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## ***Mechanisms of Collagen Homeostasis Disruption in Senescent Cells and Novel Role of Niacinamide : Enhancing Autophagy and Preventing Misfolded Collagen Accumulation with Cellular Senescence***

**Akira Hagino<sup>1</sup>, Mitsuhiro Gomi<sup>1</sup> and Hiroko Yano<sup>1</sup>**

<sup>1</sup> R&D Headquarters, KOBAYASHI Pharmaceutical Co. Ltd., Osaka, Japan

### **1. Introduction**

Cellular senescence, an irreversible arrest of the cell cycle in response to various stresses such as ultraviolet irradiation, has been implicated in the pathogenesis of age-related diseases. Senescent cells release senescence-associated secretory phenotype (SASP) factors, including inflammatory cytokines and matrix metalloproteinases, into the extracellular space, which can adversely affect the surrounding tissues [1]. Senescent cells accumulate in aged skin, particularly in the dermis [2]. Several studies have also suggested that senescent cells contribute to skin aging, such as the formation of wrinkles and sagging [2, 3, 4, 5]; however, the underlying mechanisms remain poorly understood.

The structural and qualitative changes in the dermis substantially affect wrinkle formation and sagging, which are characteristic features of skin aging. Type I collagen, the major constituent of the dermal extracellular matrix, is synthesized within dermal fibroblasts as collagen peptide chains (two Pro $\alpha$ 1 and one Pro $\alpha$ 2). These chains fold within the lumen of the endoplasmic reticulum (ER), forming a triple helical structure comprising three collagen peptide chains, and are released into the extracellular space via the Golgi network. Although proper folding within the ER, which is regulated by chaperone molecules such as heat shock protein 47 (HSP47), is crucial for normal collagen production, due to the unique features of procollagen folding, 10%-15% of the molecules are misfolded under normal conditions [6, 7]. In addition, the mutation of the gene or long-term ER stress causes misfolded collagen [8]. Misfolded collagen is usually degraded by proteasome, but aggregates formed by misfolded collagen that cannot be completely degraded by the degradation system are reportedly degraded by autophagy, contributing to the maintenance of intracellular collagen homeostasis. Autophagy, a highly conserved cellular process, plays a crucial role in damaged organelle and misfolded protein degradation, thereby maintaining cellular homeostasis [9, 10]. However, a recent study has demonstrated a decline in autophagic activity during cellular senescence [11].

Although the reduction of collagen and loss of orientation occur in the dermis of aged skin, the changes in collagen folding within the cells remain unclear. Specifically, the changes in collagen aggregate formation associated with cellular senescence have not been previously investigated. Therefore, we aimed to investigate whether the collagen homeostasis changes with cellular senescence, with a focus on misfolded collagen aggregates. Furthermore, our

study sought to elucidate and explore the potential of targeting senescent cells in preventing and treating skin aging.

## 2. Materials and Methods

### 2.1 Reagent

The following antibodies were used: anti-collagen I antibody (ab34710, abcam), anti-LC3B antibody (ab51520, abcam), anti- $\beta$ -actin antibody (#3700, Cell Signaling Technology), anti-HSP47 antibody (sc-5293, Santa Cruz Biotechnology), and anti-FAM134B (PA5-64943, Thermo Fisher Scientific).

### 2.2 Cell culture

Normal human dermal fibroblasts (NHDFs), which were purchased from Riken Cell Bank, were maintained at 37°C and 5% CO<sub>2</sub> in minimum essential medium  $\alpha$  (Thermo Fisher Scientific) containing 10% fetal bovine serum. Human umbilical vein endothelial cells (HUVECs), which were purchased from Promo Cell, were maintained at 37°C and 5% CO<sub>2</sub> in endothelial cell growth medium 2 (C-22011, Promo Cell). For senescence induction, NHDFs were treated with 200-ng/mL doxorubicin (hydrochloride) (DXR, Cayman Chemical) for 7 days and HUVEC were treated with 10 ng/mL DXR for 6 days. Senescence-associated  $\beta$ -galactosidase (SA- $\beta$  gal) staining was performed according to the  $\beta$ -galactosidase staining kit protocol (Clontech Laboratories). Following doxorubicin-induced senescence, the cells were treated with compounds for specified durations to examine the compound effects.

### 2.3 Quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cells using the ReliaPrep™ RNA Miniprep Systems (Promega). The extracted total RNA samples were reverse-transcribed using Random Primer (3801, Takara Bio) and ReverTra Ace (TRT-101, Toyobo). Quantitative PCR was performed on QuantStudio 3 (Thermo Fisher Scientific) using PowerTrack SYBR Green Master Mix (A46109, Thermo Fisher Scientific). Relative mRNA was calculated after normalization to the human TBP gene as an internal control for quantification using the  $2^{-\Delta\Delta CT}$  method.

