A novel alveolar epithelial cell sheet fabricated under feeder-free conditions for potential use in pulmonary regenerative therapy

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Abstract

Introduction

Lung transplantation is the only effective treatment option for many patients with irreversible pulmonary injury, and the demand for lung transplantation is increasing worldwide and expected to continue to outstrip the number of available donors. Regenerative therapy with alveolar epithelial cells (AECs) holds promise as an alternative option to organ transplantation. AECs are usually co-cultured with mouse-derived 3T3 feeder cells, but the use of xenogeneic tissues for regenerative therapy raises safety concerns. Fabrication of AEC sheets under feeder-free conditions would avoid these safety issues. We describe a novel feeder-free method of fabricating AEC sheets that may be suitable for pulmonary regenerative therapy.

Methods

Lung tissues excised from male outbred rats or transgenic rats expressing green fluorescent protein (GFP) were finely minced and dissociated with elastase. The isolated AECs were cultured under four different feeder-free conditions according to whether a rho kinase (ROCK) inhibitor was included in the low-calcium medium (LCM) and whether the tissue culture dish was coated with recombinant laminin-511

E8 fragment (rLN511E8). The expanded cells were cultured on temperature-responsive dishes and subsequently harvested as AEC sheets. Engraftment of GFP-AEC sheets after their transplantation onto a partially resected region of the left lung was assessed in athymic rats.

Results

AECs proliferated and reached confluence when cultured in LCM containing a ROCK inhibitor on tissue culture dishes coated with rLN511E8. When both the ROCK inhibitor and rLN511E8-coated culture dish were used, the number of AECs obtained after 7 days of culture was significantly higher than that in the other three groups. Immunohistochemical analyses revealed that aquaporin-5, surfactant protein (SP)-A, SP-C, SP-D and Axin-2 were expressed by the cultured AECs. AEC sheets were harvested successfully from temperature-responsive culture dishes (by lowering the temperature) when the expanded AECs were cultured for 7 days in LCM + ROCK inhibitor and then for 3 days in LCM + ROCK inhibitor supplemented with 200 mg/L calcium chloride. The AEC sheets were firmly engrafted 7 days after transplantation onto the lung defect and expressed AEC marker proteins.

Conclusions

AEC sheets fabricated under feeder-free conditions retained the features of AECs after transplantation onto the lung *in vivo*. Further improvement of this technique may allow the bioengineering of alveolar-like tissue for use in pulmonary regenerative therapy.

Keywords

alveolar epithelial cell, feeder-free, cell sheet, regenerative therapy

Abbreviations

AEC, alveolar epithelial cell; AECI, type I alveolar epithelial cell; AECII, type II alveolar epithelial cell; AEpiCM, alveolar epithelial cell medium; AQP-5, aquaporin-5; Ca^{2+}, ionized calcium; FBS, fetal bovine serum; GFP, green fluorescent protein; HBSS, Hanks' balanced salt solution; HE, hematoxylin and eosin; LCM, medium with a low ionized calcium concentration; PBS, phosphate-buffered saline; rLN511E8, recombinantly expressed laminin-511 E8 fragment; ROCK, rho kinase; SP, surfactant protein.

Main Text

1 Introduction

Lung transplantation is the only effective treatment option for many patients with irreversible pulmonary injury, and the demand for lung transplantation is increasing worldwide and expected to continue to outstrip the number of available donors [1]. Regenerative therapy holds great promise as a future alternative option to organ transplantation and would circumvent the limitations of conventional transplantation techniques such as the lack of donor organs, adverse effects due to immunosuppression and organ rejection [2].

Regenerative therapy for lung injury requires the transplantation of bioengineered tissue constructed from appropriate cell types. Gas exchange occurs in pulmonary alveoli, which comprise 90% of the total volume of the lungs [3]. The alveolar epithelium is lined with type I alveolar epithelial cells (AECIs), which are specialized for gas exchange, and type II alveolar epithelial cells (AECIIs), which produce surfactant proteins (SPs) involved in innate immune responses [4]. Although alveolar epithelial cell (AEC) cultures have been established previously, one of their limitations was the unwanted trans-differentiation of AECIIs to AECIs *in vitro* [5]. The

86 differentiation of AECIIs to AECIs *in vivo* normally only occurs following injury to the
87 lung and aims to replenish the damaged alveolar epithelium. Initially, AECIIs
88 proliferate to replace the lost cells, although the underlying mechanisms remain only
89 partially understood [6]. Once normal cell numbers are restored, some AECIIs
90 trans-differentiate into AECIs to re-establish the normal alveolar architecture [7].
91 Recent reports have suggested that Wnt signaling regulates the stemness of AECIIs
92 [8-10].
93 Approaches to improve the culture of cells *in vitro* include the use of feeder cells,
94 which secrete various proliferation-promoting factors, and pharmacological agents
95 such as rho kinase (ROCK) inhibitors [11,12]. Although human AECIIs proliferate
96 rapidly when cultured in the presence of mouse-derived 3T3 feeder cells and a ROCK
97 inhibitor, markers of AECIIs become downregulated after the first passage [4].
98 Furthermore, AECs are difficult to maintain in long-term culture [4]. Importantly,
99 human epithelial cell grafts co-cultured with mouse-derived 3T3 feeder cells are
100 classified by the US Food and Drug Administration as xenogeneic products, which
101 complicates their potential use as a cell therapy or regenerative therapy in the clinical
102 setting. Hence, the establishment of a feeder-free culture system for AECs would

103 facilitate the development of new regenerative therapies for patients with respiratory
104 failure due to irreversible lung injury.
105 Research is ongoing to optimize the conditions for feeder-free culture of AECs and
106 other cell types. Laminin is a high-molecular-weight protein found in the extracellular
107 matrix, and recombinant laminin-511 isoform, which is a heterotrimer consisting of α5,
108 β1, and γ1 chains, has been shown to improve the long-term culture of human
109 pluripotent stem cells in the absence of feeder cells [13]. Furthermore, recombinant
110 laminin-511 E8 fragment (rLN511E8) also has been reported to support the stable,
111 feeder-free culture of human embryonic stem cells, human induced pluripotent stem
112 cells and ocular epithelial cells [14]. In addition, ionized calcium (Ca^{2+}) is recognized
113 as a differentiation-inducing factor for epithelial cells. For example, the use of culture
114 medium containing a low concentration of Ca^{2+} (low-Ca^{2+} medium, LCM) has been
115 demonstrated to enhance the proliferation of human epidermal keratinocytes when
116 cultured in the absence of 3T3 feeder cells [15].
117 We hypothesized that it would be possible to culture AECs under feeder-free
118 conditions if a suitable combination of pharmacological agents was used. Therefore,
119 the main objectives of this study were to develop a new method of culturing AECs

under feeder-free conditions, optimize the fabrication of AEC sheets, and evaluate whether AEC sheets would engraft onto host tissue after transplantation *in vivo*. We show that AECs can be cultured under feeder-free conditions using LCM containing a ROCK inhibitor and rLN511E8-coated culture dishes. Furthermore, *in vivo* experiments in athymic rats confirmed that AEC sheets engrafted successfully after transplantation onto a lung defect.

2 Methods

All animal experiments were performed in accordance with the Guidelines of Tokyo Women's Medical University on Animal Use and consistent with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources (ILAR). The experimental procedures (isolation and culture of rat AECs followed by fabrication and transplantation of rat AEC sheets) are summarized in Fig 1.

