

# Photoprotective effect of arctiin against ultraviolet B-induced damage in HaCaT keratinocytes is mediated by microRNA expression changes

HWA JUN CHA<sup>1</sup>, GHANG TAI LEE<sup>2,3</sup>, KWANG SIK LEE<sup>2,3</sup>, KUN KOOK LEE<sup>2</sup>,  
JIN TAE HONG<sup>3</sup>, NA KYEONG LEE<sup>1</sup>, SOO-YEON KIM<sup>1</sup>, BO MI LEE<sup>1</sup>, IN-SOOK AN<sup>1</sup>,  
HYUNG JIN HAHN<sup>4</sup>, KYU JOONG AHN<sup>4</sup>, SU-JAE LEE<sup>5</sup>, SUNGKWAN AN<sup>1</sup> and SEUNGHEE BAE<sup>1</sup>

<sup>1</sup>Korea Institute for Skin and Clinical Sciences and Molecular-Targeted Drug Research Center, Konkuk University, Seoul 143-701; <sup>2</sup>Songpa R&D Center, Coreana Cosmetics Co., Ltd., Cheonan, Chungcheongnam-do 330-833;

<sup>3</sup>College of Pharmacy and Medical Research Center, Chungbuk National University, Cheongju, Chungcheongbuk-do 361-763; <sup>4</sup>Department of Dermatology, Konkuk University School of Medicine, Seoul 143-701;

<sup>5</sup>Department of Chemistry, Hanyang University, Seoul 133-791, Republic of Korea

Received September 24, 2013; Accepted April 16, 2014

DOI: 10.3892/mmr.2014.2326

**Abstract.** Human keratinocytes are located in the outermost skin layer and thus particularly vulnerable to ultraviolet B (UVB) radiation exposure. Previous studies have focused on the cellular and molecular perspectives of UVB-induced keratinocyte damage. In the present study, it was demonstrated that pretreatment with the phytochemical arctiin, one of the lignin compounds, protects human HaCaT keratinocytes from UVB-mediated damage. Biochemical assays revealed that UVB-induced cytotoxicity and cell death were significantly reduced in arctiin-pretreated HaCaT cells. In addition, arctiin promoted the wound healing and DNA repair properties of keratinocytes. The photoprotective effects of arctiin were associated with changes in the expression levels of specific microRNAs (miRNAs) in HaCaT cells. A bioinformatics analysis demonstrated that the miRNAs were functionally involved in cancer, cell cycle, and Wnt and mitogen-activated protein kinase signaling pathways. In the present study, the results from the cellular and molecular assays demonstrated a novel role for arctiin in UVB protection in keratinocytes, which is mediated by miRNA responses and the suppression of UVB-induced cell death. Furthermore, arctiin is implicated as a potential chemopreventive agent through UVB protection of keratinocytes.

## Introduction

Ultraviolet (UV) radiation induces several harmful effects, including DNA damage, reactive oxygen species (ROS) generation, cell cycle arrest, tumorigenesis, immunosuppression and apoptosis in skin cells (1). Therefore, photoprotection against UV-induced damage has an important role in maintaining skin health. UV radiation is classified as UVA, UVB and UVC. Although UVC is the most cytotoxic radiation, it is almost completely blocked by the ozone layer (1). UVA is a longer-wavelength radiation and is able to penetrate deep into skin (1). UVB is a shorter-wavelength radiation that scarcely penetrates into the dermis layer, but is more cytotoxic than UVA (1). UVB is considered to cause direct damage to DNA and indirect damage through the generation of ROS (1). Keratinocytes form the outermost tissue layer of the body and are therefore constantly exposed to UV radiation. Numerous studies have reported deleterious effects of UVB radiation on keratinocytes, including senescence, inflammation, cell death and epithelial malignancy (2). At the molecular level, UVB affects diverse signaling pathways in keratinocytes. Mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK), p38 MAPK, and c-Jun N-terminal kinase (JNK), are among the major UVB response molecules in keratinocytes (2). UVB activates MAPKs to induce either apoptosis or inflammation (2). The nuclear factor kappa B (NF- $\kappa$ B) signaling pathway is also involved in UVB-mediated responses in keratinocytes (2). UVB-induced activation of NF- $\kappa$ B pathways induces the expression of several genes that regulate the cell cycle, apoptosis and inflammation (2).

Recently, microRNAs (miRNAs) have been revealed to have pivotal roles in differentiation, senescence, cell survival and apoptosis in keratinocytes. Rivetti *et al* (3) demonstrated that miRNA-p63 feedback is important for keratinocyte senescence. Hidebrand *et al* (4) analyzed miRNA expression during keratinocyte differentiation and identified a role of miRNAs

---

*Correspondence to:* Professor Seunghee Bae, Korea Institute for Skin and Clinical Sciences and Molecular-Targeted Drug Research Center, Konkuk University, 120 Neungdong-ro, Gwangjin-gu, Seoul 143-701, Republic of Korea  
E-mail: sbae@konkuk.ac.kr

**Key words:** photoprotection, keratinocyte, ultraviolet B, microRNA, arctiin

in skin development, whereas Yang *et al* (5) demonstrated that miR-21 promotes keratinocyte migration and re-epithelialization during wound healing. Of note, it was recently reported that miRNAs are involved in UVB-mediated cellular and molecular responses in keratinocytes. Zhou *et al* (6) demonstrated that acute exposure of keratinocytes to UVB results in several specific patterns of miRNA response. Furthermore, Guo *et al* (7) revealed that protection of HaCaT keratinocytes against UVB radiation is mediated by miR-23a through the regulation of topoisomerase-1/caspase7/serine/threonine-protein kinase 4 (STK4). These results indicate that miRNAs are crucial regulators of keratinocyte development and UVB response.

