Arctiin regulates collagen type 1α chain 1 mRNA expression in human dermal fibroblasts via the miR-378b-SIRT6 axis

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Abstract. Arctiin, a lignin isolated from *Arctium lappa*, exhibits a variety of biological effects, including anti-viral, anti-inflammatory, and anti-proliferative actions, in mammalian cells. In a previous study, arctiin was demonstrated to induce procollagen type I synthesis and exhibited protective effects against ultraviolet B (UVB) radiation in normal human dermal fibroblasts (nHDFs). However, the underlying molecular mechanism of arctiin-mediated collagen synthesis remains unknown. In the present study, the mechanism for increased expression of collagen type 1α 1 chain (COL1A1) mRNA in arctiin-induced nHDFs was identified. The expression of microRNA-378b (miR-378b), downregulated by arctiin, was correlated with the expression of sirtuin-6 (SIRT6) mRNA, a regulator of COL1A1 mRNA. Furthermore, it was revealed that arctiin protected the UVB radiation-mediated decrease in COL1A1 mRNA expression, through the miR-378b/SIRT6 signaling pathway. In conclusion, these results suggest that arctiin regulates COL1A1 through the miR-378b-SIRT6 axis.

Introduction

The human skin surrounds the external surface of the human body and acts as a protector against environmental factors, including ultraviolet (UV) irradiation, destructive agents and viral infections (1,2). The skin consists of two layers, the epidermis and dermis. The epidermis forms an epidermal barrier through keratinocyte differentiation (3). The dermis consists of an extracellular matrix that is predominantly composed of type I collagen synthesized by fibroblasts (4-6). The skin loses these two functions as it ages, due to intrinsic aging and extrinsic factors (7). Intrinsic factor-induced skin aging is a natural skin aging process, caused by the loss of function of age-associated genes due to heredity or the passage of time (8). Extrinsic factor-induced skin aging is aging or photoaging, caused by environmental conditions, predominantly from exposure to UV radiation (9). UV-induced oxidative stress is accelerated by deep wrinkles caused by the loss of collagen production (10,11). Therefore, an endogenous anti-oxidant defense system is required for protection against the effects of photodamage, which contribute to skin aging (12).

Arctiin, a lignin compound isolated from *Arctium lappa*, also known as greater burdock, possesses a variety of anti-viral, anti-inflammatory and anti-proliferative effects in mammalian cells (13-15). Arctiin has recently been demonstrated to improve procollagen type I synthesis and exhibits a protective effect against ultraviolet B (UVB) radiation (16,17). An extract of *A. lappa* or isolated arctiin induces collagen synthesis in the dermis (18). However, the underlying mechanism of collagen synthesis, induced by arctiin treatment, remains unknown.

MicroRNAs (miRNAs) are small non-coding RNAs that function as important regulators of gene expression in skin aging and differentiation (19-22). These miRNAs are 16-35 nucleotides (nts) in length (median, 21-22 nts) that interact with the 3' untranslated region (3' UTR) of target messenger RNAs (mRNAs). Consequently, the complementary interaction of miRNA and its target mRNA inhibits protein translation at the post-transcriptional level (23,24). In skin aging, these miRNAs serve a key role by targeting skin aging-associated gene expression and UV protective-associated gene expression in the dermis (25-27). A previous study revealed that miRNAs serve an important role in anti-aging functions and skin stress responses of skin-derived cells, including keratinocytes and dermal fibroblasts (28). Additionally, several miRNAs have been reported to regulate melanogenesis, skin aging, and differentiation of melanocytes, keratinocytes, and dermal fibroblasts (29,30).

