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MiR-10a and miR-181c regulate collagen type I generation in hypertrophic scars by targeting PAI-1 and uPA

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ABSTRACT

Urokinase type plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) have been proposed to play key roles in extracellular matrix (ECM) deposition in hypertrophic scars (HS). Here, we found that in HS fibroblasts (HFs) miR-181c and miR-10a were differentially-expressed and targeted uPA and PAI-1, respectively. The production of Type 1 collagen (Col1) was inhibited by miR-181c knockdown or miR-10a overexpression in HFs, and this resulted in increased levels of metalloproteinase 1 (MMP1). These results suggest that the miR-181c–uPA and miR-10a–PAI-1 regulatory pathways have an integral role in HS pathogenesis.

1. Introduction

Hypertrophic scarring (HS), a fibroproliferative and abnormal wound healing process after skin injury, is characterized by an excessive dermal deposition of type 1 collagen (Col1), the main structural element of the extracellular matrix (ECM). The ECM forms a firm network of cross-linked long-chain fibers to give integrity to the scar [1–3]. In normal skin, the synthesis and degradation of ECM is controlled by numerous cytokines, but the imbalance between these pro- and anti-fibrosis related cytokines during the remodeling phase of wound healing might lead to HS.

Recently, the role of urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) in the regulation of ECM degradation has been proposed in many studies, most of which are related to fibrotic diseases [4], especially skin fibrosis, such as sclerosis and keloids. uPA and PAI-1 were reported to be involved in breast cancer metastasis via degradation of basilar membrane [5,6], but their function in breast cancer was likely an interaction with the ECM microenvironment and collagen metabolism. PAI-1, a member of the serpin family, is a main inhibitor for both urokinase-type and tissue-type plasminogen activators (uPA and tPA). The functions of uPA and PAI-1 in the regulation of ECM generation are opposite: uPA induces degradation of ECM [7,8], but PAI-1 exerts pro-fibrotic functions through inhibition of uPA [9]. Matrix metalloproteinases1 (MMP1) is believed to breakdown ECM, especially Col1 [10]. uPA positively regulates MMP1, but PAI-1 negatively regulates MMP1 [11]. Thus, a physiologic balance of uPA and PAI-1 is essential to maintain ECM proteins and tissue homeostasis [4,12]. The pro-fibrogenic function of PAI-1 in skin fibrosis, such as keloid and sclerosis has been documented [13,14]. However, the role of PAI-1 in HS remains unclear. Therefore, we hypothesize that an imbalance between PAI-1 and uPA, caused by dysregulation of their expression during the remodeling phase of wound healing, may lead to abnormal scarring and cause HS.

MicroRNAs (miRNAs) are non-coding small RNA molecules capable of silencing gene expression by inducing degradation or

Abbreviations: ECM, extracellular matrix; HS, hypertrophic scar; HF, hypertrophic scar-derived fibroblast; NF, normal skin-derived fibroblast; mRNA, messenger RNA; miRNA, microRNA; RT-PCR, reverse transcription polymerase chain reaction; UTR, untranslated region; uPA, urokinase type plasminogen activator; PAI-1, plasminogen activator inhibitor-1; MMP1, matrix metalloproteinases1.

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translational repression of target gene mRNAs, via a manner of binding complementary sequences in 3′-untranslated regions (3′-UTR) of miRNA-targeted genes [15,16]. In recent years, the roles of miRNAs in tissue fibrosis, such as cell growth, proliferation, ECM deposition, and collagen degradation has been recently reported [17,18]. To date, increasing bodies of miRNAs have been found to be dysregulated in fibrotic tissues, but only a fraction of differentially expressed miRNAs in fibrotic diseases has been functionally characterized [19]. Since the regulation of PAI-1 and uPA by differentially expressed miRNAs in HS has not been well documented, in this study, we identify differentially expressed miRNAs in HS tissues and investigated the correlation of the dysregulated miRNAs and the imbalance between PAI-1 and uPA.

Our study revealed the aberrant expression of uPA and PAI-1 at both mRNA and protein levels in hypertrophic scar-derived fibroblasts (HFs), as compared to that of normal skin-derived fibroblasts (NFs). Our study also revealed that differentially expressed miRNAs in HS are associated with dysregulated expression of uPA and PAI-1.

2. Material and methods

2.1. Tissue samples and cell culture

Hypertrophic scar and paired normal skin tissues were surgically obtained from sixteen Chinese patients (Table 1). Diagnosis of HS was confirmed by routine pathological examination. All experiments were conducted with approval of the ethics committee of the Xijing Hospital and in accordance with the Declaration of Helsinki Principles.

Primary culture of dermal fibroblasts was established as described previously [20]. Fibroblasts used in this study were obtained from the primary culture (passage 0), unless otherwise indicated.

2.2. Real-time quantitative RT-PCR

First, total RNA was extracted using TRIzol Reagent (Invitrogen, CA, U.S.), according to the manufacturer's instructions. Total RNA was then used as template for mRNA reverse-transcription, using a High-capacity RNA-to-cDNA Kit (Applied Biosystems). For quantifying miRNAs, a miScript reverse transcription kit (Qiagen, Hilden, Germany) was used for reverse transcription, followed by amplification using SYBR@Premix ExTaq™ (TaKaRa, Shiga, Japan). qRT-PCR for analysis of the expression levels of miRNA and mRNA was conducted using a Bio-Rad C1000 Thermal Cycler (Bio-Rad) and triplicate reactions were included. GAPDH and RNU6B RNA were used as internalloading controls for mRNAs and miRNAs, respectively. The following primers were used for the amplification of miRNAs by qRT-PCR: universal primer (UP) included in the miScript reverse transcription kit (Qiagen) and 5′-TACCCTGTAGATCCGAATTTGTG-3′; 5′-AACATTCAACCTGTCGGTGAGT-3′; 5′-ACGCAAATTCGTGAAGCGTT-3′ for miR-10a; miR-181c; RNU6B RNA (AUGCT, Beijing, China), respectively. Primers used in PCR for mRNA quantification are listed in Table 2.

2.3. Construction of the expression vector and siRNA

A DNA fragment containing the sequence of the PAI-1 open reading frame region was ligated into the pENTR vector (YouBio, Changsha, China) to make pENTR-PAI-1-orf and then this gene fragment was transferred into the destination vector, pcDNA™3.2-DEST (Invitrogen, U.S.). The siRNA used in our study was designed as a 19 bp RNA duplex that is complementary to the sequence of uPA mRNA. To establish an shRNA vector, paired deoxyribonucleotide oligonucleotides encoding uPA siRNA were synthesized, annealed, and cloned into the pSUPER vector (GenePharma, Shanghai, China). Stable transfection of uPA siRNA was performed according to standard protocols as recommended by the manufacturers and the empty vector was used as a control.

