

# **Eggshell membrane promotes homeostasis of elastic skin and lung tissue associated with type III collagen and decorin expression and ameliorates pulmonary fibrosis in a bleomycin mouse model**

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## Abstract

The skin and lungs are barriers to environmental threats such as toxic chemicals and microbial pathogens. The integrity of the extracellular matrix (ECM) in the dermal papillae in the skin and the interstitium in the lungs is critical for tissue homeostasis. However, it is difficult to improve the ECM integrity in the skin and lung simultaneously. Previously, we reported that eggshell membrane (ESM) provided a young ECM environment to dermal fibroblasts *in vitro* and in mouse skin and increased the elasticity of human skin. Herein, lung fibroblasts cultured on ESM showed markedly higher *type III collagen, decorin, and MMP2* levels. Oral ESM administration in mice markedly increased the *type III collagen* and *decorin* levels in lung tissues after 2 weeks, and *type III collagen, decorin, and MMP2* levels in the papillary dermis after 4 weeks. Furthermore, in a double-blind study involving 30 adults, the arm skin elasticity significantly increased after 8 weeks of ESM administration. Simultaneously, the Tiffeneau-Pinelli index, which is correlated with lung elasticity, increased also significantly. To further explore the effects of ESM on the lungs, we used a mouse model of bleomycin-induced fibrosis. In these mice, ESM significantly suppressed fibrosis at 2 weeks and increased the *type III collagen* levels in the bronchioles and *decorin* levels in the alveoli, which was implicated in the suppression of lung fibrosis. Thus, oral ESM

intake may prevent the age-dependent decline of the papillary dermis and pulmonary fibrosis by improving the extracellular environment in skin and lung tissues.

Keywords: eggshell membrane, type III collagen, decorin, lung fibrosis, bleomycin-induced mouse model

## Introduction

Barrier tissues, such as the skin and lung tissues, show susceptibility to age-dependent epithelial barrier disruption by inflammation and oxidative stress-associated threats by toxic chemicals and infections (Parrish, 2017). The integrity of the extracellular matrix (ECM), the dermal papillae in the skin, and the interstitium in the lungs, is critical for tissue homeostasis because of the dermal-epidermal crosstalk in the skin (Sorrell, et al., 2004; Lichtenberger, et al., 2016) and the epithelium-interstitial fibroblast crosstalk in the lungs (Skibinski, et al., 2007; Osei, et al., 2014; Lewis, et al., 2018). Therefore, improving the extracellular environment of the dermis and lung interstitium may represent common strategies to enhance and maintain tissue homeostasis. However, at present, it has been difficult to improve the integrity of the ECM, and consequently, enhance tissue homeostasis, in the skin and lungs simultaneously.

The naturally occurring composite material chicken eggshell membrane (ESM), barrier of the chick embryo from physical damage and microbial infection, not only has a beneficial effect on the skin but also has ameliorative effects on several disorders, such as osteoarthritis of the knee (Ruff, et al., 2009b) and joint and connective tissue disorders (Ruff, et al., 2009a), when ingested and absorbed as a supplement. Additionally, ESM has a physiological effect *in vivo* on its digestion, absorption, and metabolism; however, its underlying detailed cellular mechanisms remain unclear (Jia, et al., 2014; Jia, et al., 2017). ESM is composed of fibrous proteins cross-linked by collagen (mainly type X and

V), lysyl oxidase (LOX), and other molecules, including glycoproteins and lipids (Ahmed, et al., 2017, Akagawa, et al., 1999, Du, et al., 2015).

We have previously reported that solubilized ESM (SESM) improves the extracellular environment by inducing the expression of *decorin*, *type III collagen*, and *matrix metalloproteinase-2 (MMP2)* on SESM-specific culture surfaces (Ohto-Fujita, et al., 2011) and in the papillary layer of SESM-applied mouse skin (Ohto-Fujita, et al., 2019). Additionally, application of SESM to human skin induces an anti-aging effect by increasing arm-skin elasticity and improving wrinkles at the corners of the eyes (Ohto-Fujita et al. 2019). Therefore, in the present study, we investigated whether ingestion of ESM orally improves skin and lung within the range of homeostasis in mouse models and human subjects. In addition, since previous results predicted anti-fibrotic effects of ESM through the expression of decorin (Reed and Iozzo, 2002) and type III collagen (Liu, et al., 1997), both essential for proper fibrillogenesis of type I collagen, we also investigated whether ESM is involved in the suppression of pulmonary fibrosis.

## **Materials and methods**

### **In vitro cell studies**

#### **Cell culture**

IMR-90 lung fibroblast cells were obtained from ATCC and maintained in minimum essential media with Earle's balanced salts (MEM/EBSS; Hyclone Laboratories Inc, Logan, UT, USA) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO, USA), sodium pyruvate (Gibco, Waltham, MA, USA), non-essential amino acids (NEAA; Gibco), and penicillin-streptomycin-neomycin (PSN; Gibco). IMR-90 (passage 3,  $1 \times 10^5$  cells) were seeded in a 10 cm diameter control tissue culture dish ( $n = 3$ ) and a tissue culture dish containing a thin acrylamide gel (8 cm diameter) with SESM bound to it ( $n=3$ ) using a method described previously. The cells were cultured for 51 h.

### **Real-time PCR**

After washing the cells with PBS, total RNA was extracted with TRIzol Reagent (Life Technologies, USA) followed by Maxwell® 16 Tissue and Cell Total RNA LEV Systems (Promega Co., Ltd.). Total RNA was quantified spectrophotometrically, and an aliquot (30 ng) of total RNA was used for cDNA synthesis using the Takara Prime Script RT Reagent Kit (Perfect Real Time). The cDNA synthesis conditions were as follows: 15 min at 37 °C and 15 s at 85 °C. One microliter of cDNA at a dilution of 1/30 was used for real-time PCR. SYBR Premix Ex Taq™ II (Takara) was used for gene amplification

using the Thermal Cycler Dice Real-Time System (Takara). The primer sequences are shown in Online resource 1. *GAPDH* was used as an internal control.

## **In vivo animal studies**

### **Ethical clearance**

Animal experiments were conducted with the ethical approval of Tokyo University of Agriculture and Technology Animal Experiments Committee (No. 25-60; 2019 update).

All animal experiments were performed according to the guidelines of the Declaration of Helsinki and Tokyo University of Agriculture and Technology Animal Experiments Committee.

### **Gene expression analysis in animal lungs and skin after ingestion of ESM**

Hairless male mice (Hos: Hr, aged 8 weeks) were purchased from Hoshino Laboratory Animals, Inc. (Ibaraki, Japan) for skin layer and lung analysis. ESM (Almado Inc.) mixed with MediGel Sucralose (Clear H<sub>2</sub>O, ME, USA) was provided as a voluntary oral supplement to the mice once a day for 2 weeks for the lung experiment and 4 weeks for the skin layer analysis, during the dark phase of the light/dark cycle.

For gene expression analysis in lung, six mice were then divided into two experimental groups, with the respective amounts of ESM in each group being 0 mg (control group, n = 3), 7.3 mg/kg (ESM group, 4 tablets equivalent, n = 3)). For gene expression analysis in skin layer, nine mice were then divided into two experimental groups, with the respective amounts of ESM in each group being 0 mg (control group, n = 3), 11.0 mg/kg (ESM group, 6 tablets equivalent, n = 6)). Skin layer analysis method was previously described (Ohto-Fujita et al. 2019). The primer sequences used in this study were described in Online resource 1.

### **Mouse model of bleomycin (BLM) -induced lung fibrosis**

BLM (3 mg/kg; Nippon Kayaku Co., Ltd. Tokyo, Japan) was administered intratracheally to female 8-week-old C57BL/6 mice (Sankyo Labo Service Corporation, Inc. Tokyo, Japan) under medetomidine (Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan), midazolam (Sandoz Inc., Tokyo, Japan), and butorphanol (Meiji Seika Pharma Co., Ltd., Tokyo, Japan) by SMC Laboratories, Inc. (Tokyo, Japan). C57BL/6 mice are susceptible to pulmonary fibrosis and are often used in pulmonary fibrosis studies (Walkin, et al., 2013).

The effect of ESM on BLM-induced lung fibrosis was investigated by categorizing the mice separately into three groups; control group (n = 10), BLM group (n = 10), BLM + ESM group (n =18). The amounts of ESM (Almado Inc., Tokyo, Japan) administered in each group was 0 mg/kg/day (control group and BLM group) and 11.0 mg/kg/day (BLM and ESM group, 6 tablets equivalent), mixed with MediGel Sucralose (Clear H<sub>2</sub>O, ME, USA). It was administered orally once a day sequentially for 1 or 2 weeks. The body weight of mice was measured once a day. The mice were dissected under anesthesia induced with isoflurane. Pulmonary fibrosis score from picosirius red-stained paraffin sections of the lung was determined with blinded Ashcroft's scoring system (Ashcroft, et al., 1988).

### **Immunofluorescence studies of mouse lung sections**

Lung sections of mice were fixed in formalin and incubated with the following primary antibodies: anti-type III collagen antibody (LSL Inc. Tokyo, Japan, Cat# LB-1387; 1:50), anti-decorin antibody (Merck Millipore, Darmstadt, Germany, Cat#: ABT274; 1:50) followed by the secondary antibody, and anti-rabbit IgG Alexa Fluor 546 (Invitrogen, Carlsbad, CA, Cat# A10040; 1:50). The cell nucleus was stained with Hoechst 33342. The immunostained sections were observed under a Nikon A1 RMP confocal microscope

(Nikon Corporation). Alveolar staining of decorin was quantified by the using Nikon NIS-elements software (Nikon Corporation). The number of decorin-positive cells in the alveolar interstitium was measured using Image J. Lung paraffin sections immunostained with anti-decorin-Alexa546 were observed with a confocal microscope, and the acquired images were converted to 16 bit by Image J. After binarization, the size was set to 70-350 pixel<sup>2</sup> (4.5-22.6 μm<sup>2</sup>) and the circularity was set to (0.5-1.0), and the number of extracted objects was counted.

Interstitial fluorescence of type III collagen-Alexa546 around bronchioles (size: about 200 μm in diameter/ μm<sup>2</sup>) was obtained from two bronchioles per individual using a Nikon A1 RMP equipped with NIS-Elements software (BLM group: n = 8, BLM + ESM group: n = 11).

*Type III collagen, decorin, MMP2, type I collagen, and GAPDH* gene expression in the accessory lobe of the lung was analyzed by qPCR. Primer sequences used in this study were described in Online resource 1.

