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INVESTIGATIVE REPORT

Light-emitting Diodes at 830 and 850 nm Inhibit Melanin Synthesis *In vitro*

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Treatment of hyperpigmentation remains a challenge. Because of the positive effects of low-energy Nd:YAG lasers on the treatment of melasma, it is suggested that laser-like light-emitting diodes (LEDs) can potentially ameliorate hyperpigmentation. We evaluated the effect of seven different LED wavelengths on melanogenesis. LED irradiation at 830 nm (dose-dependent, from 1 to 20 J/cm^2) and 850 nm (1 J/cm^2) significantly reduced melanin production and tyrosinase expression, not only in a normal human melanocyte monoculture both with and without forskolin stimulation but also in a three-dimensional multiple cell type culture. It reduced melanin content via inactivation of the apoptosis signal-regulating kinase and extracellular signal-regulated kinase 1/2 pathways. The level of phosphorylated cyclic AMP response element-binding protein was also decreased by LED irradiation. Moreover, LED irradiation reduced melanogenesis through decreased expression of tyrosinase family genes (tyrosinase-related protein-1 and 2, and microphthalmia-associated transcription factor). These results indicate that LEDs could potentially be used to treat melanin-overproducing skin conditions. ***Key words: LED; reduced melanogenesis, 830 nm, melonocyte.***

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Hyperpigmentation results from an increase in melanin. Melanin biosynthesis is regulated by the melanogenic enzymes, such as tyrosinase, tyrosinase-related protein-1 (TRP-1), tyrosinase-related protein-2 (TRP-2) and microphthalmia-associated transcription factor (MITF) (1). Tyrosinase is a key enzyme in melanogenesis that catalyses the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to DOPA quinone (2). TRP-1 (5,6-dihydroxyindole-2-carboxylic acid [DHICA] oxidase) oxidizes DHICA to a carboxylated indolequinone, and TRP-2 (dopachrome tautomerase) catalyses the dopachrome to DHICA (3). Another important regulator, MITF, regulates the transcription of tyrosinase, TRP-1 and TRP-2 (4) via the activation of cyclic AMP (cAMP), including protein kinase A (PKA) and cAMP response element-binding (CREB) protein. cAMP activates the extracellular signal-regulated kinase (ERK) pathway and synthesizes melanin in melanocytes (5).

An essential role of melanin is preventing ultraviolet (UV) light-induced skin damage. Overexposure to UV radiation can lead to a pathological increase in melanin production. However, cellular reactions exhibit specificity to irradiation wavelengths (6). In fact, the selection of an appropriate wavelength from a variety of light sources is fundamental to phototherapy, which is an effective and useful therapy for a wide range of medical conditions. The benefits of light therapy have been revealed using other segments of the electromagnetic radiation spectrum with visible and near-infrared wavelengths. With the identification of photobiostimulation having effects other than carcinogenic potential, in addition to lasers, light-emitting diodes (LEDs) have been applied to treat various medical conditions (7). LEDs produce laser-like, but non-coherent and quasi-monochromatic, output with bandwidths as little as ±3 nm from the rated wavelength. When mounted in arrays, LEDs can deliver clinically useful incident energy densities (8). Beneficial effects of LED phototherapy have also been noted on certain skin diseases, such as acne vulgaris (12–15), and in wound healing (16). Interestingly, LEDs have been shown to improve UV-damaged skin conditions, including photo-aging (9–11), suggesting that the mechanism of action between LEDs and UV radiation is different. However, the inhibitory effect of LEDs on melanogenesis has rarely been mentioned.

In the present study, we determined the LED wavelength that effectively inhibits melanin biosynthesis and its molecular mechanisms underlying its effects on tyrosinase, TRP-1, TRP-2, and MITF protein expression with an intracellular signalling pathway using cultured human melanocytes. To examine the corresponding effects on skin, three-dimensional (3D) co-culture systems composed of melanocytes, keratinocytes, and fibroblasts were used, because neighbouring keratinocytes and fibroblasts may exhibit cellular interactions related to hyperpigmentation.

