Dedifferentiation of Human Epidermal Keratinocytes Induced by UV *In Vitro*

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Dedifferentiation is an important biological phenomenon and is understood as a process in which cells develop in a reverse order from a more differentiated to a less differentiated state. In the present study, we observed that after UV treatment, the surviving keratinocytes underwent the reversion from differentiated state to dedifferentiated state, evidenced by the changes from three levels, including phenotype, morphology and function. First, the mature keratinocytes acquired a dedifferentiated phenotype indicated by reexpression of transit-amplifying (TA) cell markers, including CK14 and β1 integrin. Second, the cells experienced morphological changes during the process of dedifferentiation. Cells treated with UV were small and had a high nuclear to cytoplasmic ratio, whereas cells without treatment had well-developed cellular organelles and abundant tonofilaments. Third, after UV treatment, the cells regained strong proliferation capacity. These dedifferentiation-derived stem cells formed colonies with defined edges developed and presented multiple-layer growing profile under three-dimensional culture condition. The skin equivalent (SE) produced with UV-treated keratinocytes also showed a well-organized structure with four stratifications. We also report the signaling pathway involved in the dedifferentiation process. The extracellular signal-regulated kinase (ERK) pathway regulates the phenotype reversion of keratinocytes into progenitor cells. Inhibition of ERK kinase activities with a specific inhibitor (PD98059) substantially blocked phosphorylation of ERK1/2 and human keratinocyte dedifferentiation. These data collectively provide a proof-of-concept that UV treatment of HEKs is capable of inducing a phenotype reversion from an adult differentiated state to an immature-like dedifferentiated state via ERK Mitogen-Activated Protein Kinases (MAPK)-dependent pathway. It may offer the direct evidence for the existence of dedifferentiation and the underlying mechanisms involved in the process, which may bring a new insight for the regenerative medicine.

Key words —— keratinocytes, transit-amplifying cells, dedifferentiation

INTRODUCTION

Differentiated cells are thought to be stably committed to their fate; however, there is evidence to indicate that dedifferentiation events can take place. Dedifferentiation is the progression of cells from a more differentiated to a less differentiated state. It has been shown that dedifferentiation occurs during wound repair and regeneration of plants and various vertebrates. In addition, recent studies suggest that dedifferentiation may be possible in mammalian system. It was reported that pancreatic cells,[1] renal epithelial cells,[2] retinal cells,[3] myoblasts,[4] neurons,[5] and germ cells[6] have the potential of dedifferentiation in response to appropriate signals or factors. Obviously, commitment to terminal differentiation is not an irreversible process. This process could be achieved by introducing or remodeling a microenvirnoment composed of intrinsic and extrinsic cellular molecules. In 2007, scientists from Japan and United States of America successfully induced skin fibroblasts to become pluripotent stem cells,[7, 8] which revealed the possibility of obtaining stem cells, even embryonic stem cells, from skin, thus bringing new hope for stem cell research and organ regeneration. We also

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reported that recombinant human epidermal growth factor (rhEGF) could induce epidermal cell to dedifferentiate into stem cell like-cells *in vivo* in 2001.[9] However, this event has not been identified *in vitro* and the molecular mechanisms that regulate the process remain unclear.

Mitogen-Activated Protein Kinases (MAPKs) are serine/threonine kinases that transmit signals from extracellular stimuli to multiple substrates involved in cell growth, differentiation, and apoptosis. Three major subfamilies of MAPKs, the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38, have been identified. The ERK MAPK pathway is essential for controlling cell proliferation and differentiation. Increasing evidence has shown that the ERK pathway may also play critical roles in the process of dedifferentiation. It has been demonstrated that sustained activation of the ERK MAPK kinase pathway is capable of driving the dedifferentiation of Schwann cells.[5] ERK activity in differentiated chondrocytes is dramatically increased during the course of dedifferentiation. The process is blocked after the activation of ERK has been inhibited.[10] Likewise, ERK signaling pathway correlates with dedifferentiation of smooth muscle cells (SMCs). Pretreatment SMCs with ERK inhibitor suppresses the activation of the ERK cascade, abolishes the downregulation of desmin and leads to cell cycle arrest.[11] These results suggest that the ERK-dependent signaling pathway is involved in the proliferation and dedifferentiation of differentiated cells from different tissues in a stage-specific manner. However, little is known about how the ERK MAPK kinase pathway is affected in the reversion from mature keratinocytes to a dedifferentiated immature state.

In this study, we exposed epidermal keratinocytes to UV and tested the hypothesis that UV can elicit dedifferentiation in the somatic cells. Based on morphological and immunolabeling observations and functional assessments, we show that keratinocytes can be reprogrammed by UV treatment to acquire characteristics of transit-amplifying (TA) cells or progenitor cells through activation of ERK MAPK-mediated pathways.

MATERIALS AND METHODS

Cell Culture and Cell Subculture —— Human epidermal keraticytes obtained from healthy adults were purchased from Cascade Biologics, (Cascade Biologics, Carlsbad, CA, U.S.A.) and cultured in EpiLife Medium supplemented with 1% Human Keratinocyte Growth Supplement (HKGS) and 0.2‰ Penicillin-Strepotmycin Antibiotics (PSA) (Cascade Biologics) in 25-cm^2 flasks in a 37°C/5% CO_2 incubator. Cells were passaged every 3–5 d (days). Because repeated subcultures may reduce the proportion of stem cells, we got a high proportion of differentiated keratinocytes after serial cultivation and identification. After the sixth passage, stem cells are nearly absent. All of these cells were identified as differentiated kertatinocytes, with characteristics of being positive for differentiating epidermal cell marker, CK10, and negative for epidermal stem cell and TA cell markers, including CK14, CK19, p63 and β1 integrin. In addition, at the 10th passage, no more further subcultures were possible due to replicative senescence (data not shown). So we chose the cells of the sixth passage as experimental cells for the study of dedifferentiation induction.