Arctiin is a lignin compound that has been purified from several plants, including *Arctium lappa* and *Forsythiae fructus*. Previous studies have demonstrated that arctiin exerted a protective effect against lipopolysaccharide (LPS)-induced inflammation and has anti-proliferative and anti-microbial functions (8-11). However, to the best of our knowledge, the photoprotective effect of arctiin has not been studied. In the present study, it was demonstrated that treatment with a low dose of arctiin suppresses UVB-induced keratinocyte damage though specific changes in miRNA expression.

## Materials and methods

**Cell culture and chemicals.** HaCaT keratinocytes were purchased from Cell Line Service (DKFZ, Eppelheim, Germany) and were maintained with Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Life Technologies) and antibiotics. Arctiin, a lignin isolated from *Arctium lappa*, was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich). Propidium iodide (PI), a fluorescent nuclear and chromosome counterstain, was purchased from BD Biosciences (San Jose, CA, USA).

**Cytotoxicity assay.** The cytotoxicity of arctiin on HaCaT cells was determined using a water-soluble tetrazolium (WST)-1 based cell viability assay (EZ-Cytox Cell Viability Assay kit; Itsbio, Seoul, Korea). HaCaT cells were seeded in a 96-well culture dish ( $3 \times 10^3$  cells/well) and grown for 24 h prior to the treatment with various doses of arctiin for a further 24 h. At the end of the treatment, the cells were incubated with 10  $\mu$ l of the reagent from the WST-1 assay kit for 30 min. Absorbance was recorded at 450 nm using a microplate spectrophotometer (iMark microplate reader; Bio-Rad, Hercules, CA, USA). The results are presented as the percentage of the control values.

**UVB protection assay.** The UVB protective effect of arctiin in HaCaT keratinocytes was determined using a previously described method (12). Cells were pretreated with different concentrations of arctiin for various durations, washed with phosphate-buffered saline (PBS) and irradiated with UVB (50 mJ/cm<sup>2</sup>) without lids on the plates. Following irradiation the cells were incubated for 24 h in growth media and cell viability was measured using an EZ-Cytox Cell Viability Assay kit (Itsbio). The results are presented as the percentage relative to the control (mean  $\pm$  standard deviation).

**Cell cycle analysis using flow cytometry.** Following exposure to UVB, the cells were washed twice with cold PBS and fixed by careful resuspension in cold 70% ethanol. Following fixation, the cells were washed with cold PBS and stained with PI solution (0.05 mg/ml PI, 2 mg/ml RNase A, 0.1% Triton X-100 in PBS). Fluorescence intensity (FL-2H) was measured by flow cytometry (FACScalibur; BD Biosciences) with Cell Quest software (BD Biosciences).

**In vitro scratch assay.** Cell migration *in vitro* was measured using a scratch assay. Cells were seeded densely into 60 mm culture dishes. Confluent cells were treated with arctiin (5  $\mu$ M) for 6 h and a standardized scratch was established in the monolayer using a 20  $\mu$ l loading tip. Following scratch formation the cells were irradiated with UVB (50 mJ/cm<sup>2</sup>) and incubated in growth media. The extent of scratch closure was quantified by measuring the area of the scratch prior to (0 h) and 48 h following wounding, using a phase-contrast microscope (Olympus CKX41; Olympus, Tokyo, Japan).

**DNA repair assay.** The effect on arctiin on UV-damaged plasmid was determined using a luciferase system. The pGL3 luciferase vector (Promega Corporation, Madison, WI, USA) was irradiated with UVC at the dose rate of 200 J/m<sup>2</sup>/sec for 10 sec (total 2000 J/m<sup>2</sup>). The undamaged control vector and UV-damaged pGL3 vector were co-transfected with pSV- $\beta$ -galactosidase ( $\beta$ -gal) plasmid (Promega Corporation) into HaCaT cells. Following 24 h, the luciferase activity was measured using a dual-luciferase reporter assay system as recommended by the manufacturer (Promega Corporation).

**miRNA microarray assay.** Total RNA was purified from HaCaT cells that were UVB-irradiated with or without arctiin pretreatment using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. The quality and concentration of the RNAs were analyzed using MaestroNano (Maestrogen, Las Vegas, NV, USA) and the integrity of the RNAs was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). A microarray assay was performed with the SurePrint G3 Human V16 miRNA 8x60 K array (Agilent Technologies) as previously described (12) and the results were analyzed with GeneSpring GX software version 11.5 (Agilent Technologies). Differentially expressed miRNAs were selected using the criteria of a random variance t-test, P-value <0.05 and an absolute fold change >2.

**Bioinformatics analysis of miRNAs.** Putative targets of each miRNA were identified using a DNA Intelligent Analysis (DIANA)-microT web-based bioinformatics program (<http://diana.imis.athena-innovation.gr/DianaTools/index.php>) (13). The involvement of putative target genes of each miRNA in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways was determined using the web-based bioinformatics tool Database for Annotation, Visualization and Integrated Discovery (DAVID; <http://david.abcc.ncifcrf.gov/home.jsp>) (14).

