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"Integrin ligands from the dermis: modulators of epidermal aging, including persistent pigmented spots"

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1. Introduction

Globally, there is a strong demand for skin anti-aging products, especially among females and increasingly among males. To date, anti-skin aging therapeutic strategies have primarily targeted the biological mechanisms underlying visible manifestations such as pigmented spots, wrinkles, and sagging. However, recent advances have shifted the focus toward cellular senescence, a fundamental process that is positioned upstream in the skin aging cascade. So, why has the focus of anti-skin aging strategy shifted toward cellular senescence? It depends on the nature of senescent cells, which is to show resistance to apoptosis due to the increased expression of Bcl-2 [1]. Senescent cells secrete a variety of cytokines, chemokines, growth factors, and proteases, collectively known as the senescence-associated secretory phenotype (SASP) [2]. SASP factors impair tissue homeostasis by inducing inflammation, altering the extracellular matrix, causing functional abnormalities in surrounding normal cells, and finally inducing normal cells into a senescent phase [3]. In the case of the skin, SASP factors can cause age-related structural and/or functional changes and contribute to the appearance of characteristic features of aged individuals, such as wrinkles and age spots. Thus, in the cosmetic field as well, the selective removal of senescent cells, so-called "senolysis", is becoming a popular anti-aging approach [3].

Recent studies have reported that senescent fibroblasts accumulate beneath pigmented areas of solar lentigos, which are one of the symptoms of skin aging, and contribute to the persistence of pigmented spots by activating melanocytes through SASP factors [4]. Cysteine-rich 61 (Cyr61, also known as CCN1) and connective tissue growth factor (CTGF, also known as CCN2), which are components of the SASP and act as integrin ligands [6], are thought to affect normal cells through integrin binding. Integrins, which are located on the plasma membrane, are receptors involved in cell adhesion to the extracellular matrix and are responsible for a wide range of signal transduction pathways that regulate cell proliferation, differentiation, motility, and survival. Therefore, it is easy to imagine that integrin ligands secreted by senescent fibroblasts modulate the function of melanocytes and keratinocytes through their effects

on signal transduction and contribute to the appearance of skin aging features. In fact, Cyr61 is involved in the persistence of solar lentigos through the activation of melanin synthesis in melanocytes [5].

The appearance of pigmented spots occurs through two main processes: the first is the active synthesis of melanin in melanocytes, and the second is the diffusion of melanosomes (MSs) filled with melanin in the epidermis via their incorporation into keratinocytes [6]. In those processes, the diffusion of MSs within the epidermis is a critical determinant for the visibility of pigmented spots. Even in the presence of active melanin synthesis in melanocytes, such pigmentation remains imperceptible to the naked eye if the diffusion of MSs is suppressed. Generally, the incorporation of MSs into keratinocytes is mediated by the activation of protease activated receptor-2 (PAR-2) [7].

It is widely recognized that pigmented spots are difficult to lighten once they have formed. We hypothesized that senescent fibroblasts are involved in the persistence of pigmented spots. Therefore, in this study, we investigated the effects of SASP factors secreted from senescent fibroblasts on the persistence of pigmented spots through the incorporation of MSs into keratinocytes, with a focus on the relationship between Cyr61 and PAR-2.

2. Materials and Methods

2.1. Cell culture

Normal human epidermal keratinocytes (NHEKs) and normal human dermal fibroblasts (NHDFs) were purchased from Kurabo (Osaka, Japan). NHEKs were cultured in HuMedia-KG2 (Kurabo). NHDFs were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA).

2.2. Senescent NHDFs

NHDFs were induced into a senescent phase by repeated UVA irradiation (4 J/cm^2, 4 days) or by treatment with H_2O_2 (25 μM, 4 days). The senescent phase was confirmed by the expression of senescence-associated β-galactosidase (SA-β-Gal) and p16.

