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SHORT COMMUNICATION

# Photoaging-associated changes in epidermal proliferative cell fractions in vivo

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**Abstract** The epidermis is a dynamic epithelium with constant renewal throughout life. Epidermal homeostasis depends on two types of proliferative cells, keratinocyte stem cells (KSCs), and transit amplifying (TA) cells. In the case of chronologic aging, levels of KSCs tend to decrease and change functionally. However, little is known about the effect of photoaging on epidermal proliferative subtype populations. The aim of this study was to validate involucrin/$\beta$1-integrin ratio as a molecular marker of epidermal photoaging, and to investigate the effects of photoaging caused by chronic UV exposure on the proliferative subtype populations. A total of 15 male volunteers (age range 20–24 and 77–85 years, Fitzpatrick skin phototype III–IV) provided sun-exposed and sun-protected skin samples for real-time RT-PCR, Western blot analysis and immunostaining. Fractional changes in proliferative subtype populations in photoaged and chronologically aged skins were analyzed by flow cytometry. The expression of $\beta$1-integrin was found to be significantly reduced in photoaged skin and ratios of the expressions of involucrin to $\beta$1-integrin were increased 2.6-fold only in elderly subjects. Interestingly, immunostaining of the sun-exposed skins of elderly subjects showed aberrant $\beta$1-integrin expression over the basal layer and greater numbers of Ki-67-positive cells than in sun-protected buttock skin. Flow cytometric analysis revealed that the proportion of KSCs to TA cells was reversed in sun-exposed and sun-protected skins of elderly subjects. Our results suggest that KSC numbers may be lower in photoaged skin than in chronologically aged skin and could be applied to hyperplastic pattern of photoaging. These findings suggest that the epidermis of photoaged skin is impaired in terms of its proliferative potential by attempting to repair chronic UV exposure and that photoaging may be associated with alteration in the two proliferative cell fractions.

**Keywords** Photoaging · Ultraviolet · Keratinocyte stem cells · Involucrin · $\beta$1-integrin

Damage to human skin due to repeated exposure to solar ultraviolet (UV) radiation (photoaging) and damage due to the passage of time (chronological aging) are considered to be distinct entities [1, 2]. Although many studies have focused on alterations in dermal components and the functions of fibroblasts after UV exposure, keratinocytes may be more susceptible to UV damage than any other skin cell type, and the functional properties of keratinocytes have been reported to be profoundly affected by chronic UV exposure at age 30 [3, 4]. Moreover, chronic UV exposure impairs epidermal homeostasis and upregulates the involucrin (differentiation marker) to $\beta$1-integrin (proliferation marker) ratio, which has been suggested as a molecular marker of epidermal photoaging [3].

The epidermis is in a state of dynamic epithelium and is constantly renewed. Moreover, it consists of keratinocytes that have differentiated to differing extents [5]. Cell replacement in epidermis is dependent on proliferative cells in the basal layer, which include a minor population of long-lived keratinocyte stem cells (KSCs) that cycle slowly, and a large pool of

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actively dividing, though short-lived, transit amplifying (TA) cells [5, 6]. During chronological aging or cellular senescence, levels of KSCs tend to reduce and their functionalities change, as has been demonstrated during wound healing [7, 8]. However, little attention has been paid to the effect of photoaging on proliferative subpopulations in the epidermis.

The aim of this study was to confirm that the ratio of involucrin to $\beta$1-integrin is a molecular marker of epidermal photoaging, and to investigate the effects of photoaging caused by chronic UV exposure in the KSCs population.

Seven young (age range 20–24 years) and eight elderly (77–85 years) male volunteers, whose skin types ranged from Fitzpatrick skin phototype III–IV without current or prior skin disease, provided two types of skin specimens each: (1) sun-exposed facial skin from the crow's foot area, 2 cm from the lateral canthus, and (2) sun-protected buttock skin. Two 3-mm diameter facial and two 8-mm punch-biopsy buttock specimens were obtained, respectively. Another three elderly males aged 78–84 years provided epidermal sheets. Specifically, six 7.065 $cm^2$-sized epidermal sheets were obtained from sun-exposed outer forearms and sun-protected buttocks (six samples per location per subject) using the suction blister method, involving the application of 40 mmHg of negative pressure for 3–4 h using 50 cc syringes of 3-cm diameter, respectively.