## **Human intervention studies**

### **Study subjects and study design**

Approval for the study was obtained from the Ethics Committee of Tokyo University of Agriculture and Technology (No. 27-09). Written informed consent was obtained from all study volunteers. This study was conducted in accordance with the guidelines of the Declaration of Helsinki and the Tokyo University of Agriculture and Technology. The study was pre-registered to clinical trial registry (ID: UMIN000018888) provided by University Hospital Medical Information Network (UMIN) Center, the information infrastructure for the Japanese medical community (Kiuchi and Igarashi, 2004). We investigated the effect of ESM on pulmonary function and skin elasticity. This was a double-blind, placebo-controlled study conducted from September 2015 to November 2015. Thirty healthy volunteers without any history of medical conditions including lifestyle diseases, within the age range of 21-68 years (mean age  $38.8 \pm 14.6$  years) were enrolled in the study. The subjects were divided into three groups, ESM group (n=10), placebo 1 group (n=10), and placebo 2 group (n=10).

### **ESM supplement components and ingestion**

Subjects were asked to ingest 8 ESM supplement tablets daily (4 tablets in the morning and 4 tablets in the evening) for 8 weeks. The test supplement tablet components included ESM powder (Almado Inc.) as the main ingredient (46%), lactose (39%), and

other minor ingredients, including chicken breast extract (anserine and carnosine 15% inclusion), rice germ extract, Jew's mallow powder, dried royal jelly, pig-derived elastin peptide, chicken cartilage extract, acerola powder, canola hardened oil, Japanese apricot extract, corn germ extract ceramide inclusion, dextrin, vitamin E-containing plant oil, proteoglycan-containing salmon nasal cartilage extract, corn protein, hyaluronan, cyclic oligosaccharide, lycopene-containing tomato powder, extracted carotene, eggshell calcium, vitamin C, biotin, and vitamin B2. Sugar coat components included granulated sugar, rare sugar-containing syrup, eggshell calcium, shellac, gelatin, Arabic gum, carnauba wax, and gardenia pigment. Placebo 1 group ingested vehicle tablets (crystalline cellulose, corn protein, hydrogenated rapeseed oil) and sugar coating. Placebo 2 group ingested the supplement tablets without eggshell membrane. The subjects were advised to avoid other dietary supplements and body aesthetic treatments during the experimental period.

### **Pulmonary function test**

The pulmonary function of subjects was examined using a spirometer (SPIROMETER HI-801, CHEST M. I., Inc, Tokyo, Japan) before and after 8 weeks of ESM ingestion. Pulmonary function was evaluated by Tiffeneau-Pinelli index (Tiffeneau,

1947; Gólczewski, et al., 2012), forced expiratory volume in one second/forced vital capacity ratio (FEV1/FVC) used for diagnosis of chronic obstructive pulmonary disease.

### **Skin elasticity measurements**

Skin elasticity was measured as described previously (Ohto-Fujita et al. 2019).

### **Statistical analysis**

The mean values and standard error (SE) were calculated. Student's *t*-test (two-tailed) was used for paired data obtained in the intervention studies and for the non-paired data obtained in the *in vivo* animal experiments to compare the mean values of the parameters before and after ESM ingestion. Results with  $p < 0.05$  were considered statistically significant. Data are expressed as mean  $\pm$  standard deviation (SD). A one-way analysis of variance with the Tukey-Kramer multiple comparisons test was used for comparisons. Values of  $p < 0.05$  were considered statistically significant.

## **Results**

**Human lung fibroblasts attached to ESMs induce *type III collagen, decorin, and MMP2***

The response of human lung fibroblast cell line IMR-90 to ESM was investigated in a culture environment with SESM surface as described previously (Ohto-Fujita et al. 2011). IMR-90 significantly increased the expression of *type III collagen*, *type I collagen*, *decorin*, and *MMP2* in response to SESM compared to control cells grown on SESM-free culture dishes (Fig. 1a, b, d, e). Additionally, we found that the ratio of *type III/type I collagen*, which regulates the tissue environment, increased significantly (Fig. 1c). Considering the reported fibroblast heterogeneities in lung tissue (Tsukui, et al., 2020; Xie, et al., 2018), we examined the expression of transcription factors that are highly expressed in Col14a1 matrix fibroblasts (paired-related homeobox 1, *PRRX1*, adipocyte enhancer-binding protein 1, *AEBP1*, Limb-bud-and-heart, *LBH*), which express high levels of decorin, and Col13a1 matrix fibroblasts (transcription factor 21, *TCF21*), which do not. Our results showed that human lung fibroblasts showed a significant increase in the expression levels of *PRRX1* and *LBH* upon exposure to SESM (Fig. 1f, h). In contrast, the expression levels of *AEBP1* (Fig. 1g) and *TCF21* (Fig. 1i) were not significantly different. In response to ESM, IMR-90 cells showed significantly higher expression of *decorin*, *type III collagen*, and *MMP2* compared to the cells grown without ESM, similar to dermal fibroblasts as reported in previous studies (Ohto-Fujita et al. 2011; Ohto-Fujita et al. 2019).

## **Increased *type III collagen* and *decorin* gene expression in mouse lung and skin after ESM ingestion**

Changes in gene expression were investigated in mice lungs and skin after 2- and 4 weeks of oral ESM ingestion, respectively (Fig. 2a-d and e-p). The results showed that *decorin* gene expression in the lungs in the ESM group significantly increased ( $p < 0.01$ ) compared with the control group (Fig. 2a), and a tendency of increase in *type III collagen* (Fig. 2b) and *elastin* expression (Fig. 2d) was observed. Expression of *type III collagen* (Fig. 2i, l), *decorin* (Fig. 2h, k), and *MMP2* (Fig. 2j, m) in the skin corresponding to layers 2 and 3, comprising the papillary dermis, significantly increased in the ESM group compared to the control group.

## **Ingestion of ESM increases the human lung capacity and skin elasticity**

Pulmonary function test was performed using a spirometer to investigate the effect of ESM on lung function. The result shows that Tiffeneau-Pinelli index (FEV1/FVC) significantly increased within 8-weeks of oral ESM administration in the ESM group ( $p < 0.05$ ) (Fig. 3a). No significant difference was seen in the placebo 1 and 2 groups. The mechanical property of a subject's arm skin was measured (Fig. 3b, c) to evaluate the skin

elasticity after 8 weeks of ESM intake. The results showed that the elasticity (R5 and R7) of the right inner forearm significantly increased after 8 weeks of ESM intake ( $p < 0.01$  and  $p < 0.05$ , respectively), compared with the values before intake.

### **ESM intake improved lung fibrosis score in mice**

Results of the human studies indicated that long-term ingestion of ESM supplement over 8 weeks improved respiratory function, and skin elasticity. The anti-fibrotic effect of ESM was then investigated in the BLM-induced pulmonary fibrosis mouse model (Fig. 4a). The degree of fibrosis evaluated by Ashcroft score was significantly ( $p < 0.05$ ) lower in the lungs of BLM + ESM mice than in the BLM mice after 2 weeks (Fig. 4b). Representative images of lung sections stained with picosirius red (Fig. 4c-h) showed reduced collagen deposition, fibrogenesis, and invasion of inflammatory cells such as lymphocytes near the bronchioles of BLM + ESM mice (Fig. 4h) compared to the BLM mice (Fig. 4f).

### **ESM increases decorin expression 2 weeks after BLM-induced pulmonary fibrosis is established**

Since overexpression of decorin is reported to reduce BLM fibrosis (Kolb, et al., 2001, Souma, et al., 2018), expression of *decorin* gene in the lung tissue was investigated one and two weeks after BLM treatment. *Decorin* gene expression was significantly high in BLM + ESM group compared to BLM only group at two weeks (Fig. 5a). The BLM group decreased significantly compared to the control group, but the BLM + ESM group did not decrease and was equivalent to the control group and significantly higher than the BLM group (Fig. 5b). These results are based on the image analysis (Online resource 2) from stromal cells stained with anti-decorin antibody after 2 weeks as shown in Fig. 5c-h (control group), Fig. 5i-n (BLM group) and Fig. 5o-t (BLM + ESM group). The number of alveolar stromal decorin positive cells (arrowhead in Fig. 5c, d, i, j, o, p) were counted using antibody staining images. Representative picrosirius red images of alveole are shown (Fig. 5g, h, m, n, s, t; Online resource 3). Decorin expression was observed at the interstitially as surrounded by basement membrane (Online resource 4).

### **ESM increases type III collagen expression 2 weeks after BLM-induced pulmonary fibrosis**

Lung sections of control (Fig. 6a, d, g), BLM (Fig. 6b, e, h), and BLM+ESM groups (Fig. 6c, f, i) were also examined for type III collagen expression in the bronchiolar

interstitium (Online resource 5). Type III collagen in the BLM + ESM group was found to be significantly higher than that in the BLM group two weeks after BLM treatment (Fig. 6j). Both decorin and type III collagen are reported to be essential for physiological type I collagen fibrillogenesis. Thus, their upregulation may result in enhanced healing and prevent fibrosis.

## Discussion

In the present study, we observed that two weeks of ESM intake tended to increase the expression of *decorin*, *type III collagen*, and *elastin* in animal experiments. In human experiments, ESM intake for 8 weeks significantly improved FEV1/FVC and arm skin elasticity. In mouse experiments, ESM intake for 2 weeks significantly increased the expression of *decorin* in the lung and *decorin* and *type III collagen* in the skin at 4 weeks. ESM administration in a mouse model of BLM-induced pulmonary fibrosis suppressed fibrosis at 2 weeks, significantly promoted decorin and type III collagen expression and improved extracellular environment as ESM treatment suppressed fibrosis.

The extracellular environment (ECM) not only has a structural function to support and give strength to cells in tissues, but also has essential cellular functions via cell-matrix interactions (Bruckner, 2010). ECM proteins are generally classified into four categories:

collagen, structural glycoproteins, proteoglycans, and elastin (Tsang, et al., 2010). In the skin, various collagen fibers of different types, including type I collagen and type III collagen, are the main structural components of the ECM, which give the skin its strength and elasticity. Cellular adhesion to the ECM is mediated by the physical attachment of integrin molecules, which transmit signals into the cell and are an important factor in tissue regeneration (Grzesiak, et al., 1997). Mechanical forces on the extracellular matrix regulate the expression of its proteins, and repeated stretching of the ligament enhances type I collagen and type III collagen synthesis in cells of its anterior cruciate ligament (Kim, et al., 2002) and bone marrow-derived mesenchymal cells (Zhang, et al., 2008). ECM plays an important function by interacting with numerous growth factors and signaling molecules, regulating intracellular events, such as intracellular adhesion, differentiation, motility, and survival.

