

MicroRNA-378b regulates α -1-type 1 collagen expression via sirtuin 6 interference

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Abstract. Ultraviolet (UV) light mediates skin aging and induces destruction of the dermis by modulating the expression levels of extracellular matrix-associated genes, including collagen and matrix metalloproteinases. Sirtuin 6 (SIRT6), a member of the sirtuin family of proteins, regulates collagen metabolism and is an established anti-aging protein. However, the exact underlying mechanism by which SIRT6 expression is regulated in dermal fibroblasts during the aging process is unclear. The present study demonstrated that expression of microRNA-378b (miR-378b) is induced in UVB-exposed human dermal fibroblasts (HDFs), and this was inversely associated with the mRNA expression levels of α -1-type 1 collagen (*COL1A1*). In addition, knockdown of miR-378b enhanced the mRNA expression levels of *COL1A1* in HDFs. A target analysis for miR-378b was performed, and the results revealed that SIRT6, a regulator of *COL1A1*, contains a target sequence for miR-378b in its 3'untranslated region. Notably, the present study demonstrated that an miR-378b mimic and inhibitor may directly regulate SIRT6 expression in HDFs. In conclusion, the present study suggested that miR-378b represses the mRNA expression levels of *COL1A1* via interference with SIRT6 in HDFs, and may contribute to the underlying molecular mechanism by which UVB inhibits collagen I in dermal fibroblasts.

Introduction

Skin aging is a biological process that, as for aging of other organs, is mediated by intrinsic and extrinsic factors. Features of skin aging include wrinkling, reduced elasticity, sagging,

laxity, dullness, roughness and discoloration. Deep wrinkles, in particular, are a primary consequence of ultraviolet (UV)-mediated skin aging (1-3). Histological studies have revealed that a reduction in collagen is one of the primary features of UV-mediated skin aging (1-5). This occurs via degradation of collagen and the collagen matrix, in addition to factors that interfere with their synthesis (3,6). Underlying this process are molecular mechanisms that modulate expression of collagen, matrix metalloproteinases and associated proteins (7).

The sirtuin (SIRT) family is composed of nicotinamide adenine dinucleotide-dependent protein deacetylases, which regulate aging in yeast, worms, flies and mammals (8,9). In humans, seven *SIRT* genes (*SIRT1-7*) have been identified, and these serve roles in numerous age-associated diseases, including cancer, neurodegenerative diseases, diabetes and cardiovascular disease (9-12). In mice, *SIRT1* deficiency induces destruction of the skin barrier and enhances UV-induced injury and sensitivity (10-12). Additionally, *SIRT1* may promote collagen expression in human smooth muscle cells by the deacetylation of regulatory factor X5 and alleviation of α -1-type 2 collagen (*COL1A2*) repression, and has been implicated in the inhibition of the transforming growth factor- β /mothers against decapentaplegic signaling pathway in fibroblasts during fibrosis (13-15). In mice, a deficiency in *SIRT6* led to a skin aging phenotype (16), and the expression levels of collagen 1 and 3 are reduced by knockdown of *SIRT6* in dermal fibroblasts (16). Therefore, regulation of *SIRT1* and *SIRT6* is associated with skin aging.

MicroRNAs (miRNAs) are small non-coding RNAs that are primarily generated by RNA polymerases II and III, and are processed and matured by the proteins Drosha and Dicer (17,18). These miRNAs consist of 20-24 nucleotides and associate with the miRNA-induced silencing complex. This complex targets the 3' untranslated region (3'UTR) of specific target genes, which interferes with translation and therefore down-regulates protein expression (19,20). miRNAs serve a role in numerous biological processes, including development, differentiation, the cell cycle, apoptosis, stemness and tumorigenesis (21-25).

In the present study, a novel miRNA that regulates *SIRT6* was identified that may regulate collagen expression.

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Materials and methods

Cell culture. Human dermal fibroblasts (HDFs) were purchased from Lonza (Basel, Switzerland) and cultured in Dulbecco's modified Eagle's medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences) and 1% penicillin and streptomycin. Cells were transfected with 100 nM miRNA (miR)-378b mimic (5'-ACUGGACUUGGAGGCAGAA-3'; Bioneer Corporation, Dajeon, Korea), 100 nM anti-miR-378b (5'-UUCUGCCUCCAAUCCUGU-3'; Bioneer Corporation) or 100 nM scrambled control (AccuTarget™ Negative Control siRNA; Bioneer Corporation) using Lipofectamine® RNAiMAX Transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. After transfection for 4 h, cells were washed with PBS and incubated for 24 h. Transfected HDFs were subsequently exposed to UVB using a Super light-VI UV illuminator (Boteck, Gunpo, Korea). After incubation for 24 h following UVB exposure, reverse transcription-quantitative polymerase chain reaction (RT-qPCR), western blotting and luciferase assay were performed.