**Statistical analysis.** All results were determined from three independent experiments and analyzed with the unpaired

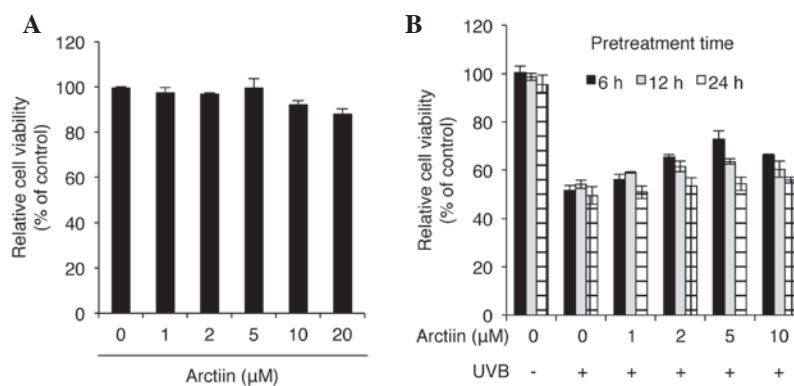


Figure 1. Arctiin inhibits UVB-mediated cytotoxicity in keratinocytes. (A) HaCaT keratinocytes were treated with different doses of arctiin for 24 h and cytotoxicity was analyzed by a WST-1 assay. (B) Cells were pretreated with various doses of arctiin for different durations prior to UVB irradiation. Cell viability was analyzed using the WST-1 assay. All data are expressed as the mean  $\pm$  standard deviation of three independent experiments. WST, water-soluble tetrazolium; UVB, ultraviolet B; miRNA, microRNA.

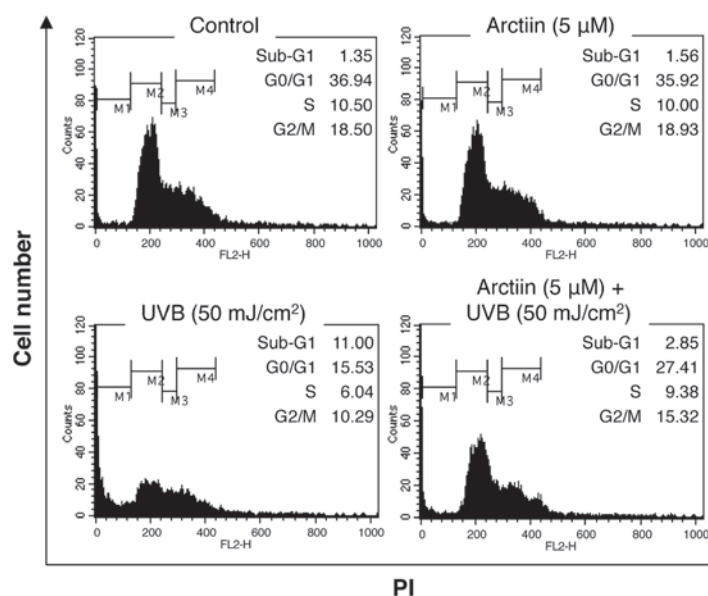


Figure 2. Arctiin inhibits the induction of HaCaT cell death by UVB irradiation. HaCaT cells were pretreated with either dimethylsulfoxide or arctiin for 6 h prior to UVB irradiation and subjected to flow cytometric cell cycle analysis. The numbers represent the percentage of cells in sub-G1, G0/G1, S and G2/M phase. UVB, ultraviolet B; miRNA, microRNA; PI, propidium iodide.

Student's t-test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Arctiin protects HaCaT cells against UVB damage.** To determine the cytotoxicity of arctiin, a cell viability assay was performed using water-soluble tetrazolium salts (WSTs) following treatment of HaCaT cells with different doses of arctiin for 24 h. Arctiin demonstrated no cytotoxicity at concentrations of 1–5  $\mu\text{M}$  and low cytotoxicity at 10 and 20  $\mu\text{M}$  (Fig. 1A). To investigate the UVB protective effect of arctiin, HaCaT cells were pretreated with arctiin at different doses and treatment durations prior to UVB (50 mJ/cm<sup>2</sup>) irradiation and a cytotoxicity assay were performed. Significant UVB protection was observed in the cells pretreated with 5  $\mu\text{M}$  arctiin for 6 h (Fig. 1B). Although other pretreatment conditions also demonstrated UVB protective activities, cell viability was not

significantly improved compared with UVB-irradiated control cells. These results identify arctiin as a novel UVB protective agent in keratinocytes.

**Arctiin pretreatment antagonizes UVB-mediated HaCaT cell death.** To determine the protective effect of arctiin on UVB-irradiated HaCaT cells, the cell cycle distribution was examined by PI staining and flow cytometry. UVB irradiation (50 mJ/cm<sup>2</sup>) increased the cell population in the sub-G1 phase, indicating the induction of cell death (Fig. 2). Pretreatment with arctiin prior to UVB treatment markedly decreased the population of cells in sub-G1 phase, suggesting that arctiin affects the UVB-mediated cellular mechanisms that induce cell death (Fig. 2).

**Arctiin pretreatment rescues UVB-induced deficiencies in wound healing and DNA repair.** To examine whether arctiin pretreatment induced UVB resistance through the regulation

