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MiR-152-3p regulates cell proliferation, invasion and extracellular matrix expression through by targeting FOXF1 in keloid fibroblasts

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ABSTRACT

Emerging evidence has revealed that microRNAs (miRNAs) play critical roles in keloid pathogenesis. However, potential molecular mechanism of keloid formation remains unclear. In the present study, our findings showed that miR-152-3p mRNA expression level was notably up-regulated in keloid tissues and keloid fibroblasts compared with that of normal skin tissues and normal skin fibroblasts, respectively. Furthermore, miR-152-3p inhibition remarkably suppressed cell proliferation, which was increased by miR-152-3p overexpression. Cell invasion was also significantly decreased by miR-152-3p inhibition, whereas was increased by miR-152-3p overexpression. The mRNA and protein expression levels of extracellular matrix components including type I collagen, type III collagen and fibronectin were decreased by miR-152-3p inhibition, but were increased by miR-152-3p overexpression. In addition, results of dual-luciferase reporter assay indicated that FOXF1 is a direct target of miR-152-3p. FOXF1 overexpression significantly inhibits cell proliferation, invasion, and extracellular matrix in keloid fibroblasts, and the suppressive effects of miR-152-3p mimic on these functions were notably partly reversed by FOXF1 overexpression. Taken together, these findings indicated that miR-152-3p regulates cell proliferation, invasion and extracellular matrix expression through targeting FOXF1 in keloid fibroblasts, suggesting that miR-152-3p is a novel and promising molecular target for keloid treatment.

1. Introduction

Keloids are defined as a proliferative scars and a dermal exuberant response to all kinds of injury to the skin including burn, abrasions, surgery, piercings and vaccinations [1,2]. Keloid tissue could spread beyond the margins of the original wound and invade into/around normal skin [3]. It often cause itching, pain, and aesthetic impairment, which leads to physical and psychological distress. Its pathogenesis is characterized by the aggressive growth, excessive extracellular matrix (ECM) synthesis and deposition [4]. Despite of great improvements in surgical excision, laser therapy, cryotherapy and systemic chemotherapy, keloids often recur and are difficult to treat [5–7]. Therefore, there is an urgent need for a better understanding of keloid pathogenesis in order to develop better treatment approaches in clinical trials.

MicroRNAs (miRNAs) belong to a family of small non-coding RNA molecules that play crucial roles in various cellular processes through binding the 3′-untranslated region (3′-UTR) of targeted genes to regulate its gene expression at the post-transcriptional level [8,9]. Recently, emerging evidences have indicated that miRNA dysregulation are involved in keloid pathogenesis. Kashiyama et al. reported [10] that miR-196a was upregulated in keloid-derived fibroblasts compared to normal fibroblasts, and its overexpression or knockdown led to a decreased or increased level of secreted type I collagen (Col-I) and type III collagen (Col-III), respectively, through directly targeting Col-I and Col-III. Feng et al. reported [11] that miR-141-3p in keloid tissues was notably decreased compared with that of normal tissues, and its overexpression decreased cell proliferation and increased cell apoptosis through targeting and inhibiting the gene expression of growth factor receptor binding 2-associated binding protein 1 (GAB1) in keloid fibroblasts. Liu et al. reported [12] that miR-21 regulated cell apoptosis in keloid fibroblasts via targeting FasL, and its mechanisms was also involved with caspase-8 and the mitochondria-mediated apoptotic signaling pathway. It has been reported that miR-152 was highly expressed in keloid tissues than in normal skin tissues [13]. However, the molecular function of miR-152 in keloid is still unknown and needs further investigated.

In this study, to investigate the molecular function of miR-152 in

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keloid, miR-152-3p expression was detected in human keloid tissues and corresponding adjacent normal skin tissue from keloid patients, and found that miR-152-3p expression was remarkably up-regulated in keloid tissues. In human keloid fibroblasts and normal skin fibroblasts, miR-152-3p expression was also detected, and found that miR-152-3p expression was also up-regulated in keloid tissues and in human keloid fibroblasts. In addition, we investigated the molecular functions of miR-152-3p in keloid fibroblasts and also explored its potential mechanism. Our findings indicated that miR-152-3p notably regulated cell proliferation, invasion, and the mRNA and protein expression levels of ECM components including type I collagen, type III collagen and fibronectin through targeting FOXF1 in keloid fibroblast. Our results suggest that miR-152-3p is a promising molecular target for keloid treatment.

2. Materials and method

2.1. Patient samples

Keloid samples and corresponding adjacent normal skin tissue samples from patients (26 cases, 18–41 years old), who received surgery at The First Affiliated Hospital of Xi'an Jiaotong University, were obtained from October 2016 to March 2018. Keloids were identified and confirmed by the physicians in our hospital. These patients had no other skin diseases and other organic diseases, and had also not received topical and systemic therapy. Before surgery, informed consent was provided by each patient recruited. This research was approved by the Ethics Committee of The First Affiliated Hospital of Xi'an Jiaotong University.

