Mitochondrial dynamics shoot an arrow at skin aging

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Abstract

Background: A decreased quality of mitochondria (MT) in fibroblasts has been observed in photoaged dermis. We previously reported that the insufficient amounts of dermal fibers formed by fibroblasts were caused by decreased mitochondrial quality. However, it remained unclear how the decreased quality of MT causes the insufficient formation of dermal fibers. Thus, this study was conducted to emphasize the importance of maintaining mitochondrial quality to prevent photoaging focusing on the relevance between the decreased quality of MT and endoplasmic reticulum (ER) stress.

Methods: Protein levels of mitochondrial ubiquitin ligase (MITOL), which regulates mitochondrial quality, and matrix metalloprotease-1 (MMP-1) in UVA-irradiated normal human dermal fibroblasts (NHDFs) were measured. ER stress in NHDFs was evaluated by measuring levels of spliced X-box binding protein 1 (sXBP1) and inositol-requiring enzyme 1α (IRE1α).

Results: MITOL levels in NHDFs were decreased by UVA irradiation and by culture on carbonylated protein (CP) -scaffolds. The knock-down (KD) of MITOL in NHDFs caused ER stress and also increased MMP-1 secretion. Furthermore, NHDFs treated with tunicamycin to cause ER stress had increased MMP-1 secretion. N-acetyl cysteine (NAC) rescued the MITOL and ER stress, and suppressed MMP-1 secretion in UVA-irradiated NHDFs.

Conclusion: We conclude that the decreased quality of MT is caused by MITOL depletion, and that the resulting ER stress enhances MMP-1 secretion. Furthermore, NAC rescues those

phenomena, which suggests that intracellular oxidative stress triggers the decrease in MITOL expression. Thus, this study emphasizes that antioxidation care is effective to prevent photoaging by maintaining MT quality.

Keywords: photoaging, dermal fiber, mitochondria, ER stress

Introduction

Skin conditions are constantly affected by the internal and external environments, and stresses that continuously occur to respond against those influences promote skin aging. In particular, chronic sun-exposure containing ultraviolet light (UV) alters the dermal structure and eventually leads to photoaged skin.

Photoaged dermis is characterized by a decrease of collagen fibers and a loss of oxytalan fibers. One reason for those phenomena is the dysfunction of fibroblasts in matrix reconstruction following UV radiation. That dysfunction of fibroblasts is also caused by alterations of their surrounding conditions. In fact, carbonylated proteins (CPs) and glycated proteins (AGEs), which are types of oxidized proteins, are found at higher levels in the dermis at sun-exposed sites [1]. We have previously reported that CPs reduce the ability of fibroblasts for matrix reconstruction [2]. Thus, it is important to understand the details of cellular dysfunction in matrix reconstruction in order to propose solutions to prevent skin aging.

Mitochondria (MT) are essential intracellular organelles that interact with the endoplasmic reticulum (ER) and peroxisomes in order to maintain each other's functions through their dynamics. MT might be responsible for the progression of photoaged skin because there is often a decreased quality of MT in photoaged skin. In general, the quality of MT is maintained by the dynamics of their balance between fission and fusion, which is regulated by mitochondrial ubiquitin ligase (MITOL) [3]. Our previous study revealed that the knock-down (KD) of MITOL in fibroblasts decreases the quality of MT characterized by the excess fission of MT, decreases of intracellular ATP and increases of mitochondrial reactive oxygen species (mtROS). Also, MITOL-KD fibroblasts had a decreased formation of collagen and fibrillin-1 fibers [4]. Those results strongly suggested that a decrease of MITOL in fibroblasts might cause the characteristics of photoaged dermal structure.

However, several important questions remain as follows: what situations cause the decrease of MITOL following UVA irradiation and how does the decrease of MITOL decreases the formation of dermal fibers. Thus, this study was conducted to discover clues to prevent photoaging by elucidating the answers to those questions.

