Differentiation of embryonic stem cells into lung-like cells using lung-derived matrix sheets

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Abstract

Various extracellular matrix (ECM) in the lungs regulate tissue development and homeostasis, as well as provide support for cell structures. However, few studies regarding the effects of lung cell differentiation using lung-derived ECM (LM) alone have been reported. The present study investigated the capability of lung-derived matrix sheets (LMSs) to induce lung cell differentiation using mouse embryonic stem (ES) cells. Expressions of lung-related cell markers were significantly upregulated in ES-derived embryoid bodies (EBs) cultured on an LMS for two weeks. Moreover, immunohistochemical analysis of EBs grown on LMSs revealed differentiation of various lung-related cells. These results suggest that an LMS can be used to promote differentiation of stem cells into lung cells.

Keywords

extracellular matrix; stem cell; matrix sheets; differentiation; lung

1. Introduction

The lungs have important roles related to exchange of gas and prevention of infection from viruses or bacteria in inhaled air, are essential organ for life support [1]. However, lung tissue is difficult to regenerate as compared to that of other organs [2], thus lung diseases, such as chronic obstructive pulmonary disease (COPD) [3] and idiopathic pulmonary fibrosis (IPF) [4], are difficult to treat effectively. Once lung structures are destroyed in individuals with these diseases, the condition is irreversible and the only curative treatment currently available is lung transplantation [5,6]. Therefore, investigations of lung stem cells and lung regeneration have recently been performed [7-9].

Multipotent stem cells such as embryonic stem (ES) cells and induced-pluripotent stem (iPS) cells have an ability to differentiate into various cell types [10-12]. Several studies that examined differentiation of multipotent stem cells into various lung cell types, such as alveolar epithelial cells [13], ciliated cells [14,15], and club cells [16], have been reported. Most of those were performed using combinations of cytokines, hormones, and transgenes, while examinations of induction of various lung-lineage cells using an extracellular matrix (ECM) alone are quite limited.

Decellularized tissues can be obtained using various decellularization techniques, and retain ECMs with three-dimensional structures [17]. ECMs mainly consist of two types of polymers; fibrillar proteins and proteoglycans [18], though their composition and functions in various organs differ. In the lungs, ECMs support cell structure and regulate developmental organogenesis, homeostasis, and injury-repair responses [19]. Therefore, lung derived-ECMs may be effective for differentiation of stem cells into lung cells.

Our previous study examined use of a lung-derived ECM (LM) for differentiation of ES

cells into lung cells. ES cell-derived embryoid bodies (EBs) transplanted in LMs were induced to differentiate into lung cell-like cells [20]. In the present study, induction of lung cell differentiation using lung-derived matrix sheets (LMSs) was investigated and the findings indicated promotion of differentiation into various lung-related cells.

2. Material and methods

2.1. Cells

Mouse ES cells [20,21] (G4-2; a kind gift from Dr. Hitoshi Niwa, Kumamoto University, Kumamoto, Japan) were maintained in ES cell medium (ES-M) in gelatin-coated dishes without feeder cells. ES-M was composed of DMEM supplemented with 10% FBS (PAA Laboratories, Germany), 0.1 mM 2-mercaptoethanol (FUJIFILM Wako Chemical, Osaka, Japan), 0.1 mM nonessential amino acids (Invitrogen, Waltham, MA), 1 mM sodium pyruvate (FUJIFILM Wako Chemical), 0.1% penicillin/streptomycin (FUJIFILM Wako Chemical), and 1000 U/ml LIF (FUJIFILM Wako Chemical). G4-2 ES cells carried the enhanced green fluorescent protein (EGFP) gene driven by the CAG promotor.

2.2. Mice

Inbred female ICR mice were purchased from Japan SLC (Hamamatsu, Japan) and housed in group cages at the animal facilities of Nara Medical University. Lung tissues from 12-week-old adult female ICR mice were used for the experiments. The animal experimental protocols were approved by The Animal Care and Use Committee at Nara Medical University (no. 13245). All animal experiments including the surgical steps were performed in accordance with the guidelines of Nara Medical University and Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines.

