"Senescent Keratinocytes as "Melanin Vacuums": Targeting the Melanin-accumulated Cells for Advanced Skin Brightening"

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1. Introduction

Melanin plays a crucial role in protecting skin cells from ultraviolet (UV) radiation. It is synthesized in melanocytes and transferred to keratinocytes, forming a protective "melanin cap" over the nucleus to shield genomic DNA from UV damage. In addition to its photoprotective function, melanin is essential in determining skin pigmentation. In healthy skin, UV exposure stimulates both melanin production in melanocytes and melanin uptake by keratinocytes, leading to skin darkening, a process known as tanning. However, this darkening is temporary, as melanin is gradually eliminated through epidermal turnover once UV exposure ends.

In contrast, senile lentigo (SL) is a common pigmentary disorder associated with aging. Histologically, it is characterized by excessive melanin accumulation in basal keratinocytes. SL does not fade spontaneously; it worsens with age, becoming a common cosmetic concern, particularly among mature women. Consequently, many cosmetic researchers have devoted years to uncovering its underlying mechanisms. Traditionally, SL formation has been attributed primarily to the overproduction of melanin. Recent studies suggest that dysfunctions in keratinocyte differentiation and proliferation could also play an important role in SL pathogenesis.

Since the accumulation of melanin in basal keratinocytes is a characteristic feature of SL, many researchers have attempted to elucidate the mechanism of SL by observing changes in the behavior of cultured keratinocytes after the addition of melanin [1,2]. However, melanin uptake by keratinocytes is also upregulated during tanning, which is a transient and physiologically normal process that resolves spontaneously. Therefore, changes in keratinocyte behavior induced by melanin uptake alone may be insufficient to fully explain the pathogenesis of SL.

While conducting a similar investigation into keratinocyte behavior following melanin uptake, we unexpectedly observed that certain keratinocytes accumulated noticeably more melanin than others. This observation led us to hypothesize that a specific subpopulation of

keratinocytes may contribute to the melanin accumulation and persistence characteristic of SL. To explore this possibility, we conducted a series of experiments to characterize the unique properties of these cells.

2. Materials and Methods

2.1. Cell culture

Normal human epidermal keratinocytes (NHEKs) were obtained from Kurabo (Japan) and cultured in EpiLife® medium (Thermo Fisher Scientific, USA) supplemented with Human Keratinocyte Growth Supplement (Kurabo) at 37°C in a humidified atmosphere with 5% CO_2. The human melanoma cell line MNT-1 was obtained from the American Type Culture Collection (ATCC, USA). MNT-1 cells were maintained in Dulbecco's modified Eagle's medium (Gibco, USA) supplemented with 20% fetal bovine serum, 10% AIM-V medium (Thermo Fisher Scientific), and 0.1 mM nonessential amino acids (Thermo Fisher Scientific), at 37°C in a humidified atmosphere with 5% CO_2. Melanosomes, the specialized organelles where melanin pigment is produced, were isolated from MNT-1 cells as previously described [3]. Fluorescent beads (Fluoresbrite® YO Carboxylate Microspheres 0.50 μm) were purchased from Polysciences (USA).

2.2. Senescence analysis

NHEKs were seeded into 96-well plates at a density of 6,000 cells per well and cultured for 24 h. The culture medium was then replaced with fresh medium containing either a melanosome suspension (equivalent to 0.82 μg of synthetic melanin) or fluorescent beads (final concentration: 0.01%). After 48 h of incubation, cells were stained with Hoechst 33342 (Dojindo, Japan), and images were acquired using an APX100 microscope (Evident, Japan). Senescence was evaluated by measuring Senescence-Associated β-Galactosidase (SA-β-Gal) activity using the Senescence β-Galactosidase Staining Kit (Cell Signaling Technology, USA). Cell outlines were extracted using Cellpose, an artificial intelligence (AI)-based image segmentation tool (open-source software) [4]. Melanin content (measured as the melanin-masked area), bead content (measured as integrated density), and SA-β-Gal activity (measured as the SA-β-Gal-masked area) were quantified using ImageJ software (NIH).