2.1 Isolation of rat AECs

AECs were isolated from 4-8 week-old male outbred rats (Slc:SD; Japan SLC Inc., Shizuoka, Japan) or transgenic rats (SD-Tg[CAG-EGFP]; Japan SLC Inc.) expressing

137 green fluorescent protein (GFP) and weighing 100–150 g. The chest wall was incised
138 along the entire length of the sternum and anterior portion of the diaphragm under
139 inhalation anesthesia with isoflurane. The right ventricle and left atrium were
140 cannulated with 20-gauge plastic catheters, and the lungs were perfused with
141 phosphate-buffered saline (PBS; Fujifilm Wako Pure Chemical Corporation, Osaka,
142 Japan) until white in color to ensure that they had been cleared of blood. Lung tissues
143 were carefully excised from the chest cavity and finely minced. Dissociated cells were
144 obtained by gently shaking the lung tissue in Hanks' balanced salt solution (HBSS;
145 Fujifilm Wako Pure Chemical Corporation) containing 1 mg/mL elastase (Worthington
146 Biochemical Corporation, Lakewood, NJ, USA) at 37ºC for 60 min. After quenching
147 the enzymatic activity with fetal bovine serum (FBS; Thermo Fisher Scientific,
148 Waltham, MA, USA), the digested tissue was filtered through 100-μm and 40-μm cell
149 strainers (Corning Inc., Corning, NY, USA) to collect a cell suspension. After
150 centrifugation at 400×*g* for 10 min at room temperature, the cell pellet was
151 resuspended in HBSS and layered on a Percoll gradient (1.083 g/mL; Sigma-Aldrich,
152 St. Louis, MO, USA). After centrifugation at 400×*g* for 30 min at room temperature,
153 the layer of AECs generated on the Percoll gradient was collected.

2.2 Culture of rat AECs

The isolated AECs were suspended in LCM comprising Alveolar Epithelial Cell Medium (AEpiCM; ScienCell, Carlsbad, CA, USA) supplemented with 2% FBS. Primary AECs were cultured under four different feeder-free conditions (see Fig. 2A) according to whether 10 μM Y-27632 (a ROCK inhibitor; Fujifilm Wako Pure Chemical Corporation) was included in the LCM and whether the tissue culture dish was coated with rLN511E8 (iMatrix-511; Matrixome, Osaka, Japan). AECs were seeded at a density of 2.0×10^4 cells/cm^2 onto tissue culture dishes (35 mm in diameter) and cultured for 7–10 days at 37ºC in a humidified atmosphere containing 5% CO_2. Primary cultured cells were subsequently harvested by treatment with 0.05% trypsin-ethylenediamine tetraacetic acid for 5 min at 37ºC. The expanded cells were seeded at a density of 5.0×10^4 cells/cm^2 onto temperature-responsive culture dishes (35 mm in diameter; UpCell, CellSeed Inc., Tokyo, Japan) and cultured for 7–10 days at 37ºC.

2.3 Fabrication and transplantation of rat AEC sheets

AECs were cultured on temperature-responsive culture dishes until confluency. In some experiments, the cells were further cultured in medium supplemented with 200

171 mg/L calcium chloride (to increase the Ca^{2+} concentration) for 3 days. A support
172 membrane (Cell Shifter; Cell Seed Inc.) was placed into the culture dish, and the
173 culture medium was aspirated. Following incubation at 20ºC for 30 min, the
174 membrane was slowly peeled from the periphery of the dish to harvest the AECs as
175 an intact sheet.
176 The rat GFP-AEC sheets were transplanted onto the lungs of male athymic rats
177 (F344/NJcl-rnu/rnu; CLEA Japan Inc., Tokyo, Japan) under isoflurane inhalation
178 anesthesia and mechanical ventilation. The rat was placed in the right lateral
179 decubitus position, and a left lateral thoracotomy was performed. A 5mm lung incision
180 with 3mm depth was made in the left lung using scissors, and three GFP-AEC sheets
181 were transplanted onto the incised region of the left lung. After the GFP-AEC sheets
182 were transplanted, no air leakage from lung was confirmed and the wound closed
183 without chest tube drainage. Seven days later, the rat was euthanized by
184 exsanguination under isoflurane anesthesia, and the left lung including the
185 transplanted cell sheets was resected for histological analyses.
186 **2.4 Histology and immunohistochemistry**
187 For immunohistochemistry, cultured AECs were fixed in 4% paraformaldehyde and

188 blocked with 5% FBS in PBS for 60 min. Then, the cells were incubated with
189 anti-SP-A rabbit polyclonal antibody (1:300 dilution; bs-10265R; Bioss, Woburn, MA,
190 USA), anti-SP-C rabbit polyclonal antibody (1:300 dilution; bs-10067R; Bioss),
191 anti-SP-D rabbit polyclonal antibody (1:300 dilution; bs-1583R; Bioss),
192 anti-aquaporin-5 (AQP-5) rabbit polyclonal antibody (1:300 dilution; bs-1554R; Bioss),
193 anti-Axin-2 rabbit polyclonal antibody (1:300 dilution; GTX31822; Genetex, Irvine, CA,
194 USA) or anti-E-cadherin rabbit polyclonal antibody (1:300 dilution; GTX477; Genetex)
195 at 4ºC overnight. On the following day, the AECs were incubated with Alexa Fluor 488
196 goat anti-rabbit IgG antibody (1:500; A11008; Invitrogen, Carlsbad, CA, USA) as the
197 secondary antibody at 20–30ºC for 60 min.
198 The harvested AEC sheets were fixed in 4% paraformaldehyde and either
199 processed into 5-μm-thick paraffin wax-embedded sections (for cross-sectional
200 observations) or not further processed (planar observations). The sections were
201 treated with anti-SP-A rabbit polyclonal antibody (1:200 dilution; bs-10265R; Bioss),
202 anti-SP-C rabbit polyclonal antibody (1:100 dilution; bs-10067R; Bioss), anti-SP-D
203 rabbit polyclonal antibody (1:200 dilution; bs-1583R; Bioss), anti-AQP-5 rabbit
204 polyclonal antibody (1:200 dilution; bs-1554R; Bioss), anti-Axin-2 rabbit polyclonal

antibody (1:100 dilution; GTX31822; Genetex) or anti-E-cadherin rabbit polyclonal antibody (1:100 dilution; GTX477; Genetex) at 4ºC overnight. Subsequently, the sections were incubated with Alexa Fluor 488 goat anti-rabbit IgG antibody (1:500 dilution; A11008; Invitrogen) as the secondary antibody at 20–30ºC for 30 min. In addition, cross-sectional observations of the cell sheets were made after staining with hematoxylin and eosin (HE) using conventional methods.

Resected lung tissues were fixed in 4% paraformaldehyde and routinely processed into 5-μm-thick paraffin-embedded sections. For histology, HE staining was performed by conventional methods. For immunohistochemistry, the tissues were blocked with 5% FBS in PBS for 60 min and then incubated with anti-GFP rabbit polyclonal antibody (1:100 dilution; ab290; Abcam, Cambridge, UK), anti-SP-A rabbit polyclonal antibody (1:100 dilution; bs-10265R; Bioss), anti-SP-C rabbit polyclonal antibody (1:100 dilution; bs-10067R; Bioss), anti-SP-D rabbit polyclonal antibody (1:100 dilution; bs-1583R; Bioss), anti-AQP-5 rabbit polyclonal antibody (1:100 dilution; bs-1554R; Bioss) or anti-Axin-2 rabbit polyclonal antibody (1:100 dilution; GTX31822; Genetex) at 4ºC overnight. Then, the tissues were incubated with Opal Polymer Anti-Rabbit HRP (1:5 dilution) and either the Opal 520 (1:100 dilution) or

Opal 570 (1:100 dilution) fluorophore (NEL840001KT; Akoya Biosciences Inc., Marlborough, MA, USA) at 20–30ºC for 10 min. Subsequently, microwave treatment was used to remove the primary and secondary antibodies and non-specific staining and to reduce tissue autofluorescence. Another round of staining for additional targets was performed after microwave treatment without the risk of antibody cross-reactivity. The primary antibody was omitted in negative control experiments. Tissue sections stained using immunohistochemical methods were observed using a confocal laser scanning microscope (FluoView FV1200; Olympus Corporation, Tokyo, Japan).