2.3. Preparation of lung-derived matrix sheets (LMSs)

To prepare lung-derived matrixes, lungs were collected from ICR mice and decellularized using the following SDS treatment procedure [22]. After washing the isolated lungs with PBS,

they were treated with 0.01% and 0.1 % SDS, respectively for every 24 hours. Next, treatment was performed with 1% SDS for 48 hours and the lungs were thoroughly washed with PBS containing 0.1% penicillin/streptomycin. Thereafter, 500, 750, and 1000 μm lung-derived matrix sheets (LMSs) were prepared using a tissue chopper device (McIlwain, Lafayette, IN) (Fig. 1A).

2.4. Differentiation into lung-like cells

Undifferentiated ES cells were dissociated and cultured in hanging drops with a density of 500 cells per 20 μL ES-M without LIF for four days to prepare EBs (Fig. 1B). Ten EBs were collected and placed on an LMS in a culture insert with 8-μm sized pores (BD, Franklin Lakes, NJ) using a 200 μL-tip, then cultured using an air-liquid interface (ALI) method for two weeks (Fig. 1C). Control experiments were performed by cultivation of ten EBs with ES-M without LIF in 35-mm gelatin-coated plastic dishes. Half of the culture medium was replaced with new medium every three days. The procedures used for these experiments are shown in Figure 1D.

2.5. Real-time qRT-PCR

Total RNA was prepared from cultured samples using TRIzol reagent (Invitrogen), then reverse transcription and qPCR were performed using a SYBR PrimeScript RT-PCR kit II (TaKaRa Bio Inc., Otsu, Japan), according to the manufacturer's instructions. Gene-specific primers were purchased from TaKaRa Bio Inc. and shown in Supplementary Table S1. Target gene PCR product amounts were calculated relative to the internal control (β-actin), then compared between the experimental and control groups using the ΔΔCT method.

2.6. Histology and immunohistochemistry

After culturing for two weeks, tissues were collected and fixed in 4% PFA for two days. Next, they were treated with 20% sucrose for one day and then embedded in OCT compound. Histology examinations were performed with hematoxylin-eosin (H&E) staining using a general protocol with 6-μm sections prepared with a cryostat. Immunohistochemistry was performed according to the following procedure. Sections were immersed in PBS, then blocked with 5% normal donkey serum (NDS) or normal goat serum (NGS). After washing with PBS, they were treated with primary antibodies (diluted 1:100 with 1% NDS or NGS) overnight at 4˚C. The primary antibodies (anti-Nkx-2.1, anti-SFTPC, anti-CC10, anti-AQP5) were purchased from Santa Cruz, Inc. (Santa Cruz, CA). After washing twice with PBS, the sections were treated with Alexa Fluor 546 conjugated anti-rabbit or anti-goat secondary antibodies (Invitrogen) diluted 1:200 with 1% NDS or NGS, then washed with PBS and stained with DAPI (Dojindo, Kumamoto, Japan). Fluorescence was detected using a BZ-X710 fluorescence microscope (Keyence, Kyoto, Japan).

2.7. Statistical analysis

Data are expressed as the mean ± SD of three independent experiments. Statistical significance was tested using Student's *t* test, with a *p* value <0.05 considered to indicate significance.

3. Results

3.1. Cell outgrowth dependent on LMS thickness

Tissues were cultured on LMSs with a thickness of 500, 750, or 1000 μm for two weeks, then collected and fixed with 4% PFA. Sections were prepared using a cryostat, then H&E staining was performed for cell outgrowth analysis (Fig. 2A). Calculations performed with BZ-X710 software showed that cell growth areas were significantly greater with the 1000-μm LMSs (Fig. 2B), thus that thickness was used for the following experiments.

3.2. Observation of EB outgrowths cultured on LMS

Ten EBs were cultured on LMSs for two weeks using the ALI method. Samples were observed on Day 0, 3 ,7 and 14 using fluorescence microscopy (Fig. 2C). EB outgrowths on the LMSs showed fluorescence, which expanded with a longer culture period, thus demonstrating that EBs on the LMSs survived and proliferated.

3.3. Gene expression analysis of lung-related cell markers

Gene expressions of lung-related cell markers were examined using a real-time qRT-PCR method. Total RNA samples were prepared from undifferentiated ES cells, EBs, differentiated EB outgrowths cultured in gelatin-coated dishes, and EB outgrowths cultured on LMSs (ES, EB, 2D, LMS, respectively; Fig. 3). The expressions of *Nkx-2.1*, *AQP5*, *SFTPC*, and *CC10* were analyzed as lung cell markers. Expressions of all examined lung-related cell markers in EB outgrowths cultured on LMSs were significantly increased as compared to the ES, EB, and 2D samples. These results indicated that EBs cultivated on an LMS were able to effectively differentiate into lung-related cells.

