

# miR-181b-5p promotes proliferation and inhibits apoptosis of hypertrophic scar fibroblasts through regulating the MEK/ERK/p21 pathway

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**Abstract.** Hypertrophic scar (HS) is a common skin disorder occurring during the wound healing process, and the pathogenesis of HS remains unclear. Previous studies indicated that miRNAs may be involved in the onset and progression of HS. In the present study, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting were used to investigate the expression of miR-181b-5p and decorin in HS tissues. Direct interaction between miR-181b-5p and decorin was confirmed using a dual-luciferase assay. Human HS fibroblasts (HSFbs) were cultured and transfected with miR-181b-5p mimics, and MTT assay and Annexin V fluorescein isothiocyanate/propidium iodide staining were performed to investigate the role of miR-181b-5p in the proliferation and apoptosis of HSFbs. Subsequently, the expression levels of mitogen-activated protein kinase kinase (MEK), phospho (p)-extracellular signal-regulated kinase (ERK) and p21 were determined in HSFbs transfected with miR-181b-5p mimics and untransfected cells using RT-qPCR and western blotting. The results indicated upregulation of miR-181b-5p and down-regulation of decorin expression in HS tissues compared with normal skin samples. miR-181b-5p may regulate the expression of decorin through direct binding to the 3'-untranslated region, as demonstrated by the results of the dual-luciferase assay. Transfection with miR-181b-5p mimics in HSFbs enhanced cell proliferation, reduced apoptosis and increased the expression of MEK, p-ERK and p21. Furthermore, treatment with MEK inhibitor in HSFbs transfected with miR-181b-5p mimics partially inhibited miR-181b-5p-induced antiapoptotic

effects. Taken together, increased expression of miR-181b-5p may serve important roles in the pathogenesis of HS through regulating the MEK/ERK/p21 pathway, suggesting that miR-181b-5p may be a therapeutic target for the treatment of HS.

## Introduction

Hypertrophic scar (HS) is a common fibroproliferative disorder that may develop following thermal or traumatic injuries involving reticular dermis, characterized by excessive extracellular matrix (ECM) deposition and dysregulated activation of fibroblasts (1). Pathological scars frequently affect a patient's quality of life due to functional and/or aesthetic reasons (2). Although the mechanisms underlying the pathogenesis of HS are not well understood, excessive proliferation and apoptosis resistance of HS fibroblasts (HSFbs) serve roles in HS formation (3,4).

MicroRNAs (miRNAs/miRs) are a group of small non-coding RNAs (18-22 nucleotides in length), which regulate the expression of target mRNAs through binding to their 3'-untranslated region (UTR) (5). Previous studies indicated that miRNAs serve roles in the pathophysiological processes of certain skin disorders (6,7). Therefore, the role of miRNAs in HS has been previously investigated (8,9). A recent study indicated that HS tissues exhibit increased levels of miR-181b-5p compared with normal skin (10). Inhibition of miR-181b-5p reverses transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1)-induced myofibroblast differentiation in HS, suggesting that miR-181b-5p may be involved in the pathological process of HS (10). A number of studies demonstrated that miR-181b-5p is associated with proliferation of various types of cells, including vascular smooth muscle cells (11), tumor cells (12-14) and metanephric mesenchymal cells (15). Therefore, the present study aimed to investigate whether miR-181b-5p serves a role in the regulation of proliferation and apoptosis of HSFbs.

A search for genes targeted by miR-181b-5p using TargetScan database has revealed a potential binding site for miR-181b-5p in the 3'-UTR of decorin. A previous study confirmed that miR-181b-5p can directly regulate the expression of decorin

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by binding to the predicted target site (10). Decorin, a member of the small leucine-rich proteoglycan family, is an abundant protein in the ECM, where it inactivates profibrotic TGF- $\beta$ 1 and regulates cellular functions involved in wound healing and scar formation (16,17). Decreased decorin expression level was identified in HS tissues compared with normal skin samples (10), and recombinant decorin was demonstrated to induce an *in vitro* and *in vivo* anti-fibrotic effect in HS development (18-20). Decorin exerts its beneficial functions partially through inhibiting proliferation and inducing apoptosis of HSFbs (19,20). By targeting decorin, miR-181b-5p may serve a role in proliferation and apoptosis of HSFbs during HS formation.

The present study aimed to confirm the dysregulated expression of miR-181b-5p and its target decorin in HS tissues and the direct interaction between miR-181b-5p and decorin. Furthermore, the effect of miR-181b-5p on proliferation and apoptosis of HSFbs was also determined in the present study. The results of the present study indicated that the mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK)/p21 pathway may serve a role in the mechanisms underlying the effect of miR-181b-5p on HSFbs. These results may improve the understanding of the molecular mechanisms of HS and provide novel diagnostic and predictive markers for HS therapy.

