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Inhibition of melanogenesis by tyrosinase siRNA in human melanocytes

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Tyrosinase (TYR) plays a critical role in cellular melanogenesis and, thus, has been the major target of pharmacological approaches for the control of skin pigmentation. This study examined an alternative molecular approach using TYR-small interfering RNA (siRNA) to control melanogenesis in the human melanocytes. Both the mRNA and protein levels of TYR were significantly lowered by TYR-siRNA treatment, whereas TYR-related protein 1 and TYR-related protein 2 displayed no such changes. TYR-siRNA treatment inhibited the cellular melanin synthesis from the externally supplied TYR substrate L-tyrosine. TYR-siRNA also suppressed melanin synthesis and decreased the viability of cells exposed to ultraviolet radiation, supporting a critical role of melanin in protection against ultraviolet radiation. These results suggest that molecular approaches using siRNA targeted to the enzymes of melanogenic pathway may provide a novel strategy for the control of cell pigmentation. [BMB reports 2009; 42(3): 178-183]

INTRODUCTION

Skin pigmentation in mammals is regulated by specialized cells termed melanocytes. Melanocytes express multiple enzymes that act in a cascade to synthesize melanin in melanosomes (1). Tyrosinase (TYR) initiates melanin synthesis by catalyzing the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (DOPA) and oxidation of DOPA to DOPAquinone. DOPAquinone is spontaneously oxidized to DOPA chrome. Tyrosinase-related protein 2 (TRP2) converts DOPAchrome to 5,6-dihydroxyindole-2-carboxylic acid. Tyrosinase-related protein 1 (TRP1) possesses 5,6-dihydroxyindole-2-carboxylic acid oxidase activity. Although these three and other enzymes are involved in melanin biosynthesis, TYR is major target of pharmacological approaches to control melanin synthesis. These approaches involve the regulation of TYR expression at the transcription and translation levels, TYR maturation via asparagine-linked oligosaccharide processing, TYR degradation and the modulation of TYR catalytic activity (2, 3). Our previous studies have demonstrated that plant constituents including p-coumaric acid inhibit cellular melanogenesis through inhibition of TYR expression and/or enzyme activity (4-6).

Although the hyper- or abnormal accumulation of melanin is an issue of cosmetic concern, melanin is an excellent photoprotectant against harmful ultraviolet (UV) radiation causing skin cancer development (7). Melanin absorbs UV radiation and dissipates the energy in the form of heat. In fact, artificial melanin synthesis by forskolin, which induces TYR expression by a cAMP-dependent mechanism, reduces the incidence of skin cancer in mice (8). Consistently, the frequency of malignant melanoma is significantly higher in lighter skinned people (9). Indeed, TYR activity is closely related with human skin color (10, 11). Therefore, specific regulation of TYR expression or melanin synthesis is an important issue in skin cancer research as well as in cosmetics.

RNA interference has emerged as a potent tool for post-transcriptional gene silencing (12, 13). Small interfering RNA (siRNA) assembles with proteins to form an RNA-induced silencing complex that targets complementary mRNA for degradation (14). Because this tool allows the specific down-regulation of the given gene and observation of the responses, it has been widely used in studies of cellular function of a specific gene (15). RNA interference has also been successfully used in melanogenic cells. For example, siRNA targeted to microphthalmia-associated transcription factor (MITF) down-regulates MITF and its transcriptional target, TYR (16). The siRNA-mediated specific down-regulation of TYR inhibits melanin synthesis in fish embryos (17). However, studies involving siRNA targeted to human TYR (TYR-siRNA) have not been published. The present study was undertaken to assess TYR-siRNA as a novel tool for the control of melanogenesis in human melanocytes.

RESULTS

Effect of TYR-siRNA on TYR expression at the mRNA level

TYR-siRNA with the nucleotide sequences corresponding to a part of the coding region of a human tyrosinase gene transcript (NCBI GenBank accession number, NM_000372) was used in