3.4. Immunohistochemical analysis of EB outgrowths cultured on LMS

Following cultivation for two weeks on LMSs using an ALI method, immunohistochemical analysis with lung-related cell markers was performed (Fig. 4). All cells in sections from EB outgrowths on LMSs were positive for both DAPI and GFP. Moreover, cells showing immunopositivity for Nkx-2.1, AQP5, SFTPC, and CC10 were detected among the GFP-positive cells. These results demonstrated that use of an LMS induced differentiation of ES cells into various types of lung-related cells.

4. Discussion

Results of tissue engineering that used extracellular matrix (ECM) from various organs have been reported [17], though those ECMs were predominantly used as materials for cell scaffolds [22,23]. In our previous study, ES cell-derived embryoid bodies (EBs) were transplanted into lung-derived ECM (LM) prepared by decellularizing mice lungs, and the results showed induction of differentiation into lung cells [20]. In the present study, examinations were performed to determine whether LMs were capable of inducing lung cell differentiation by using LMSs, with the results showing that use of an LMS for EB cultures with the ALI method expressed various lung-related cell markers.

EBs cultured on LMSs outgrew toward the lower of sheet neighboring liquid phase (Fig. 2A), with greater sized outgrowths noted with increased sheet thickness (Fig. 2B). The thinnest LMS (500 μm) was thought to provide a dry environment, because of the lack of tissue volume absorbed from the liquid phase. On the other hand, the thickest LMS (1000 μm) showed swelling, and was found suitable for cell growth and survival. Thus, in the present study, 1000-μm LMSs were utilized to culture EBs with an ALI method (Fig. 2C). Although several studies of cell differentiation and tissue engineering using cell sheets have been reported [24,25], the effects of cell sheet thickness were not investigated. The present results demonstrated that a thick LMS had positive effects on cells residing in phases between air and liquid. Furthermore, use of LMSs with different thicknesses may provide a more appropriate environment for growth and differentiation of lung cells.

In the present study, we examined the expressions of lung cell-related markers. EB outgrowths cultured on LMSs expressed significantly higher levels of the lung lineage marker *Nkx2.1* (Fig. 3), the earliest progenitor marker of lung epithelial specification [26,27]. Since

Nkx2.1 is also expressed in cells during the early stages of thyroid and ventral forebrain progenitor formation [28], lung cell development was confirmed by use of *AQP5* [29], *SFTPC* [30], and *CC10* [31], markers of alveolar type 1 and type 2 cells, and club cells, respectively. Gene expression analysis with qRT-PCR demonstrated that each of those lung cell-related markers was significantly upregulated in EB outgrowths cultured on LMSs (Fig. 3). Furthermore, immunohistochemical analysis indicated that ES cell-derived GFP positive cells simultaneously expressed their markers (Fig. 4). There were no distinct differences regarding the distribution of cells that expressed each lung cell-related marker in EB outgrowths cultured on LMSs. These results indicated that use of an LMS provides synchronous promotion of differentiation into various types of lung cells.

ECMs regulate cellular growth, migration, and differentiation [32], while dynamic changes in their components have important roles, including regulation of airway branching and cell differentiation during lung development [33]. The present study performed culture experiments using only LMSs prepared from adult mouse lungs. However, it is thought that ECM components change during lung development. Therefore, use of a suitable LMS prepared from lung tissue in each stage of development may be able to appropriately control differentiation into lung cells.

The present findings demonstrated that ES cell-derived EBs cultured with LMSs using an ALI method underwent differentiation into various lung-related cells. It is thus considered that an LMS is useful for handling and technical modifications. Additional refinement of LMS use, i.e., sheet layers prepared from various lung development stages, may be able to more precisely mimic differentiation into lung-related cells and lung development.

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CRediT authorship contribution statement

Tomotaka Kitamura: Data curation, Formal analysis, Investigation, Visualization, Writing – original draft. **Masayasu Misu**: Data curation, Investigation, Methodology. **Masahide Yoshikawa**: Supervision, Writing – review & editing. **Yukiteru Ouji**: Conceptualization, Data curation, Formal analysis, Supervision, Visualization, Funding acquisition, Writing – review & editing.