2.3. Incorporation of pseudo-melanosomes (pMSs), fluorescent beads, into NHEKs

NHEKs were incubated with HuMedia-KB2 (Kurabo) supplemented with fluorescent beads, which served as pMSs in place of natural MSs [8], for 4 h. The amounts of fluorescent beads incorporated into the NHEKs were determined by measuring fluorescence intensity (Ex: 535 nm, Em: 590 nm) after lysing the cells.

2.4. Quantification of Cyr61 secretion

Cyr61 secreted into the medium by NHDFs was quantified using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the instruction manuals.

2.5. RNA interference

NHEKs were treated with 100 nM Cyr61-siRNA (siCyr61) (Invitrogen, Carlsbad, CA, USA) or a non-targeting siRNA (siControl) (Ambion, Austin, TX, USA) using Lipofectamine RNAiMAX (Invitrogen) for 24 h.

2.6. Ex vivo study

Human skins (Biopredic, Rennes, France) were cultured with DMEM/Ham F17 (Nacalai, Kyoto, Japan) in the presence of Cyr61 (Abcam, Cambridge, UK) after UVB irradiation (100 mJ/cm²). Melanin granules (MGs) were visualized by Fontana-Masson staining.

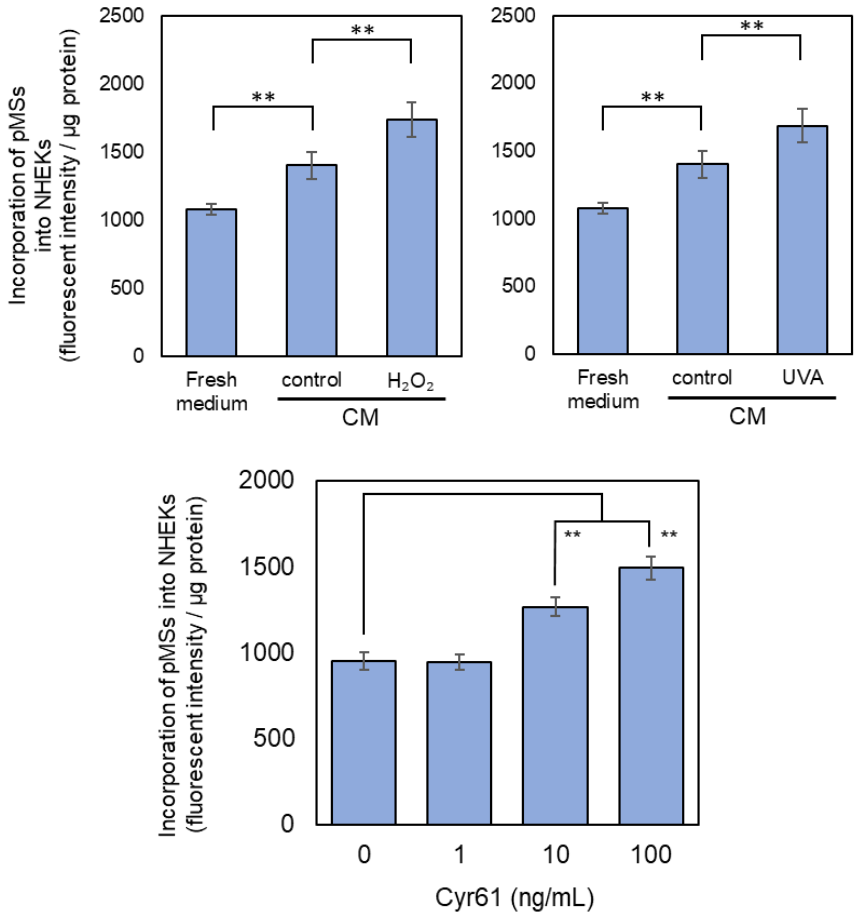
2.7. Quantitative PCR

Total RNA extracted from NHEKs was reverse-transcribed into cDNA using a Power SYBR Green Cells-to-CT kit (Invitrogen). Real-time PCR was performed with SYBR Green Master Mix (Invitrogen) using an Applied Biosystems Step-One (Applied Biosystems, Foster City, CA, USA). Data were analyzed according to the ΔΔCt method.