2.3. Statistical analysis

Pearson's correlation coefficient (r) was used to assess the relationship between cell size and each parameter (melanin content, bead content, and SA-β-Gal activity). *P*-values less than 0.01 were considered statistically significant.

2.4. Time-lapse imaging

NHEKs were seeded into 96-well plates at a density of 6,000 cells per well and cultured for 24 h. The culture medium was then replaced with fresh medium containing fluorescent beads (final concentration: 0.01%). Time-lapse imaging was initiated at the time of bead addition (designated as 0 h). Images were captured using an APX100 microscope every 30 min for 100 h to monitor cellular behavior and bead uptake dynamics.

2.5. Immunostaining

NHEKs were cultured with fluorescent beads for 1 week, as described in section 2.2. After washing with phosphate-buffered saline (PBS), the cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature, permeabilized with 0.02% Triton X-100, and then blocked with 3% bovine serum albumin (BSA) for 30 min. For immunostaining, the cells were incubated at room temperature for 1 h with the anti-PAR-2 (protease-activated receptor-2) antibody

(Santa Cruz Biotechnology, USA; 1:100 dilution). After washing, the cells were incubated for 1 h with the Alexa Fluor® 488-conjugated Goat Anti-Mouse IgG (Abcam, UK). Nuclei were counterstained with DAPI Fluoromount-G® (SouthernBiotech, USA). Images were acquired using an APX100 microscope.

2.6. Evaluation of cosmetic ingredients on bead-accumulated keratinocytes

NHEKs were seeded into 24-well plates at 30,000 cells per well and cultured. After 24 h, the culture medium was replaced, and fluorescent beads (final concentration: 0.01%) were added. The cells were further cultured for an additional 72 h to ensure sufficient bead incorporation and to facilitate the emergence of senescent cells. Subsequently, cosmetic ingredients were applied and incubated for 72 h. After treatment, the nuclei were stained with Hoechst 33342 (Dojindo), and images were acquired using an APX100 microscope. To evaluate cell area, the actin cytoskeleton was stained with Alexa Fluore^{TM}488 phalloidin (Thermo Fisher Scientific). Cell outlines were extracted using Cellpose, and the area of each individual cell was quantified using ImageJ software (NIH).
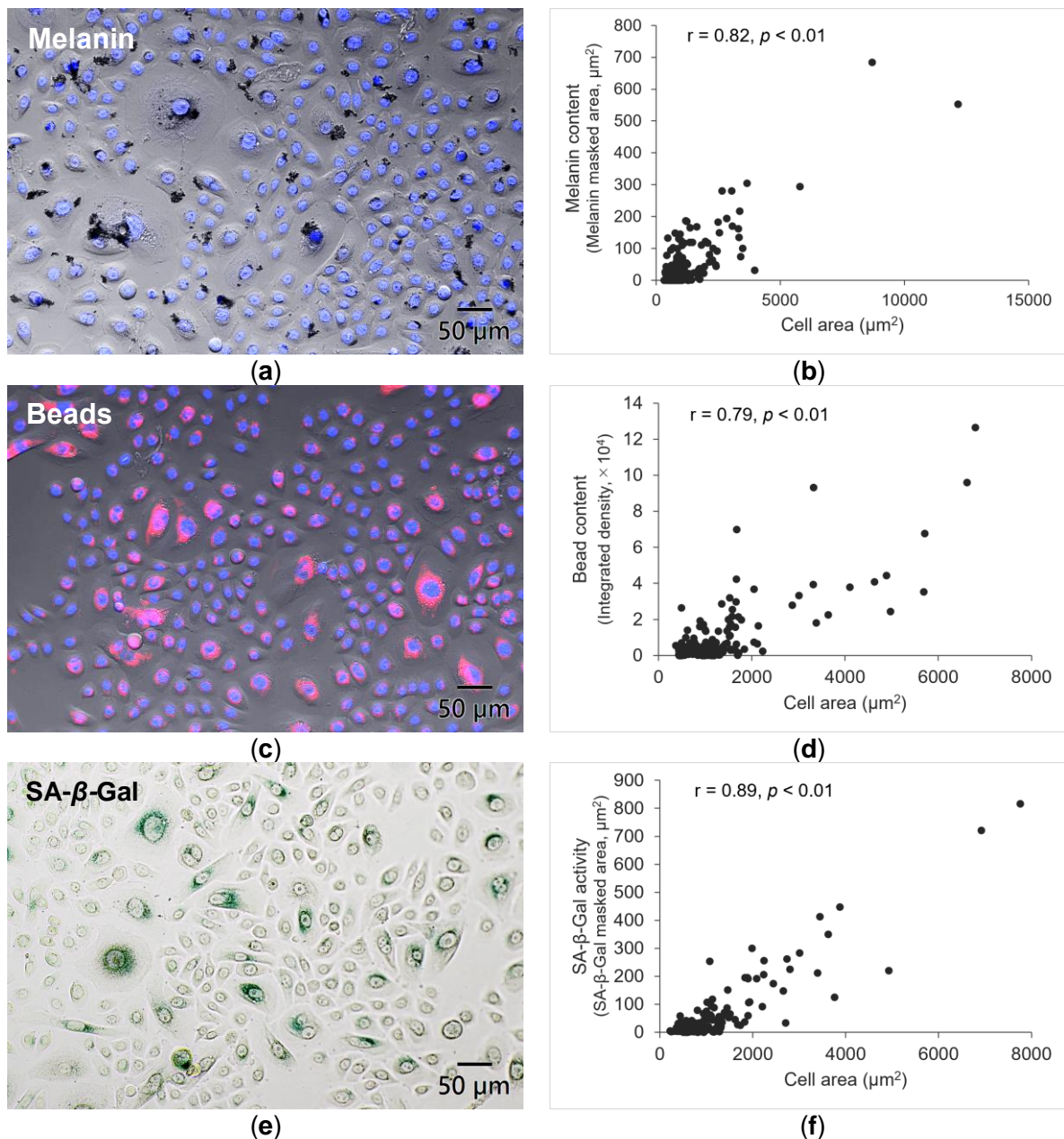
3. Results