2.2. Cell culture

Human keloid fibroblasts and normal skin fibroblasts were isolated from keloid tissues and normal skin tissues following described previously [10]. Briefly, these tissues were washed with PBS, cut into 1 mm^3 pieces. After cells were digested with 0.15% collagenase (Roche Applied Science, Indianapolis, USA), the suspension was collected, filtered, centrifuged, resuspended, and then cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 1% penicillin/streptomycin at 37 °C in a humidified incubator containing 5% CO_2. The cells of fibroblasts at passages 3 to 5 were used to further experiment.

2.3. Cell transfection

The miR-152-3p mimic (5′-ucagugcaugacagaacuugg-3′) and its mimic control (5′-uucuccgaacgugucacgutt-3′), miR-152-3p inhibitor (5′-ccaaguucugucaugcacuga-3′) and its inhibitor control (5′-aauccgacuauaacuttguga-3′), pcDNA3.1/FOXF1 plasmid (pcFOXF1) and its negative control plasmid (pcControl) were designed by Genepharma (Shanghai, China). For transfection, these miRNAs or plasmids were transiently transfected into cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After 24 h of transfection, cells were collected and the efficiency of transfection was detected.

2.4. Cell proliferation assay

Cell proliferation was examined using the Cell Counting Kit-8 (CCK-8) kit according to the manufacturer's protocol (Dojindo Laboratories, Kumamoto, Japan). Briefly, cells were seeded into 96-well plates, and then cultured for 48 h. Next, 20 μL of CCK-8 solution was added into each well and continued to incubated for another 2 h. Subsequently, a microplate reader (Bio-Rad Laboratories, Inc., USA) was used to read the absorbance optical density (OD) values of each well at the wavelength of 450 nm. At least three times in triplicates was repeated in this experiment.

2.5. Dual-luciferase reporter assay

The FOXF13′-UTR fragment containing the wild type or mutated miR-152-3p binding sites were amplified and subcloned into pGL-3 vector (Promega, Madison, WI, USA) to generate the wild type vector (WT-FOXF1) or mutant vector (MUT-FOXF1). The pRL-TK containing Renilla luciferase (Promega, Madison, WI, USA) was used as an internal control. For Luciferase assays, 293T cells (2.5×10^4/well) were plated into 12-well plates for 12 h, and then co-transfected with vectors and miRNAs using Lipofectamine 3000 (Invitrogen, Carlsbad, CA). After further cultivation for 24 h, cells were harvested and the luciferase activities were measured using the Dual-Luciferase Reporter Assay (Promega, Madison, WI, USA) according to the manufacturer's instruction. Relative luciferase activities were calculated by normalizing to the Renilla luciferase activities. Each experiment was repeated three times in duplicate.

2.6. Cell invasion assay

Cell invasion was examined by transwell insert chambers (Costar, Cambridge, MA, USA). The upper chamber of cell invasion was coated with Matrigel (8 μm). After transfection with miRNAs or plasmids, cells with serum-free medium were seeded into the upper chamber. The medium containing 10% FBS was only added into the bottom chamber. After incubation for 24 h, cells that did not invade through the pores were completely removed using a cotton swab. Subsequently, cells that invaded to the basement membrane of the transwell insert were stained with 0.1% crystal violet. The number of stained cells was counted in six independent fields of view under a microscope.

2.7. Western blotting

Cells with previous treatment were lysed using radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Waltham, MA, USA). Lysates were then collected, and protein concentrations were detected using the Bradford Protein Assay kit. Equal amounts of protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride membranes. The membranes were then blocked with 5% bovine serum albumin for 1 h at room temperature. Subsequently, the membranes were incubated with primary antibodies against FOXF1, Col-I, Col-III, fibronectin, and β-actin (Abcam, Cambridge, MA, USA) overnight at 4 °C. After washed wish TBST, the membranes were incubated at room temperature for 1 h with horseradish peroxidase-conjugated secondary antibody (Abcam, Cambridge, MA, USA). The bands were visualized by enhanced chemiluminescence (Pierce; Thermo Fisher Scientific, Inc.). The relative band intensity was quantified by Image J software. β-Actin was used as internal reference to normalize protein expression

2.8. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from tissues and cells with various treatments above using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA concentration was measured using a NanoDrop-1000 (Thermo Fisher Scientific, Waltham, MA, USA), and then equal RNA was reverse-transcribed to complementary DNA (cDNA) using TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) or PrimeScript reverse transcriptase Reagent kit (TaKaRa, Dalian, China). Then, the mRNA expression level was performed by RT-PCR using Taq-Man miRNA assays or a SYBR Green PCR Kit (Takara, Kyoto, Japan) according to the manufacturers' protocols. All reactions were performed in triplicate. U6/β-actin was used as internal control.

