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Breaking the senescence chain: Identification of cellular senescence inducing microRNA

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1. Introduction

Aging is the one of the greatest challenges facing humanity. In most organisms, aging is an inevitable process that alters biological functions, leading to a decline in quality of life. In the skin, numerous age-related changes have been reported, including wrinkles, sagging, a decline in elasticity, and delayed wound healing [1, 2]. In addition, senescent cells are known to accumulate *in vivo*, and these cells can induce a chain reaction of senescence to the surrounding cells, causing not only the cells but also their surrounding tissue to age [3, 4].

In recent years, extracellular vesicles (EVs) have been attracting attention as a means of intercellular communication. EVs are approximately 50-150 nm in size and are released from cells, encapsulating various biological molecules such as nucleic acids, proteins, and lipids. They play a critical role in delivering molecules, whose contents depends on the state of supplier cells, resulting in changes of recipient cell behavior [5, 6]. MicroRNA (miRNA), approximately 20 nucleotides in length, is one of the components in EVs that suppresses the expression of target genes by cleaving target sequences or inhibiting their translation. Several miRNAs, including miR-152, are known to exhibit increased intracellular expression levels associated with aging [7]. However, few studies have reported on the involvement of EVs and their encapsulated miRNAs in aging cascades through the intercellular communication, and many aspects remain unclear. Therefore, the objective of this study is to explore novel factors associated with aging cascades by targeting the miRNAs encapsulated in EVs and to elucidate their functions.

2. Materials and Methods

2.1. Cell culture

Adult human skin fibroblasts were obtained from the upper arm of a 36-year-old male donor and maintained in MEM (Shimadzu Diagnostics Corporation) supplemented with 10% fetal

bovine serum (FBS). Approval for this surgery was obtained from the Ethics Committee of Nihon Pharmaceutical University (approval no. 27-04).

Normal human dermal fibroblasts (NHDF) (KURABO) were maintained in DMEM (Shimadzu Diagnostics Corporation), supplemented with 10% FBS. Human primary epidermal keratinocytes (HPEK) (CELLnTEC) were maintained in CnT-PR (CELLnTEC). Human umbilical vein endothelial cells (HUVEC) (Promocell) were maintained in endothelial basal medium with supplement (Promocell).

2.2. Knockdown of *EFEMP2*

5 pmol of negative control (CTR) (Thermo Fisher Scientific) or siRNA of *EFEMP2* (si*EFEMP2*) (Bioneer) was transfected by Lipofectamine™ RNAi MAX (Thermo Fisher Scientific) into fibroblasts. 48 h after treating, knockdown of *EFEMP2* was confirmed by real-time qPCR.

2.3. EVs purification and micro array analysis

EFEMP2 knockdown fibroblasts were cultured in the medium containing exosome-free FBS (System Biosciences, LLC) for 96 h. Supernatant was collected and EV purification was performed using MagCapture™ Exosome Isolation Kit PS Ver. 2 and EV-Save™ Extracellular Vesicle Blocking Reagent (FUJIFILM Wako Pure Chemical Corp) or Cell Culture Media Exosome Purification and RNA isolation (NORGEN). The Purified EVs were confirmed using Nano Sight LM10 (Malvern Panalytical), transmission electron microscope (TEM) and Western blotting. MiRNAs in culture supernatant derived EV was analyzed using 3D gene microarray analysis (TORAY).

2.4. Treatment of miR-570-3p mimic

15 nM of mirVana™ miRNA mimic, negative control #1 (CTR) or mirVana® miRNA mimic of miR-570-3p (mimic) (Thermo Fisher Scientific) were transfected by lipofectamine into NHDF and HPEK as described in Section 2.2. After 48 h, cells were harvested using TRIzol (Invitrogen) or QIAzol (QIAGEN) for RNA extraction, and RIPA Lysis and Extraction Buffer (FUJIFILM Wako Pure Chemical Corp) containing a cOmplete™ Mini protease inhibitor cocktail (Roche) for protein extraction.