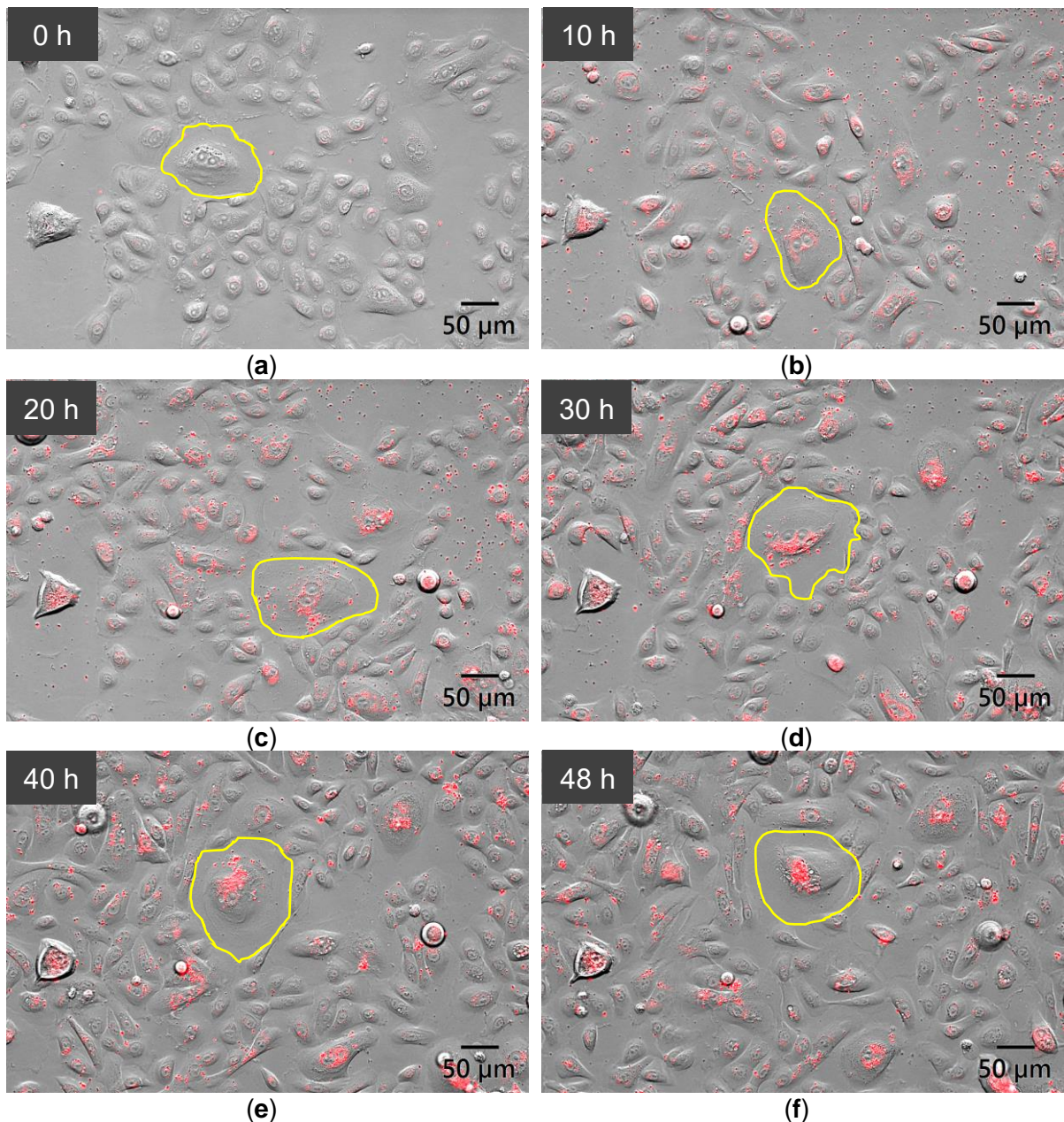### 3.1. Enlarged cells accumulate melanin and beads and exhibit senescence

