Induction of lung-like cells from mouse embryonic stem cells by decellularized lung matrix

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A B S T R A C T

Decellularization of tissues is a recently developed technique mostly used to provide a 3-dimensional matrix structure of the original organ, including decellularized lung tissues for lung transplantation. Based on the results of the present study, we propose new utilization of decellularized tissues as inducers of stem cell differentiation. Decellularized lung matrix (L-Mat) samples were prepared from mouse lungs by SDS treatment, then the effects of L-Mat on differentiation of ES cells into lung cells were investigated. ES cell derived-embryoid bodies (EBs) were transplanted into L-Mat samples and cultured for 2 weeks. At the end of the culture, expressions of lung cell-related markers, such as TTF-1 and SP-C (alveolar type II cells), AQP5 (alveolar type I cells), and CC10 (club cells), were detected in EB outgrowths in L-Mat, while those were not found in EB outgrowths attached to the dish. Our results demonstrated that L-Mat has an ability to induce differentiation of ES cells into lung-like cells.

1. Introduction

Lungs have important roles in the respiratory system, including their function as waste management for the body [1]. It was previously thought that lung tissues are highly quiescent and have limited re-generation potential [2], while more recent findings have shown them to possess a remarkable reparative capacity after lung injury, such as scarring or fibrosis [3,4]. A variety of different immature cells, such as alveolar stem/progenitor cells, are involved in regeneration after lung injury, as they differentiate into mature lung cells including those of the injured tissue along with acquisition of multiple paracrine factors [5,6]. Moreover, several preclinical studies that used adult stem cells such as bone marrow derived-mesenchymal stem cells (MSCs) have been conducted [7].

Embryonic stem (ES) and induced-pluripotent stem (iPS) cells have abilities to differentiate into various cell types [8–11]. Previous studies have reported successful methods for differentiation of those into various lung cell types, such as type I and II alveolar epithelial cells [12], ciliated cells [13,14], club cells [15], and basal cells [16]. However, most of those studies were performed to find a specific differentiation method for a certain cell type, while few examined simultaneous induction of various lung-lineage cells.

Recently, decellularization has been shown to be a promising technique for repair and transplantation of organs and tissues (e.g.,

urinary bladder, small intestine, skin, amnion) [17–20]. Decellularized tissues (scaffolds) retain various extracellular matrixes (ECMs) as well as the gross anatomy of the original tissue/organ [21,22]. The ECM interacts with cells to regulate diverse functions, including proliferation, migration, and differentiation, thus we speculated that decellularized tissues may have potential to induce ES cells and iPS cells to differentiate toward residential cells of the organs from which the tissues were derived.

In the present study, we investigated the capability of decellularized lung scaffolds obtained from adult mice to induce ES cells to differentiate into various types of lung cells. Our results showed induction of lung cell-related markers of ES cell-derived cells in decellularized lung matrix (L-Mat) samples, indicating an important role of L-Mat for inducing ES cells to differentiate into lung cell-like cells.