RNA extraction and RT-qPCR. Total RNA was isolated from cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA was synthesized using 2 µg RNA with the miScript II RT kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol. Following cDNA synthesis, the mRNA expression levels of *COL1A1* and the gene encoding β-actin were detected using the StepOnePlus Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.), using EvaGreen™ premix (Solis BioDyne, Tartu, Estonia) and the following specific primers: Forward, 5'-AGGGCCAAGACATC-3' and reverse, 5'-AGATCACGTCATCGCACAACA-3' for human *COL1A1*; and forward, 5'-GGATTCCTATGTGGGCGACGA-3' and reverse, 5'-CGCTCGGTGAGGATCTTCATG-3' for human β-actin. Expression levels of miR-378b were detected with miR-378b specific primers (Qiagen GmbH) using the hsa-mir-378b miScript Primer assay (cat. no. MI0014154) and Hs_RNU6-2_11 miScript Primer assay (cat. no. MS00033740) from Qiagen GmbH, and the miScript SYBR® Green PCR kit (Qiagen GmbH) with the StepOnePlus Real-Time PCR system. Forward primers were included in the miScript Primer assay kits and reverse primers were included in the miScript SYBR® Green PCR kit. The expression levels of *COL1A1* and miR-378b were normalized to β-actin and U6 respectively, using the $2^{-\Delta\Delta C_q}$ method (26). All RT-qPCRs were performed as follows: Initialization step at 94°C for 5 min, followed by 40 cycles (denaturing, 94°C for 30 sec; annealing, 60°C for 30 sec; polymerization, 72°C for 30 sec) and a final elongation step at 72°C, for 5 min. All experiments were repeated three times. Data was analyzed with Excel 2016 (Microsoft Corporation, Redmond, WA, USA) and presented as the mean value of viable cells ± standard deviation.

Target prediction and identification of miR-378b. Predicted targets of miR-378b were identified using the bioinformatic analysis tool, microRNA.org (www.microRNA.org). A luciferase reporter construct containing the predicted target sequence of

miR-378b in the *SIRT6* 3'UTR was generated by ligating a region (+1,312 to +1,329) of the human *SIRT6* gene into the *Xba*I restriction site, downstream of the luciferase gene in the pGL3 vector (Promega Corporation, Madison, WI, USA). HDFs were subsequently transfected with the 1 µg reporter assay vector and the 0.2 µg pSV-β-galactosidase control plasmid (Promega Corporation), with or without an miR-378b mimic or anti-miR-378b, using Lipofectamine RNAiMAX Transfection reagent (Invitrogen; Thermo Fisher Scientific) for 4 h, and subsequently incubated at 37°C for 24 h. After incubation for 24 h, luciferase and β-galactosidase assays were performed using Luciferase Assay Reagent (Promega Corporation) and the β-galactosidase Detection kit II (Clontech Laboratories, Inc., Mountainview, CA, USA), according to the manufacturer's protocol. Luciferase results were normalized using β-galactosidase activity.

Western blot analysis. Cells were lysed in radioimmunoprecipitation assay buffer, containing 1% NP-40, 150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and complete protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). Extracted proteins (20 µg) were loaded onto 12% gels and separated by electrophoresis. Proteins were subsequently transferred onto nitrocellulose membranes (EMD Millipore, Billerica, MA, USA) and blocked with blocking buffer [(5% skim milk in TBS-Tween-20 buffer (50 mM Tris, 150 mM NaCl, 0.1% Tween 20)] at 25°C for 1 h. Protein expression levels of SIRT6 and β-actin were detected using rabbit anti-SIRT6 (1:2,000; D8D12; cat. no. 12486; Cell Signaling Technology, Inc., Danvers, MA, USA) anti-β-actin (1:10,000; N-21; cat. no. sc-130656; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) primary antibodies, followed by anti-mouse IgG horseradish peroxidase (HRP)-conjugated (1:5,000; cat. no. 7076; Cell Signaling Technology, Inc.) and anti-rabbit IgG HRP-conjugated antibody (1:3,000; cat. no. 7074; Cell Signaling Technology, Inc.) secondary antibodies. Membranes were incubated with primary antibody at 25°C for 4 h, followed by incubation with secondary antibody at 25°C for 1 h. Proteins were visualized using SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.). The intensity of each band was measured using ImageJ software Version 1.50 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Statistical significance was calculated using one-way analysis of variance with Tukey's post-hoc test. $P < 0.05$ was considered to indicate a statistically significant difference using Excel 2016 (Microsoft Corporation, Redmond, WA, USA). Data are presented as the mean ± standard error.