### 2.4 Immunofluorescence microscopy

Non-senescent human dermal fibroblasts (nSDFs) or senescent human dermal fibroblasts (SDFs) were grown on a cover glass and treated with or without niacinamide and fixed in 4% paraformaldehyde for 15 min. The cells were washed using a phosphate-buffered saline (PBS) solution to remove the excess fixatives. After washing, the cells were permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) for 15 min and blocked with 1% fetal bovine serum in PBS for 1 hour at room temperature. After blocking, the cells were incubated with the primary antibody and PROTEOSTAT (Enzo Life Science), which can detect protein aggregates, for 1 hour at 4°C, washed thrice, and incubated for 1 hour with appropriate secondary antibodies conjugated with fluorophore. For nuclear staining, we used Hoechst33342 (Dojindo). The images were acquired using the All-in-one Fluorescence Microscope (BZ-X800, Keyence). The intensity of co-localization signals of collagen and the protein aggregates in the images were calculated in an automated manner using the BZ-X800 analyzer software (Keyence).

### 2.5 Immunoblotting

nSDFs or SDFs were seeded in six-well plates at a density of  $3.0 \times 10^5$  cells/well and cultured for 24 h. Thereafter, the cells were cultured with or without niacinamide or chloroquine diphosphate (Fujifilm Wako Chemicals) for the indicated times and then lysed on ice in RIPA buffer supplemented with protease inhibitor cocktail (P8340-1ML, Sigma). After

centrifugation, the supernatant's protein concentration was measured using a Takara BCA Protein Assay kit (T9300A, Takara Bio) and dissolved in the sample buffer. The proteins were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 5% skim milk for 1 h and probed with the following primary antibodies. Subsequently, we incubated the membranes with secondary antibodies (Cell Signaling Technology), detected the signals by chemiluminescence using an ECL prime kit (RPN2232, Fisher Scientific), and captured images using LAS500 (Cytiva).

## **2.6 mCherry-GFP tandem fluorescent-tagged LC3 (tfLC3) assay**

Hela cells were transfected with a plasmid encoding tfLC3 using Lipofectamine 3000 (Thermo Fisher Scientific). The selected cells stably expressing tfLC3 were treated with or without niacinamide for 24 hours. After treatment, the cells were fixed with 4% paraformaldehyde, including Hoechst 33342 for 15 minutes, and mCherry-GFP-LC3 puncta were observed by fluorescence microscopy (BZ800, Keyence). The intensity of GFP and mCherry were measured using the BZ-X800 analyzer software (Keyence), and the autophagy flux was determined by calculating the GFP/mCherry rates.

## **2.7 Statistical analysis**

Statistical data were analyzed using Prism10 (GraphPad Software). All quantitative data were expressed as means  $\pm$  standard deviation, and significant differences were determined by the two-tailed unpaired student's t-test or ordinary one-way analysis of variance followed by Dunnett's test. *P*-values of  $<0.05$  were considered significant.

