



miR-211-5p inhibits the proliferation, migration, invasion, and induces apoptosis of human hypertrophic scar fibroblasts by regulating TGF β R2 expression

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Background: Hypertrophic-scar (HS) is the most common pathological healing phenomenon after trauma, especially after deep burns. We aimed to investigate the expression and role of microRNA-211-5p (miR-211-5p) in HS and explore its underlying mechanism.

Methods: Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the expression of miR-211-5p in 15 cases of HS tissues and normal skin tissues, as well as its expression in human hypertrophic scar fibroblasts (hHSFs) and normal fibroblasts. At the same time, the cell counting kit-8 (CCK-8), scratch test, cell invasion test, and flow cytometry were used to determine cell proliferation, migration, invasion, and apoptosis, respectively. Western blot assay was used to determine the expression of proteins. TargetScan was performed to predict the potential binding sites between miR-211-5p and TGF β R2, which was then verified by western blotting and luciferase reporter gene experiments. Also, co-transfection of plasmids that overexpress miR-211-5p and TGF β R2 were used to observe the reversal effect of miR-211-5p.

Results: The level of miR-211-5p in HS tissues and hHSFs cells was significantly down-regulated (both $P < 0.05$). The TGF β R2/Smad3 signaling pathway was activated (both $P < 0.05$). Furthermore, the overexpression of miR-211-5p could inhibit the proliferation ($P < 0.05$), migration ($P < 0.05$), and invasion ($P < 0.05$) of hHSFs cells, and induce their apoptosis ($P < 0.05$), and could also regulate the expression of related proteins (all $P < 0.05$). Moreover, the overexpression of miR-211-5p could also inhibit the accumulation of ECM and the activation of the TGF- β R2/Smad3 pathway (all $P < 0.05$), while the opposite effect (all $P < 0.05$) was observed when the level of miR-211-5p was interfered with. Finally, it was confirmed that miR-211-5p could target TGF β R2 (all $P < 0.05$), and when hHSFs cells simultaneously overexpressed miR-211-5p and TGF β R2, the promotion effect of TGF β R2 on cells was reversed by miR-211-5p (all $P < 0.05$).

Conclusions: miR-211-5p can inhibit the activation of the TGF- β R2/Smad3 signaling pathway by targeting TGF β R2, thereby suppressing the proliferation, migration, invasion, and ECM production of hHSFs, and inducing their apoptosis, suggesting that miR-211-5p can become a potential target for the treatment of HS.

Keywords: miR-211-5p; hypertrophic scar (HS); fibroblasts; proliferation; apoptosis; TGF β R2

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Introduction

Hypertrophic scar (HS) is a common disease caused by abnormal skin tissue repair after burns or surgical trauma (1). It is characterized by abnormally increased activity of numerous cells (such as fibroblasts), excessive proliferation of cells, and increased collagen deposition (2). The reduced elasticity of scar tissue destroys the normal anatomical structure of the skin tissue on the body surface, thereby affecting the appearance of the skin, and leading to varying degrees of tissue and organ dysfunction. This negatively impacts the normal life and work of the patient, injures their body, and affects their positive mentality (3,4). At present, the treatment for HS mainly includes comprehensive therapies such as surgery, silica gel, radiotherapy, cryotherapy, and compression therapy, however there is no particularly effective treatment method (5). Abnormal proliferation and activation of fibroblasts are considered to be the main feature of HS (2). However, the pathogenesis of HS requires further study, which is a huge challenge for clinical treatment (6). In recent years, researchers have conducted numerous in-depth studies on the underlying mechanisms associated with the pathogenesis of HS. It has been shown that the prevention and treatment of HS should avoid or reduce the occurrence and development of abnormal scars at the source. Identifying the genetic defects of HS and correcting them at the genetic level is the premise for the breakthrough treatment of the HS (7-9).

MicroRNAs (miRNAs) can recognize and bind related target genes in a completely or incompletely complementary fashion (10), and cut or inhibit the translation of the target gene, which regulates the expression of genes and/or proteins after transcription (11,12). Studies have shown that miRNAs can play a vital role in the occurrence and development of HS through a variety of mechanisms (13-15). According to previous studies, miR-211-5p is a pro-survival miRNA (16), but also a pro-apoptotic miRNA (17). MiR-211-5p is decreased in osteosarcoma, could regulate cell functions (18). Similarly, miR-211-5p is also decreased in breast cancer tissues, and its overexpression could suppress the development of breast cancer (19). Shang *et al.* found that miR-211-5p can reduce ischemia/reperfusion-induced kidney injury by regulating the activation of TGF β R2/TGF- β /Smad3 pathway (20). Nonetheless, the roles of miR-211-5p in the occurrence and development of HS are still unclear.

Therefore, in this study, we aim to investigate the expression of miR-211-5p in HS tissues and human HS fibroblasts (hHSFs), and to further elucidate the roles

of miR-211-5p in hHSFs and explore its underlying mechanism.

We present the following article in accordance with the MDAR reporting checklist (available at <http://dx.doi.org/10.21037/atm-21-1806>).

Methods

Clinical sample collection

Fifteen HS tissues and 15 normal skin tissue samples from patients undergoing scar resection and repair surgery in our hospital from January to October 2020 were collected. The clinical tissue samples in this study were only used for research purposes. All participants had signed the informed consent form before participating in the study. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of West China Hospital, Sichuan University (No.: ChiCTR1800019082).

Cell culture

Briefly, HS tissues were rinsed with phosphate-buffered saline (PBS), and Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) containing 0.1% type I collagenase (Sigma, USA) was used to incubate the minced tissue at 37 °C for 3 h. Next, the hHSFs were cultured in DMEM medium containing 10% fetal bovine serum (FBS, Gibco), 1% penicillin, and 1% streptomycin double antibiotics (Invitrogen, USA) at 37 °C in a humidified atmosphere with 5% CO₂. In this study, third to fifth generations of hHSFs that reached 70–80% confluence were used for subsequent experiments.