## Materials and methods

**Tissue samples.** A total of 30 pairs of HS tissues and adjacent normal skin tissues were obtained from patients who underwent plastic surgery in the Department of Dermatology, The First Hospital of China Medical University (Shenyang, China), and the diagnosis of HS was confirmed using routine pathological approaches as previously described (21). All experiments were approved by the Ethics Committee of Eastern Liaoning University (Dandong, China), and written informed consent was obtained from all patients.

**Cell culture.** Human HS fibroblasts (HSFbs; Shanghai Bioleaf Biotech, Co., Ltd., Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) and 1% (w/v) penicillin/streptomycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in a humidified incubator with 5% CO<sub>2</sub> at 37°C.

**Cell transfection.** HSFbs were transfected with 50 nmol miR-181b-5p mimics or negative control (NC) miRs (Shanghai GenePharma Co., Ltd., Shanghai, China) using Lipofectamine<sup>®</sup> 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Cells were harvested 48 h following transfection for further analysis. To investigate the association between miR-181b-5p and the MEK/ERK/p21 signaling pathway, cells transfected with 50 nmol miR-181b-5p mimics were treated with MEK inhibitor trametinib (Selleck Chemicals, Houston, TX, USA). The sequences of the miR-181b-5p mimics and NC were as follows: miR-181b-5p mimics, 5'-AACAUUCAUUGCUGCGGUGG GU-3'; miR-181b-5p mimics NC, 5'-UUCUCCGAACGUGUC ACGUTT-3'.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc.) and reverse transcription was performed using PrimeScript<sup>™</sup> RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China) at 37°C for 15 min and 85°C for 5 sec. qPCR was performed using SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> kit (Takara Biotechnology Co., Ltd.) with an ABI 7300 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to manufacturer's protocol. The following thermocycling conditions were used for the qPCR: Initial denaturation at 95°C for 30 sec; 40 cycles of 95°C for 5 sec and 60°C for 30 sec. Primers were synthesized by GenScript (Nanjing, China) and are listed in Table I. Relative expression of p21 in each sample was normalized to the level of GAPDH using the 2<sup>- $\Delta\Delta$ C<sub>q</sub></sup> method (22). The expression of miR-181b-5p was determined using the Hairpin-it<sup>™</sup> miRNAs qPCR Quantitation kit (Shanghai GenePharma Co., Ltd.) according to manufacturer's protocol, and RNA, U6 small nuclear 6, pseudogene (GenScript Corporation, Piscataway, NJ, USA) was used for normalization.

**Western blotting.** Proteins were extracted from the harvested cells using radioimmunoprecipitation assay (Beyotime Institute of Biotechnology, Haimen, China), and protein concentration was determined using Pierce<sup>®</sup> BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.). Proteins were separated using SDS-PAGE (10% gel), and transferred onto polyvinylidene difluoride membranes. Subsequently, the membranes were blocked at room temperature for 1 h using 5% non-fat milk in tris-buffered saline and incubated with primary antibodies: Anti-decorin (cat. no. ab175404), anti-MEK (cat. no. ab178876), anti-ERK (cat. no. ab54230), anti-p-ERK (cat. no. ab222493) and anti-p21 (cat. no. ab109520; all 1:1,000; Abcam, Cambridge, MA, USA) at 4°C overnight. The membranes were washed and incubated with secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit; cat. no. ab6721; 1:5,000; Abcam). Bands were visualized by incubation with Novex<sup>®</sup> ECL Chemiluminescent Substrate Reagent kit (Thermo Fisher Scientific, Inc.), and the signals were detected using ChemiDoc<sup>™</sup> XRS+ imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Cell apoptosis assay.** Cell apoptosis was evaluated using Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA). Cells were washed with ice-cold PBS, resuspended in cold Annexin V binding buffer, and incubated with Annexin V/FITC and PI for 15 min at room temperature. The percentage of apoptotic cells was analyzed using BD FACSVerse<sup>™</sup> flow cytometer (BD Biosciences). The data was analyzed by FlowJo (version 7.6.5; Tree Star, Inc., Ashland, OR, USA).

**Cell proliferation assay.** Cell proliferation was measured using MTT assay (Sigma-Aldrich; Merck KGaA) in a 96-well plate according to the manufacturer's protocol. MTT solution was added to each well and the plate was incubated at 37°C for 4 h. The optical density of each well was measured using a microplate reader at a wavelength of 490 nm.