Conflicts of interest

None of the authors have conflicts of interest to declare.

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Figure legends

Fig. 1. Preparation of lung-derived matrix sheets (LMSs) and differentiation of ES cells into lung-like cells by cultivation on LMSs.

(A) LMSs were prepared by use of a tissue chopper device to cut from decellularized lungs.

(B) Preparation of embryoid bodies (EBs) from ES cells cultured for four days using a hanging drop method.

(C) Ten EBs were cultured on LMSs using an air-liquid interface (ALI) method for two weeks.

(D) Procedure used for differentiation of ES cells into lung-like cells on LMSs with ALI method. Control experiments were performed by cultivation of EBs on gelatin-coated dishes.

Fig. 2. Effects of LMS thickness on EB outgrowths and observation of EB outgrowths cultured on LMSs using fluorescence microscopy.

(A) HE staining of sections prepared from EB outgrowths cultured on LMSs for two weeks. ES derived-cell areas were detected by use of a software package (green areas in Analysis images). Scale bars = 200 μm.

(B) Total area of EB outgrowth on LMS (*p <0.05).

(C) EB outgrowths cultured on LMSs were observed on Day 0, 3, 7, and 14 using fluorescence microscopy. Fluorescence was detected and found to expand with longer culture periods. Scale bar = 200 μm.

Fig. 3. Gene expression analysis of lung-related cell markers.

Gene expressions of lung-related cell markers were examined using a real-time qRT-PCR method. Total RNA samples were prepared from undifferentiated ES cells, EBs, differentiated

EB outgrowths cultured in gelatin-coated dishes (2D), and EB outgrowths cultured on LMSs. Expression levels of all lung-related cell markers in EB outgrowths cultured on LMSs were significantly increased (*p <0.05).

Fig. 4. Immunohistochemical analysis of EB outgrowths cultured on LMSs.

Immunohistochemical analysis of EBs cultured on LMSs for determining lung-related cell markers was performed. All cells in sections from EB outgrowths on LMSs were DAPI- and GFP-positive (blue and green, respectively). Cells showing immunopositivity for Nkx-2.1, AQP5, SFTPC, and CC10 simultaneously expressed GFP (red). All insets show enlarged images of each section. Scale bar: original images, 400 μm; inset images, 100 μm.

Fig. 1.