MATERIALS AND METHODS

Light sources and treatment

The LED device used (Electronics and Telecommunications Research Institute, Daejeon, Korea) produces light at wavelengths of 415, 530, 630, 660, 830, 850 and 940 nm. The emission spectrum of the peak wavelength was confirmed by referring to the datasheets describing the characteristics of the LED lamps that are commercially available (Table I). To minimize heat generation, the LED modules have heat sinks, such as electric cooling fans, at the rear of the arrayed lamps.

All cultures were irradiated with an LED at 415 nm (54.8 mW/cm^2), 530 nm (15.8 mW/cm^2), 630 nm (22.2 mW/cm^2), 660 nm (17.1 mW/cm^2), 830 nm (96 mW/cm^2), 850 nm (114 mW/cm^2) and 940 nm (55.5 mW/cm^2). To avoid absorption by the coloured culture medium, the cells were rinsed twice with phosphate-buffered saline (PBS) and irradiated 2 cm away from the LED arrays. Protective eyewear was worn for all treatments (17).

Normal human epidermal melanocyte culture

Skin specimens obtained from repeated Caesarean sections and circumcisions were used for cultures. The epidermis was separated from the dermis after a 1-h treatment with 2.4 U/ml dispase (Roche, Mannheim, Germany). The cells were suspended in Medium 254 (Cascade Biologics, Portland, OR, USA) supplemented with bovine pituitary extract, foetal bovine serum (FBS), bovine insulin, hydrocortisone, basic fibroblast growth factor (bFGF), bovine transferrin (Cascade Biologics), heparin and phorbol myristate acetate (Cascade Biologics) at 37ºC in a humidified atmosphere containing 5% CO_2.

Mixed-cell 3D culture

Culturing was performed using the collagen gel model, as described previously (18). Briefly, primary cultured human fibroblasts (5×10^5 cells) suspended in 1 ml collagen matrix (Invitrogen, Frederick, MD, USA) were seeded onto the outer six-well culture dish (Corning Inc., Corning, NY, USA) and incubated at 37ºC for 1 h. Mixtures of cultured melanocytes (2.5×10^5 cells) and keratinocytes (5×10^5 cells) were then seeded onto BioCoat cell culture inserts (Corning Inc.). The mixed cells were cultured in a mixture of keratinocyte growth medium (KGM; 2.9 ml) and the above-mentioned melanocyte growth medium (0.1 ml). The KGM was composed of EpiLife Medium (Cascade Biologics) supplemented with bovine pituitary extract, bovine insulin, hydrocortisone, human epidermal growth factor and bovine transferrin (Cascade Biologics).

Melanin content assay

Primary cultured human melanocytes were seeded in a 60-mm culture dish at a density of 5×10^5 cells/well. The cells were irradiated with different LED wavelengths, and then incubated for 40 h. The irradiated cells were dissolved in 1 N NaOH at 70ºC for 1 h, and the relative melanin content was measured at an absorbance of 475 nm with the Spectra Max 384 PLUS microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Assessment of cell viability

Human melanocytes (5×10^3 cells/well) were seeded on 12-well plates. The next day, the cells were irradiated with LEDs at different wavelengths and subsequently incubated for 48 h. The ratio of cytotoxicity was assessed using an MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for 4 h, following which the precipitated formazan was dissolved with dimethyl sulphoxide, and the optical density was measured at 540 nm using a spectrophotometer. The optical relative data are presented as percentages relative to the control (mean ± SD).

Western blot analysis

Blots were probed using primary antibodies against ERK1/2 and phosphorylated ERK1/2 (1:1000; Cell Signaling, Beverly, MA, USA), AKT and phosphorylated AKT (1:1000; Cell Signaling), tyrosinase (1:1000; Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA) or β-actin (1:1000; Santa Cruz Biotechnology Inc.). Tyrosinase family genes, including TRP-1, TRP-2 and MITF (1:1000; Santa Cruz Biotechnology Inc.), were also blotted. The intensities of the bands were measured by LAS-3000 (Fujifilm, Tokyo, Japan).