Results and discussion

UVB exposure enhances miR-378b and reduces *COL1A1* expression levels in HDFs. The expression levels of various miRNAs alter during skin aging and in UVB-exposed cells (27). Therefore, to determine whether miR-378b is modulated by UVB, miR-378b expression was measured by RT-qPCR in UVB-exposed HDFs. miR-378b expression levels were significantly enhanced in cells exposed to 5–25 mJ/cm² UVB in a dose-dependent manner, compared to untreated cells (0 mJ/cm² UVB; $P < 0.05$; Fig. 1A). In addition, in HDFs exposed to the

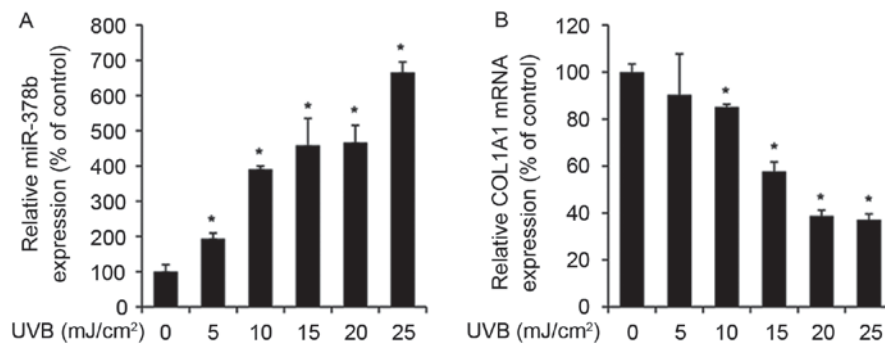


Figure 1. UVB treatment enhances miR-378b and reduces *COL1A1* mRNA expression levels in HDFs. HDFs were exposed to various doses of UVB and reverse transcription-quantitative polymerase chain reaction was performed following a 24-h incubation. Expression levels of (A) miR-378b and (B) *COL1A1*. Data are expressed as the mean \pm standard error of three independent experiments, as percentage of the untreated control. * $P < 0.05$ vs. untreated cells (0 mJ/cm²). UVB, ultraviolet B; miR-378b, microRNA-378b; *COL1A1*, α -1-type 1 collagen; HDFs, human dermal fibroblasts.

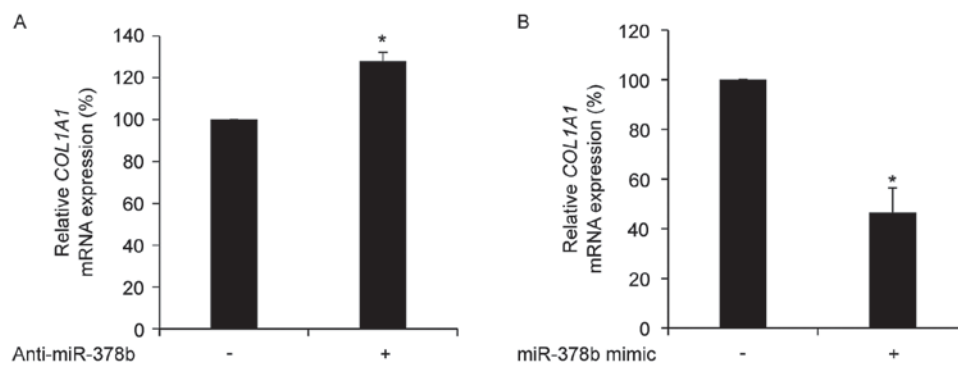


Figure 2. *COL1A1* is negatively regulated by miR-378b in HDFs. HDFs were transfected with (A) anti-miR-378b or (B) an miR-378b mimic, and reverse transcription-quantitative polymerase chain reaction was performed to detect the mRNA expression levels of *COL1A1*. Data are expressed as the mean \pm standard error of three independent experiments, as a percentage of the scramble control-transfected cells. * $P < 0.05$ vs. scramble control. *COL1A1*, α -1-type 1 collagen; miR-378b, microRNA-378b; HDFs, human dermal fibroblasts.

same doses of UVB, expression levels of *COL1A1* were reduced in a dose-dependent manner compared with untreated cells, and were inversely associated with miR-378b expression levels (Fig. 1B).