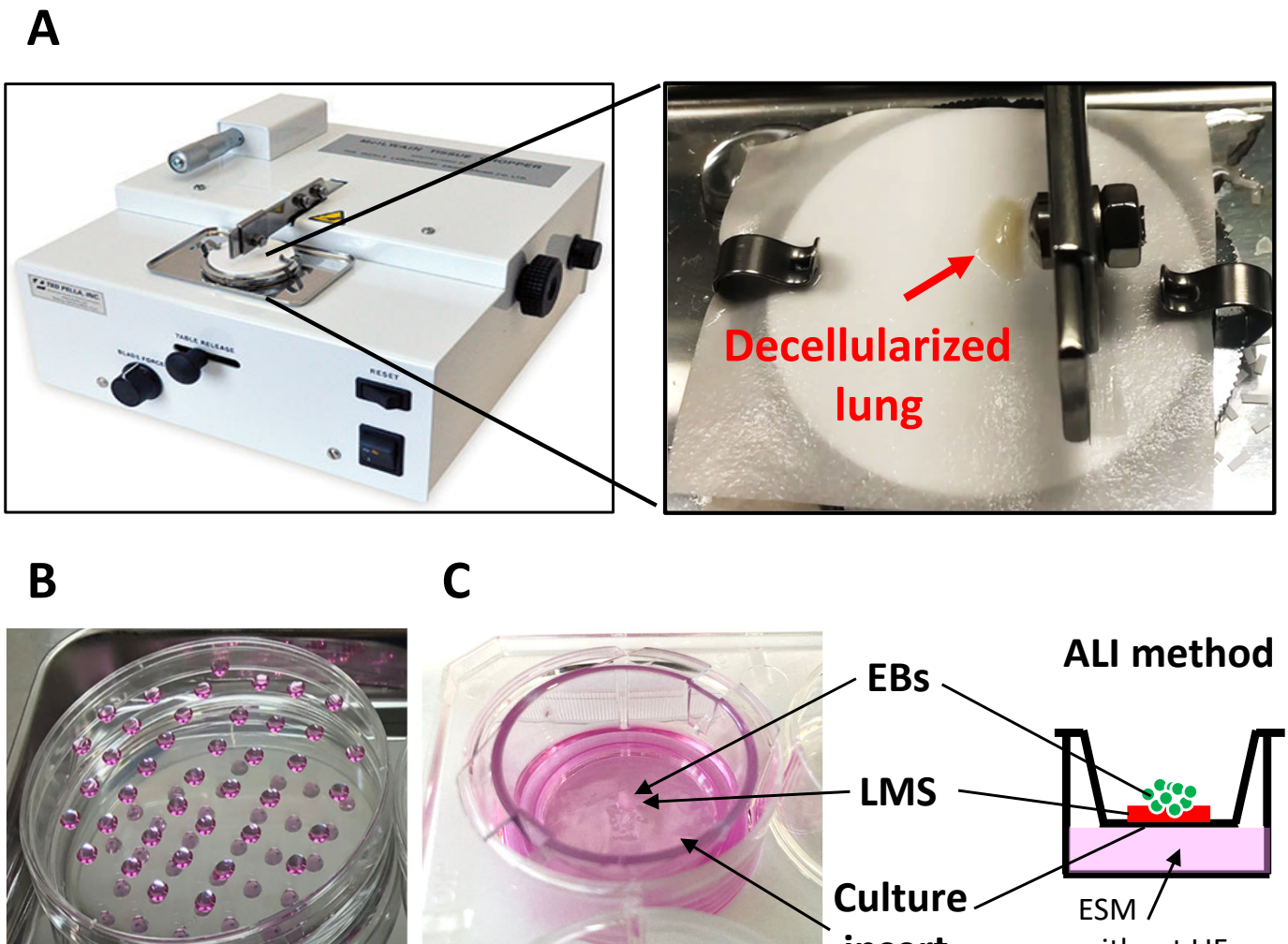
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Fig. 2.