Data were analyzed using the $2^{-\Delta\Delta Ct}$ method. The relative mRNA expression level was normalized to the level of U6/β-actin mRNA. The primer sequences used were as follows: miR-152-3p: 5′-acactccagctgggtcagtgcatgacag-3′ (forward) and 5′-ctcaactggtgtcgtggagtcggcaattcagttgagccaagtt-3′ (reverse); FOXF1: 5′-tatctgcaccagaacagccacaac-3′ (forward) and 5′-actcctttcggtcacacatgct-3′ (reverse); collagen I: 5′-ttctgtacgcaggtgattgg-3′ (forward) and 5′-catgttcagctttgtggacc-3′ (reverse); Collagen III: 5′-gctctgcttcatcccactatta-3′ (forward) and 5′-tgcgagtcctcctactgctac-3′ (reverse); Fibronectin: 5′-tgactggccttaccagaggg-3′ (forward) and 5′-catctgtaggctggttcaggc-3′ (reverse); β-actin: 5′-ggacttcgagcaagagatgg-3′ (forward) and 5′-agcactgtgttggcgtacag-3′ (reverse); U6: 5′-gcttcggcagcacatatactaaaat-3′ (forward) and 5′-cgcttcacgaatttgcgtgtcat-3′ (reverse).

2.9. Statistical analysis

Each experiment was independently performed at least three times. Data are expressed as the mean ± standard deviation. Statistical analysis was performed using SPSS version 19.0 (IBM Corporation, Armonk, NY, USA). The differences between two groups were analyzed using Student's *t*-test. Correlation analysis was performed using multiple linear regression and spearman's correlation analysis. $P < 0.05$ was considered to be a significant difference.

3. Results

3.1. MiR-152-3p is up-regulated in keloid tissues and fibroblasts

To explore the role of miR-152-3p in keloid pathogenesis, miR-152-3p expression was detected by qRT-PCR in human keloid tissues ($n = 26$) and corresponding adjacent normal skin tissue from keloid patients (n = 26). The results showed (Fig. 1A) that the mRNA expression level of miR-152-3p was notably upregulated in keloid tissues compared with that of normal skin tissues. In addition, the miR-152-3p expression was also examined by qRT-PCR in keloid fibroblasts and normal skin fibroblasts isolated from keloid tissues and normal skin tissues, and found its expression was also notably increased (Fig. 1B). These data indicated that miR-152-3p was related to keloid pathogenesis.

3.2. MiR-152-3p regulates cell proliferation, invasion, and extracellular matrix expression in keloid fibroblasts

To analyze the function of miR-152-3p in keloid fibroblasts, was transfected into keloid fibroblasts, gain-of-function and loss-function experiments were performed using miR-152-3p inhibitor and its mimic, respectively. The transfection efficiency was detected by qRT-PCR (Fig. 2A). Subsequently, cell proliferation of the transfected keloid fibroblasts was detected by CCK-8 assay, the results showed that cell viability was remarkably increased by miR-152-3p mimic, but inhibited by miR-152-3p inhibitor compared with its control (Fig. 2B and C). And then, cell invasion ability was examined by transwell assays. The results showed that miR-152-3p mimic remarkably increased the number of cell invasion, which was inhibited by miR-152-3p inhibitor compared with its control (Fig. 2D–E). We also analyzed extracellular matrix expression in keloid fibroblasts by detecting the gene expression of Col-I, Col-III and Fibronectin using Western blotting and qRT-PCR. As shown in Fig. 2F–H, the protein and mRNA levels of Col-I, Col-III and Fibronectin were significantly down-regulated by miR-152-3p inhibitor, but upregulated by miR-152-3p mimic compared with its control. These findings suggest that miR-152-3p regulates cell proliferation, invasion, and extracellular matrix expression in keloid fibroblasts.