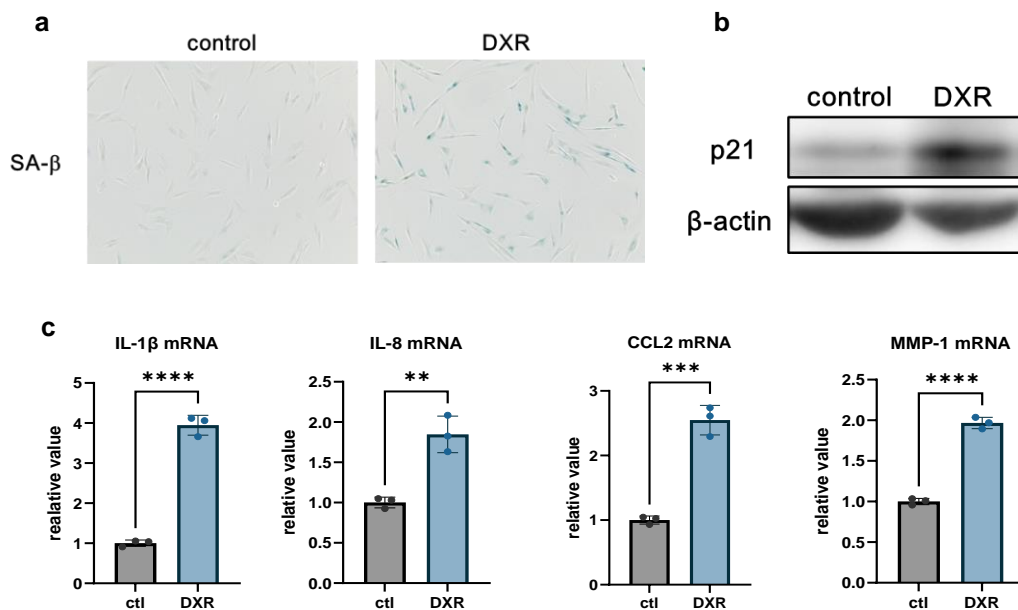
# **3. Results**

## **3.1 Intracellular collagen homeostasis is disrupted in senescent fibroblasts**

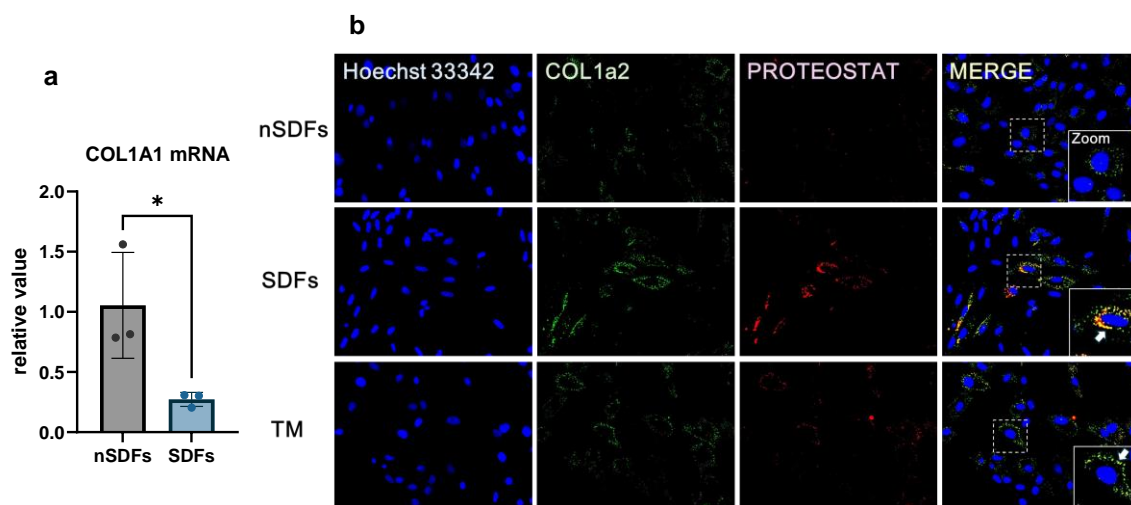
To establish a cellular senescence model, we treated human dermal fibroblasts with doxorubicin and confirmed the induction of cellular senescence by assessing the SA- $\beta$  gal staining results and the increased expression of senescence markers, including p21 and SASP factors (Figure 1a-c). Having established this model, we next sought to explore whether intracellular collagen homeostasis changes with cellular senescence. To this end, we first examined the gene expression of type I collagen  $\alpha$ 1chain in SDFs. As reported previously [12], compared with nSDFs, the level of gene expression was lower in SDFs (Figure 2a). Next, to investigate whether collagen folding changes during cellular senescence, we observed the misfolded collagen aggregates in SDFs by immunofluorescence against type I collagen and protein aggregates. The protein aggregates were detected using PROTEOSTAT. Remarkably, we found that the misfolded collagen aggregates, demonstrated by the colocalization of type I collagen  $\alpha$ 1 (green) and protein aggregates (red), accumulated in the SDFs. Furthermore, the extent of these aggregates was greater than that observed in the misfolded collagen aggregates induced by tunicamycin, an ER stress inducer (Figure 2b). Thus, the collagen homeostasis in senescent cells is thought to be disrupted.

## **3.2 HSP47 expression and autophagic activity are reduced in senescent fibroblasts**

The observed accumulation of misfolded collagen aggregates in SDFs led us to hypothesize that the function of collagen folding within the ER and the degradation of misfolded collagens are affected by cellular senescence. To examine this, we first investigated whether the expression of HSP47, a collagen-specific molecular chaperone, changes with cellular senescence. As a result, we found that the gene and protein expressions of HSP47 were reduced