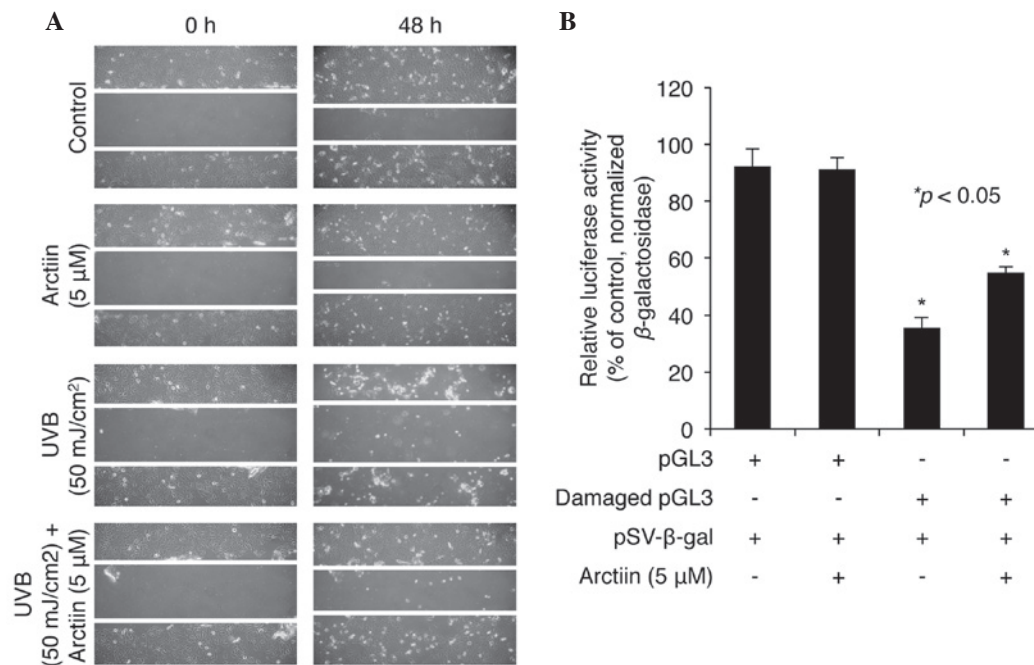


Figure 3. Effect of arctiin on scratch wound healing and DNA repair in UVB-irradiated HaCaT cells. (A) Arctiin rescues the UVB-induced wound-healing defect. Confluent cells were pretreated with arctiin and mechanically scratched with a pipette tip prior to UVB irradiation. Phase-contrast micrographs of scratched HaCaT cells were taken at 0 and 48 h following irradiation. (B) Arctiin enhances DNA repair. Cells were transfected with either untreated pGL3 or UV-irradiated pGL3 and then treated with arctiin for 24 h. Following incubation, the luciferase activity in the cell lysate was measured and normalized to  $\beta$ -gal activity. Data are expressed as the mean  $\pm$  standard deviation of three independent experiments. \* $P < 0.05$  vs. control cells (undamaged plasmid). UVB, ultraviolet B; miRNA, microRNA; gal, galactosidase.

of the cell migration associated with wound healing, a series of scratch assays were performed in the HaCaT cells. Firstly, marked closure of the scratch was demonstrated in the untreated control cells following 48 h incubation with DMSO vehicle (Fig. 3A, top panel). A similar extent of cell migration was observed in the cells treated with arctiin (5  $\mu$ M; Fig. 3A, second panel). However, UVB-irradiated cells demonstrated only a weak change in the extent of cell migration between 0 and 48 h (Fig. 3A, third panel). Of note, the UVB-mediated defect in cell migration was rescued by pretreatment with arctiin to a level of scratch closure similar to that in the control cells, suggesting that arctiin protects against the UVB-induced loss of migration in HaCaT cells (Fig. 3A, bottom panel).

To further examine the effect of arctiin on the repair of UV-induced DNA damage in HaCaT cells, a DNA repair assay was performed using the luciferase system and a damaged pGL3 luciferase plasmid that had been irradiated with UVC (2000 mJ/cm<sup>2</sup>). The control or damaged pGL3 were co-transfected into HaCaT cells with the  $\beta$ -gal plasmid, followed by treatment with arctiin for 24 h. In the absence of arctiin, the luciferase activity of damaged pGL3 was ~40% of that measured for the undamaged control pGL3 (Fig. 3B). However, the decrease in luciferase activity for the damaged plasmid was significantly reduced in the cells treated with arctiin following transfection, implying that arctiin enhances DNA repair in HaCaT cells (Fig. 3B).

*UVB protective functions of arctiin are associated with changes in miRNA expression profiles in HaCaT cells.* Next, the present study focused on exploring whether the UVB

protective function of arctiin in HaCaT cells was associated with changes in miRNA expression profiles by miRNA microarray analysis. UVB radiation following arctiin pretreatment altered the expression levels of several miRNAs compared with UV treatment alone (Fig. 4A). Four miRNAs were significantly upregulated and 62 miRNAs were significantly downregulated >2-fold (Fig. 4B), implying that arctiin modulates the expression of several miRNAs to protect cells from UVB-induced HaCaT cell damage. The full list of dysregulated miRNAs is shown in Table I. miR-21-3p was the most highly upregulated (3.85-fold) and miR-513c-5p was the most downregulated (13.32-fold) miRNA during the arctiin-mediated UVB protective response in HaCaT cells.

Following this, the biological mechanisms by which altered miRNA expression mediates the UVB protection effect were investigated. miRNAs target mRNAs by directly interacting with complementary sequences, so in the present study, putative target genes of each miRNA were analyzed using the DIANA bioinformatics tool. The resultant target genes were then analyzed using the bioinformatics database DAVID. The results generated by the KEGG pathway-based enrichment analysis program in DAVID indicated that these miRNAs may be involved in the regulation of pathways in cancer, the cell cycle, Wnt and MAPK signaling pathways (Tables II and III). Targets of the majority of upregulated miRNAs had biological functions in pathways involved in cancer, the cell cycle and Wnt signaling as described in Table II, whereas the majority of the targets of downregulated miRNAs were involved in the regulation of the actin cytoskeleton, cancer pathways, and MAPK, Axon, ErbB and insulin signaling pathways (Table III). Previous studies



Table I. Changes in miRNA expression in UVB-irradiated HaCaT cells pretreated with arctiin<sup>a</sup>.