3. Results

3.1. Cyr61 from senescent NHDFs and their effects on NHEKs

To identify the influence of SASPs secreted from senescent NHDFs on NHEKs, we first examined whether Cyr61 is present in the culture medium and found that senescent NHDFs secrete large amounts of Cyr61. Treatment with the conditioned medium (CM) from senescent NHDFs or the addition of Cyr61 significantly enhanced the incorporation of pMSs into NHEKs (Figure 1a) and also significantly increased PAR-2 mRNA expression in NHEKs (Figure 1b).



(a)

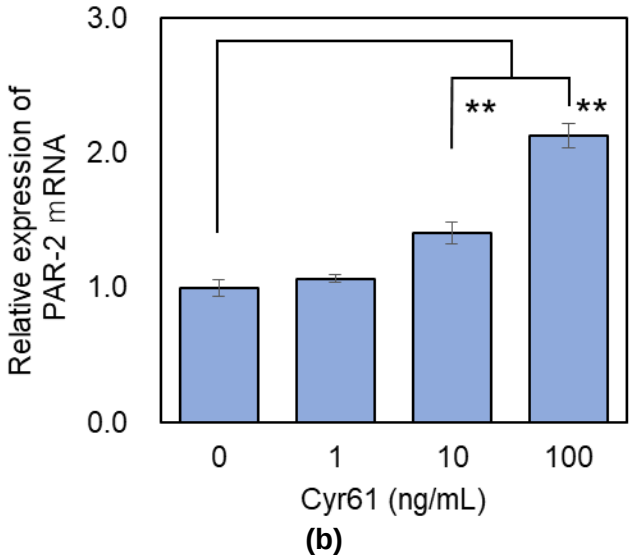


Figure 1. Cyr61 from senescent NHDFs and its effects on NHEKs. **(a)** Treatment with the conditioned medium from senescent NHDFs or the addition of Cyr61 enhanced the incorporation of pMSs into NHEKs. **(b)** Cyr61 increased PAR-2 mRNA expression in NHEKs. Bars indicate means ± S.D. (n= 3). Significance: **: $p < 0.01$.

3.2. Cyr61 knockdown in senescent NHDFs reduces MS incorporation by NHEKs through PAR-2

To confirm whether Cyr61 is a predominant factor that enhances the incorporation of pMSs into NHEKs and/or in the upregulation of PAR-2 mRNA expression, we examined the effects of CM from Cyr61-knockdown NHDFs (Cyr61-KD NHDFs) on those behaviors. Both behaviors induced by the CM of senescent NHDFs were significantly reduced by Cyr61 knockdown (Figure 2). These results indicate that Cyr61 in the CM of senescent NHDFs is a critical factor that enhances the incorporation of MSs into NHEKs, and that its mechanism may involve an increase in PAR-2 production.

