Altered miRNA expression profiles are involved in the protective effects of troxerutin against ultraviolet B radiation in normal human dermal fibroblasts

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Abstract. The aim of this study was to investigate the mechanisms by which troxerutin protects cells against ultraviolet B (UVB) radiation. First, we demonstrate that pre-treatment with troxerutin protects normal human dermal fibroblasts (nHDFs) against UVB-induced cytotoxicity. As shown by migration assay and DNA repair analysis, troxerutin increased cell migration and DNA repair activity in the nHDFs. Subsequently, we analyzed microRNA (miRNA) expression profiles in the nHDFs. miRNAs are 19- to 24-nucleotide (nt) non-coding RNA molecules that regulate the translation of target genes through RNA interference. In UVB-exposed cells, miRNAs act on crucial functions, such as apoptosis and cellular senescence. miRNA expression is significantly altered during the protective process induced by phytochemicals. Therefore, understanding changes that occur in miRNA expression profiles may help to elucidate the protective mechanisms of troxerutin. We identified 11 miRNAs that were significantly (>2-fold) upregulated and 12 that were significantly downregulated (>2-fold) following treatment of the nHDFs with troxerutin. In addition, we investigated the biological functions of these miRNAs through the prediction of miRNA targets and Gene Ontology analysis of the putative targets. Overall, our findings indicate that pre-treatment with troxerutin increases the viability of UVB-exposed nHDFs through the alteration of the miRNA expression profiles.

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Introduction

In the dermis, fibroblasts that are derived from mesenchymal cells play a critical role in maintaining homeostasis (1,2). Dermal fibroblasts synthesize and secrete extracellular matrix (ECM) components, such as collagen and elastin (3). The ability to synthesize ECM components is repressed by chronic and acute solar ultraviolet (UV) exposure (4,5), and UV radiation also indirectly causes the destruction of the ECM through the induction of matrix metalloproteinase (MMP) secretion by dermal fibroblasts. The impaired ECM forms a non-elastic matrix in the dermis, which leads to aging (6). Furthermore, UV triggers apoptosis and growth arrest in fibroblasts (7,8). Therefore, as dermal fibroblasts play an important role in the construction and homeostasis of the dermis and UV induces a decrease in the number of dermal fibroblasts and the destruction of the ECM, UV radiation is one of the major inducers of disorders of the dermis.

microRNAs (miRNAs) are 19- to 24-nucleotide (nt) non-coding RNA sequences that regulate target gene expression through RNA interference. miRNAs have been shown to modulate various cellular processes, such as apoptosis, proliferation, cell cycle progression and differentiation (9). Several lines of evidence suggest that miRNAs play important roles in the dermis. miRNA-152 and miRNA-181 act on adhesion proteins and ECM proteins in senescent dermal fibroblasts (13). The expression of miRNA-92a leads to a decreased expression of MMP1 and the accumulation of collagen (14), and miRNA-196a and miRNA-150 directly target collagen type I in scleroderma

dermal fibroblasts (15). Moreover, the expression of miRNAs is sensitively altered in response to UV radiation (10-12). In a recent study, we demonstrated that phytochemicals protect various cell types against UV radiation, suggesting a role for miRNAs in this protective mechanism in dermal fibroblasts (16). However, little is known about the underlying mechanisms.

Troxerutin is a trihydroxyethylated derivative of the natural bioflavonoid, rutin, which is one of the phytochemical components extracted from *Sophora japonica* (17). In addition, as troxerutin has been shown to have radioprotective, anti-inflammatory and antioxidant effects, it seems likely that troxerutin may protect cells against UV-induced oxidative stress and DNA damage (18,19).

In this study, we aimed to determine whether troxerutin induces a resistance response in dermal fibroblasts exposed to UVB radiation and, if so, to determine whether miRNAs are involved in the protective effects of troxerutin against UVB radiation. We confirm that the treatment of human dermal fibroblasts (HDFs) with troxerutin induces resistance to UVB-induced cell damage. In addition, we characterize the miRNA expression profiles associated with the protective effects of troxerutin against UVB radiation.