Using our previously described photographic photodamage scale [9], all young subjects belonged to wrinkle grade 1 with no wrinkles and all the elderly subjects belonged to grade 7 of thick, severely photodamaged skin with coarse wrinkles. Sunscreen with SPF 15, covering a broad-spectrum from both UVA and UVB rays, was provided and worn during the day, and the subjects were counseled to avoid strong sunlight at least for 2 weeks [10]. The Institutional Review Board at Seoul National University Hospital approved this study and all subjects gave written informed consent.

Samples used for immunostaining were placed immediately into a Cryomatrix (Shandon, Pittsburgh, PA) or fixed in 10% buffered formaldehyde and then embedded in paraffin. Specimens for real-time RT-PCR and Western blot analyses were snap frozen in liquid nitrogen, and epidermal sheet specimens for flow cytometric analysis were soaked in normal saline until cell isolation.

Total RNA was extracted from tissues using Trizol (Invitrogen, Carlsbad, CA), and 1 μg of total RNA was converted to cDNA using First Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD). mRNA levels of procollagen $\alpha$1(I) and endogeneous reference 36B4 were quantified by real-time RT-PCR, as previously described [11]. Sequence-specific PCR primer sets and FAM-labeled TaqMan MGB probe were purchased from Applied Biosystems. Western blot analysis was performed as follows. To separate samples at the dermo-epidermal junction, they were incubated in 1 M NaCl for 24 h at 4°C, and then the epidermis and dermis were peeled apart [12]. Proteins were extracted and then, 20 μg of protein per sample was electrophoresed and blotted onto nitrocellulose membrane. The membranes were incubated with anti-rabbit polyclonal involucrin (BT601, Biomedical Tech, Stoughton, MA), anti-mouse monoclonal $\beta$1-integrin (610468, BD Biosciences, San Jose, CA) and anti-goat polyclonal $\beta$-actin (SC1616, Santa Cruz, CA) at 4°C, overnight. Membranes were then incubated with anti-mouse (1:2,000) or anti-rabbit IgG–HRP conjugate (1:2,000) for 1 h at room temperature and antibody–antigen complexes were detected using an ECL system (Amersham, Little Chalfont, UK).

Serial sections of 6 μm thickness were mounted onto silane-coated slides (Dako, Glostrup, Denmark) and acetone fixed at −20°C for 15 min. Sections were incubated with anti-human $\beta$1-integrin antibody in a humidified chamber at 4°C overnight, and then with secondary FITC-conjugated anti-mouse IgG (Dako, Carpinteria, CA) for 1 h at room temperature. Immunofluorescent staining was monitored under a fluorescence microscope (Olympus, Tokyo). For immunohistochemical staining, sections were incubated with anti-human Ki-67 antibody (M7240, Dako, Glostrup, Denmark) and then with biotinylated secondary antibody, followed by streptavidin peroxidase. Reactions were visualized using DAB chromogen and finally, the sections were counterstained with Mayer's hematoxylin.

To obtain relatively lower viable keratinocytes, epidermal sheets were incubated with 0.25% trypsin (Sigma Chemical Co., St Louis, MO) for 20 min at 37°C [13]. Cell suspensions were prepared in 0.01% DNase (Sigma) and 10% FBS (Hyclone Laboratories, Logan, UT) by gentle teasing and then filtered through a 40 μm cell strainer (BD Falcon, Bedford, MA). Cells were then resuspended in 1 ml HBSS and slowly pipetted into 30 ml of 70% cold ethanol [13]. Dual immunostaining was performed using PE-conjugated anti-$\alpha$6-integrin antibody (MCA1457PE, Serotec, NC) and FITC-conjugated anti-CD71 monoclonal antibody (F0829, Dako) at 10 μg/ml, as previously described [8]. Labeling reactions were performed under subdued light for 30 min at 4°C. Cells were then resuspended in PBS containing 2% FBS at 6–8 $\times$ $10^5$ cells/ml. Flow cytometric analyses were performed using a FACS Calibur (Becton-Dickinson, Franklin Lakes, NJ). Experiments were performed in triplicate and statistical significance was determined using the Mann–Whitney test. $P$ values of less than 0.05 were considered statistically significant.