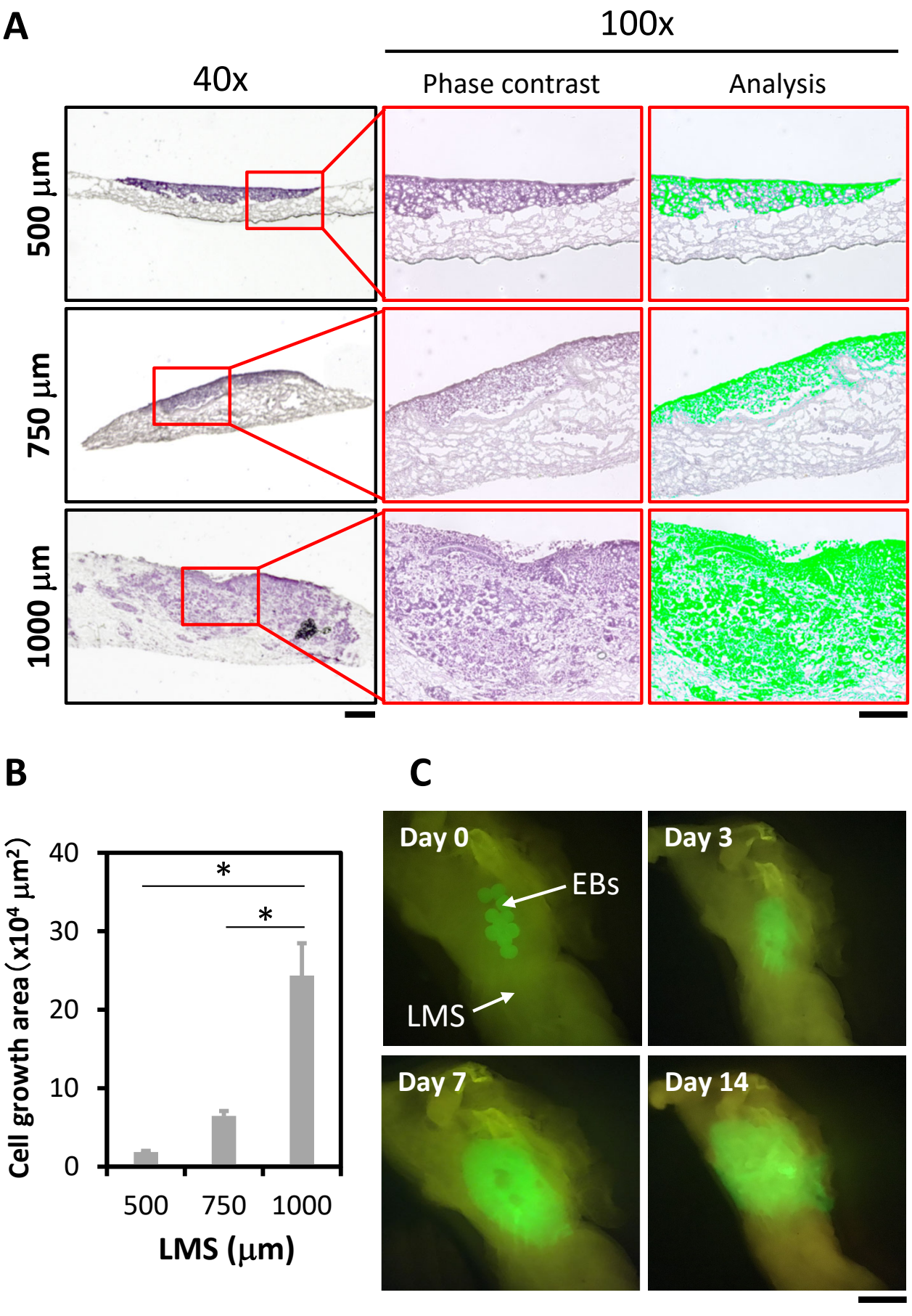
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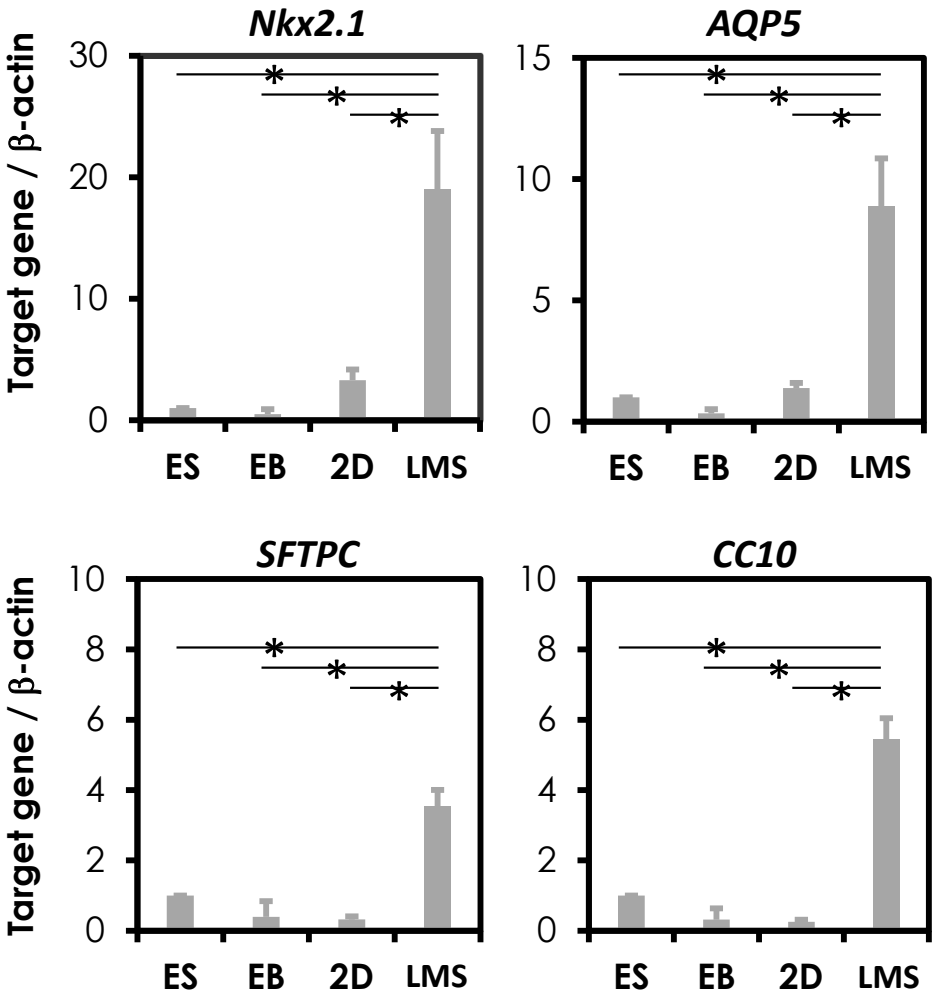
Fig. 3.