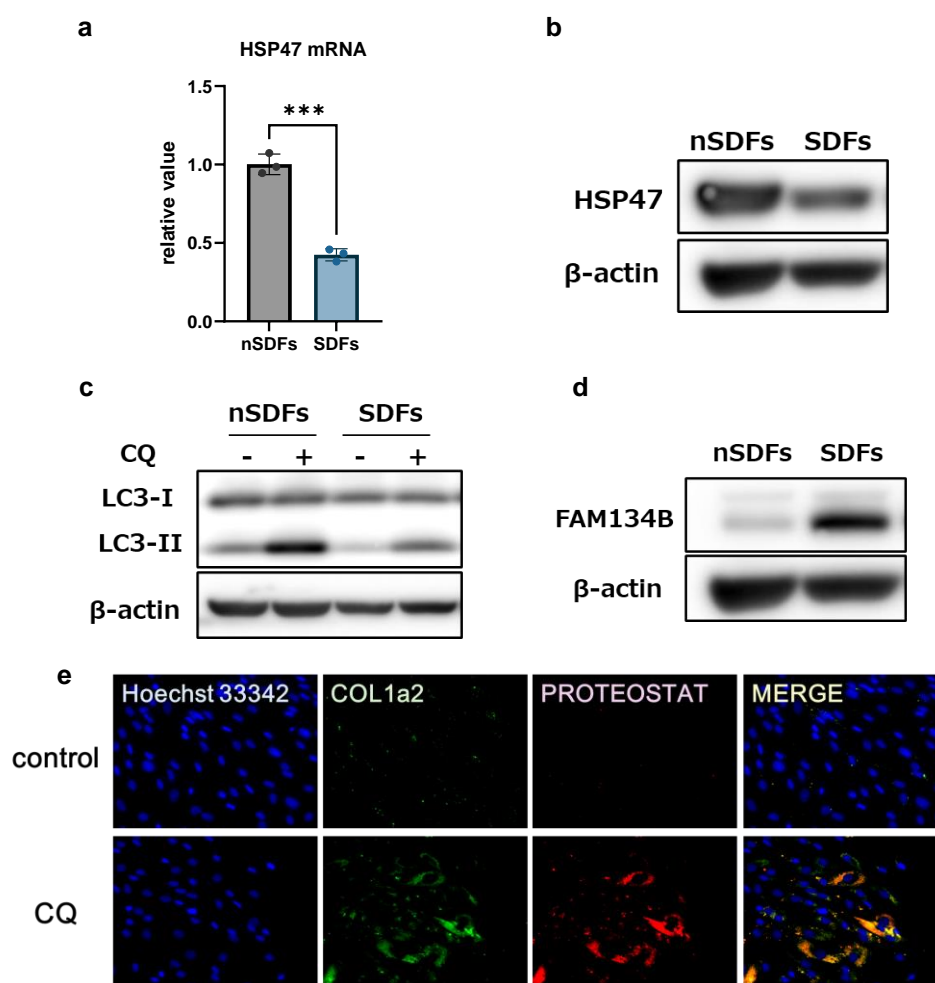
**Figure 1. Doxorubicin induces cellular senescence.** Normal human dermal fibroblasts were treated with or without 200 ng/mL doxorubicin for 7 days. After treatment, the senescence-associated  $\beta$ -galactosidase (SA- $\beta$  gal) activity (a), protein expression of p21 (b), and gene expression of IL-1 $\beta$ , IL-8, CCL2, and MMP-1 (c) were analyzed. Data are presented as mean  $\pm$  standard deviation (SD). Statistical analysis was performed with two-tailed unpaired *t*-test (*N* = 3). \*\**P* < 0.01, \*\*\**P* < 0.001, \*\*\*\**P* < 0.0001.



**Figure 2. Collagen homeostasis is disrupted in senescent fibroblasts.** (a) Collagen type I  $\alpha$ 1 chain (COL1A1) gene expression in non-senescent fibroblasts (nSDFs) and senescent fibroblasts (SDFs) was determined by quantitative polymerase chain reaction (qPCR). (b) Non-senescent human dermal fibroblasts (nSDFs) or senescent human dermal fibroblasts (SDFs) treated with tunicamycin (TM) were immunolabeled for type I pro-collagen  $\alpha$ 2 (COL1a2, green) and PROTEOSTAT, which detected protein aggregates (red). Arrow indicates colocalization of COL1a2 and protein aggregates. Data are presented as mean  $\pm$  standard deviation (SD). Statistical analysis was performed with two-tailed unpaired *t*-test (*N* = 3). \**P* < 0.05.

in SDFs (Figure 3a, b). Then, to investigate whether the ability to degrade collagen aggregates also changes with cellular senescence, we examined the activity of autophagy, which reportedly degrades the aggregates of misfolded collagens, in SDFs. To assess the

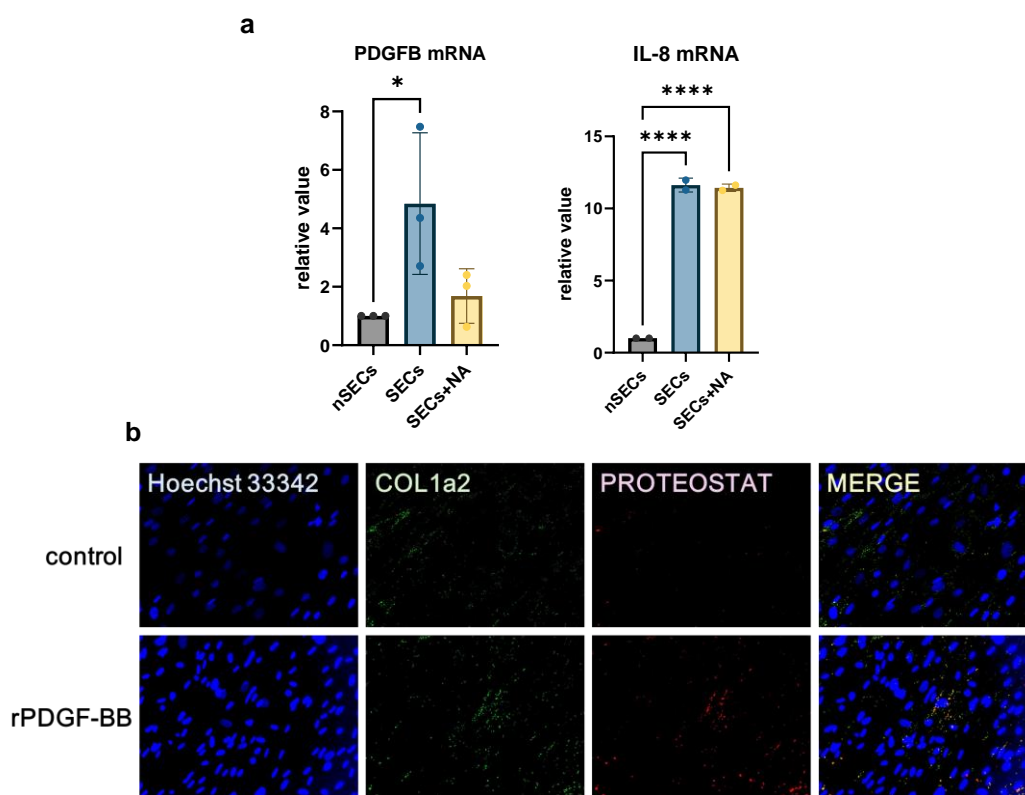
autophagy flux, we measured the levels of LC3-II, an autophagy marker that binds to the autophagosome membrane and is degraded upon fusion with lysosomes, with and without lysosome inhibitor (chloroquine) treatment. The lower increase in LC3-II levels upon lysosome inhibition in SDFs as compared with nSDFs indicated a decrease in autophagic flux, suggesting a reduced autophagosome degradation rate in SDFs (Figure 3c). A recent study has shown that misfolded collagens within the ER are recognized by FAM134B, an autophagy receptor protein, and then misfolded collagens- FAM134B were degraded by autophagy [13]. The FAM134B expression in SDFs was also increased (Figure 3d), suggesting that the decline in autophagy caused FAM134B accumulation in the senescent cells. Furthermore, to explore whether the decline in autophagic activity contributes to the accumulation of misfolded collagen aggregates, we conducted the experiment using chloroquine, an inhibitor of autophagy. As a result, we observed a remarkable increase in collagen aggregates in the cells treated with chloroquine (Figure 3e).