The expressions of procollagen $\alpha$1(I) mRNA were similar in both sun-exposed and sun-protected areas of the young subjects. The levels of $\alpha$1(I) procollagen mRNA expressions in photoaged and intrinsically aged skins of elderly subjects were significantly lower than those of young buttock skins, which reflect the phenomenon of photodamage rather than general wound healing as previously reported [14] ($P < 0.05$, Fig. 1a).

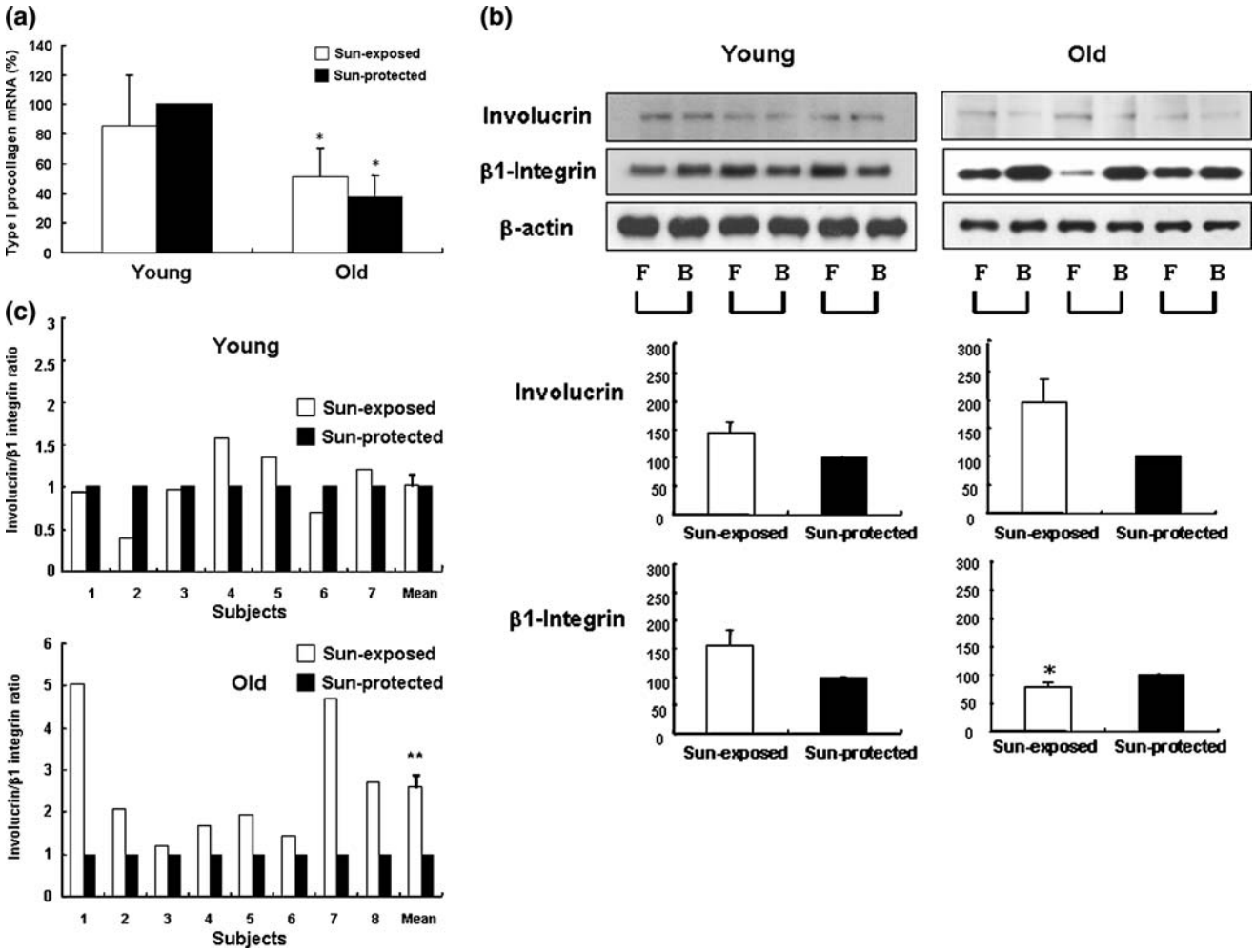


**Fig. 1** The expressions of procollagen α1(I) mRNA and involucrin/β1-integrin protein in sun-exposed and sun-protected skin. **a** The procollagen α1(I) mRNA levels were determined by real-time RT-PCR. Data are presented as percentage of type I procollagen levels relative to those of sun-protected buttock skins from young subjects. * $P < 0.05$, compared with young buttock skin, mean ± SE. **b** The expression of involucrin and β1-integrin. In young subjects, the expressions of involucrin and β1-integrin were increased in sun-exposed epidermis. On the other hand, in elderly subjects, involucrin expression was greater than in young subjects but expression of β1-integrin was significantly lower in sun-exposed epidermis than in sun-protected epidermis. **c** Ratio of involucrin to β1-integrin as a molecular marker of epidermal photoaging. In young subjects, this ratio varied on an individual basis, whereas in elderly subjects, it was constantly higher in sun-exposed epidermis than in sun-protected epidermis. * $P < 0.05$, ** $P < 0.01$. Data are expressed as mean ± SE. *F* face (sun-exposed skin), *B* buttock (sun-protected skin). The relative densities in