RNA extraction and real-time fluorescent quantitative polymerase chain reaction (qRT-PCR)

The cellular RNA was extracted with TRIzol reagent (Takara, Japan) strictly according to the manufacturer's instructions. The miScript reverse transcription kit (Qiagen, German) was used to synthesize the cDNA for the detection of miRNA, and PrimeScript™ Reverse Transcription Kit (TaKaRa, Japan) was used to synthesize the cDNA for mRNA detection. The SYBR Green PCR kit (TaKaRa, Japan) was used for specific amplification of cDNA on the ABI7500 real-time PCR system (Applied Biosystems, USA). U6 and GAPDH were used as the internal loading controls

Table 1 Primer sequences of target genes

Target gene	Forward primer (3'-5')	Reverse primer (3'-5')
<i>miR-211-5p</i>	CCGGAATTCGGTTTTACAACACCCCATTTCCACC	CGCGGATCCGCGCGAGCAACAGAGTAGAACAGG
<i>U6</i>	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTTCAT
<i>TGFβR2</i>	GTAGCTCTGATGAGTGCAATGAC	CAGATATGGCAACTCCCAGTG
<i>GAPDH</i>	CTTTGGTATCGTGAAGGACTC	GTAGAGGCAGGGATGATGTTCT

TGFGAG, transforming growth factor receptor 2.

for miRNA and mRNA, respectively. The $2^{-\Delta\Delta C_t}$ method was used to determine the relative quantification of genes. Primer sequences are shown in *Table 1*.

Cell transfection

Plasmids of miR-211-5p mimic (mimic), inhibitor (inhibitor), and negative control (NC), as well as the plasmids that overexpressed the transforming growth factor β receptor 2 (TGF β R2) and its control (Vector), were all synthesized by Shanghai Jima Pharmaceutical Technology Co., Ltd. (China). Lipofectamine 2000 (Invitrogen, USA) was used for cell transfections according to the manufacturer's instructions.

Cell proliferation measurement

Cell proliferation was determined using the cell counting kit-8 (CCK-8, Dojindo, Japan). First, cells transfected with the miR-211-5p mimic, inhibitor, NC, or TGF β R2, Vector for 48 h were seeded into 96-well plates (1×10^4 cells/well). After 0, 12, 24, 36, and 48 h, 10 μ L of CCK-8 were added to each well. After continuous culturing for 2 h at 37 °C, the absorbance at 450 nm was detected with a spectrophotometer (BioTek Instruments, USA).

Caspase-3 activity determination

Caspase-3 activity detection kit (Biyuntian Biotechnology Co., Ltd., China) was used to detect cell apoptosis. Briefly, the lysed cells were incubated with 2 mM Ac DEVD pNA in the reaction buffer at 37 °C for 2 h. Subsequently, a spectrophotometer was used to detect the absorbance value at 405 nm.

Flow cytometry to measure cell apoptosis

hHSFs cells were first digested with trypsin and resuspended

in PBS, and the Annexin V-FITC Kit (Invitrogen, USA) was then used to measure apoptosis strictly according to the manufacturer's instructions. Specifically, 48 h after transfection of the miR-211-5p mimic, inhibitor, and NC, the hHSFs were treated with 0.25% trypsin without EDTA. The cells were then washed twice with pre-chilled PBS and resuspended in the binding buffer. Annexin V-FITC and PI were then added to the cell suspension for a 15-min incubation at room temperature in the dark. Flow cytometry (FACS Calibur, BD Bioscience, USA) was then used to determine the apoptosis of the samples, and the Flowjo software (Treestar, Inc., USA) was used to analyze the apoptosis rate of each group of cells.

Dual luciferase experiment

The 3'-untranslated region (UTR) fragments of wild type (WT) or mutant type (MUT) TGF β R2 containing the predicted miR-211-5p targeting sites was cloned into the dual luciferase miRNA target expression vector pmirGLO vector (Promega, USA). The constructed vector and miR-211-5p mimic or inhibitor were then transfected into the hHSFs. The transfected hHSFs were cultured at 37 °C for 48 h, and the luciferase activity of the cells was then analyzed with the dual-luciferase reporter analysis system (Promega, USA).

Scratch test

The stably-transfected hHSFs (3×10^5 cells/well) were inoculated into a six-well plate. When the cells reached contact inhibition, a 10- μ L pipette tip was used to vertically draw three parallel lines on the plate. The exfoliated cells were rinsed off with PBS, and were then cultured in a serum-free medium. The scratches were observed, photographed, and recorded under a microscope (Olympus, Japan) at 0 and 48 h. The migration distance based on the scratch area of the cell at 0 and 48 h was then calculated.

Cell invasion test

The transwell plate chamber (3428, Corning, USA) was pretreated with matrigel glue (Corning, USA) at 4 h, and placed overnight at 37 °C. A number of 2×10^3 cells/well was then seeded in the upper chamber of the transwell chamber containing serum-free medium, and 600 μ L of normal medium containing 10% FBS was added to the lower chamber. After 24 h of incubation, the liquid was discarded, and the cells were fixed with 4% methanol (Biyuntian Biotechnology Co., Ltd., China) at room temperature for 15 min. After staining with 0.1% crystal violet (Biyuntian Biotechnology Co., Ltd., China) at room temperature for 20 min, the number of cells was counted from 10 randomly chosen areas under a microscope.

Detection of protein expression level with western blot

The cells were lysed with a strong RIPA lysis solution (Biyuntian Biotechnology Co., Ltd., China), and then the protein concentration of the sample was determined using the BCA kit (Biyuntian Biotechnology Co., Ltd., China) according to the manufacturer's instructions. A total of 40 μ g of protein extract from each sample was separated on a 10% SDS-PAGE gel and then transferred to a PVDF membrane (Millipore, USA), which was then blocked with 5% skim milk at 37 °C for 1 h, and then incubated with primary antibody overnight at 4 °C. Primary antibodies included transforming growth factor receptor 2 (TGF β R2, ab184948, abcam, UK), G1/S-specific cyclin-D1 (CCND1, ab16663, abcam, UK), C-myc (ab32072, abcam, UK), Bcl2-Associated X (Bax, ab32503), B-cell lymphoma-2 (Bcl-2, ab182858, abcam, UK), matrix metalloproteinase 3 (MMP3, ab52915, abcam, UK), matrix metalloproteinase 9 (MMP9, ab76003, abcam, UK), α -smooth muscle actin (α -SMA, ab5831, abcam, UK), type 1 collagen (Col I, ab34710, abcam, UK), Col III (ab184993, abcam, UK), Smad3 (ab40854, abcam, UK), phosphorylated Smad3 (ab52903, abcam, UK), and GAPDH (ab8245, abcam, UK). The next day, the membrane was incubated with the appropriate secondary antibody (Sigma, USA) for 1 h at room temperature. Electrochemi-luminescence (ECL) Kit (Thermo Fisher Scientific, USA) was used to observe protein bands in the imaging system, and the relative protein expression level of cells was analyzed according to the gray value.

Statistical analysis

The data were represented as mean \pm standard deviation,

and SPSS19.0 statistical software (SPSS, USA) was used for data analysis. The difference between different groups was compared with the *t*-test, and one-way analysis of variance was used to compare groups under the same conditions. The difference was statistically significant when $P < 0.05$.