Microscopic observations revealed an uneven distribution of melanin and melanin-mimicking beads among the keratinocyte population (Fig. 1a, c). Scatter plots based on measurements of cell area and intracellular melanin and bead content demonstrated that enlarged keratinocytes accumulated higher levels of both (Fig. 1b, d). Since cell enlargement is a characteristic morphological change associated with cellular senescence [5], we performed staining using the well-established senescence marker SA-β-Gal. The results confirmed that these enlarged cells were indeed senescent (Fig. 1e, f). These findings suggest that keratinocytes with large amounts of melanin and bead accumulation are senescent and that cell size may serve as a reliable indicator of keratinocyte senescence. Since the intracellular uptake behavior of melanin and melanin-mimicking fluorescent beads was consistent, we used the more visible beads in subsequent experiments.

3.2. Senescent keratinocytes acted as "vacuums"

Although Figure 1 demonstrates that senescent keratinocytes accumulate substantial amounts of melanin and beads, the mechanism underlying this accumulation is unclear. To address this, we conducted time-lapse imaging to observe particle uptake dynamics in real-time. Time-lapse imaging revealed that enlarged senescent keratinocytes exhibited vacuum-like behavior, internalizing beads that remained on the well bottom (Fig. 2). These observations suggest that senescent cells actively internalize extracellular particles, which may contribute to the observed melanin accumulation.