sun-exposed epidermis are quoted as percentages of those of sun-protected epidermis

In young subjects, the expression of involucrin, the terminal differentiation marker, was elevated in epidermis of sun-exposed skin by 145 ± 17% (five of seven subjects, mean ± SE) compared with that of sun-protected skin. In the photoaged epidermis of elderly subjects, its expression tended to be higher than in intrinsically aged epidermis by 196 ± 25% (six of eight subjects)(Fig. 1b). On the other hand, whereas the β1-integrin expression, the proliferation marker, was increased by 155 ± 29% in sun-exposed epidermis in young subjects compared with sun-protected epidermis of their counterparts (five of seven subjects), in elderly subjects, β1-integrin expression was significantly lower in epidermis of photoaged skin (eight of eight subjects, 80 ± 12%)($P < 0.05$) (Fig. 1b).

The ratio of involucrin to β1-integrin expression has been suggested to be a molecular marker of photoaging [3]. For each subject, we calculated these ratios in sun-exposed and sun-protected skin. Whereas sun-exposed skin in younger subjects had a slightly higher mean ratio of 1.1 ± 0.2 (mean ± SE), photoaged skin in elderly subjects displayed significantly higher ratio of 2.6 ± 0.5 versus chronologically aged skin ($P < 0.01$) (Fig. 1c).

Immunostaining findings for β1-integrin and Ki-67 were similar for the epidermis of sun-protected skin in both the age groups. However, β1-integrin expression was more diffuse in the basal and suprabasal layers of sun-exposed skins of elderly subjects compared with that of young subjects (Fig. 2a). Ki-67-positive keratinocytes were uniformly