Results

The level of miR-211-5p was reduced in HS tissues and hHSFs

This study firstly used qRT-PCR to determine the level of miR-211-5p in HS tissues. As shown in *Figure 1A*, the expression level of miR-211-5p in HS tissue was significantly reduced ($P < 0.05$) compared to the normal control. Moreover, as shown in *Figure 1B*, this study also found that the expression level of miR-211-5p in fibroblasts derived from HS was also significantly lower than that in fibroblasts derived from normal skin ($P < 0.05$). In addition, this study also detected the activation of TGF β R2 and Smad3 proteins in human normal and HS tissues, as well as normal skin and HS fibroblasts. As shown in *Figure 1C and D*, this study found that HS tissues and TGF β R2 levels in cells significantly increased compared with normal tissues and normal cells ($P < 0.05$). In addition, the phosphorylation level of Smad3 also increased significantly ($P < 0.05$).

miR-211-5p inhibited cell functions and induced apoptosis in hHSFs

As shown in *Figure 2A*, this study first verified the effectiveness of the miR-211-5p mimic and inhibitor (both $P < 0.05$). The CCK-8 method was then used to determine the effect of miR-211-5p on cell proliferation. The overexpression of miR-211-5p in hHSFs cells significantly inhibited cell proliferation ($P < 0.05$), while interference with the expression of miR-211-5p reversed the above effects ($P < 0.05$) (*Figure 2B*). Cell transfection with the miR-211-5p mimic increased Caspase-3 activity ($P < 0.05$), and markedly increased the cell apoptosis rate ($P < 0.05$) (*Figures 2C,D*). Also, after transfection of the miR-211-5p mimic inhibitor, the Caspase-3 activity and apoptosis rate of cells were notably reduced (both $P < 0.05$) (*Figure 2C,D*). Furthermore, miR-211-5p significantly inhibited the migration and invasion of hHSFs (both $P < 0.05$) (*Figure 2E,F*). It is known that CCND1, C-myc, Bax, Bcl-2, MMP3, and MMP9 are important biological markers of cell proliferation, apoptosis, migration, and invasion, respectively. In this study, the expression levels of these proteins in cells were

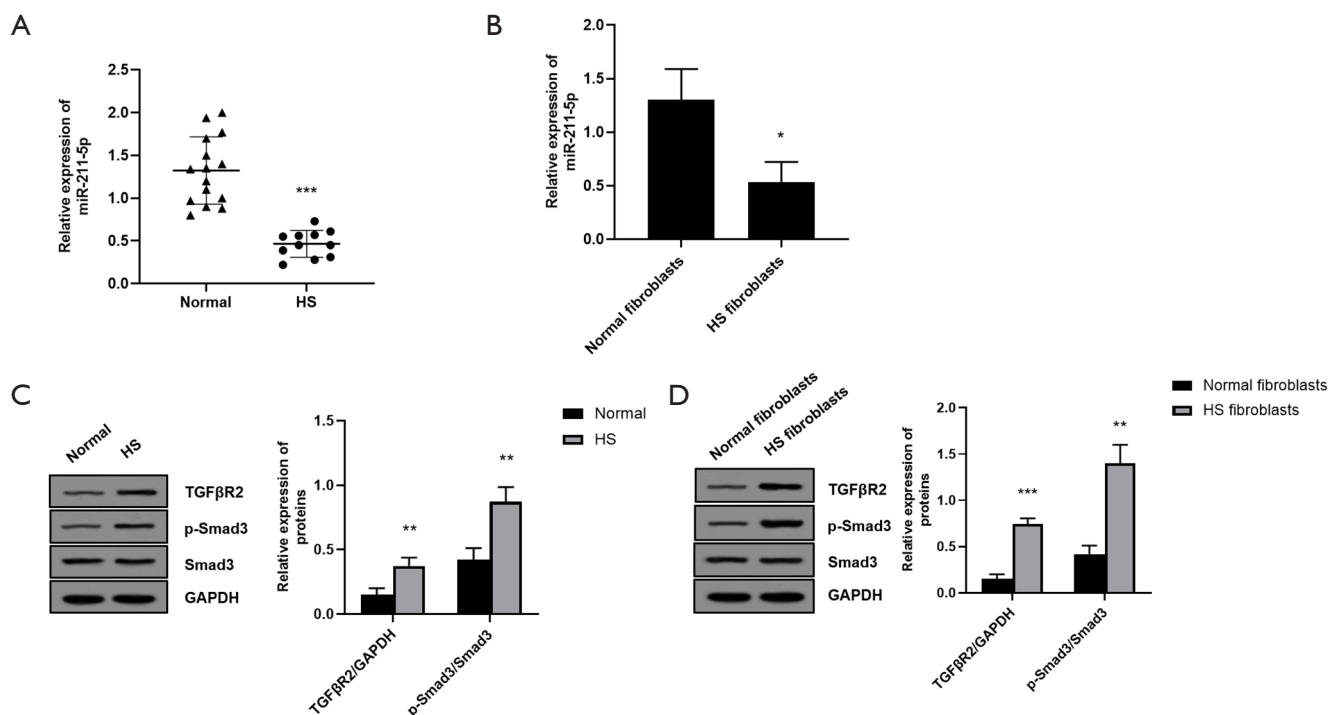


Figure 1 The level of miR-211-5p in HS tissues and hHSFs was decreased and the TGFβR2/Smad3 pathway was activated in HS tissues and HS fibroblasts. (A) The relative levels of miR-211-5p in HS tissues (n=15) and normal control tissues (normal, n=15) were determined by qRT-PCR. ***, P<0.001 *vs.* normal group; (B) the relative quantification of miR-211-5p in hHSFs and normal fibroblasts (n=3) were determined by qRT-PCR. *, P<0.05 *vs.* normal fibroblasts. (C) Western blot detection of TGFβR2, Smad3 and p-Smad3 protein expression levels in HS tissues (n=15) and normal control tissues (normal, n=15). **, P<0.01 *vs.* normal group; (D) Western blot detection of TGFβR2, Smad3 and p-Smad3 protein expression levels in HS tissue and normal tissue fibroblasts. **, P<0.01. ***, P<0.001 *vs.* normal fibroblasts. HS, hypertrophic scar; hHSFs, human hypertrophic scar fibroblasts.

measured by western blot. As shown in *Figure 2G*, when the cells overexpressed miR-211-5p, the expression levels of CCND1, C-myc, Bcl-2, MMP3, and MMP9 were markedly reduced (all P<0.05), while the Bax level increased notably (all P<0.05). However, interference with miR-211-5p reversed the expression level changes of the above-mentioned proteins (all P<0.05).

miR-211-5p inhibited collagen deposition and activation of the TGF-βR2/Smad3 signaling pathway

As shown in *Figure 3A*, we found that, compared with the control group, the protein levels of α-SMA, Col I, and Col III in hHSFs transfected with the miR-211-5p mimic were significantly down-regulated (all P<0.05), while cells transfected with the miR-211-5p inhibitor were markedly up-regulated (all P<0.05). We also determined the activation of the TGF-βR2/Smad3 signaling pathway, which plays an

important role in the occurrence and development of HS. As shown in *Figure 3B*, when the cells overexpressed miR-211-5p, the activation of the TGF-βR2/Smad3 pathway was inhibited (P<0.05). Conversely, when the level of miR-211-5p in cells was decreased, the pathway was activated (P<0.05).