Figure 1. Enlarged Keratinocytes Accumulate More Melanin and Beads and Exhibit Senescence-Associated Features. (a) Merged gradient contrast and fluorescence microscopy images of NHEKs after 48 h of incubation with melanin. Blue: nuclei; black: melanin. **(b)** Scatter plot showing the relationship between cell area and melanin content per cell. Melanin content is measured as a melanin-masked area. **(c)** Merged gradient contrast and fluorescence microscopy images of NHEKs after 48 h of incubation with fluorescent beads. Red: beads; blue: nuclei. **(d)** A scatter plot shows the relationship between cell area and bead content per cell. Bead content is quantified as the integrated fluorescence intensity (integrated density) of the red channel. **(e)** Brightfield microscopy image of SA-β-Gal staining. SA-β-Gal-positive cells are stained blue. **(f)** Scatter plot showing the relationship between cell area and SA-β-Gal activity per cell. SA-β-Gal activity is measured as SA-β-Gal-positive area. Pearson's correlation coefficient (r) was used to assess the relationships shown in (b), (d), and (f). In all cases, **$P < 0.01$. Images in (a), (c), and (e) were acquired at 20× magnification.
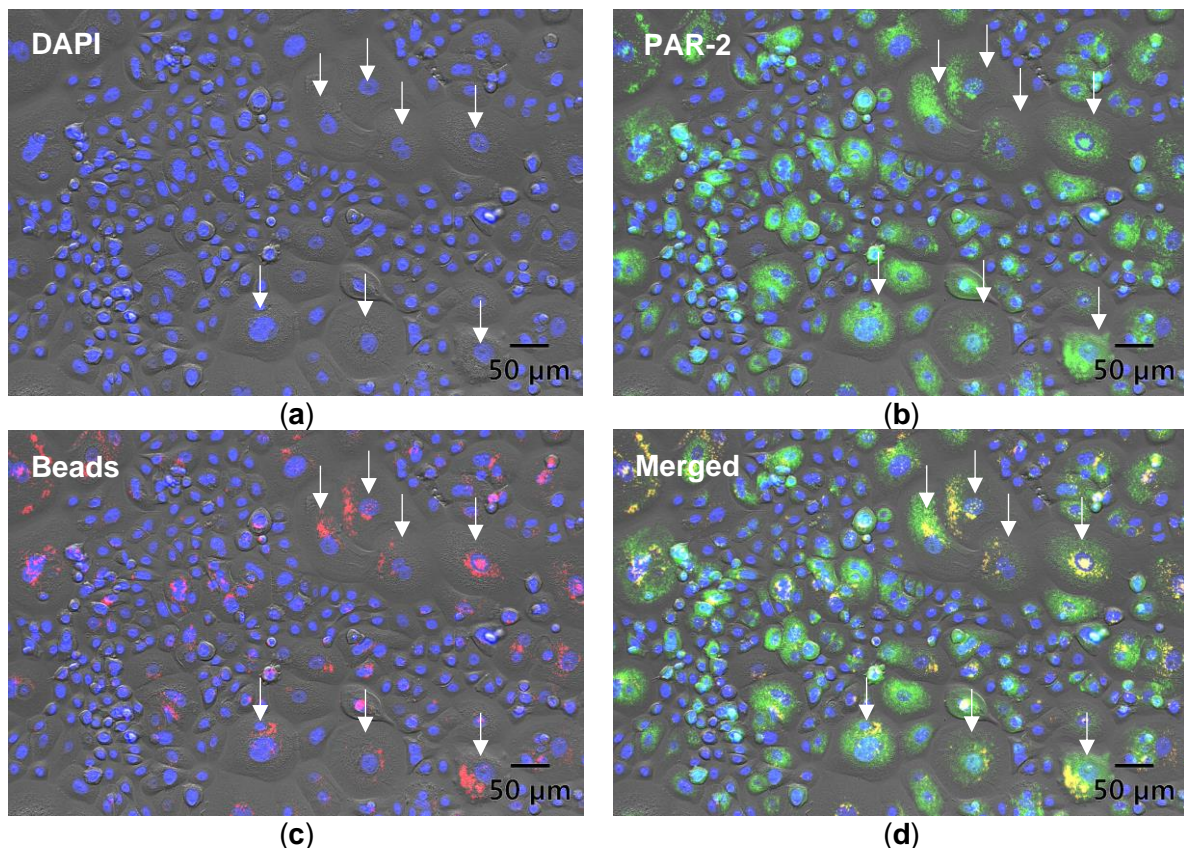
Figure 2. Uptake and Accumulation of Fluorescent Beads in Senescent Keratinocytes.
Panels (a–f) show merged gradient contrast and fluorescence microscopy images of keratinocytes cultured with fluorescent beads for 48 h. **(a)** Immediately after bead addition, the beads are mostly floating in the medium, with very few adhering to the well bottom. **(b)** 10 h, **(c)** 20 h, **(d)** 30 h, **(e)** 40 h, and **(f)** 48 h post-bead addition. Red fluorescence represents the beads, and yellow circles highlight the same senescent cells across time points, characterized by cellular enlargement. Images were acquired at 20× magnification.