Materials and Methods

Ultraviolet A (UVA) irradiation

Normal human dermal fibroblasts (NHDFs; Kurabo) were irradiated with 2 J/cm^2 UVA in Dulbecco's Modified Eagle Medium (DMEM) without phenol red and were then further cultured in DMEM. FL20S BLB lamps (Toshiba) were used as a UVA source and irradiation energies of UVA were measured with a UVX radiometer (UVP).

Preparation of CP-scaffolds by carbonylation of collagen with acrolein

Type I bovine atelocollagen (collagen; KOKEN) was coated on the bottom of culture dishes by treatment at a concentration of 300 μg/ml at 4ºC overnight. Collagen treated without or with acrolein (Tokyo Chemical Industry) at 37ºC for 72 h was used as a control-scaffold or a CP-scaffold, respectively.

Knock-down (KD) of MITOL

NHDFs were seeded in multi-well plates or in culture dishes in DMEM containing 5% fetal bovine serum (FBS), then were incubated with 100 nM siMITOL or siControl in the presence of Lipofectamine™ RNAiMAX (Thermo) for 24 h.

Western blotting

Proteins secreted from NHDFs into the culture medium and cellular proteins extracted with a protein extract reagent containing protease inhibitors were separated using SDS-PAGE, and then were transferred to polyvinylidene difluoride membranes. The membranes were then incubated with primary antibodies specific for MITOL, β-actin and matrix metalloprotease-1 (MMP-1). Immunoreactive protein bands were then visualized using EzWestBlue and were analyzed using Image J.

Quantification of interleukin 6 (IL-6)

IL-6 protein levels in the culture medium were quantified by ELISA (R&D systems) according to the manual.

Real-time PCR

Total RNAs in NHDFs were extracted, after which cDNAs were synthesized using a Power SYBR Green Cells-to-CT kit (Thermo). Real-time PCR was performed using a Real-Time PCR system with specific primers.

Immunostaining

NHDFs were fixed with 4% paraformaldehyde for 15 min at room temperature. After blocking with 1% IgG-free bovine serum albumin (BSA), NHDFs were incubated with primary antibodies against inositol-requiring enzyme 1α (IRE1α) and were then further incubated with a secondary antibody conjugated to Alexa Fluor. Nuclei were stained with Hoechst 33342. Images were obtained using a fluorescence microscope.

Mitochondrial staining

NHDFs were fixed with 4% paraformaldehyde for 15 min at room temperature. Mitochondria in NHDFs were visualized using a MITO-ID® Green detection kit (Enzo Life Sciences), which is a specific dye to detect mitochondrial signals. Fluorescence images were obtained using a Floid Cell Imaging Station (Thermo Fisher Scientific).

Results

MITOL protein levels in NHDFs are decreased by UVA irradiation and by culture on CP-scaffolds

UVA triggers photoaging by affecting fibroblasts. Additionally, in aged dermis, fibroblasts are in higher oxidative stress conditions surrounded by oxidative proteins such as CPs [5]. Thus, we first examined the influence of UVA and high oxidative conditions on MITOL expression in fibroblasts. The results show that MITOL protein levels in NHDFs were decreased at 1 h after UVA irradiation (Fig. 1a), and were also decreased by culture on CP-scaffolds for 48 h (Fig. 1b).

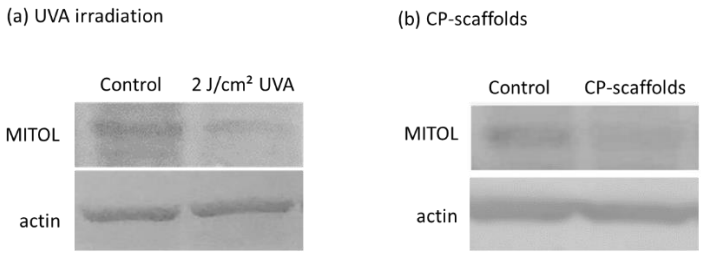

Fig. 1 The influence of UVA irradiation and CP-scaffolds on MITOL protein expression in NHDFs. Time after 1 hour for (a) and 48 hours for (b).in NHDFs.