Table I. Sequences of the primers used in the reverse transcription-quantitative polymerase chain reaction analysis.

Name	Primer sequence (5'-3')	
	Forward	Reverse
miR-181b	GCGGATCATTCATTGCTGTCG	ATCTGGTGGCTCTCGGAGTAA
Decorin	CGGAATTCACATGAAGGCCACTATCATCCT	CGGGATCCTTATAGTTTCCGAGTTGAATG
MEK	GACGACCAGTGGGGAGAGTA	ACAGCGGCTCAACTCTCAAA
p21	ATGTCAGAACCGGCTGGGGA	TTAGGGCTTCCTCTTGAGAGA
GAPDH	AAGTATGACAACAGCCTCAAGA	CACCACCTTCTTGATGTCATCA
U6	CTCGCTTCGGCAGCACATA	GTGCAGGGTCCGAGGT

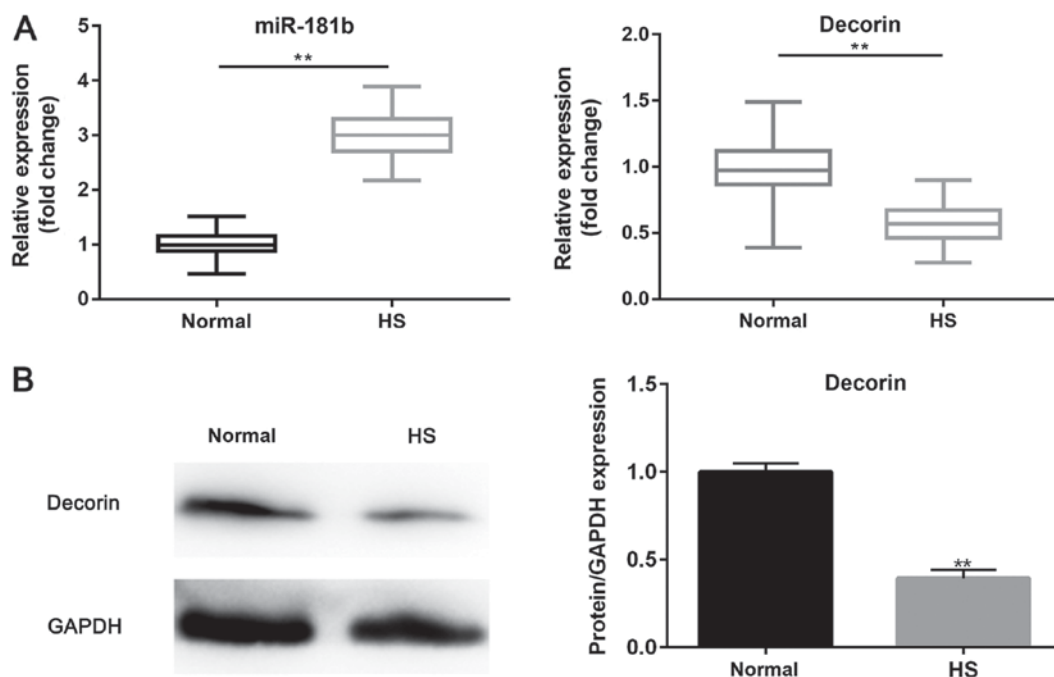


Figure 1. miR-181b-5p is upregulated and negatively associated with decorin expression in HS tissues. (A) Reverse transcription-quantitative polymerase chain reaction analysis of miR-181b-5p and decorin mRNA expression in HS tissues and normal control samples. (B) Representative western blot analysis of the protein expression of decorin. GAPDH was used as the loading control. Data were normalized to the expression level of GAPDH. Results are presented relative to the value of normal control samples. \*\* $P < 0.01$  vs. the normal control samples. HS, hypertrophic scar.

**Prediction of target gene of miR-181b-5p.** To predict the target gene of miR-181b-5p, the authors used the online website TargetScan ([www.targetscan.org](http://www.targetscan.org)). miR-181b-5p has a binding site at the 3'-UTR of decorin.

**Dual luciferase activity assay.** The 3'-UTR of decorin with the predicted miR-181b-5p binding site was amplified by PCR from genomic DNA, and inserted into the pMIR-REPORT<sup>™</sup> luciferase reporter vector (Thermo Fisher Scientific, Inc.) to obtain the wild-type luciferase reporter plasmid (p-decorin-wt). Several nucleotides were mutated in the predicted binding region of miR-181b-5p using PCR, which used the Easy-Load<sup>™</sup> PCR Master Mix (Beyotime Institute of Biotechnology); the thermo profile was as follows: Initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and elongation at 72°C for 1 min, and final elongation at 72°C for 1 min. This fragment was cloned into the pMIR-REPORT

luciferase reporter vector to generate a mutant reporter plasmid (p-decorin-mut). All constructed plasmids were verified through DNA sequencing. 293T cells were cultured in 96-well plates and co-transfected with miR-181b-5p or NC, and luciferase reporter plasmid using Lipofectamine 3000. After 24 h, the cells were lysed and their luciferase activities were assayed using Dual-Luciferase<sup>®</sup> Reporter Assay System (Promega Corporation, Madison, WI, USA). Values were normalized to *Renilla* luciferase activity.