Reverse-transcription PCR

PCR amplification was conducted using a DNA Thermal Cycler 9600 (Applied Biosystems, Foster City, CA, USA) using the following settings: 35~40 cycles at 94ºC for 1 min, 55ºC~62ºC for 1 min, and 72ºC for 1 min. The primers used were as follows: MITF-M, 5′-ATGCTGGAAATGCTAGAATATAAT-3′ (forward) and 5′-ATCATCCATCTGCATACAG-3′ (reverse); Trp-2, 5′-GCACACATGTAACCTCTGTG-3′ (forward) and 5′-TCATATAAGCAGGCTTGGCC-3′ (reverse); Trp-1, 5′-TGGCAAAGCGCACAACTCACCC-3′ (forward) and 5′-AGTGCAACCAGTAACAAAGCGCC-3′ (reverse); tyrosinase, 5′-TGGCATAGACTCTTCTTGTTGCGG-3′ (forward) and 5′-CAAGGAGCCATGACCAGATCCG-3′ (reverse); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5’-TCCACCACCCTGTTGCTGTA-3′ (forward) and 5′-ACCACAGTCCATGCCATCAC-3′ (reverse).

Statistical analysis

Student’s *t*-test was used for comparisons. $p < 0.05$ was considered statistically significant. Statistical analyses of data were performed using SPSS for Windows software (version 15.0; SPSS Inc., Chicago, IL, USA).

RESULTS

LED wavelengths of 830, 850 and 940 nm inhibit the production of tyrosinase in primary cultured human melanocytes

Before the effects of LED irradiation on tyrosinase expression were examined, the cytotoxic effects of LED irradiation were determined by an MTT assay. When primary cultured human melanocytes were irradiated with various LED wavelengths (415, 530, 630, 660,

Table I. *The peak wavelength is the same as the number in the names of the fabricated light-emitting diode (LED) modules. The full width at half-maximum (FWHM) of the LED light sources ranged from 15 nm to 50 nm depending on the wavelength of the LED module. In general, the FWHM of a short wavelength LED is narrow*

Fabricated LED modules	LED415	LED530	LED630	LED660	LED830	LED850	LED940
Peak wavelength (nm)	415	535	630	660	830	860	940
FWHM (nm)	15	35	20	18	40	40	50

830, 850 and 940 nm), none of the wavelengths, with the exception of 415 nm, exerted cytotoxic effects (Fig. S1; available from: http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1319).

The tyrosinase expression in melanocytes was significantly ($p < 0.05$) reduced by LED irradiation at 830 nm in a dose-dependent manner and at 850 and 940 nm with low energy (1 or 2 J/cm^2) compared with non-irradiated cells (control, arbitrary value of 1) (Fig. 1).

Forskolin-stimulated melanogenesis is decreased by LED irradiation at 830, 850 and 940 nm

Because 830 nm (20 J/cm^2), 850 nm (1 J/cm^2) and 940 nm (2 J/cm^2) wavelengths inhibited tyrosinase expression in the cultured cells without stimulation of melanogenesis, the effects of these wavelengths were examined under melanogenesis stimulation. In addition to UV and α-melanocyte-stimulating hormone (19), melanogenesis can be stimulated by forskolin (10 μM) (20), which was used in this study. After stimulation with forskolin, irradiation at the above-mentioned three wavelengths clearly reduced the melanin contents in the cell pellets (Fig. 2A). The absorbance was also significantly ($p < 0.05$) decreased at these three wavelengths compared with the control group (Fig. 2A) without influencing cell viability (data not shown). Tyrosinase contents also decreased significantly ($p < 0.05$) with irradiation at the aforementioned three wavelengths compared with the forskolin-treated controls. The tyrosinase was reduced more by repeated irradiation (Fig. 2B). In addition, LED irradiation at these wavelengths significantly ($p < 0.05$) inhibited tyrosinase activity (L-DOPA content and L-DOPA stainability), although the tyrosinase activities reduced by irradiation were not down to values of the forskolin non-treated control (Figs 2C and D).