3.3. Senescent keratinocytes exhibit elevated PAR-2 expression and bead uptake

Fluorescence immunostaining with an anti-PAR-2 antibody was performed on keratinocytes treated with fluorescent beads (Fig. 3a–d). As shown in Fig. 3b, the enlarged senescent cells exhibited higher PAR-2 expression levels than the surrounding smaller non-senescent cells. Furthermore, the distribution of keratinocytes containing fluorescent beads (Fig. 3c) was generally consistent with that of cells expressing high levels of PAR-2 (Fig. 3b), and partial colocalization was observed in the merged image (Fig. 3d). PAR-2 is known to be a receptor deeply involved in melanosome uptake *[6]*, and these results suggest that bead incorporation may be associated with PAR-2 expression.
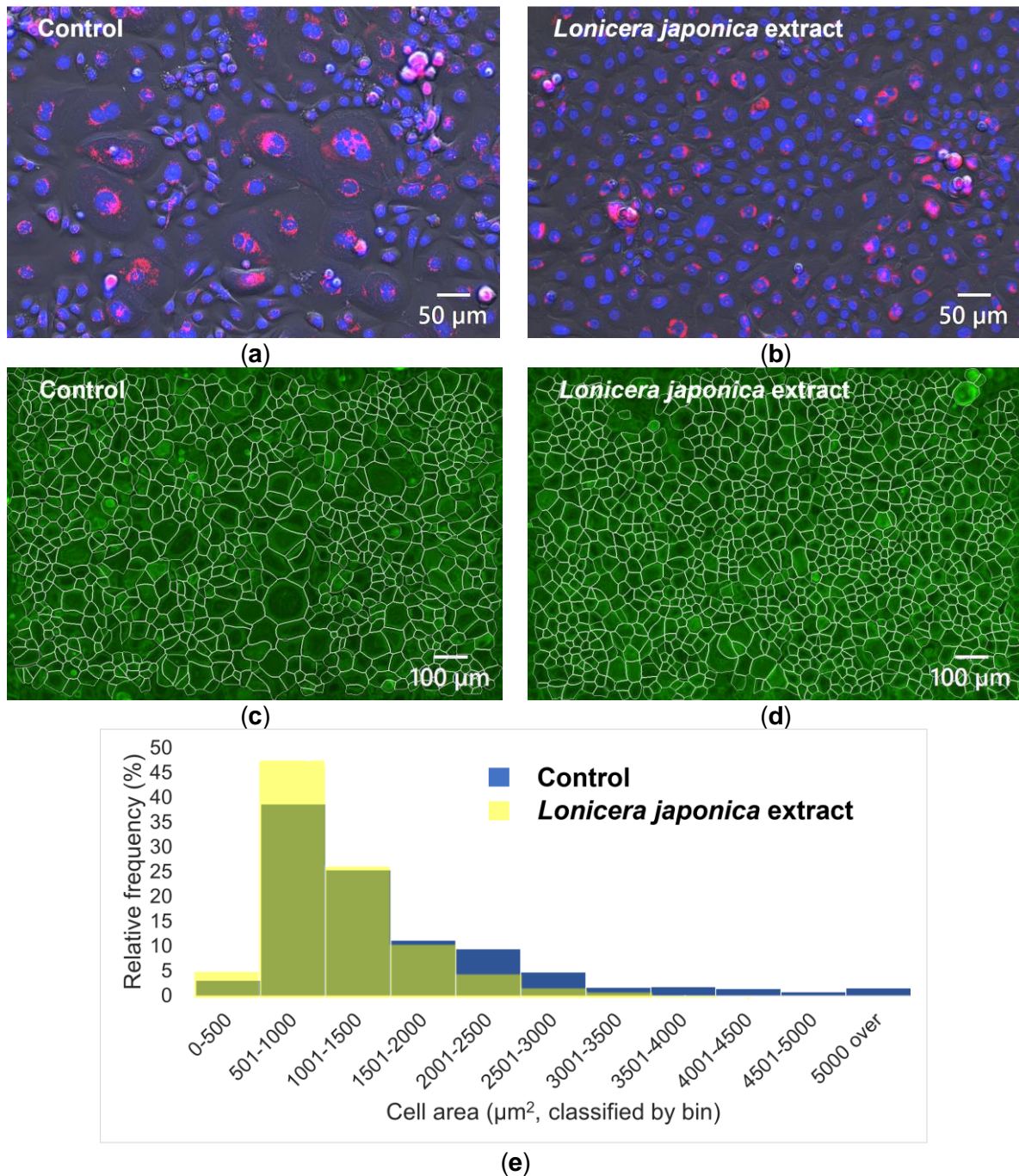