**Statistical analysis.** All experiments were performed in triplicate. Data were analyzed using SPSS 20.0 software (IBM Corp., Armonk, NY, USA) and presented as the mean  $\pm$  standard deviation. Statistical significance between two groups was analyzed using Student's t-test, and the comparisons among multiple groups were performed using analysis of variance followed by Turkey's post hoc test.  $P < 0.05$  was considered to indicate a statistically significant difference.

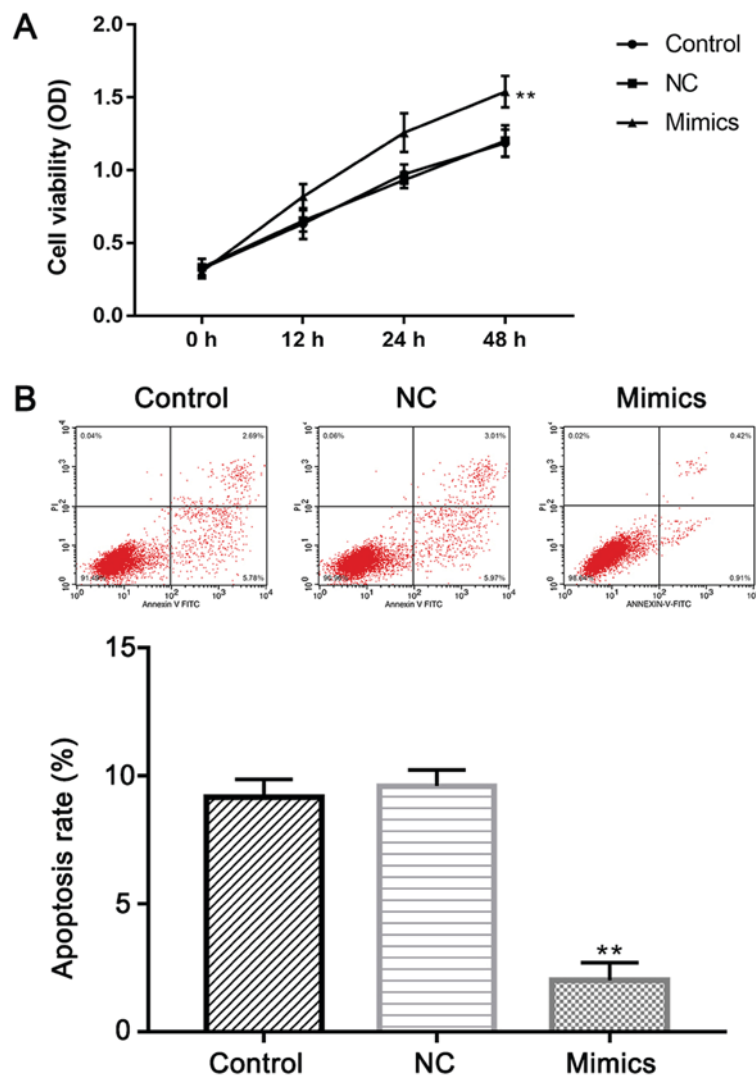


Figure 2. miR-181b-5p increases the proliferation and inhibits apoptosis of human HSFbs. Primary human hypertrophic scar fibroblasts were transfected with miR-181b-5p mimics or NC miRs. Untransfected cells were used as the normal control. (A) MTT assay was used to examine cell proliferation with OD values measured at a wavelength of 490 nm. Data are presented as the mean  $\pm$  standard deviation of three independent experiments. (B) Representative scatter plots of cell apoptosis assessed using flow cytometry following Annexin V-FITC/PI staining. Apoptotic rates were calculated and are presented as the mean  $\pm$  standard deviation from three independent experiments. \*\* $P < 0.01$  vs. the control group. HSFbs, hypertrophic scar fibroblasts; NC, negative control; miR, microRNA; OD, optical density; FITC, fluorescein isothiocyanate; PI, propidium iodide.