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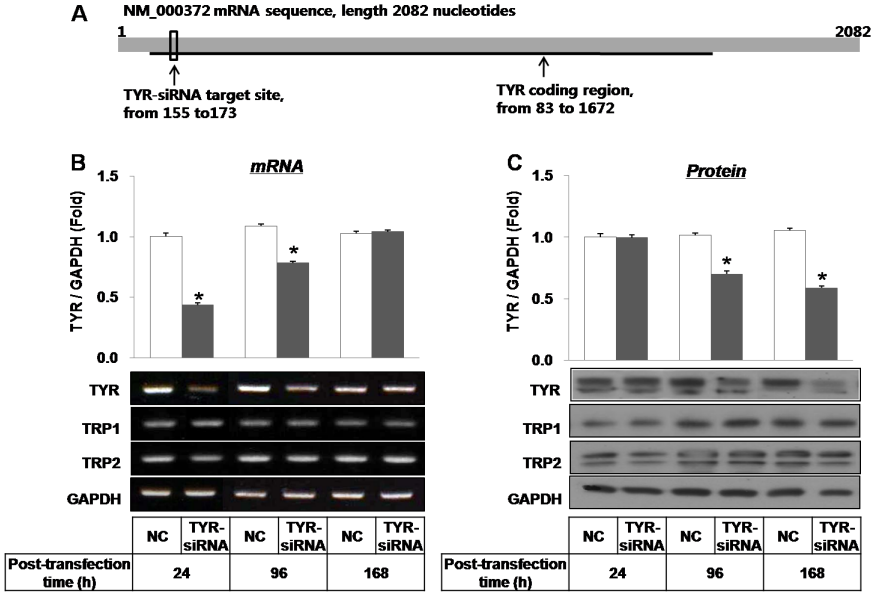

Fig. 1. Effect of TYR-siRNA on the expression of TYR, TRP1 and TRP2 at the mRNA and protein levels. (A) The TYR coding region and TRY-siRNA target site along with the gene transcript are shown. The numbers indicate the nucleotide position along the transcript sequence. HEMs were transfected with 20 nM TYR-siRNA or NC with scrambled sequences for 3 h and maintained in a growth medium for the indicated time. (B) Total cellular RNA was subjected to semi-quantitative RT-PCR analysis. Typical DNA gel images are shown. (C) Cell lysates were subjected to Western blotting analysis. Typical blot images are shown. The expression levels of TYR were normalized based on the GAPDH levels. Data are presented as relative fold changes (mean ± SE, n = 3). *P < 0.05 versus NC.

this study (Fig. 1A). To examine whether TYR-siRNA can selectively knockdown TYR gene expression, human epidermal melanocytes (HEMs) were transfected with TYR-siRNA or a negative control oligoribonucleotide duplex (NC), and change of TYR mRNA level was examined by reverse transcription-polymerase chain reaction (RT-PCR) in comparison with those of TRP1 and TRP2. Cells transfected with TYR-siRNA displayed a significantly lower level of TYR mRNA than NC cells (Fig. 1B). In contrast, the mRNA levels of TRP1 and TRP2 were not significantly affected by TYR-siRNA transfection indicating a selective inhibitory effect of TYR-siRNA on TYR expression. The effect of TYR-siRNA treatment on TYR mRNA level was transient, as expected. A maximal effect was noted the day following transfection, after which it lessened over time.

Effect of TYR-siRNA on TYR protein expression

The effect of TYR-siRNA on the expression of TYR, TRP1 and TRP2 protein was examined by Western blotting using specific antibodies. TYR-siRNA treatment led to a significant decline of TYR protein whereas the protein levels of TRP1 and TRP2 were unaltered (Fig. 1C). Together with the mRNA results, this result was consistent with a selective down-regulation of TYR expression by specific siRNA.

Effect of TYR-siRNA on melanin synthesis from L-tyrosine

Because TYR enzyme and its L-tyrosine substrate are required for cellular melanin synthesis, the effect of TYR gene silencing was examined in the absence and presence of L-tyrosine. HEMs transfected with TYR-siRNA or NC cells were maintained in a growth medium supplemented with vehicle or 1.0 mM L-tyrosine for 7 days. The cells were subjected to Fontana-Masson staining of melanin or used for spectrophotometric determination of intracellular melanin content. As shown in Fig. 2A, L-tyrosine caused apparent cell pigmentation which was prevented by TYR-siRNA. The L-tyrosine-mediated increase in intracellular melanin content was substantially abrogated by TYR-siRNA (Fig. 2B). The selective gene silencing effect of TYR-siRNA was confirmed by Western blotting (Fig. 2C).

Effect of TYR-siRNA on melanin synthesis and cell viability loss due to UV exposure

Taking advantage of the TYR-siRNA approach, we further sought to determine whether melanin is essential for the protection of cells against ultraviolet radiation. On the day after transfection with TYR-siRNA or NC duplex, cells were exposed to UV radiation (200 μW/cm^2 for 7.5-15 s) followed by incubation in a growth medium for 3 days. As expected, melanin synthesis was increased while viable cell number was decreased by UV exposure (Figs. 3A-3C). The TYR-siRNA treatment appeared to suppress melanin synthesis and decrease the viability of cells exposed to UV radiation (Figs. 3A-3C). The selective gene silencing effect of TYR-siRNA under this experimental condition was verified (data not shown).