**Figure 3. The heat shock protein (HSP47) expression and autophagic activity are reduced in senescent fibroblasts.** (a, b) The HSP47 gene and protein expressions in non-senescent human dermal fibroblasts (nSDFs) and senescent human dermal fibroblasts (SDFs) induced by doxorubicin were determined by quantitative polymerase chain reaction (qPCR) and immunoblotting, respectively. (c) nSDFs and SDFs were subjected to autophagy flux assay. (d) FAM134B protein expression in nSDFs and SDFs was determined by immunoblotting. (e) Normal human dermal fibroblasts treated with or without 10-μM chloroquine (CQ) for 4 days were subjected to immunofluorescence against type I pro-collagen α2 (COL1a2, green) and PROTEOSTAT (red). Data are presented as mean ± standard deviation (SD). Statistical analysis was performed with two-tailed unpaired *t*-test (N = 3). \*\*\**P* < 0.001.

### 3.3 Plated-derived growth factor expression is increased in senescent vascular endothelial cells and induces the accumulation of misfolded collagen aggregates

To investigate the effect of senescent cells other than fibroblasts on collagen folding in fibroblasts, we conducted an experiment using vascular endothelial cells. First, we confirmed that the gene expression of inflammatory cytokines and platelet-derived growth factor subunit b (PDGFB), which functions as a PDGF-BB dimeric protein, increased in doxorubicin-induced senescent vascular endothelial cells (Figure 4a). Given that excessive PDGF-BB is known to promote fibrosis by stimulating collagen production [14], we hypothesized that it might also disrupt the delicate process of collagen folding within the fibroblasts. Therefore, we examined the possibility that PDGF-BB might affect collagen folding. As a result, collagen aggregates accumulated in nSDFs treated with recombinant PDGF-BB (Figure 4b). This finding suggested that the senescent cells, other than the dermal fibroblasts, are also involved in the disruption of collagen homeostasis.