2.4. Luciferase reporter assay

The 3′untranslated region (UTR) fragments of uPA and PAI-1 containing the miRNA binding sites were amplified by PCR using the cDNA template obtained from RNA sample of HFs. The wide-type 3′UTRs of uPA and PAI-1 as well as mutant 3′UTRs with nucleotide substitutions in the putative binding sites corresponding to the seed sequence of miR-181c and miR-10a were cloned downstream of the firefly luciferase gene in the pGL3 vector (Promega, Madison, WI). The reporter construct of uPA-3′UTRs was co-transfected with miR-181c or a control miRNA into HEK293T cells. Similarly, the PAI-1-3′UTRs reporter was also co-transfected with miR-10a or a control miRNA into HEK293T cells. 48 h after transfection, cells were rinsed in PBS and their luciferase activity was measured by a luminometer (Promega), using dual luciferase reporter assay system.

Table 1
The profile of each sample for primary culture.

HFs and NFs from 16 patients	Sex	Age (years)	Biopsy site	Duration of the lesion (months)	Etiology	Previous treatment
Cell types						
HF/NF 1[a]	Male	21	Shoulder	6	Burn	Both[b]
HF/NF 2	Female	16	Chest	8	Trauma	Topi cal silicon gel
HF/NF 3	Female	27	Arm	13	Scald	Topical silicon gel
HF/NF 4	Female	43	Shoulder	11	Burn	Pressure garment
HF/NF 5	Male	38	Chest	17	After ope.	_
HF/NF 6	Female	24	Shoulder	9	Burn	Pressure garment
HF/NF 7	Male	36	Buttock	7	Burn	Topical silicon gel
HF/NF 8	Male	12	Cheek	10	After ope.	Topical silicon gel
HF/NF 9	Male	21	Shoulder	5	Scald	Both[b]
HF/NF 10	Female	16	Chest	14	Scald	Topical silicon gel
HF/NF 11	Female	27	Cheek	12	Burn	Pressure garment
HF/NF 12	Female	43	Back	8	Trauma	_
HF/NF 13	Male	38	Chest	17	After ope.	Resection and electron beam
HF/NF 14	Male	24	Shoulder	24	Scald	Pressure garment
HF/NF 15	Female	44	Back	20	Scald	Topical silicon gel
HF/NF 16	Male	19	Arm	15	Electric injury	Topical silicon gel

Abbreviations: HF, hypertrophic scar derived fibroblast; NF, normal skin fibroblast; ope, operation.
[a] The indicated person derived samples were used for Affymetrix miRNA microarray.
[b] Both: treatment methods included Topi cal silicon gel and Pressure garment.

Table 2
The primers used in Real-time PCR.

	Primer	Sequence (5′ → 3′)
Real-time PCR	uPA-forward	AAATGCTGTGTGCTGCTGAC
	uPA-reverse	AGGCCATTCTCTTCCTTGGT
	PAI-1-forward	CGCCTCTTCCACAAATCAG
	PAI-1-reverse	ATGCGGGCTGAGACTATGA
	GAPDH-forward	TCACCAGGGCTGCTTTTAAC
	GAPDH-reverse	GACAAGCTTCCCGTTCTCAG

2.5. Western blot analysis

Western blotting was performed as previously described [19], using an anti-uPA rabbit monoclonal antibody (ab169754, 1:1000 dilution, Abcam, U.S.), anti-PAI-1 mouse monoclonal antibody (ab125687, 1:1000 dilution, Abcam, U.S.) and anti-beta-actin mouse monoclonal antibody (ab133626, 1:2000 dilution, Abcam, U.S.). Signals were recorded using an Odyssey Infrared Imaging System (LI-COR Biosciences, NE, U.S.).

2.6. Immunofluorescence of tissue samples

Skin tissues were fixed in 4% paraformaldehyde. For immunofluorescence analysis, the sections were first blocked with 1% bovine serum albumin and then incubated with both polyclonal antibody against uPA (ab24122, 1:1000, Abcam, U.S.) and PAI-1 (ab66705, 1:1000, Abcam, U.S.), washed and incubated with secondary antibodies: both Cy3 Donkey Anti-Goat IgG (cw0216, 1:500, CW Biotech) and FITC Goat Anti-Rabbit IgG (cw0114, 1:50, CW Biotech). Finally, the sections were mounted using Vectashield with DAPI (BD5010, 2 μg/ml, Bioworld) and fluorescence was observed with microscopy (FV10i, Olympus).

2.7. ELISA for detection of collagen type 1 (Col1) and MMP1 levels

The culture medium was replaced with conditioned medium containing AA-2P (Sigma, U.S.), at the sixth hour after transfection. Then every 24 h, the medium was changed. The collected medium was used to detect the concentration of total MMP1 and collagen type I triple helix by human MMP-1 (Merck, Japan) and human collagen type I (ACEL, Japan) enzyme-linked immunosorbent assay (ELISA) kits, respectively.

2.8. Statistical analysis

All experiments were performed in triplicate and all data are presented as mean ± standard deviation (S.D.). Two-tailed unpaired Student's *t*-test (except where otherwise stated) was used for statistical analysis. P values < 0.05 were considered statistically significant and are indicated with a single asterisk (*). In addition, P values < 0.01 are indicated with a double asterisk (**) and P values < 0.001 are indicated with a triple asterisk (***).