2.5 Statistical analysis

JMP Pro 14.0.0 (SAS Institute, Cary, NC, USA) was used for data analysis. Cell counts are presented as boxplots (showing the median, interquartile range and range) and were compared between groups using the Steel-Dwass test. A p-value less than 0.05 ($p < 0.05$) was considered statistically significant.

3 Results

3.1 Optimization of the conditions for feeder-free culture of rat AECs

239 Primary AECs were cultured in AEpiCM under feeder-free conditions in the
240 absence or presence of a ROCK inhibitor and in the absence or presence of
241 rLN511E8 as a coating on the tissue culture dish (i.e., four experimental groups; see
242 Fig. 2a). When both the ROCK inhibitor and rLN511E8-coated culture dish were used,
243 the number of AECs obtained after 7 days of culture was significantly higher than that
244 in the other three groups (Fig. 2b). Phase-contrast microscopy demonstrated that the
245 cultured AECs exhibited a polygonal, cobblestone-like morphology characteristic of
246 epithelial cells (Fig. 2c). Furthermore, immunohistochemical analyses (Fig. 2d–h)
247 revealed that the cultured AECs expressed AQP-5 (a marker of AECIs), SP-A, SP-C,
248 SP-D (markers of AECIIs) and Axin-2 (a marker of a subpopulation of AECIIs capable
249 of trans-differentiation into AECIs) [8,16,17].

250 **3.2 Optimization of the conditions for fabrication of rat AEC sheets**

251 In our initial attempts to fabricate rat AEC sheets, AECs were subcultured on
252 temperature-responsive culture dishes in AEpiCM containing a ROCK inhibitor.
253 However, after 7 days of subculture, confluent AECs could not be harvested as a cell
254 sheet from the temperature-responsive culture dish when the temperature was
255 reduced from 37ºC to 20ºC for 30 min (Fig. 3a). Therefore, we altered the protocol by

adding an additional stage. First, the AECs were subcultured for 7 days as described above and confirmed to be confluent. Then, the AECs were cultured for a further 3 days in AEpiCM containing a ROCK inhibitor and 200 mg/L calcium chloride. Using this approach, it was possible to harvest the AECs from the temperature-responsive culture dish as an intact cell sheet by reducing the temperature from 37ºC to 20ºC for 30 min (Fig. 3b). Staining with HE showed that the AEC sheet was composed of a single layer of cells (Fig. 3c). Immunohistochemical analyses revealed that the cells expressed E-cadherin when cultured in the presence of calcium chloride but not when cultured in the absence of calcium chloride (Fig. 3a,b). Additionally, the AEC sheets expressed AQP-5, SP-A, SP-C, SP-D and Axin-2 (Fig. 3d–h).

3.3 Transplantation of rat AEC sheets

Seven days after the transplantation of three GFP-AEC sheets onto a partially resected region of the left lung of an athymic rat, illumination of the transplantation region with fluorescent light (488 nm) demonstrated the presence of GFP-positive cells that were derived from the transplanted cell sheets (Fig. 4a,b). Staining with HE revealed that the transplanted AEC sheets were tightly adhered to the lung tissue with no air spaces found between the cell sheets and pulmonary parenchyma (Fig. 4c).

Immunohistochemical analyses revealed that cells on the surface of the lung expressed GFP and that the same region also expressed AQP-5, SP-A, SP-C, SP-D and Axin-2 (Fig. 4d–h). Positive staining was not observed when the primary antibodies were omitted in negative control experiments (Fig. 4i). Immunohistochemical analyses of lung tissues without transplanted GFP-AEC sheets are shown in Supplemental Fig. 1.

4 Discussion

The present study has described the successful fabrication of AEC sheets under feeder-free conditions. Furthermore, the fabricated cell sheets continued to express AEC-specific proteins after transplantation onto the lung. We propose that the AEC sheets bioengineered using our technique may have potential for development into a new regenerative therapy for lung injury.

One of the challenges facing researchers striving to develop regenerative therapies for respiratory diseases is the structural complexity of the pulmonary system, which is highly specialized for gas exchange. Previously, we reported that temperature-responsive culture dishes could be used to fabricate transplantable cell

290 sheets without the need for additional synthetic or biological materials such as
291 scaffolds [18,19]. Various cell types can attach to, proliferate on and spread along
292 these unique surfaces when cultured under standard conditions at 37°C, with cell
293 growth comparable to that seen on ordinary tissue culture dishes. However, a major
294 advantage of temperature-responsive dishes is that cultured cells together with their
295 extracellular matrix can be harvested non-invasively as intact sheets (without the
296 need for proteolytic enzymes) simply by reducing the temperature to 20°C. This
297 non-destructive method of harvesting maintains the cell-to-cell junctions and
298 extracellular matrix proteins in the cell sheet construct [18,19]. Notably, cell
299 sheet-based regenerative therapies for various organs have been applied in the
300 clinical setting [20-22]. Furthermore, cell sheet-based therapy was reported to be
301 superior to cell infusion-based therapy in terms of cell viability, number of engrafted
302 cells and transplanted cell function [23]. Our previous research in the field of
303 respiratory medicine showed that autologous dermal fibroblast sheets harvested from
304 temperature-responsive culture dishes could be used as a pleural substitute to seal
305 intraoperative air leaks in the lungs in both animal models and clinical studies [24-27].
306 The AEC sheet described in the present study was fabricated using cell sheet-based

307 tissue engineering. These results indicate that further development of this technique
308 will allow AEC sheets to be used clinically to promote lung regeneration and thereby
309 treat pulmonary disorders that currently require lung transplantation.
310 Because safety is of paramount importance, major concerns have been raised
311 about regenerative therapies based on cells derived from different species. Therefore,
312 the objectives of this study were to develop a method for feeder-free cultivation of
313 AECs and to find suitable conditions for the fabrication of AEC sheets for
314 transplantation *in vivo*. Feeder-free culture on an rLN511E8-coated dish in LCM
315 containing a ROCK inhibitor promoted rapid AEC growth. In the culture method of this
316 study, AECII did not completely trans-differentiate into AECI in vitro and remained
317 after transplantation, possibly preserving the characteristics of both AECI and AECII.
318 ROCK-induced Rho signaling leads to the phosphorylation and activation of
319 non-muscle myosin light chain II and hence muscle contraction [28,29]. Non-muscle
320 myosin light chain II is a key modulator of cell behavior that has been implicated in
321 cell migration, proliferation and differentiation [30,31]. ROCK inhibitors have been
322 widely used in the culture of several cell types because of their ability to suppress
323 apoptosis, and pharmacological inhibition of ROCK has been shown to enhance the

proliferation of keratinocytes from foreskin, ectocervical and vaginal tissues [32]. Ca^{2+} induces calmodulin-mediated signaling that regulates the activity of the myosin light chain [33]. Ca^{2+} is also recognized as a trans-differentiation-inducing factor for epithelial cells and is essential for cell–cell adhesion via E-cadherin [34,35]. LCM prevents the differentiation of epithelial cells and promotes cell expansion [36]. Remarkably, the combination of LCM and a ROCK inhibitor has been reported to cause a 10^{12}-fold increase in epithelial cell numbers [37]. rLN511E8 strongly supports the long-term, feeder-free culture of several cell types. For example, a previous study utilized a feeder-free culture system and recombinant laminin-coated dishes to generate human pluripotent stem cells that could be differentiated into retinal pigment epithelial cells and corneal limbal epithelial stem cells, and the authors developed cryopreservation protocols for all three cell types [14]. In the present study, AECs were also able to grow rapidly on rLN511E8-coated dishes when cultured in LCM containing a ROCK inhibitor under feeder-free conditions. This new method of culturing AECs under feeder-free conditions is straightforward and would circumvent safety issues regarding the use of xenogeneic feeder cells if used as the basis of a regenerative therapy.