TGFβR2 is the target of miR-211-5p

To explore the potential role of miR-211-5p, the target of miR-211-5p was predicted through TargetScan bioinformatics analysis. As shown in *Figure 4A*, it was predicted that the 3'-UTR of TGFβR2 contained the predicted target site for miR-211-5p. To verify whether miR-211-5p could directly target the 3'-UTR of TGFβR2, we used the 3'-UTR reporter vector of TGFβR2 to conduct the dual-luciferase analysis. We found that the overexpression of miR-211-5p significantly reduced the

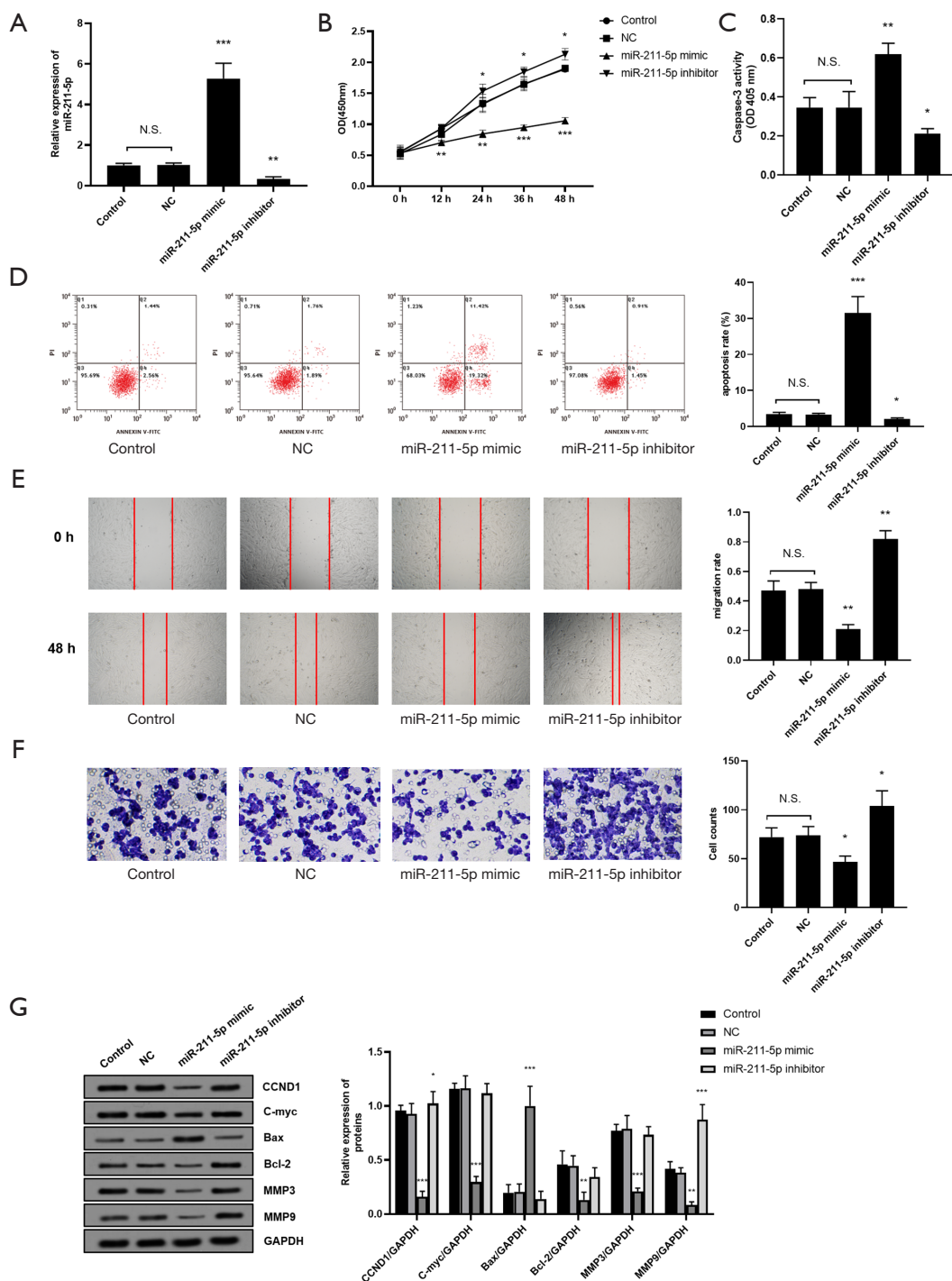


Figure 2 miR-211-5p inhibits the functions of hHSFs, and induces their apoptosis. (A) The effectiveness of the miR-211-5p mimic and inhibitor were verified by qRT-PCR; (B) the CCK-8 method was used to detect cell proliferation after transfection of the miR-211-5p mimic and inhibitor; (C) measurement of cell Caspase-3 activity; (D) changes in the cell apoptosis rate were detected by flow cytometry; (E) the scratch test was used to measure cell migration; magnification, 40 \times ; (F) the cell invasion test was used to evaluate cell invasion; cells were stained with 0.5% crystal violet solution; magnification, 100 \times ; (G) western blotting was used to determine the expression levels of proliferation-, migration-, invasion-, and apoptosis-related proteins in cells. $n=3$. N.S., $P>0.05$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ vs. Control group. hHSFs, human hypertrophic scar fibroblasts; CCK-8, cell counting kit-8.

