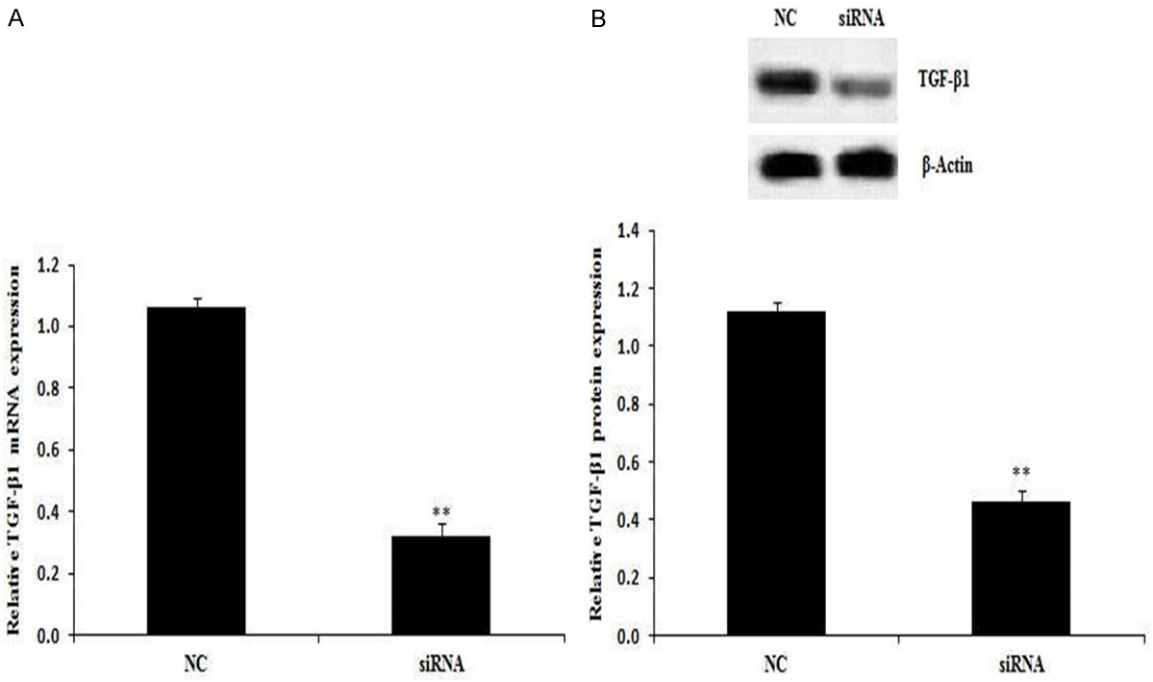

Figure 2. TGF-β1 mRNA and protein expression in fibroblasts prior to and after silencing of TGF-β1 by small-interfering RNA (siRNA). Mean ± SEM (n = 6); **P<0.01 compared to NC.

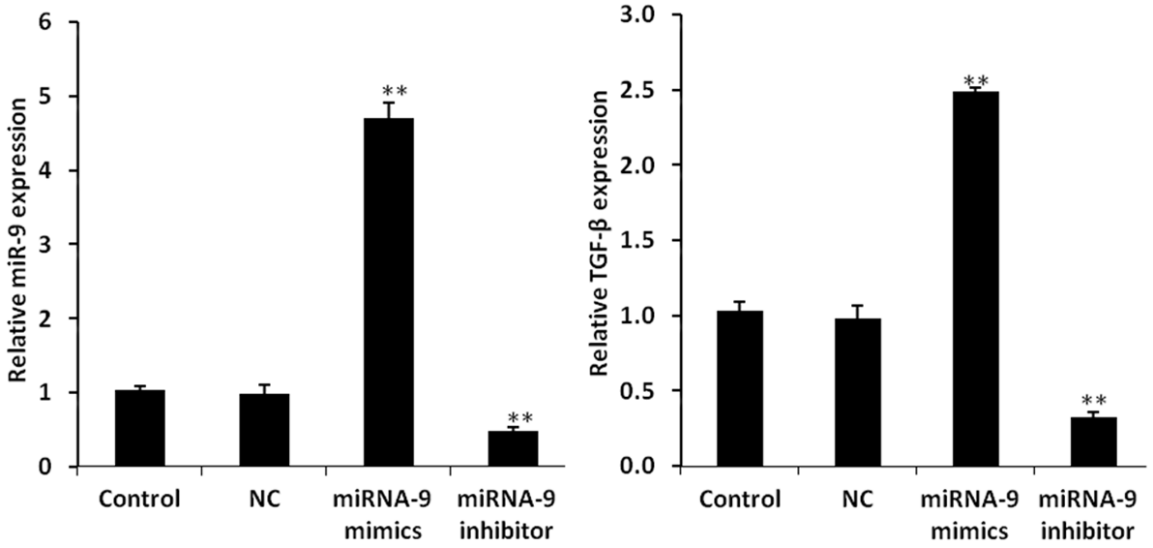

Figure 3. TGF-β1 mRNA and miR-9 expression in fibroblasts prior to and after transfection with miR-9 mimic and inhibitor. Mean ± SEM (n = 6); **P<0.01 compared to NC and control.