AECs cultured in LCM containing a ROCK inhibitor could not be harvested as a cell sheet from the temperature-responsive culture dish due to insufficient cell–cell adhesion. Since Ca^{2+} is essential for cell–cell adhesion via E-cadherin, we altered the protocol so that the confluent AECs were further cultured in LCM supplemented with Ca^{2+}. AECs cultured in LCM containing additional Ca^{2+} exhibited expression of E-cadherin (unlike cells not cultured in Ca^{2+}-supplemented medium) and could be harvested as a cell sheet, likely due to the strengthening of cell-cell adhesions via E-cadherin. A potential disadvantage of this approach is that Ca^{2+} is a differentiation-inducing factor for epithelial cells. However, immunohistochemical analyses showed that the cell sheets harvested after culture in Ca^{2+}-supplemented medium for 3 days exhibited localized expression of AEC-specific proteins.

GFP-AEC sheets transplanted onto rat lung continued to express AQP-5 (a marker of AECIs), SP-A, SP-C, SP-D (markers of AECIIs) and Axin-2 (a marker of a subpopulation of AECIIs capable of trans-differentiation into AECIs), which indicates that the cell sheets retained the characteristics of AECs after transplantation. There is a paucity of data regarding the fate of AECs after transplantation onto the lung *in vivo*. Lung fibroblasts reside beneath the alveolar epithelium and make contact with AECs

358 through gaps in the basement membrane [38]. Pulmonary fibroblasts secrete
359 important soluble factors that have been reported to limit the trans-differentiation and
360 promote the proliferation of AECs [39]. In addition, trans-differentiation is slowed
361 when AECs are grown under air-liquid interface conditions *in vitro* [4]. Therefore,
362 environmental factors such as the presence of pulmonary fibroblasts and an air-liquid
363 interface likely inhibit the differentiation of AECs *in vivo*.
364 This study has some limitations. First, AEC sheet engraftment was only evaluated
365 7 days after transplantation *in vivo*, so it remains unclear whether the transplanted
366 cells would survive as AECs for a longer period of time. Whether AEC sheets are
367 maintained long-term after transplantation will require additional evaluation. Second,
368 although engraftment of the AEC sheet onto the lung was demonstrated from a
369 structural perspective, the functionality of the AEC sheet after transplantation was not
370 assessed. Further research will be needed to establish whether transplanted AEC
371 sheets exert functional effects such as improvements in pulmonary function or
372 suppression of lung disease progression. In addition, although feeder-free culture on
373 an rLN511E8-coated dish in LCM containing a ROCK inhibitor promoted rapid AEC
374 growth, the effects of each on AECs and differentiation have not been investigated.

Pharmacological aspects of the effects on AECs will be needed to investigate.

5. Conclusions

We have bioengineered AEC sheets under feeder-free conditions that retain the morphological features of AECs after transplantation onto the lung *in vivo*. The ultimate objective of our research is to develop a regenerative therapy that can reconstruct the alveolar structure in an injured lung, and the research described in this study is part of that process. One of our future aims is to fabricate three-dimensional lung tissue using AEC sheets and enlarge it step-by-step *in vitro*. Furthermore, we will investigate whether the transplantation of AEC sheets onto decellularized pulmonary tissue would generate lung constructs that could be used as a regenerative therapy.

Declaration of competing interests

Tatsuya Shimizu is a shareholder of CellSeed Inc. Tokyo Women's Medical University received research funding from CellSeed Inc. The other authors have no competing interests to declare.

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Figure legends

Figure 1. Schematic diagram of the methods used to fabricate and transplant alveolar epithelial cell (AEC) sheets. Lung tissues were carefully excised from male rats and finely minced, and cells were dissociated with elastase. A Percoll gradient was used to separate AECs from other cell types in the cell suspension. The isolated AECs were cultured under feeder-free conditions, and the expanded cells were seeded and cultured on temperature-responsive culture dishes. After subculture, the cells were harvested from the temperature-responsive dish as an AEC sheet by reducing the temperature from 37ºC to 20ºC for 30 min. Each AEC sheet was transplanted onto a partially resected region of the left lung or onto the left gluteal musculature of an athymic rat.

Figure 2. Alveolar epithelial cells (AECs) cultured under feeder-free conditions. (a) Primary AECs were cultured under feeder-free conditions in the absence or presence of a rho kinase (ROCK) inhibitor (10 mM Y-27632) and in the absence or presence of recombinantly expressed laminin-511 E8 fragment (rLN511E8) as a coating on the tissue culture dish (i.e., four experimental groups). The cultured AECs

were harvested 7 days later, and the number of cells was counted. (b) Comparison of the number of AECs expanded under each feeder-free condition. The data are displayed as box plots showing the median, interquartile range and range (n = 7). * p < 0.05. (c) Phase-contrast microscopy of AECs cultured on an rLN511E8-coated dish in alveolar epithelial cell medium (AEpiCM) containing a ROCK inhibitor. The cultured AECs exhibited a polygonal, cobblestone-like morphology characteristic of epithelial cells. (d–h) Immunohistochemical analyses of AECs cultured on rLN511E8-coated dishes in AEpiCM containing a ROCK inhibitor. The cultured AECs were immunostained using primary antibodies against aquaporin-5 (AQP-5), surfactant protein (SP)-A, SP-C, SP-D and Axin-2.

Figure 3. Fabrication of alveolar epithelial cell (AEC) sheets. (a) AECs were subcultured on 35-mm-diameter temperature-responsive culture dishes in alveolar epithelial cell medium (AEpiCM) containing a rho kinase (ROCK) inhibitor (10 mM Y-27632). However, after 7 days of subculture, confluent AECs could not be harvested as a cell sheet when the temperature was reduced from 37ºC to 20ºC for 30 min. Immunohistochemical analysis revealed that cells cultured under these

conditions did not express E-cadherin. (b) AECs were subcultured on 35-mm-diameter temperature-responsive culture dishes in AEpiCM containing a ROCK inhibitor, and the cells were confirmed to be confluent after 7 days. Then, the AECs were cultured for a further 3 days in AEpiCM containing a ROCK inhibitor and supplemented with 200 mg/L calcium chloride. Under these conditions, the AECs could be harvested as a cell sheet by reducing the temperature from 37ºC to 20ºC for 30 min. Immunohistochemical analysis revealed that cells cultured using this protocol expressed E-cadherin. (c–h) Hematoxylin and eosin (HE) staining and immunofluorescence staining of AEC sheets fabricated under the feeder-free conditions described in (b). Positive staining for aquaporin-5 (AQP-5), surfactant protein (SP)-A, SP-C, SP-D and Axin-2 showed that the cultured cells were AECs. Cross-sectional (upper panel) and planar (lower panel) views were obtained for the immunohistochemical analyses.