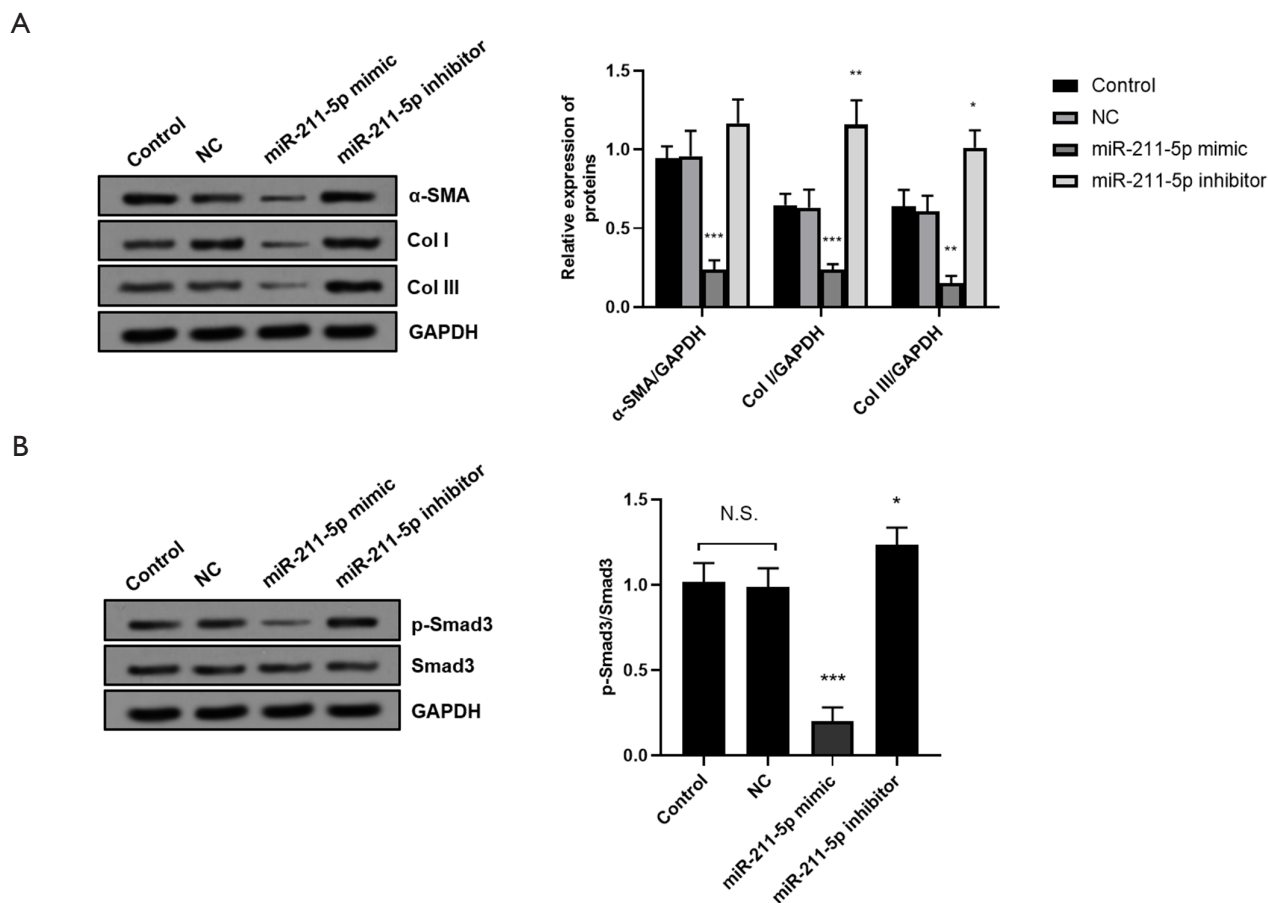


Figure 3 miR-211-5p inhibited collagen deposition and the activation of the TGFβ2/Smad3 signaling pathway. (A) Western blot was used to determine the protein expression levels of ECM marker proteins including α-SMA, Col I, and Col III; (B) Western blot was used to measure the phosphorylation level of smad3. N.S., $P>0.05$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ vs. Control group. ECM, extracellular matrix.

luciferase activity of the WT TGFβ2 3'-UTR reporter vector ($P<0.05$), while there was no notable effect on the luciferase activity of the TGFβ2 3'-UTR reporter vector containing the miR-211-5p mutation binding site ($P>0.05$) (Figure 4B). To further confirm that TGFβ2 is the target gene of miR-211-5p, the effect of miR-211-5p on the expression of TGFβ2 was examined. As shown in Figure 4C, the overexpression of miR-211-5p markedly reduced the protein expression level of TGFβ2 ($P<0.05$), while the inhibition of miR-211-5p expression had the opposite effect ($P<0.05$).

miR-211-5p reversed the effects of TGFβ2 on HS fibroblasts

We also verified the effectiveness of the TGFβ2

overexpression plasmid (all $P<0.05$) (Figure 5A,B). Subsequently, the CCK-8 method was used to measure the effects of co-transfection of miR-211-5p and TGFβ2 on cell proliferation. The overexpression of TGFβ2 significantly promoted hHSFs proliferation ($P<0.05$), and when miR-211-5p was overexpressed at the same time, the proliferation activity was reversed ($P<0.05$) (Figure 5C). In terms of apoptosis, the overexpression of miR-211-5p reversed the apoptosis-inhibiting effect of TGFβ2 overexpression (all $P<0.05$) (Figure 5D,E). In terms of cell migration and invasion, the overexpression of miR-211-5p in hHSFs cells reversed the enhancement of migration and invasion caused by the overexpression of TGFβ2 (both $P<0.05$) (Figure 5F,G). Furthermore, we also measured the expression of cell function-related proteins by western blot. As shown in Figure 5H, the overexpression of miR-