tion with miR-9 mimic and inhibitor. The results indicated that neither miR-9 nor TGF-β1 mRNA expression was altered in the NC group compared to the control group. However, the miR-9 mimic treatment group showed increased levels of miR-9 and TGF-β1 mRNA expression compared to both the NC and control groups. The levels of miR-9 and TGF-β1 mRNA expression were reduced in the miR-9 inhibitor treatment group compared to both the NC and control groups.

miR-9 inhibitor treatment suppresses cell proliferation and enhances apoptosis

Figure 4 shows the effects of miR-9 mimic and inhibitor on the apoptosis of cells at 48 hours after transfection, and the percentage of cell

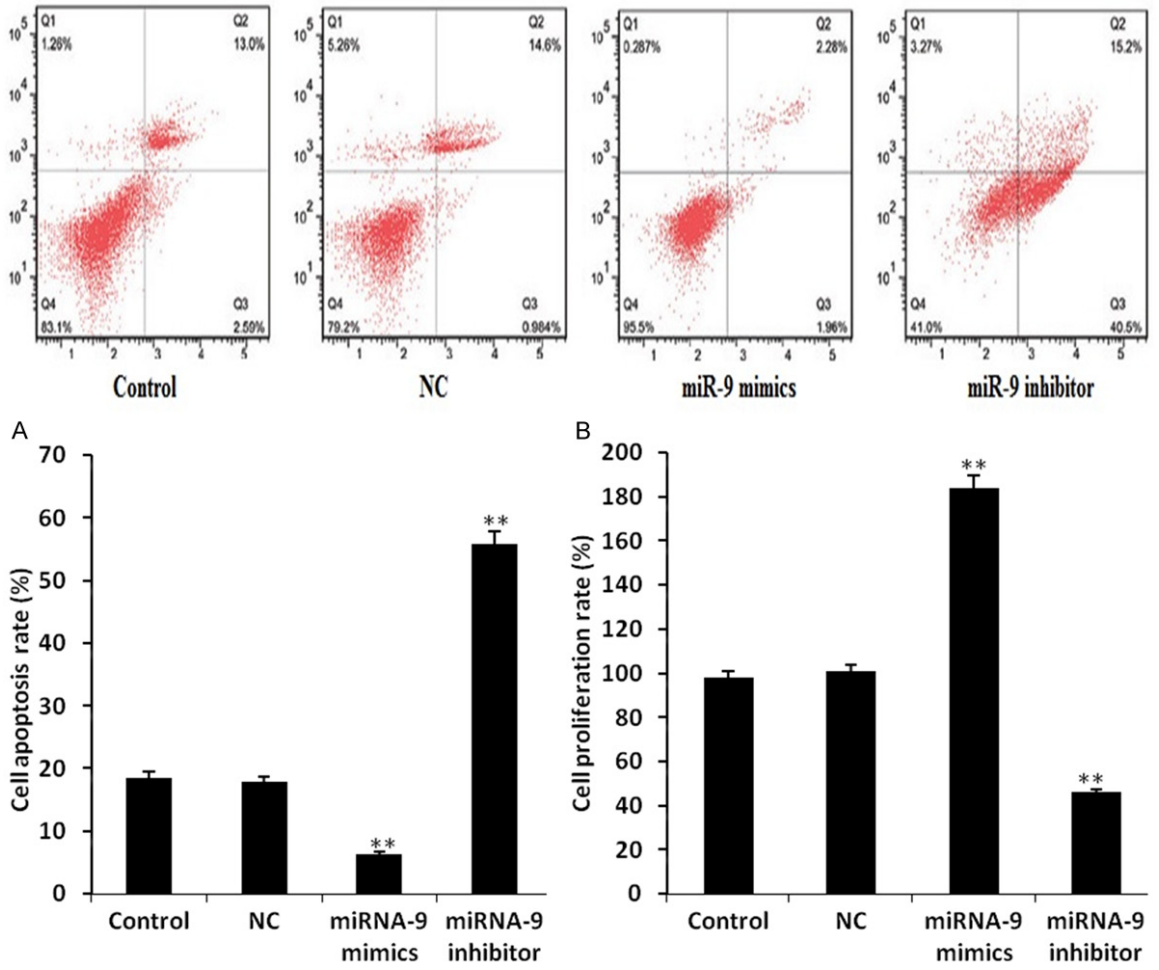

Figure 4. A. Effects of miR-9 on apoptosis at 48 hours after transfection as determined by flow cytometry. B. Effects of miR-9 on cell proliferation determined by Cell Counting Kit-8 (CCK-8) assay. Mean ± SEM (n = 6); **P<0.01 compared to NC and control.

proliferation. The results indicated that the percentage of apoptotic cells was reduced, and cell proliferation was enhanced, in the miR-9 mimic group compared to the NC and control groups. However, miR-9 inhibitor treatment increased the percentage of apoptotic cells and decreased cell proliferation compared to the NC and control groups.