3. Results

3.1. Aberrant expression of uPA and PAI-1 in HFs

To compare the expression levels of uPA and PAI-1 between HFs and NFs, 16 pairs of tissue samples from HS and normal skin were

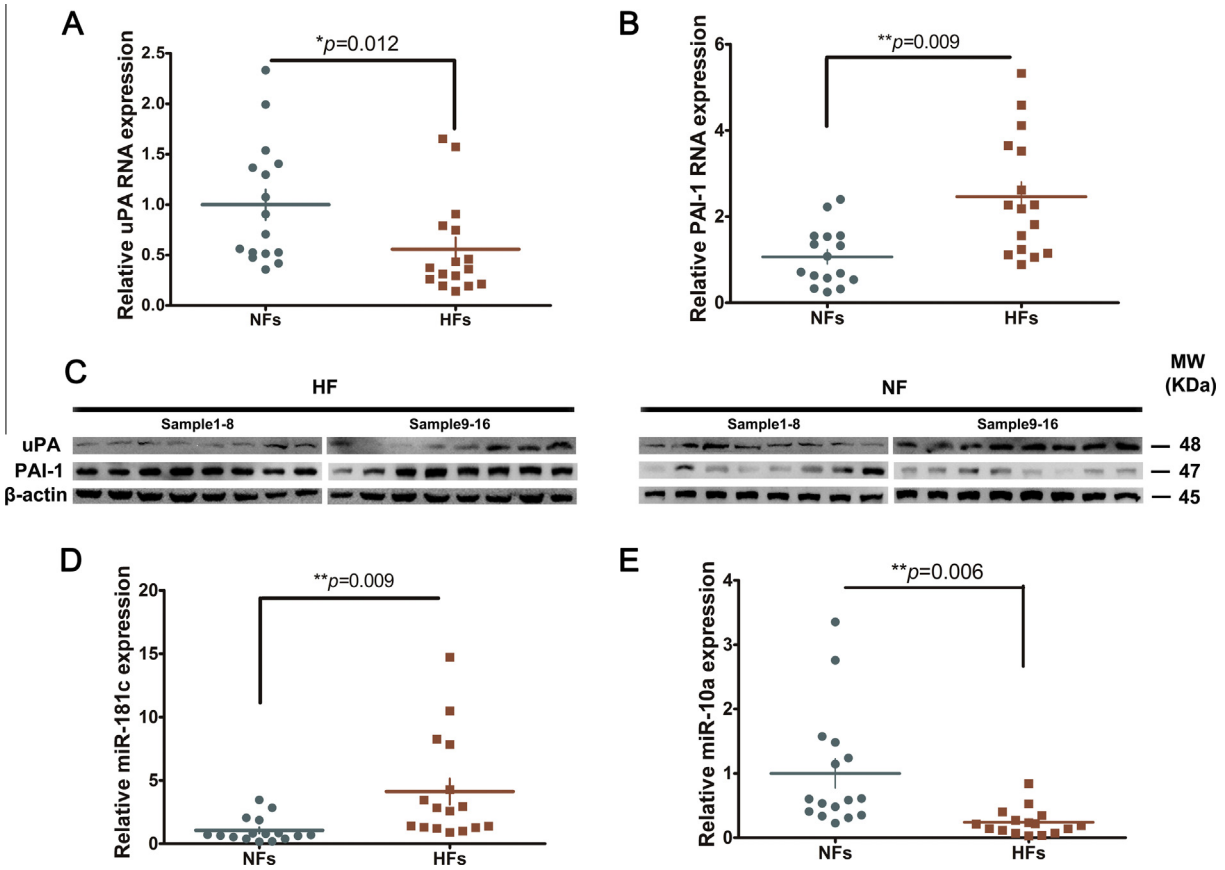

Fig. 1. Expression of uPA and PAI-1, miR-181c and miR-10a in hypertrophic scar-derived fibroblasts (HFs) and normal fibroblasts (NFs). (A, B) Comparison of mRNA expression of uPA and PAI-1 between HFs and NFs (passage 0) using 16 paired tissue samples. (C) Western-blot analysis for the protein expression of uPA and PAI-1 in HFs and NFs (passage 0–2), using 16 paired tissue samples. β-actin was used as a loading control. (D, E) Validation of miR-181c and miR-10a expression in HFs and NFs using qRT-PCR. Bars represent the mean ± S.D. (*P < 0.05, **P < 0.01).

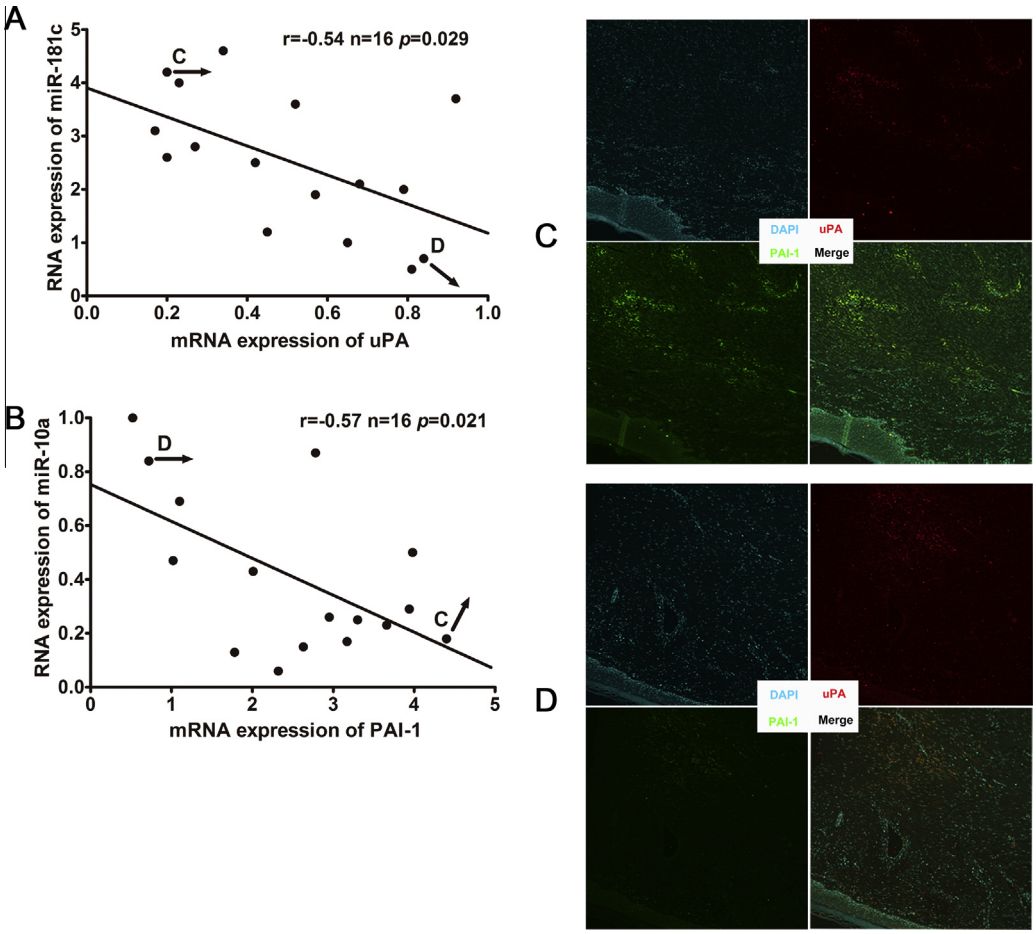

Fig. 2. Correlations between miR-181c expression and uPA level as well as miR-10a and PAI-1. (A) An inverse correlation of miR-181c expression with uPA level was found in HS tissue samples. (B) An inverse correlation of miR-10a and PAI-1 was found in HS tissue samples. (C, D) Examination of uPA and PAI-1 expression in HS tissue samples (C: case #5 and D: case #9) by immunofluorescence staining: uPA-positive (red) and PAI-1-positive (green).

collected and cultured (Table 1). Our results showed that the expression of uPA was decreased in HFs, as compared to that of NFs at mRNA and protein levels (Fig. 1A and C). In contrast, an elevated PAI-1 expression was found in cultured HFs (Fig. 1B and C) compared to NFs.