Figure 4. Analyses of green fluorescent protein (GFP)-expressing alveolar epithelial cell (AEC) sheets (GFP-AEC sheets) after transplantation onto the lung. (a) Three GFP-AEC sheets were transplanted onto a partially resected area of

559 the left lung of an athymic rat. (b) Seven days later, illumination of the transplantation
560 region with fluorescent light revealed the presence of GFP-positive cells, which were
561 derived from the transplanted cell sheets. (c–i) Histological and immunohistochemical
562 analyses of lung tissues and transplanted GFP-AEC sheets. The lung tissues and
563 transplanted GFP-AEC sheets were subjected to HE staining or immunofluorescence
564 staining for GFP, aquaporin-5 (AQP-5), surfactant protein (SP)-A, SP-C, SP-D or
565 Axin-2. Staining with HE demonstrated that the transplanted AEC sheets were tightly
566 adhered to the lung tissue, and no air spaces were found between the cell sheets and
567 the pulmonary parenchyma (arrowheads). The immunohistochemical analyses
568 revealed that cells on the lung surface expressed GFP (white arrows), and the same
569 region also expressed AQP-5, SP-A, SP-C, SP-D and Axin-2. No staining was
570 observed when the primary antibodies were omitted in negative control experiments.
571
572 **Supplemental figure 1. Immunohistochemical analyses of lung tissues without**
573 **transplanted green fluorescent protein (GFP)-expressing alveolar epithelial cell**
574 **(AEC) sheets.** (a) No staining was observed when the primary antibodies were

575 omitted in negative control experiments. (b–e) Immunofluorescence staining of lung

576 tissues for aquaporin-5 (AQP-5), surfactant protein (SP)-A, SP-C, SP-D and Axin-2.

Figure 1

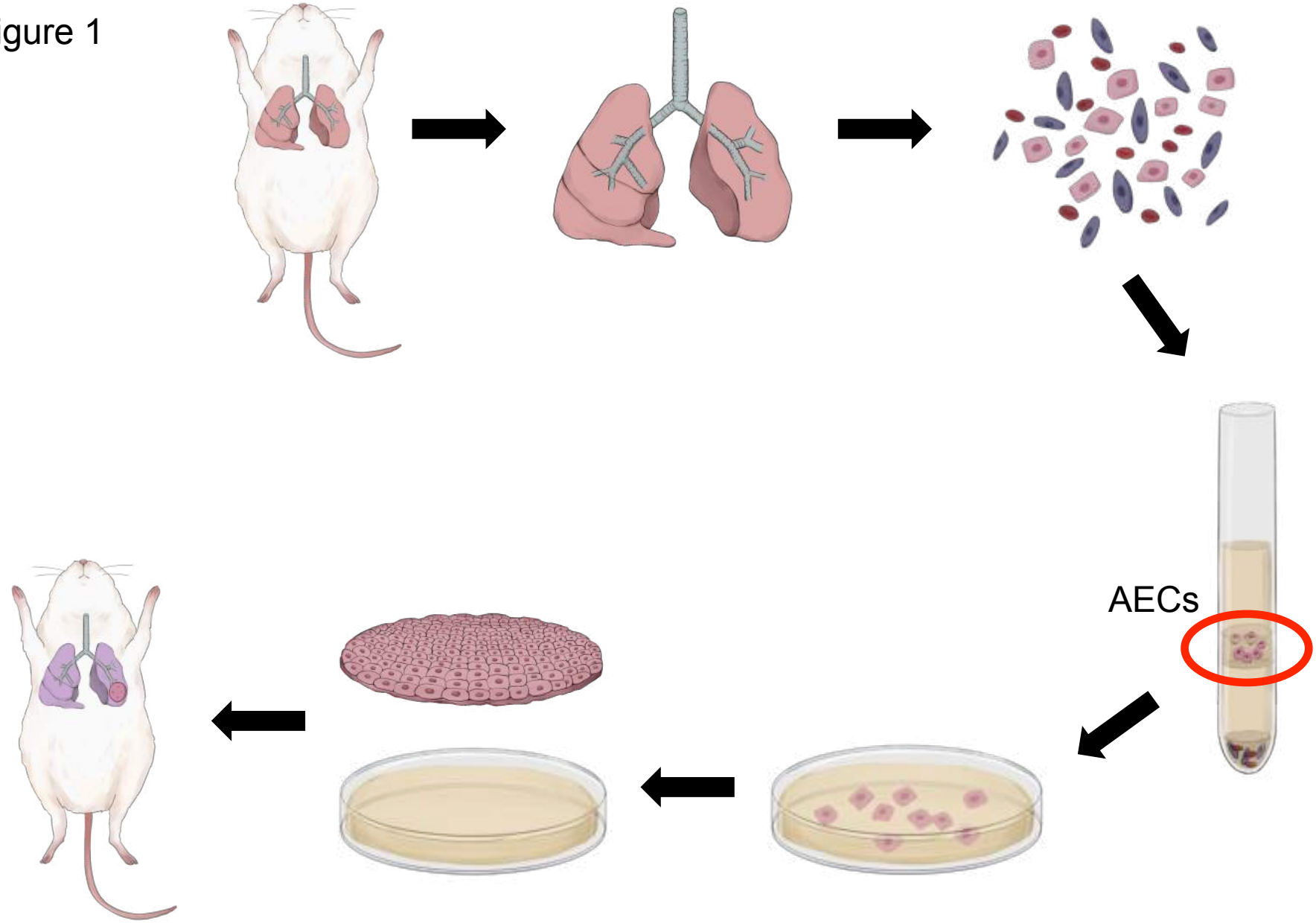
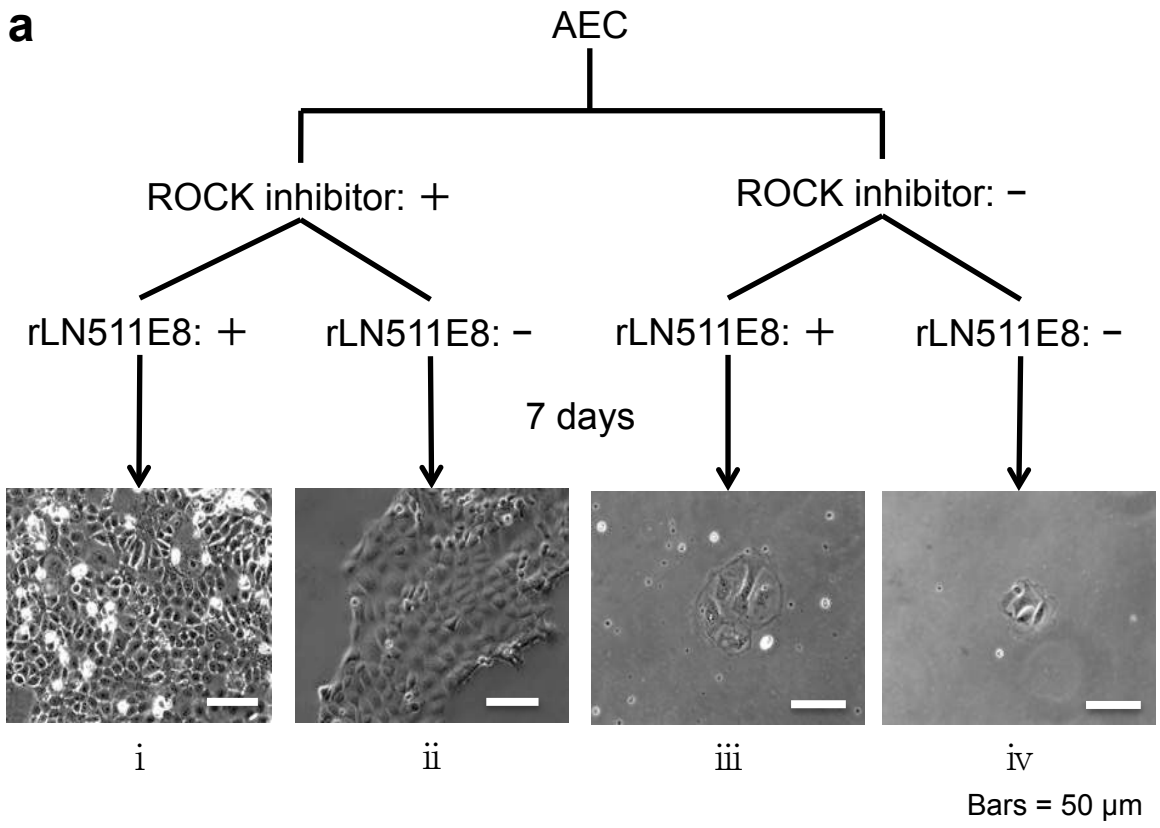
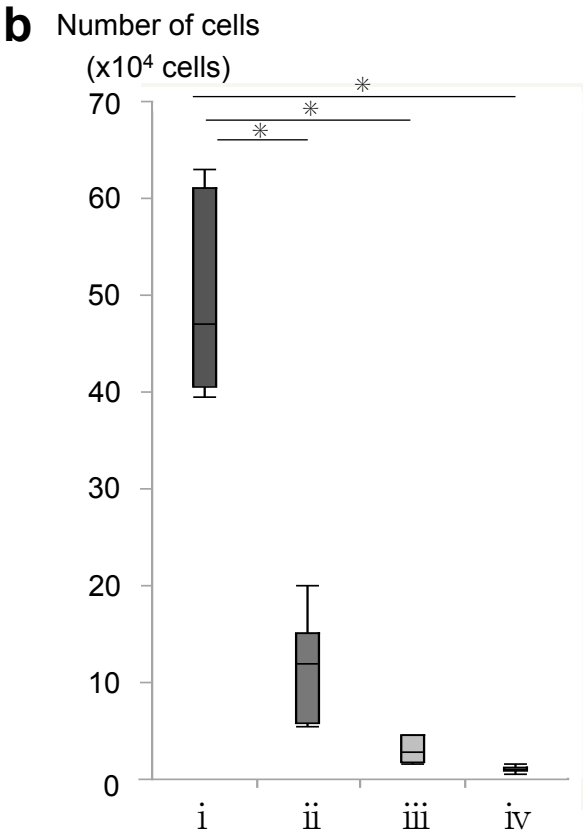
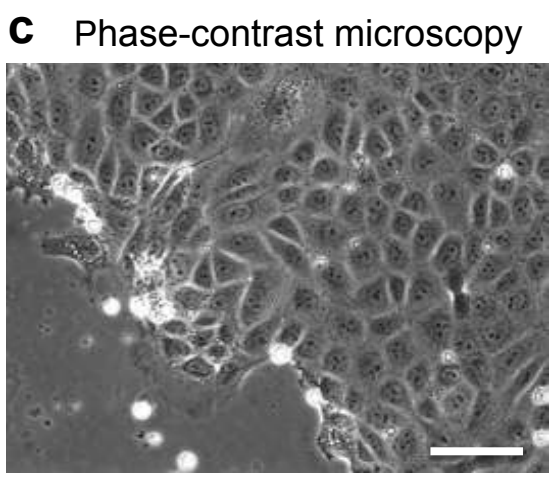
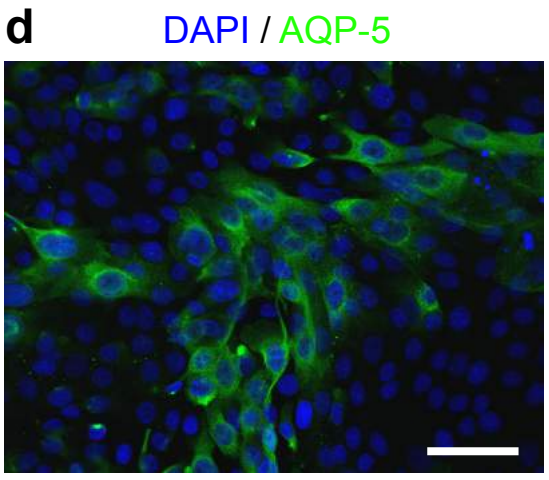