Cell Treatment —— For experiment of dedifferentiation, cells were plated 1 d before induction at a density of 1×10^4 cells/well in 6-well culture plates. These cells were grown in medium as described previously. For UV radiation, cells plated in 6-well culture plates were rinsed with phosphate-buffered saline (PBS) and irradiated with UVC using a portable germicidal UVC lamp (single wavelength output 254 nm; Shanghai SIGMA High-tech Co., Shanghai, China) to a dose of 8 J m^{-2} in the presence or absence of ERK specific inhibitor PD98059 (10 μM, Promega, Madison, WI, U.S.A.). Following UV treatment, fresh medium was added to the plates and the cells were cultured for 7 d and 14 d respectively under the condition of 37°C/5%CO_2/95%O_2 until harvest. After 14 days of culture, the UV-treated cells were used for three-dimensional and Organotypic cultures. The non-treated cells were performed with same procedures as the UV-treated cells.

Immunocytochemistry —— Both non- and UV-treated cells cultured for 7 d and 14 d were fixed in acetone for 30 min at room temperature. Endogenous peroxidases were blocked by incubation in 3% hydrogen peroxide. Cells were incubated in anti-CK10, -CK14 and -integrin β1 monoclonal antibodies (Sigma, St. Louis, MO, U.S.A.) at a 1 : 100 dilution, followed by the appropriate horseradishperoxidase (HRP)-conjugated goat anti-mouse IgG (Vector, Burlingame, CA, U.S.A.) as secondary antibody at 1 : 100. The im-

munoreactivity of these antigens was visualized as a brown precipitate after these cells were developed in 3,3′-diaminobenzidine (DAB), counterstained with hematoxylin, and mounted in resin according to the power vision two-step immunostaining kit (Zymed Laboratories Inc., South San Francisco, CA, U.S.A.).

Western Blot Analysis —— The non-treated cells cultured for 14 d and UV-treated cells cultured for 7 d and 14 d were collected. Cells were washed twice with PBS, incubated with lysis buffer (50 mM TRIS, 150 mM sodium chloride, 1 mM EDTA, 2.5 mM sodium pyrophosphate, 1% Nonidet P40, 0.1% Sodium Dodecyl Sulfate (SDS), 50 mM sodium fluoride, 1 mM phenylmethyl sulfonylfluoride, 1 mM sodium vandate, 1 mg/ml leupeptin, pH 7.4) for 30 min with gentle shaking, scraped from the tissue culture flask, and then incubated overnight at 4°C with gentle agitation. Supernatants were collected after centrifugation at 12000 rpm for 15 min at 4°C, and the total protein was quantified. Samples (100 μg) reduced with β-mercaptoethanol were electrophoresed by using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane (Millipore, Billerica, MA, U.S.A.) by using a Western transfer system (Invitrogen). Membranes were blocked with 5% skimmed milk in TRIS-buffered saline-Tween (TBST) for 1 hr, incubated with mouse anti-CK14 and anti-β1 integrin antibodies (Sigma), polyclonal rabbit anti-human ERK1/2 and p-ERK1/2 antibodies (Cell Signaling Technology, Danvers, MA, U.S.A.) overnight at 4°C, washed in TBST, incubated with HRP-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies (Vector) for 2 hr, washed again, and then developed with ECL (electrochemiluminescence, Gene, Invitrogen, Carlsbad, CA, U.S.A.). Proteins were visualized by using Kodak film (Kodak, Rochester, NY, U.S.A.).

Flow Cytometric Analysis —— The antigen expression of non-treated cells and UV-treated cells cultured for 14 d was determined by flow cytometric analysis (FACS). The collection of treated cells and non-treated cells was performed as described above. These cells were rinsed with 0.01 mol/l PBS three times and then incubated with mouse antihuman primary monoclonal antibodies against CK10, CK14 and β1 integrin (Sigma) at a 1 : 50 dilution at 4°C for 30 min. Samples in which PBS was substituted for primary monoclonal antibodies were used as negative controls. After being washed with PBS, the cells were incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG secondary antibody (Vector) at a 1 : 50 dilution at 4°C for 30 min. Dual staining was performed using FITC-conjugated α6 integrin monoclonal antibody and PE-conjugated CD71 monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.). Mouse IgG: FITC/mouse IgG: RPE antibody was used as an isotype-matched negative control. After two more washes, flow cytometric analysis was performed on a flow cytometer (BD Biosciences, San Jose, CA, U.S.A.) and CellQuestk software with 20000 events being recorded for each sample. Experiments were repeated at least twice under the same conditions and settings.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Spectrophotometry —— Non- and UV-treated cell were planted into 96-well plates and divided into 4 groups: non-treated cells cultured for 7 d, non-treated cells cultured for 14 d, UV-treated cells cultured for 7 d, and UV-treated cells cultured for 14 d. After culture for 24 hr, MTT solution was added to every group, and the cells were continued to culture for 4 hr. The medium was discarded, and then DMSO was added to dissolve the crystal. Ten minutes later, the absorbance at 490 nm was measured. The experiments were carried out three times independently, in triplicate each time, and the average values of the three independent experiments were calculated.