COL1A1 is negatively regulated by miR-378b in HDFs. To determine whether the expression of *COL1A1* is directly affected by miR-378b, the mRNA expression levels of *COL1A1* in HDFs treated with anti-miR-378b or an miR-378b mimic were measured. Transfection with anti-miR-378b significantly up-regulated *COL1A1* mRNA expression levels ($P < 0.05$; Fig. 2A), whereas transfection with an miR-378b mimic resulted in a significant reduction in *COL1A1* mRNA expression levels compared with cells transfected with a scramble control ($P < 0.05$; Fig. 2B). However, the 3'UTR of *COL1A1* does not contain a predicted binding site for miR-378b. In addition, the miR-378b mimic did not directly modulate the luciferase activity of a luciferase-*COL1A1* 3'UTR fusion construct in HDFs (data not shown). Therefore, a target prediction for miR-378b was performed using microRNA.org, which revealed that *SIRT6* contained a binding site for the miR-378b seed sequence in its 3'UTR. *SIRT6* has been identified as an anti-aging protein due to its ability to regulate the mRNA expression levels of *COL1A1* and *COL3A1* in HDFs (16). Therefore, it was hypothesized that miR-378b may directly regulate *SIRT6* expression.

miR-378b negatively regulates SIRT6 by binding to its 3'UTR. The majority of miRNAs regulate target genes by binding to their 3'UTRs (28,29). It was therefore determined whether the miR-378b mimic directly interacted with the *SIRT6* 3'UTR, by measuring the luciferase activity from a luciferase-*SIRT6* 3'UTR fusion construct. It was determined, using microRNA.org, that the seed sequence from miR-378b matched a region between +1,312 and +1,324 bp of the *SIRT6* 3'UTR (Fig. 3A). The *SIRT6* 3'UTR was subsequently cloned into a luciferase plasmid and transfected into HDFs. Co-transfection of an miR-378b mimic significantly reduced luciferase activity compared with pGL3-*SIRT6*-3'UTR only ($P < 0.05$; Fig. 3B), whereas co-transfection with an miR-378b mimic and anti-miR-378b significantly enhanced luciferase activity compared with cells transfected with *SIRT6* 3'UTR and an miR-378b mimic ($P < 0.05$; Fig. 3B). Thus, these results suggested that miR-378b directly binds to, and interferes with, *SIRT6* mRNA.

miR-378b represses endogenous SIRT6 expression in HDFs. It was subsequently determined whether the miR-378b mimic affected endogenous *SIRT6* protein expression by western blot analysis. Overexpression of miR-378b reduced the expression levels of *SIRT6* (Fig. 4A). Transfection with anti-miR-378b had the reverse effect and enhanced *SIRT6*