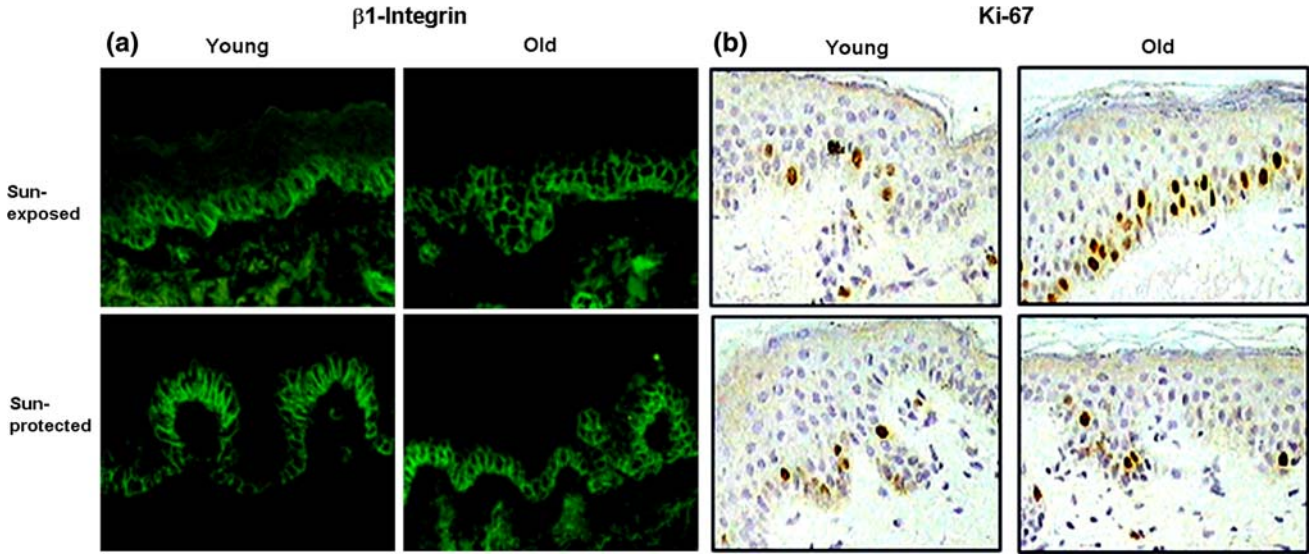


**Fig. 2** Immunostaining of sun-exposed facial and sun-protected buttock epidermis in young and elderly subjects. **a** Immunofluorescent staining showed little difference between sun-exposed and sun-protected young skins in terms of $\beta$1-integrin expression. However, sun-exposed elderly epidermis showed enhanced basal and suprabasal $\beta$1-integrin expression versus sun-protected epidermis. **b** Immunohistochemical study of Ki-67 expression in sun-exposed and sun-protected epidermis. Little difference was observed between these two site types in terms of the number of Ki-67-positive cells in young subjects. However, in elderly subjects, sun-exposed skin showed significantly greater numbers of Ki-67-positive cells in epidermis than in sun-protected skins (Original magnification $\times$400, images were representative of the two age groups and obtained from the same 23-year-old and 78-year-old subjects, respectively)

distributed in the basal layer of sun-protected epidermis in both groups and a few Ki-67-positive cells were observed just above the basal cell layer. Interestingly, in sun-exposed elderly skins, the number of Ki-67 positive keratinocytes ($24.0 \pm 4.8$, mean $\pm$ SE) per millimeter length of epidermis was significantly higher than in the skins of young subjects ($8.4 \pm 2.3$)($P < 0.05$) (Fig. 2b).

Keratinocytes from the sun-exposed forearm and sun-protected buttock skins of three elderly donors were subjected to fractionation experiments. The dot graph in Fig. 3 shows a plot of $\alpha$6-integrin ($y$-axis) versus CD71 expression ($x$-axis). Two arbitrary horizontal and vertical grids were drawn on the graph, and cells were grouped into three populations as described previously [8]: R1 represents $\alpha6^{bri}CD71^{dim}$ enriched with candidate KSCs; R2 represents $\alpha6^{bri}CD71^{bri}$ cells—candidate TA cells; and R3 represents $\alpha6^{dim}CD71^{dim}$ cells—the post-mitotic differentiating cells (Fig. 3a). Dot plot analysis revealed that the average R1 of sun-exposed epidermis ($0.08 \pm 0.02\%$) tended to be lower than that of sun-protected epidermis ($0.18 \pm 0.02\%$), whereas the average of R2 from sun-exposed epidermis ($1.91 \pm 0.25\%$) was greater than that of sun-protected epidermis ($0.84 \pm 0.06\%$). A histogram plot also showed that CD71 expression showed increased fluorescence in sun-exposed epidermis than in sun-protected epidermis (Fig. 3b).

Cutaneous aging can be divided into two simultaneous processes, chronologic aging and photoaging. Photoaging is a premature aging process that is superimposed on chronologic (or intrinsic) aging, which is characterized primarily by functional alterations rather than gross morphological changes [1, 2]. Epidermis of photoaged skin may be hyperplastic or severely atrophic compared with sun-protected sites in the same individuals [15]. The hyperproliferative state of epidermis in photoaged skin is generally regarded to be a consequence of a chronic condition induced by persistent UV exposure and chronic repair. This hyperproliferative state is, perhaps, a reaction that ultimately extinguishes itself and results in epidermal atrophy [3].

Involucrin is a well-established keratinocyte differentiation marker and is known to be upregulated in photoaged skin as a consequence of an altered keratinocyte terminal differentiation [3]. In addition, the amount of $\beta$1-integrin in keratinocytes decreases with age and in the elderly, keratinocytes have a low $\beta$1-integrin content, low self-renewal capacities, and altered adhesion properties [4]. Consequently, the upregulation of the involucrin/$\beta$1-integrin ratio by chronic sun exposure is viewed to be due to a disturbance of epidermal homeostasis, and thus, has been suggested to be a useful molecular marker of photoaging [3]. Our finding that this ratio is significantly increased only in elderly subjects supports its usefulness as a biomarker of photoaging.