## Results

*miR-181b-5p is overexpressed and negatively associated with decorin expression in human HS tissues.* RT-qPCR was used to investigate the expression of miR-181b-5p. As indicated in Fig. 1A, miR-181b-5p was present in normal tissue samples; however, its expression increased 2-fold in HS tissues ( $P < 0.01$ ). Decorin was identified as a candidate target gene of miR-181b-5p based on the results of bioinformatics analysis. RT-qPCR and western blot analysis of decorin expression were performed using the tissue samples. HS tissues exhibited a significantly reduced decorin expression, both at the mRNA and protein level ( $P < 0.01$ ; Fig. 1A and B). These data suggested an inverse association between the expression of miR-181b-5p and its target decorin, and are consistent with a previous study (10).

*miR-181b-5p induces proliferation and inhibits apoptosis of HSFbs.* Increased proliferation and reduced apoptosis contribute to the development of HS (9). To clarify

the biological roles of miR-181b-5p in HSFbs, MTT assay and Annexin V-FITC/PI staining were performed following transfection with miR-181b-5p in HSFbs (Fig. 2). Transfection with miR-181b-5p mimics resulted in a 3-fold increase in miR-181b-5p miRNA expression (Fig. 3A). MTT assay revealed that miR-181b-5p mimics promoted cell proliferation in a time-dependent manner (Fig. 2A). miR-181b-5p mimics increased cell proliferation at 12 and 24 h post-transfection compared with untreated control cells or NC miRs. A significant increase in cell proliferation was observed at 48 h post-transfection ( $P < 0.01$ ). Flow cytometry analysis using Annexin V-FITC/PI staining (Fig. 2B) indicated that miR-181b-5p significantly inhibited cell apoptosis compared with the control and NC miR groups ( $P < 0.01$ ). The percentage of apoptotic HSFbs cells transfected with miR-181b-5p mimics decreased ~4-fold compared with the NC miR group.

*miR-181b-5p may downregulate the expression of decorin via the MEK/ERK/p21 pathway.* As demonstrated above,