In a previous study, the authors revealed that arctiin inhibited microRNA-378b (miR-378b) expression in UVB-irradiated normal human dermal fibroblasts (nHDFs) (16). Therefore, in the present study, it was investigated whether this alteration of

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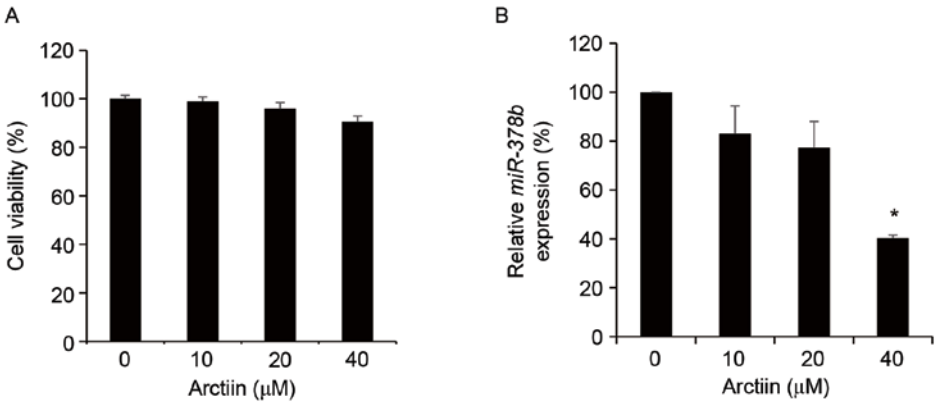

Figure 1. Arctiin decreases miR-378b expression in nHDFs. (A) Arctiin causes no significant cytotoxicity in nHDFs. Cells were seeded in 96-well culture plates and treated with the indicated doses of arctiin (0, 10, 20 or 40 μM). After 24 h of incubation, a water-soluble tetrazolium salt-based cytotoxicity assay was performed. Each bar represents the mean + standard deviation from three independent experiments. (B) Analysis of relative miR-378b expression in arctiin-treated nHDFs. Cells were treated with the indicated doses of arctiin (0, 10, 20, or 40 μM) for 24 h. The level of miR-378b expression was determined using the reverse transcription-quantitative polymerase chain reaction. Results were normalized to the U6 small nuclear RNA expression level. The data are representative of three independent experiments. *P<0.05 vs. control. miR-378b, microRNA-378b; nHDFs, normal human dermal fibroblasts.

miR-378b contributes to the enhanced procollagen synthesis induced by treatment with arctiin in nHDFs. A detailed mechanism by which arctiin stimulates collagen synthesis in nHDFs, by inhibiting miR-378b expression that, in turn, increases expression of its target sirtuin-6 (SIRT6) through reduced miRNA-mediated repression is demonstrated.

Materials and methods

Cell culture, chemicals, and reagents. nHDFs (Lonza Group, Ltd., Basel, Switzerland) were cultured in Dulbecco's Modified Eagle medium containing 10% fetal bovine serum (both HyClone; GE Healthcare Life Sciences, Logan, UT, USA) and antibiotics in a humidified incubator under 5% CO_2 at 37˚C. Arctiin was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and dissolved in dimethyl sulfoxide.

Arctiin treatment and UVB exposure. For arctiin treatment alone, nHDFs ($1x10^5$ cells in 60 mm culture dish and $3x10^3$ cells in 96-well plates) were incubated with arctiin at 37˚C for 24 h, and then a cell viability assay and reverse transcription-quantitative polymerase chain reaction were performed. At UVB exposure, nHDFs ($1x10^5$ cells in 60 mm culture dish and $3x10^3$ cells in 96-well plates) was pre-incubated with arctiin at 37˚C for 3 h, and then 30 mJ/cm^2 UVB was exposed to arctiin-pretreated nHDFs. After UVB exposure, the nHDFs were incubated at 37˚C for 24 h and then western blotting and RT-qPCR were performed.