3.2. Aberrant expression of miR-181c and miR-10a in HFs

To validate miRNA microarray data (Fig. S4) and the prediction of TargetScan, the expression of miRNAs in 16-paired fibroblasts was examined by qRT-PCR assay (Table 1). As compared to NFs, overexpression of miR-181c was found in HFs (Fig. 1D). On the other hand, downregulation of miR-10a was also detected in HFs (Fig. 1E).

3.3. Correlation of altered miRNAs and aberrant expression of uPA and PAI-1 in HFs

To evaluate the correlation of miR-181c or miR-10a with uPA and PAI-1 in HS, their expression was examined in 16 tissue samples of HS by qRT-PCR. As shown in Fig. 3A and B, a negative correlation was found between level of endogenous miR-181c and uPA mRNA, and between miR-10a and PAI-1.

Immunofluorescence staining also confirmed the conclusion drawn from PCR data of HS tissues and NS tissues (Fig. 2A–D and S.2F). Among all HS tissues, case #5 (Fig. 2C) was characteristic of a relatively high level of miR-181c, but low level of miR-10a. Another case (#9) (Fig. 2D) showed a relatively low level of miR-181c, but a high level of miR-10a. Compared to #9, the fluorescence signals of PAI-1 proteins with a positive immunostaining were much higher in #5; however, the signals of immunostained uPA proteins were much less.

3.4. Identification of uPA and PAI-1 genes as direct targets of miR-181c and miR-10a

The high conservation, across different species, of the putative binding sites of miR-181c or miR-10a in the region of 3′-UTR of uPA or PAI-1, respectively, was found by bioinformatics analysis (Figs. 3A and 4A). To verify the binding sites, the region containing the wild type or the mutated seed-sequence of miR-181c or miR-10a in the 3′UTR of uPA or PAI-1, respectively, was cloned into the luciferase reporter plasmids (Figs. 3B and 4B). A dual luciferase reporter gene assay showed that in the presence of miR-181c or miR-10a, the luciferase activity of the wild-type reporter in the transfected HEK-293T cells was inhibited, but those miRNA-mediated inhibitory effects were not observed in the mutant reporter-transfected cells (Figs. 3C and 4C). These results indicate that uPA or PAI-1 is a direct target gene of miR-181c or miR-10a, respectively.

To verify the role of miR-181c or miR-10a in regulating uPA or PAI-1 expression, respectively, qRT-PCR and Western blot assays were performed using the samples from both HFs and NFs. Because of the relatively higher level of miR-181c and lower level of miR-10a in HFs as compared to NFs, we transfected miR-181c mimics and miR-10a inhibitors into NFs. In contrast, the miR-181c inhibi-