Gene name	Fold change	Regulation	Chr	Gene name	Fold change	Regulation	Chr
hsa-miR-125a-5p	2.74	Up	chr19	hsa-miR-3656	-4.21	Down	chr11
hsa-miR-205-3p	2.51	Up	chr1	hsa-miR-3663-3p	-3.31	Down	chr10
hsa-miR-21-3p	3.85	Up	chr17	hsa-miR-3665	-3.01	Down	chr13
hsa-miR-29b-1-5p	2.81	Up	chr7	hsa-miR-3679-5p	-4.16	Down	chr2
hsa-miR-1181	-2.38	Down	chr19	hsa-miR-371a-5p	-2.26	Down	chr19
hsa-miR-1202	-3.40	Down	chr6	hsa-miR-3911	-3.00	Down	chr9
hsa-miR-1207-5p	-3.81	Down	chr8	hsa-miR-424-3p	-4.00	Down	chrX
hsa-miR-1224-5p	-2.70	Down	chr3	hsa-miR-4257	-4.71	Down	chr1
hsa-miR-1225-5p	-4.11	Down	chr16	hsa-miR-4270	-4.41	Down	chr3
hsa-miR-1234-3p	-2.90	Down	chr8	hsa-miR-4271	-3.72	Down	chr3
hsa-miR-1246	-4.23	Down	chr2	hsa-miR-4281	-3.08	Down	chr5
hsa-miR-125a-3p	-2.86	Down	chr19	hsa-miR-4298	-2.31	Down	chr11
hsa-miR-1268a	-3.12	Down	chr15	hsa-miR-4299	-3.21	Down	chr11
hsa-miR-1275	-3.94	Down	chr6	hsa-miR-4327	-3.48	Down	chr21
hsa-miR-1290	-4.59	Down	chr1	hsa-miR-452-5p	-2.72	Down	chrX
hsa-miR-134	-4.14	Down	chr14	hsa-miR-483-5p	-5.72	Down	chr11
hsa-miR-135a-3p	-3.92	Down	chr3	hsa-miR-494	-3.35	Down	chr14
hsa-miR-1469	-5.69	Down	chr15	hsa-miR-513a-5p	-5.23	Down	chrX
hsa-miR-150-3p	-3.28	Down	chr19	hsa-miR-513b	-4.68	Down	chrX
hsa-miR-188-5p	-4.11	Down	chrX	hsa-miR-513c-5p	-13.32	Down	chrX
hsa-miR-1915-3p	-2.80	Down	chr10	hsa-miR-572	-5.49	Down	chr4
hsa-miR-1973	-2.63	Down	chr4	hsa-miR-575	-4.10	Down	chr4
hsa-miR-28-5p	-2.20	Down	chr3	hsa-miR-629-3p	-2.33	Down	chr15
hsa-miR-2861	-4.08	Down	chr9	hsa-miR-630	-4.37	Down	chr15
hsa-miR-3127-5p	-2.00	Down	chr2	hsa-miR-638	-4.40	Down	chr19
hsa-miR-3141	-2.33	Down	chr5	hsa-miR-642b-3p	-3.03	Down	chr19
hsa-miR-3162-5p	-3.08	Down	chr11	hsa-miR-663a	-5.28	Down	chr20
hsa-miR-3196	-2.96	Down	chr20	hsa-miR-718	-4.48	Down	chrX
hsa-miR-320c	-2.75	Down	chr18	hsa-miR-762	-2.73	Down	chr16
hsa-miR-345-5p	-10.76	Down	chr14	hsa-miR-765	-6.70	Down	chr1
hsa-miR-3646	-2.75	Down	chr20	hsa-miR-874	-3.09	Down	chr5
hsa-miR-3648	-4.46	Down	chr21	hsa-miR-939-5p	-2.01	Down	chr8
hsa-miR-3652	-8.13	Down	chr12	hsa-miR-940	-2.39	Down	chr16

<sup>a</sup>selected miRNAs exhibiting a >2-fold expression change. Chr, chromosome; miR, microRNA; UVB, ultraviolet B; hsa, *Homo sapiens*.

have reported that these pathways are affected by UVB radiation (2,15-17). In summary, these results identified the cellular processes involved in arctiin-mediated UVB protection.

## Discussion

Keratinocytes are the predominant cell type in the outermost skin layer, the epidermis, and have a pivotal role in primary protection against environmental insults, including UV radiation. UV radiation is the major inducer of photoaging of keratinocytes and may result in cellular aging, senescence, apoptosis or cancer by inducing damage to intracellular molecules, in particular DNA. In the present study, biochemical and genetic analyses were utilized to identify the phytochemical arctiin as a novel photoprotective agent against UVB-mediated keratinocyte

damage. The data revealed that arctiin itself had a low cytotoxicity in HaCaT cells. Notably, arctiin pretreatment reduced UVB-induced cytotoxicity and suppressed UVB-mediated cell death. It was also demonstrated that arctiin enhanced wound healing and DNA repair in UVB-exposed HaCaT keratinocytes.

Significant changes in miRNA expression profiles in cells that were pretreated with arctiin prior to UVB irradiation were observed, as compared with those in the cells that only received UVB irradiation, with 66 miRNAs exhibiting altered expression. Of note, almost all of the dysregulated miRNAs were down-regulated (62/66) and only four miRNAs were upregulated. This expression pattern differs from the results of a previous study by our group in which the titrated extract of *Centella asiatica* (TECA) was demonstrated to protect keratinocytes against UVB-induced damage through upregulation of 46 miRNAs and downregulation of 36 miRNAs (12). In addition, Zhou *et al* (6)

Table II. Functional annotation chart for miRNAs that were upregulated in arctiin-pretreated UVB-irradiated HaCaT keratinocytes.