(a)   (b)
(c)   (d)

Figure 3. Co-localization of PAR-2 and Fluorescent Beads. Panels (a–d) show merged gradient contrast and fluorescence microscopy images of keratinocytes cultured with fluorescent beads for 1 week, followed by immunostaining for PAR-2. White arrows indicate senescent cells, characterized by an enlarged cell morphology. **(a)** Blue indicates nuclei, **(b)** green indicates PAR-2 expression and **(c)** red indicates fluorescent beads. **(d)** Merged image of nuclear staining (blue), fluorescent beads (red), and PAR-2 staining (green). The yellow to orange signals in (d) represent the colocalization of PAR-2 and beads, suggesting a close spatial association in senescent cells. Images were acquired at 20× magnification.

3.4. *Lonicera japonica* extract selectively reduces enlarged senescent cells in the keratinocyte population.

Given that senescent keratinocytes actively phagocytose and accumulate beads in the cells, screening was conducted to identify compounds capable of selectively removing these bead-

accumulated enlarged cells from the cell population. Microscopic observations revealed that treatment with *Lonicera japonica* extract led to a noticeable reduction in the number of enlarged, flattened cells compared to the untreated control (Fig. 4a, b). Cell outlines were extracted from the images (Fig. 4c, d) to quantify this effect, and the cell area distributions were analyzed. In Fig. 4e, the histogram shows the cell area distribution of the control group (blue) overlaid onto that of the *Lonicera japonica*-treated group (yellow). A clear shift toward smaller cell sizes was observed in the treated group. These findings suggest that the *Lonicera japonica* extract selectively promotes the elimination of senescent keratinocytes.

Figure 4. Selective Removal of Enlarged Keratinocytes by *Lonicera japonica* Extract. NHEKs were cultured with 0.01% fluorescent beads for 72 h to ensure sufficient bead incorporation,

during which time, senescent NHEKs were observed, as shown in Figure 1c. The cells were then treated with water control or *Lonicera japonica* extract for 72 h. **(a, b)** Merged gradient contrast and fluorescence images of live cells treated with water (a) and *Lonicera japonica* extract (b), showing the nuclei (blue) and internalized beads (red) at 20× magnification. **(c, d)** Gradient contrast images of cells treated with water (c) and *Lonicera japonica* extract (d) under bead-free conditions. Cells were stained with phalloidin, and cell outlines were extracted using Cellpose (white lines) and overlaid onto the original images. The magnification is 10×. **(e)** Cell area distributions from (c) and (d) were measured using ImageJ. The control group (e, blue) and *Lonicera japonica*-treated group (e, yellow) show a reduction in enlarged cells after treatment.

4. Discussion

Understanding the mechanisms underlying SL has long been a challenge in cosmetic research. Although various active ingredients have been developed to improve SL, their efficacy remains limited, particularly in treating pre-existing pigmentation. This may be because most of these ingredients primarily target melanogenesis in melanocytes. However, recent studies suggest that SL is also associated with abnormalities in the differentiation and proliferation of basal keratinocytes. This implies that effective treatments would need to specifically target the melanin-rich basal keratinocytes characteristic of SL. Nonetheless, the precise nature of these keratinocyte abnormalities remains unclear.

During our experiments, we observed that certain keratinocytes in the cultured population accumulated notably more melanin than others. This interesting finding led us to hypothesize that identifying the characteristics of these melanin-accumulated keratinocytes could be crucial to understanding the mechanisms behind abnormal melanin deposition and persistence in SL.