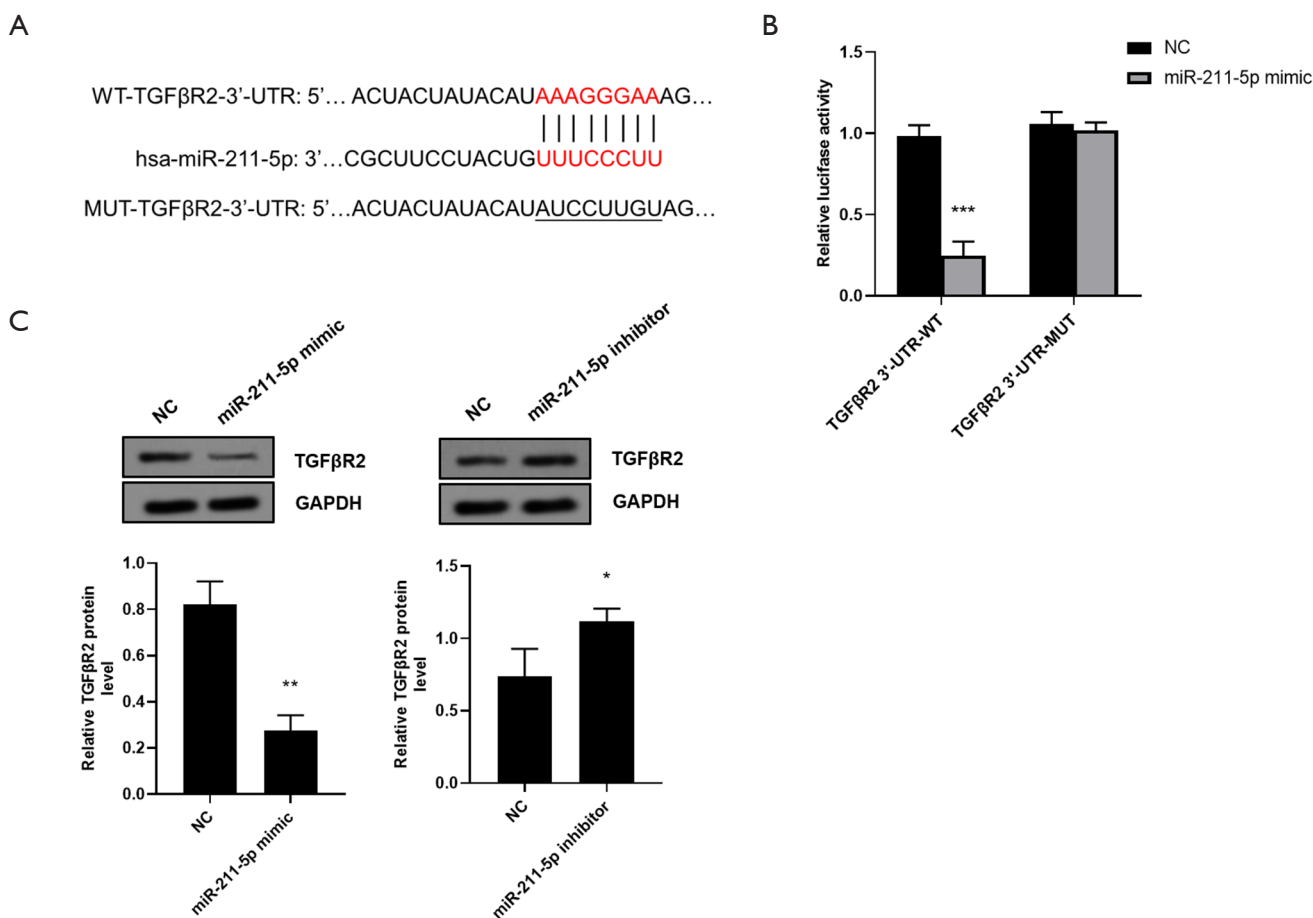


Figure 4 TGFβR2 is the target of miR-211-5p. (A) Prediction of the binding site between the 3'-UTR of TGFβR2 and miR-211-5p; (B) detection of luciferase activity of the reporter gene transfected either with TGFβR2 3'-UTR-WT or TGFβR2 3'-UTR-MUT binding site. NC was used as the control, and the difference in luciferase activity after transfection of the miR-211-5p mimic was measured; (C) Western blot was used to measure the levels of TGFβR2 protein in cells transfected with NC, miR-211-5p mimic or inhibitor, or miR-NC. *, P<0.05; **, P<0.01; ***, P<0.001 vs. NC group.

211-5p reversed the changes in the expression levels of CCND1, C-myc, Bax, Bcl-2, MMP3, and MMP9 caused by the overexpression of TGFβR2 (all P<0.05). The above results suggest that miR-211-5p can reverse the regulation of TGFβR2 on the proliferation, migration, invasion, and apoptosis of hHSFs.

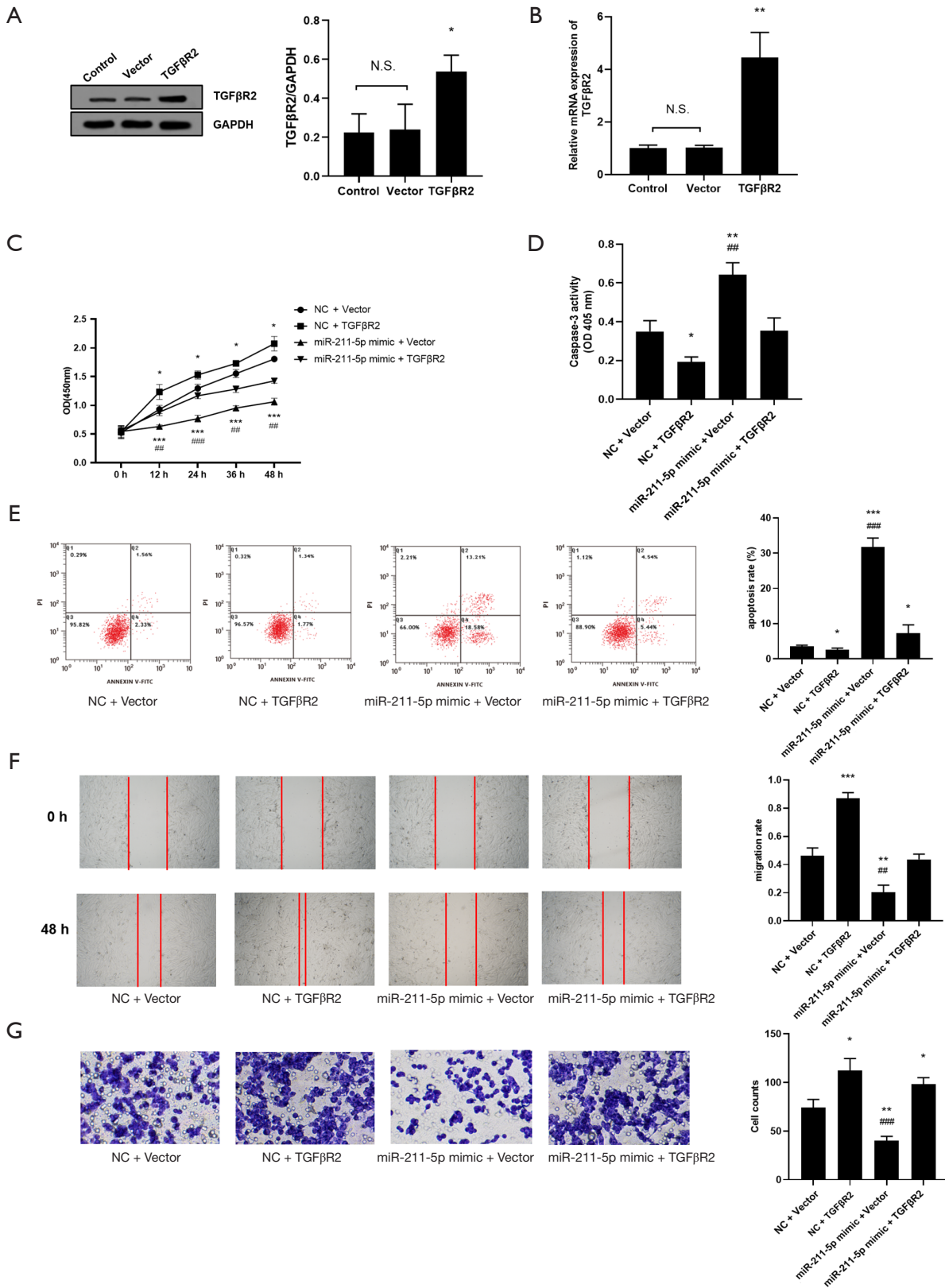
miR-211-5p reversed TGFβR2's promotion of collagen deposition and the activation of the TGFβR2/Smad3 signaling pathway