2. Materials and methods

2.1. Cells

Undifferentiated ES cells (G4-2) [23,24] were maintained in gelatin-coated dishes without feeder cells in DMEM (Wako, Kyoto, Japan) supplemented with 10% FBS (PAA), 0.1 mM 2-mercaptoethanol (Wako), 0.1 mM nonessential amino acids (GIBCO), 1 mM sodium pyruvate (Wako), 0.1% penicillin/streptomycin, and 1000 U/ml of LIF
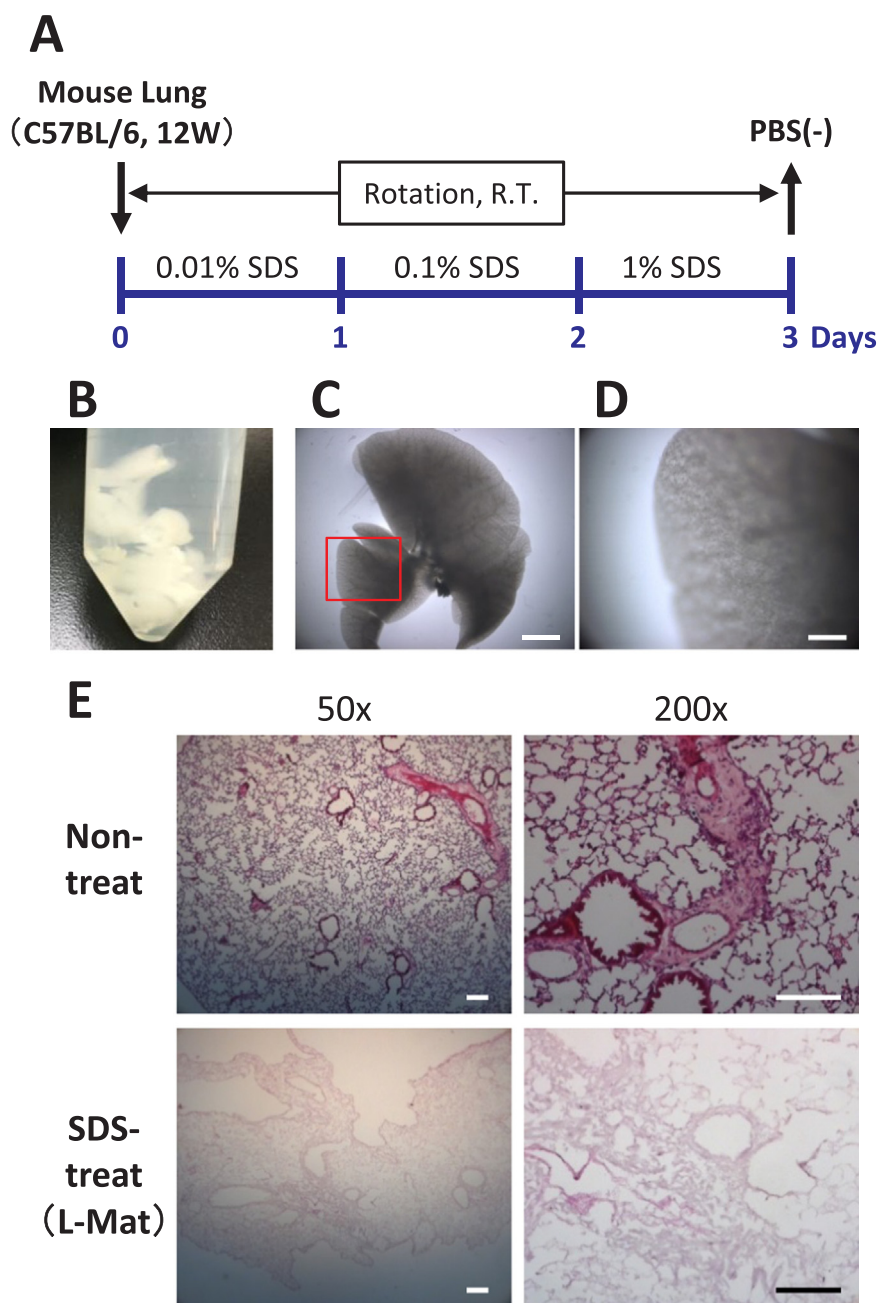
Fig. 1. Preparation of decellularized lung matrix (L-Mat) using mouse whole lung tissues. (A) Protocol for preparation of L-Mat samples. A 3-step method using the surfactant SDS was employed to create decellularized mouse lungs. (B) The color of the decellularized lungs was changed to clear white. (C, D) Hierarchical branching structures of airways and vasculature in L-Mat samples shown by stereo-microscopy. Scale bar = 1 mm. (E) Microscopic images following H&E staining of normal lung tissues (Non-treat) and lung tissues after decellularization with SDS (SDS-treat, L-Mat) (50×, 200×). Scale bar = 100 μm.