LED irradiation reduces tyrosinase family gene expression

Because LED irradiation decreased melanin synthesis and tyrosinase expression, the expression of tyrosinase family genes was examined in the human melanocytes treated with forskolin. Irradiation at 830 nm (20 J/cm^2) and 850 nm (1 J/cm^2) significantly ($p < 0.05$) decreased the protein expression of MITF, TRP-1 and TRP-2 compared with the forskolin-treated controls (Fig. 3A). On the other hand, irradiation at 940 nm did not significantly reduce the expression level of the tyrosinase family genes (Fig. 3A). In addition to protein expression, the mRNA expression of tyrosinase, MITF, TRP-1 and TRP-2 also significantly ($p < 0.05$) decreased by LED irradiation at 830 nm (20 J/cm^2) and 850 nm (1 J/cm^2), whereas irradiation at 940 nm (2 J/cm^2) did not (Fig. 3B).

LED reduces melanogenesis via the cAMP, AKT and ERK1/2 signalling pathways

Tyrosinase activates melanogenesis through the stimulation of the cAMP and ERK1/2 pathways (21); therefore, we examined whether the irradiation influenced the activation of ERK1/2, CREB and AKT after forskolin stimulation, which is a cAMP-dependent protein kinase A- and ERK1/2-activator (21). Phosphorylation of CREB significantly ($p < 0.05$) decreased upon irradiation with LED wavelengths of 830 nm (10 J/cm^2 and 20 J/cm^2), 850 nm (1 J/cm^2) and 940 nm (2 J/cm^2) compared with the forskolin-treated controls (Fig. 3C). ERK1/2 activation was also significantly ($p < 0.05$) inhibited by LED irradiation at 830 and 850 nm, but not at 940 nm (Fig. 3C). In addition, the activation of AKT was significantly ($p < 0.05$) inhibited by LED irradiation at 830 nm (20 J/cm^2) (Fig. 3C).

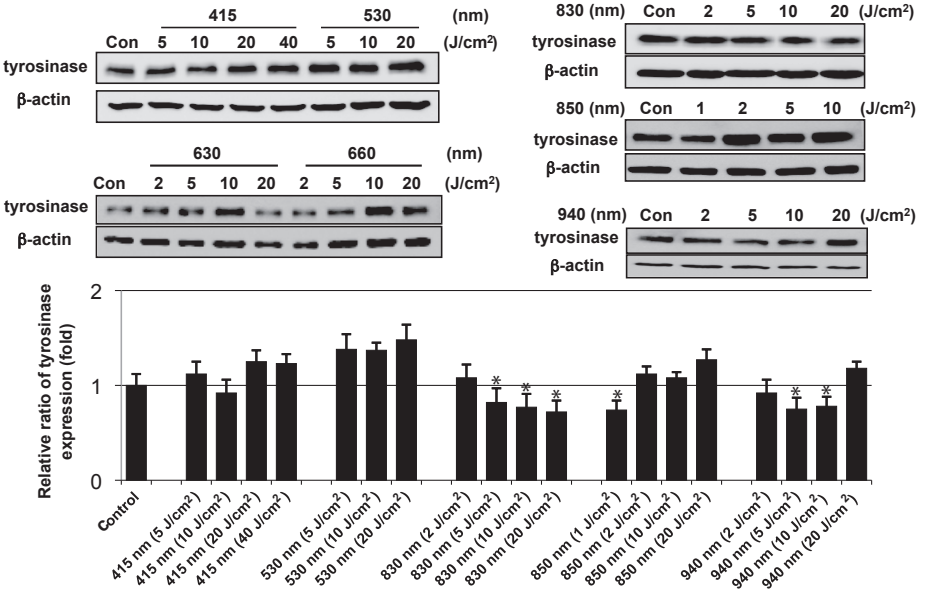

Fig. 1. Wavelength of light-emitting diodes (LED) effective on tyrosinase expression in human melanocytes, as determined using various wavelengths by western blot analysis. Data are expressed as ratios relative to the non-irradiated controls and presented as the mean ± SD of three separate experiments. *p<0.05 compared with control.