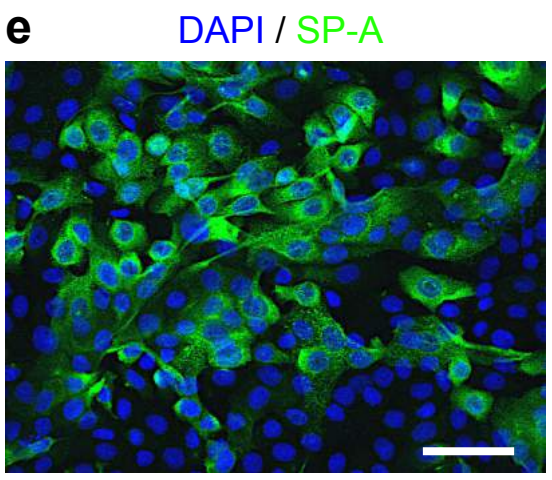
Figure 2

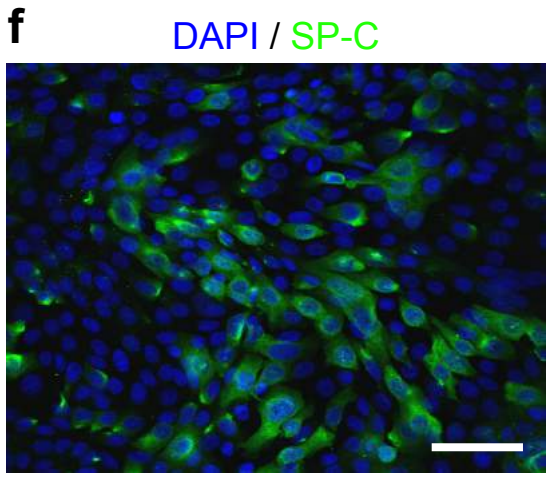

a
AEC
ROCK inhibitor: +
ROCK inhibitor: −
rLN511E8: +
rLN511E8: −
rLN511E8: +
rLN511E8: −
7 days
i
ii
iii
iv
Bars = 50 μm

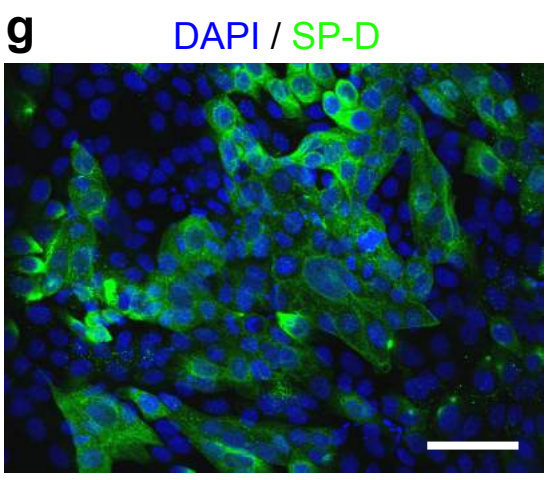

b
Number of cells
(x10⁴ cells)
70
60
50
40
30
20
10
0
i
ii
iii
iv


c
Phase-contrast microscopy


d
DAPI / AQP-5


e
DAPI / SP-A


f
DAPI / SP-C


g
DAPI / SP-D

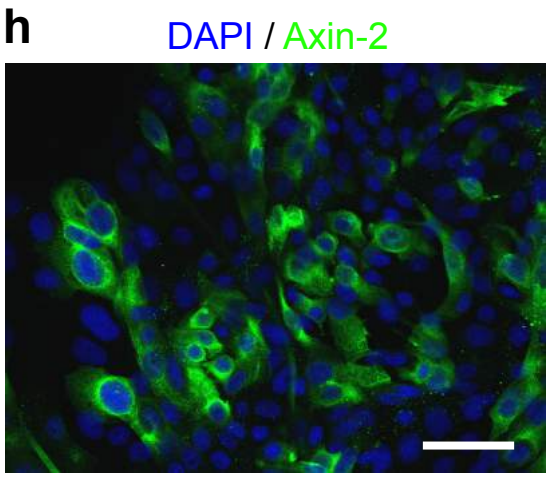
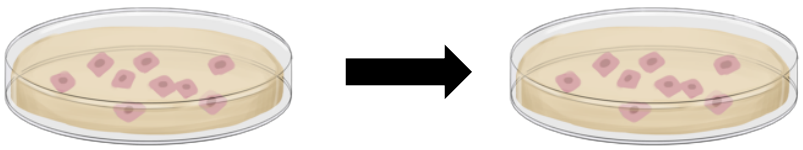
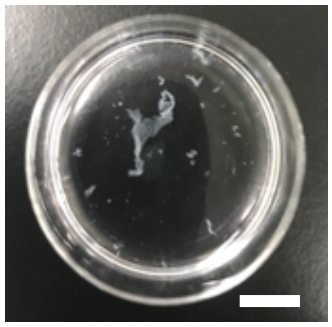

