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

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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-
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ed with 10% foetal bovine serum (FBS; Hy-
clone, 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 cri-
teria were applied as follows: no hormone treat-
mend 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 Biote-
ch Co., Ltd. (Shanghai, China), and cultured in
medium supplemented with penicillin/strepto-
mycin 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 tis-
sue 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 vol-
ume to the sample to terminate the digestion
process. Fibroblasts at a density of 4 × 10^4/
cm² were added to the culture plates and cul-
tured at 100% humidity in an atmosphere con-
taining 5% CO₂. The medium was replaced with
fresh medium every day, and cells were pas-
saged 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, fibro-
blasts were transfected with small-interfering
RNA (siRNA) targeting TGF-β1. As negative con-
trols, treatment was performed with scrambled
siRNA, miR-9 mimic, and miR-9 inhibitor. Lipofe-
tectamine 2000 (1 μl; Thermo Fisher Scientific)
and miR-9 mimic plasmid (1.25 μl) were seed-
ed 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 nor-
mal 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 reac-
tion mixtures containing cDNA (2 μl), 0.5 μl of
each forward and reverse primer, and Super-
Real 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^ΔΔCq method.

Western blotting

Radioimmunoprecipitation assay lysis buffer
(200 μl) was mixed with 100 mg of ground tis-
sue sample for 30 minutes for lysis and the
mixture was centrifuged for 15 minutes at 4°C,
12,000 rpm. A bicinechonic acid (BCA) kit (Th-
ermo 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) mem-
branes. The membranes were incubated with pri-
mary antibodies, i.e. rabbit anti-human TGF-β1
and β-actin, overnight at 4°C, followed by wa-
sing with PBS and incubation overnight with
polycyonal goat anti-rabbit horseradish peroxi-
dase-conjugated secondary antibody. A chemi-
luminescence detection kit was used to devel-
op the enhanced images of the membranes
and image analysis was performed using Image
Lab software (ver. 3.0; Bio-Rad, Hercules, CA,
USA).
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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 (A450) 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 transfection.
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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.

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...
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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
miR-9 inhibition attenuates hyperplastic scar 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

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Disclosure of conflict of interest

None.

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