Materials and methods

Cell lines and cell culture. Normal HDFs (nHDFs) were purchased from Lonza (Basel, Switzerland) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin/streptomycin (Gibco Invitrogen) in a humidified atmosphere of 95% air/5% CO_2 at 37˚C.

WST-1 assay. Cell viability was determined using a water-soluble tetrazolium salt-1 (WST-1) assay. nHDFs were seeded in a 96-well plate (SPL Life Sciences, Pocheon, Korea) at a density of $4x10^3$ cells/well. The nHDFs were incubated for 24 h under growth conditions and then pre-treated with the indicated concentrations of troxerutin for 4, 8 and 12 h. At each time point, the cells were exposed to 100 mJ/cm^2 UVB radiation using a G8T5E lamp (Sankyo Denki, Toshima-ku, Japan) and a UV light meter (Lutron UV-340; Lutron Electronic Enterprise Co., Taipei, Taiwan). Following exposure to UVB radiation, the nHDFs were incubated for 24 h prior to the addition of WST-1 solution (EZ-Cytox cell viability assay kit; Itsbio, Seoul, Korea) for 1 h. The optical density of the colored WST-1 was measured at 450 nm and normalized using optical density at 620 nm. The results are expressed as the mean percentage ± standard error (SE) of experiments performed in triplicate. Statistical significance was determined using a two-tailed t-test compared with untreated nHDFs or UVB-exposed nHDFs not treated with troxerutin.

Cell cycle and DNA repair analyses. Cell cycle distribution was determined by measuring the DNA content. nHDFs were seeded in 60-mm culture dishes (SPL Life Sciences) at a density of $7x10^5$ cells/dish. The cells were incubated for 24 h under growth conditions and then pre-treated with 10 μM troxerutin for 8 h. At various time points, the cells were exposed to 100 mJ/cm^2 UVB and incubated for a further 24 h. Following incubation, the nHDFs were fixed with 70% ethanol at 4˚C for 3 h and incubated with staining solution containing 50 μg/ml PI (Sigma-Aldrich), 0.5% Triton X-100 (Bioshop, Burlington, Canada) and 100 μg/ml RNase (Bioshop) at 37˚C for 1 h. DNA content was measured using the FL2-H channel of a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). DNA repair activity was determined by transfection with a damaged plasmid as previously described (21). Before transfection, the pGL3 luciferase reporter plasmids were damaged with 2,000 J/m^2 UVC radiation. The nHDFs were seeded at $7x10^5$ cells in 60-mm culture dishes and transiently transfected with either the control pGL3 or the damaged pGL3 plasmids along with pSV-β-galactosidase plasmid (as a transfection control). The transfected cells were treated with 10 μM troxerutin for 24 h. After the treatments, DNA repair activity was determined by using the Luciferase assay system (Promega, Madison, WI, USA). The results were normalized to β-galactosidase activity and are presented as the means ± SE of the percentage of the control. Results shown are the averages of 3 independent experiments.

Migration assay. The migration rate was measured as described in a previous study (20). Briefly, $7x10^5$ nHDFs were seeded in a 60-mm culture dish and grown to >90% confluence. The cells were pre-treated with 10 μM troxerutin for 8 h and then exposed to 100 mJ/cm^2 UVB. The cell monolayer was scraped in a straight line with a p200 pipet tip. At 0 and 48 h after wounding, the distance between the cells was compared by imaging using a phase contrast microscope.

miRNA microarray. Total RNA was extracted from each sample using RiboEX (GeneAll, Seoul, Korea) and labeled using Agilent miRNA labeling kits (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. The labeled RNA was purified by a Micro Bio-Spin P-6 column (Bio-Rad Laboratories, Hercules, CA, USA) and hybridized with the SurePrint Human v16 miRNA microarray kit (based on miRBase Release 19.0; Agilent Technologies) as recommended by the manufacturer. The microarray contained 1,368 probes and detected 1,205 miRNAs. The fluorescence intensity of each probe was measured using Scanner and Feature Extraction software (Agilent Technologies). The digitalized fluorescence intensity was analyzed using GeneSpring GX version 11.5 (Agilent Technologies).