Vertebrates are known to express 28 types of collagen (Fidler, et al., 2018). Type I collagen alone cannot form normal collagen fibers. *Type III collagen* knockout mice models exhibited abnormalities in type I collagen in heterozygous mice upon electron microscopy showing uneven fibers with fiber diameter not being constant. They also exhibited fragile skin and blood vessels, abnormal type I collagen formation with abnormalities in the lungs observed by electron microscopy (Liu et al. 1997), and scar

tissue formation (Volk, et al., 2011). These results suggest that type III collagen is essential for the regulation of cell motility required for tissue formation and regeneration. A large amount of type III collagen is contained in soft tissues, such as blood vessels and skin. The lungs are also relatively soft tissues. Elevated type III collagen in this study appears to underlie anti-fibrotic effects. In African spiny mice, which have a high capacity for skin regeneration, the type III collagen ratio is higher than in B6 mice (Seifert, et al., 2012). The lungs are elastic tissues with well-developed capillaries that are continuously subjected to the mechanical stress of stretching and contraction associated with respiration. Since type III collagen expression is upregulated along with type I collagen in pulmonary fibrosis, there seems to be a strong general understanding that increased type III collagen in the lung is pathological. However, recent cell aging studies of the lung have shown that type III collagen and decorin decrease with age in the lung as well, suggesting that the tissue environment of the lung and dermal papillary layer may be similar. Considering this fact, the increase in type III collagen and decorin in the lung due to ESM intake may promote tissue repair and supply a younger extracellular environment. A homeostatic effect of type III collagen in the lung should be further studied.

Diminished *type III collagen* expression in *type III collagen* haploinsufficient (*Col3<sup>+/-</sup>*) mice promotes myofibroblast differentiation, skin wound closure and scar

formation (Volk et al. 2011). And fetal scarless wounds also contain more type III collagen than type I collagen (Karppinen, et al., 2019). This supports a shift in the previously accepted paradigm for the role of type III collagen in tissue maintenance and repair, and proposes that in addition to forming extracellular scaffolds that serve a structural purpose, type III collagen also plays a vital role in coordinating cellular activity during tissue formation and regeneration (Volk et al. 2011). The expression of type III collagen in the pulmonary fibrosis model found in this study appears to prevent the entry of myofibroblasts leading to scar formation (i.e., fibrosis) and modulates tissue remodeling. The present study supports this possibility. The inhibitory effect of enhanced type III collagen expression on pulmonary fibrosis could be validated by enhanced fibrosis in a BLM mouse model using haploid-sufficient (*Col3<sup>+/-</sup>*) mice. A direct relationship between type III collagen and the mechanism of the preventive effect of ESM on fibrosis should also be elucidated in the future.

Recently, a comprehensive classification of fibroblast populations in the lung has been made in relation to our understanding of the pathogenesis of fibrosis (Xie et al. 2018). In lung fibrosis, direct differentiation from mesenchymal progenitor cells to Col14a1 matrix fibroblasts, which express high levels of decorin, is reportedly suppressed. To alter the toxic cell lineages back to normal differentiation, enhanced expression of transcription

factors that highly expressed in Col14a1 matrix fibroblasts (*PRRX1*, *AEBP1*, *LBH*) may have an advantage. In this study, Col14a1 matrix fibroblasts and Col13a1 matrix fibroblasts (*TCF21*) were studied using human lung cell line. We found a significant increase in expression of *PRRX1* and *LBH*, suggesting that the ESM may contribute to normal differentiation from mesenchymal progenitor cells leading to suppression of lung fibrosis.

Since ESM is a natural material that protects the development of chicken embryo from various stresses, its role is protective against tissue damage. ESM-derived peptides can potentially use as anti-oxidant (Huang et al., 2010; Shi et al., 2014) therefore may work as a redox-fibrosis therapeutics (Richter et al., 2015). Decorin is known to be an anti-fibrotic gene in the lung (Kolb, et al., 2001; Souma, et al., 2018). Since lungs and skin are soft tissues, type III collagen and decorin are required for adequate response to passive pressures on tissues and are essential for the formation of normal type I collagen fibers (Reed and Iozzo, 2002; Schonherr, et al., 1993). Type III collagen and decorin are located just below the basement membrane and may repress epithelial mesenchymal transition (EMT) in epithelial cells. Physiological stretching increases the expression of both genes (Kim et al. 2002; Chen et al. 2017). The distribution of the molecular composition of type IV collagen in the basement membrane beneath the tissue epithelium is different from

tissue to tissue and is consistent with tissue-specific functions, and in bronchi the basement membrane beneath the pseudostratified columnar cells shows a pattern similar to that of skin (Saito, et al., 2000). It is possible that basement membrane type IV collagen, type III collagen and decorin function to respond to the mechanical loading for tissues, and ESM may influence these molecules to improve tissue function. Since ESM intake of type III collagen and decorin is effective and contributes to homeostasis not only in the skin but also in the lung in cells, mice and humans, it is likely that some common mechanism exists in the skin and lung.

Capillaries in peripheral tissues play a major role in tissue homeostasis. In the chick embryo, erythrocytes exchange gas with the outside world through the layers of ESM, chorion epithelium, and sub-chorionic capillary sinus layers of the Chorioallantoic Membrane (Chen, et al., 2021). It would not be surprising to expect that the ingested ESM has some effect on vascular endothelial cells. The effects of eggshell membrane to basement membrane-vascular endothelial cell communication (Imamura, et al., 1991) in the lung and papillary dermis will need to be investigated in the future.

ESM is a water-insoluble membrane of fibrous proteins cross-linked by LOX. Our previous reports have shown that the development of soluble eggshell membranes has enabled us to study their direct effects on cells (Ohto-Fujita, et al., 2011; Ohto-Fujita, et

al., 2019). In addition, this study showed that human consumption of ESM powder as a dietary supplement can produce ECM effects such as skin elasticity and improved respiratory function. Since skin aging is characterized by structural and functional changes such as wrinkles and loss of elasticity, and skin stiffness reflects reorganization of the ECM in the dermis (Runel et al. 2020), mechanical properties and functional changes in the skin and lungs by ESM ingestion may indicate a shift to a younger ECM. The mechanism by which cells in skin and lung tissues responded to ESM digests is a very interesting question for the future. This study implies that ESM, a complex fibrous membrane, is surprisingly digestible and absorbable in humans, and reaches peripheral tissues via the bloodstream. In the future, more research will be conducted on the functions of natural eggshell membrane powder in contributing to human health.

In this study, after 8 weeks of the oral intake of eggshell membrane, the FEV1/FVC and forearm elasticity showed a marked increase, suggesting that the ESM supplementation-induced increase in the expression of type III collagen and decorin exerts protective effects, at least in the skin and lungs directly exposed to the outside world. Considering the effects of improving lung function in humans in this study, and anti-fibrosis effect on lung by mouse model, eggshell membrane may be a new anti-fibrotic agent that aids in systemic tissue homeostasis in humans.

## **Compliance with ethical standards**

## **Conflicts of interest**

E. Ohto-Fujita, M. Shimizu, and Y. Atomi are endowed chairs funded by Almodo, Inc.

Y. Hasebe is the founder of Almodo. All other authors declare no potential conflict of interest.

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## **Informed consent**

Written informed consent was obtained from all participants prior to inclusion in the study.

## **Ethical approval**

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals

were in accordance with the ethical standards of Tokyo University of Agriculture and Technology Animal Experiments Committee (No. 25-60 (2019 update)).

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

The study protocol was approved by the Ethics Committee of Tokyo University of Agriculture and Technology (No. 27-09).

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## **Data availability**

All data generated or analyzed during this study are included in this published article and its Supplementary Information files.

## References

- Ahmed TA, Suso HP, Hincke MT (2017) In-depth comparative analysis of the chicken eggshell membrane proteome. *J Proteomics* 155:49-62
- Akagawa M, Wako Y, Suyama K (1999) Lysyl oxidase coupled with catalase in egg shell membrane. *Biochim Biophys Acta* 1434:151-160
- Ashcroft T, Simpson JM, Timbrell V (1988) Simple method of estimating severity of pulmonary fibrosis on a numerical scale. *J Clin Pathol* 41:467-470
- Bruckner P (2010) Suprastructures of extracellular matrices: paradigms of functions controlled by aggregates rather than molecules. *Cell Tissue Res* 339:7-18
- Chen L, Wang S, Feng Y, Zhang J, Du Y, Zhang J, Ongeval CV, Ni Y, Li Y (2021) Utilisation of Chick Embryo Chorioallantoic Membrane as a Model Platform for Imaging-Navigated Biomedical Research. *Cells* 10: 463
- Chen S, Peng C, Wei X, Luo D, Lin Y, Yang T, Jin X, Gong L, Li H, Wang K (2017) Simulated physiological stretch increases expression of extracellular matrix proteins in human bladder smooth muscle cells via integrin  $\alpha_4/\beta_1$ -FAK-ERK1/2 signaling pathway. *World J Urol* 35:1247-1254
- Du J, Hincke MT, Rose-Martel M, Hennequet-Antier C, Brionne A, Cogburn LA, Nys Y,

- Gautron J (2015) Identifying specific proteins involved in eggshell membrane formation using gene expression analysis and bioinformatics. *BMC Genomics* 16:792
- Fidler AL, Boudko SP, Rokas A, Hudson BG (2018) The triple helix of collagens - an ancient protein structure that enabled animal multicellularity and tissue evolution. *J Cell Sci* 131:jcs203950
- Gólczewski T, Lubiński W, Chciałowski A (2012) A mathematical reason for FEV1/FVC dependence on age. *Respiratory Research* 13:57
- Grzesiak JJ, Pierschbacher MD, Amodeo MF, Malaney TI, Glass JR (1997) Enhancement of cell interactions with collagen/glycosaminoglycan matrices by RGD derivatization. *Biomaterials* 18:1625-1632
- Huang X, Zhou Y, Ma M, Cai Z, Li T (2010) Chemiluminescence Evaluation of Antioxidant Activity and Prevention of DNA Damage Effect of Peptides Isolated from Soluble Eggshell Membrane Protein Hydrolysate. *Journal of Agricultural and Food Chemistry* 58:12137-12142
- Imamura T, Tokita Y, Mitsui Y (1991) Contact with basement membrane heparan sulfate enhances the growth of transformed vascular endothelial cells, but suppresses normal cells. *Cell Struct Funct* 16:225-230

Jia H, Aw W, Saito K, Hanate M, Hasebe Y, Kato H (2014) Eggshell membrane ameliorates hepatic fibrogenesis in human C3A cells and rats through changes in PPARgamma-Endothelin 1 signaling. *Sci Rep* 4:7473