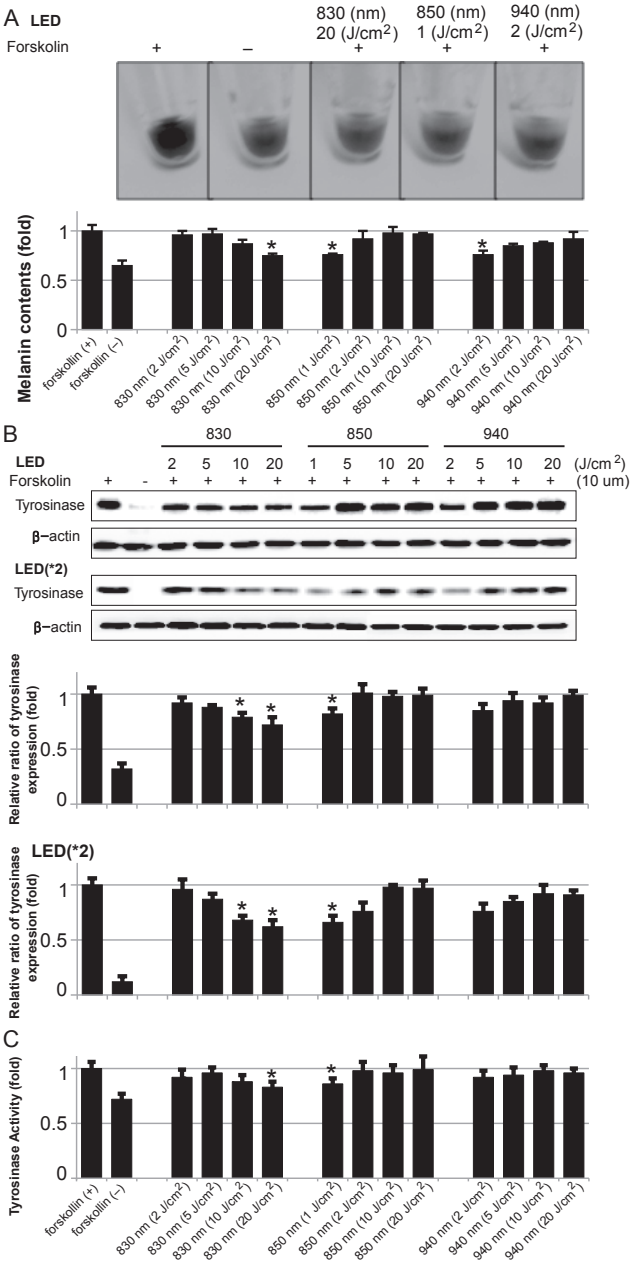

Fig. 2. Effects of various light-emitting diode (LED) wavelengths on melanogenesis with or without stimulation by 10 μM forskolin were evaluated in human melanocytes. Data are expressed as ratios relative to the forskolin-treated controls and presented as the mean ± SD of three separate experiments. $*p < 0.05$ compared with the control. (A) Melanin contents in pellets were determined by absorbance at 475 nm with the Spectra Max 384 PLUS microplate reader. (B) Western blot analysis of tyrosinase expression after single exposure to LED at wavelengths (*upper*). Tyrosinase expression after repeated irradiation with the same LED wavelengths (*lower*). Effects of LED wavelengths on cellular tyrosinase activity were determined according to L-DOPA oxidase activity (C) and L-DOPA stainability (D; available from: http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1319) in melanocyte cells.