2.5. Real-time PCR

Total RNA was isolated from the cells using the RNeasy Plus Mini Kit (QIAGEN). cDNAs were synthesized using the iScript™ Advanced DNA Synthesis Kit for RT-qPCR (BIO-RAD) followed by a real-time PCR analysis for mRNAs using SsoAdvanced™ Universal SYBR® Green Supermix (BIO-RAD) with the target gene specific primers. MiRNA was isolated using the miRNeasy Mini Kit (QIAGEN). MiRNAs were then reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit and TaqMan MicroRNA Assay (Thermo Fisher Scientific).

2.6. Western Blotting

The cell lysates were resolved using gel electrophoresis, and transferred to a PVDF membrane (BIO-RAD). Proteins on the membrane were detected with rat monoclonal antibody (mAb) against CD9, mouse mAb against CD63, rat mAb against CD81 (FUJIFILM Wako Pure Chemical Corp), rabbit polyclonal antibody against SIRT1 (Proteintech), and mouse mAb against β-actin (Sigma-Aldrich) and appropriate secondary antibodies according to the host of primary

antibodies (Cytiva). The intensity of protein bands were quantified using the FUSION software (M&S instruments Inc.).

2.7. Senescence-associated β-galactosidase (SA-β-gal) stains

To detect senescent cells, cells were stained with SA-β-gal using the Senescence Cells Histochemical Staining Kit (Sigma-Aldrich). The cells were imaged using a light microscope, and the number of senescent cells were obtained manually.

2.8. *Ex vivo* culture of fresh human skins

Human skin samples were obtained from a 36 year old Caucasian subject who underwent abdominal plastic surgery, with informed consent provided in accordance with applicable ethical guidelines and regulations in CTIBiotech (Meyzieu, France). The explants were maintained in culture medium at 37 °C in 5% CO_2. Only the dermis was in contact with the culture medium while the epidermis was exposed to air. Skin explants were treated with 100 µM of miR-570-3p mimic containing medium from the basal side of the skins. Explants were harvested at day 7 and 9, and elastin fragmentation were analyzed by immunofluorescence staining using mouse mAb against elastin (Merck Millipore).

2.9. Statistical analysis

A statistical analysis was performed with the GraphPad Prism8 software (GraphPad software). The data were analyzed using Student's *t*-test. Differences were considered significant at *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$.

3. Results

3.1. MiR-570-3p is increased by cellular senescence in dermal fibroblasts derived EVs

We first investigated the miRNAs within EVs whose quantities fluctuate in an aging-dependent manner. In adult human skin fibroblasts obtained from the upper arm of a 36-year-old male donor, we suppressed the expression of the previously identified aging-suppressing factor *EFEMP2* to enrich senescent cells [8]. We then collected the conditioned media and purified the EVs. The particle size of the purified EVs was verified using a Nano Sight LM10, which yielded a single peak around 100-150 nm. The morphology of spherical EVs was also confirmed with TEM. Furthermore, Western blotting of the purified EVs revealed the existence of EV marker proteins, CD9, CD63 and CD81. These results confirm that the EVs were effectively purified. We then subjected the purified EVs to microarray analysis to comprehensively examine the miRNAs contained within the EVs (Fig. 1a). As demonstrated in the MA plot, the amount of miR-570-3p in EVs showed the most significant increase by cellular aging resulting from *EFEMP2* knockdown (Fig. 1b). In order to confirm whether this phenomenon is also observed in a cell line different from that used in the microarray analysis, we examined the changes in the levels of miR-570-3p using real-time PCR following *EFEMP2* knockdown in a commercially available normal human dermal fibroblast. Furthermore, we investigated whether intra-EV miR-570-3p increase could also be observed in replicative senescence, which is another aging model. The results from these experiments consistently showed a statistically significant increase in the amount of miR-570-3p not in the cells but in the EVs (Fig. 1c-f). Based on these findings, we identified intra-EV miR-570-3p as not only an aging-related factor but also a candidate senescence chain related factor.