Cell viability assay. The cytotoxic effects of arctiin on nHDFs were determined using a water-soluble tetrazolium salt (WST-1) assay (EZ-Cytox cell viability assay kit; ITS Bio, Seoul, Korea). nHDFs were seeded at a density of $3x10^3$ cells in 96-well plates and incubated for 24 h. The cells were then incubated with 0-40 μM arctiin for 24 h. The WST-1 assay solution was added to the cells at a 1/10 volume of the total culture medium and were incubated at 37˚C for 1 h. Cell viability was evaluated by measuring the absorbance at 450 nm, using an iMark microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

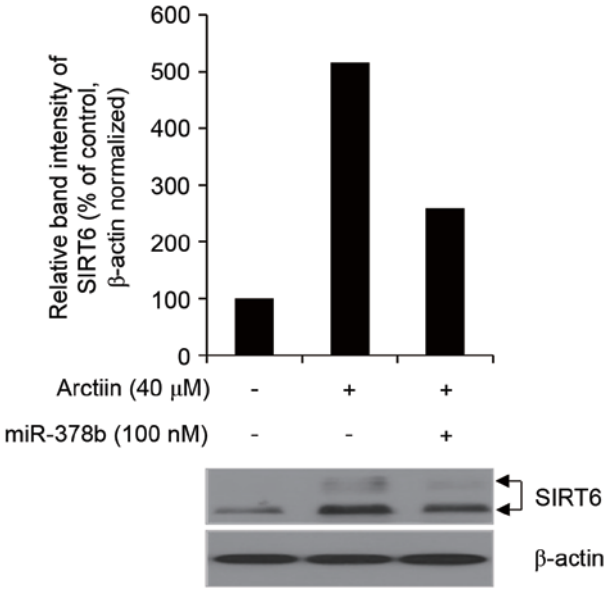

Figure 2. Arctiin increases SIRT6 protein expression in nHDFs through miR-378b. Western blot analysis of SIRT6 expression in nHDFs transfected with miR-378b or negative control miRNA and incubated in the presence or absence of arctiin (40 μM). After 24 h of incubation, the levels of SIRT6 proteins were evaluated by western blotting analysis. β-actin was used as an internal control. Sirtuin-6, SIRT6; nHDFs, normal human dermal fibroblasts; microRNA-378b, miR-378b.

Transfection of a miR-378b mimic and anti-miR-378b. An *miR-378b* mimic (100 nM; 5'-ACUGGACUUGGA GGCAGAA-3'), an anti-*miR-378b* (100 nM; 5'-ACUGGA CUUGGAGGCAGAA-3'), and 100 nM scrambled control (AccuTarget™ negative control siRNA; Bioneer Corporation, Daejeon, Korea) were purchased from Qiagen GmbH (Hilden, Germany). miRNAs were dissolved in nuclease-free water (USB Biochemicals; Affymetrix, Inc., Santa Clara, CA, USA). nHDFs were seeded at a density of $1x10^5$ cells in 60 mm culture dish and incubated for 24 h. Cells were transfected with the miRNA mimic, anti-miRNA of *miR-378b*, or the negative control using Lipofectamine RNAiMAX (Invitrogen; Thermo