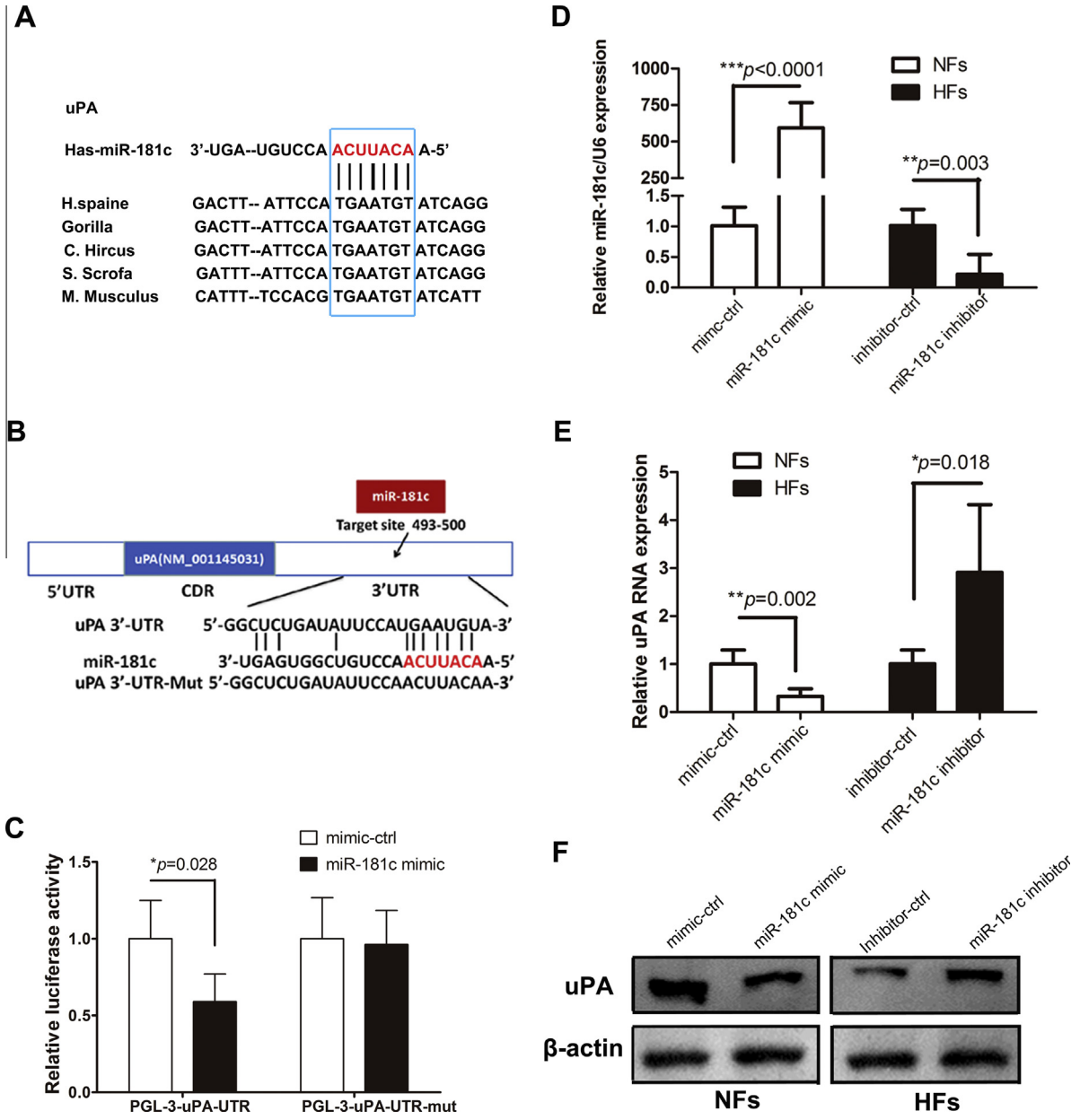

Fig. 3. Identification of uPA as a target of miR-181c. (A) Putative binding site of miR-181c within the 3′UTR of uPA. The binding site sequences are highly conserved across different species. (B) According to the sequence of the miR-181c binding site within the 3′UTR of human uPA, the luciferase reporter with either wild-type or mutant sequences of uPA 3′UTR was constructed, using pGL3 vectors. (C) 48 h after transfection, relative luciferase activity in HEK-293T cells was measured. The data represents firefly luciferase activity units (relative light units, RLU) from three separate experiments. RLU were normalized to renilla luciferase activity. (D) NFs were transfected with miR-181c mimic and mimic control (mimic-ctrl), and HFs were transfected with miR-181c inhibitor and inhibitor control (inhibitor-ctrl). The miR-181c levels in the transfected cells were examined by qRT-PCR analysis. (E) At 48 h after transfection, qRT-PCR and (F) Western-blot analyses were used to evaluate the expression level of uPA in NFs (+miR-181c mimic) and HFs (+miR-181c inhibitor). All values are normalized to either the mimic control (mimic-ctrl) or inhibitor control (inhibitor-ctrl).

tors and miR-10a mimics were transfected into HFs. Results showed that the mRNA and protein levels of uPA or PAI-1 were decreased in the cells transfected with either miR-181c mimics or miR-10a mimics, as compared with their levels in the control groups (Figs. 3D–F and 4D–F). These results suggest that miR-181c and miR-10a may regulate the gene expression of uPA and PAI-1, respectively, at both the mRNA and protein levels.

3.5. Altered miRNAs in HFs significantly impact production of type I collagens and MMP1

In our study, collagen type I as the most abundant collagen within the abnormal ECM deposits in HS [1–3,10,21], was selected to evaluate the role of miR-181c and miR-10a in the regulation of ECM degradation. As uPA and PAI-1 are well-known cytokines implicated in ECM degradation and their expression is regulated by miRNAs, we examined whether miRNAs treatment could reduce Col1 expression in HFs and NFs cells. Through the transfection of miR-181c inhibitor and miR-10a mimic, a reduced Col1 expression in HFs (Fig. 5A–B), but not in NFs (S.1A–D), was detected by ELISA. Furthermore, co-transfection of miR-10a mimics and miR-181c inhibitors together in HFs was still decreased the expression of Col1 (S.2A–E).

The MMP1 expression levels in HFs transfected with either miR-181c inhibitors or miR-10a mimics were determined by ELISA. As shown in Fig. 5C–D, both miR-181c inhibitors and miR-10a mimics