Bioinformatics analysis of miRNAs with significant changes in expression. Putative targets of miRNAs were identified using bioinformatics miRNA target prediction software, TargetScan (www.targetscan.org). The prediction of targets was performed using a conserved database for all miRNAs that were either up- or downregulated by >2-fold. The targets of each significant miRNA were collected in a target pool and Gene Ontology (GO) of the target pool and were analyzed using DAVID (http://david.abcc.ncifcrf.gov/). After GO analysis, the significant GO terms were filtered by a >5% 'percentage of count', which represented the percentage of each GO term-related genes.

Statistical analyses. Statistical significance was determined by a Student's t-test. Values of $p<0.05$ were considered to indicate statistically significant differences.

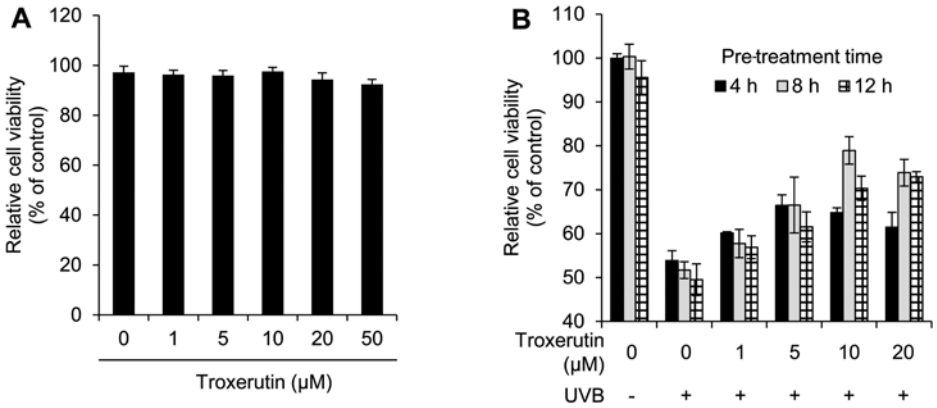

Figure 1. Troxerutin protects normal human dermal fibroblasts (nHDFs) against UVB-induced cytotoxicity. (A) Effects of troxerutin on nHDF viability. nHDFs were seeded in 96-well plates and then treated with various concentrations of troxerutin (0-50 μM) for 24 h. (B) Protective effects of troxerutin against cytotoxicity induced by UVB radiation in nHDFs. nHDFs ($4x10^4$ cells/well) were seeded in 96-well plates and pre-treated with 10 μM troxerutin for 4, 8 and 12 h. Following pre-treatment, the nHDFs were exposed to 100 mJ/cm^2 UVB and incubated for 24 h. Cell viability was measured by WST-1 assay. The results are presented as the means ± SE of the percentage of control optical density (OD) from experiments performed in triplicate.