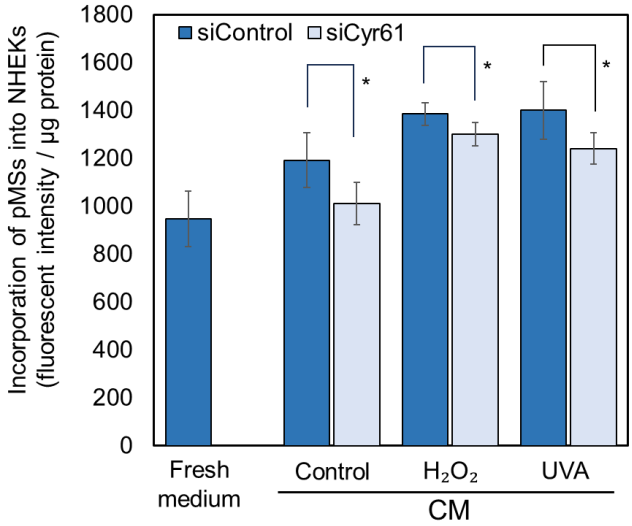
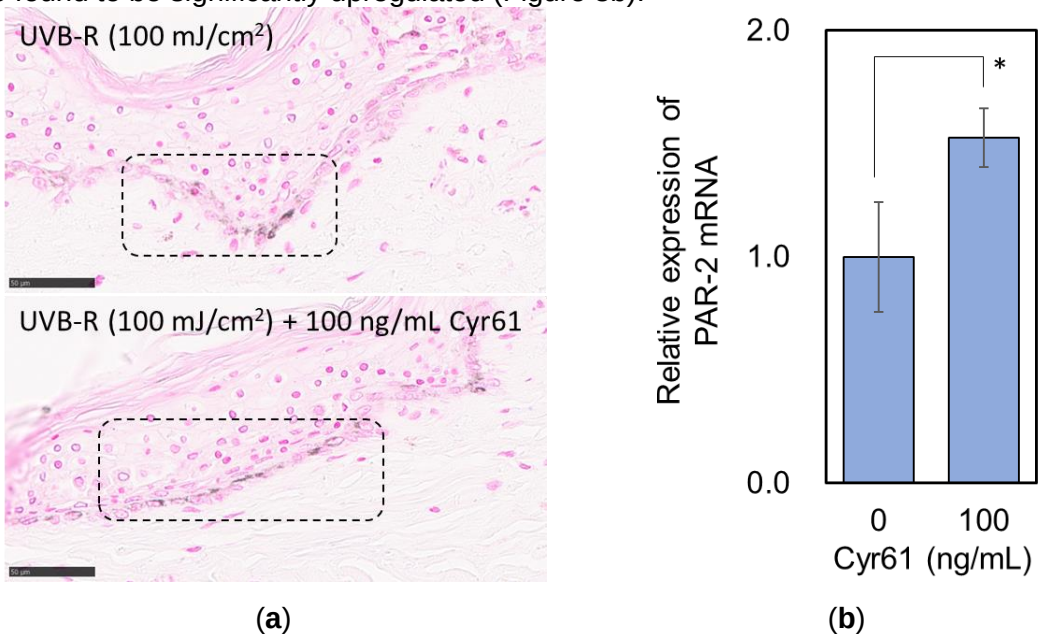


Figure 2. Effect of Cyr61 produced by senescent NHDFs on the incorporation of pMSs by NHEKs**.** Incorporation of pMSs into NHEKs treated with the CM of senescence-induced NHDFs with Cyr61 KD. Bars indicate means ± S.D. (n= 3). Significance: *: $p < 0.05$.

3.3. Effects of Cyr61 on the distribution of MGs and PAR-2 expression in human epidermis

To verify whether the biological events that occur in human skin *in situ* are reflected by the findings obtained from the *in vitro* experiments, we performed an *ex vivo* study. In an *ex vivo* study, to promote melanin synthesis, we first irradiated excised human skin with UVB and then added Cyr61 to the culture medium used for the skin in order to observe the distribution of MGs in the epidermis. MGs in human skin cultured in the presence of Cyr61 after UVB irradiation were widely dispersed in the basal layer (Figure 3a), and PAR-2 mRNA expression was also found to be significantly upregulated (Figure 3b).



(**a**) (**b**)

Figure 3. Effect of Cyr61 on melanosome transfer in an *ex vivo* model of human skin. **(a)** Melanocyte diffusion in UVB-irradiated human explanted skin after repeated treatment with UVB and Cyr61; Fontana-Masson stain. Scale bars, 50 µm. **(b)** PAR-2 gene expression in human explanted skin treated repeatedly with Cyr61. Bars indicate means ± S.D. (n= 3). Significance: *: $p < 0.05$.