Transmission Electron Microscopy —— The ultrastructure of cells being treated with and without UV was observed through the procedure as previous described.[12] Briefly, separated cells were gently centrifuged at 800 × g for 5 min, and cell pellets were fixed in freshly prepared fixative solution (2.5% glutaraldehyde in 0.1 M pH 7.2 phosphate buffer) for 72 hr. After washing with PBS, the cells were postfixed in 2% OsO_4 in PBS for 1 hr, dehydrated in graded ethanol, and embedded in Epon. Sections were cut using ultramicrotome and stained with saturated solution of uranyl acetate in acetone and then with a solution of 0.2% lead citrate. Samples were observed under a JEM-100CX transmission electron microscope.

Colony Forming Assay —— To assess clonogenic capacity of keratinocytes induced by UV, cells at the density of 1×10^4 cells per well were seeded in 6-well plates. After being exposed to UV, cells were grown under the condition of 37°C/5%CO_2/95%O_2 in EpiLife Medium supplemented with 1% HKGS and 0.2 PSA. After 7 and 14 d, culture medium in the dishes was removed

and the colony forming situation of these treated cells was observed using a phase contrast microscope (Olympus, Tokyo, Japan) and photographed using digital camera (Olympus). The non-treated cells cultured for 14 d were performed with same procedures as the UV-treated cells.

Three-dimensional Cell Cultures —— Three-dimensional cell cultures were constructed using mouse embryonic fibroblasts (MEFs) feeder layer. Briefly, collagen type I was paved at the bottom of 30 mm culture dish at the concentration of 3 mg/ml. Then, MEFs were seeded at the dried collagen layer at the density of $0.5 \times 10^4/cm^2$ after their being treated with mitomycin C (10 μg/ml) for 4 hr and thoroughly washed to remove mitomycin C. After the feeder layer was well prepared, non-treated and UV-treated epidermal keratinocytes after 14 days of culture were seeded at the density of $1 \times 10^4/cm^2$ onto the feeder layer. The keratinocyte medium was added and changed three times per week. The cell growth situation was observed on 10 d and 20 d using a phase contrast microscope (Olympus) and photographed using digital camera (Olympus).

Organotypic Cultures —— Organotypic cultures were constructed using a previously established system consisting of a collagen type I lattice populated with fibroblasts known as dermal equivalents (DE). Briefly, the DE were prepared by polymerizing 1 ml acellular bovine collagen I, followed by 3 ml of collagen matrix containing 5×10^5 human dermal fibroblast (HDF) per well. The DE were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, U.S.A.) for 7 days and then treated with mitomycin for 1 hr to repress the growth of fibroblasts, then repeatedly washed with D-Hanks solution. Subsequently, non-treated and UV-irradiated epidermal keratinocyptes after 14 days of culture were seeded at 1×10^4 cells onto the dermal substitute. The keratinocyte medium was added and changed three times per week. The cell growth situation was observed on 10 d and 20 d under a phase contrast microscope (Olympus) and photographed (Olympus).

Statistical Analysis —— All experiments were repeated at least three times, unless otherwise indicated. Data are presented as mean ± S.D. Statistical analysis involved use of the Student *t*-test. A $p < 0.05$ was considered significant.

RESULTS

The Phenotypical Reversion of Keratinocytes after UV Treatment

Dedifferentiation of the HEKs is characterized by the conversion of the phenotype. After UV injury, about 25% of the cells died as indicated by the 3-(4,5-dimethlthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) (data not shown). The remaining cells were collected and detected with relative antibodies for the expressions of CK14 and β1 integrin, the TA cell markers, using immunocytochemical analysis. As shown in Fig. 1, the non-treated cells grew dispersedly. They were negative for CK14 and β1 integrin, and still positive for CK10. In contrast, the expression of CK14 and

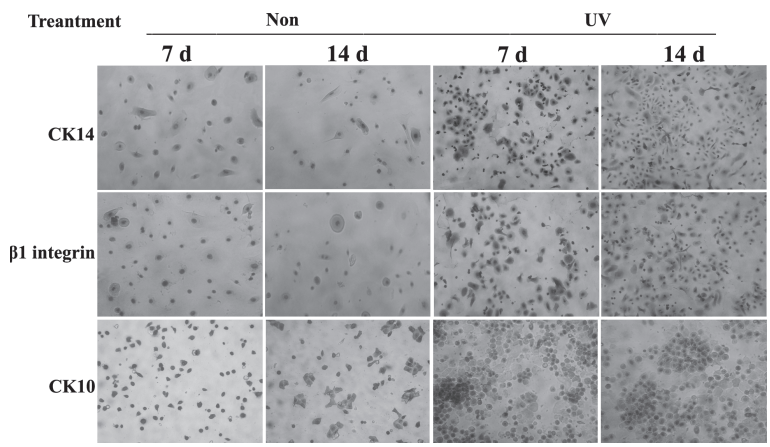

Fig. 1. Immunocytochemical Staining of CK10, CK14 and β1 Integrin in Cultured Differentiated HEKs Treated with and without UV

Positive staining is brown and nuclei counterstaining is blue. The non-treated cells (Non) for 7 d and 14 d grew dispersedly. They were negative for CK14 and β1 integrin and still positive for CK10. In contrast, the expression of CK14 and β1 integrin were significantly increased at different level on 7 d and 14 d in the treated cells with UV.