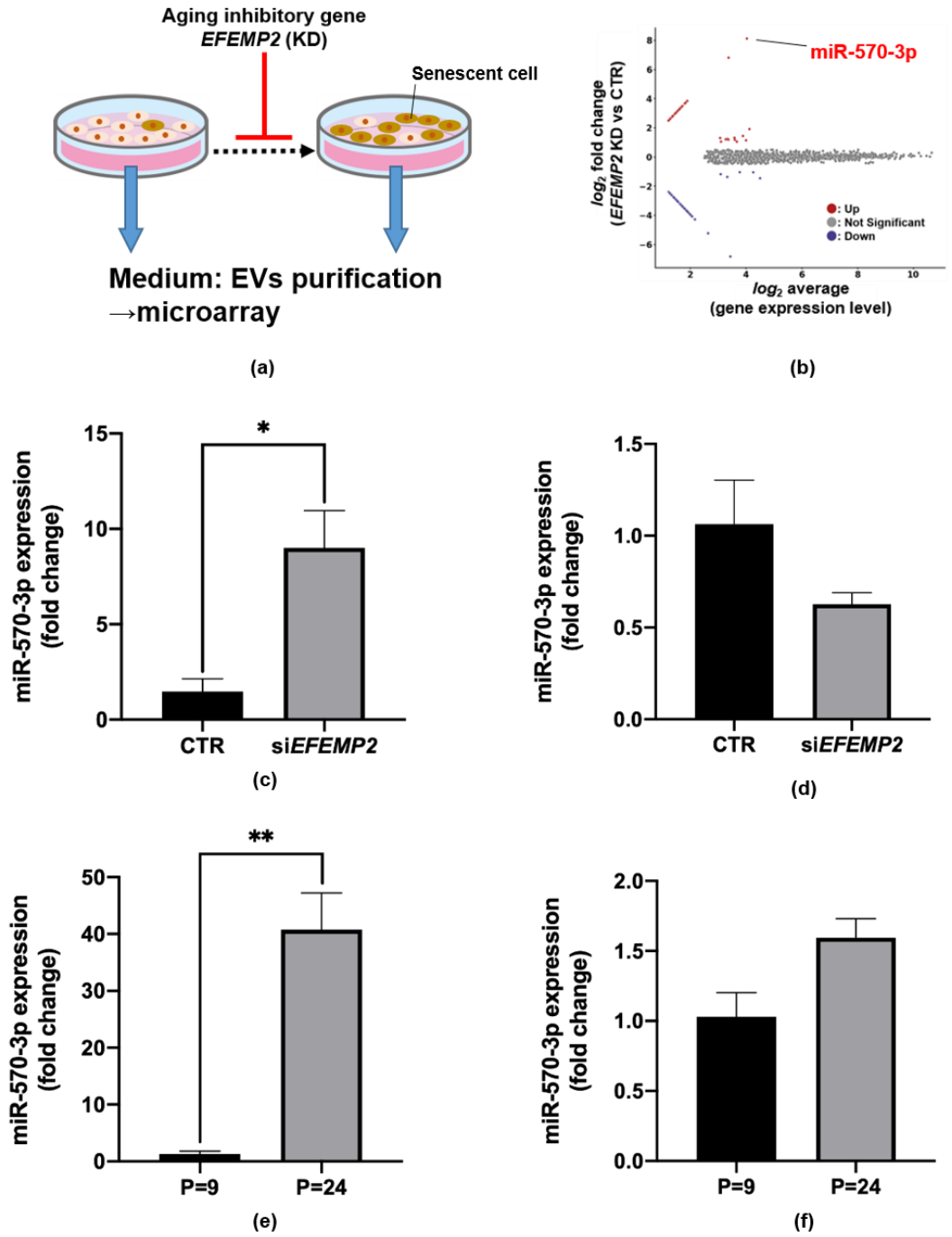

Fig. 1 Alteration of miRNAs in dermal fibroblasts-derived EVs due to aging.
(a) Overview of the experiment. (b) Changes in miRNA encapsulated in EVs induced by *EFEMP2* knockdown using microarray analysis. (c, d) Changes in miR-570-3p induced by EFEMP2 knockdown. (EVs: (c), cells: (d)). (e, f) Changes in miR-570-3p induced by in vitro aging (EVs: (e), cells: (f)). Data are represented as mean ± SEM. *: p <0.05, **: p <0.001.