3.4. Involvement of PAR-2 in Cyr61 in promoting the incorporation of MSs into NHEKs

To identify whether the upregulation of PAR-2 is involved in the increased incorporation of MSs into NHEKs, we investigated the incorporation of MSs into NHEKs treated with a PAR-2-neutralizing antibody. At 24 h after treatment with Cyr61, the incorporation of MSs into NHEKs was significantly decreased in the presence of a PAR-2 neutralizing antibody (Santa Cruz Biotechnology, Dallas, TX, USA) (Figure 4). Furthermore, treatment with Cyr61 induced the polymerization of actin filaments in NHEKs (Figure 5).

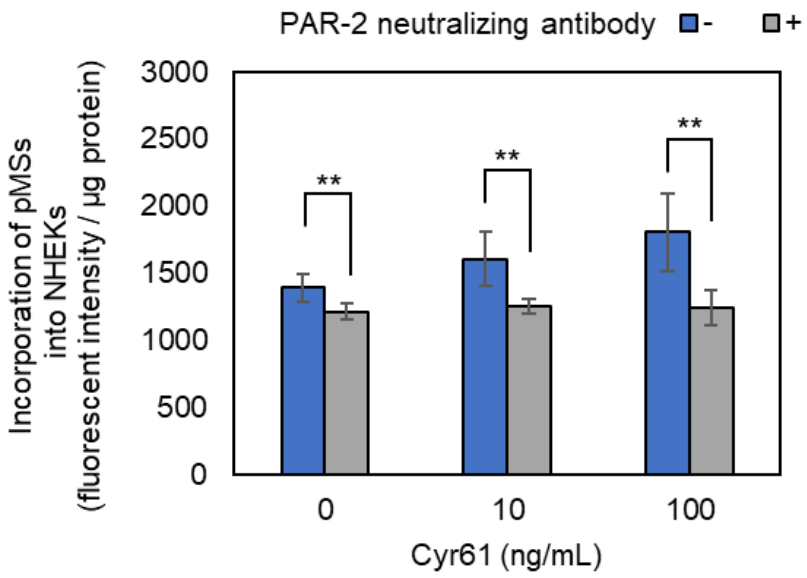


Figure 4. Involvement of PAR-2 in Cyr61–induced melanosome uptake in NHEKs. Treatment with Cyr61 followed by treatment with a specific PAR-2 neutralising antibody inhibited the Cyr61-enhanced incorporation of pMSs into NHEKs. Bars indicate means ± S.D. (n= 3). Significance: **: $p < 0.01$.

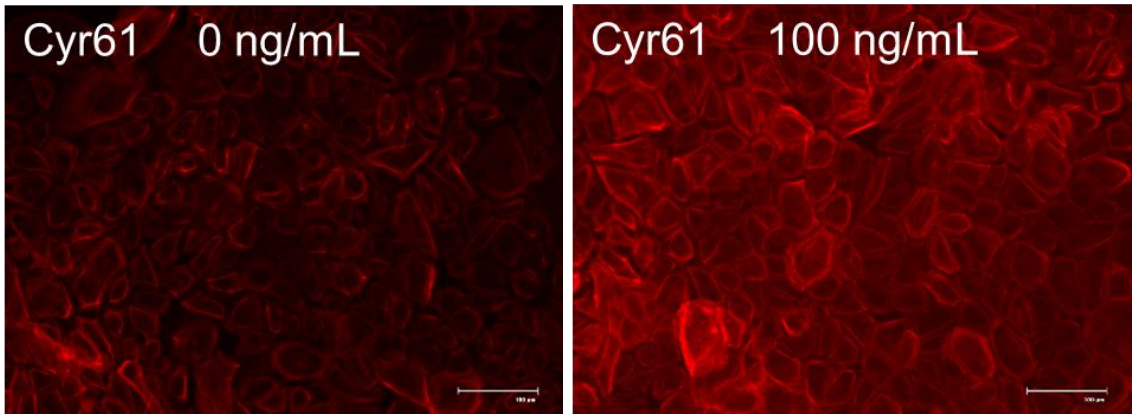


Figure 5. Cyr61 induces the polymerization of actin filaments in NHEKs. Actin filaments of NHEKs treated with Cyr61 for 24 h were stained with phalloidin. Scale bars, 100 µm.