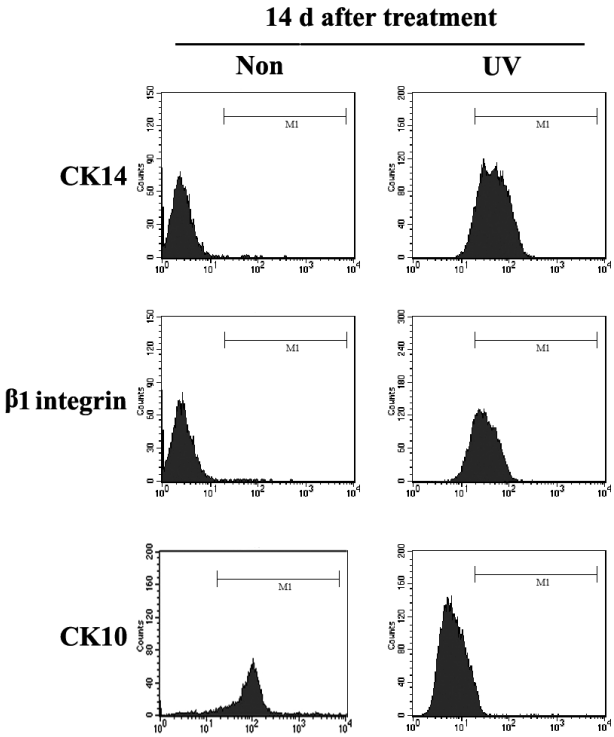

Fig. 2. Flow Cytochemistry Analysis of the Percentages of CK10, CK14 and β1 Integrin Positive Cells in Cultured Differentiated HEKs Treated with and without UV

After 14 days of culture, the percentages of CK14, β1 integrin and CK10 positive cells were 0.17%, 0.25% and 92.32% respectively in the non-treated cells (Non). The numbers were 91.35%, 84.53% and 11.72% respectively in the UV-treated cells (UV).

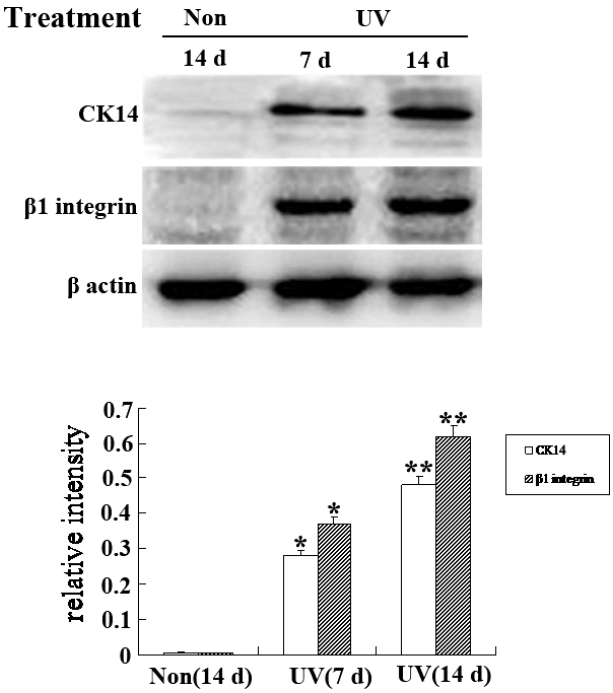

Fig. 3. Western Blot Analysis of CK14 and β1 Integrin in Cultured Differentiated HEKs Treated with and without UV

The expression of CK14 and β1 integrin was undetectable in non-treated cells (Non) on 14 d, and was increased significantly on 7 d and 14 d after these cells were exposed to UV. The relative intensity was determined by the ratio of the specific marker to β-actin as measured by densitometry. The experiments were carried out three times independently, in triplicate each time. Data are presented as mean ± S.D. Bar with * is significantly different ($p < 0.05$); **, $p < 0.01$.

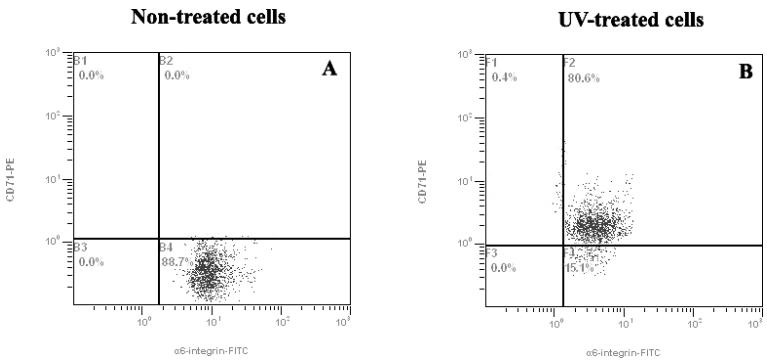

Fig. 4. Two-color Flow Cytochemistry Analysis of α6 Integrin and CD71 Expression in Cultured Differentiated HEKs Treated with and without UV

Dot plots showed the results of FCAS of non-treated cells (A) and UV-treated cells (B) double-labeled with anti-α6 integrin and anti-CD71 antibodies. B1 and F1: α6 integrin^{bri}CD71^{dim} cell population; B2 and F2: α6 integrin^{bri}CD71^{bri} cell population; B4 and F4: α6 integrin^{dim} cell population. B4 represented a low level of α6 integrin and a high level of CD71 (A). F2 represented a high level of α6 integrin and a high level of CD71 expression (B).