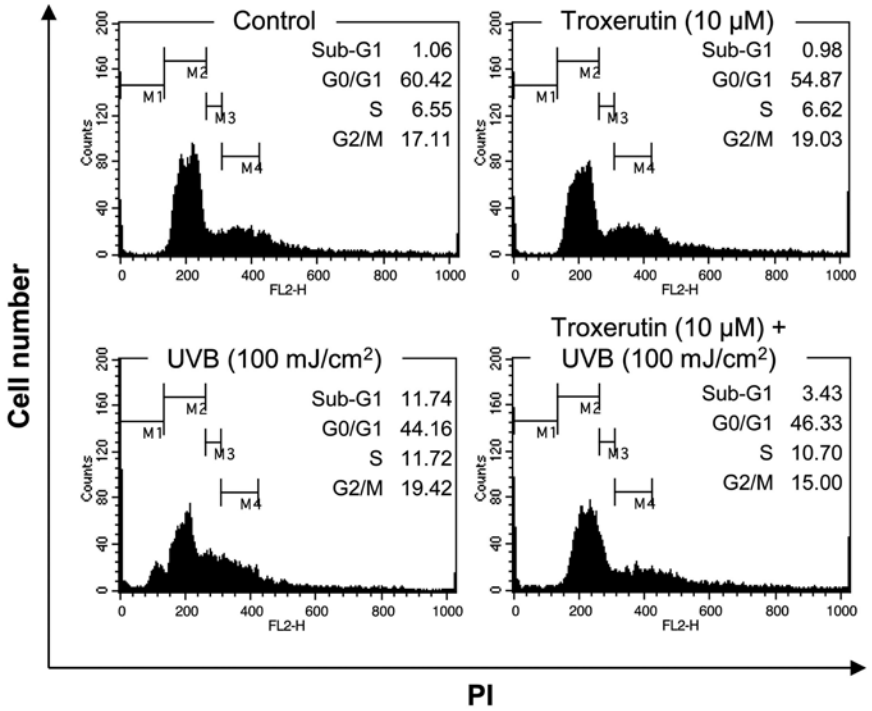

Figure 2. UVB-induced cell death was reduced by troxerutin. Normal human dermal fibroblasts (nHDFs; $7x10^5$ cells) were seeded in 60-mm culture dishes, treated with troxerutin for 8 h, and then exposed to 100 mJ/cm^2 UVB. Follwoing treatment, the cells were incubated for 24 h and collected for PI staining of DNA. Fluorescence intensity distribution of the PI-stained cells was analyzed by a flow cytometer. Sub-G1, G1, S and G2/M phases were separated using the 4 indicated gates (M1, M2, M3 and M4).

Results

Cytotoxicity of troxerutin in nHDFs and protective effects against UVB radiation. To determine the cytotoxicity of troxerutin we first performed cell viability assays for nHDFs treated with the indicated concentrations of troxerutin. Overall, there was no significant toxicity observed for concentrations up to 50 μM and treatment with 50 μM troxerutin decreased cell viability to 92.40% (Fig. 1A). Therefore, we used troxerutin at concentrations <50 μM in the subsequent experiments.

We then investigated the protective effects of troxerutin against UVB radiation. nHDFs were pre-treated with 0-20 μM troxerutin for 4, 8 and 12 h, and then exposed to 100 mJ/cm^2 UVB. As shown in Fig. 1B, pre-treatment with troxerutin reversed the UVB-induced inhibitory effects on cell growth. In particular, pre-treatment with 10 μM troxerutin for 8 h restored cell viability to 78.95% compared with 51.68% for the UVB-exposed nHDFs. We then examined whether troxerutin increases cell viability in nHDFs through the regulation of the cell cycle or cell death. To investigate cell cycle arrest we performed a flow cytometric cell cycle analysis using PI staining. As shown in Fig. 2, exposure to 100 mJ/cm^2 UVB increased the number of cells in the sub-G1 phase, increasing the population of dead cells from 1.06 to 11.74%. However,