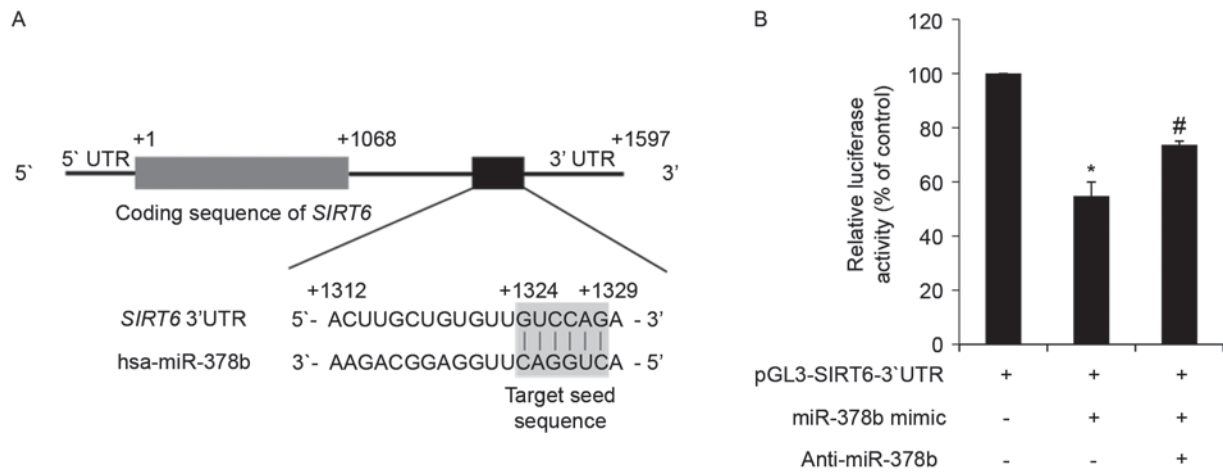


Figure 3. miR-378b directly targets *SIRT6* mRNA and represses its translation. (A) *In silico* analysis of *SIRT6* mRNA and the predicted target sequence of miR-378b. The ATG start codon is indicated by +1, and the 3' end of the *SIRT6* coding region is indicated by +1,068. The region of the 3'UTR containing the miR-378b recognition sequences is located from +1,312 to +1,329 in the *SIRT6* transcript. (B) HDFs were transfected with a reporter construct containing the miR-378b recognition sequences from *SIRT6* fused to luciferase, and pSV- β -galactosidase, which was the control vector for normalization. Additional groups were co-transfected with an miR-378b mimic and anti-miR-378b. Following 24 h transfection, cells were harvested and luciferase assays were performed. Data are expressed as the mean \pm standard error of three independent experiments, as a percentage of the pGL3-*SIRT6*-3'UTR-transfected group. * $P < 0.05$ vs. pGL3-*SIRT6*-3'UTR only-transfected group. # $P < 0.05$ vs. pGL3-*SIRT6*-3'UTR and miR-378b mimic co-transfected group. miR-378b, microRNA-378b; *SIRT6*, sirtuin 6; UTR, untranslated region; HDFs, human dermal fibroblasts.

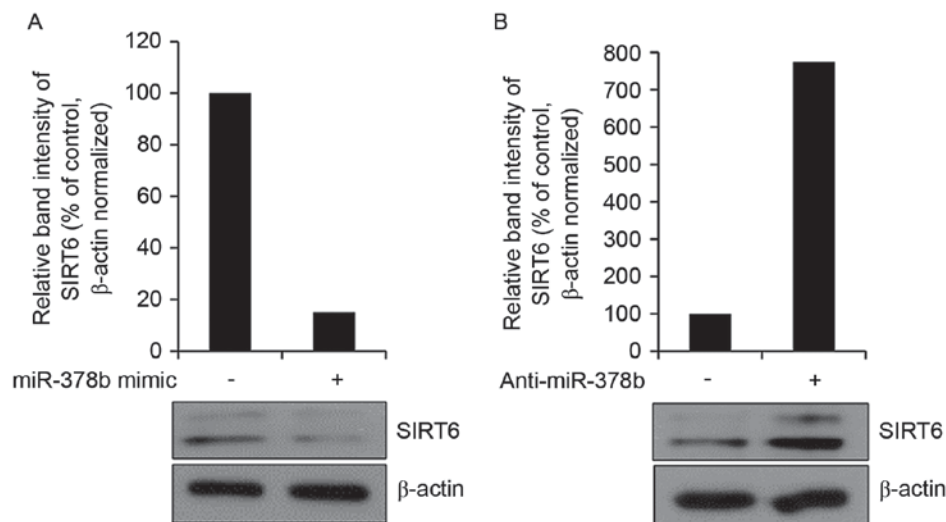


Figure 4. miR-378b downregulates protein expression levels of SIRT6 in HDFs. HDFs were transfected with (A) an miR-378b mimic or (B) anti-miR-378b, and western blot analysis was performed to detect SIRT6 protein expression levels. Image J analysis software was utilized for quantification of SIRT6 western blot band density, which was normalized to the β -actin loading control. Representative results from triplicate experiments are presented. miR-378b, microRNA-378b; SIRT6, Sirtuin 6; HDFs, human dermal fibroblasts.

protein expression levels (Fig. 4B). These results suggested that miR-378b may inhibit *COL1A1* mRNA expression via effects on *SIRT6*. In addition, a previous study demonstrated that SIRT6 regulates the expression of genes associated with stress and aging (30). In HDFs from younger individuals, SIRT6 was highly activated compared with HDFs from older individuals (31). Therefore, miR-378b, which was upregulated by UVB in the present study (Fig. 1A), may regulate aging and collagen expression via interference of translation of *SIRT6* mRNA. Investigation of the association between miR-378b and skin aging in human skin samples is required in future studies to indicate whether miR-378b is a marker of photo-aging.

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