h
DAPI / Axin-2
Bars = 50 μm

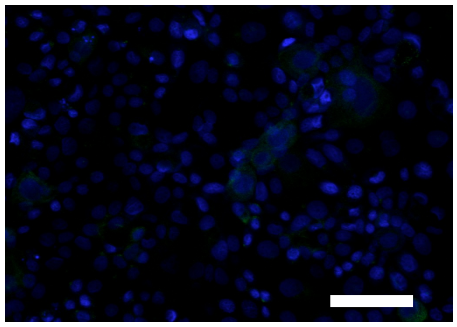
Figure 3

a

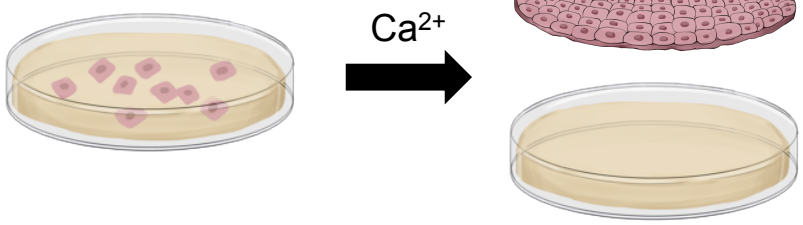


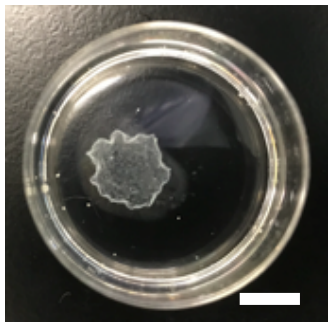


DAPI / E-cadherin

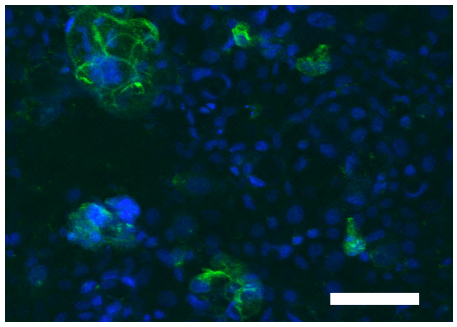


b





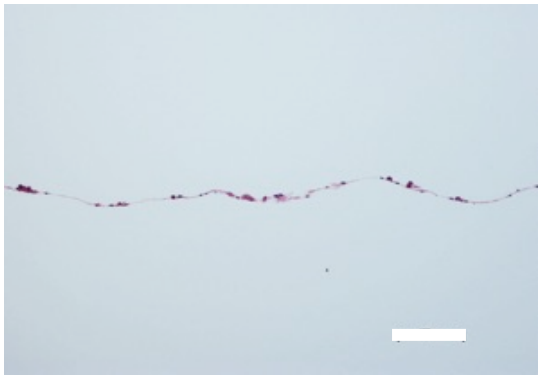
DAPI / E-cadherin



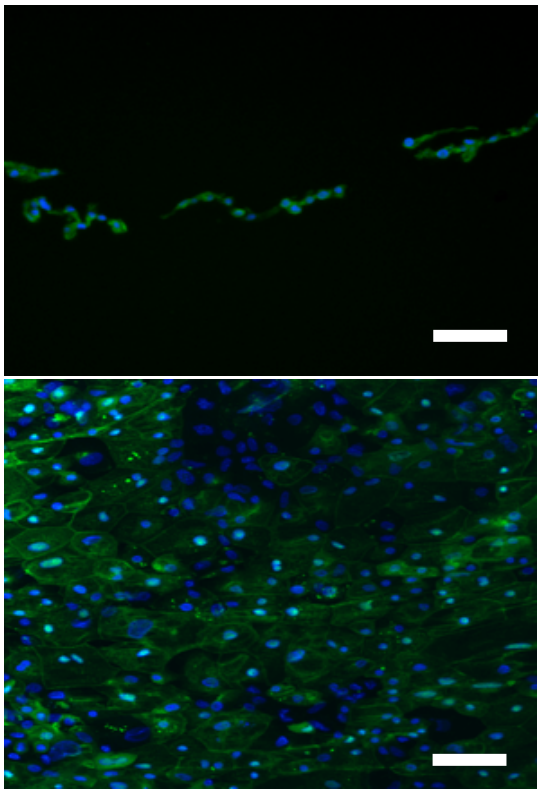
Bars = 10 mm

Bars = 50 μm

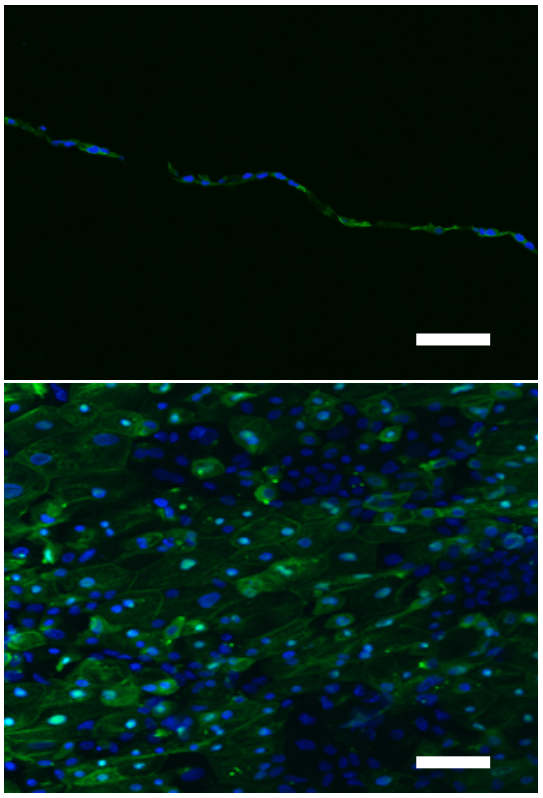
c HE



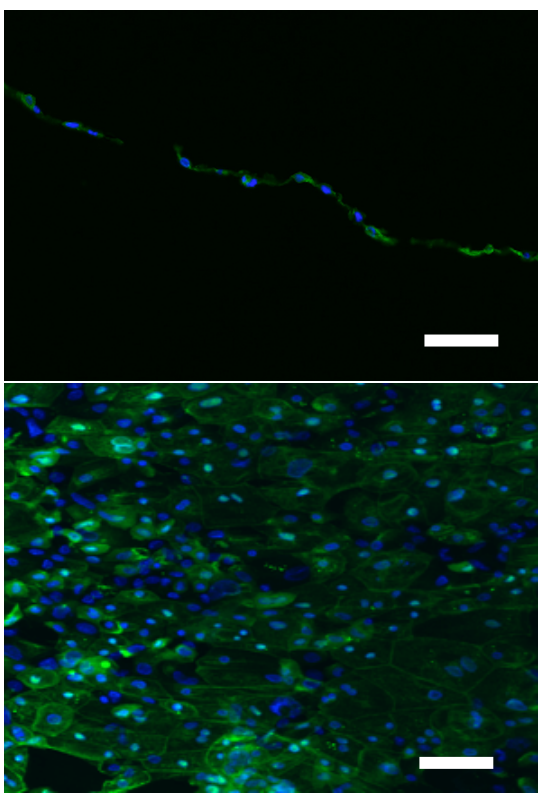