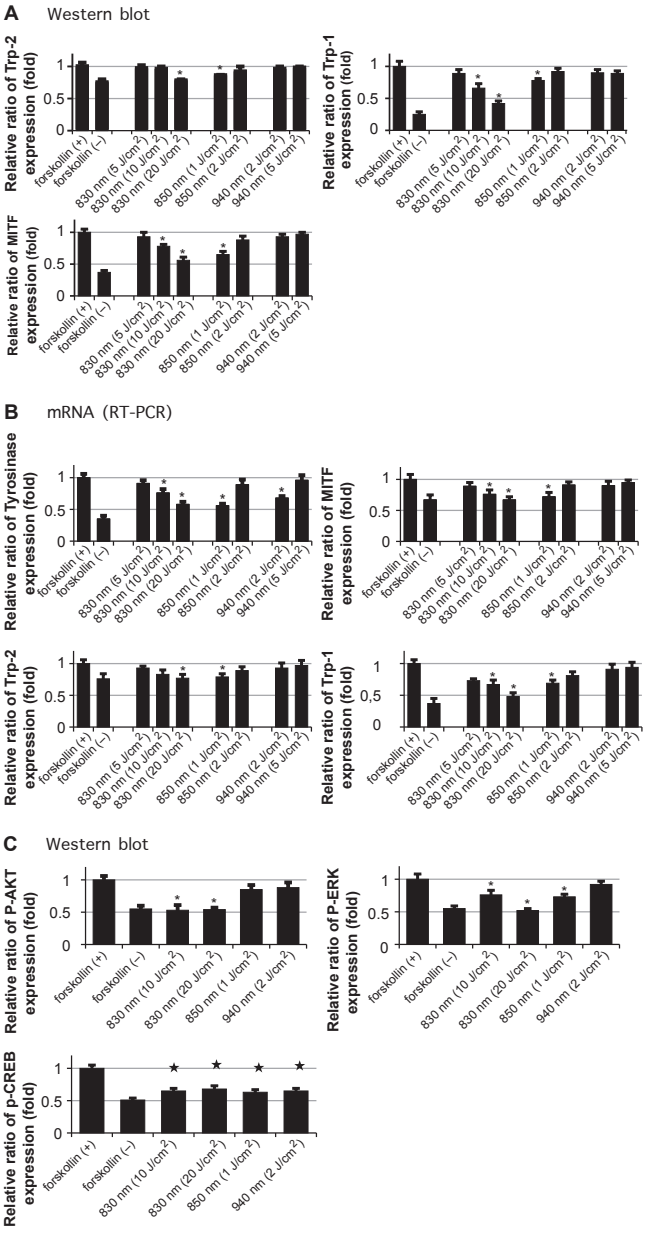

Fig. 3. In the presence of 10 μM forskolin, human melanocyte cells were irradiated with the indicated light-emitting diode (LED) wavelengths. Data are expressed as ratios relative to the forskolin-treated controls and presented as the mean ± standard deviation of 3 separate experiments. $*p < 0.05$ compared with the control. (A) Western blot analysis using anti-tyrosinase-related protein-1 (TRP-1), tyrosinase-related protein-2 (TRP-2) and microphthalmia-associated transcription factor (MITF) antibodies. β-actin was used as an internal control. (B) Results of reverse-transcriptase (RT)-PCR for the levels of the mRNA expression of tyrosinase family genes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a reference gene. (C) Inhibitory effects of LEDs on forskolin-induced cyclic AMP (cAMP), extracellular signal-regulated kinase (ERK) and AKT levels examined by western blot analysis. The complete figure is available from http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1319.