DISCUSSION

Molecular approaches using siRNA allow highly selective gene silencing due to specific base pairing between the siRNA oligoribonucleotide and the target gene (12, 14). In addition, the gene specificity of siRNA can be conveniently predicted by BLAST search over the entire human genome. The oligoribonucleotide sequence of human TYR-siRNA presently used matched completely and solely with the human TYR gene. The selective gene silencing effect of TYR-siRNA was also ex-

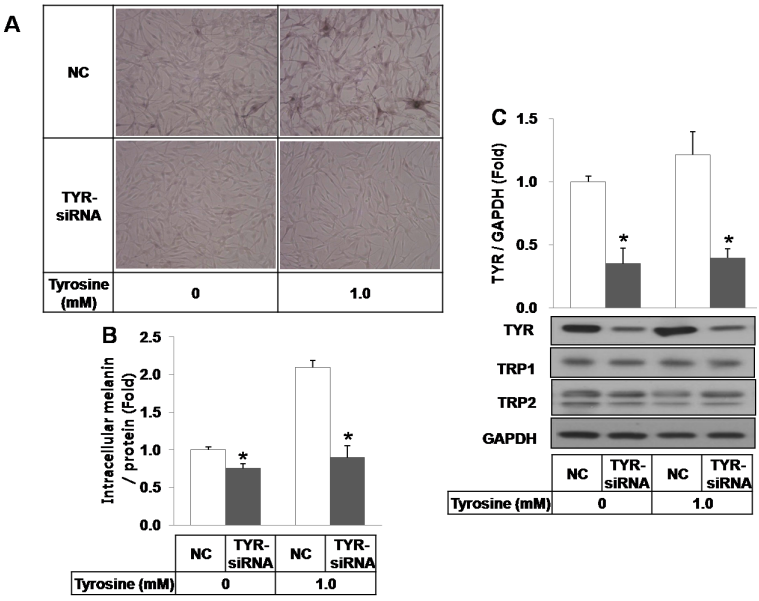

Fig. 2. TYR-siRNA inhibits cellular melanin synthesis from externally added L-tyrosine. HEMs were transfected with 20 nM TYR-siRNA or NC with scrambled sequences for 5 h and maintained in a growth medium with or without 1.0 mM L-tyrosine for 7 days. Cells were subjected to Fontana Masson staining of melanin (A) or spectrophotometric determination of intracellular melanin content (B). Melanin content was normalized for total protein content. (C) Expression of TYR, TRP1 and TRP2 at the protein levels was monitored by Western blotting; typical blot images from three independent experiments are shown. The TYR expression levels were normalized based on the GAPDH levels. Data are presented as relative fold changes (mean ± SE, n = 3). *P < 0.05 versus NC.

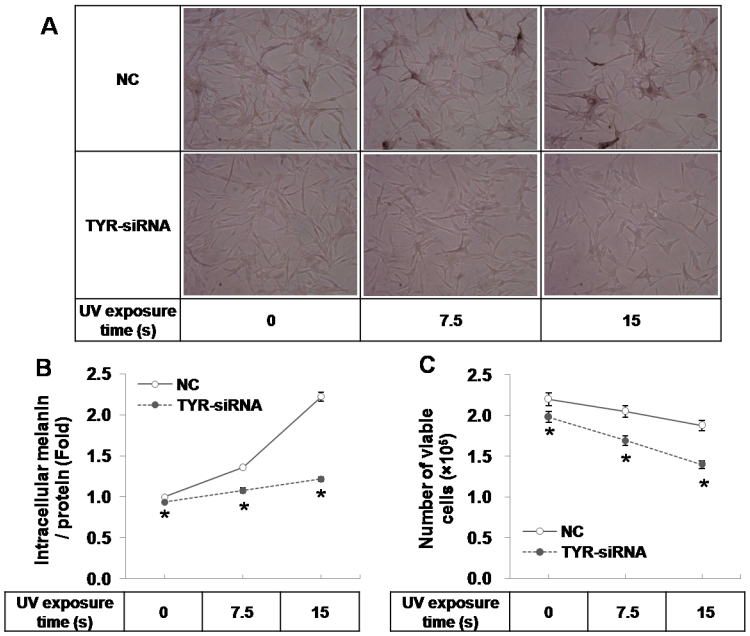

Fig. 3. TYR-siRNA decreases melanin synthesis and viability of cells exposed to UV radiation. HEMs were transfected with 20 nM TYR-siRNA or NC with scrambled sequences for 5 h and maintained in a growth medium for 1 day. Then, cells were exposed to UV radiation (200 μW/cm^2) for the indicated time, followed by incubation for 3 days. Cells were subjected to Fontana-Masson staining of melanin (A) or spectrophotometric determination of intracellular melanin content (B). Melanin content was normalized for total protein content and data are presented as relative fold changes. (C) Cell viability was determined by the exclusion of Trypan Blue. Data are presented as the means ± SE (n = 3). *P < 0.05 versus NC.

perimentally verified at the mRNA and protein levels (Fig. 1).