3.3. FOXF1 is a direct target of miR-152-3p

To investigate the mechanism underlying the effects of miR-152-3p on keloid pathogenesis, FOXF1 was identified as a likely target of miR-152-3p using bioinformatics tools (TargetScan and miRanda) to predict its potential target genes (Fig. 3A). Next, the target relationship with FOXF1 and miR-152-3p was detected by the dual-luciferase reporter assay. Our results showed (Fig. 3B) that, in cells transfected with wild type vector, the luciferase activity was notably decreased by miR-152-3p mimic, but remarkably increased by miR-152-3p inhibitors when compared with its corresponding control. In contrast, miR-152-3p had no effect on the luciferase activity in mutant FOXF1-3′-UTR groups. Then, this target relationship was further confirmed by qRT-PCR and Western blotting. As shown in Fig. 3C and D, the mRNA and protein expression levels of FOXF1 in keloid fibroblasts were notably decreased by miR-152-3p mimic, but increased by miR-152-3p inhibitor when compared with its corresponding control. In addition, the significant down-regulation of FOXF1 was examined by qRT-PCR in human keloid tissues and corresponding adjacent normal skin tissue from keloid patients (Fig. 3E). We also analyzed the correlation of FOXF1 mRNA expression level with miR-152-3p expression level, and found that miR-152-3p expression was negatively correlated with FOXF1 mRNA expression level in keloid tissues (Fig. 3F). These findings indicated that FOXF1 is a direct target of miR-152-3p in keloid fibroblasts.

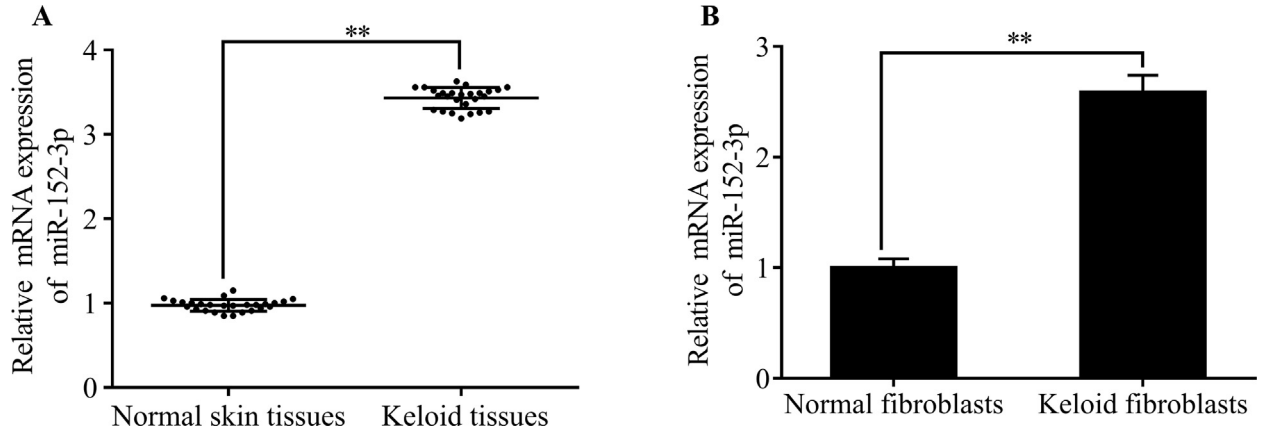

Fig. 1. MiR-152-3p is up-regulated in keloid tissues and fibroblasts. (A) MiR-152-3p mRNA expression level was examined by qRT-PCR in human keloid tissues and corresponding adjacent normal skin tissue from keloid patients ($n = 26$). (B) The miR-152-3p expression was also examined by qRT-PCR in keloid fibroblasts and normal skin fibroblasts isolated from keloid tissues and normal skin tissue. Each experiment was independently performed at six times. The error bars represent for standard deviation. $^{**}P < 0.01$.