β1 integrin were significantly increased at different levels on 7 d and 14 d in the treated cell, showing a definite feature of dedifferentiation. The result was also confirmed by flow cytochemetry analysis (Fig. 2) and Western blot analysis (Fig. 3). Figure 4 showed a representative dot plot in which cells are represented by the expressions of α6 integrin (x-axis) and CD71 (y-axis). In Fig. 4A, B4 represented a low level of α6 integrin and a high level of CD71, which is the feature of differentiated epidermal cells. In Fig. 4B, F2 represented a high level of α6 integrin and a high level of CD71 expression, which is the characteristic of TA cells.

Morphology, Proliferation and Redifferentiation Potential of Keratinocytes Enhanced by UV Treatment

The A490 value in UV-treated cell groups were higher than that in the non-treated cell group ($p < 0.01$) (Fig. 5). As shown in Fig. 6, UV-treated keratinocytes became smaller, with fewer cellular organelles and a higher ratio of nucleus to cytoplasm (Fig. 6B, C) than differentiated epidermal keratinocytes (Fig. 6A). To investigate the proliferative potential of the keratinocytes, we observed the clonogenic growth after these cells had been induced by UV. Within 7 d, the treated cells produced colonies with defined edges, which gradually enlarged after another 7 d (Fig. 6E, F), while the non-treated cells grew dispersedly after 14 days of culture (Fig. 6D).

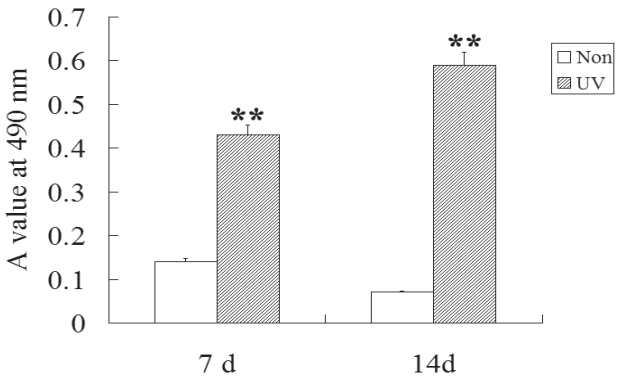

Fig. 5. Proliferation Capacity of HEKs Treated with and without UV Measured by MTT

Bar with ** is significantly different *vs.* the non-treated cells ($p < 0.01$).

Under three-dimensional culture, the non-treated cells could not form obvious cell growing layers within 20 days (Fig. 7A). In contrast, ten days after UV treatment, the cells presented multiple-layer growing profile (Fig. 7B) and after another 10 days, the cells grew upward continuously and formed a ridge-like structure (Fig. 7C). Because epidermal reconstruction of epidermis is the best way to examine the usefulness of progenitor cells, we wondered whether these UV-treated cells could form a relative complete and stratified epidermis. SEs consisting of fibroblasts and collagen were prepared with use of non-treated and UV-treated epidermal keratinocytes to determine the epidermal regenerative capacity of these different cell populations. The SEs produced by non-treated epidermal keratinocytes showed a thin epidermis without the distinct multilayered structure within 20 days (Fig. 7D). Interestingly, the SEs generated by UV-treated epidermal keratinocytes showed a relatively well-organized structure of about four stratifications (Fig. 7E, F). The successful construction of epidermal structure through organotypic cultures reconfirmed that these UV-treated cells regained the strong proliferation ability to self-renew and terminally differentiate to form a relatively complete epidermis.

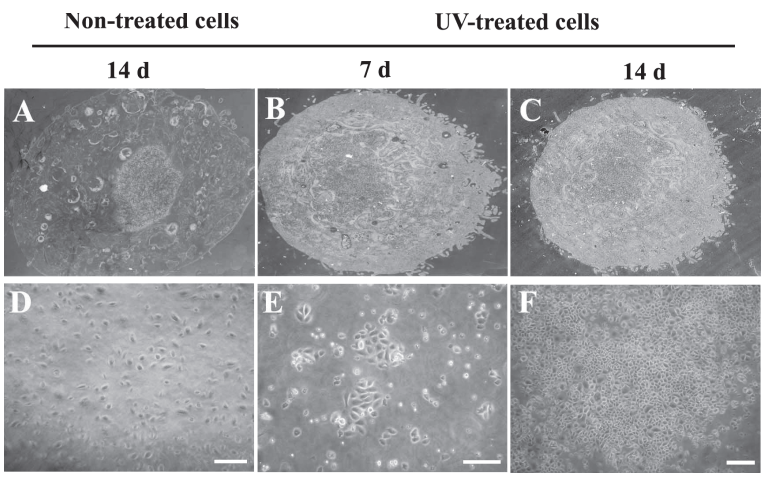

Fig. 6. Cell Size, N/C Ratio, and the Ultrastructures of Cultured Differentiated HEKs Treated with and without UV

Transmission electron microscopy of non-treated and UV-treated cells was performed (× 2700). UV-treated cells were found to have smaller size, higher N/C ratio and more number of organelles 7 d and 14 d after induction (B, C) than non-treated cells (A). Colony forming activities and multiple-layer growing profiles of HEKs treated with and without UV. Under two-dimensional culture, the non-treated cells grew dispersedly (D), whereas the UV-treated cells formed colonies with defined edges on 7 d (E) and the colonies gradually enlarged to form a large colony on 14 d (F).