(Wako). G4-2 ES cells carried the enhanced green fluorescent protein (EGFP) gene under control of the CAG expression unit.

2.2. Mice

Inbred 12-week-old C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan) and housed in group cages at the animal facilities of our institution. Following euthanasia, lung tissues were isolated and L-Mat samples prepared, as described below. All animal procedures were conducted in accordance with the guidelines of Nara Medical University for animal experimentation.

2.3. Preparation of L-Mat

A 3-step method was used to obtain decellularized mouse lungs (Fig. 1A) [25]. First, whole lungs were isolated and treated with 0.01% SDS in a phosphate-buffered saline (PBS) solution for 24 h, then treated with 0.1% SDS in PBS for 24 h. For the final step, lung tissues were subjected to 1% SDS for 24 h and washed with PBS containing 0.1% penicillin/streptomycin for at least 3 days. The resulting tissues were prepared and used as L-Mat samples.

2.4. Differentiation

Differentiation of undifferentiated ES cells into lung cells was performed using the following procedure. Briefly, ES cells were dissociated

A

B
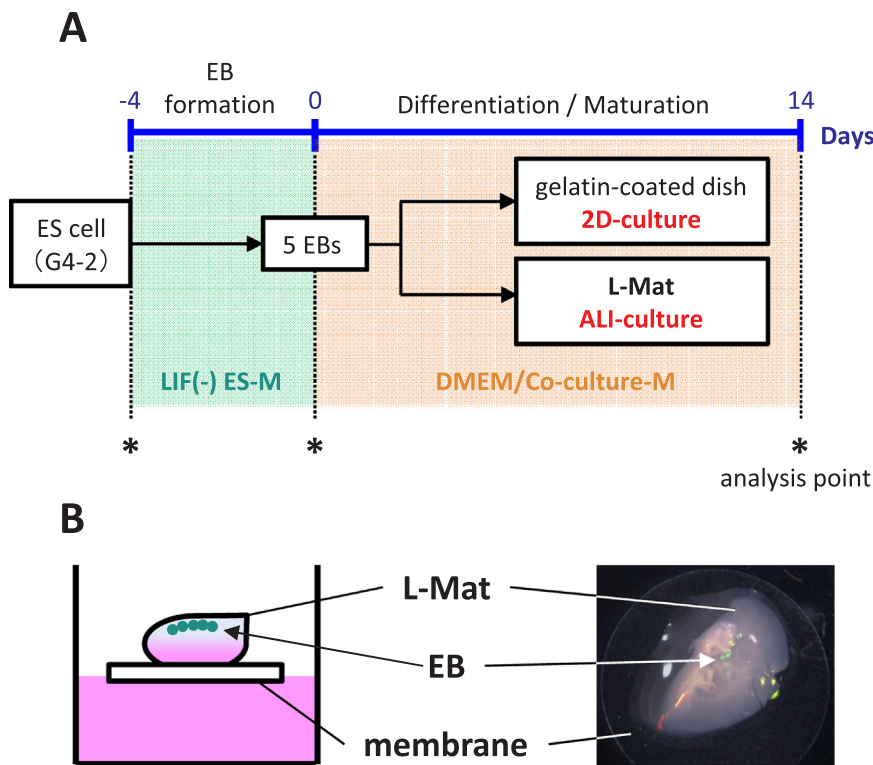
Fig. 2. Cultivation of mouse ES cells using L-Mat. (A) Experimental protocol for differentiation of ES cells into lung cell-like cells. After EB formation for 4 days, ES-derived EBs were cultured for 2 weeks in gelatin-coated dishes (2D culture) or with L-Mat samples using an air liquid interface (ALI) method. (B) Using the ALI method, 5 EBs from each culture were transplanted into individual L-Mat samples on a membrane and cultured in differentiation medium. Representative image shows EBs with GFP fluorescence at start of cultivation.