Decrease of MITOL stimulates MMP-1 secretion via NF-κB-IL-6 signaling

We next examined the influence of MITOL depletion on MMP-1 secretion by MITOL-KD NHDFs. MMP-1 secretion was significantly increased in MITOL-KD NHDFs (Fig. 2a). It is known that MMP-1 production is induced by nuclear factor-kappa B (NF-κB)-IL-6 signaling [6, 7]. MITOL-KD NHDFs had increased IL-6 secretion compared with the siControl (Fig. 2b). The increase of MMP-1 secretion in MITOL-KD NHDFs was suppressed by neutralizing with an antibody to IL-6 (Fig. 2a). Furthermore, IL-6 protein levels in MITOL-KD NHDFs were decreased by JSH23, which is an inhibitor of NF-κB activation (Fig. 2b). The sum of these results indicates that MMP-1 secretion stimulated by MITOL depletion was increased via NF-κB-IL-6 signaling.

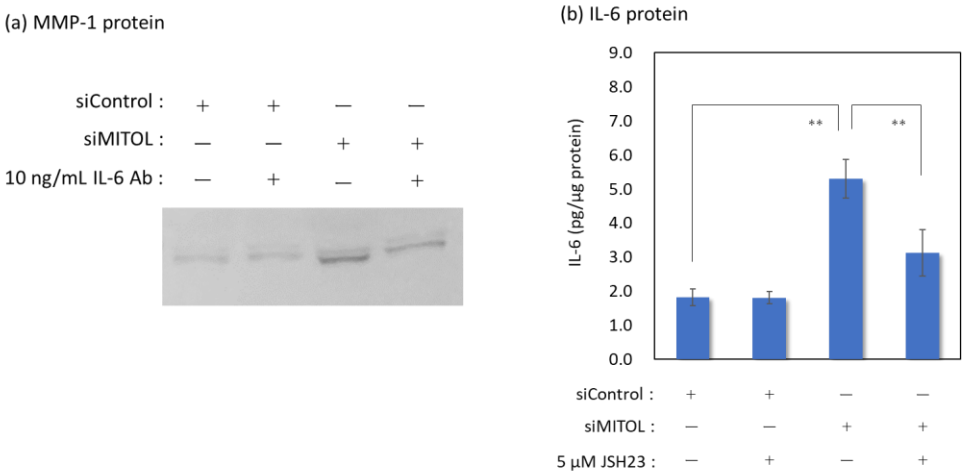

Fig. 2 The influence of MITOL knock-down on MMP-1 (a) and IL-6 (b) protein levels in NHDFs. (n=3, **p<0.01)

MITOL depletion causes ER stress, which is a trigger to increase MMP-1 secretion

It is known that ER stress activates NF-κB by oligomerization of IRE1α, an ER stress marker [8]. On the other hand, MT suppresses ER stress by binding to the ER through interactions between mitofusin2 (Mfn2) and MITOL [9]. Thus, we examined the effects of ER stress levels in MITOL-KD NHDFs. Interestingly, IRE1α protein levels in NHDFs were increased by MITOL-KD and the mRNA expression level of sXBP1, which is another ER stress marker, also increased (Fig. 3), indicating that MITOL depletion causes ER stress in NHDFs.

Furthermore, tunicamycin, which is an inducer of ER stress, also increased MMP-1 secretion associated with an increase of IL-6, and the increase of MMP-1 was abrogated by neutralizing IL-6 with an antibody (Fig. 4a). In addition, because the increase of IL-6 protein caused by tunicamycin was abrogated by JSH23 (Fig. 4b), ER stress induces MMP-1 secretion via NF-κB-IL-6 signaling. Thus, we consider that the decrease of MITOL initially causes ER stress, after which MMP-1 secretion through NF-κB-IL-6 signaling is activated by the ER stress.