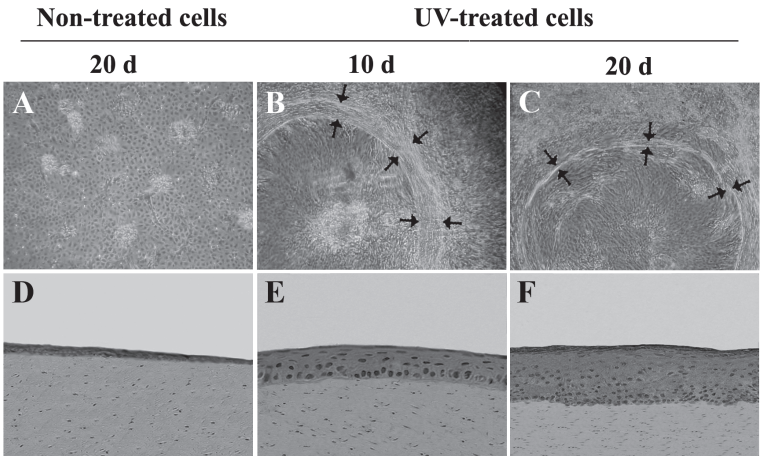

Fig. 7. Proliferation and Redifferentiation Capacity of HEKs Treated with and without UV

Under three-dimensional culture, the non-treated cells did not form obvious cell growing layers (A) within 20 days. Ten days after UV treatment, the cells presented multiple-layer growing profile and the arrows point to the area of the cell layer (B). 14 days after UV treatment, the cells grew upward continuously and formed a ridge-like structure (C). The arrows point to the growing peak. UV-treated cells have a higher proliferative potential than non-treated cells. Structure of skin equivalents (SEs) derived from non-treated and UV-treated epidermal keratinocytes. SEs derived from UV-treated cells showed relative thick structure with about four stratifications (E, F), compared with the thin structure produced with non-treated cells (D).

The Potential Signaling Pathway in the Phenotype Reversion of Keratinocytes Induced by UV

To understand the mechanism of the phenotype reversion of keratinocytes, we determined whether the ERK is required for formation of these phenotype changes after UV injury. After UV treatment, the cells were harvested and the cell lysates were analyzed by Western blot analysis using specific antibodies against either phospho-ERK1/2 or total ERK1/2. As shown in Fig. 8, UV treatment resulted in a transient activation of ERK1/2. After ending UV treatment, phosphorylated ERK1/2 was increased by 30 m and peaked by 120 m; the activation remained unchanged until 12 hr; and then slowly decreased by 24 hr and finally to nearly undetectable level by 36 hr. The total ERK1/2 protein did remain unchanged before and after the UV treatment. Thus, the time point of 120 m after ending the UV treatment was chosen for the subsequent experiments.

To confirm specificity of the UV-induced activation of ERK MAPK and the causality of this activation in UV-induced expression of epidermal TA cell markers, the keratinocytes were pretreated with PD98059 for 30 m and then followed by the UV treatment. By 120 m after UV induction, the cells were harvested and the cell lysates were analyzed by Western blot analysis using specific antibodies against phospho-ERK1/2, total ERK1/2, CK14 and β1 integrin. The results showed that pretreatment of the cells with PD98059 resulted in strong inhibition

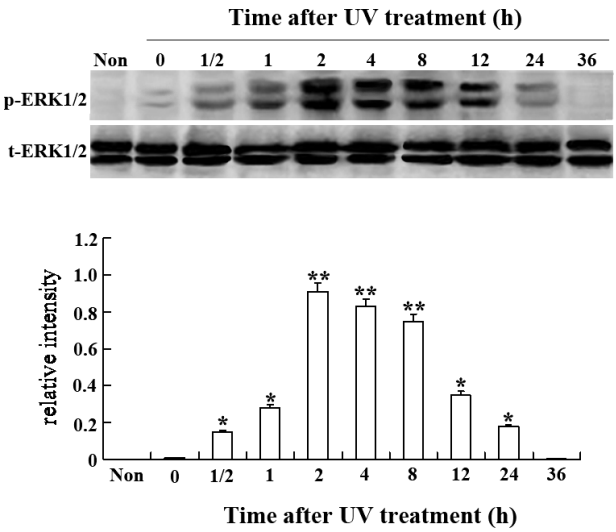

Fig. 8. UV Treatment Activates ERK1/2 in Cultured Differentiated HEKs in a Time-dependent Manner

After UV treatment, the cells were harvested and the cell lysates were analyzed by Western blot analysis using specific antibodies against either phospho-ERK1/2 or total ERK1/2. The relative intensity was determined by the ratio of phospho-ERK1/2 to total ERK1/2 as measured by densitometry. The experiments were carried out three times independently, in triplicate each time. Data are presented as mean ± S.D. Bar with * is significant different ($p < 0.05$); **, $p < 0.01$.

of the phosphorylation of ERK1/2 and phenotypical reversion of the HEKs induced by the UV (Fig. 9).