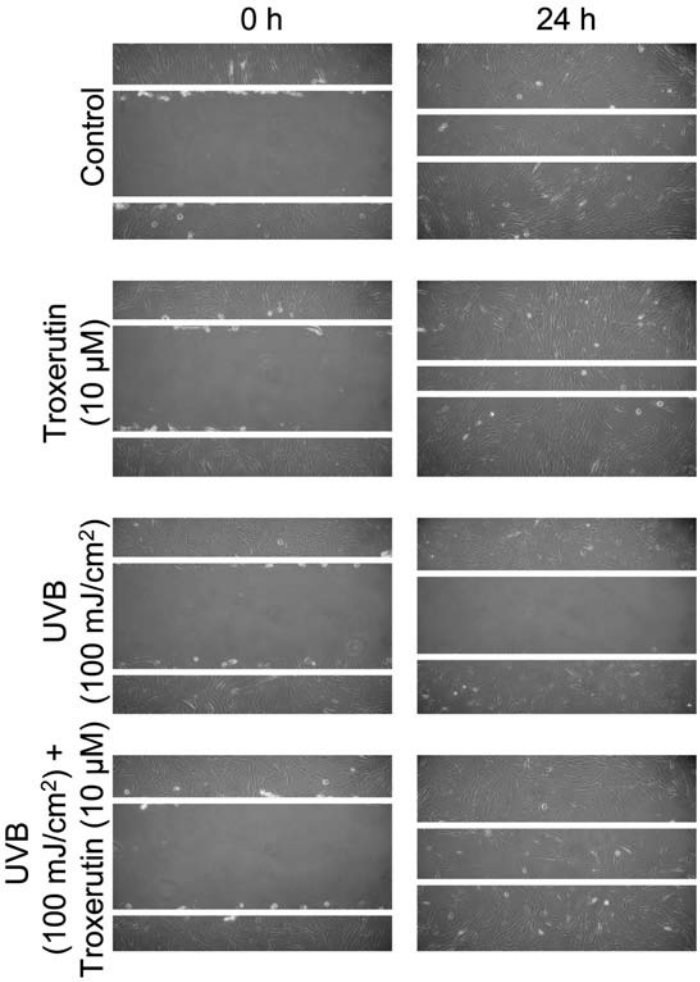

Figure 3. UVB-induced decrease in migration rate was reversed by troxerutin. Normal human dermal fibroblasts nHDFs ($7x10^5$ cells) were seeded in 60-mm culture dishes, treated with troxerutin for 8 h, and then exposed to 100 mJ/cm^2 UVB. Following exposure to UVB radiation, a wound was created by scratching the cell monolayer. At 0 and 24 h, the wound was photographed under a phase contrast microscope (magnification, x200).

pre-treatment with 10 μM troxerutin for 8 h reversed the UVB-induced increase in the number of cells in the sub-G1 phase (percentage of cells in sub-G1 phase decreased to 3.43%). Therefore, troxerutin blocked the UVB-induced decrease in cell viability by repressing cell death.

Troxerutin enhances cell migration and DNA repair activity. To investigate the effects of troxerutin on the migration rate we performed a migration assay using a scratch line. nHDFs were grown to >90% confluence and pre-treated with 10 μM troxerutin for 8 h. Following pre-treatment, the nHDFs were exposed to 100 mJ/cm^2 UVB and scraped to form a scratch. As shown in Fig. 3, treatment with troxerutin enhanced the cell migration rate compared with the untreated (control) nHDFs and those exposed to UVB radiation and not treated with troxerutin. In addition, we examined whether troxerutin increases DNA repair activity. A plasmid containing the luciferase gene was damaged by UVC and then transfected into the nHDFs. The damaged vector was broken by endonucleases in the nHDFs, whereas the repaired vector remained and expressed luciferase, as shown in a previous study (21). Therefore, an increase in luciferase activity indicates enhanced DNA repair activity. As shown in Fig. 4, troxerutin increased luciferase activity in the nHDFs transfected with the damaged vector.