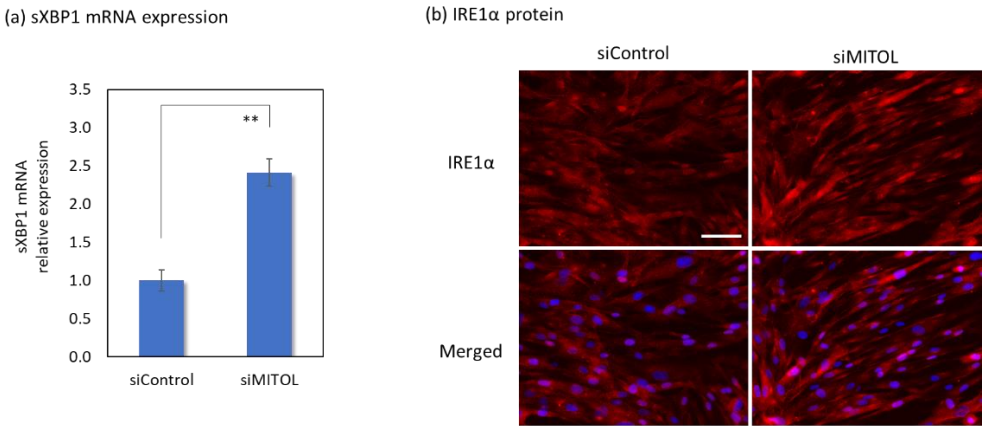

Fig. 3 The influence of MITOL knock-down on ER stress in NHDFs.
(a; n=3, **p<0.01, b; red: IRE1α, blue: nuclei, scale bar: 100 μm)

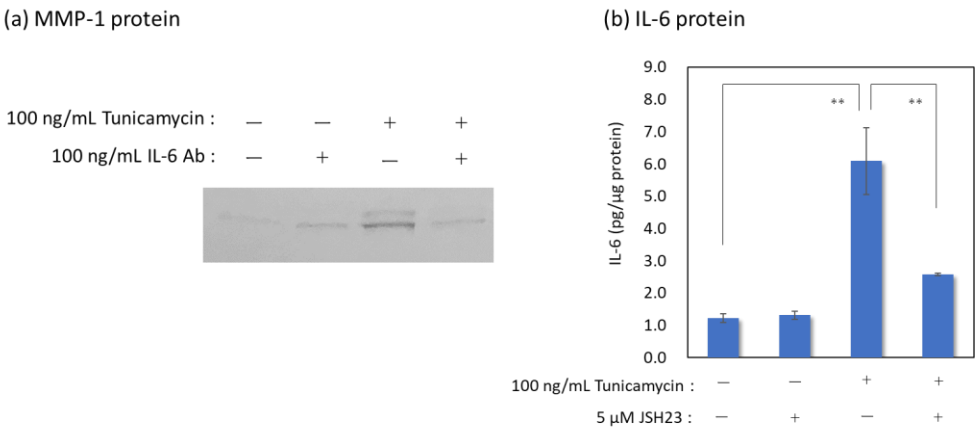

Fig. 4 MMP-1 (a) and IL-6 (b) protein levels in NHDFs treated with tunicamycin. (n=3, **p<0.01)

The potential of maintaining mitochondrial quality with antioxidants

Since MITOL was decreased in higher oxidative conditions (Fig. 1), the involvement of reactive oxygen species (ROS) on the decrease of MITOL was anticipated. Thus, we hypothesized that antioxidants maintain mitochondrial quality by avoiding MITOL decreases and results in ameliorating insufficient dermal fiber formation by the suppression of MMP-1 secretion. To verify that hypothesis, we evaluated the effects of N-acetyl cysteine (NAC), which is a typical antioxidant, on phenomena involved in the quality of MT and the secretion of MMP-1 in NHDFs. The UVA-induced events, including the decrease of MITOL, the excess fission of MT, IL-6 secretion and the accumulation of IRE1α, were restored by NAC (Figs. 5, 6, 7, 8). Of course, MMP-1 secretion induced by UVA also was suppressed by NAC (Fig. 8).