As shown in *Figure 6A*, transfection with the miR-211-5p mimic reversed the changes in the protein levels of α-SMA, Col I, and Col III in hHSFs caused by the overexpression

of TGFβR2 (all P<0.05). The activation of the TGFβR2/Smad3 pathway caused by TGFβR2 was also suppressed by the overexpression of miR-211-5p (P<0.05), as shown in *Figure 6B*.

Discussion

The present study found that the expression of miR-211-5p was decreased in HS tissues and hHSFs cells, and that inhibited hHSF proliferation, migration, and invasion by inhibiting the expression of TGFβR2. MiR-211-5p also induced the occurrence of apoptotic responses, reduced the accumulation of ECM, and inhibited the activation of TGFβR2/Smad3 pathway. The above results indicate that



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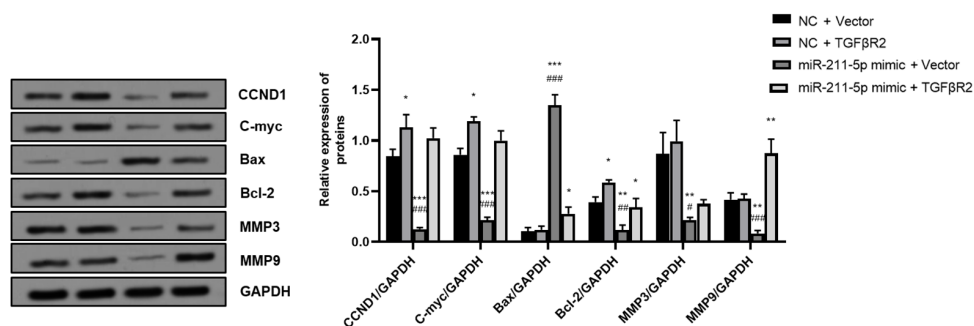


Figure 5 miR-211-5p reversed the effects of TGFβR2 overexpression on HS fibroblasts. (A,B) The effectiveness of cells transfected with the TGFβR2 overexpression plasmid was verified by qRT-PCR and western blot; (C) cell proliferation after transfection with the empty plasmid (Vector) and TGFβR2 overexpression plasmid was measured using the CCK-8 method; (D) measurement of cell Caspase-3 activity; (E) changes in cell apoptosis rate were determined by flow cytometry; (F) the scratch test was performed to measure cell migration; magnification, 40×; (G) the cell invasion test was used to evaluate cell invasion; cells were stained with 0.5% crystal violet solution; magnification, 100×; (H) Western blot was used to determine the levels of proliferation-, migration-, invasion-, and apoptosis-related proteins in cells. n=3. N.S., P>0.05; *, P<0.05; **, P<0.01; ***, P<0.001 vs. Control group; #, P<0.05; ##, P<0.01; ###, P<0.001 vs. miR-211-5p + TGFβR2 group. CCK-8, cell counting kit-8.

the miR-211-5p/TGFβR2 axis may be a potential target for the treatment of HS.

The healing process of normal and pathological wounds is complex (21,22). Histologically, HS is characterized by excessive proliferation of fibroblasts and mast cells, accompanied by excessive accumulation of ECM (23). However, the exact pathogenesis of HS is still unclear, and the current treatment methods are also limited (5). In recent years, studies have found that abnormal miRNA expression plays a key role in the progression of skin fibrosis (24). Previous studies have demonstrated the anti-cancer or tumor-promoting effects of miR-211-5p in various tumors. For example, miR-211-5p can promote cancer cell survival by inhibiting the circadian rhythm regulators and protein synthesis (25). MiR-211-5p may also be able to enhance the proliferation, colony formation, and invasion of non-small cell lung cancer (NSCLC) cells by targeting sarcoma (SRC) kinase signaling inhibitor 1 (26). However, in cervical cancer, miR-211-5p can inhibit the proliferation, invasion, and migration of cancer cells by targeting secreted protein acidic and rich in cysteine (SPARC) (27). Furthermore, miR-211-5p has been shown to promote the sensitivity of platinum drugs by blocking the DNA damage response in ovarian cancer (28). Finally, in ischemia/reperfusion kidney injury, Shang *et al.* found that TGFβR2 is the target gene of miR-211-5p, where it can reduce cell apoptosis and injury by inhibiting TGF-βR2/Smad3 signal activation (20). However, there are no relevant reports on the role of miR-211-5p in

HS. Therefore, this study first measured the levels of miR-211-5p in HS tissues and hHSFs cells and subsequently explored its biological effects.

By measuring the level of miR-211-5p in HS tissues and hHSFs cells, we discovered that the level of miR-211-5p was significantly down-regulated, and the TGFβR2/Smad3 signaling pathway was activated. By overexpressing and inhibiting the expression level of miR-211-5p in hHSFs cells, miR-211-5p had the effects of inhibiting fibroblast proliferation, invasion, and migration, and inducing fibroblast apoptosis. It is known that CCND1 and C-myc are important factors that regulate the cell cycle and cell proliferation (29). Furthermore, Bax and Bcl-2 are key proteins that promote apoptosis and anti-apoptosis, respectively (30). Also, MMP3 and MMP9, which can regulate the synthesis and degradation of ECM at the wound site, are protein factors that can regulate cell migration and invasion (31). In this study, the expression levels of the above-mentioned proteins in the cells were measured by western blot, and it was found that their expression levels were consistent with the results of cell function. That is, the expression levels of proliferation-promoting proteins including CCND1 and C-myc, the pro-invasion and migration-promoting proteins including MMP3 and MMP9, and the anti-apoptosis protein, Bcl-2, were decreased, while the expression of the pro-apoptotic protein, Bax, was up-regulated. Furthermore, we also measured the expression levels of ECM marker proteins