LED inhibits melanogenesis in co-culture systems

To determine whether the inhibitory effects of LED irradiation at 830 nm (dose-dependent), 850 nm (1 J/cm^2) and 940 nm (2 J/cm^2) on tyrosinase and melanin synthesis in human melanocyte monocultures occur in a skin equivalent, we used 3D mixed cell-culture system, as described in the Materials and Methods section (Fig. 4A). The levels of tyrosinase expression were significantly ($p < 0.05$) reduced by LED irradia-

tion at all the aforementioned wavelengths, compared with the non-irradiated controls (Fig. 4B). However, the expressions of TRP-1 and TRP-2 were significantly ($p < 0.05$) inhibited by irradiation at 830 nm and 850 nm, compared with the non-irradiated controls (Fig. 4B). Moreover, MITF expression was significantly ($p < 0.05$) inhibited only by irradiation at 830 nm, compared with the non-irradiated controls (Fig. 4B).

DISCUSSION

The results of this study show that various LED wavelengths can affect melanogenesis in normal human melanocytes. When the samples were exposed to the LED light source, we considered the irradiation dose in J/cm^2. The unit mW/cm^2 represents the optical power of the LED light source measured by a detector. The irradiation dose was calculated as follows: J/cm^2 = optical power × time (s). A long exposure time is required for low-power LEDs (e.g. 15.8 mW/cm^2). By adjusting the exposure time, the irradiation dose produced by LEDs at different wavelengths and optical power was standardized for all samples.

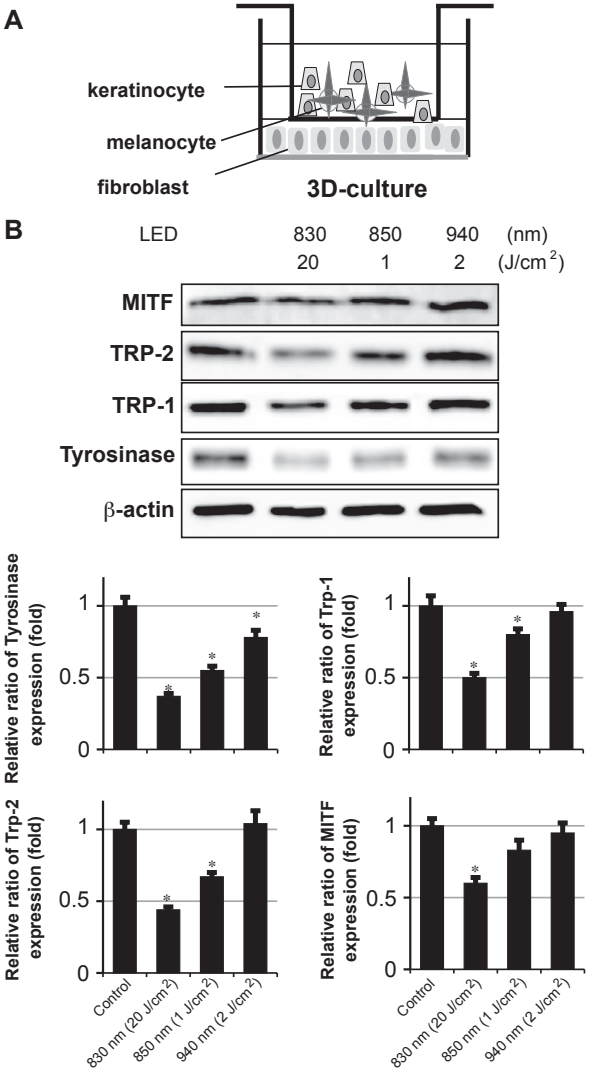

Fig. 4. (A) Three-dimensional (3D) multiple cell type co-culture system, including human melanocytes, fibroblasts and keratinocytes, for skin-equivalent conditions, as described in the Materials and Methods section. (B) The expression levels of tyrosinase and its related genes evaluated by western blotting after light-emitting diode (LED) irradiation at 830, 850 and 940 nm. Values shown are the mean ± standard deviation of 3 independent experiments. *$p < 0.05$ compared with the control.