d DAPI / AQP-5



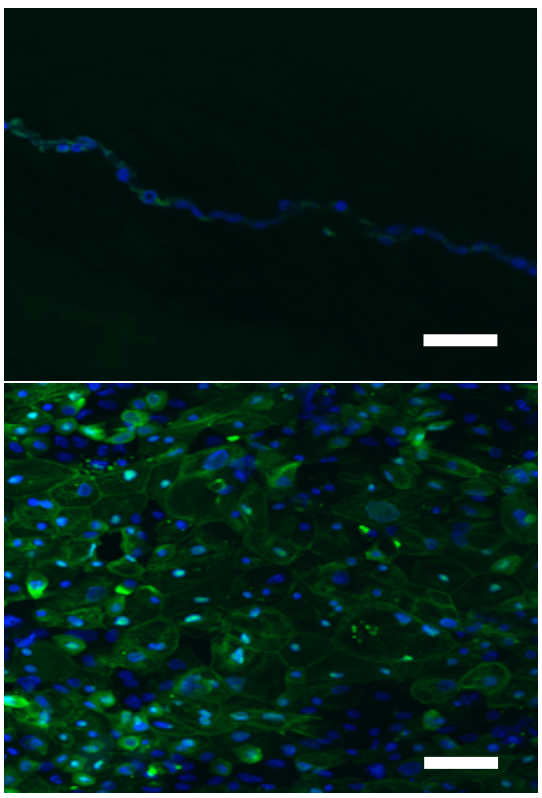
e DAPI / SP-A



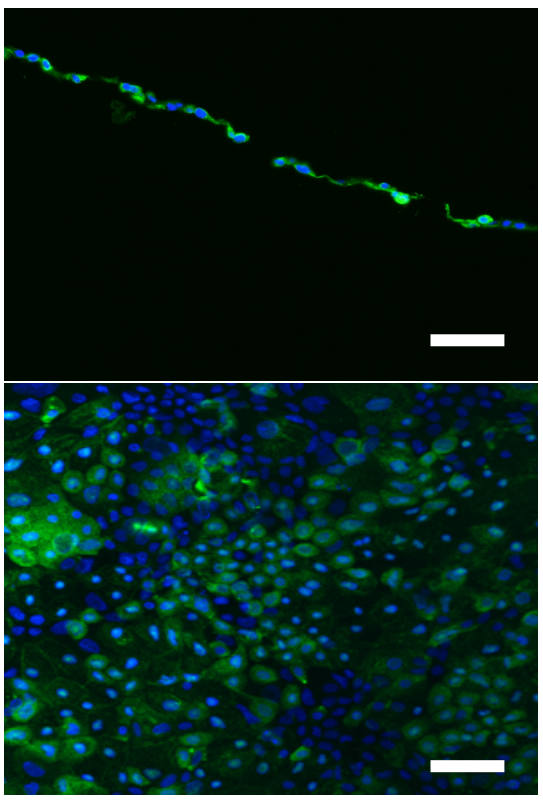
f DAPI / SP-C



g DAPI / SP-D

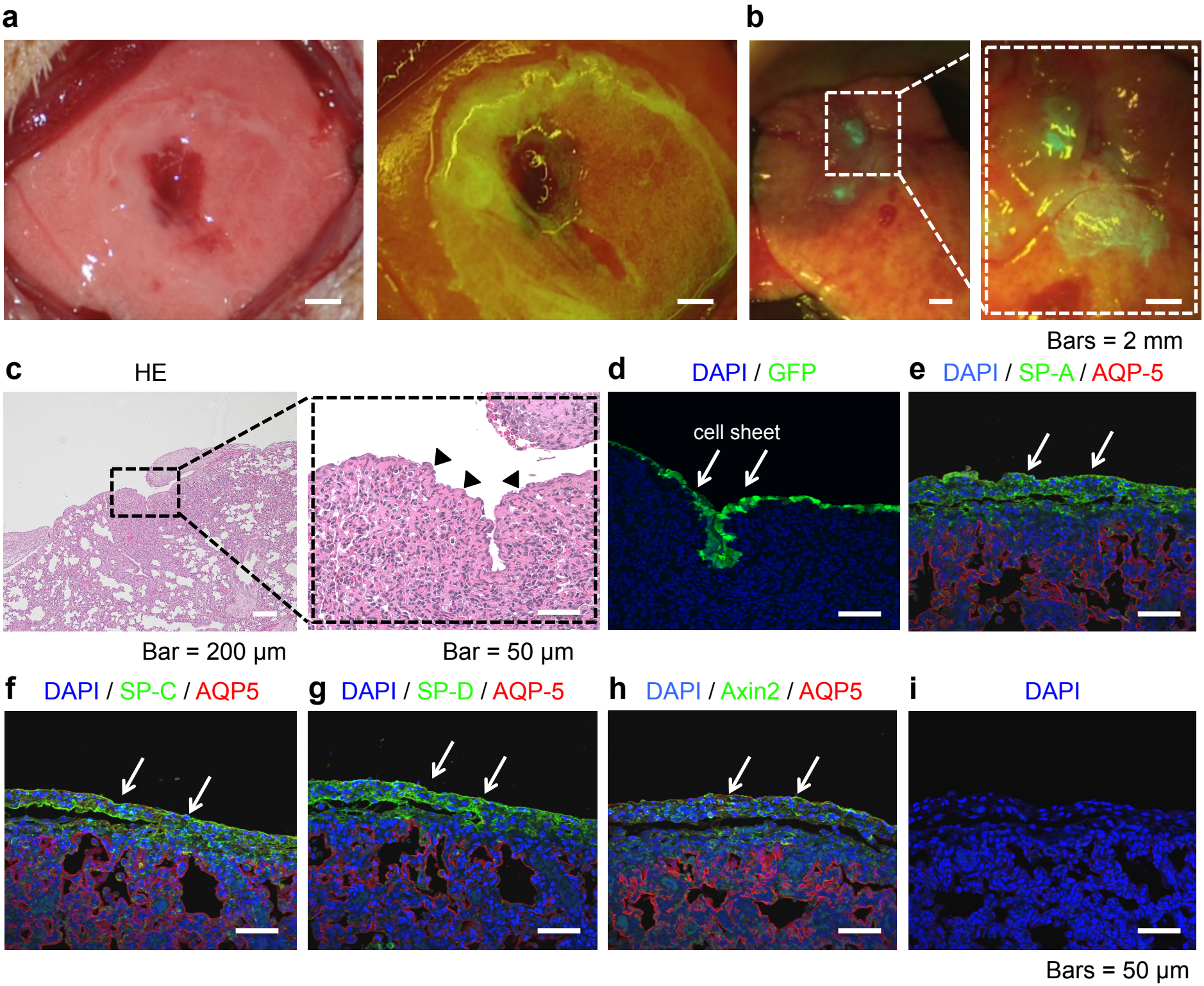


h DAPI / Axin-2



Bars = 50 μm

Figure 4


a
b
Bars = 2 mm
c
HE
Bar = 200 μm
Bar = 50 μm
d
DAPI / GFP
cell sheet
e
DAPI / SP-A / AQP-5
f
DAPI / SP-C / AQP5
g
DAPI / SP-D / AQP-5
h
DAPI / Axin2 / AQP5
i
DAPI
Bars = 50 μm

Supplemental Figure 1