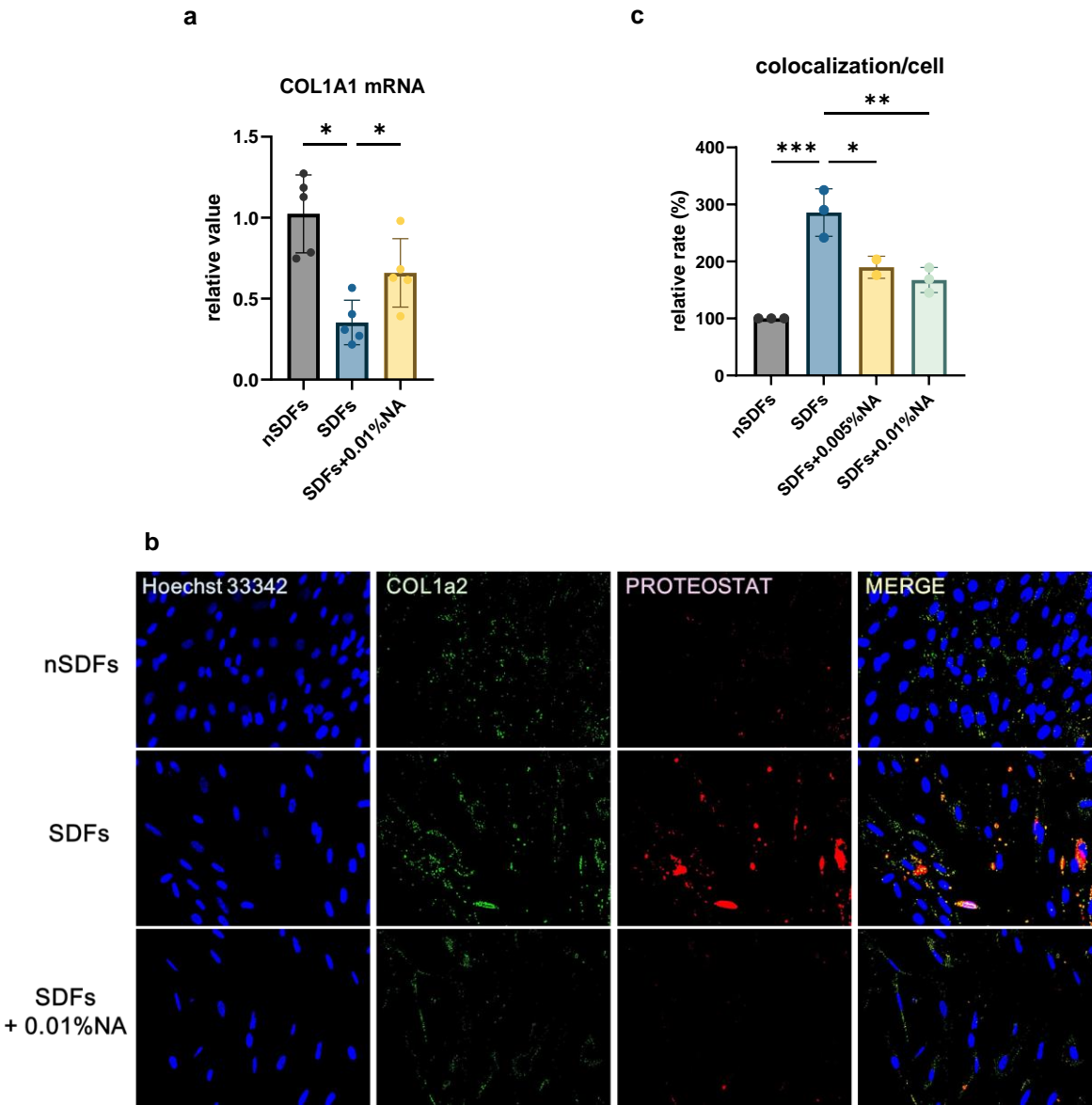


**Figure 4. The expression of platelet-derived growth factor subunit B (PDGFB) is increased in the senescent endothelial cells, and the molecule induces the accumulation of misfolded collagen in the dermal fibroblasts.** (a) The gene expression of PDGFB and IL-1 $\beta$  in non-senescent human umbilical vein endothelial cells (nSECs) and senescent human umbilical vein endothelial cells (SECs) treated with or without 0.01% niacinamide (NA) was determined by quantitative polymerase chain reaction (qPCR). (b) Human dermal fibroblasts treated with or without recombinant PDGF-BB were immunolabeled for type I collagen  $\alpha$ 2 (COL1a2, green) and PROTEOSTAT (red). Data are presented as mean  $\pm$  standard deviation (SD). Statistical analysis was performed with ordinary one-way analysis of variance (ANOVA) followed by Dunnett's test (N = 3). \* $P$  < 0.05, \*\*\*\* $P$  < 0.0001.