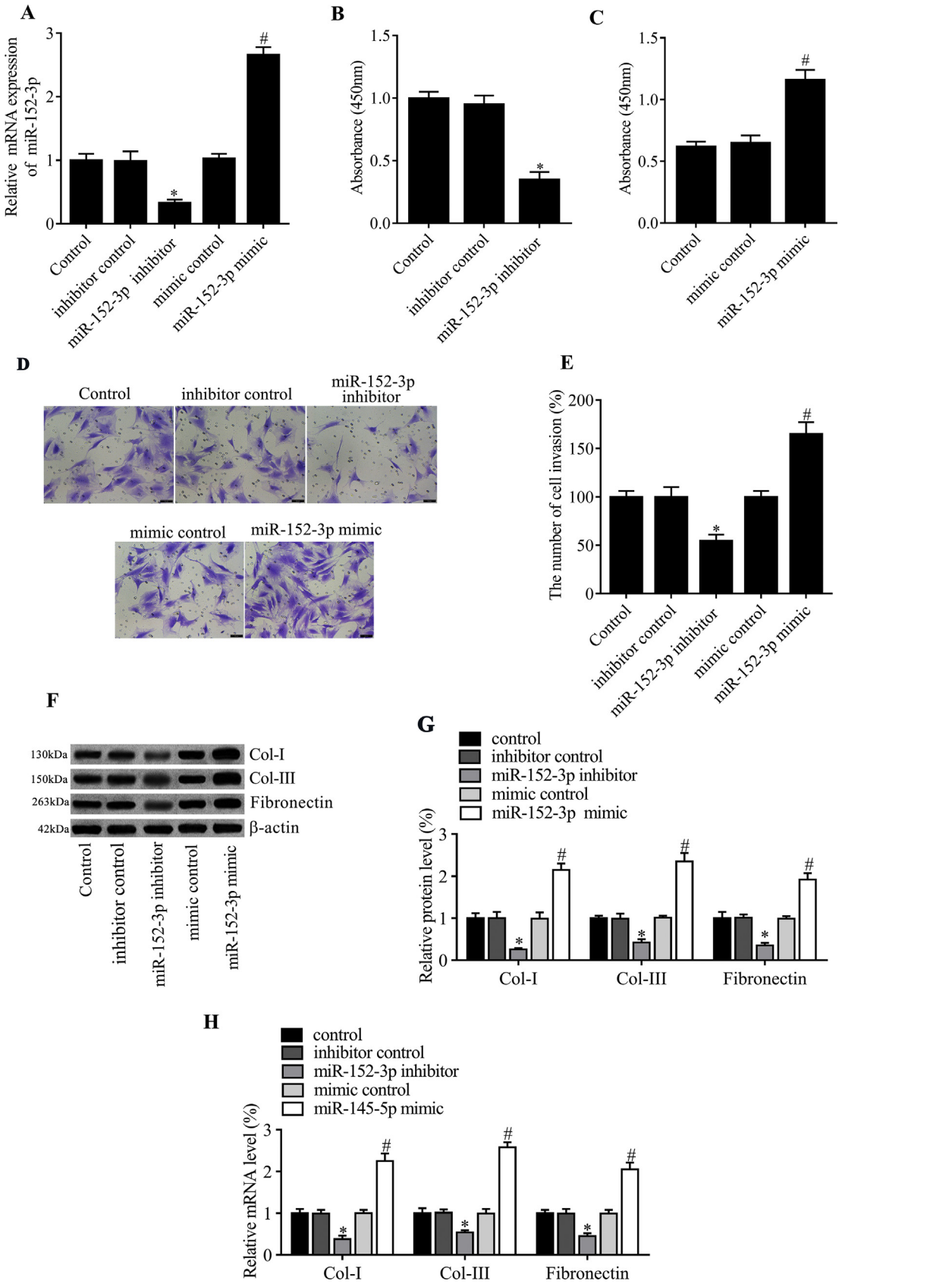

Fig. 2. MiR-152-3p regulates cell proliferation, metastasis, and extracellular matrix expression in keloid fibroblasts. Cells were transfected with miR-152-3p mimic, mimic control, miR-152-3p inhibitor or inhibitor control. (A) MiR-152-3p mRNA expression level was examined by qRT-PCR in human keloid fibroblasts. (B–C) Cell proliferation was examined by CCK-8 assay in human keloid fibroblasts. Cell invasion (D–E) was examined using transwell assay in human keloid fibroblasts. The mRNA and protein expression levels of extracellular matrix components including type I collagen, type III collagen and Fibronectin were detected by Western blot (F) and qRT-PCR (H) in human keloid fibroblasts. The relative protein expression (G) was quantified using Image J software. Each experiment was independently performed at six times. The error bars represent for standard deviation. *$P < 0.05$ as compared with inhibitor control, #$P < 0.05$ vs. mimic group.