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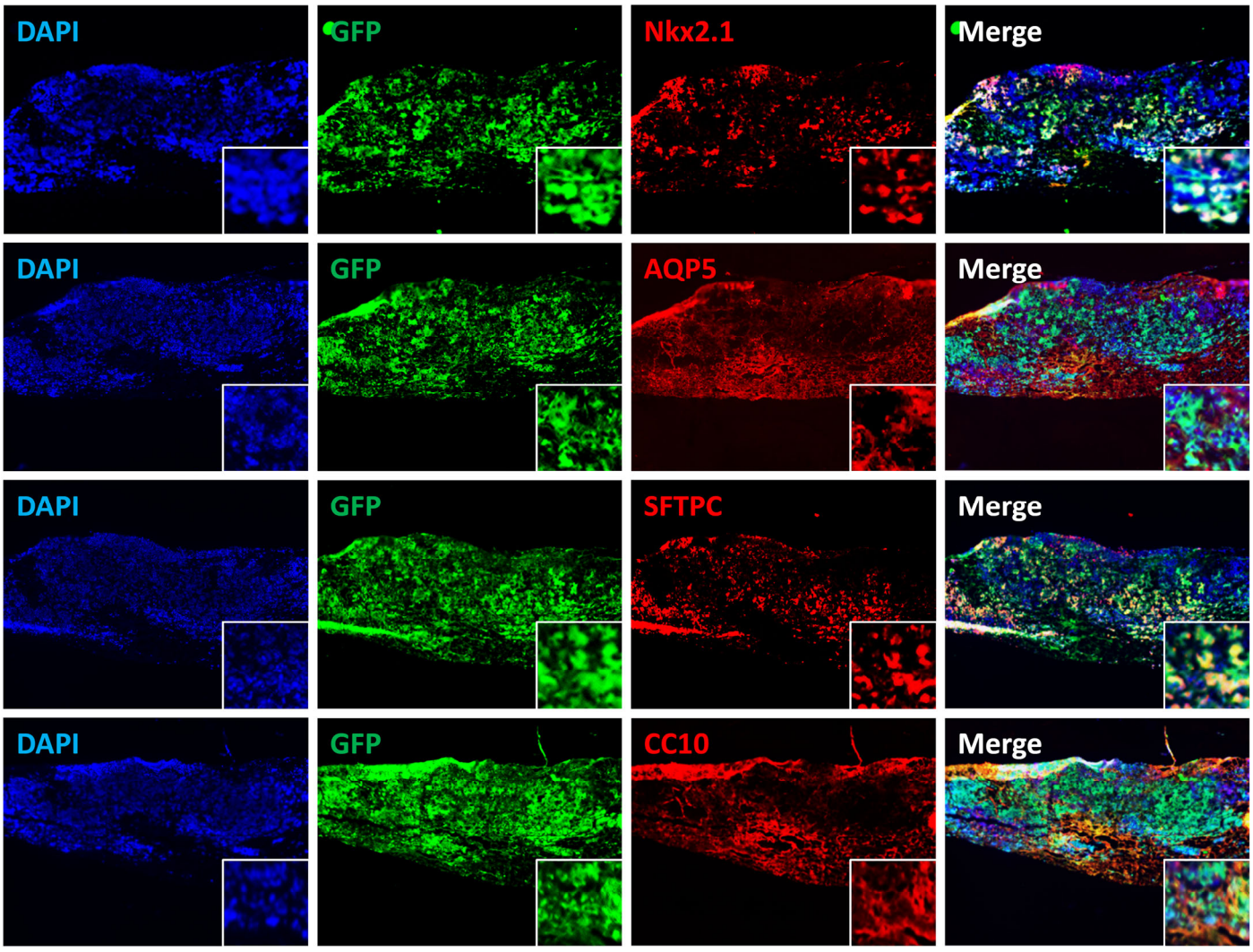
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