by trypsin and cultured in hanging drops to form embryoid bodies (EBs) [26], with a cell density of 500 cells per 20 µl of ES cell medium in the absence of LIF (ES-M) for each drop (Fig. 2A). After 4 days, 5 EBs were collected from each hanging drop culture and transplanted into an L-Mat sample placed on a membrane with a pore size of 8 µm (Millipore) at a depth of 20 mm from the top of the L-Mat, then cultured in differentiation medium [DMEM/Co-culture medium (CELLnTEC) (1:1)] for 2 weeks using an air liquid interface (ALI) method (Fig. 2B). Reference experiments were performed with a 2-dimensional (2D) culture method, in which 5 EBs were collected and seeded into 35-mm gelatin-coated plastic dishes filled with differentiation medium, then cultured for 2 weeks, with half of the culture medium exchanged with new medium every 2 days.

2.5. Real-time RT-PCR

Total RNA (1 µg) was extracted from cultured cells 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, with primers purchased from TaKaRa Bio Inc. (Supplementary Table S1). The amounts of target gene PCR products 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

All samples were cultured for 2 weeks, then fixed with 4% PFA for 2 days at 4 °C, followed by 20% sucrose in PBS for 12 h. Next, the samples were embedded in OTC compound, and sections (6 µm thick) were prepared using a cryostat (Thermo) and stained with hematoxylin-eosin (H&E) with a standard protocol. For immunohistochemistry, the sections were permeabilized with 0.1% Triton X-100 in PBS (TPBS) containing 1% BSA. The primary antibodies and dilutions in TPBS were as follows: anti-αtubulin (1:100, Santa Cruz), anti-TTF-1 (1:100, Santa Cruz), anti-SP-C (1:100, Santa Cruz), anti-AQP5 (1:100, Santa Cruz), and anti-CC10 (1:100, Santa Cruz). After incubation overnight at 4 °C

and washing with TPBS 3 times, AlexaFluor 488 or 546 conjugated anti-goat, anti-rabbit, or anti-mouse secondary antibodies (Molecular Probes, Invitrogen) were used to detect primary antibodies. All nuclei were stained with DAPI (Dojin). Following 1 h of incubation at room temperature and washing with TPBS 3 times, fluorescence was examined using a fluorescence microscopy system (Zeiss Axiovert 200).

2.7. Statistical analysis

Data are expressed as the mean ± SD of 3 or 5 independent experiments. Statistical significance was examined using an unpaired *t*-test, unless specified otherwise.

3. Results

3.1. L-Mat verification

Lungs obtained from adult mice were decellularized by sequential treatments with SDS (Fig. 1A), then L-Mat samples were prepared. Each L-Mat specimen was found to be colored white and microscopically transparent (Fig. 1B). Furthermore, the hierarchical branching structures of the airways and vasculature in the specimens seemed to be conserved when viewed by stereo-microscopy (Fig. 1C, D). To confirm the structures and complete removal of cellular components, we analyzed sections of L-Mat samples prepared from frozen blocks and stained with H&E (Fig. 1E). Mesh-like structures were maintained as in normal lungs, whereas cellular components such as cytoplasm and nuclei were completely absent.