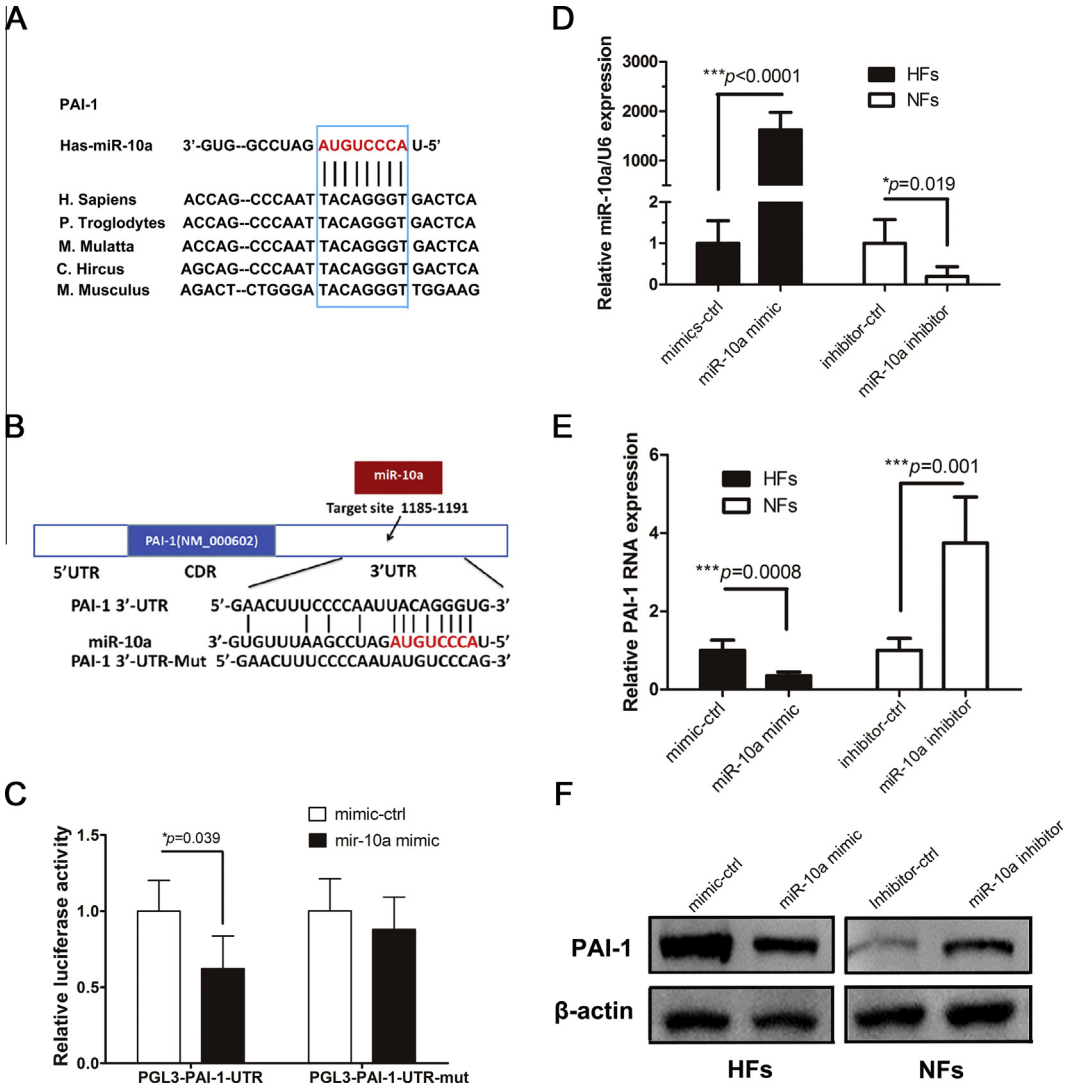

Fig. 4. Identification of PAI-1 as a target of miR-10a. (A) Putative binding site of miR-10a within the 3′UTR of PAI-1. The binding site sequences are highly conserved across different species. (B) According to the sequence of the miR-10a binding site within the 3′UTR of human PAI-1, the luciferase reporter with either wild-type or mutant sequences of PAI-1 3′UTR was constructed, using pGL3 vectors. (C) 48 h after transfection, the relative light units (RLU) of HEK-293T cells were examined and normalized to renilla luciferase activity. To confirm that miR-10a specifically binds to PAI-1 3′UTR, the luciferase activities of pGL3-PAI-1-wt and pGL3-PAI-1-mut in the presence of miR-10a mimic were determined and compared to the RLU of cells transfected with mimic-ctrl. (D) HFs were transfected with miR-10a mimic and mimic control (mimic-ctrl), and NFs were transfected with miR-10a inhibitor and inhibitor control (inhibitor-ctrl). miR-10a expression levels in transfected cells were evaluated by qRT-PCR. (E) At 48 h after transfection, qRT-PCR and (F) Western-blot analyses were used to evaluate the expression level of PAI-1 in HFs (+miR-10a mimic) and NFs (+miR-10a inhibitor). All values are normalized to either the mimic control (mimic-ctrl) or inhibitor control (inhibitor-ctrl).

upregulated MMP1 expression in HFs. However, there was no significant change in MMP1 expression in the transfected NFs (S.3A–D).

3.6. The decreased expression of Col1 could be rescued by uPA inhibition or PAI-1 overexpression in HFs

As shown in Fig. 6A and B, the up-regulated mRNA and protein expression of uPA induced by miR-181c-inhibitors could be significantly inhibited by siRNA-uPA transfection, leading to a decreased MMP1 and increased Col1 production (Fig. 6C and D). Since PAI-1 expression was down-regulated in HFs by transfection of miR-10a mimics (Fig. 6E and F), our study showed that reintroduction of PAI-1 using a eukaryotic expression vector, significantly restored the level of PAI-1 in those cells and induced a significant decrease in MMP1 and increase in Col1 levels in the culture media (Fig. 6G and H).

4. Discussion

Collagen metabolism disorder after skin damage is the major pathogenesis in the production of HS [22]. PAI-1 expression has been shown to be remarkably increased in the process of wound healing [23,24], which may accelerate wound repair. However, the persistent overexpression of PAI-1 will cause collagen metabolism impairment, promoting collagen deposition and leading to various organ fibrosis. The role of cytokines, especially PAI-1, in the pathogenesis of keloid scars has been reported [25]. Meanwhile, the differential expression of uPA and PAI-1 was found at the edge of wounds in mice [26]. The changes in the expression of those genes may be involved in the migration of keratinocytes and connective tissue cells during re-epithelialization and tissue remodeling in wound healing. However, the wounded mouse is not capable of developing HS at the end stage of wound healing. In addition, HS can only happen after the wound is closed [20].

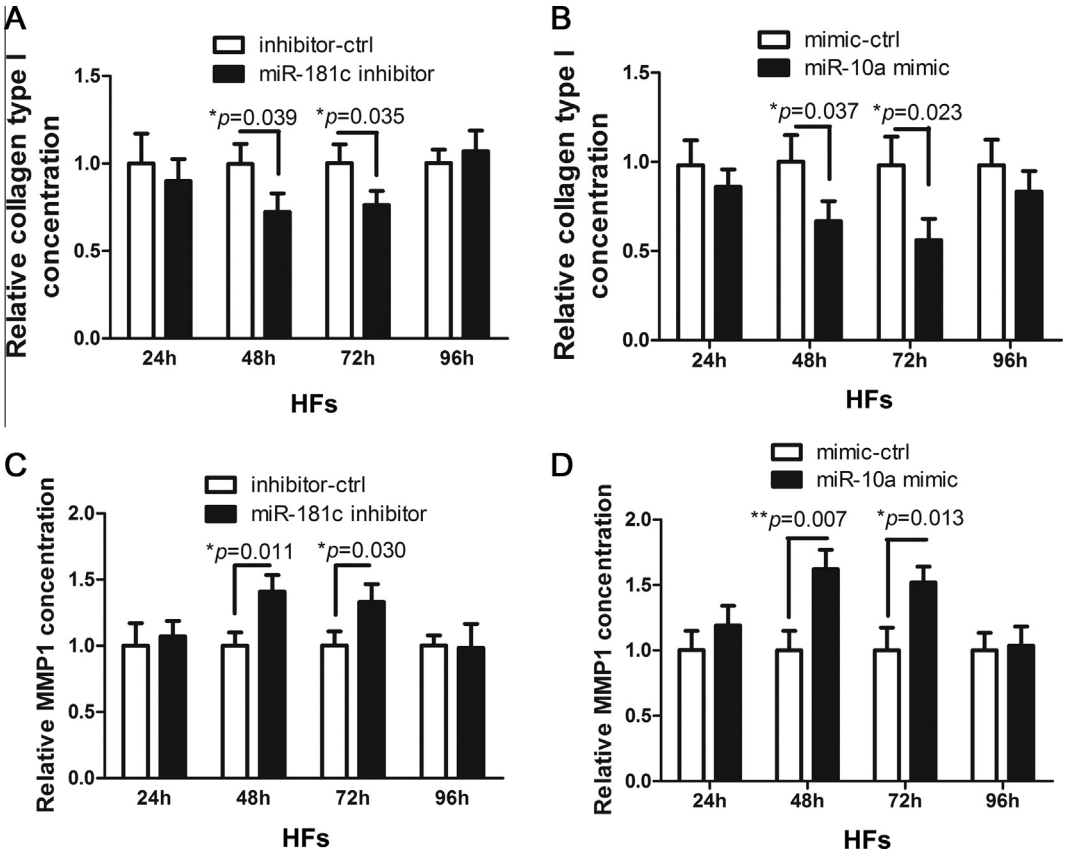

Fig. 5. Effect of miR-181c and miR-10a on Col1 and MMP1 expression in HFs. At 48 h after transfection of miR-181c inhibitors, (A) the decreased amount of collagen type I (Col1) and (C) increased amount of MMP1 was detected in the supernatant of cultured HFs as compared to inhibitor-ctrl. (B) Transfection of miR-10a mimics significantly inhibited Col1 expression at 48 and 72 h after transfection. (D) In contrast, the amount of MMP1 was increased after transfection of miR-10a mimics, as compared to mimic-ctrl. The concentration of Col1 and MMP1 in conditioned medium was measured by ELISA every 24 h after transfection. Bars represent the mean ± S.D. ($^{*}P < 0.05$, $^{**}P < 0.01$, $n = 3$).