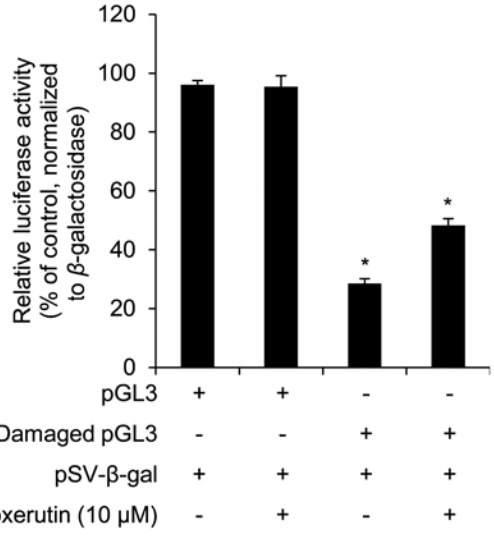

Figure 4. Troxerutin enhances the repair of DNA damage. Normal human dermal fibroblasts (nHDFs; $7x10^5$ cells) were seeded in 60-mm culture dishes and transfected with a damaged luciferase vector and pSV-β-gal. Following transfection, the cells were treated with 10 μM troxerutin for 24 h and DNA repair activity was determined by luciferase activity. A β-galactosidase assay was performed for normalization. The results are presented as the means ± SE of the percentage of luciferase activity in triplicate samples. Asterisks denote a significant difference compared with nHDFs transfected with the damaged pGL3 vector alone ($^*p<0.05$).

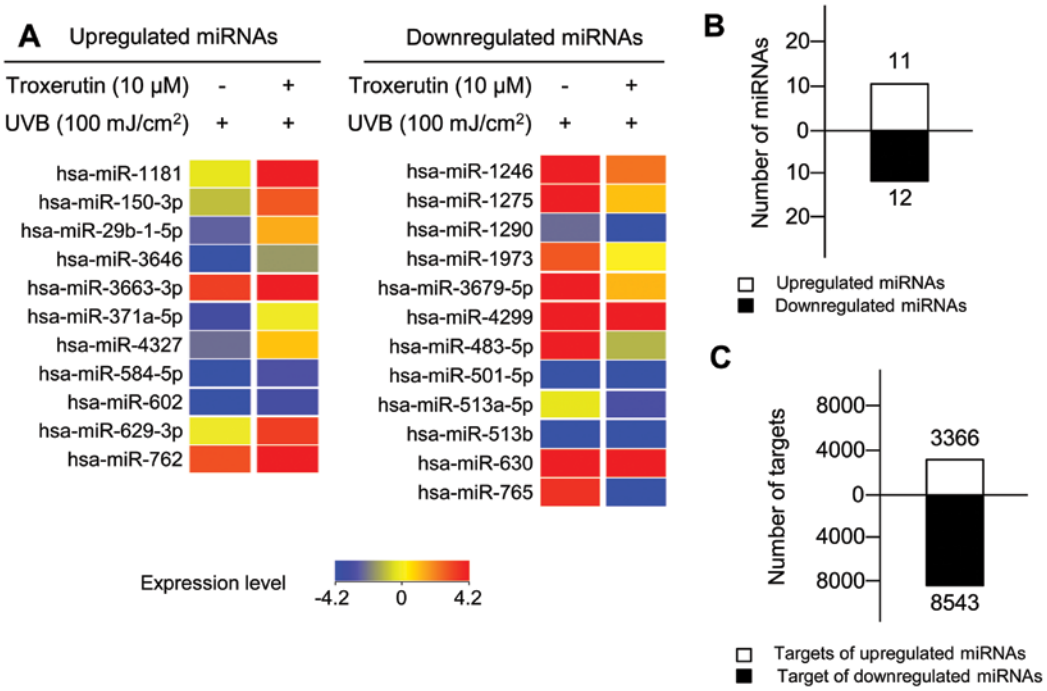

Figure 5. Troxerutin alters miRNA expression profiles. (A) Heat map of troxerutin-regulated miRNAs. Normal human dermal fibroblasts (nHDFs; $7x10^5$ cells) were seeded in 60-mm culture dishes, treated with troxerutin for 8 h, and then exposed to 100 mJ/cm^2 UVB. Follwoing treatment with troxerutin and exposure to UVB radiation, miRNA expression was determined using miRNA microarray. Expression levels are indicated in the legend bar. (B) Total number of miRNAs that were either up- or downregulated by troxerutin in nHDFs. (C) Total number of targets of miRNAs that were either up- or downregulated by troxerutin in nHDFs.