According to the present study, LED irradiation at 830, 850 and 940 nm decreased tyrosinase expression (Fig. 4) without exerting any cytotoxic effects (Fig. S1). Although the inhibitory power differed depending on the wavelength and irradiation dose, it was similar in both the presence (Fig. 2B) and absence (Fig. 1) of forskolin. Dose-dependent reduction of the expression levels of tyrosinase protein (Fig. 2B) and mRNA (Fig. 3B) occurred with LED irradiation at 830 nm up to 20 J/cm^2, but not at 850 or 940 nm. Although melanogenesis was inhibited at lower doses in case of irradiation at 850 and 940 nm, repeated exposure to the same doses at these wavelengths reduced tyrosinase expression more remarkably than by a single exposure (Fig. 2B); this can be attributed to the synergistic or combined effects of repeated irradiation.

In addition to tyrosinase expression levels, tyrosinase activity levels, which were examined with respect to the levels of L-DOPA production (2), showed a similar, although less remarkable, pattern in response to irradiation (Fig. 2C). Irradiation at 830 nm reduced the activity only with a dose of 20 J/cm^2, although irradiation at 850 nm reduced the activity with the same dose that inhibited the expression level. Irradiation at 940 nm did not result in a significant change in tyrosinase activity (Fig. 2C). The inhibition of TRP-1, TRP-2 and MITF gene expression was also less sensitive compared with that of tyrosinase. Similar to the results of tyrosinase activity, LED irradiation at 830 nm (20 J/cm^2) and 850 nm (1 J/cm^2) showed a significant inhibition of the expressions of TRP-1, TRP-2 and MITF at both protein (Fig 3A) and mRNA levels (Fig. 3B), whereas irradiation at 940 nm did not. Although irradiation at 940 nm reduced tyrosinase synthesis, it may inhibit melanin synthesis less remarkably than irradiation at other wavelengths.

The activation levels of ERK1/2, CREB and AKT were examined because they are the signalling pathways involved in melanogenesis. The phosphorylation of CREB decreased at all three wavelengths (Fig. 3C). CREB protein is a common downstream target for melanocyte differentiation and proliferation. CREB leads to the upregulation of MITF expression (22), and MITF upregulates the expressions of tyrosinase, TRP-1 and TRP-2 (23, 24). Therefore, proper doses of irradiation with all three wavelengths were expected to have inhibitory effects on MITF, TRP-1 and TRP-2. However, irradiation at 940 nm did not significantly reduce the activations of TRP-1, TRP-2 or MITF, which is similar to its effects on ERK1/2 activation. ERK1/2 activation

was not inhibited at 940 nm, whereas it was at 830 and 850 nm (Fig. 3C). Although the activation of ERK1/2 is sequentially connected to the activation of CREB protein (25), the mechanism and implications of the insignificant inhibition of tyrosinase-related genes at 940 nm are unclear. Interestingly, the results for tyrosinase and its related genes in the monolayer cell culture were similar in the 3D mixed-cell culture system (Fig. 4A), in that the tyrosinase and tyrosinase family genes were significantly inhibited at 830 and 850 nm, while only tyrosinase was inhibited at 940 nm (Fig. 4B). Taken together, the results suggest that LED wavelengths of 830 and 850 nm could be considered useful for ameliorating skin conditions involving hyperpigmentation. However, it is unclear why only irradiation at 830 nm, but neither 850 nor 940 nm, significantly inhibited the activation of AKT, whose pathway is also involved in survival and differentiation of melanocytes (25).

In conclusion, we demonstrated that LED irradiation at wavelengths of 830, 850 and 940 nm effectively reduce melanin synthesis, not only in a normal human melanocyte monoculture, but also in a 3D multiple cell type co-culture model, without any cytotoxic effects. Therefore, these LED wavelengths, particularly 830 nm, might be helpful therapeutic tools for treating patients with hyperpigmentation. However, further experiments, including animal testing, clinical efficiency and safety evaluations, are required to identify all the effects.

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The authors declare no conflicts of interest.

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