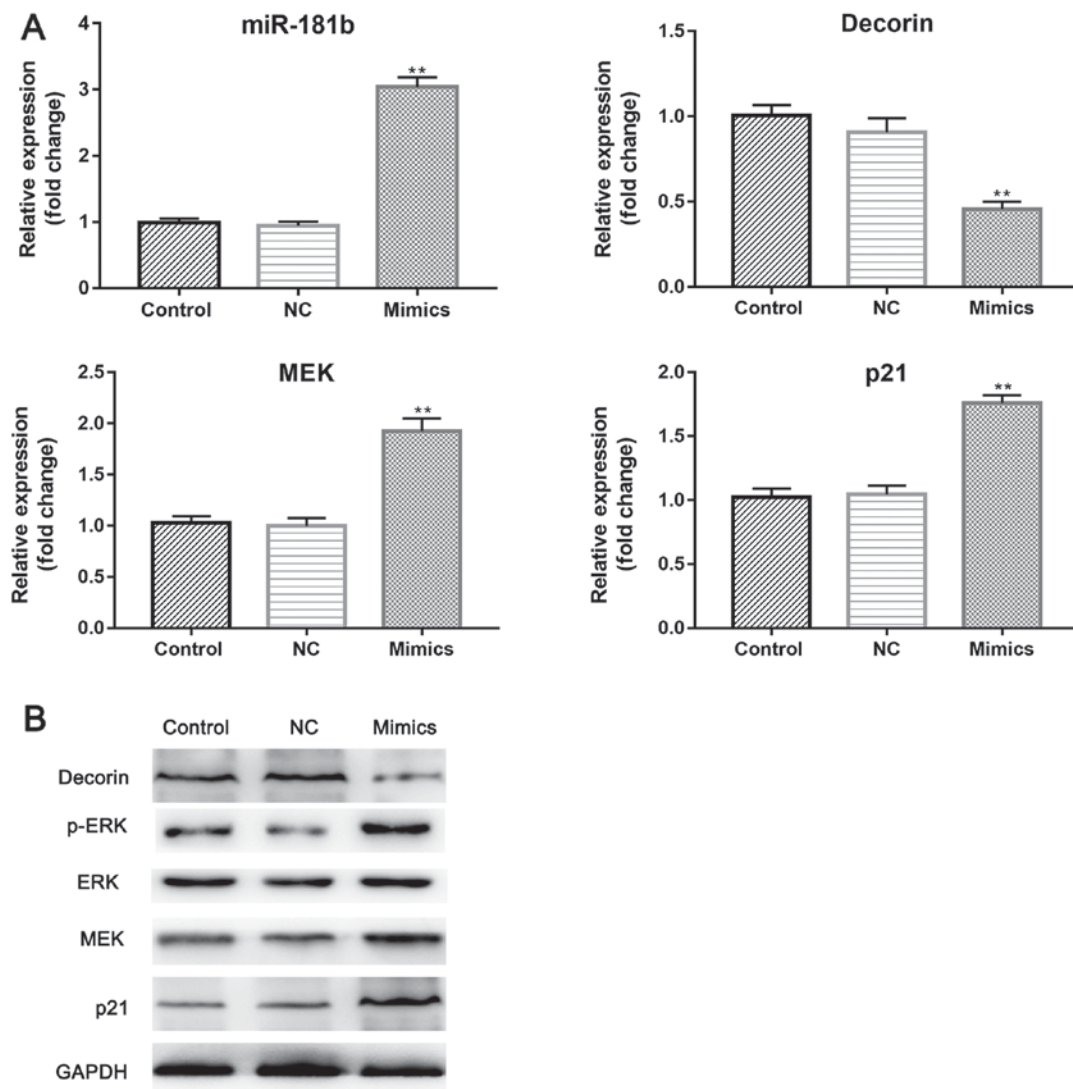


Figure 3. miR-181b-5p downregulates the expression of decorin via the ERK/MEK/p21 pathway. (A) Reverse transcription-quantitative polymerase chain reaction analysis of miR-181b-5p, decorin, MEK and p21 mRNA expression in human HSFbS transfected with miR-181b-5p mimic or NC miRs and untransfected cells as the normal control. Data were normalized with the level of GAPDH control. Results were expressed relative to the value of normal controls that were assigned a value of 1. \*\* $P < 0.01$  vs. the control group. (B) A representative western blot presenting the protein expression of decorin, p-ERK, ERK, MEK and p21 in human HSFbS transfected with miR-181b-5p mimic or NC miRs, and untransfected cells as the normal control. GAPDH was used as the loading control. HSFbS, hypertrophic scar fibroblasts; NC, negative control; miR, microRNA; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase.

miR-181-5p expression was negatively associated with decorin expression. Therefore, the present study aimed to further determine whether the expression of decorin was suppressed following transfection with miR-181b-5p mimics. Transfection with miR-181b-5p mimics induced a significant decrease in decorin expression compared with the NC miR group, both at the mRNA and protein level ( $P < 0.01$ ; Fig. 3).

The present study further aimed to analyze the possible mechanisms underlying miR-181b-5p-mediated cellular responses. The MEK/ERK signaling pathway has been reported to upregulate the expression of p21 (23) and subsequent cellular processes including cell proliferation and apoptosis. It was reported that the activation of MEK/ERK signaling pathway increased the proliferation and decreased the apoptosis of HSFbS (9). To investigate the contribution of miR-181b-5p to the regulation of the MEK/ERK/p21 signaling pathway, the present study determined the expression of MEK,

ERK, p-ERK and p21 in cells transfected with miR-181b-5p using RT-qPCR and western blotting. miR-181b-5p mimics induced a significant increase in mRNA expression of MEK and p21 compared with the NC group ( $P < 0.01$ ; Fig. 3), and similar effects were observed for protein expression of MEK, p-ERK and p21. miR-181b-5p had no effect of the expression of total ERK. Furthermore, cells transfected with miR-181b-5p were treated with MEK inhibitor (Fig. 4). MEK inhibitor partially inhibited the antiapoptotic effects of miR-181b-5p. These results suggest that miR-181b-5p may exert its cellular functions partially through the activation of the MEK/ERK/p21 pathway.

*Decorin is a direct target of miR-181b-5p.* The present study aimed to confirm whether the observed reduction in decorin mRNA and protein expression resulted from a direct interaction between miR-181b-5p and 3'-UTR of decorin.