Gene silencing efficiency of siRNA in a particular cell type varies with turnover rates of the mRNA and protein of the target genes. In the case of TYR, the mRNA level was rapidly lowered by siRNA treatment while the protein level exhibited a rather slower change, indicative of a relatively slow turnover rate of TYR protein (Fig. 1). The latter seemed to be associated with the prolonged effect of TYR-siRNA suppression of melanin synthesis stimulated by L-tyrosine and UV radiation (Figs. 2 and 3).

In addition to genetic background of the organism (18), non-genetic factors including hormonal change, chronic inflammation, aging and UV radiation affect skin pigmentation by stimulating the expression of TYR and other enzymes involved in melanogenesis (19). Proopiomelanocortin-derived peptides including α-melanocyte stimulating hormone (α-MSH) are expressed, processed and secreted by various skin cells in response to UV radiation or inflammatory cytokines (20, 21). α-MSH acts on the G protein-coupled melanocortin 1 receptor

and stimulates adenylate cyclase, which leads to the production of cAMP that in turn leads to the activation of cAMP responsive element binding protein transcription factor (CREB) (22). CREB stimulates the expression of MITF (19), which in turn induces the gene expression of TYR and other enzymes involved in melanin synthesis (23).

Because UV radiation is cytotoxic, UV radiation-induced melanin synthesis can be regarded as an adaptive response. Presently, UV radiation caused cell viability loss while increasing melanin synthesis (Fig. 3). Cell viability loss was significantly increased by TYR gene silencing, which also suppressed melanin synthesis in response to UV radiation (Fig. 3). These results are consistent with the notion that melanin provides a protective mechanism against UV radiation (9). The minor cytotoxic effect of TYR-siRNA in the absence of UV radiation (Fig. 3), however, implies that melanin might play essential roles for cell survival that are not limited to UV absorption. Consistent with this notion, a recent study has demonstrated that the gene silencing of MITF in murine melanoma cells leads to the inhibition of melanin synthesis and the increase of apoptosis in the absence of UV radiation (16).

In conclusion, this study demonstrates the selective inhibitory effect of TYR-siRNA on TYR gene expression and melanin synthesis in human melanocytes. TYR-siRNA may provide a novel experimental tool for the control of skin cell pigmentation. However, its use as a hypo-pigmenting agent for cosmetic purposes would require careful consideration of other aspects of this approach because the depletion of TYR enzyme and the inhibition of melanin synthesis could lower cell survival, especially under UV radiation.

MATERIALS AND METHODS

Cell culture and treatments

HEMs derived from moderately pigmented neonatal foreskin were obtained from Cascade Biologics (Portland, OR, USA). The cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Growth medium was Medium 254 containing human melanocyte growth supplement (Cascade Biologics), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B. Cells treated with siRNA as described below were maintained in the growth medium for up to 7 days, with replacement of the medium every 3 days. When specified, cells were either supplied with L-tyrosine (Sigma-Aldrich, St. Louis, MO, USA) or exposed to UV radiation. UV treatment was done in a cell culture hood at a distance of 100 cm from Model G40T10 low-pressure mercury lamps (Sankyo Denki, Kanagawa, Japan). UV intensity at the surface of the culture plate was 200 μW/cm^2. Cell viability was determined by the exclusion of Trypan Blue in a standard procedure.

siRNA transfection

TYR-siRNA and NC cells with scrambled sequences were purchased from Bioneer (Daejeon, Korea). Their nucleotide sequences were: TYR-siRNA, 5′-GUC UCC UCU AAG AAC CUG A(dTdT)-3′ (sense) and 5′-UCA GGU UCU UAG AGG AGA C(dTdT)-3′ (antisense); NC, 5′-CCU ACG CCA CCA AUU UCG U(dTdT)-3′ (sense) and 5′-ACG AAA UUG GUG GCG UAG G(dTdT)-3′ (antisense). Transfection of cells with siRNA was performed using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA). In brief, HEMs were seeded on 6-well plates, grown for 1 day to reach 30-50% confluency, washed with Opti-MEM (Invitrogen) and treated with a mixture of 20 nM siRNA and 2 μl Lipofectamine RNAiMAX in 100 μl Opti-MEM for 3 h.