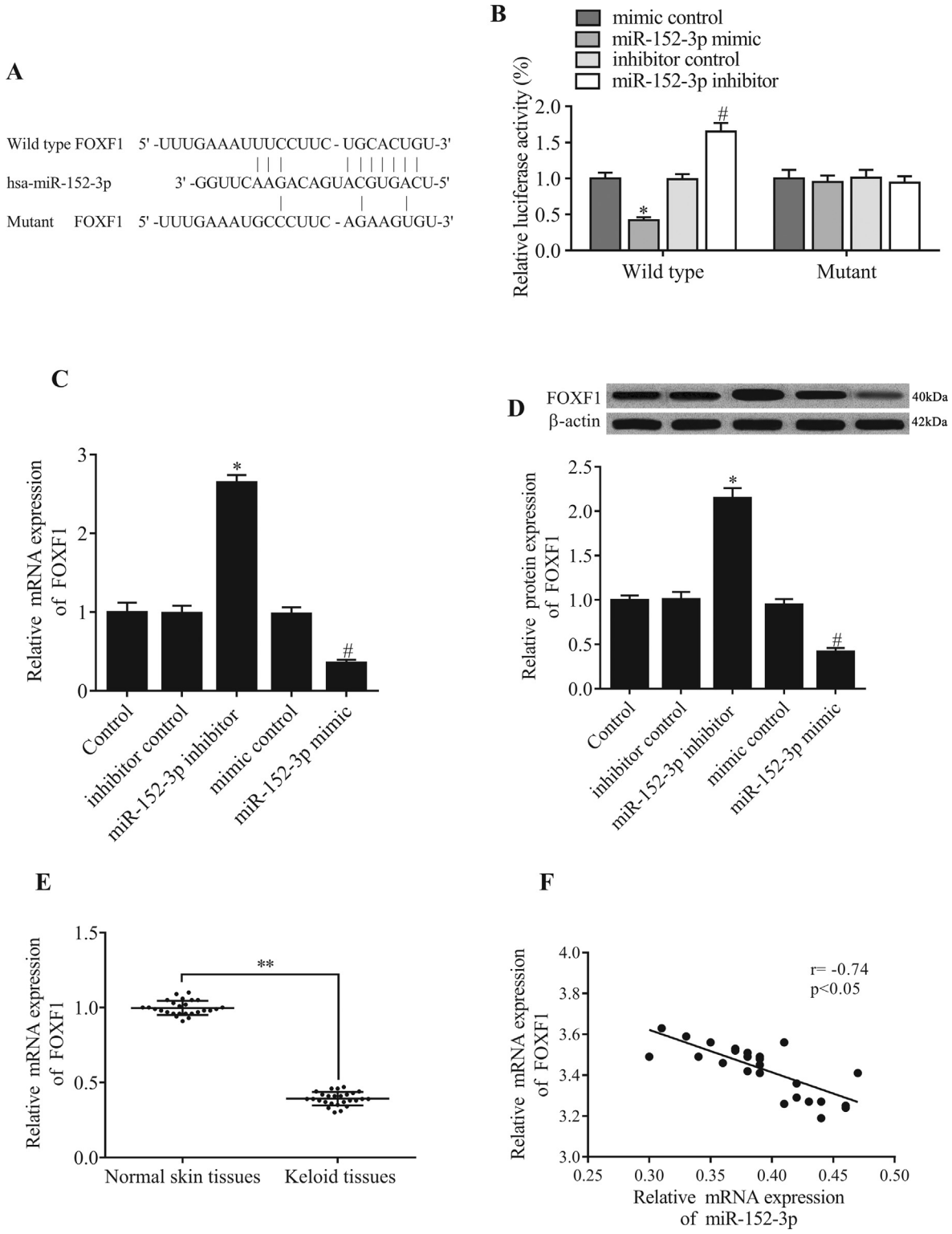

Fig. 3. FOXF1 is a direct target of miR-152-3p. (A) The sequences of FOXF1 mRNA 3′-UTR including wild type and mutant are shown with the miR-152-3p sequence. (B) The relative luciferase activity was examined using a dual luciferase reporter assay in 293T cells. The mRNA (C) and protein (D) expression levels of FOXF1 were examined by qRT-PCR and Western blotting in human keloid fibroblasts transfected with miR-152-3p mimic, mimic control, miR-152-3p inhibitor or inhibitor control. (E) The FOXF1 mRNA expression level was examined by qRT-PCR in human keloid tissues and corresponding adjacent normal skin tissues from keloid patients ($n = 26$). (F) The correlation of FOXF1 mRNA expression level with the miR-152-3p mRNA expression level was analyzed in keloid tissue using multiple linear regression and spearman's correlation analysis. Each experiment was independently performed at six times. The error bars represent for standard deviation. *P $<$ 0.05 as compared with inhibitor control group, #P $<$ 0.05 vs. mimic control group.