### 3.4 Niacinamide alleviates disrupted intracellular collagen homeostasis in senescent cells

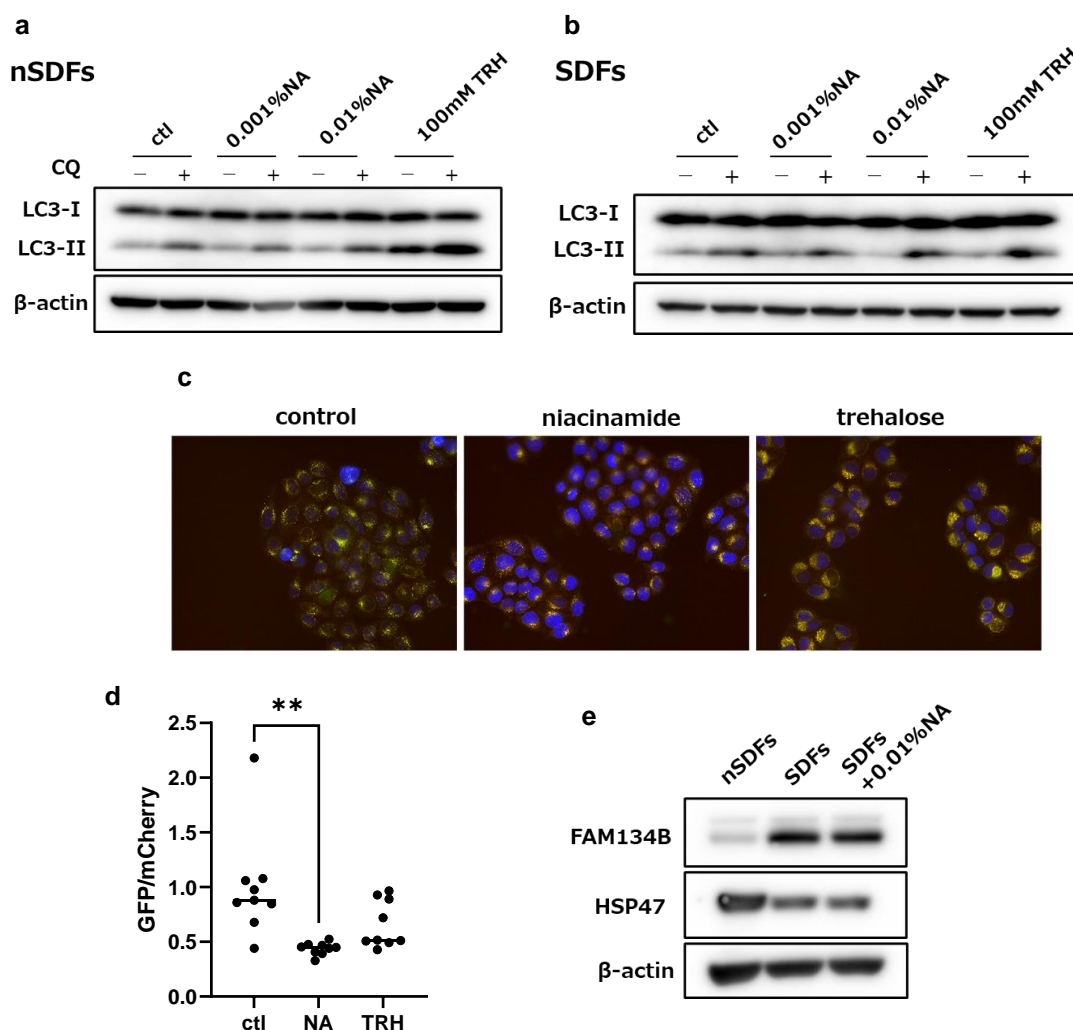
Given the possibility that collagen homeostasis is disrupted by cellular senescence, we next investigated the effects of niacinamide, which has various skin effects and is widely used as

a cosmetic ingredient, on senescent cells. First, we confirmed that niacinamide treatment restored the decreased collagen gene expression in the SDFs (Figure 5a). Furthermore, this treatment also reduced the formation of collagen aggregates in senescent cells (Figure 5b, c). Next, we examined the mechanism by which niacinamide reduced collagen aggregates. Niacinamide did not affect the HSP47 expression in SDFs (Figure 6e). However, by immunoblotting, we confirmed that niacinamide enhanced the autophagic activity in both nSDFs and SDFs (Figure 6a, b). The enhancing effect of niacinamide on autophagy was also confirmed by the tFLC3 assay using HeLa cells, which allows for the detection of autophagy flux by monitoring the pH-dependent quenching of GFP fluorescence within the acidic lysosomal environment, while mCherry fluorescence remains stable (Figure 6c, d) [15]. Moreover, we included trehalose, a known autophagy activator, as a positive control in our experiments, which also demonstrated increased autophagic activity (Figure 6a- d). Additionally, FAM134B expression was decreased in the niacinamide-treated SDFs (Figure 6e). Finally, we investigated the effect of niacinamide treatment on PDGFB expression in the senescent vascular endothelial cells. Niacinamide treatment suppressed the increased PDGFB gene expression in these cells (Figure 4a).



**Figure 5. Niacinamide alleviates disrupted collagen homeostasis in senescent fibroblasts.**

(a) The gene expression of type I collagen  $\alpha 1$  chain (COL1A1) in non-senescent human dermal fibroblasts (nSDFs) and senescent human dermal fibroblasts (SDFs) treated with or without niacinamide (NA) for 4 days was determined by quantitative polymerase chain reaction (qPCR). (b) nSDFs and SDFs treated with or without niacinamide were immunolabeled for type I collagen  $\alpha 2$  (green) and PROTEOSTAT (red). (c) The obtained images were analyzed to calculate the intensity of the colocalization signals of COL1a1 and PROTEOSTAT per cell using the BZ-X800 analyzer software (Keyence). Data are presented as mean  $\pm$  standard deviation (SD). Statistical analysis was performed with ordinary one-way analysis of variance (ANOVA) followed by Dunnett's test (a: N = 5, c: N = 3). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

**Figure 6. Niacinamide enhances the activity of autophagy.**

(a, b) non-senescent human dermal fibroblasts (nSDFs) and senescent human dermal fibroblasts (SDFs) were treated with or without niacinamide (NA) or trehalose (TRH) for 48 hours. After treatment, these cells were subjected to immunoblotting to examine the autophagy flux. (c) HeLa cells stably expressing mCherry-GFP tandem fluorescent-tagged LC3 were treated with or without 0.01% NA or 100-mM TRH for 24 hours, and fixed. Images were acquired using the All-in-one Fluorescence Microscope (BZ-X800, Keyence). The intensity of mCherry and GFP in the obtained images (c) were measured and the rates of GFP/mCherry were calculated using an imaging analyzer (Keyence). (e) The expression of heat shock protein 47 and FAM134B in nSDFs and SDFs treated with or without NA were determined by immunoblotting. Data are presented as mean  $\pm$  standard deviation (SD). Statistical analysis was performed with ordinary one-way analysis of variance (ANOVA) followed by Dunnett's test (N = 9). \*\* $P < 0.01$ .