miRNA ( <i>Homo sapiens</i> )	Putative target genes	KEGG pathway	Genes involved in the term	% of involved genes/total genes	P-value
miR-125a-5p	162	Pathways in cancer	8	4.9	3.60x10 <sup>-2</sup>
		Cell cycle	4	2.5	1.20x10 <sup>-1</sup>
miR-205-3p	944	Pathways in cancer	19	2	2.50x10 <sup>-1</sup>
		MAPK signaling pathway	17	1.8	1.70x10 <sup>-1</sup>
		Wnt signaling pathway	15	1.6	9.20x10 <sup>-3</sup>
miR-21-3p	210	CAMs	7	3.3	4.70x10 <sup>-3</sup>
		Ubiquitin mediated proteolysis	6	2.9	2.30x10 <sup>-2</sup>
		Long-term potentiation	5	2.4	8.60x10 <sup>-3</sup>
		Oocyte meiosis	5	2.4	4.20x10 <sup>-2</sup>
miR-29b-1-5p	265	Insulin signaling pathway	5	1.9	8.50x10 <sup>-2</sup>
		Cell cycle	4	1.5	2.00x10 <sup>-1</sup>
		Wnt signaling pathway	4	1.5	2.90x10 <sup>-1</sup>
		Jak-STAT signaling pathway	4	1.5	3.00x10 <sup>-1</sup>

CAMs, cell adhesion molecules; UVB, ultraviolet B; MAPK, mitogen-activated protein kinase; miR, microRNA; KEGG, Kyoto Encyclopedia of Genes and Genomes.

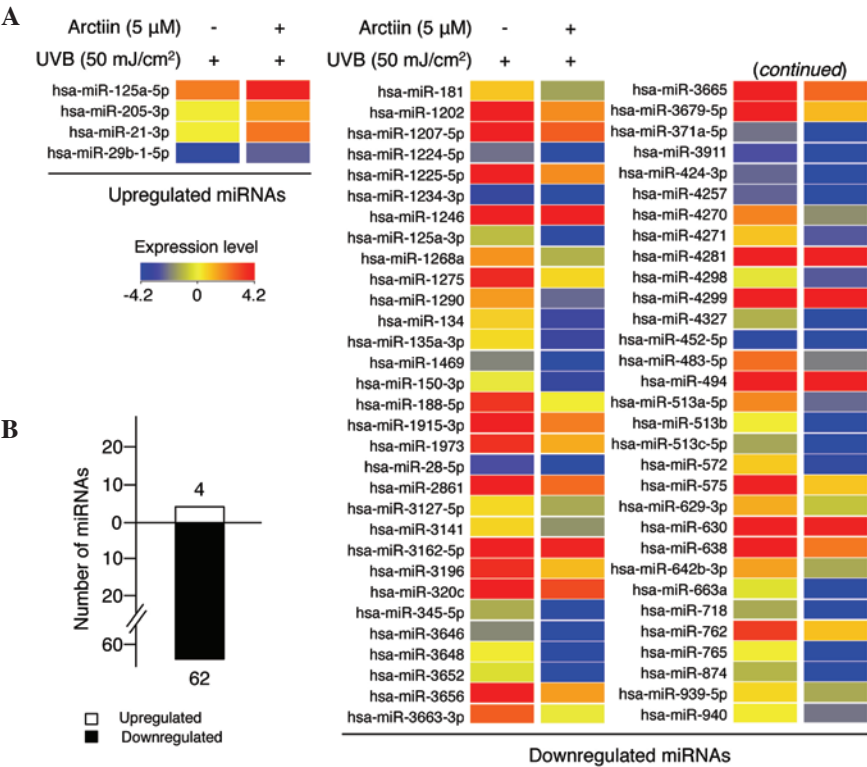


Figure 4. Comparison of miRNA expression in UVB-irradiated keratinocytes with or without arctiin pretreatment. (A) Results of miRNA microarray assays. HaCaT cells were treated with arctiin for 6 h prior to irradiation with UVB. Following 24 h, total RNAs were isolated and subjected to microarray assay. Left panel, miRNAs that were upregulated >2-fold; right panel, miRNAs that were downregulated >2-fold. (B) Bar chart revealing the number of up- and downregulated miRNAs based on microarray data. UVB, ultraviolet B; miR/miRNA, microRNA; hsa, *Homo sapiens*.

recently characterized UVB-responsive miRNAs in human keratinocytes using microRNA microarray analysis and identified that the expression of 44 miRNAs was up- or downregulated by >2-fold compared with that in non-irradiated keratinocytes. There appears to be no correlation between the UVB-responsive

miRNAs and the arctiin-induced UVB protective miRNAs; however, certain miRNAs identified in the present study were also dysregulated in other studies. miR-3652 and miR-494, which were downregulated by 8.31- and 3.35-fold in the present study, were also downregulated in keratinocytes with TECA-induced

Table III. Functional annotation chart for miRNAs that were downregulated in arctiin-pretreated UVB-irradiated HaCaT keratinocytes.<sup>a</sup>

