Appendix S1

MATERIALS AND METHODS

Patients

Eleven female melasma patients (age range 38–60 years, mean age 49.8 years) with cadherin11 (CDH11) upregulation were included in the present study. The study was approved by the Institutional Review Board of Dongguk University Ilsan Hospital and conducted according to the principles of the Declaration of Helsinki. After obtaining informed written consent from each patient, pairs of hyperpigmented and adjacent normally pigmented skin specimens located on the lateral side of the upper cheek were biopsied with a 3-mm diameter punch for immunohistochemistry or real-time PCR. Skin specimens from 7 patients were used for histological and immunohistochemical analyses, and those from the other 4 were for real-time PCR.

Normal human epidermal cell culture

Adult skin specimens obtained from Caesarean sections and circumcisions were used to establish cells in culture. The epidermis was separated from the dermis, and suspensions of individual epidermal cells were prepared.

For the keratinocytes culture, the individual cells were suspended in EpiLife Medium (#M-EPI-500-CA; Invitrogen, Carlsbad, CA, USA) supplemented with bovine pituitary extract (BPE), bovine insulin (BI), hydrocortisone, human epidermal growth factor, and bovine transferrin (BT) (#S-001-5; Invitrogen). The cells were used from passages 3 or 5 only. For fibroblasts, individual dermal cells were suspended in DMEM (Gibco/BRL, Grand Island NY, USA) supplemented with 10% foetal bovine serum (Gibco/BRL), 100 U/ml penicillin (Gibco/BRL), and 0.1 mg/ml streptomycin (Gibco/BRL). Cells were used at passage numbers between 5 and 10. All procedures were performed 3 times for each of the 3 cell lines, and thus a total of 9 procedures were performed for each experiment.

CDH11 overexpression and knockdown

For CDH11 overexpression, the transfection of cells with pCMV containing the CDH11 gene (human clone; ORIGENE, Rockville MD, USA) was carried out using Lipofectamine 2000 according to the manufacturer's protocol. For CDH11 downregulation, cultured cells in 6-well plates were transfected with 100 nM or 50 nM siRNA for human CDH11 or a negative control (ON-TARGETplus SMARTpool or Non-targeting siRNA; Dharmacon, Lafayette CO, USA) using the TransIT-siQUEST transfection reagent (Mirus, PanVera, Madison WI, USA) according to the manufacturer's instructions. The cells were used for the experiments 24 h post-transfection.

UV light source

The NB-UVB light source for cultured cells was a COSMOLUX N-UVB 1000 U apparatus (Choyang Medical, Seongnam, South Korea) equipped with the UVB Narrowband TL lamps (Royal Philips, Amsterdam, The Netherlands). The lamps emitted a range of wavelengths between 305 and 315 nm with peak at 311 nm. The irradiance at the 30 cm distance was 3.5 mW/cm^2.

Real-time PCR

The levels of mRNA expression relative to glyceraldehyde-3-phosphate dehydrogenase were measured by quantitative PCR using the LightCycler Real-Time PCR System (Roche, Mannheim, Germany). The primers used were as follows: collagen type IVα1 5-GGGAGAAAAGGGTGAAGCA-3 (forward) and 5-CCAAAGGTCCTGTGCCTATAA-3 (reverse); collagen type IVα2 5-GAGAAGGCGCACAACCAG-3 (forward) and 5-CAGTACAGGGGCATGG-3 (reverse).

Western blot analysis

Equal amounts of extracted proteins (20 mg) were resolved using 10% acrylamide sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and were subsequently transferred to nitrocellulose membranes. The membranes were incubated with antibodies against collagen I, MMP-3, MMP-9, TIMP1, TIMP2 (goat polyclonal; Santa Cruz Biotechnology, Santa Cruz CA, USA), CDH11, MMP-1, MMP-2, VEGF-A, VEGF-D (rabbit polyclonal; Santa Cruz Biotechnology), collagen IV (mouse monoclonal; Dako, Santa Clara CA, USA), β-actin (mouse monoclonal; Sigma, St Louis, MO, USA). After washing, the membranes were incubated further with anti-rabbit or anti-mouse horseradish peroxidase-conjugated antibodies (Santa Cruz Biotechnology), and then treated with an enhanced chemiluminescence solution (Thermo, Rockford IL, USA). The signals were captured on an Image Reader (LAS-3000; Fuji Photo Film, Tokyo, Japan), and protein bands were analysed by densitometry.

Immunohistochemistry

For immunofluorescence staining, after deparaffinization and rehydration, the sections were pre-incubated with 3% bovine serum albumin (BSA). For double staining, the cultured cells or sections were sequentially incubated with an anti-CDH11 antibody and 1:200 Alexa Fluor-labelled goat anti-rabbit IgG (488; Molecular Probes, Eugene OR, USA), and then with anti-collagen I, MMP-2, MMP-9, VEGF-A, or VEGF-D antibody and Alexa Fluor-labelled goat anti-mouse or donkey anti-goat IgG (594; Molecular Probes). The nuclei were counterstained with Hoechst 33258 (Sigma-Aldrich). Each stained specimen was photographed at fixed conditions, which were determined in an image analysis system (Dp Manager 2.1; Olympus Optical Co., Tokyo, Japan). The staining intensity was compared in 3 microscopic fields (×400) of paired skin specimens. The intensity of selected images was measured by means of Qwin V3 image processing and analysis software (Leica Microsystems Ltd, Bellinzona, Switzerland).

Gelatin zymography

The enzymatic activity of MMP-2 and MMP-9 was determined by SDS-PAGE, which was carried out at 4°C under non-reducing conditions using gels containing 0.1% gelatin. Gels were incubated with 2.5% Triton X-100 at room temperature for 30 min and subsequently at 37°C with buffer containing 50 mM Tris (pH 7.4), 5 mM $CaCl_2$ and 1 mM $ZnCl_2$ for 24 h. MMP activity was visualized by staining with Coomassie Blue R-250 (Bio-Rad). The stained specimens were evaluated using an image analysis system (Dp Manager 2.1; Olympus Optical Co., Tokyo, Japan).

Histological analysis

Histological sections (each 5-μm thick) were stained with Masson's trichrome or Verhoeff. The specimens were examined using light microscopy (DM LB Microscope; Leica Microsystems, Wetzla, Germany) equipped with a camera (DC300F; Leica Microsystems, Heerburgg, Switzerland), and analysed with the aid of a software image analyser (Qwin V3 Image processing and analysis software; Leica Microsystems).

Statistical analysis

Statistical analysis was performed using Student's *t*-test. The results were expressed as mean ± SD. A p-value < 0.05 was considered significant.