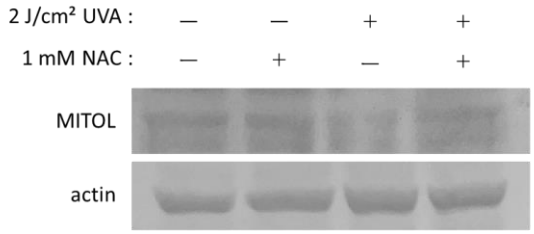

Fig. 5 MITOL protein expression in UVA-irradiated NHDFs treated with NAC.

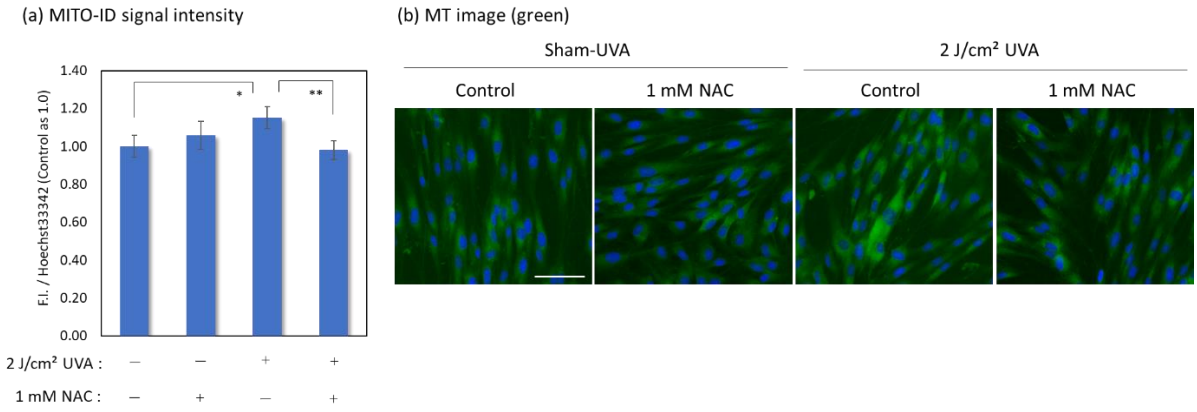

Fig. 6 Mitochondrial fission in UVA-irradiated NHDFs treated with NAC.
(a; n=3, *p<0.05, **p<0.01, b; green: mitochondria, blue: nuclei, scale bar: 100 µm)