4. Discussion

This study was conducted to clarify why pigmented spots in the skin such as solar lentigines are difficult to lighten, taking into account the microenvironment of pigmented skin areas. Histological studies have reported that senescent fibroblasts accumulate beneath pigmented areas. Thus, we considered the influence of SASPs released from senescent fibroblasts on persistent pigmented spots, with a particular focus on Cyr61 and its effect on MS incorporation into keratinocytes. To achieve that purpose, we first examined whether the CM from senescent

NHDFs affects the incorporation of MSs into NHEKs, using fluorescent beads as pMSs. In fact, Cyr61 was found to be present in large amounts in the CM of senescent NHDFs. In addition, the CM from senescent NHDFS as well as the addition of Cyr61 promoted the incorporation of pMSs into NHEKs (Figure 1a). Those results suggest that SASPs released from senescent fibroblasts may be involved in the diffusion of melanin granules in the epidermis by promoting the incorporation of MSs into keratinocytes. Furthermore, interestingly, Cyr61 that is included in the CM of NHDFs stimulated the expression of PAR-2 (Figure 1b), which is a receptor involved in the incorporation of MSs. These *in vivo* results were successfully reproduced in human skin in an *ex vivo* study (Figure 3). Gathering these results, the mechanism of MS incorporation into keratinocytes promoted by the CM of senescent fibroblasts is hypothesized to involve Cyr61, one of the components in the CM, which promotes the process through the increase of PAR-2 expression in keratinocytes.

To test that hypothesis, we needed to confirm that Cyr61 is a predominant factor in the CM that is responsible for these results. Thus, we investigated the influence of the CM from Cyr61 KD NHDFs on the incorporation of MSs and PAR-2 expression in NHEKs. These behaviors observed with the CM of senescent NHDFs were abolished by Cyr61 KD. These results indicated that Cyr61 is the causal factor in the promotion of MS incorporation and the increase of PAR-2 expression in NHEKs that occur following treatment with the CM of senescent NHDFs. Next, we need to determine whether PAR-2, which is increased by Cyr61, contributes to the incorporation of MSs in NHEKs. Indeed, blocking PAR-2 with a specific antibody significantly reduced the incorporation of MSs into NHEKs. In addition, since Cyr61 is a ligand for integrins, it is considered that the binding of Cyr61 to integrins alters the actin structure in keratinocytes. Moreover, the structural transformation of actin into filaments is essential for the incorporation of foreign substances into cells [7]. As expected, treatment with Cyr61 caused the polymerization of actin into filaments. Taken together, our findings support the hypothesis that Cyr61 in the CM from senescent fibroblasts promotes the diffusion of melanin pigment by enhancing MS incorporation into keratinocytes through the upregulation of PAR-2.

5. Conclusion

The results of this study demonstrate that Cyr61, a member of the SASP family, is responsible for promoting the incorporation of MSs into keratinocytes and also for the upregulation of PAR-2. Additionally, Cyr61 has been reported to increase melanin synthesis in melanocytes. These facts suggest that Cyr61, a component of SASPs that are released from senescent fibroblasts, stimulates melanocytes to increase melanin synthesis. Furthermore, Cyr61 promotes the diffusion of melanin granules by upregulating PAR-2. Thus, we conclude that the presence of senescent fibroblasts is one reason why pigmented spots in the skin like solar lentigines are difficult to lighten by promoting the diffusion of melanin granules.

6. References

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