Recently, it was reported that stem cells appear to be incapable of being passed infinitely [16]. Moreover, although substantial numbers of stem cells can be sustained throughout life, their endogenous replicative potentials

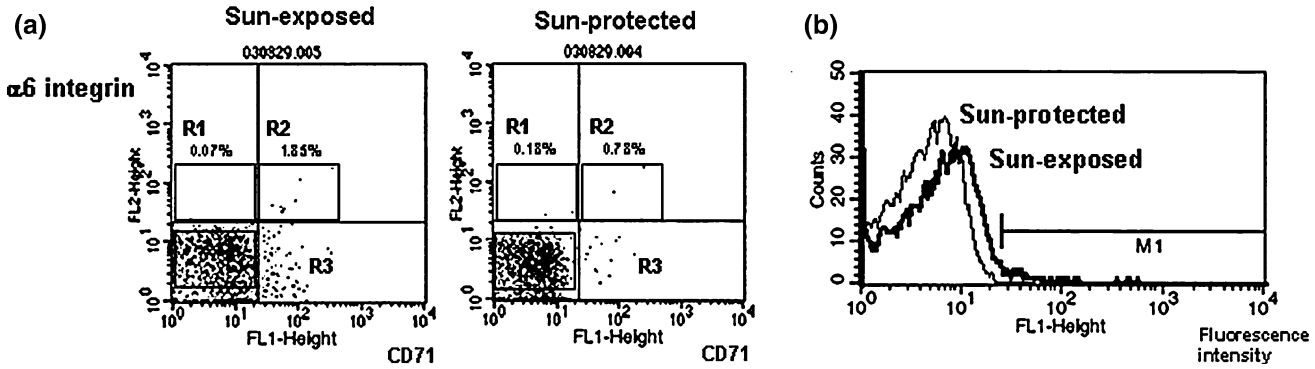


**Fig. 3** Flow cytometric analysis of epidermal sheets from sun-exposed and sun-protected skins from an 82-year-old volunteer. **a** Distributions of α6-integrin and CD71 fluorescence in keratinocytes. $\alpha6^{bri}$ $CD71^{bri}$ cell (TA cell) numbers were higher in sun-exposed skin than in sun-protected skin. **b** CD71 expressions in sun-exposed and sun-protected skins. The plot of the sun-exposed skin showed a shift to the right, showing that the proportion of $\alpha6^{bri}$ $CD71^{bri}$ cells was greater in sun-exposed epidermis. The figure represents the results of repetitive flow cytometry in triplicate

have been shown to decline sharply with age [17]. Furthermore, extrinsic factors that induce stimuli like reactive oxygen species, enhance a pathway that causes stem cells to exit their normal quiescent state and eventually may lead to their exhaustion [16]. In the present study, it was found that photoaging might be associated with alterations of the two proliferative cell fractions in the same individuals. Our results show that KSCs numbers tend to be lower in photoaged skin than in chronologically aged skin. Previously, Webb et al. [5] reported that the numbers of KSCs in sun-exposed skin samples of one group were significantly lower than in sun-protected areas of the other group or in neonatal tissues. It is possible that this difference is due to anatomical variations at different body sites, but KSC numbers have been reported to be insensitive to anatomical differences in a mouse model [18]. Increases in TA cell numbers in photoaged epidermis have been hypothesized to be a consequence of a compensatory mechanism to maintain epidermal homeostasis [18] and this is supported by our findings concerning the increased expression of $\beta$1-integrin in suprabasal keratinocytes and increased numbers of Ki-67-positive proliferative cells in epidermis. The expression of $\beta$1-integrin, which is usually confined to basal keratinocytes in normal undamaged epidermis, may be induced in suprabasal, differentiating cell layers associated with hyperproliferation during wound healing or in psoriatic lesions [19]. Therefore, our results could be applied to hyperplastic pattern of photoaging induced by persistent UV exposure and attempt to repair.

The mechanisms of photoaging are not fully understood and although the present study is limited by the small number of samples analyzed, we are encouraged to speculate that photoaging may be associated with alterations in the two proliferative cell fractions. However, future studies are required to elucidate the mechanisms that control KSCs and TA cells during photoaging.

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