3.2. MiR-570-3p inhibits SIRT1 expression in skin cells

MiR-570-3p has been reported to suppress the expression of SIRT1, a known longevity gene, in airway epithelial cells [9]. However, there have been few reports regarding miR-570-3p in skin cells. In order to determine whether miR-570-3p also inhibits SIRT1 in skin cells, we analyzed the impact of an artificial mimic of miR-570-3p against dermal fibroblasts and epidermal

keratinocytes. In dermal fibroblasts, the addition of miR-570-3p mimic resulted in a decrease in SIRT1 at both the gene and protein expression level (Fig. 2a, b). Furthermore, in epidermal keratinocytes, a similar decrease in SIRT1 gene and protein was observed through an addition of miR-570-3p mimic (Fig. 2c, d) indicating that miR-570-3p suppresses the expression of SIRT1 in skin cells.

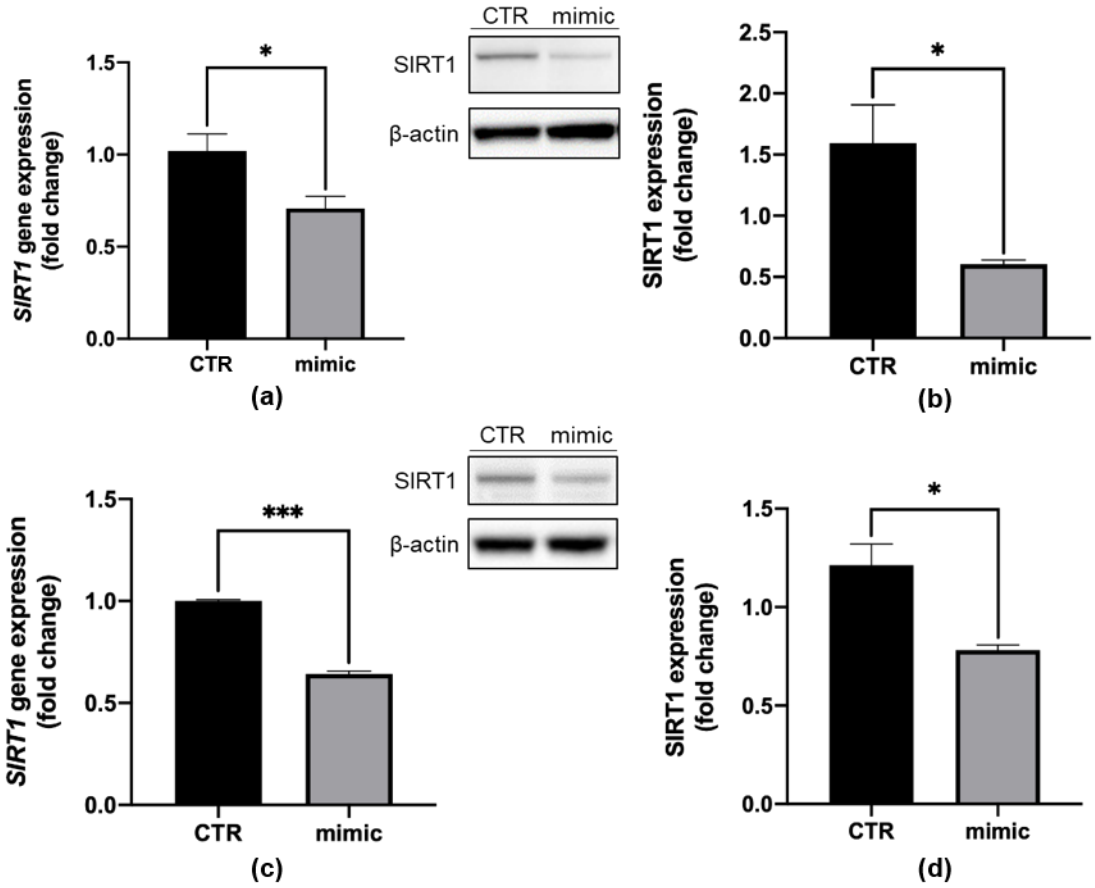

Fig. 2 Changes in SIRT1 expression in skin cells induced by miR-570-3p mimic.
(a, b) Changes in SIRT1 expression induced by miR-570-3p mimic in dermal fibroblasts. (c, d) Changes in SIRT1 expression induced by miR-570-3p mimic in epidermal keratinocytes. Data have been represented as mean ± SEM. *: p <0.05, ***: p <0.001.

3.3. MiR-570-3p induces cellular senescence in skin cells

Since SIRT1 is also known as a longevity gene, next, we decided to investigate the effect of miR-570-3p on cellular senescence in skin cells. First, we examined the impact of miR-570-3p mimic to dermal fibroblasts and found that the addition of miR-570-3p mimic reduced cell proliferation by approximately 35% of that observed in the control (Fig. 3a). Furthermore, staining with the senescence marker SA-β-gal revealed that the addition of miR-570-3p mimic resulted in a higher proportion of SA-β-gal-positive cells compared to the control (Fig. 3b). It was also found that the expression of *LMNB1*, which is known to decrease with aging, was reduced to about half of that in the control when mimic was applied (Fig. 3c). A similar analysis was conducted on epidermal keratinocytes, and the addition of miR-570-3p mimic was found to reduce the cell proliferation rate to approximately 70% (Fig. 3d). The addition of miR-570-3p mimic led to a higher proportion of SA-β-gal-positive cells compared to the control and the expression of