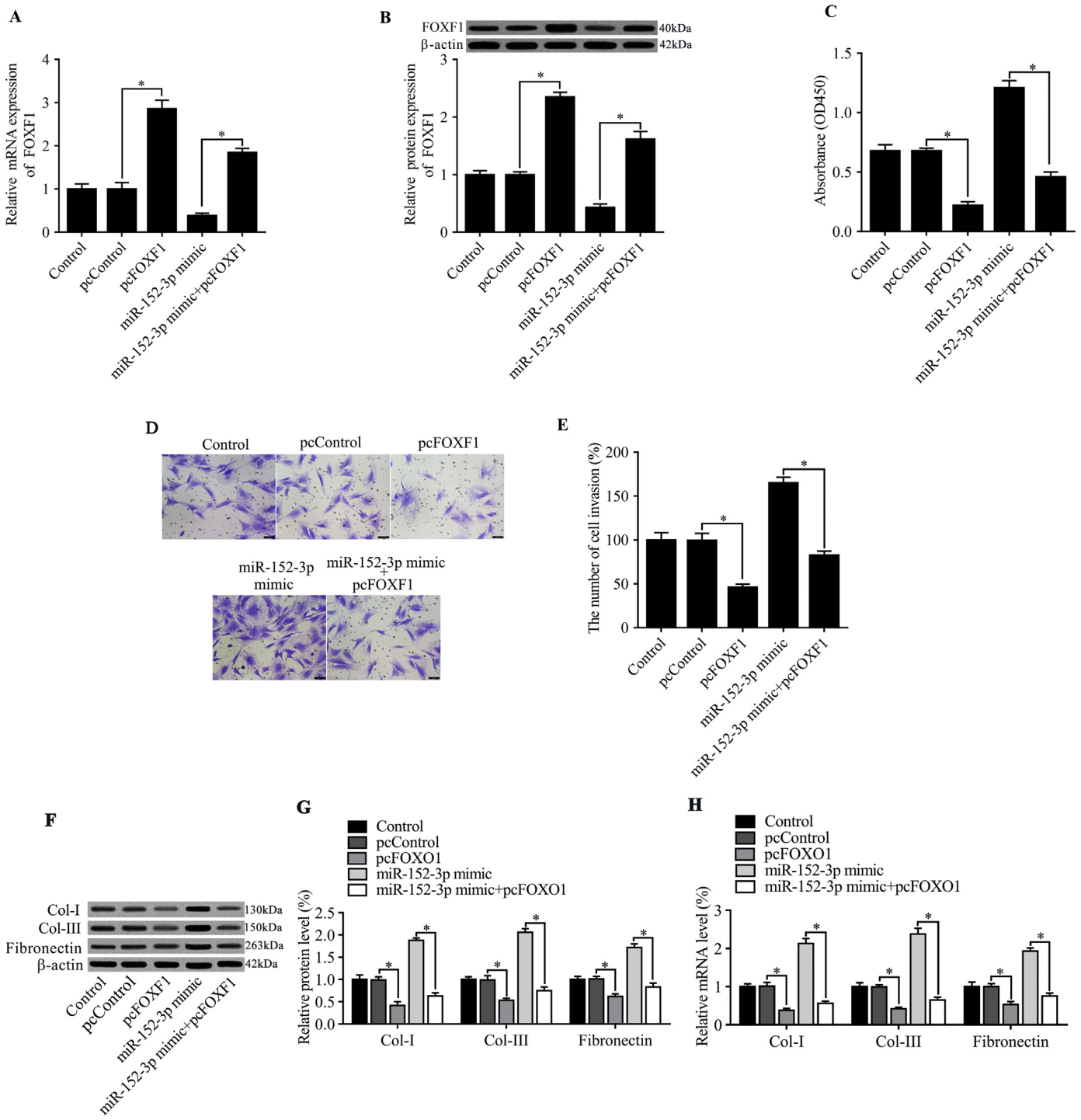

Fig. 4. FOXF1 overexpression partly reverses the suppressive effect of miR-152-3p mimic. The mRNA (A) and protein (B) expression levels of FOXF1 were examined by qRT-PCR and Western blotting in human keloid fibroblasts transfected with miR-152-3p mimic, mimic control, pcDNA3.1-FOXF1 (pcFOXF1) or its negative control plasmid (pcControl). (C) Cell proliferation was examined by CCK-8 assay in human keloid fibroblasts. Cell invasion (D–E) was examined using transwell assay in human keloid fibroblasts. The mRNA and protein expression levels of extracellular matrix components including type I collagen, type III collagen and Fibronectin were detected by Western blot (F) and qRT-PCR (H) in human keloid fibroblasts. The relative protein expression (G) was quantified using Image J software. Each experiment was independently performed at six times. The error bars represent for standard deviation. *$P < 0.05$.

3.4. FOXF1 overexpression partly reverses the suppressive effect of miR-152-3p mimic

To confirm that miR-152-3p regulates cell proliferation, metastasis, and extracellular matrix expression in keloid fibroblasts through targeting FOXF1 gene expression, cells were transfected with pcDNA3.1-FOXF1 or miR-152-3p mimic. Results showed that FOXF1 gene expression both mRNA and protein levels was remarkably upregulated by pcDNA3.1-FOXF1 in keloid fibroblasts, and the suppressive effect of miR-152-3p mimic on FOXF1 expression was significantly reversed by FOXF1 overexpression (Fig. 4A–B). Furthermore, FOXF1 overexpression significantly inhibits cell proliferation, invasion, and extracellular matrix expression in keloid fibroblasts, and the suppressive effect of miR-152-3p mimic on these functions was notably partly

reversed by FOXF1 overexpression (Fig. 4C–H). These results indicated that miR-152-3p regulates cell proliferation, invasion, and extracellular matrix expression in keloid fibroblasts through targeting FOXF1 gene expression.