All of these data suggested that UV could stimulate the reversion of keratinocytes from a more differentiated state to a less differentiated state, and that ERK1/2 mediated this phenotype change.

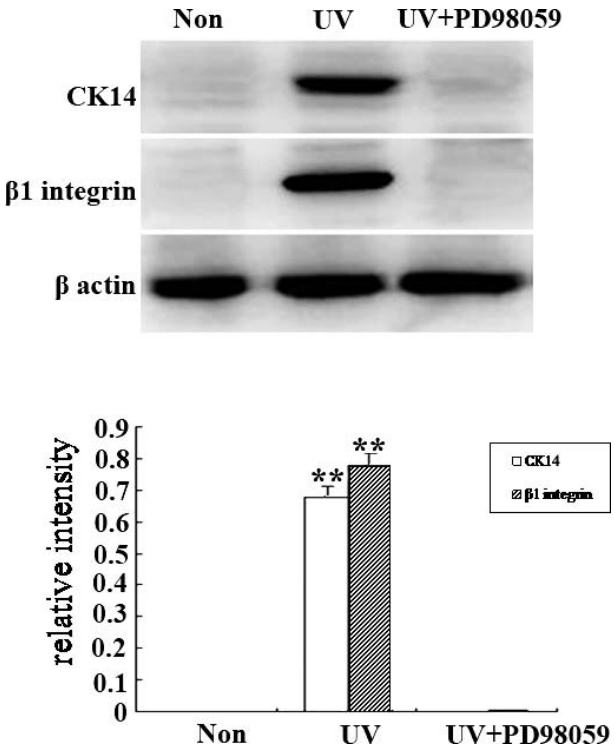

Fig. 9. Pretreatment of the Cells with PD98059 Resulted in Inhibition of Phenotypical Reversion of the HEKs Induced by UV

The cells were pretreated with PD98059 for 30 m and then followed by the UV treatment. By 120 m after UV induction, the cells were harvested and the cell lysates were analyzed by Western blot analysis using anti-CK14 and anti-β1 integrin antibodies. The relative intensity was determined by the ratio of the specific marker to β-actin as measured by densitometry. The experiments were carried out three times independently, in triplicate each time. Data are presented as mean ± S.D. Bar with ** is significantly different ($p < 0.01$).

DISCUSSION

Epidermal stem cells and its progeny TA cells are promising clinical candidates for the treatment of skin orders, including burns, chronical wounds, and ulcers. They may hopefully become the favored cells in skin regenerative medicine, but achieving this reality involves many obstacles, both technical and practical. Thus, exploring a new source of endogenous progenitor cells for regeneration in skin seems imperative for regenerative medicine. Dedifferentiation, which has been observed in various plant and animal cells, is a new source of stem cells and has provoked great interest in biology and medicine. It is understood as a process in which cells develop in a reverse order from a more differentiated to a less differentiated state. With an efficient dedifferentiation process, abundant, healthy and easily accessible epidermal stem cells could be used to generate different types of functional cells for repair of damaged skin.

The phenomenon of dedifferentiation can be observed at different levels, including gene, protein, morphology and function.[13] First, the cell undergoes the reversion from a differentiated cell gene expression profile to a progenitor gene expression profile. During the process, the development-related genes are closed, which is accompanied by the opening of genes which can keep the cell in the undifferentiated state. Second, the phenomenon of dedifferentiation can also be observed at protein level, as evidenced by the up-regulation of progenitor cell-related proteins and down-regulation of differentiated cell-related proteins. Third, the cell experiences morphological changes during dedifferentiation. Compared to mature cells, the dedifferentiated cells present smaller cell size, less number of organelles and higher nuclear to cytoplasmic ratio. Fourth, the cell regains the proliferation capacity, which means that a postmitotic cell can reenter the cell cycle. Meanwhile mature cells or lineage-committed cells might become multipotent or pluripotent progenitor cells with the potential of differentiating into new phenotypes. All of these changes at four levels may occur in a certain dedifferentiation process. Although many researchers declared that they had observed the phenomenon of dedifferentiation, such as dedifferentiation of pancreatic cells, retinal cells, renal cells and germ cells, all of these findings were merely based on one or two of these manifestations concluded above.

Also, although we have observed the phenomenon of dedifferentiation in human regenerative epidermis, the process is observed merely from the protein level *in vivo* and there seems to be more evidence need be collected to confirm the phenomenon sufficiently. First, we need establish a stable and efficient dedifferentiation-inducing model *in vitro*. By doing this, the study of dedifferentiation of epidermal cells will become easier and its manipulation more efficient. Second, we need confirm the dedifferentiation of epidermal cells from more than one level. More sufficient evidence of these changes from differentiated epidermal cells to dedifferentiated progenitor cells need be collected. Third, tight control of the dedifferentiation signaling pathways must be required so that it can be incited to initiate the dedifferentiation process and subsequently shut off to allow redifferentiation to take place.