Jia H, Hanate M, Aw W, Itoh H, Saito K, Kobayashi S, Hachimura S, Fukuda S, Tomita M, Hasebe Y, Kato H (2017) Eggshell membrane powder ameliorates intestinal inflammation by facilitating the restitution of epithelial injury and alleviating microbial dysbiosis. *Sci Rep* 7:43993

Karppinen SM, Heljasvaara R, Gullberg D, Tasanen K, Pihlajaniemi T (2019) Toward understanding scarless skin wound healing and pathological scarring. *F1000Res* 8: F1000 Faculty Rev-787

Kim SG, Akaike T, Sasagawa T, Atomi Y, Kurosawa H (2002) Gene expression of type I and type III collagen by mechanical stretch in anterior cruciate ligament cells. *Cell Struct Funct* 27:139-144

Kiuchi T, Igarashi T (2004) UMIN--current status and future perspectives. *Stud Health Technol Inform* 107:1068-1072

Kolb M, Margetts PJ, Galt T, Sime PJ, Xing Z, Schmidt M, Gauldie J (2001) Transient transgene expression of decorin in the lung reduces the fibrotic response to bleomycin. *Am J Respir Crit Care Med* 163:770-777

- Lewis KJR, Hall JK, Kiyotake EA, Christensen T, Balasubramaniam V, Anseth KS (2018) Epithelial-mesenchymal crosstalk influences cellular behavior in a 3D alveolus-fibroblast model system. *Biomaterials* 155:124-134
- Lichtenberger BM, Mastrogiannaki M, Watt FM (2016) Epidermal beta-catenin activation remodels the dermis via paracrine signalling to distinct fibroblast lineages. *Nat Commun* 7:10537
- Liu X, Wu H, Byrne M, Krane S, Jaenisch R (1997) Type III collagen is crucial for collagen I fibrillogenesis and for normal cardiovascular development. *Proc Natl Acad Sci U S A* 94:1852-1856
- Ohto-Fujita E, Konno T, Shimizu M, Ishihara K, Sugitate T, Miyake J, Yoshimura K, Taniwaki K, Sakurai T, Hasebe Y, Atomi Y (2011) Hydrolyzed eggshell membrane immobilized on phosphorylcholine polymer supplies extracellular matrix environment for human dermal fibroblasts. *Cell Tissue Res* 345:177-190
- Ohto-Fujita E, Shimizu M, Sano S, Kurimoto M, Yamazawa K, Atomi T, Sakurai T, Murakami Y, Takami T, Murakami T, Yoshimura K, Hasebe Y, Atomi Y (2019) Solubilized eggshell membrane supplies a type III collagen-rich elastic dermal papilla. *Cell Tissue Res* 376:123-135
- Osei ET, Brandsma C-A, Noordhoek JA, Timens W, Postma D, Heijink I (2014) Crosstalk

- between epithelium and fibroblasts; implications for COPD. *European Respiratory Journal* 44:P3899
- Parrish AR (2017) The impact of aging on epithelial barriers. *Tissue Barriers* 5:e1343172
- Reed CC, Iozzo RV (2002) The role of decorin in collagen fibrillogenesis and skin homeostasis. *Glycoconj J* 19:249-255
- Richter K, Konzack A, Pihlajaniemi T, Heljasvaara R, Kietzmann T (2015) Redox-fibrosis: Impact of TGF $\beta$ 1 on ROS generators, mediators and functional consequences. *Redox Biol* 6:344-352
- Ruff KJ, DeVore DP, Leu MD, Robinson MA (2009a) Eggshell membrane: a possible new natural therapeutic for joint and connective tissue disorders. Results from two open-label human clinical studies. *Clin Interv Aging* 4:235-240
- Ruff KJ, Winkler A, Jackson RW, DeVore DP, Ritz BW (2009b) Eggshell membrane in the treatment of pain and stiffness from osteoarthritis of the knee: a randomized, multicenter, double-blind, placebo-controlled clinical study. *Clinical rheumatology* 28:907-914
- Runel G, Cario M, Lopez-Ramirez N, Malbouyres M, Ruggiero F, Bernard L, Puisieux A, Caramel J, Chlasta J, Masse I (2020) Stiffness measurement is a biomarker of skin ageing in vivo. *Exp Dermatol* 29:1233-1237

Saito K, Naito I, Seki T, Oohashi T, Kimura E, Momota R, Kishiro Y, Sado Y, Yoshioka H, Ninomiya Y (2000) Differential expression of mouse alpha5(IV) and alpha6(IV) collagen genes in epithelial basement membranes. *J Biochem* 128:427-434

Schonherr E, Beavan LA, Hausser H, Kresse H, Culp LA (1993) Differences in decorin expression by papillary and reticular fibroblasts in vivo and in vitro. *Biochem J* 290 ( Pt 3):893-899

Seifert AW, Kiama SG, Seifert MG, Goheen JR, Palmer TM, Maden M (2012) Skin shedding and tissue regeneration in African spiny mice (*Acomys*). *Nature* 489:561-565

Shi Y, Kovacs-Nolan J, Jiang B, Tsao R, Mine Y (2014) Peptides derived from eggshell membrane improve antioxidant enzyme activity and glutathione synthesis against oxidative damage in Caco-2 cells. *Journal of Functional Foods* 11:571-580

Skibinski G, Elborn JS, Ennis M (2007) Bronchial epithelial cell growth regulation in fibroblast cocultures: the role of hepatocyte growth factor. *American Journal of Physiology-Lung Cellular and Molecular Physiology* 293:L69-L76

Sorrell JM, Baber MA, Caplan AI (2004) Site-matched papillary and reticular human dermal fibroblasts differ in their release of specific growth factors/cytokines and

in their interaction with keratinocytes. *J Cell Physiol* 200:134-145

Souma K, Shichino S, Hashimoto S, Ueha S, Tsukui T, Nakajima T, Suzuki HI, Shand FHW, Inagaki Y, Nagase T, Matsushima K (2018) Lung fibroblasts express a miR-19a-19b-20a sub-cluster to suppress TGF-beta-associated fibroblast activation in murine pulmonary fibrosis. *Sci Rep* 8:16642

Tiffeneau R (1947) Air circulant et air captif dans l'exploration de la fonction ventilatrice pulmonaire. *Paris medical* 37:624-628

Tsang KY, Cheung MC, Chan D, Cheah KS (2010) The developmental roles of the extracellular matrix: beyond structure to regulation. *Cell Tissue Res* 339:93-110

Tsukui T, Sun KH, Wetter JB, Wilson-Kanamori JR, Hazelwood LA, Henderson NC, Adams TS, Schupp JC, Poli SD, Rosas IO, Kaminski N, Matthay MA, Wolters PJ, Sheppard D (2020) Collagen-producing lung cell atlas identifies multiple subsets with distinct localization and relevance to fibrosis. *Nat Commun* 11:1920

Volk SW, Wang Y, Mauldin EA, Liechty KW, Adams SL (2011) Diminished type III collagen promotes myofibroblast differentiation and increases scar deposition in cutaneous wound healing. *Cells Tissues Organs* 194:25-37

Walkin L, Herrick SE, Summers A, Brenchley PE, Hoff CM, Korstanje R, Margetts PJ (2013) The role of mouse strain differences in the susceptibility to fibrosis: a

systematic review. *Fibrogenesis Tissue Repair* 6:18

Xie T, Wang Y, Deng N, Huang G, Taghavifar F, Geng Y, Liu N, Kulur V, Yao C, Chen P,

Liu Z, Stripp B, Tang J, Liang J, Noble PW, Jiang D (2018) Single-Cell

Deconvolution of Fibroblast Heterogeneity in Mouse Pulmonary Fibrosis. *Cell*

Rep 22:3625-3640

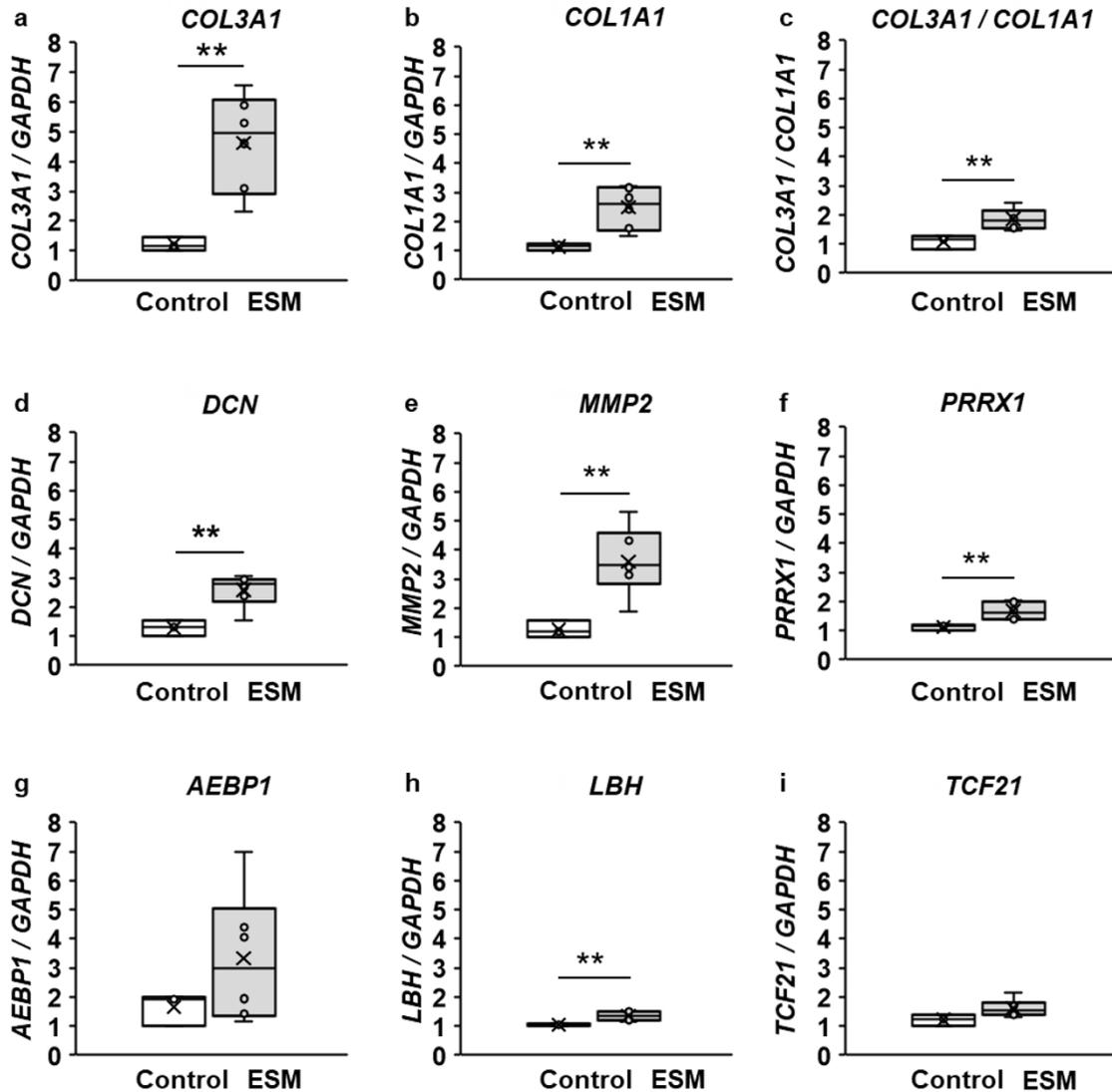
Zhang L, Tran N, Chen HQ, Kahn CJ, Marchal S, Groubatch F, Wang X (2008) Time-