RT-PCR

PCR primer sets specific for TYR, TRP1, TRP2 and D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed using Primer3 software (http://frodo.wi.mit.edu/) (24) on the basis of sequences deposited in the NCBI GenBank database. The sequences of the primers (Bioneer; Daejeon, Korea) were: TYR (accession number, NM_000372), 5′-CTC AAA GCA GCA TGC ACA AT-3′ (sense) and 5′-GCC CAG ATC TTT GGA TGA AA-3′ (antisense); TRP1 (accession number, NM_000550), 5′-CCG AAA CAC AGT GGA AGG TT-3′ (sense) and 5′-TCT GTG AAG GTG TGC AGG AG-3′ (antisense); TRP2 (accession number, NM_001922), 5′-GGT TCC TTT CTT CCC TCC AG-3′ (sense) and 5′-AAC CAA AGC CAC CAG TGT TC-3′ (antisense); GAPDH (accession number, NM_002046) 5′-GCC AAA AGG GTC ATC ATC TC-3′ (sense) and 5′-GTA GAG GCA GGG ATG ATG TTC-3′ (antisense). Total cellular RNA was isolated using RNeasy mini kit (QIAGEN, Valencia, CA, USA) as per the manufacturer's instructions. RT-PCR was performed using GeneAmp® PCR system 9700 (Applied Biosystems, Foster City, CA, USA) in a 20 μl reaction mixture containing *Maxime* RT-PCR PreMix (iNtRON Biotechnology, Seongnam, Korea), 500 ng RNA and 20 pmole of gene-specific primer sets. RT for the first strand cDNA synthesis was performed at 45°C for 30 min followed by heat inactivation of reverse transcriptase at 95°C for 5 min. PCR was carried out for 32 cycles of 45 s at 95°C, 45 s at 55°C and 60 s at 72°C, with a final extension step of 5 min at 72°C. Preliminary PCR runs ascertained that the reactions were in the exponential phase suitable for the quantitative analysis of TYR, TRP1, TRP2 and GAPDH mRNAs. Amplification products were electrophoresed in an 1.2% agarose gel with a 100 bp DNA ladder as a size marker. The gel was ethidium bromide-stained and band intensities were quantified using a Gel Doc system (Bio-Rad Laboratories, Hercules, CA, USA).

Western blotting

Cells were washed twice with ice-cold phosphate buffered saline and resuspended in lysis buffer (10 mM Tris-Cl, pH 7.4, 120 mM NaCl, 25 mM KCl, 2 mM EGTA, 1 mM EDTA, 0.5% Triton X-100 and protease inhibitor cocktail) in a pre-cooled tube. After 45 min of incubation on ice, each homogenate was centrifuged at 15,000 × g for 15 min at 4°C to obtain the clear

cell lysate. Aliquots of the lysates containing 20 μg protein were then diluted in Laemmli buffer containing 2% SDS and 1.2% dithiothreitol, and heated at 95°C for 5 min to denature the proteins. Proteins were separated by 15% SDS-PAGE at 120 V for 2 h and the separated proteins were transferred to a polyvinylidene fluoride membrane. The membrane was incubated with a primary antibody overnight at 4°C , and then with a secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature. The bands were detected using Amersham enhanced chemiluminescence (ECL) Western blotting detection reagents (GE Healthcare, Chalfont St Giles, Bucks, UK) according to the manufacturer's instruction and subjected to densitometry analysis. Primary antibodies for TYR, TRP1, TRP2 and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Determination of intracellular melanin content

For the extraction of intracellular melanin, cells were lysed in 0.1 M NaOH at 60°C for 60 min. Cell lysates were centrifuged at 15,000 × g for 10 min and the clear supernatant was used for the optical density determination of melanin content at 490 nm. Melanin content was normalized for protein content, which was determined by the Bio-Rad DC assay (Bio-Rad).

Fontana-Masson staining

Cells were fixed in 4% paraformaldehyde for 10 min at room temperature and stained for melanin using a Fontana-Masson staining kit from American Master*Tech Scientific (Lodi, CA, USA) according to the manufacturer's instructions. In brief, cells were stained with an ammoniacal silver solution for 30 min at 60°C, incubated in 0.1% gold chloride and then in 5% sodium thiosulfate. Cell morphology and pigmentation were examined using an Eclipse TS100 phase-contrast microscope (Nikon Instruments, Melville, NY, USA).

Statistical analysis

Data are expressed as means ± SE from a minimum of three experiments. Statistical analysis was performed by unpaired Student t-test. A P value < 0.05 was considered statistically significant.

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