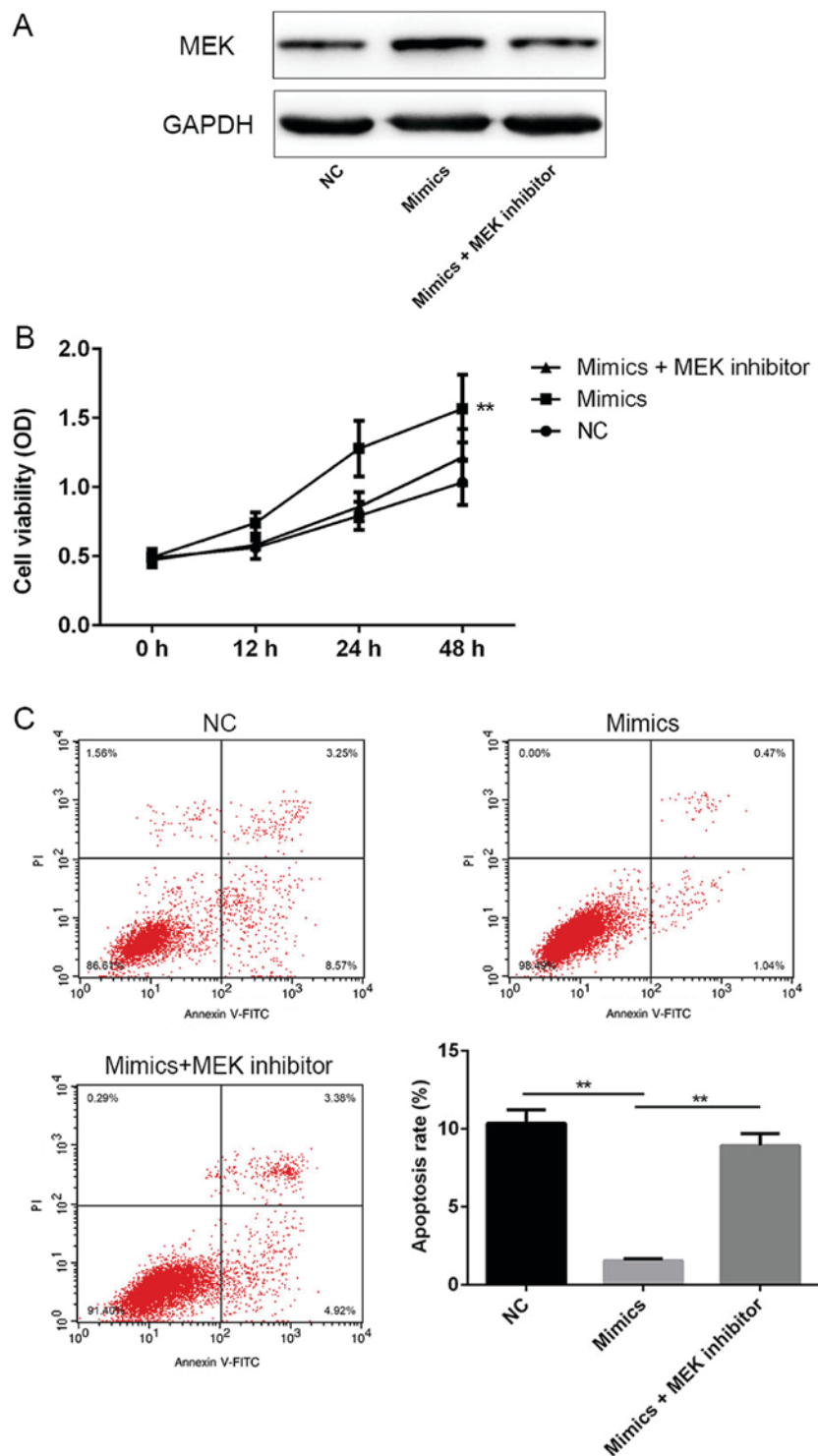


Figure 4. MEK inhibitor partially inhibits miR-181b-5p-induced antiapoptotic effects. (A) Effect of MEK inhibitor on the expression of MEK in cells transfected with miR-181b-5p. (B) Effect of MEK inhibitor on the proliferation of cells transfected with miR-181b-5p. \*\* $P < 0.01$  vs. the NC group. (C) Effect of MEK inhibitor on the apoptosis of cells transfected with miR-181b-5p. \*\* $P < 0.01$  as indicated. Data are presented as the mean  $\pm$  standard deviation of three independent experiments. MEK, mitogen-activated protein kinase kinase; NC, negative control; miR, microRNA; OD, optical density; FITC, fluorescein isothiocyanate; PI, propidium iodide.

P-decorin-wt and p-decorin-mut were co-transfected with either miR-181b-5p mimic or NC into 293T cells and luciferase activity was measured 24 h following transfection. Co-transfection with miR-181b-5p mimics and p-decorin-wt significantly decreased the relative luciferase activity compared with the group transfected with NC siR (Fig. 5),

whereas a reversal of relative luciferase activity was observed in experiments where miR-181b-5p was co-transfected with reporter construct p-decorin-mut (Fig. 5). The above results indicate that miR-181b-5p could specifically bind to the 3'-UTR of decorin and these results are consistent with a previous study (10).