related changes in expression of collagen types I and III and of tenascin-C in rat

bone mesenchymal stem cells under co-culture with ligament fibroblasts or

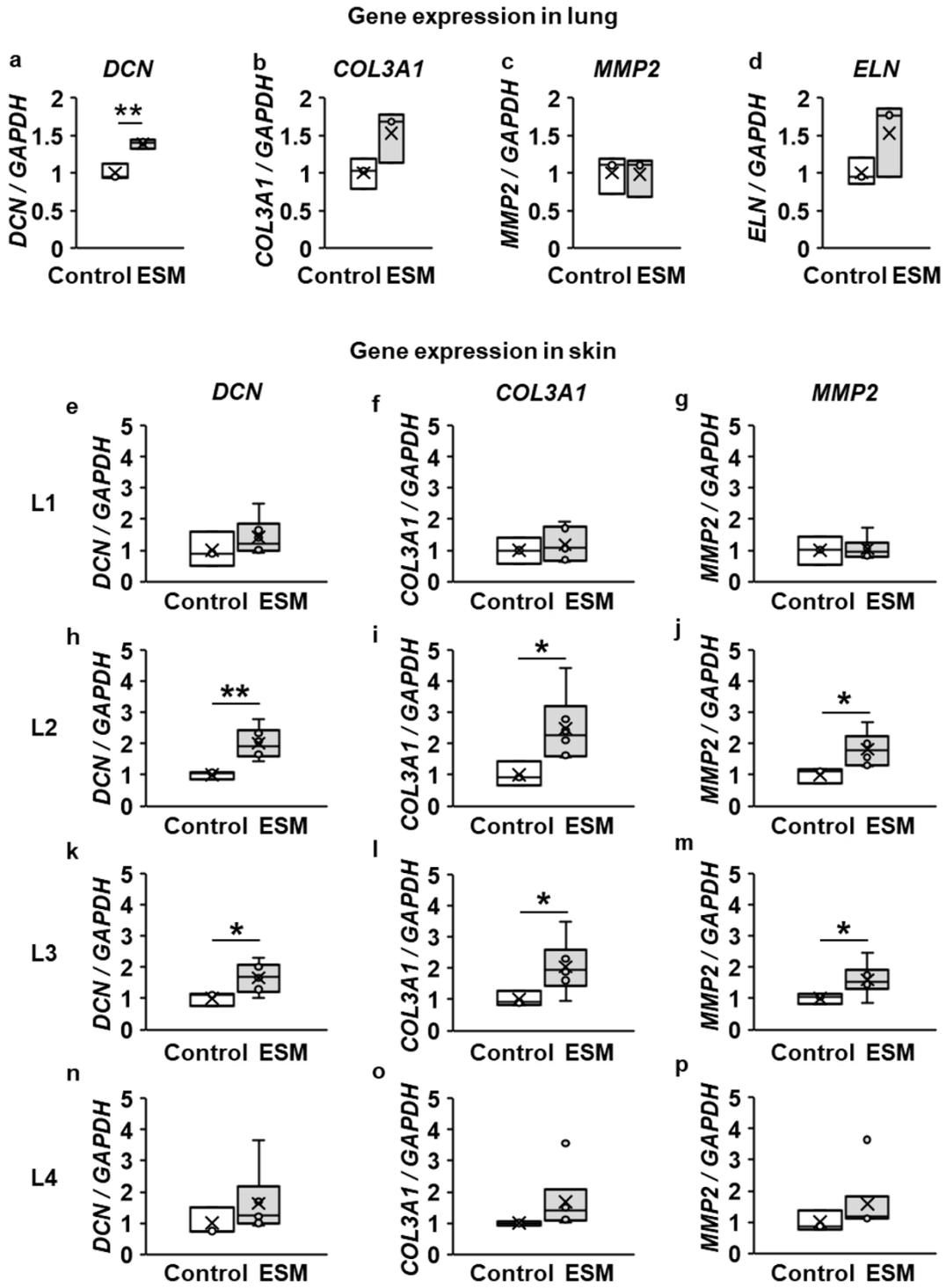
uniaxial stretching. *Cell Tissue Res* 332:101-109

Figure 1



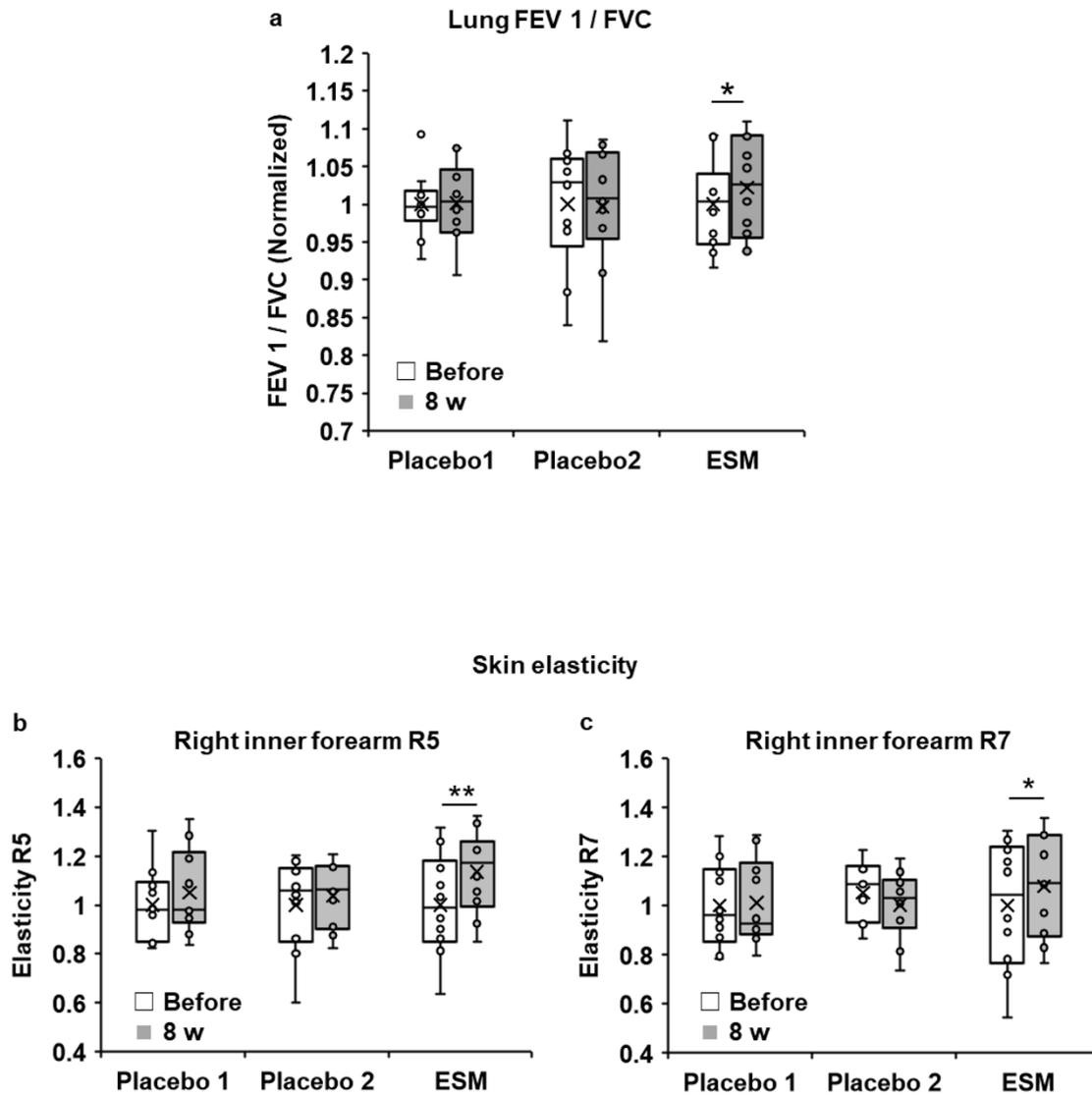
**Fig. 1** Human normal lung fibroblast cell line induce *type III collagen*, *decorin*, *MMP2*, and the marker transcription factors for high decorin and coll4a1 matrix fibroblast. Gene expression of normal lung fibroblast cell line (IMR-90) on ESM surface (a-i). ESM significantly induces *type III collagen* (*COL3A1*) (a), *type I collagen* (*COL1A1*) (b), *decorin* (*DCN*) (d), *MMP2* (e), *PRRX1* (f), and *LBH* (h) expression. High *type III collagen* versus *type I collagen* expression on ESM was confirmed (c)

Figure 2



**Fig. 2** Increased *type III collagen* and *decorin* gene expression in mouse lung and skin by oral ingestion of ESM. (a-d) Changes in gene expression of *decorin (DCN)* (a), *type III collagen (COL3A1)* (b), *MMP2* (c) and *elastin (ELN)* (d) after 2-weeks ingestion of ESM were analyzed by RT-PCR. ESM: ESM intake group. Control: control group. (n=3 for each group). \* : p<0.05 is considered significant. (e-p) Skin layer analysis. (d) Gene expression changes of *decorin (DCN)* (e, h, k, n) *type III collagen (COL3A1)* (f, I, l, o), *MMP2* (g, j, m, p) in mice skin layers after ESM intake. Gene expression changes in the fourth layer of skin in hairless mice after 4-weeks of ESM ingestion analyzed by RT-PCR. Skin biopsy skin samples of hairless mice after 4 weeks of ESM intake were sectioned into 100 µm thick sections by vibratome, and gene expression changes in layer 1 (L1) (e-g) corresponding to epidermis, layer 2 (L2) (h-j) corresponding to dermal papillary layer, layer 3 (L3) (k-m), and layer 4 (L4) (n-p) corresponding to dermal reticular layer were analyzed by RT-PCR. ESM significantly induces gene expression of *decorin (DCN)*, *type III collagen (COL3A1)*, and *MMP2* in L2 and L3. ESM: ESM intake group. Control: control group. (Control: n=3, ESM: n=6). \*: p<0.05, \*\*: p<0.01