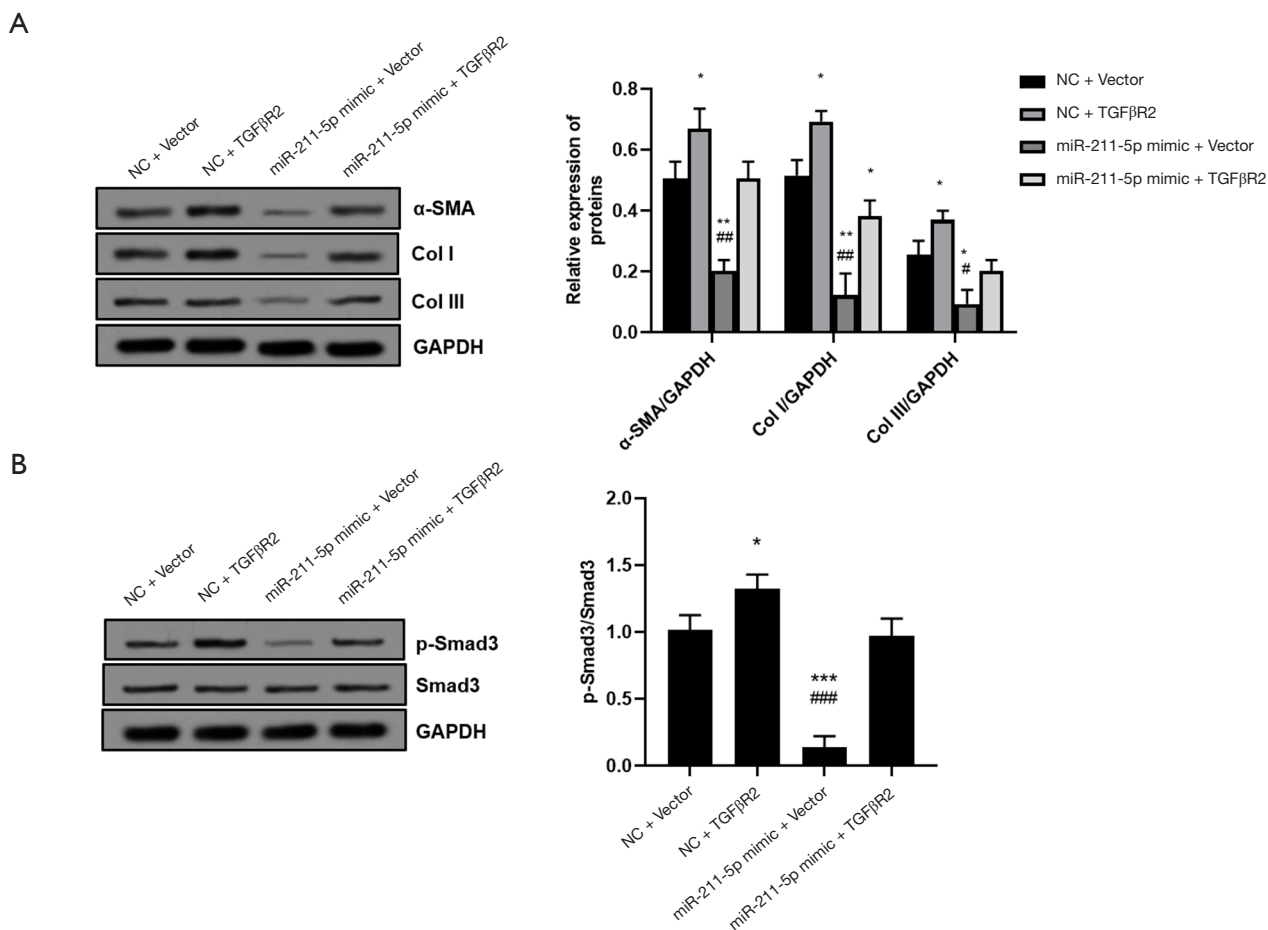


Figure 6 miR-211-5p reversed TGF β R2's promotion of collagen deposition and the activation of the TGF β /Smad3 signaling pathway. (A) Western blot was used to determine the protein expression levels of ECM marker proteins including α -SMA, Col I, and Col III; (B) Western blot was performed to measure the phosphorylation level of smad3. N.S., $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. Control group. #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ vs. NC+ TGF β R2 group.

including α -SMA, Col I, and Col III, and found that miR-211-5p could suppress ECM accumulation. MiR-211-5p may exert the above-mentioned effects by inhibiting the activation of the TGF- β R2/Smad3 signaling pathway.

Shang *et al.* found that miR-211-5p could target TGF β R2 (20). We predicted the possible binding site between miR-211-5p and the 3'-UTR of TGF β R2 through bioinformatic analysis, and found that the up-regulation of miR-211-5p in hHSFs significantly inhibited the luciferase activity of TGF β R2 with the 3'-UTR-WT, which is consistent with previous studies. It is known that TGF β R2 is a transmembrane receptor of the TGF β /Smad signaling pathway, which can induce phosphorylation of Smad2/3 and inhibit cell apoptosis (32). TGF- β can also initiate and terminate tissue repair, and its continued production will

promote the development of fibrosis (33). Moreover, it has been established that TGF- β is involved in various fibrous diseases, which can affect the functions of many organs (34). TGF- β plays a role by binding to TGF β R2. Recently, it was shown that TGF- β R2/Smad signaling plays an important role in the formation of normal and HSs (35). Also, Smad3, as a member of the Smad family, can mediate the production of collagen by dermal fibroblasts stimulated by TGF- β (35). Liu *et al.* found that blocking the TGF- β R2/Smad signaling pathway could prevent the formation of HS (36). To verify these findings, we demonstrated that the up-regulation of miR-211-5p inhibited the levels of TGF β R2 protein, while suppressing the expression of miR-211-5p increased the TGF β R2 protein expression level, thereby further verifying the target relationship between miR-211-5p and TGF β R2.

Subsequently, hHSFs cells were co-transfected with a miR-211-5p mimic and a TGFβR2 overexpression plasmid to observe the reversal effect of miR-211-5p on TGFβR2. Specifically, the overexpression of TGFβR2 enhanced cell proliferation, invasion, and migration, while apoptosis was inhibited, ECM production was reduced, and the TGF-βR2/Smad3 signaling pathway was activated. On the other hand, when miR-211-5p was co-transfected, the above effects were reversed, suggesting that the overexpression of miR-211-5p can eliminate the effects of TGFβR2.

In summary, this study found, for the first time, that miR-211-5p could deactivate the TGF-βR2/Smad3 signaling pathway by targeting TGFβR2, regulate cell proliferation, invasion, migration, apoptosis, and genes related to ECM collagen production. It could also inhibit hHSF proliferation, invasion, migration, and promote its apoptosis. Therefore, miR-211-5p is expected to become a potential target for the treatment of HS.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of West China Hospital, Sichuan University (No.: ChiCTR1800019082) and informed consent was taken

from all the patients.

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