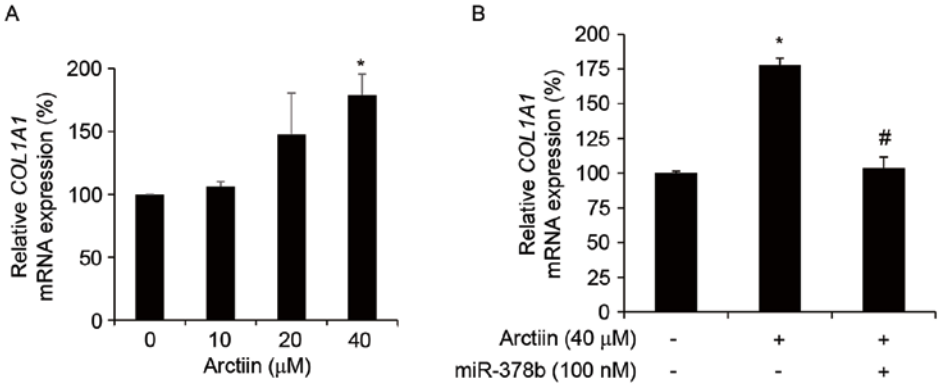

Figure 3. Arctiin increases COL1A1 mRNA expression in nHDFs through inhibition of miR-378b. (A) Analysis of relative COL1A1 mRNA expression in arctiin-treated nHDFs. Cells were treated with the indicated doses of arctiin (0, 10, 20, or 40 μM) for 24 h. The level of COL1A1 mRNA was determined by RT-qPCR. (B) Analysis of miR-378b-mediated COL1A1 mRNA expression in arctiin-treated nHDFs. After a 24-h incubation, the level of COL1A1 mRNA was determined by RT-qPCR. Values represent the mean + standard deviation of three independent experiments. *P<0.05 vs. control; #P<0.05 vs. arctiin-treated nHDFs. nHDFs, normal human dermal fibroblasts; COL1A1, collagen type 1α chain 1; miR-378b, microRNA-378b; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol.

RNA preparation and RT-qPCR. Total RNA was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The purity and concentration of the RNA samples were estimated using MaestroNano® (Maestrogen, Inc., Las Vegas, NV, USA). cDNA was synthesised from total RNA using the SuperScript™ III Reverse Transcriptase kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. To evaluate collagen type 1^{α} 1 chain (COL1A1) mRNA expression, qPCR was performed using COL1A1 primers (human COL1A1 forward: 5'-AGGGCCAAGACATC-3'; and reverse: 5'-AGATCACGTCATCGCACAACA-3') and EvaGreen premix (Solis BioDyne, Tartu, Estonia) with the StepOnePlus™ real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Expression was normalized to β-actin (human β-actin forward: 5'-GGATTCCTA TGTGGGCGACGA-3' and reverse: 5'-GCTCGGTGAGGA TCTTCATG-3'). Additionally, cDNAs for miR-378b detection were synthesized using the miScript II RT Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. qPCR was performed using a miR-378b-specific primer, Hs_ miR-1290_1 miScript Primer Assay (Qiagen Inc., Valencia, CA, USA), and the miScript SYBR-Green PCR Kit (Qiagen Inc., Valencia, CA, USA) with the StepOnePlus™ real-time PCR system. MiR-378b expression was normalized to U6 small nuclear RNA. The $2^{-\Delta\Delta Cq}$ method was used to calculate the relative expression level of COL1A1 and miR-378b (31). Cycling conditions were as follows: An initial predenaturation step at 94˚C for 5 min, followed by 40 cycles of denaturation at 94˚C for 30 sec, annealing at 60˚C for 30 sec, extension at 72˚C for 30 sec and a final extension step at 72˚C for 5 min. All experiments were repeated three times. Data was analysed using Microsoft Excel 2016 (Microsoft Corporation, Redmond, WA, USA) and presented as the mean ± standard deviation.

Western blot analysis. Cells were analysed using radioimmunoprecipitation assay buffer (50 mM Tris-Cl pH=8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitors (EASYpack; Roche Applied Science, Mannheim, Germany). The protein concentration was measured using the Bradford assay (Bio-Rad Laboratories, Inc.). A total of 50 μg cellular protein was dissolved in SDS sample buffer. Proteins were separated by SDS-PAGE on a 10% gel and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked in 5% skimmed milk buffer (in 50 mM Tris, 150 mM NaCl and 0.1% Tween 20) for 1 h at room temperature and were probed with rabbit anti-SIRT6 (1:2,000; catalog no. 12486; Cell Signalling Technology, Inc., Danvers, MA, USA) or anti-β-actin (1:10,000; N-21; catalog no. sc-130656; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) primary antibodies at 4˚C for 18 h. Subsequently, these proteins were