3.2. Cultivation of mouse ES cells in L-Mat samples using ALI method

Four-day EBs were transplanted into L-Mat samples and cultured using ALI for 2 weeks, while others were cultured in gelatin-coated dishes using a 2D culture protocol (Fig. 2A, B). Although no cells were directly observable by microscopy in the L-Mat samples during culture, GFP signals from cells in the L-Mat samples implanted with EBs were detected. In the 2D cultures, cellular growth was observed, with a large
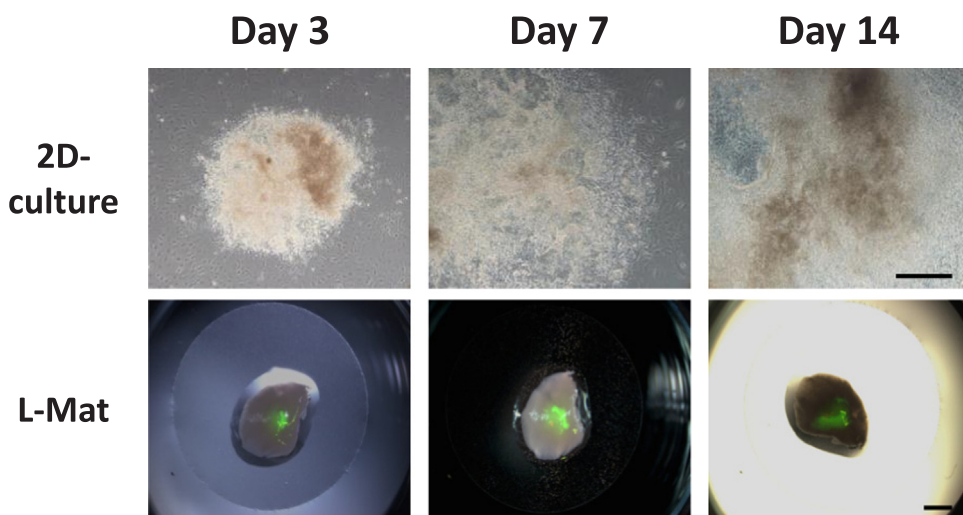
Fig. 3. EB outgrowths grown in 2D cultures or with L-Mat for 2 weeks. Representative microscopic images show EBs after 3, 7, and 14 days of culture. Upper panel: 2D culture. Large numbers of heterogeneous shaped cells were observed proliferating around the originally attached EBs (2D). Lower panel: L-Mat culture. EBs showed slight proliferation in a direction upward from the site of EB transplantation (L-Mat). Scale bar = 200 μm.

number of heterogeneous shaped cells located around the originally attached EBs (Fig. 3, 2D culture). We also noted that EBs had proliferated in the ALI cultures, with growth seemingly in a upward direction from the site of transplantation (Fig. 3, L-Mat; Supplemental Fig. S1).

3.3. EBs cultured in L-Mat samples show induction of lung cell marker genes

After 2 weeks of cultivation, gene expressions in EBs related to lung cells were assessed using real time RT-PCR analysis, with *TTF-1* and *SP-C* (alveolar type II cells) [27], *AQP5* (alveolar type I cells) [28], and *CC10* (club cells) [29] used as lung cell differentiation markers. RNA samples were prepared from undifferentiated ES cells, 4-day EBs, EB outgrowths in 2D cultures, and EB outgrowths cultured with L-Mat samples (ES, EB, 2D, L-Mat, respectively) (Fig. 4A). Neither the undifferentiated ES cells nor EBs expressed lung cell markers, while samples from the 2D cultured EB outgrowths showed very limited expressions of *TTF-1*, *SP-C,* and *AQP5*. In contrast, EBs cultured with L-Mat samples expressed all of the examined lung cell markers at significantly upregulated levels.

3.4. L-Mat induces differentiation of ES cells into lung cell-like cells

After 2 weeks of culture with L-Mat samples, EB growths were fixed with 4% PFA, then frozen sections were prepared for histology. First, we examined cell localization using H&E staining and found that EB-derived cells were located in the L-Mat samples, in areas ranging from the air-interface surface to deep inside (Supplemental Fig. S2). Next, we used immunohistochemical analysis to examine various lung cell-related markers in frozen sections. Cells showing immunopositivity for TTF-1 and SP-C (alveolar type II cells), tubulin (ciliate cells) [30], AQP5 (alveolar type I cells), and CC10 (club cells) were detected in sections of EBs cultured with the L-Mat samples (Fig. 4B, L-Mat; Supplemental Fig. S3), whereas no cells in EB outgrowths from the 2D cultures were immunopositive for those markers (Fig. 4B, 2D). These results suggest that L-Mat has a capability to induce differentiation of EB-derived cells into lung cell-like cells.