In order to address these questions mentioned above, we designed the current study. First, based on the study of dedifferentiation reported world-

wide, we concluded that the induction factors of dedifferentiation for mammals could be mainly divided into four ways. The first is physical way; for example, heat treatment.[6] The second is chemical way, for example, oxidant injury.[3] The third is biological way, including newt regeneration extract,[14] carcinoma extract and embryonic stem cell extract.[15] Finally and most recently, transgenic way is widely adopted to induce adult cells to dedifferentiate into pluripotent stem cells with the characteristics of embryonic stem cells.[16] At the same time, the proliferation of keratinocytes caused by UV is thought to be correlated with dedifferentiation of these cells to produce stem cells and then redifferentiate into a number of keratinocytes. In our laboratory, we have been trying to find proper induction factors to establish dedifferentiation model of keratinocytes *in vitro* after our getting the evidence of dedifferentiation of skin cells *in vivo* in 2001.[9] These attempts include such treatments as UV, heat, oxidant injury, embryonic tissue extract; and preliminary data have been acquired. Based on these results, we found that UV treatment was relatively stable for these cells to dedifferentiate and the induction procedure was easily controllable. So UV was chosen for our present study. So, we exposed epidermal cells of the sixth passage to UV and compared the differences between non-treated cells and treated cells according to the changes at three levels, including protein, morphology and proliferative ability. Stem cell and TA cell markers, including CK14, CK19, p63, CD71, α6 integrin and β1 integrin were chosen as indicators to illustrate the protein level changes. Morphological changes included cell size, number of organelles and nuclear to cytoplasmic ratio of these two kinds of cells. We also observed the proliferation and redifferentiation capacity of these two cell populations, according to the colony formation, multiple-layer growing profile and reconstruction of epidermis. The results showed that after induction, the surviving differentiating keratinocytes acquired the phenotype of TA cells, which was indicated by the fact that TA cell markers, including CK14, β1 integrin and CD71$^{bri}\alpha$6 integrin^{bri}, were not seen in cells without UV treatment, but were highly expressed in UV-treated cells through immunocytochemical, Western blot and flow cytometry analyses. However, the expression of putative epidermal stem cell marker, such as CK19, p63 and CD71$^{dim}\alpha$6 integrin^{bri}, did not change with and without treatment (data not shown), indicating that these treated cells underwent just one step from final differentiated stage to TA stage and not further reversed to epidermal stem cell stage. Second, these treated cells also experienced morphological changes to present smaller cell size, less number of organelles and higher nuclear to cytoplasmic ratio as compared with non-treated cells through electron microscopic observation. In addition, cells treated with UV regained the proliferation and redifferentiation capacity to form certain number of colonies, present multiple-layer growing profile, and reconstruct a well-formed epidermis with regular stratification, which means that postmitotic cells reentered the cell cycle. All of these changes from three levels showed a definite feature of dedifferentiation for epidermal cells.

For the third question, the answer is that ERK1/2 signaling pathway plays a role in dedifferentiation of epidermal cells. Although the signaling pathways involved in dedifferentiation have not been completely identified, increasing evidence has shown that such signaling pathways as MAPK, Wnt/β-catenin,[17–19] Janus kinase-signal transducer and activator of transcription (Jak-STAT),[20] Notch,[21] and BMP/Nogging[2] and other unidentified signaling pathway,[22] play some critical roles in the process. Among them, MAPK signaling pathway is studied most widely and deeply. MAPKs are serine/threonine kinases that transmit signals from extracellular stimuli to multiple substrates and are composed of three major subfamilies, including ERKs, JNKs, and p38.[23] Most likely, ERK is highly responsive to mitogen stimulation (*e.g.*, by growth facors), while JNK and p38 are activated by a variety of genotoxic stresses, including cell cycle arrest, DNA repair, and apoptosis.[23] All three subfamilies of MAPKs are reported to be involved in the process of dedifferentiation.[3, 11, 24] At the same time, UV exposure has been reported to be involved in the activation of ERK and JNK.[25] So we determined all three subfamilies of MAPKs in induction of keratinocytes to dedifferentiation by UV treatment. During the process, dedifferentiation induced by UV is strongly activated with the expression of phospho-ERK. Inhibition of ERK kinase activities by treatment with a selective chemical inhibitor of ERK (PD098059) substantially abrogates the UV induction of cells. In contrast, a p38 kinase inhibitor (SB203580) and the dominant negative mutant JNK1 have little effect on keratinocyte induction by UV (data not shown).

These data presented here supported the following conclusions. First, after treatment of UV, the

surviving keratinocytes undergo the reversion from differentiated state to dedifferentiated state, as evidenced by the changes from three levels, including protein (reexpression of TA cell markers), morphology (smaller cell size, less number of organelles and higher nuclear to cytoplasmic ratio) and function (regaining the powerful proliferation and redifferentiation activity). Second, the effects of UV on the differentiating keratinocytes may dependent on the activation of ERK1/2 MAPK pathway, which was indicated by the facts that the expression of phospho-ERK1/2 were significantly increased during the process and pretreatment of epidermal cells with PD98059 resulted in strong inhibition of the dedifferentiation induced by UV.

Thus, this study illustrates for the first time in the keratinocytes that UV induce a phenotype reversion of keratinocytes from an adult-differentiated state to an immature-like dedifferentiated state through activation of ERK MAPK, which may have important implication of finding an alternative route to reap an abundant source of epidermal progenitor cells/TA cells. However, we have to admit that UV is a DNA-damaging agent and has the risk to cause skin cancers. It should be cautious when we use UV as an external factor to induce the dedifferentiation of epidermal cells for the purpose of getting large number of epidermal progenitor cells. Therefore, tumor-related indexes or factors should be determined and estimated during the process of dedifferentiation in order to evaluate the safety problem for its use in clinical settings.

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