#### 4. Discussion

It is well-established that senescent cells accumulate in the skin, particularly in the dermis, with aging. However, the association between senescent cell accumulation and skin aging remains poorly understood. In the present study, we demonstrated that intracellular collagen homeostasis is disrupted by cellular senescence. We also showed that niacinamide, which is an ingredient widely used in cosmetics, contributes to the maintenance of collagen homeostasis. Our findings provide novel insights into the relationship between senescent cells and skin aging.

Consistent with the results of a previous study showing decreased collagen in aged skin [16], our experiments revealed reduced collagen gene expression in senescent cells. Furthermore, for the first time, we discovered that collagen aggregates accumulate within the senescent cells. This strongly suggests that the reduced collagen in aged skin not only is a result of decreased collagen expression but also considerably involves impaired collagen folding within the ER during cellular senescence, leading to a decrease in the amount of properly folded collagen for release into the extracellular space. We found a significant decrease in the expression of HSP47, a collagen-specific molecular chaperone, in the senescent cells. Previous studies using HSP47 knockout cells have demonstrated that misfolded collagen aggregates are degraded through autophagy, an intracellular degradation mechanism [17]. In addition, the accumulation of protein aggregates due to decreased autophagy has been reported in neurons, which has been implicated as a cause of cognitive decline [18]. Crucially, our findings also revealed a considerable reduction in the autophagic activity in senescent fibroblasts. Consistent with this result, we observed the accumulation of FAM134B, a receptor protein that selectively recognizes misfolded collagen and targets it for autophagic degradation, in senescent cells. To further investigate the role of autophagy in the clearance of misfolded collagen aggregates, we conducted an experiment using chloroquine, an inhibitor of autophagy. The results showed a remarkable increase in collagen aggregates in the cells treated with chloroquine. These data strongly suggest that the decreased HSP47 expression during cellular senescence leads to the increased formation of misfolded collagen aggregates, which accumulate within the senescent cells due to impaired autophagy-mediated degradation.

The dermis comprises various cell types beyond fibroblasts, and vascular endothelial cells have also been reported to undergo senescence with aging [19]. In our experiments, senescent vascular endothelial cells exhibited increased PDGFB expression. Although PDGF-BB is known to enhance collagen expression, its excessive production can lead to fibrosis [14]. We observed collagen aggregate accumulation in fibroblasts treated with recombinant PDGF-BB. This may be attributed to the exceeding of the collagen folding capacity due to the excessively elevated collagen expression. These results suggest that senescence in cells other than fibroblasts within the dermis also contributes to the formation of collagen aggregates. It is highly plausible that not only PDGF-BB but also the other SASP factors released from the senescent vascular endothelial cells, as well as SASP factors secreted by the other senescent cell populations within the skin, contribute to the disruption of collagen homeostasis in fibroblasts. To fully elucidate the complex interplay of various SASP factors and their specific contributions to collagen dysregulation, further comprehensive studies are warranted.

Niacinamide possesses various effects, including the inhibition of melanosome transfer and enhancement of barrier function [20], making it a widely used cosmetic ingredient. Although its collagen production-promoting effect has been previously reported, we found for the first time that it restores decreased collagen gene expression in senescent cells. Furthermore, niacinamide reduced the misfolded collagen aggregates that have accumulated in senescent

cells. Although niacinamide did not affect the HSP47 expression, it enhanced autophagic activity. This suggests that autophagy-mediated degradation is among the mechanisms by which niacinamide reduces collagen aggregates in senescent cells. In line with our findings on niacinamide's ability to promote autophagy, other studies have shown that niacinamide can enhance mitophagy, a specific form of autophagy responsible for eliminating damaged mitochondria, in aged skin fibroblasts [21]. This suggests a broader role for niacinamide in maintaining homeostasis in senescent cells by improving both protein and organelle quality control. Finally, we found that niacinamide suppressed the expression of PDGFB in senescent vascular endothelial cells, indicating that niacinamide may prevent the disruption of collagen homeostasis associated with cellular senescence by acting on various dermal cells, not just fibroblasts.

## 5. Conclusion

Our study data suggest that, in senescent cells, collagen expression decreases, and misfolded collagen accumulates owing to the decreased HSP47 expression and reduced autophagic activity, leading to the disruption of collagen homeostasis. We also demonstrated the new roles of niacinamide, which include promoting the degradation of misfolded collagen that has accumulated in the fibroblasts by autophagy and inhibiting PDGFB production in the senescent endothelial cells, thereby maintaining the collagen homeostasis. This newly identified mechanism highlights the potential of niacinamide as a valuable preventative agent for skin aging through serotherapy, targeting senescent cells.

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