Although differentially expressed uPA and PAI-1 are found in either wound healing or HS, the changes in the expression of those genes may be involved in different biological and pathological processes. Therefore, our findings might have novelty that will lead to a better understanding of the development of HS. In our study, we investigated and found that PAI-1 expression was up-regulated in the primary fibroblasts isolated from HS, as compared to that in NFs. Furthermore, we also revealed that re-introduction of PAI-1 into HFs indeed increased the expression of Col1, accompanied with decreased MMP1, which is consistent with previous reports about the role of PAI-1 in collagen synthesis of keloid [23,27]. It is anticipated that an increased PAI-1 expression in HFs could be one of the causes that contributes to the pathogenesis of HS, which might be considered a promising target for the treatment of skin fibrosis.

uPA, a target molecule of PAI-1, was previously reported to be involved in the protein degradation of the basement membrane; up-regulated uPA expression causes ECM degradation of tissues surrounding tumors, thereby promoting tumor metastasis [6]. Although little is known about the anti-fibrosis function of uPA, some studies revealed that overexpression of uPA is involved in collagen degradation, alleviating liver fibrosis [28], and that uPA can accelerate the invasion of retinal pigment epithelial cells through collagen gels [29]. In terms of idiopathic pulmonary fibrosis, Gharaee-Kermani et al. suggested that an approach to target uPA may be a promising new therapeutic strategy [30]. On the other hand, the pro-fibrosis function of uPA was found in cardiac fibrosis, for example, macrophage-derived uPA increases collagen level following myocardial ischemia [31]. The above information indicates that either the pro-fibrosis or anti-fibrosis functions of uPA depend on tissue specificity. In our study, we found decreased expression of uPA in HFs, as compared to that in NFs. Moreover, we revealed that the anti-fibrosis function of uPA is predominant in HS, because transfection of HFs with siRNA-uPA can promote the production of Col1, accompanied with decreased MMP1, implicating that a decreased uPA expression in HFs may be associated with the pathogenesis of HS.

Aberrant expression of miRNAs is involved in wound healing and underlying mechanisms are related to posttranscriptional regulation of the miRNA-targeting gene expression [32,33]. Therefore, targeting a specific mRNA that is aberrantly expressed and linked to a particular disease may offer clinicians new therapeutic options [34]. Our study showed that either miR-181c or miR-10a was differentially expressed in HFs, as compared to the paired NFs derived from the same person. Meanwhile, we also found that up-regulated miR-181c or down-regulated miR-10a expression in HFs was inversely correlated with the level of uPA or PAI-1, respectively. The relationships between miR-181c and uPA as well as miR-10a and PAI-1 were examined by computational analysis and luciferase reporter assay using reporter plasmids containing wild-type or mutant 3′-UTR of uPA and PAI-1. In addition, the methods for overexpression or inhibition of these two miRNAs in HFs or NFs were also used to verify the computational prediction and results of reporter assay. All of our results supported our hypothesis that uPA and PAI-1 genes are direct targets of miR-181c and miR-10a, and those two target genes are regulated by differentially expressed miR-181c and miR-10a in HS.