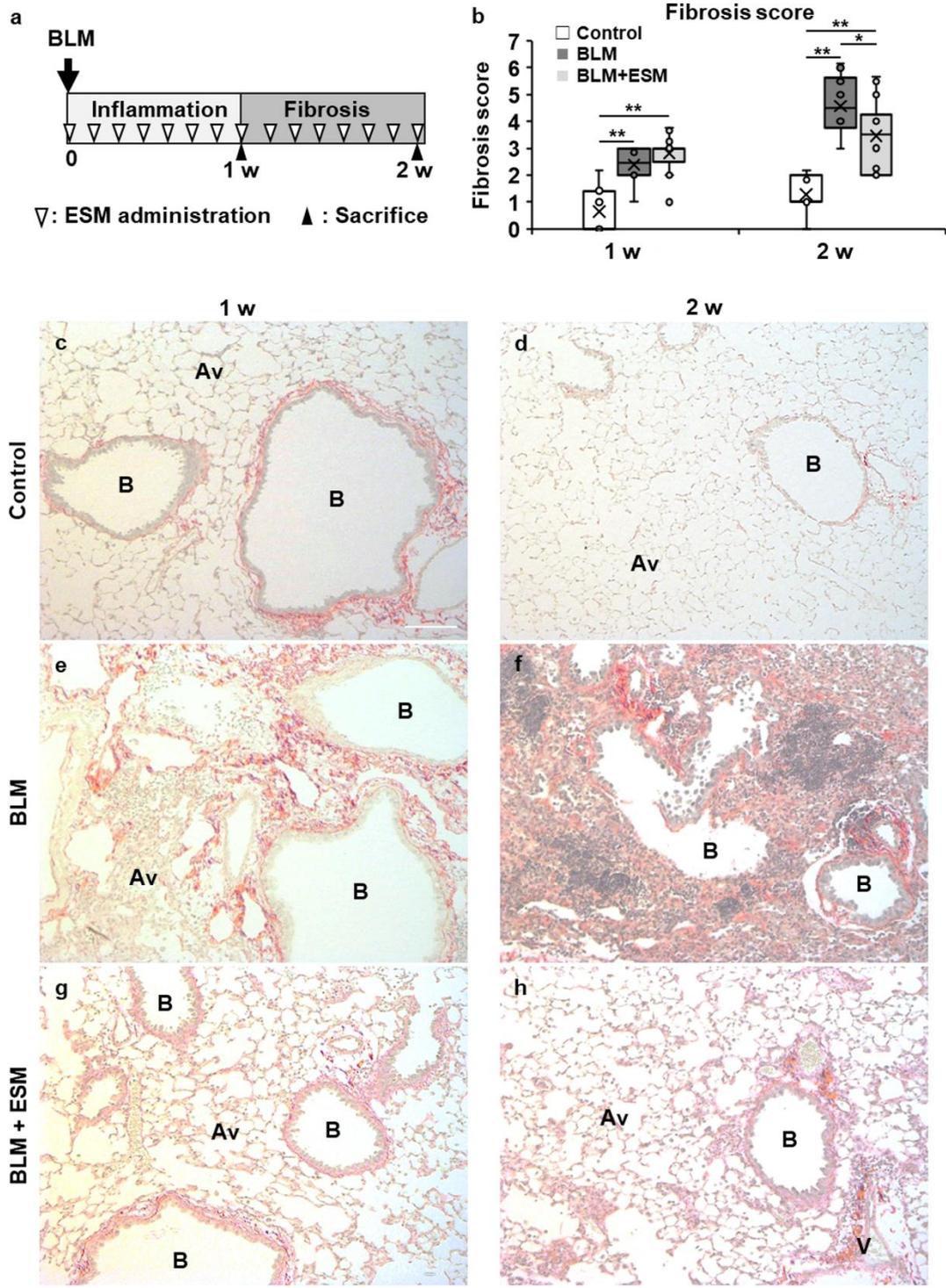
**Figure 3**



**Fig. 3** Oral Ingestion of ESM increases the human lung FEV1/FVC and skin elasticity.

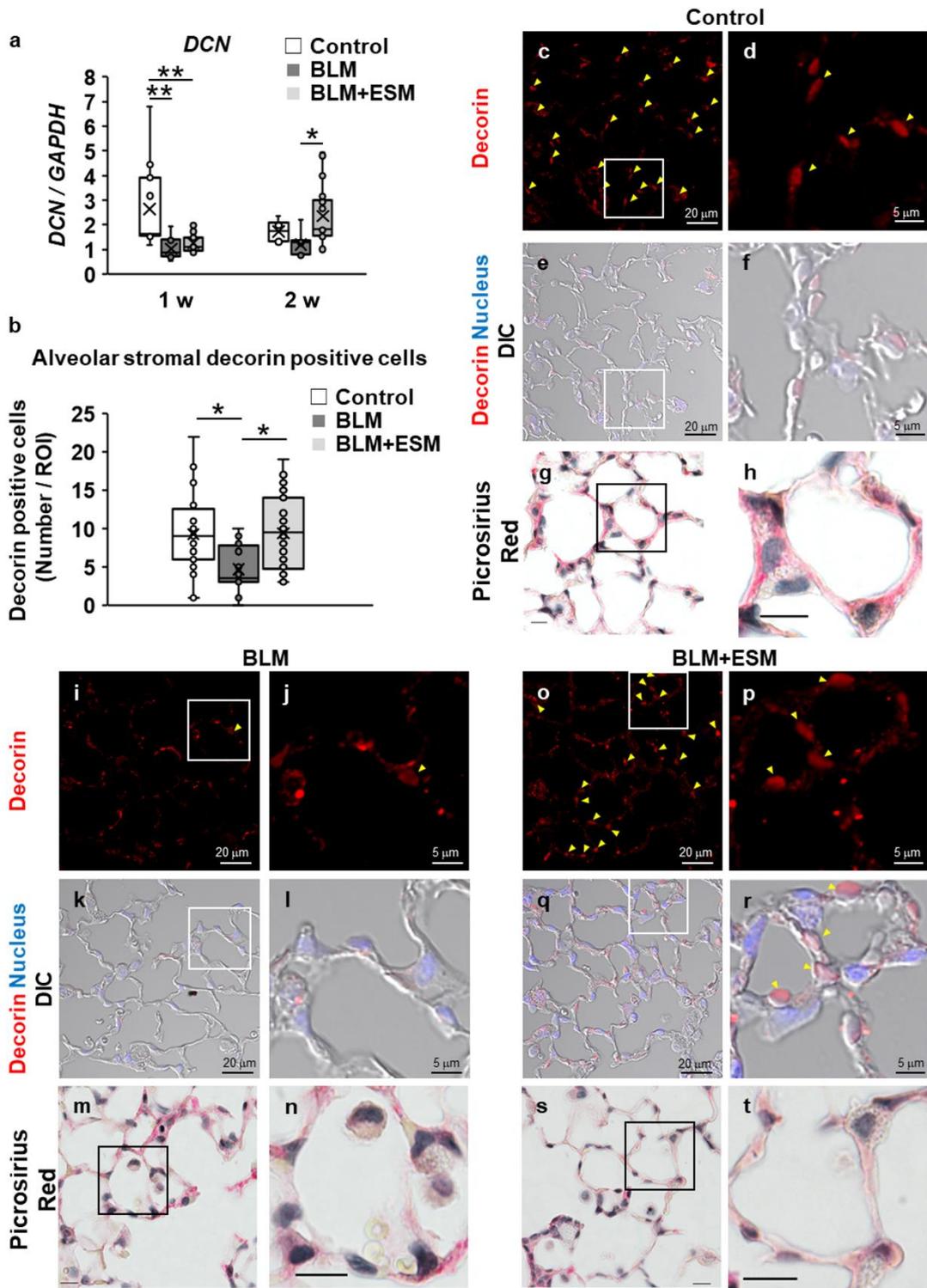
(a) Pulmonary function test results showing that FEV1/FVC significantly increased within 8-weeks of ESM intake. Significant differences were not seen in the placebo 1 and 2 groups. Data presented as mean  $\pm$  SE. (n=10) (b, c) Increase in elasticity (R5 and R7) of right inner forearm skin after ESM intake within 8-weeks. Data presented as mean  $\pm$  SE. (n=10)

**Figure 4**



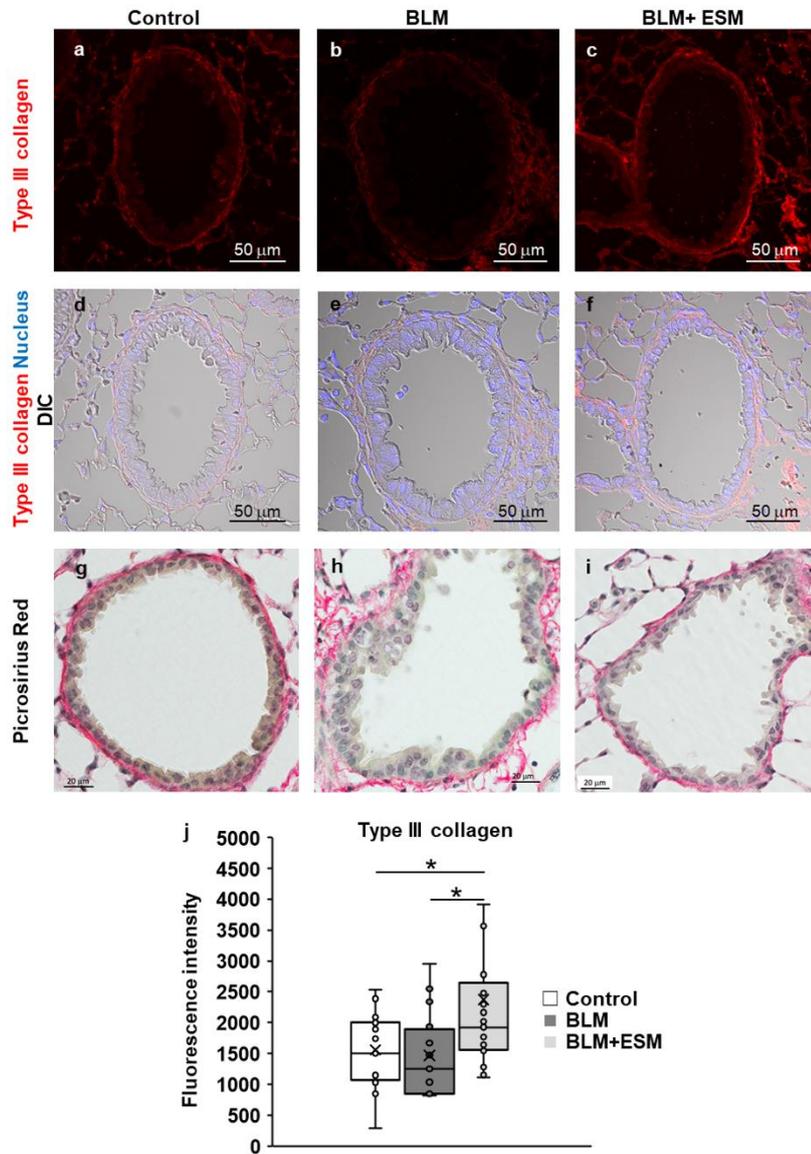
**Fig. 4** ESM ameliorates pulmonary fibrosis in a bleomycin mouse model. (a) Experimental schedule. (b) Fibrosis score. After 2 weeks of oral intake of ESM, fibrosis was significantly ameliorated in the ESM group compared to the BLM group. Control group (n=10), BLM group (n=10), BLM+ESM group (n=18). \* :  $p < 0.05$ , \*\* :  $p < 0.01$  (c-h) Lung histochemistry of picosirius red-stained images from each group. Bar = 100  $\mu\text{m}$ . Av: alveoli, V: vessel, B: bronchiole