miRNA ( <i>Homo sapiens</i> )	Putative target genes	KEGG pathway	Genes involved in the term	% of involved genes/total genes	P-value
miR-513c-5p	142	Tight junction	4	2.8	3.30x10 <sup>-2</sup>
		Phosphatidylinositol signaling system	3	2.1	5.80x10 <sup>-2</sup>
		CAMs	3	2.1	1.50x10 <sup>-1</sup>
miR-345-5p	94	Axon guidance	3	3.2	8.20x10 <sup>-2</sup>
		Regulation of actin cytoskeleton	3	3.2	1.90x10 <sup>-1</sup>
		Type II diabetes mellitus	2	2.1	1.60x10 <sup>-1</sup>
		Arginine and proline metabolism	2	2.1	1.80x10 <sup>-1</sup>
		VEGF signaling pathway	2	2.1	2.50x10 <sup>-1</sup>
		TGF- $\beta$ signaling pathway	2	2.1	2.80x10 <sup>-1</sup>
miR-3652	195	Axon guidance	5	2.6	3.30x10 <sup>-2</sup>
		CAMs	5	2.6	3.50x10 <sup>-2</sup>
		Insulin signaling pathway	5	2.6	3.80x10 <sup>-2</sup>
		ErbB signaling pathway	4	2.1	4.80x10 <sup>-2</sup>
		MAPK signaling pathway	4	2.1	4.70x10 <sup>-1</sup>
miR-765	548	Cytokine-cytokine receptor interaction	11	2	2.00x10 <sup>-1</sup>
miR-483-5p	32	Focal adhesion	2	6.2	1.50x10 <sup>-1</sup>
miR-1469	2	-	-	-	-
miR-572	6	-	-	-	-
miR-663a	33	-	-	-	-
miR-513a-5p	980	MAPK signaling pathway	25	2.6	1.00x10 <sup>-2</sup>
		Pathways in cancer	24	2.4	1.30x10 <sup>-1</sup>
		Regulation of actin cytoskeleton	20	2	2.50x10 <sup>-2</sup>
miR-1290	593	Pathways in cancer	17	2.9	4.00x10 <sup>-2</sup>
		Focal adhesion	14	2.4	7.90x10 <sup>-3</sup>
		Insulin signaling pathway	13	2.2	7.60x10 <sup>-4</sup>
		Regulation of actin cytoskeleton	12	2	6.30x10 <sup>-2</sup>
		MAPK signaling pathway	12	2	1.90x10 <sup>-1</sup>
		ErbB signaling pathway	11	1.9	2.80x10 <sup>-4</sup>
miR-718	40	-	-	-	-
miR-3648	13	-	-	-	-
miR-4270	423	Axon guidance	8	1.9	5.50x10 <sup>-3</sup>
		Regulation of actin cytoskeleton	8	1.9	6.70x10 <sup>-2</sup>

<sup>a</sup>The list shows only the top 15 downregulated miRNAs displaying >2-fold expression changes. KEGG, Kyoto Encyclopedia of Genes and Genomes; CAMs, cell adhesion molecules; UVB, ultraviolet B; MAPK, mitogen-activated protein kinase; miR/miRNA, microRNA; VEGF, vascular endothelial growth factor; TGF, transforming growth factor; ErbB, epidermal growth factor receptor.

UVB protection (12). miR-125a-5p was previously reported to be expressed at significantly lower levels in UVB-irradiated mouse epidermis than in control epidermis (18), although the results of the present study demonstrated that the miRNA was significantly upregulated (2.74-fold). In addition, miR-1246 (4.23-fold downregulation in this study) was reported to be a target of the p53 transcription factor, which is significantly upregulated by UVB irradiation in keratinocytes (19), and miR-125a-3p (2.86-fold downregulation) has been demonstrated to reduce cell proliferation and migration by targeting Fyn kinase, which is activated by UVB irradiation in keratinocytes (20,21). In summary, the

microarray data of the present study suggested that although the dysregulated miRNAs were specifically induced by arctiin treatment, they may be involved in the regulation of general UVB-mediated cellular transduction. Although miR-513c-5p and miR-345-5p were the most highly dysregulated miRNAs in the present study (13.32- and 10.76-fold downregulated, respectively), their biological functions have not been reported. It is possible that these miRNAs also regulate UVB-mediated cytotoxicity in keratinocytes.

The biological significance of the dysregulated miRNAs identified in the present study was further examined. For this,

the putative target genes of the miRNAs were predicted using DIANA, a web-based bioinformatics program, and the target genes involved in KEGG pathways were then analyzed using the DAVID database. The results indicated that the MAPK signaling as the pathway that was most affected by arctiin-induced UVB protection in keratinocytes. miR-205-3p, miR-3652, miR-513a-5p and miR-1290 were predicted to affect target genes involved in the MAPK pathway. Indeed, MAPK activation is one of the main signaling pathways activated by UVB exposure. In keratinocytes, UVB stimulates immediate activation of JNK, p38 MAPK and ERK1/2 (2). Activated JNK and p38 MAPK mediate both cell survival and death in UVB-irradiated keratinocytes (2). In addition, it was demonstrated that UVB-induced production of ROS (including H<sub>2</sub>O<sub>2</sub>) activates p38 MAPK and JNK signaling in keratinocytes, suggesting that UVB-induced ROS may affect signal transduction through MAPK pathways (2). Furthermore, it has been suggested that p38 MAPK and JNK have a pivotal role in the UVB-induced inflammatory response by regulating cyclooxygenase-2 (COX-2) activity in keratinocytes (2). Of note, UV radiation does not regulate the mRNA levels of *JNK*, *p38 MAPK* and *ERK1/2*, but rather the levels of post-translational modification by protein phosphorylation. This indicates that the activation of MAPK proteins by UVB radiation is not transcriptionally induced (2). However, recent studies have demonstrated that post-transcriptional regulators, including miRNAs, may regulate MAPK activity. Although the miRNAs do not directly regulate mRNA levels of MAPKs, upstream or downstream members of MAPK signaling pathways are post-transcriptionally regulated by miRNAs. Zhang *et al* (22) demonstrated that miR-451 regulates p38 MAPK signaling by targeting of *Ywhaz*, and Antoon *et al* (23) demonstrated that increased p38 MAPK activity results in part from differential miRNA expression. Therefore, arctiin-induced UVB protective miRNAs may regulate members of MAPK signaling pathways, rather than the mRNAs of MAPKs themselves.