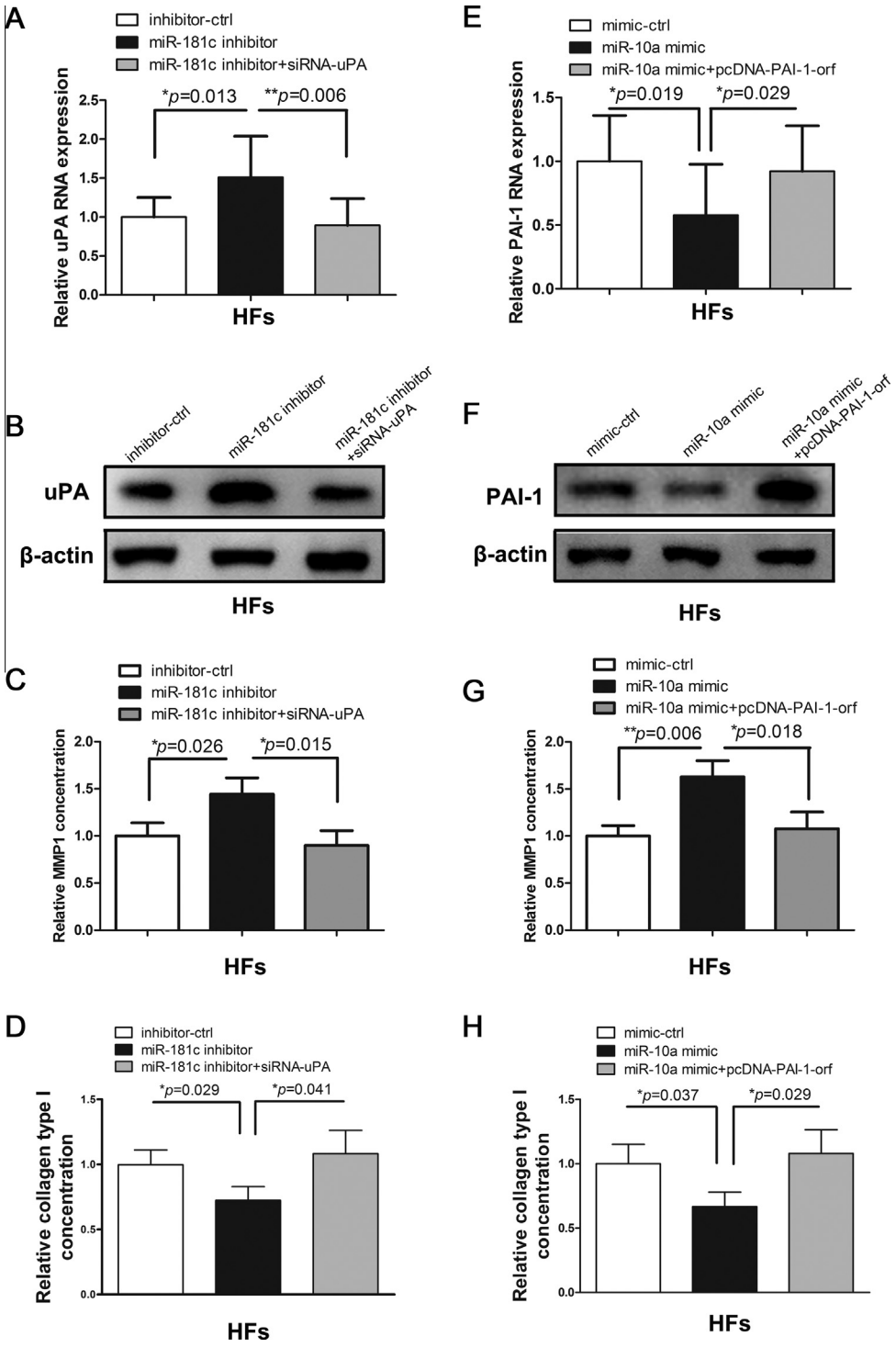

Fig. 6. Inhibition of uPA or overexpression of PAI attenuated the effect of miRNAs (miR-181c or miR-10a) on Col1 expression. siRNA-uPA transfection restored the expression level of uPA mRNA (A) and protein (B), which had been upregulated by miR-181c inhibitors. (C) siRNA-uPA transfection restored the level of MMP1 in HFs, which had been up-regulated by miR-181c inhibitors. (D) siRNA-uPA transfection also restored the level of Col1 in HFs, which had been reduced by miR-181c inhibitors. Transfection of pcDNA-PAI-1-orf restored PAI-1 mRNA (E) and protein (F) expression that had been suppressed by miR-10a mimic. (G) Overexpression of PAI-1 in HFs transfected with miR-10a mimic rescued the increased MMP1 expression induced by miR-10a mimics. (H) Overexpression of PAI-1 in HFs transfected with miR-10a mimic restored the decreased level of Col1 caused by miR-10a mimics. The concentration of MMP1 and Col1in conditioned medium was measured by ELISA at 48 h after transfection. Bars represent the mean ± S.D. ($^{*}P < 0.05$, $^{**}P < 0.01$, $n = 3$).

It is required to breakdown ECM for cell migration and cancer metastasis [35]. On the other hand, the inhibition of miR-181c expression could increase cell migration, and invasion in neuroblastoma cells [36], whereas overexpression of miR-10a enhanced the metastatic potential of many cancer cells [37,38]. These results support our findings that miR-181c inhibition and miR-10a overexpression may downregulate Col1 production. It was previously reported that Col1 production was induced by overexpression of PAI-1 [27]. Similarly, we found that ectopic expression of miR-10a could inhibit PAI-1 expression and up-regulate MMP1 expression,

thereby reducing the production of Col1 in HFs. miR-181c inhibition led to up-regulation of uPA and MMP1 expression in HFs, resulting in a decrease in Col1 production. Although Col1 production was decreased by only 20% in miR-181c inhibitors-treated HFs, it is acceptable because the effect of miR-181c inhibitors on uPA expression might be limited and ECM degradation involves multiple targets of uPA, except for Col1. In contrast, although miR-181c mimics can decrease uPA expression and miR-10a inhibitors can induce PAI-1 expression in NFs, those changes did not show much effect on Col1 production (S2.A-D). Moreover, MMP1 as the Col1 regulatory protease was also not affected by the transfection of miRNAs mimics and inhibitors. It seems that the expression of MMP1 cannot be regulated by miR-181c or miR-10a under normal conditions. These contradictory results between HFs and NFs are not unique in our experiments; the phenomenon was also observed in a study reported by Aoki et al. [39]. In their study, knockdown of the tissue inhibitor ofMMP1 in NFs using siRNA did not lead to degradation of Col1 or decreased cell viability, as compared to the efficacy of the same siRNA in keloid fibroblasts. These findings indicated that there might be a crucial difference between HFs and NFs, and it may implicate that these altered expression of PAI-1 or uPA has less effect on skin fibroblasts under normal condition.

In terms of gene therapy, miRNAs have the potential to treat fibrotic diseases by taking advantage of small molecules and negative regulation of specific target genes. Recently, several miRNAs were found to be involved in pro-fibrogenic pathways [40,41]. Previous studies also revealed that miR-29 family members and miR-101 exert their anti-fibrotic functions through the inhibition of the transforming growth factor-β (TGF-β) pathway [42,43], which is usually thought to be a pro-fibrotic pathway [44]. We found that up-regulated miR-181c inhibited uPA expression in HS tissues. Our findings are consistent with other study, in which miR-181c was also found to inhibit Smad7 expression in neuroblastoma cells [36]. These results suggest that miR-181c may exert its pro-fibrotic functions through activation of TGFβ/Smad pathway, because smad7 plays a suppressive role in the TGF-β pathway. Collectively, all of these data indicate that the regulation of miRNA expression by manipulating the intracellular levels of specific miRNA could be a potential treatment strategy for HS. Although a smaller sample size in this study is acceptable, it is necessary to increase the number of patients enrolled in our future studies [40]. In addition, as to the enhanced secretion of total MMP1 within HFs after treatment, it is unknown whether it this resulted from a functional change or aberrant behavior; therefore, this needs to be further clarified. Finally, to test our hypothesis about the roles of two miRNAs in HS, the therapeutic effects of miR-181c inhibitor and miR-10a mimic on scar reduction were investigated through in vitro assays using cultured HFs. However, in future studies, methods using either HS organ culture or an HS animal model should be considered [45].

In summary, the correlation between differentially expressed miRNAs and the aberrant expression of genes related to the production of pro-fibrotic cytokine production was investigated in our study. Two particular molecular series were characterized, including the pathways of miR-181c/uPA and miR-10a/PAI-1. Those pathways are partially responsible for fibroblast-mediated collagen deposition. Our data suggest that miR-181c and miR-10a play a role in the regulation of uPA as well as PAI-1 expression, respectively, in HFs, and changes in these two miRNAs contribute to significant alteration in Col1 secretion in HS.

Conflict of interest

The authors have no conflicts of interest to declare.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.12.024.

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