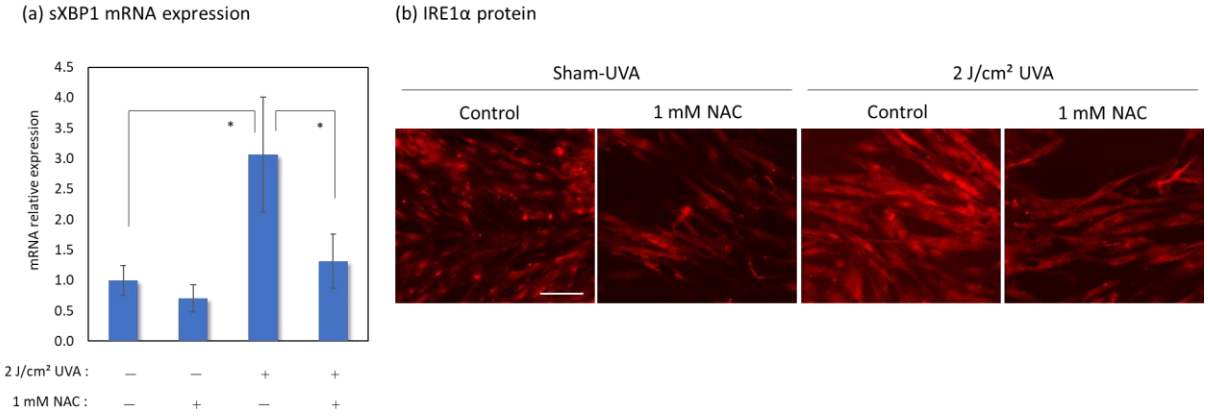

Fig. 7 ER stress level in UVA-irradiated NHDFs treated with NAC.
(a; n=3, *p<0.05, b; red: IRE1α, scale bar, 100 µm)

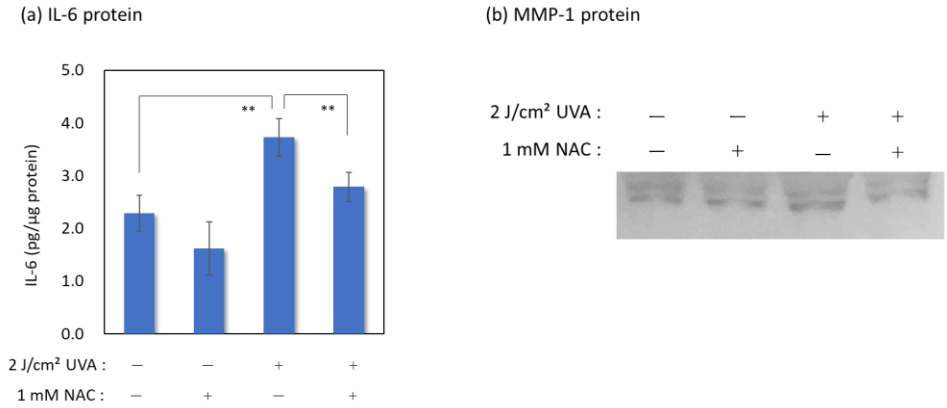

Fig. 8 IL-6 (a) and MMP-1 (b) protein levels in NHDFs treated with NAC. (n=3, **p<0.01)

Discussion

MT are intracellular organelles that are susceptible to injury by UVA because they contain chromophores of UVA. Injured MT, which have a lower quality, have reduced levels of ATP synthesis, releasing excess ROS due to lowering of the membrane potential, which results in the induction of apoptosis. Physiologically, the quality of MT is maintained by the dynamic behavior of fission and fusion. In general, MT are reduced in size by fission, which is regulated by dynamin-related protein 1 (Drp1) and decreases the potential to produce ATP and increases the release of ROS. MITOL, which is a ubiquitin ligase located at the outer membrane of MT, modulates the level of Drp1 by decomposition through ubiquitination to suppress the abnormal fission of MT. Thus, MITOL plays an important role in maintaining the quality of MT. In addition, a recent study demonstrated that MITOL is also a key molecule in the suppression of ER stress by stabilizing the interaction between ER and MT through the ubiquitination of Mfn2 [9].

On the other hand, it is known that ER stress activates NF-κB signaling, which is a transcription factor that modulates inflammation. The decrease of collagen fibers, which is a typical characteristic of photoaged dermis, is caused by the excess production of MMP-1. NF-κB signaling contributes to the excess production of MMP-1 in UV-exposed cells via activation of the IL-1-IL-6-ERK1/2 pathway. Gathering these facts, we speculate that the decrease of MITOL enhances MMP-1 production/secretion through the activation of ER stress-NF-κB-IL-6 signaling.