4. Discussion

A variety of methods used for differentiation of pluripotent stem cells, such as ES and iPS cells, into various types of lung cells (e.g., alveolar type I and II cells, club cells, ciliated cells, others) have been reported [12–16]. However, most were composed of multi-step processes, and found to be time consuming and complicated. In the present study, we attempted to develop a simple culture method for differentiation of ES cells into lung cells. First, we performed cultures of attached EBs in 2D plastic dishes without additional specific chemical substances or cocultured cells to assist with differentiation into lung cells. Some gene markers related to lung cells, such as *TTF-1*, *SP-C*, and *AQP5,* were found expressed in those EB outgrowths, though the amounts were limited or nearly undetectable (Fig. 4A), while cells showing immunopositivity for lung cell markers were absent (Fig. 4B, 2D). These results suggested that EBs grown in 2D cultures without cytokines or reagents are not able to achieve differentiation into lung cells.

Techniques for decellularization of organs and tissues have been recently developed [21]. As a result, decellularized organs and tissues are now being utilized as transplant scaffolds or reservoirs for bridge-use [31–33], because they have been demonstrated to retain the ECM (e.g., collagen, fibronectin) as well as a 3-dimensional structure. As for lung tissues, successful decellularization has been reported in small animals including mice and rats [34,35], as well as in pigs and humans [36,37]. Furthermore, in transplantation experiments that used a variety of different types of implanted cells, such as ES cells, iPS cells [38], primary or immortalized airway and alveolar cells [6], and bone marrow-derived mesenchymal stem cells (MSCs) [39], cellular remobilization in decellularized lungs was shown. In those studies, L-Mat samples were only considered as structures for cell mobilization and inhabitance, and not examined as an inducer of differentiation. In the present study, we examined differentiation of ES cells into lung cells with L-Mat samples used as an inducer. Our novel findings revealed that EBs cultured with L-Mat specimens showed significant expression of lung cell-related marker genes (*TTF-1*, *SP-C*, *AQP5*, *CC10*) and were also immunopositive for tubulin, TTF-1, SP-C, AQP5, and CC10 (Fig. 4). This is the first report of this *in vitro* capability of L-Mat for induction of ES cell differentiation into various types of lung cell-like cells. Nevertheless, it will be necessary to investigate whether this capability to induce lung cell-like cells is associated with L-Mat alone and not other organ-derived decellularized matrix scaffolds.

In conclusion, we investigated the effects of ECM samples obtained from lung tissues after SDS treatment for decellularization (L-Mat) on the differentiation of ES cells into lung cell-like cells. Our results showed induction of expressions of various lung cell-related gene and protein markers. Although the mechanisms of such L-Mat samples as related to differentiation of ES cells require additional analysis, we concluded that SDS-treated ECM has potential to play important roles in induction of differentiation of lung cells.