**Figure 5**



**Fig. 5** ESM increases decorin expression at the 2-week alveoli of bleomycin fibrosis induction model. (a) Changes in *decorin* (*DCN*) gene expression in the lung after 1 and 2 weeks of BLM treatment were examined by RT-PCR. 2 weeks showed that ESM significantly induced decorin gene expression. Control group (n=10), BLM group (n=10), BLM+ESM group (n=18). \* p<0.05, \*\* p<0.01. (b) Quantification of the number of alveolar stromal decorin positive cells (arrowhead) at week 2. ESM significantly increases the number of alveolar decorin staining. Control group (n=8), BLM group (n=8), BLM+ESM (n=15) \* p<0.05. (c-t)) Section images of alveoli in non-inflamed area. (c-h) Control, (i-n) BLM, (o-t) BLM+ESM. (c, d, i, j, o, p) Decorin staining. (e, f, k, l, q, r) Decorin, nuclear staining, Differential interference contrast (DIC) superimposition. (f, n, t) Magnified view of black square in the left panel. (g, m, s) Picrosirius red images and (h, n, t) magnified view of black square in the left panel

**Figure 6**



**Fig. 6** ESM increases type III collagen expression at the 2-week mark in the small bronchiole of bleomycin-induced fibrosis mouse model. (a-c) Type III collagen staining images of peribronchial bronchi at week 2, (d-f) superimposition of type III collagen, nuclei and Differential interference contrast (DIC) images, (g-i) picrosirius red staining images. (j) Quantification of type III collagen staining of peribronchial bronchi at week 2. ESM significantly increases bronchial type III collagen staining. \*  $p < 0.05$

## Electronic supplementary file

### Eggshell membrane promotes homeostasis of elastic skin and lung tissue associated with type III collagen and decorin expression and ameliorates pulmonary fibrosis in a bleomycin mouse model

#### Cell and Tissue Research

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#### Online resource 1 Primer sequences (5'-3')

For amplification in human IMR-90

Gene	Forward primer	Reverse primer
<i>GAPDH</i> <sup>*1</sup>	CGACAGTCAGCCGCATCTTC	CGCCAATACGACCAAATCCG
<i>COL3A1</i> <sup>*2</sup>	GGACCTCCTGGTGCTATAGGT	CGGGTCTACCTGATTCTCCAT
<i>COL1A1</i> <sup>*2</sup>	GGGATTCCCTGGACCTAAAG	GGAACACCTCGCTCTCCA
<i>DCN</i> <sup>*2</sup>	GGAGACTTTAAGAACCTGAAGAACC	CGTTCCAACCTTCACCAAAGG
<i>MMP2</i> <sup>*3</sup>	CCCCTGCGTTTTTCTCGAAT	CAAAGGGGTATCCATCGCCAT
<i>PRRX1</i> <sup>*4</sup>	TGATGCTTTTGTGCGAGAAGA	AGGGAAGCGTTTTTATTGGCT
<i>AEBP1</i> <sup>*5</sup>	GGTTACGTGATCCCCAACTACG	CTGCCCACTTGTCGGTCTC
<i>LBH</i> <sup>*6</sup>	GCCCCGACTATCTGAGATCG	GCGGTCAAATCTGACGGGT
<i>TCF21</i> <sup>*7</sup>	TCCTGGCTAACGACAAATACGA	TTTCCCGGCCACCATAAAGG

\*<sup>1</sup>Designed by Sigma-Genosys, Japan; \*<sup>2</sup> (Ohto-Fujita, et. al., 2011); \*<sup>3</sup>(Zhao, et al., 2017); \*<sup>4</sup>(Loh, et al., 2016); \*<sup>5</sup> PrimerBank ID 53692188c2 (Spandidos, et al., 2009); \*<sup>6</sup>(Jiang, et al., 2019); \*<sup>7</sup>PrimerBank ID 208609971c1 (Spandidos, et al., 2009)

For amplification in mouse lung and skin

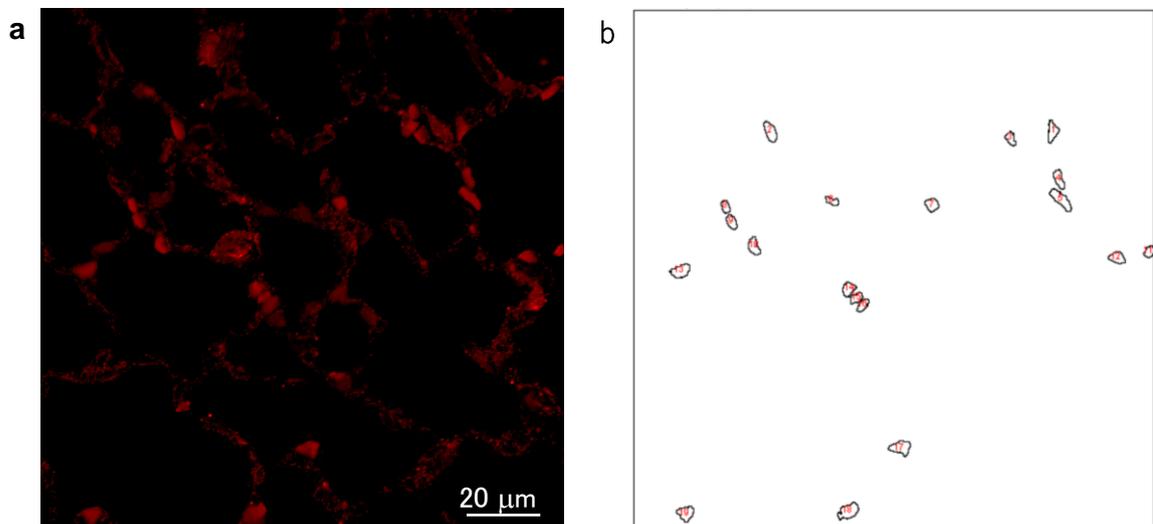
Gene	Forward primer	Reverse primer
<i>GAPDH</i> <sup>*8</sup>	AGCTTGTCATCAACGGGAAG	TTTGATGTTAGTGGGGTCTCG
<i>COL3A1</i> <sup>*8</sup>	TCCCCTGGAATCTGTGAATC	TGAGTCGAATTGGGGAGAAT
<i>MMP2</i> <sup>*8</sup>	GGAGAAGGCTGTGTTCTTCG	AGGCTGGTCAGTGGCTTG
<i>DCN</i> <sup>*8</sup>	GAGGAGAAGTGAGGGGAGACA	GATTATCTCATGTATTTTCACGACCTT
<i>ELN</i> <sup>*8</sup>	TGGAGCAGGACTTGGAGGT	CCTCCAGCACCATACTTAGCA

\*<sup>8</sup> (Ohto-Fujita E, et al., 2019)