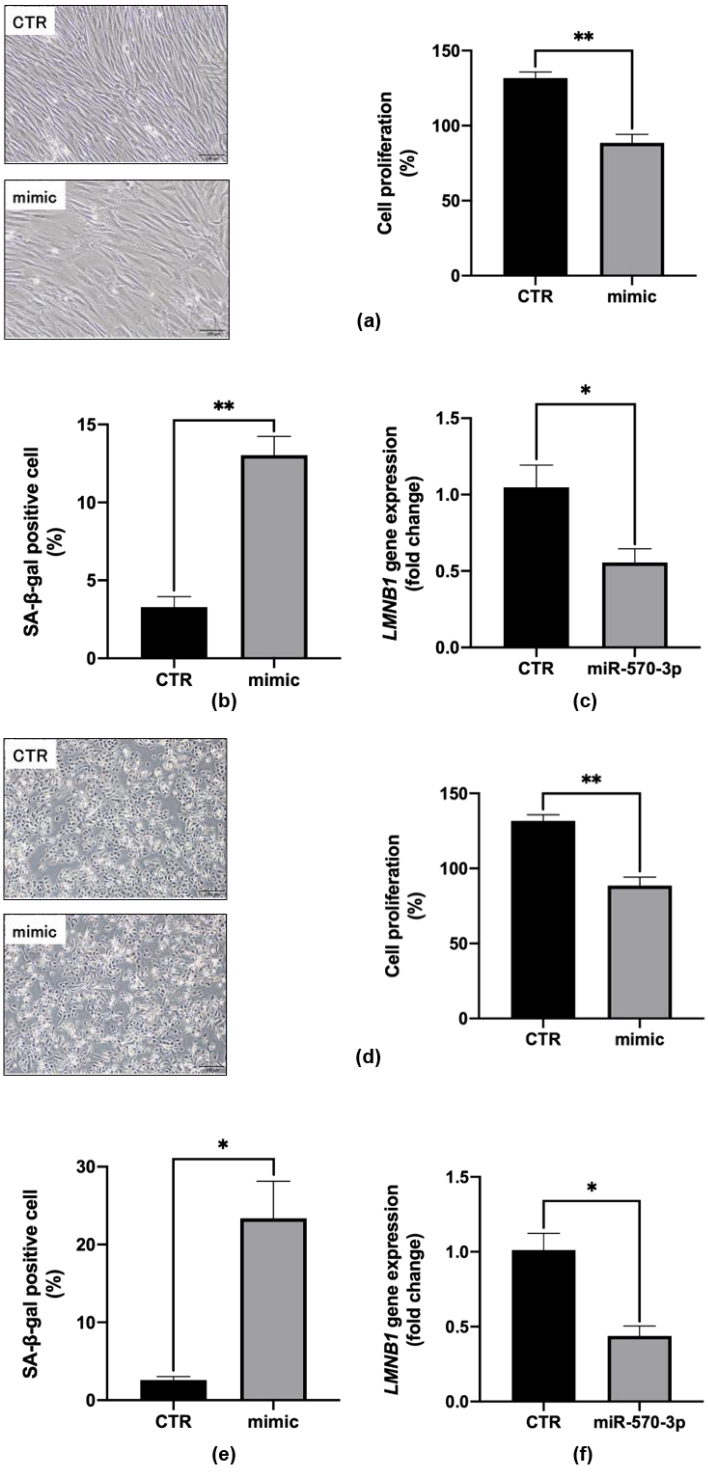

Fig. 3 MiR-570-3p mimic induced cellular aging in skin cells.

a) Changes in cell proliferation induced by miR-570-3p mimic in dermal fibroblasts. (b) Changes in the proportion of SA-β-gal-positive cells in dermal fibroblasts. (c) miR-570-3p-dependent downregulation of *LMNB1* gene expression in dermal fibroblasts. (d) Changes in cell proliferation induced by miR-570-3p mimic in epidermal keratinocytes. (e) Changes in the proportion of SA-β-gal-positive cells in epidermal keratinocytes. (f) miR-570-3p-dependent downregulation of *LMNB1* gene expression in epidermal keratinocytes. Data have been represented as mean ± SEM. *: p <0.05, **: p <0.01, ***: p <0.0001.

LMNB1 decreased with the addition of miR-570-3p mimic (Fig. 3e, f). These findings demonstrate that miR-570-3p induces senescence in both dermal fibroblasts and keratinocytes.

3.4. MiR-570-3p induces cellular senescence in vascular cells

Based on the results obtained thus far, we have clarified that miR-570-3p induces senescence in dermal fibroblasts and epidermal keratinocytes within the skin. However, it should be noted that the skin is surrounded by various tissues, including blood vessels. Blood vessels, in particular, play a crucial role in supplying nutrients and oxygen to the skin. Therefore, we investigated the effects of miR-570-3p on vascular cells as well. The addition of miR-570-3p mimic to HUVECs significantly reduced cell proliferation capability (Fig. 4a). The application of miR-570-3p mimic resulted in a higher proportion of SA-β-gal-positive cells compared to the control (Fig. 4b). Furthermore, analysis of the expression levels of *CDKN1A*, known as senescence marker genes, revealed that significantly upregulated by the addition of mimic (Fig. 4c). These results suggest that miR-570-3p induces aging not only in skin cells but also in vascular cells.