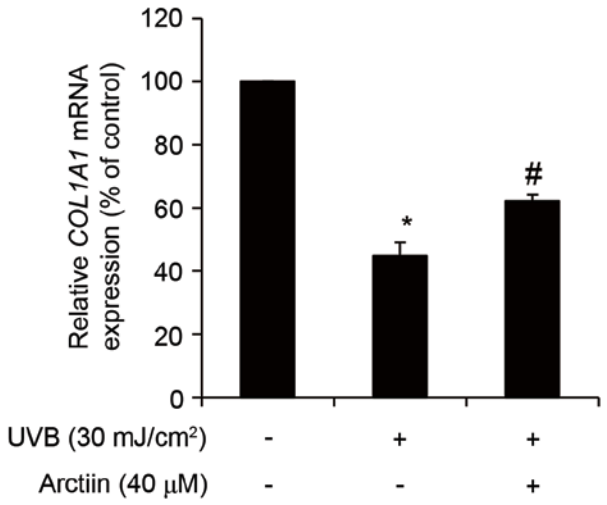

Figure 4. Arctiin attenuates UVB-mediated decrease in COL1A1 mRNA in nHDFs. nHDFs were pretreated with arctiin for 6 h and then exposed to 30 mJ/cm^2 UVB. After 24 h, COL1A1 mRNA expression was determined by the reverse transcription-polymerase chain reaction. Values represent the mean + standard deviation of three independent experiments. *P<0.05 vs. controls; #P<0.05 vs. UVB-treated nHDFs. UVB, ultraviolet B; COL1A1, collagen type 1α chain 1 chain; nHDFs, normal human dermal fibroblasts.

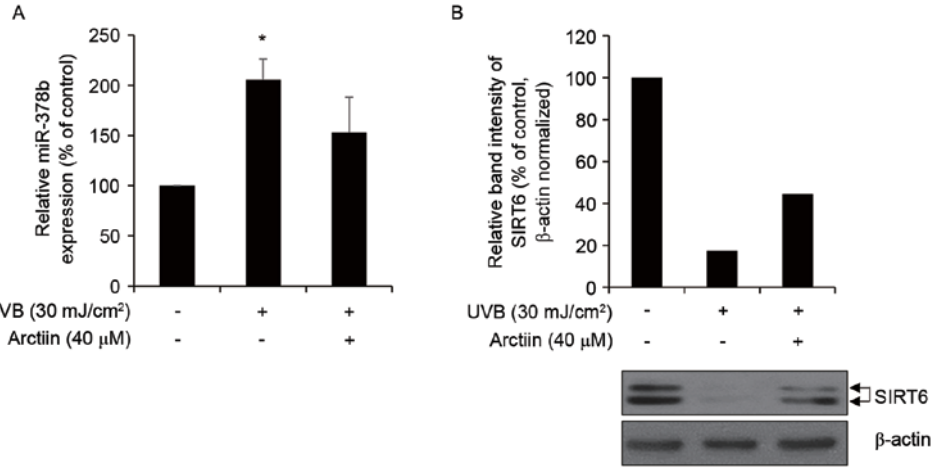

Figure 5. Arctiin regulates the miR-378b-SIRT6 axis in UVB-exposed nHDFs. (A) miR-378b expression was restored by arctiin in 30 mJ/cm^2 UVB-exposed nHDFs. Cells were treated with UVB radiation either following the exposure to arctiin or in its absence. The level of miR-378b expression was determined by the reverse transcription-polymerase chain reaction. Results were normalized to U6 small nuclear RNA expression level. The data are representative of three independent experiments. *P<0.05 vs. controls; $^\#$P<0.05 vs. UVB-treated nHDFs. (B) SIRT6 protein expression was restored by treatment with arctiin in UVB-exposed nHDFs. Cells were treated with UVB radiation following either the presence or the absence of arctiin. The level of SIRT6 was determined by western blotting. β-actin was used as a protein loading control. nHDFs, normal human dermal fibroblasts; miR-378b, microRNA-378b; UVB, ultraviolet B; SIRT6, sirtuin-6.

visualized using an anti-mouse IgG horseradish peroxidase (HRP) (1:5,000; catalog no. 7076; Cell Signalling Technology, Inc.) and anti-rabbit IgG HRP (1;3,000; catalog no. 7074; Cell Signalling Technology, Inc.) secondary antibodies at 25˚C for 1 h. A SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.) and a ChemiDoc Touch Imaging system (Bio-Rad Laboratories) were used to visualise protein bands.