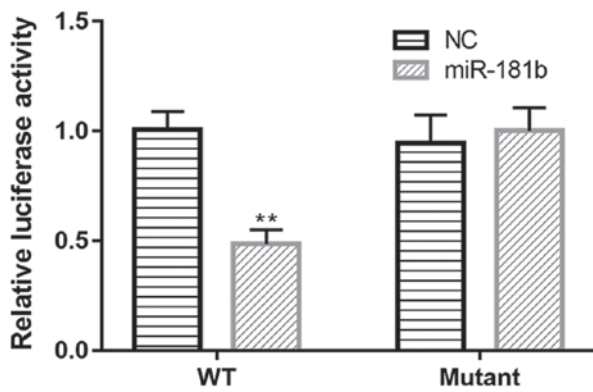


Figure 5. Decorin is a direct target of miR-181b-5p. Each reporter construct (WT or mutant) was co-transfected with either miR-181b-5p mimic or NC miR in 293T cells and a dual luciferase assay was performed after 24 h. Luciferase activity was normalized to *Renilla* and presented as relative to miR NC (assigned a value of 1). Data are presented as the mean  $\pm$  standard deviation of three independent experiments. \*\* $P < 0.01$  vs. the NC group. miR, microRNA; NC, negative control; WT, wild type.

## Discussion

Fibroblasts serve roles in tissue repair and wound healing processes. Enhanced proliferation and reduced apoptosis of HSFbs have been reported in the development of HS (3,4). Elucidation of the molecular mechanisms underlying proliferation and apoptosis of HSFbs may aid in the development of novel therapeutic strategies that minimize fibrosis and reduce HS formation.

Recently, miRNAs have been demonstrated to regulate various pathological processes in HS (8,9). miR-181b-5p and its target decorin have been hypothesized to be involved in the pathological process of HS (10,18-20). The expression of decorin was markedly downregulated and the mRNA levels of miR-181b-5p were upregulated in HS tissues compared with normal control samples used in the present study. Furthermore, mRNA and protein expression of decorin decreased in HSFbs transfected with miR-181b-5p mimics. The direct interaction between miR-181b-5p and decorin was confirmed using a dual-luciferase assay. The results of the present study confirm previously reported data (10) and suggest that miR-181b-5p may downregulate the expression of decorin via direct binding to the 3'-UTR, contributing to the development of HS.

Considering the proapoptotic and antiproliferative effects of decorin on HSFbs (19,20), the present study hypothesized that miR-181b-5p may exhibit opposite cellular functions. MTT and Annexin V-FITC/PI staining assays were performed to confirm this hypothesis. Significantly enhanced apoptosis and reduced proliferation were observed following transfection with miR-181b-5p mimics in HSFbs. The results of the present study and previously published data (10) indicate that miRNA-181b-5p may be involved in the pathogenesis of HS by promoting fibroblast-to-myofibroblast differentiation, and promoting proliferation and inhibiting apoptosis of fibroblasts.

The intracellular signaling pathway MEK/ERK regulates a number of cellular functions including differentiation, proliferation and apoptosis in numerous types of cells (24,25). Activation of the MEK/ERK pathway leads to the accumulation of p53 and p21, which promotes proliferation and

inhibits apoptosis (9,24). Previous studies indicated that the MEK/ERK/p21 pathway is involved in the proliferation and apoptosis of fibroblasts and is associated with the pathogenesis of HS (9,26). Furthermore, the association between miR-181b-5p and the MEK (25) and ERK (26) signaling pathways has been previously studied. Therefore, the present study aimed to investigate the potential effect of miR-181b-5p on the MEK/ERK/p21 pathway in HS. The results of RT-qPCR and western blot analysis demonstrated that transfection with miR-181b-5p mimics activated the expression of MEK, and p21 at mRNA and protein levels. Furthermore, treatment with MEK inhibitor partially inhibited the antiapoptotic effects of miR-181b-5p. These results suggested that miR-181b-5p may exert its function in HS via the MEK/ERK/p21 pathway.

The present study confirmed the increased expression of miR-181b-5p in HS tissues and the direct interaction between miR-181b-5p and decorin, as previously reported (10,18). Furthermore, in the present study, miR-181b-5p mimics significantly inhibited apoptosis, enhanced proliferation of HSFbs and activated the MEK/ERK/p21 signaling pathway. The results of the present study suggest a novel mechanism underlying HS, and miR-181b-5p may be a therapeutic target for prevention and treatment of this disease.

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## Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

## Authors' contributions

BL performed the experiments and wrote part of the manuscript. ZG performed some of the experiments. WG designed the study and wrote most of the experiments. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

## Ethics approval and consent to participate

The present study was approved by the ethics committee of Eastern Liaoning University and written informed consent was obtained from all patients.

## Patient consent for publication

The consent for publication was obtained from the patients.

## Competing interests

The authors declare that they have no competing interests.

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