Table I. Troxerutin-induced upregulated miRNAs in UVB-exposed nHDFs.

Accession no.	miRNA	Fold change	Chromosome
MIMAT0005826	hsa-miR-1181	3.45	chr19
MIMAT0004610	hsa-miR-150-3p	2.82	chr19
MIMAT0004514	hsa-miR-29b-1-5p	2.95	chr7
MIMAT0018065	hsa-miR-3646	2.29	chr20
MIMAT0018085	hsa-miR-3663-3p	2.30	chr10
MIMAT0004687	hsa-miR-371a-5p	2.43	chr19
MIMAT0016889	hsa-miR-4327	2.54	chr21
MIMAT0003249	hsa-miR-584-5p	2.77	chr5
MIMAT0003270	hsa-miR-602	5.24	chr9
MIMAT0003298	hsa-miR-629-3p	2.51	chr15
MIMAT0010313	hsa-miR-762	3.60	chr16

Table II. Troxerutin-induced downregulated miRNAs in UVB-exposed nHDFs.

Accession no.	miRNA	Fold change	Chromosome
MIMAT0005898	hsa-miR-1246	-3.31	chr2
MIMAT0005929	hsa-miR-1275	-2.95	chr6
MIMAT0005880	hsa-miR-1290	-2.32	chr1
MIMAT0009448	hsa-miR-1973	-2.02	chr4
MIMAT0018104	hsa-miR-3679-5p	-3.20	chr2
MIMAT0016851	hsa-miR-4299	-2.42	chr11
MIMAT0004761	hsa-miR-483-5p	-10.23	chr11
MIMAT0002872	hsa-miR-501-5p	-2.38	chrX
MIMAT0002877	hsa-miR-513a-5p	-2.13	chrX
MIMAT0005788	hsa-miR-513b	-2.49	chrX
MIMAT0003299	hsa-miR-630	-3.86	chr15
MIMAT0003945	hsa-miR-765	-13.79	chr1

Identification of troxerutin-induced alterations in miRNA expression in UVB-exposed nHDFs and target prediction of these miRNAs. We identified 23 troxerutin-specific miRNAs whose expression was altered by >2-fold in the troxerutin-treated nHDFs (Fig. 5A and B), including 11 upregulated miRNAs (Table I) and 12 downregulated miRNAs (Table II). To elucidate the biological functions of the troxerutin-specific miRNAs, we identified putative targets using TargetScan, a seed sequence-based miRNA target prediction system. Since the miRNAs and miRNA target sites are conserved in various species (22), we performed TargetScan in the conserved database. Through TargetScan, we identified 3,366 targets of troxerutin-specific upregulated miRNAs and 8,543 targets of troxerutin-specific downregulated miRNAs (Fig. 5C).

Biological functions of putative targets of troxerutin-specific miRNAs. To identify the biological functions of the putative targets of miRNAs that were either up- or downregulated in response to troxerutin, we performed GO analysis using DAVID and obtained the GO distribution of the putative targets.

Table III. Gene Ontology (GO) analysis of potential target genes of troxerutin-regulated miRNAs.

GO no.	GO term	Percentage of count
Targets of upregulated miRNAs		
GO:0045449	Regulation of transcription	17.5
GO:0010604	Positive regulation of macromolecule metabolic processes	5.5
GO:0043067	Regulation of programmed cell death	5.2
GO:0007049	Cell cycle	5.0
Targets of downregulated miRNAs		
GO:0045449	Regulation of transcription	15.9
GO:0007242	Intracellular signaling cascade	7.6
GO:0006793	Phosphorus metabolic processes	5.9
GO:0008104	Protein localization	5.5
GO:0010604	Positive regulation of macromolecule metabolic processes	5.4
GO:0042127	Regulation of cell proliferation	5.0

As shown in Table III, GO analysis of the putative targets of troxerutin-specific upregulated miRNAs revealed the following distribution of biological functions: regulation of transcription (17.5%, percentage of GO term-related gene in the putative target pool), positive regulation of macromolecule metabolic processes (5.5%), the regulation of programmed cell death (5.2%) and cell cycle (5.0%). GO analysis of putative targets of troxerutin-specific downregulated miRNAs revealed the following distribution of biological functions: regulation of transcription (15.9%), intracellular signaling cascades (7.6%), phosphorus metabolic processes (5.9%), protein localization (5.5%), positive regulation of macromolecule metabolic processes (5.4%) and the regulation of cell proliferation (5.0%).