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 Microsoft Excel 2016 (Microsoft Corporation). Data are presented as the mean ± standard error.

Results and Discussion

Arctiin reduces miR-378b expression in nHDFs. Arctiin has been reported to possess a variety of biological effects, including anti-viral, anti-inflammatory and anti-proliferative, in mammalian cells (32,33). Binic *et al* (34) revealed that arctiin induces collagen synthesis in nHDFs. These results have provided a novel insight into the anti-aging effect of arctiin on the skin. However, the underlying mechanism of collagen induction by treatment with arctiin remains unknown. In a previous study, it was revealed that arctiin markedly inhibited miR-378b in UVB-irradiated nHDFs (16). Therefore, in the present study, the miRNA-dependent mechanism with which COL1A1 is induced in arctiin-treated nHDFs was investigated.

Cell viability in nHDFs was not affected at a concentration of 0-40 μM arctiin (Fig. 1A). Furthermore, in nHDFs, miR-378b levels decreased by treatment with arctiin in a dose-dependent manner (Fig. 1B). Treatment with 40 μM arctiin significantly downregulated miR-378b by 40.34% compared with control cells (P<0.05). Therefore, in the subsequent experiments a dose of arctiin <40 μM was used.

Arctiin increases COL1A1 expression through the miR-378b-SIRT6 axis. Our ongoing study revealed that miR-378b repressed COL1A1 expression, interfering with SIRT6 expression (data unpublished). Therefore, it is hypothesized that *miR-378b* downregulates COL1A1 indirectly by interfering with the translation of *SIRT6* mRNA. Additionally, SIRT6 silencing is implicated in the regulation of COL1A1 expression and skin aging in human dermal fibroblasts (35). Treatment with 40 μM of arctiin increased SIRT6 protein expression in nHDFs in Fig. 2, demonstrating that arctiin regulates SIRT6 in nHDFs. Furthermore, *miR-378b* inhibited the arctiin-mediated increase in SIRT6 protein expression (Fig. 2). These results indicate that arctiin elevates SIRT6 protein expression indirectly, through inhibition of *miR-378b*. Furthermore, the arctiin-mediated increase of COL1A1 gene was antagonized by the addition of exogenous *miR-378b* (Fig. 3B). Arctiin regulates COL1A1 via the *miR-378b*-SIRT6 axis.

Arctiin protects against UVB-induced decrease in COL1A1 levels in nHDFs. Previous studies have indicated that arctiin has protective effects against UVB radiation in nHDFs (16). It was examined whether this protective effect of arctiin against UVB opposed the photoaging-dependent decrease in COL1A1 levels in nHDFs. nHDFs were pretreated with 40 μM arctiin for 6 h and then exposed to 100 mJ/cm^2 UVB radiation. Pre-treatment with arctiin alleviated the UVB-induced inhibitory effects on COL1A1 mRNA expression (Fig. 4). Furthermore, UVB treatment increased miR-378b expression and decreased SIRT6 protein expression in nHDFs (Fig. 5A and B). Conversely, arctiin treatment decreased miR-378b expression and increased SIRT6 in UVB-exposed nHDFs. These inverse correlations indicated that arctiin elevates COL1A1 transcription indirectly in UVB-exposed nHDFs through loss of miR-378b-mediated inhibition of SIRT6. Alteration of Col1A1 expression, by enhanced SIRT6, is induced by repression of NF-κB activation (35). In various aging mechanism, activation NF-κB signaling is a key

mediator of aging (36,37). In UVB-induced photoaging, NF-κB activity is implicated, since NF-κB is indirectly and directly regulated by collagens and matrix metalloproteinases (38). Therefore, the results suggest that miR-378b is useful in preventing aging through blocking of SIRT6.

In conclusion, the study demonstrated that the arctiin-induced upregulation of COL1A1 expression is regulated by the miR-378/SIRT6 signaling pathway. Furthermore, it was revealed that arctiin is able to prevent the UVB-induced reduction of COL1A1 expression, at least partially, through this same pathway.

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