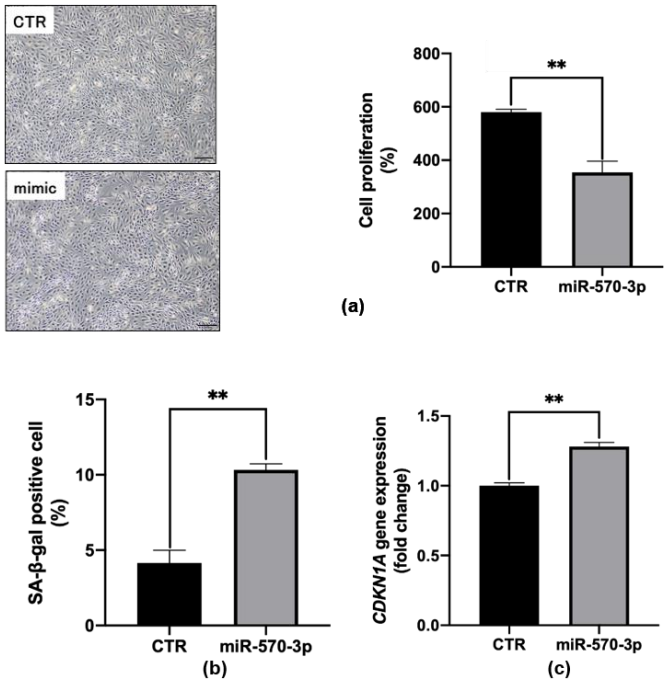

Fig. 4 MiR-570-3p mimic induced cellular aging in HUVEC.
(a) Changes in cell proliferation induced by miR-570-3p mimic in HUVEC. (b) Changes in the proportion of SA-β-gal-positive cells in HUVEC. (c) miR-570-3p-dependent upregulation of *CDKN1A* gene expression in HUVEC. Data have been represented as mean ± SEM. **: p <0.01.

3.5. MiR-570-3p induces fragmentation of elastin fibers in *ex vivo* human skin

Finally, we investigated the effects of miR-570-3p on actual human skin. We cultured *ex vivo* human skin with the addition of miR-570-3p mimic for 7-9 days. Immunostaining of elastin fibers in the dermis revealed that the addition of miR-570-3p led to fragmentation of these fibers on both day 7 and day 9, with particularly pronounced changes observed on day 9 (Fig. 5). It has been reported that elastin fibers undergo fragmentation due to aging in human skin [10]. The addition of miR-570-3p mimic to *ex vivo* human skin resulted in the observation of a phenotype similar to that seen in human skin aging.

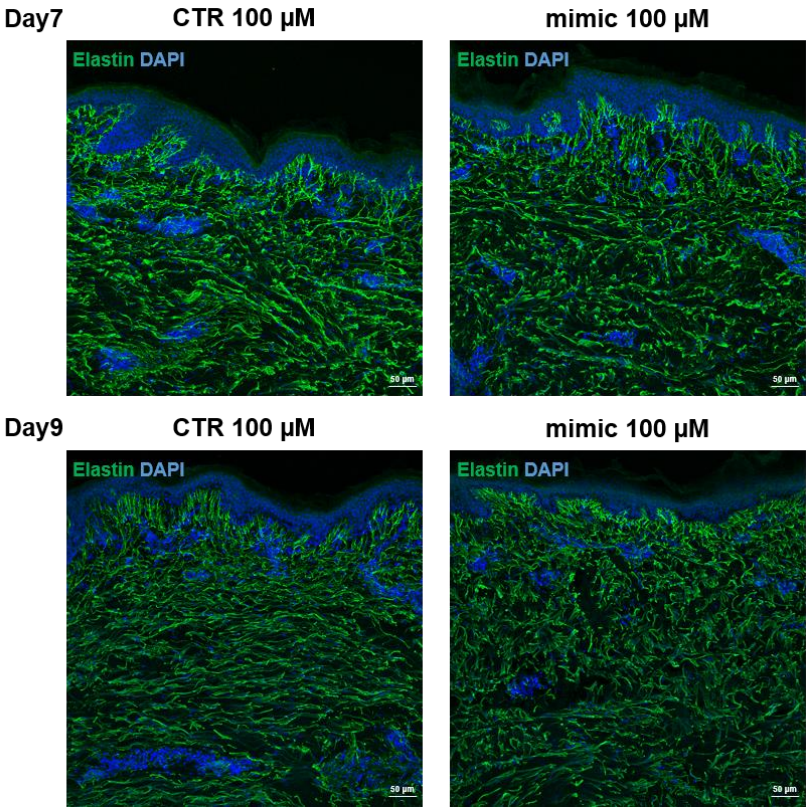

Fig. 5 MiR-570-3p induces elastin fragmentation in *ex vivo* human skin.
Elastin immunostaining of *ex vivo* human skin treated for 7 or 9 days with 100 µM of miR-570-3p mimic.