In fact, we found that MITOL levels in fibroblasts are decreased 1 h after UVA irradiation and by culture on CP-scaffolds (Fig. 1). Furthermore, examinations using MITOL-KD and an ER-inducer, tunicamycin, demonstrated the validity of the cascade of ER stress-NF-κB-IL-6 signaling on MMP-1 secretion in MITOL-KD NHDFs and also in UVA-irradiated NHDFs (Figs. 2, 3, 4). Thus, these results answer the question of how a decrease of MITOL causes the formation of insufficient dermal fibers.

On the other hand, the question remained as to what situations cause the decrease of MITOL following UVA irradiation. It is well known that UVA is an enhancer of oxidative stress through the generation of excess ROS by the photosensitization reaction and the activation of NADPH oxidase. In addition, we have reported that culture on CP-scaffolds increases intracellular CPs through the increase of ROS [10]. Fibroblasts in both situations

showed a decrease of MITOL (Fig. 1). These results indicate that oxidative stress caused by excess ROS must be one trigger for the decrease of MITOL. In fact, NAC, which is a typical antioxidant, rescued the decrease of MITOL, improved the quality of MT and restored MMP-1 secretion in UVA-irradiated NHDFs (Figs. 5, 6, 7, 8). Thus, the answer to this question is that oxidative stress caused by excess ROS is a trigger of the lower expression of MITOL.

Gathering these results, we consider that the decrease of MITOL caused by exposure to oxidative stress triggers the decrease in collagen fibers, which is a typical feature of photoaged dermis, by enhancing their decomposition due to the increased levels of MMP-1.

Conclusion

MITOL regulates MT dynamics and plays a central role in the progression or suppression of photoaging. We conclude that antioxidant care is an effective approach to maintain the quality of MT through the protection of MITOL, and results in the prevention and improvement of photoaging.

Conflict of Interest Statement

NONE.

References

1. Ogura Y, Kuwahara T, Akiyama M, et al (2011) Dermal carbonyl modification is related to the yellowish color change of photo-aged Japanese facial skin. J Dermatol Sci. 64:45-52.
2. Yamawaki Y, Mizutani T, Okano Y, et al (2019) The impact of carbonylated proteins on the skin and potential agents to block their effects. Exp Dermatol. Suppl 1:32-37.
3. Nagashima S, Tokuyama T, Yonashiro R, et al (2014) Roles of mitochondrial ubiquitin ligase MITOL/MARCH5 in mitochondrial dynamics and diseases. J Biochem. 155:273-279.
4. Katsuyama Y, Yamawaki Y, Sato Y, et al (2021) The dysfunction of dermal fibers in photoaging is caused by the impairment of mitochondrial function. The 26th IFSCC Mexico Conference: Proceedings.
5. Varani J, Dame MK, Rittie L, et al (2006) Decreased collagen production in

chronologically aged skin: roles of age-dependent alteration in fibroblast function and defective mechanical stimulation. Am J Pathol. 168:1861-1868.

6. Wlaschek M, Heinen G, Poswig A, et al (1994) UVA-induced autocrine stimulation of fibroblast-derived collagenase/MMP-1 by interrelated loops of interleukin-1 and interleukin-6. Photochem Photobiol. 59:550-556.
7. Kida Y, Kobayashi M, Suzuki T, et al (2005) Interleukin-1 stimulates cytokines, prostaglandin E2 and matrix metalloproteinase-1 production via activation of MAPK/AP-1 and NF-kappaB in human gingival fibroblasts. Cytokine. 29:159-168.
8. Tam AB, Mercado EL, Hoffmann A, et al (2012) ER stress activates NF-κB by integrating functions of basal IKK activity, IRE1 and PERK. PLoS One. 7:e45078.
9. Sugiura A, Nagashima S, Tokuyama T, et al (2013) MITOL regulates endoplasmic reticulum-mitochondria contacts via Mitofusin2. Mol Cell. 51:20-34.
10. Yamawaki Y, Mizutani T, Okano Y, et al. (2019) An oxidative environment surrounding fibroblasts is one cause for the progression of skin photoaging. The 25th IFSCC Milan Conference: Proceedings.