#### Online resource references

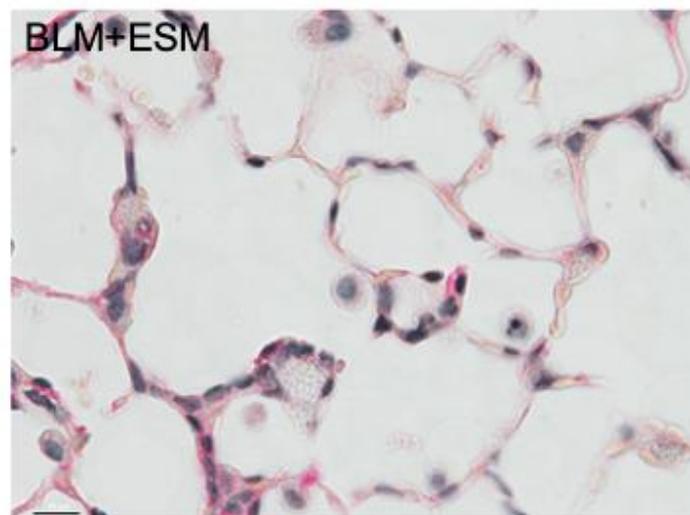
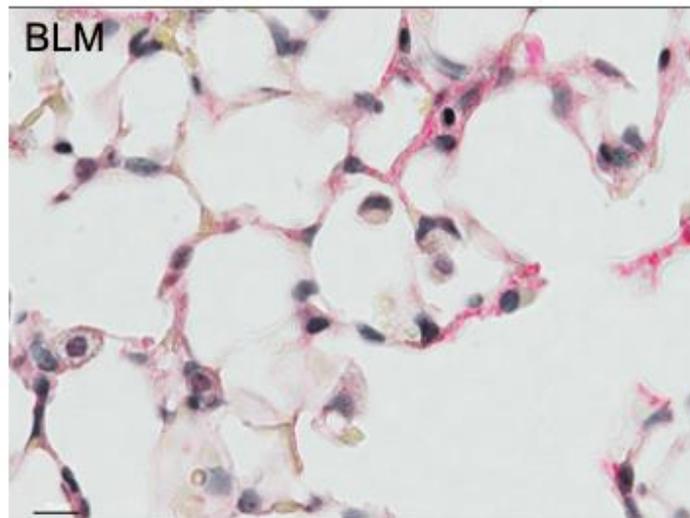
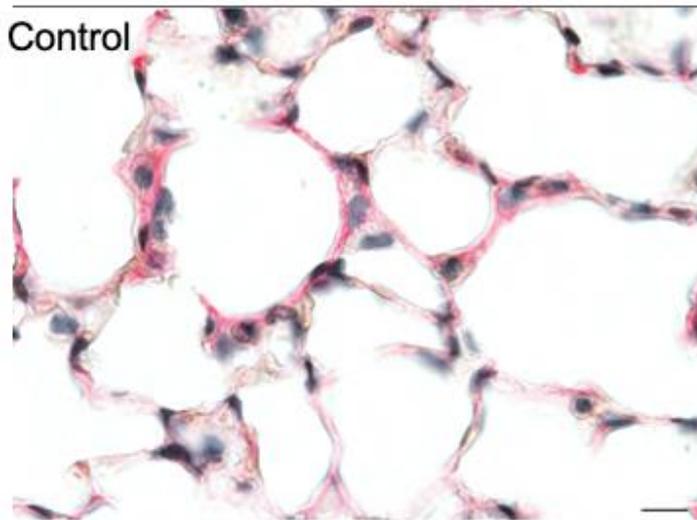
- Jiang Y, Zhou J, Zou D, Hou D, Zhang H, Zhao J, Li L, Hu J, Zhang Y, Jing Z (2019) Overexpression of Limb-Bud and Heart (LBH) promotes angiogenesis in human glioma via VEGFA-mediated ERK signalling under hypoxia. *EBioMedicine* 48:36-48
- Loh KM, Chen A, Koh PW, Deng TZ, Sinha R, Tsai JM, Barkal AA, Shen KY, Jain R, Morganti RM, Shyh-Chang N, Fernhoff NB, George BM, Wernig G, Salomon REA, Chen Z, Vogel H, Epstein JA, Kundaje A, Talbot WS, Beachy PA, Ang LT, Weissman IL (2016) Mapping the Pairwise Choices Leading from Pluripotency to Human Bone, Heart, and Other Mesoderm Cell Types. *Cell* 166:451-467
- Ohto-Fujita E, Konno T, Shimizu M, Ishihara K, Sugitate T, Miyake J, Yoshimura K, Taniwaki K, Sakurai T, Hasebe Y, Atomi Y (2011) Hydrolyzed eggshell membrane immobilized on phosphorylcholine polymer supplies extracellular matrix environment for human dermal fibroblasts. *Cell Tissue Res* 345:177-190
- Ohto-Fujita E, Shimizu M, Sano S, Kurimoto M, Yamazawa K, Atomi T, Sakurai T, Murakami Y, Takami T, Murakami T, Yoshimura K, Hasebe Y, Atomi Y (2019) Solubilized eggshell membrane supplies a type III collagen-rich elastic dermal papilla. *Cell Tissue Res* 376:123-135
- Spandidos A, Wang X, Wang H, Seed B (2009) PrimerBank: a resource of human and mouse PCR primer pairs for gene expression detection and quantification. *Nucleic Acids Research* 38:D792-D799
- Zhao S, Zhao Y, Guo J, Fei C, Zheng Q, Li X, Chang C (2017) Downregulation of MMP1 in MDS-derived mesenchymal stromal cells reduces the capacity to restrict MDS cell proliferation. *Sci Rep* 7:43849

**Online resource 2** Quantification of the number of alveolar stromal decorin-positive cells by Image J.

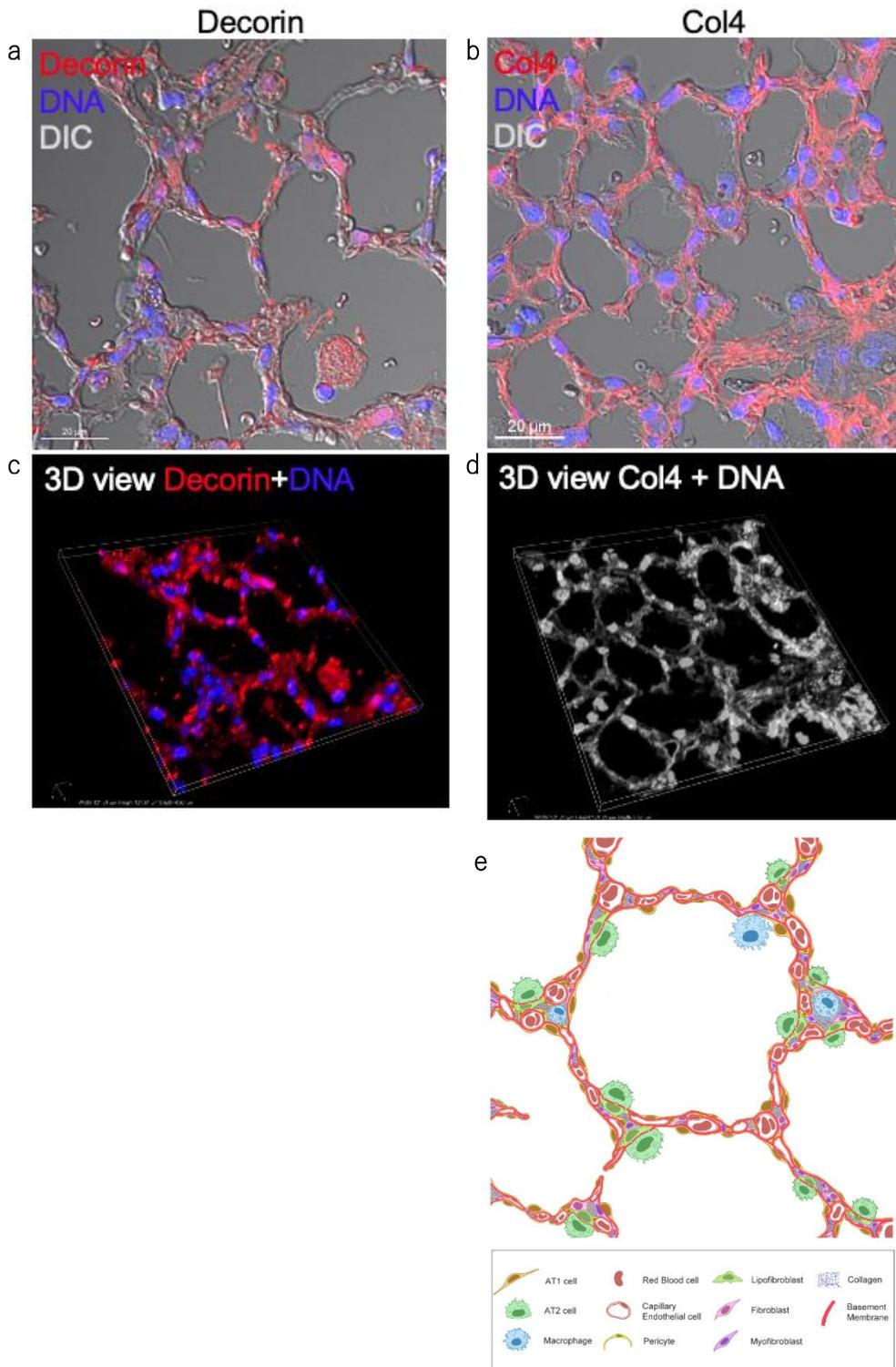


The number of decorin-positive cells in the alveolar interstitium was measured using Image J. Lung paraffin sections immunostained with anti-decorin-Alexa546 were observed with a confocal microscope, and the acquired images (a) were converted to 16 bit by Image J. After binarization, the size was set to 70-350 pixel<sup>2</sup> (4.5-22.6 mm<sup>2</sup>) and the circularity was set to (0.5-1.0), and the number of extracted objects was counted (b).

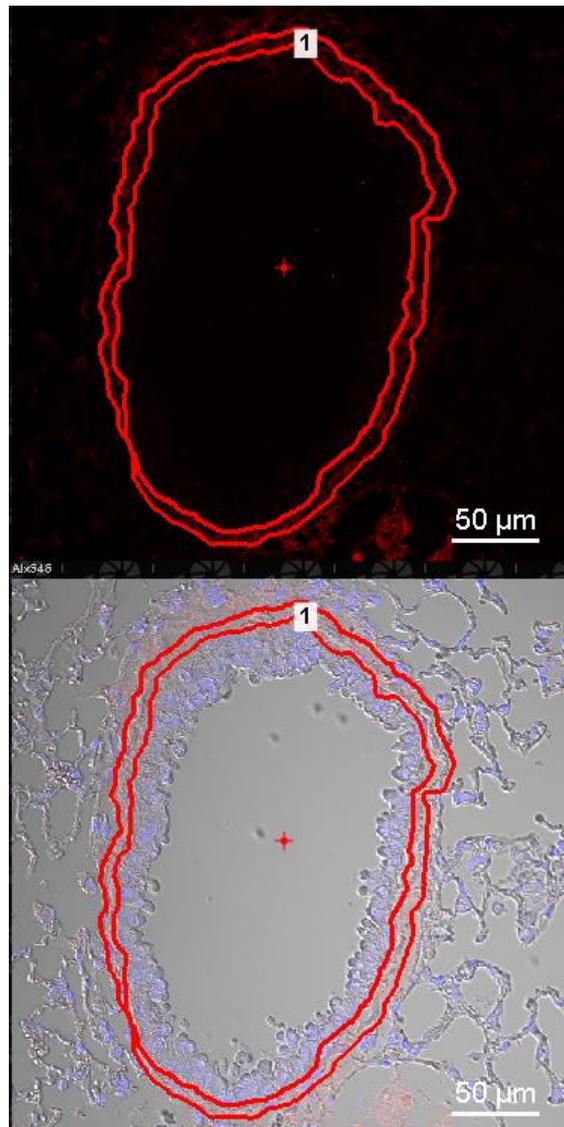
**Online resource 3** Large view of Figure 5 (picrosirius red staining images).  
Bar is 10 mm.



**Online resource 4.** Alveolar staining of decorin (a, c) and Col4 (basement membrane) (b, d). (e) Illustration of cells and basement membrane in alveolar area.



**Online resource 5** Quantification of fluorescence intensity of type III collagen-stained area around bronchi in lung tissue sections



Interstitial fluorescence of type III collagen-Alexa546 around bronchioles (size: about 200  $\mu\text{m}$  in diameter/  $\mu\text{m}^2$ ) was obtained from two bronchioles per individual using a Nikon A1 RMP equipped with NIS-Elements software