4. Discussion

In our study, both the knockdown of *EFEMP2* and replicative senescence, which are different types of aging model, resulted in an age-dependent increase in the miR-570-3p content within EVs, suggesting that intra-EV miR-570-3p increases due to dermal fibroblasts aging. On the other hand, the lack of a significant quantitative change in miR-570-3p within the cells suggests that, while the expression of miR-570-3p increases due to cellular aging, it has the characteristic of being released from the cell as an EVs cargo rather than remaining within the cells. Further validation of this characteristic is necessary, but this implies that miR-570-3p has the potential to induce aging in other cells rather than in the cells themselves.

While there have been reports of miRNAs that increase in expression with aging in human skin [7, 11], there have been very few reports on miRNAs that can induce aging or trigger an aging cascade in other cells. In this study, miR-570-3p was found to induce an aging phenotype in primary skin cells such as dermal fibroblasts and epidermal keratinocytes through the suppression of the expression of SIRT1, which is known as an anti-aging gene. Taken together with our data showing that EV-encapsulated miR-570-3p secretion increases with dermal fibroblast aging, these findings strongly suggest that miR-570-3p is one of the factors involved in the vicious cycle of skin aging. Furthermore, we used TargetScan (Whitehead Institute for Biomedical Research) to identify candidate genes, other than those related to cell aging, whose expression is suppressed by miR-570-3p, and found that various collagen-related genes and tight junction-related genes such as *COL4A1* and *OCLN* are potential targets

regulated by this miRNA. It is possible that in addition to inducing cellular aging through SIRT1, miR-570-3p may also contribute to the decline of the fundamental skin structure and function, such as suppression of ECM production and the weakening of skin barriers [12].

The addition of miR-570-3p mimic to HUVEC did not result in any significant changes in the expression levels of SIRT1. On the other hand, the proportion of SA-β-gal-positeive cells and the expression levels of *CDKN1A*, both are commonly used aging markers, significantly increased with the addition of miR-570-3p mimic. Therefore, it was possible that aging was induced through a pathway that did not involve SIRT1. It has been reported that the Klotho beta (*KLB*) gene, known as an anti-aging gene, is decreased in aging HUVECs [13]. The *KLB* gene is one of the candidate genes whose expression is suppressed by miR-570-3p based on TargetScan prediction. Although further validation is needed, it is suggested that in HUVECs, miR-570-3p may induce aging by suppressing the expression of the *KLB* gene. From the above results, it can be inferred that miR-570-3p could promote the aging of blood vessels other than the local skin environment. Blood vessels serve as the conduits for nutrient supply, thus, this could lead to a further deterioration of skin condition.

In *ex vivo* human skin study, the addition of miR-570-3p mimic was observed to cause fragmentation of elastin fibers. It is known that the expression of multiple matrix metalloproteinases (MMPs) increases in dermal fibroblasts with aging [14]. In our study, we demonstrate that miR-570-3p induces senescence in dermal fibrobasts *in vitro* culture. The results observed in *ex vivo* human skin study suggest that miR-570-3p may cause dermal fibroblast senescence also in the tissue environment and trigger an increase in MMPs. Taken together, these findings suggest that miR-570-3p induces aging not only in skin cells but also potentially in skin tissues.

5. Conclusion

In this study, we demonstrated that the abundance of miR-570-3p within EVs increases with aging in dermal fibroblasts, and that miR-570-3p induces aging in various types of skin cells such as dermal fibroblasts, epidermal keratinocytes, and vascular endothelial cells. In addition, miR-570-3p may potentially influence aging of *in vivo* human skin. Based on these results, we identified miR-570-3p as a chain factor of aging. Our findings suggest that pharmacological inhibition of miR-570-3p, along with procedural interventions, could expand the scope of aesthetic and therapeutic approaches to anti-aging treatments from the cellular level to the tissue level. This novel strategy offers a promising approach to effectively disrupt molecular cascades involved in intercellular communication and the aging processes of tissues, thereby extending the lifespan of skin tissue.

6. References

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