Discussion

The pathogenesis of HS has not been elucidated completely, but extracellular matrix deposition and fibroblast proliferation are known to be increased in HS tissues [8]. Moreover, the pathogenesis of HS was shown to be due to an imbalance between decomposition and synthesis of collagen fibres [9]. HS results in extreme contracture, which causes functional disorder, pain, and other abnormalities conditions in the body. TGF-β1 has been shown to inhibit cell proliferation [10]. Moreover, healing of skin in the foetus showing a reduced level of TGF-β1 expression was reported to differ from healing of adult skin, in terms of a lack of scar tissue formation, reduced collagen content, and faster healing. It has also been suggested that TGF-β1 expression plays an important role in the process of healing [11]. The results of this previous study showed that TGF-β1 expression was elevated in HS, and that primary fibroblasts transfected with siRNA targeting TGF-β1 showed enhanced proliferative activity. Therefore, HS formation was suggested to occur due to elevated TGF-β1 expression.

Moreover, miR-9 plays a vital role in the proliferation and growth of tissue in the body, as well

as a role in the pathogenesis of cancer [12]. In the present study, miR-9 inhibitor increased the percentage of apoptosis and reduced cell proliferation compared to the NC and control groups, whereas miR-9 mimic reduced the percentage of apoptosis and increased cell proliferation compared to the NC and control groups. A previous study indicated that expression of miR-9 is altered in TGF-β1-induced lung cancer. Excess fibrous tissue growth was reported to be due to traumatic injury in HS tissue [13]. The present study estimated the role of miR-9 in the development of HS. The results presented here showed that the level of TGF-β1 expression was lower in the miR-9 inhibitor group than the NC and control groups.

Conclusion

In conclusion, the results of the present study showed that miR-9 plays an important role in fibroblast proliferation by regulating TGF-β1 in HS tissue. Inhibition of miR-9 decreased fibroblast proliferation and may represent a useful therapeutic target for the management of HS.

Acknowledgements

The authors are grateful to Xiangyang Central Hospital, Hubei University of Arts and Science, China, for providing the necessary facilities to conduct the present study.

Disclosure of conflict of interest

None.

Address correspondence to: Qun Xie and Qiang Hu, Department of Plastic Surgery, Xiangyang Central Hospital of Hubei University of Arts and Science, NO.136 Jingzhou Road, Xiangyang 441021, Hubei, P. R. China. Tel: 0086-15071520020; Fax: 00-86-15071520020; E-mail: xohur@yahoo.com (QX); q3i880@163.com (QH)

References

[1] Xue M, Jackson CJ. Extracellular matrix reorganization during wound healing and its impact on abnormal scarring. Adv Wound Care (New Rochelle) 2015; 4: 119-136.

[2] Edriss AS, Mesták J. Management of keloid and hypertrophic scars. Ann Burns Fire Disasters 2005; 18: 202-210.

[3] Bonnans A, Chou J and Werb Z. Remodelling the extracellular matrix in development and disease. Nat Rev Mol Cell Biol 2014; 15: 786-801.

[4] Gauglitz GG, Korting HC, Pavicic T, Ruzicka T, Jeschke MG. Hypertrophic scarring and keloids: pathomechanisms and current and emerging treatment strategies. Mol Med 2010; 17: 113-125.

[5] Andrews JP, Marttala J, Macarak E, Rosenbloom J, Uitto J. Keloids: the paradigm of skin fibrosis - pathomechanisms and treatment. Matrix Biol 2016; 51: 37-46.

[6] Manikandan J, Aarthi JJ, Kumar SD, Pushparaj PN. Oncomirs: the potential role of non-coding microRNAs in understanding cancer. Bioinformation 2008; 2: 330-4.

[7] Duan Y, Chen Q. TGF-β1 regulating miR-205/miR-195 expression affects the TGF-β signal pathway by, respectively, targeting SMAD2/SMAD7. Oncol Rep 2016; 36: 1837-1844.

[8] Frangogiannis NG. Fibroblast-extracellular matrix interactions in tissue fibrosis. Curr Pathobiol Rep 2016; 4: 11-18.

[9] Zhang Y, Lin X, Zhang L, Hong W, Zeng K. MicroRNA-222 regulates the viability of fibroblasts in hypertrophic scars via matrix metalloproteinase 1. Exp Ther Med 2017; 15: 1803-1808.

[10] Herrera RE, Mäkelä TP, Weinberg RA. TGF-beta-induced growth inhibition in primary fibroblasts requires the retinoblastoma protein. Mol Biol Cell 1996; 7: 1335-1342.

[11] Wang L, Qin W, Zhou Y, Chen B, Zhao X, Zhao H, Mi E, Mi E, Wang Q, Ning J. Transforming growth factor β plays an important role in enhancing wound healing by topical application of povidone-iodine. Sci Rep 2017; 7: 991.

[12] Nowek K, Wiemer EAC, Jongen-Lavrencic M. The versatile nature of miR-9/9* in human cancer. Oncotarget 2018; 9: 20838-20854.

[13] Han L, Wang W, Ding W, Zhang L. MiR-9 is involved in TGF-β1-induced lung cancer cell invasion and adhesion by targeting SOX7. J Cell Mol Med 2017; 21: 2000-2008.