4. Discussion

In the present study, miR-152-3p expression was detected by qRT-PCR in human keloid tissues and fibroblasts isolated from keloid tissues, and found its expression was notably up-regulated compared with that of normal skin tissues and normal skin fibroblasts isolated from normal skin tissues. Moreover, the molecular function of miR-152-3p in keloid fibroblast and its mechanism was investigated. Results indicated that miR-152-3p inhibition notably decreased cell proliferation, invasion, and the mRNA and protein expression levels of ECM components including type I collagen, type III collagen and Fibronectin. In contrast, miR-152-3p mimic showed the opposite effects in keloid fibroblast. In addition, we found that FOXF1 is a direct target of miR-152-3p. FOXF1 overexpression partly reverses the suppressive effect of miR-152-3p mimic. Taken together, our findings suggest that miR-152-3p regulates cell proliferation, invasion and extracellular matrix expression in keloid fibroblasts through targeting FOXF1 gene expression.

A growing body of evidence has revealed that many miRNAs were differentially expressed in skin keloid tissues and regulated its pathologic process such as miR-31 [14], miR-181a [15] and miR-637 [16]. On the basis of previous miRNA microarray studies, miR-152 was up-regulated in keloid tissues [17], but its functions in keloid pathogenic process was still unclear. MiR-152, located on chromosome 17q21.32, is a member of the miR-148/152 family that includes miR-148a, miR-148b and miR-152. MiR-152-5p and miR-152-3p are different mature sequences of miR-152. The former is excised from the 3′ arm of the hairpin precursor, and has been detected in more species than miR-152-5p [18]. So, in this study, miR-152-3p was selected to be examined in keloid tissues and fibroblast, and found that its expression was also remarkably increased.

Keloid formation is related with abnormal fibroblast proliferation, invasiveness beyond surrounding skin, and the excessive accumulation of ECM components such as type I collagen, type III collagen and fibronectin [19,20]. Invasion refers to the process in which cells leave the primary tissue, invade adjacent tissues and then continue to grow. Inhibiting cell invasion of fibroblast is crucial for keloid treatment. Combined our results and previous miRNA microarray studies, miR-152-3p is involved in keloid pathogenesis. It has been reported that miR-152 inhibit tumor cell growth and metastasis in various cancers [21–23]. Mancini M et al. reported that miR-152 contribute to fibroblast adhesion in human dermal fibroblasts [24]. So we speculate that miR-152-3p may participate in regulating keloid fibroblast proliferation, invasiveness and ECM production. In line with our speculation, our findings indicated that miR-152-3p inhibitor notably inhibited cell proliferation, invasion, and the mRNA and protein expression levels of ECM components including type I collagen, type III collagen and fibronectin, whereas miR-152-3p mimic showed the opposite effects. These results indicated that miR-152-3p inhibition was involved in decreasing keloid fibroblast proliferation, invasion and ECM expression, suggesting that inhibiting miR-152-3p expression could decrease keloid formation.

FOXF1, located on chromosome 16q24.1, is a member of the FOX-containing transcription factor family, which regulates cell growth, tissue repair, and homeostasis [25–27]. FOXF1, in various cancers, has been reported to be an important role including cell growth, cell invasion and migration [28–30]. Black M. Et al reported that FOXF1 inhibited pulmonary fibrosis, and its deletion promoted myofibroblast invasion and collagen secretion [31]. However, the molecular function of FOXF1 in keloid tissue is still not clear. In our study, we found that FOXF1 was a direct target of miR-152-3p. FOXF1 expression was notably decreased in keloid tissue. FOXF1 overexpression inhibited cell proliferation, invasion and extracellular matrix expression in keloid fibroblasts. Also, we found that FOXF1 overexpression abrogated the suppressive effect of miR-152-3p mimic. These findings indicated that miR-152-3p regulated cell proliferation, invasion and extracellular matrix expression in keloid fibroblasts through targeting FOXF1 gene, suggesting that miR-152-3p is a potential biomarker for keloid treatment.

In addition, our studies have some limitations. On the one hand, future research should confirm whether miR-152-3p targets other genes that mediate cell proliferation, invasion and extracellular matrix expression in keloid fibroblasts. On the other hand, further experimentation will be performed in a keloid animal model [32] to verify the role of miR-152-3p.

5. Conclusion

In conclusion, our findings clearly demonstrated that miR-152-3p inhibition decreased cell proliferation, invasion and extracellular matrix expression, whereas miR-152-3p overexpression has an opposite effects, the underlying mechanism of which is mediated, at least partially, through targeting FOXF1 in keloid fibroblasts. These results indicated that inhibiting miR-152-3p expression could decrease keloid formation, which provide a new therapy for keloid treatment.

Declaration of competing interest

All authors declare no competing interests.

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