In this study, our investigations revealed that keratinocytes accumulating melanin and beads exhibited characteristics of cellular senescence, such as increased cell area and the expression of the senescence-associated marker, SA-β-Gal (Fig. 1). These findings suggest that keratinocytes with substantial melanin and bead accumulation are senescent, and that cell area could serve as a reliable indicator of keratinocyte senescence. Since the behavior of melanin and fluorescent beads was similar, we used fluorescent beads that mimic melanin uptake in subsequent experiments to aid visualization and tracking.

While Fig. 1 provides a static snapshot showing that senescent keratinocytes accumulate substantial amounts of melanin and beads, the mechanism underlying this accumulation remained unclear. To investigate this further, we performed time-lapse imaging to observe particle uptake in real-time. Surprisingly, senescent keratinocytes actively phagocytosed beads that remained on the surface of the culture well (Fig. 2). Given their non-proliferative nature and presumed low metabolic activity, we initially hypothesized that melanin accumulation simply resulted from the inability to divide and dilute cellular contents. However, the observed active engulfment suggests that senescent cells retain unexpected phagocytic activity despite their typically static nature.

To help explain why senescent cells exhibit active phagocytosis, we identified the possible reason for the enhanced phagocytic activity observed in senescent cells: increased expression of PAR-2 (Fig.3), a receptor involved in melanosome uptake [6]. Recent studies have reported that PAR-2 may also promote cellular senescence [7], suggesting that its inhibition could offer

the dual benefit of suppressing both melanin uptake and senescence progression. However, if the pigmentation in SL results from pre-existing melanin-rich senescent keratinocytes, targeting PAR-2 may help prevent future pigmentation but may not resolve existing lesions. Therefore, rather than inhibiting PAR-2, we considered that selectively eliminating these senescent keratinocytes containing melanin could be a more effective strategy for improving SL.

Through screening of cosmetic ingredients, we identified *Lonicera japonica* extract as a promising candidate. In mixed keratinocyte cultures, which included both non-senescent and senescent cells, the extract reduced the number of enlarged, senescent cells, suggesting selective clearance of senescent cells (Fig. 4). In preliminary experiments, *Lonicera japonica* extract did not exhibit any senescent cell eliminating effects in cultures composed solely of senescent keratinocytes induced by doxorubicin (data not shown). This suggests that the extract may not directly act on senescent cells, but it is likely working through surrounding cells; however, the mechanism behind this selective clearance remains unclear, and further studies are needed to elucidate it.

This study found that keratinocytes accumulating melanin are senescent and exhibit active engulfment. Two key characteristics of senescent cells are their resistance to being removed from the tissue and their ability to promote senescence in neighboring cells through the secretion of senescence-associated secretory phenotype (SASP) factors [5]. We propose that senescent cells play a key role in SL pathogenesis. Specifically, repeated UV exposure generates a small number of senescent basal keratinocytes, which, over time, spread senescence to neighboring cells through SASP, gradually forming a larger population. With increased phagocytic ability, these senescent cells accumulate melanin and resist clearance, leading to visible hyperpigmentation in SL and sustained pigmentation.

Regarding the relevance of cellular senescence in SL lesions, several studies have reported the presence of senescent keratinocytes in SLs based on immunostaining for p16^{INK4a} in excised tissue sections [8,9]. However, the findings remain controversial due to variability in marker expression and detection sensitivity [10]. In contrast, a recent study has revealed reduced mitochondrial activity in keratinocytes in SL lesions [2], further supporting the presence of senescent cells in these lesions.

Based on our model, effective SL treatments should focus on selectively eliminating melanin-accumulated senescent keratinocytes. The *Lonicera japonica* extract identified in this study removes senescent cells from mixed populations, including both non-senescent and senescent cells. Unlike traditional brightening treatments, this approach specifically targets melanin-rich keratinocytes in the basal layer of SL lesions, offering a promising strategy for promoting an even skin tone and improving SL.

5. Conclusion

Senescent keratinocytes accumulate melanin and beads due to active uptake, which is a process likely mediated by PAR-2. Keratinocyte senescence is expected to be a key factor in the pathogenesis of SL. Therefore, we propose that *Lonicera japonica* extract, which selectively eliminates senescent cells *in vitro*, may promote the clearance of melanin-rich keratinocytes from SL lesions and offer a novel strategy for improving SL.

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