In conclusion, the present study suggested that arctiin may be a novel photoprotective agent that confers resistance to UVB-mediated keratinocyte damage by altering miRNA expression profiles. In particular, the MAPK signaling pathway appears to be a major target of the miRNAs that are affected by arctiin. Further studies are required to determine the exact mechanisms by which arctiin induces a UVB protective effect in keratinocytes.

## Acknowledgements

The authors are grateful to all other members of Coreana Cosmetics Co., Ltd. for their support. This paper was supported by the KU Research Professor Program of Konkuk University and grant from the Ministry of Science, ICT and Future Planning (no. 20110028646) of the Republic of Korea.

## References

- Wondrak GT, Jacobson MK and Jacobson EL: Endogenous UVA-photosensitizers: mediators of skin photodamage and novel targets for skin photoprotection. *Photochem Photobiol Sci* 5: 215-237, 2006.
- Muthusamy V and Piva TJ: The UV response of the skin: a review of the MAPK, NFκappaB and TNFalpha signal transduction pathways. *Arch Dermatol Res* 302: 5-17, 2010.
- Rivetti di Val Cervo P, Lena AM, Nicoloso M, *et al*: p63-microRNA feedback in keratinocyte senescence. *Proc Natl Acad Sci USA* 109: 1133-1138, 2012.
- Hildebrand J, Rütze M, Walz N, *et al*: A comprehensive analysis of microRNA expression during human keratinocyte differentiation in vitro and in vivo. *J Invest Dermatol* 131: 20-29, 2011.
- Yang X, Wang J, Guo SL, *et al*: miR-21 promotes keratinocyte migration and re-epithelialization during wound healing. *Int J Biol Sci* 7: 685-690, 2011.
- Zhou BR, Xu Y, Permatasari F, *et al*: Characterization of the miRNA profile in UVB-irradiated normal human keratinocytes. *Exp Dermatol* 21: 317-319, 2012.
- Guo Z, Zhou B, Liu W, *et al*: miR-23a regulates DNA damage repair and apoptosis in UVB-irradiated HaCaT cells. *J Dermatol Sci* 69: 68-76, 2013.
- Lee S, Shin S, Kim H, *et al*: Anti-inflammatory function of arctiin by inhibiting COX-2 expression via NF-κB pathways. *J Inflamm (Lond)* 8: 16, 2011.
- Hirose M, Yamaguchi T, Lin C, *et al*: Effects of arctiin on PhIP-induced mammary, colon and pancreatic carcinogenesis in female Sprague-Dawley rats and MeQx-induced hepatocarcinogenesis in male F344 rats. *Cancer Lett* 155: 79-88, 2000.
- Hayashi K, Narutaki K, Nagaoka Y, Hayashi T and Uesato S: Therapeutic effect of arctiin and arctigenin in immunocompetent and immunocompromised mice infected with influenza A virus. *Biol Pharm Bull* 33: 1199-1205, 2010.
- Matsuzaki Y, Koyama M, Hitomi T, *et al*: Arctiin induces cell growth inhibition through the down-regulation of cyclin D1 expression. *Oncol Rep* 19: 721-727, 2008.
- An IS, An S, Choe TB, *et al*: Centella asiatica protects against UVB-induced HaCaT keratinocyte damage through microRNA expression changes. *Int J Mol Med* 30: 1349-1356, 2012.
- Maragkakis M, Reczko M, Simossis VA, *et al*: DIANA-microT web server: elucidating microRNA functions through target prediction. *Nucleic Acids Res* 37: W273-W276, 2009.
- Huang da W, Sherman BT and Lempicki RA: Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 4: 44-57, 2009.
- Misovic M, Milenkovic D, Martinovic T, Ciric D, Bumbasirevic V and Kravic-Stevovic T: Short-term exposure to UV-A, UV-B, and UV-C irradiation induces alteration in cytoskeleton and autophagy in human keratinocytes. *Ultrastruct Pathol* 37: 241-248, 2013.
- Madson JG and Hansen LA: Multiple mechanisms of Erbb2 action after ultraviolet irradiation of the skin. *Mol Carcinog* 46: 624-628, 2007.
- Yamada T, Hasegawa S, Inoue Y, *et al*: Wnt/β-catenin and kit signaling sequentially regulate melanocyte stem cell differentiation in UVB-induced epidermal pigmentation. *J Invest Dermatol* 133: 2753-2762, 2013.
- Zhou BR, Xu Y and Luo D: Effect of UVB irradiation on microRNA expression in mouse epidermis. *Oncol Lett* 3: 560-564, 2012.
- Henseleit U, Zhang J, Wanner R, Haase I, Kolde G and Rosenbach T: Role of p53 in UVB-induced apoptosis in human HaCaT keratinocytes. *J Invest Dermatol* 109: 722-727, 1997.
- Ninio-Many L, Grossman H, Shomron N, Chuderland D and Shalgi R: microRNA-125a-3p reduces cell proliferation and migration by targeting Fyn. *J Cell Sci* 126: 2867-2876, 2013.
- He Z, Cho YY, Ma WY, Choi HS, Bode AM and Dong Z: Regulation of ultraviolet B-induced phosphorylation of histone H3 at serine 10 by Fyn kinase. *J Biol Chem* 280: 2446-2454, 2005.
- Zhang Z, Luo X, Ding S, *et al*: microRNA-451 regulates p38 MAPK signaling by targeting of *Ywhaz* and suppresses the mesangial hypertrophy in early diabetic nephropathy. *FEBS Lett* 586: 20-26, 2012.
- Antoon JW, Nitzchke AM, Martin EC, *et al*: Inhibition of p38 mitogen-activated protein kinase alters microRNA expression and reverses epithelial-to-mesenchymal transition. *Int J Oncol* 42: 1139-1150, 2013.