Acknowledgements
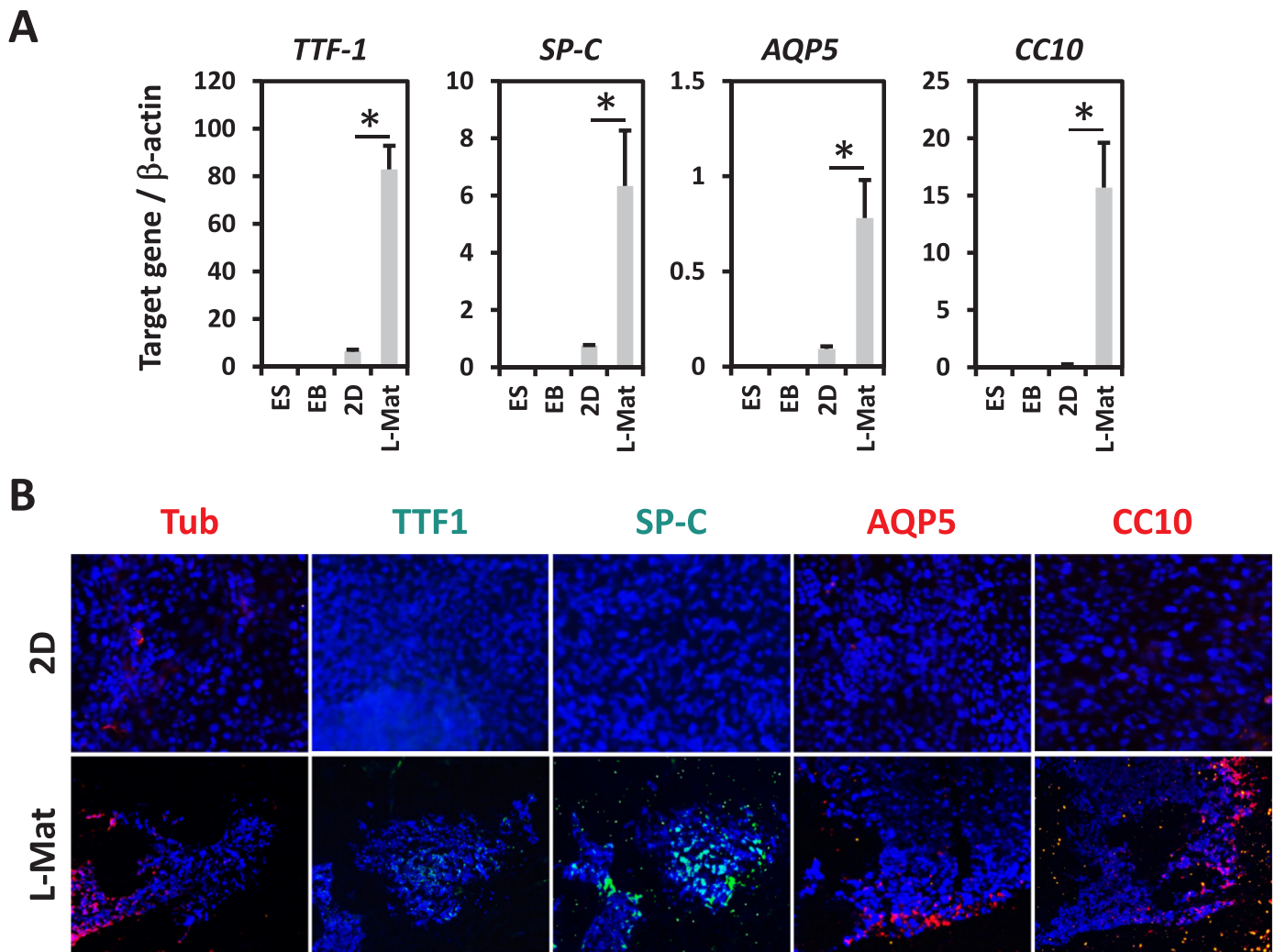
Fig. 4. L-Mat induces differentiation of ES cells into lung cell-like cells. (A) Gene expressions of lung cell-related markers, including *TTF-1* and *SP-C* (alveolar type II cells), *AQP5* (alveolar type I cells), and *CC10* (club cells), in EB outgrowths cultured with 2D method or L-Mat samples were analyzed using a real-time RT-PCR method. (B) After 2 weeks of differentiation in cultures with L-Mat samples, EB outgrowths were analyzed using an immunohistochemical method for lung cell-related markers, including tubulin (ciliate cells), TTF-1 and SP-C (alveolar type II cells), AQP5 (alveolar type I cells), and CC10 (club cells). Scale bar = 20 μm.

Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2018.06.005.

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