Discussion

In the present study, we investigated the protective effects of troxerutin against UVB radiation in nHDFs and showed that the beneficial effects of troxerutin were mediated by miRNAs. These findings provide new insight into the protective mechanisms against UVB-induced damage in dermal fibroblasts. UVB radiation is linked to the majority of skin disorders, including cancer, photoaging, sunburn and hyperpigmentation (23). In the dermis, UVB exposure induces disorders, such as inflammation and photoaging through cellular senescence and apoptosis (7,24-28). Thus, protection from UVB radiation is important to maintain the physiological functions of the dermis.

We first demonstrated that troxerutin markedly protected nHDFs from UVB-induced cell growth arrest and death, as indicated by the restoration of cell viability and an increase in the sub-G1 cell population following exposure to UVB radiation (Figs. 1B and 2). In addition, we demonstrated that troxerutin enhanced cell migration and increased DNA repair activity in the nHDFs (Fig. 4). Under the same conditions, our data indicated that troxerutin altered the expression of 23 miRNAs by at least 2-fold in UVB-exposed nHDFs.

The targets of several of these miRNAs have been reported in previous studies. The overexpression of miR-602 (5.24-fold upregulation by troxerutin) has been shown to induce proliferation and to target RASSF1A and p73, a member of the p53 family that represses proliferation and induces cell cycle arrest (29). miR-483-5p (downregulated 10.23-fold by troxerutin) has been shown to suppress proliferation through interference with ERK1 translation (30). miR-630 (3.86-fold downregulation by troxerutin) has been shown to target BCL2, BCL2L2, YAP1 and IGF-1R (31,32). BCL2 and BCL2L2, members of the pro-apoptotic BCL-2 family, repress intrinsic apoptosis by blocking the release of cytochrome *c* from the mitochondria (33). YAP1 is a co-transcription factor that increases the expression of growth-stimulated protein (34). Lastly, IGF-1R has been implicated in growth factor-mediated cell growth in various cells (31). In addition, in cisplatin-induced apoptosis, the expression of miR-630 has been shown to be increased by p63, a member of the p53 tumor suppressor family (32). Thus, it is reasonable to suggest that troxerutin mediates its UVB protective effects through the regulation of the expression of these miRNAs.

We then demonstrated biological function by GO analysis of the putative targets of troxerutin-specific miRNAs using DAVID (Table III). The putative targets of the troxerutin-specific upregulated miRNAs were involved in 'regulation of transcription', 'positive regulation of macromolecule metabolic processes', 'regulation of programmed cell death' and 'cell cycle'. Targets of troxerutin-specific downregulated miRNAs were involved in 'regulation of transcription', 'intracellular signaling cascade', 'phosphorus metabolic processes', 'protein localization', 'positive regulation of macromolecule metabolic processes' and 'regulation of cell proliferation'. In particular, GO analysis revealed that the protective effects of troxerutin against UVB-induced cell death were mediated by alterations in the expression of miRNAs that targeted genes within specific functional categories, including programmed cell death, cell cycle and cell proliferation.

Overall, our study provides information on a number of putative miRNA-target associations. Further studies are required to mechanistically explore the involvement of these miRNAs in the protective effects of troxerutin against UVB-induced cell damage in nHDFs. In addition, the discovery of previously

unidentified functional associations may lead to the development of novel therapeutic approaches in UVB-induced skin disorders.

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