Master's Thesis

Investigation of Induced-Lung Progenitor Cell Differentiation Methods

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Introduction

Chronic Obstructive Pulmonary Disease (COPD)

COPD is the third cause of death around the world, which cause around 2.5 million deaths every year [1]. It is usually associated with inflammation of small airway, bronchitis, and eradication of lung parenchymal, emphysema. The characteristic of this disease is airflow limitation due to airway narrowing, and emphysema [2].

The causes of COPD are cigarette (or tobacco) smoking and long-term exposure to toxic chemical, which recruit inflammatory cells into airway and cause chronic inflammation. Another factor that causes COPD is the imbalance of proteolytic and anti-proteolytic molecules in the lung. Increasing of proteolytic activity eliminates healthy lung tissue and leads to development of emphysema. The third factor is oxidative stress caused by the excess of the reactive oxygen species. Smoking also increases the production of oxidants. The oxidative stress leads to impairment in cellular functions and promotes the damage of this organ. In healthy lung tissue, the protective proteins such as α-1 anti-trypsin (α-1AT) and tissue inhibitor of metalloproteinase (TIMPs) counteract against these exogenous stress, and therefore tissue homeostasis is maintained[3].

Bronchitis is the lung infectious disease in infants and children caused by virus infection in bronchioles. The inflamed bronchioles induce increased production of mucus and their plugging, which reduce air flow to the lung. The inflammation also damages the ciliated cells that are responsible for the protection of airways from germ and irritants[4]. Emphysema is a chronic disease of the lung caused by abnormal, permanent enlargement of the air sacs by the destruction of their walls[5].

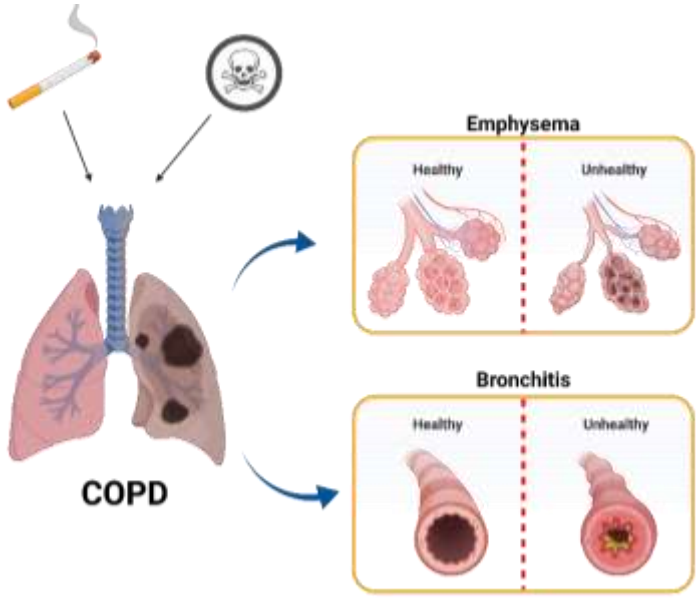


Figure 1: Chronic Obstructive Pulmonary Disease (COPD), which usually caused by the smoking or long-term exposure of the toxic chemical. COPD consist of two types of diseases, which are emphysema and bronchitis.

The development of both human and mouse lung can be divided into 5 stages [6]. During the embryonic stage, the lung is started to develop as lung buds protruded from foregut endoderm, which are true lung primordium, and first recognizable as a laryngotracheal groove. The lung buds that consist of single layer of epithelium surrounded with mesenchyme begin to divide and give rise to conducting airways and five primordial lung lobes[7]. In the next pseudoglandular stage, respiratory duct, tubule acinus gland, and air-conducting bronchial tree are developed. The air-conducting bronchial tree are coated with the cuboidal epithelial cells, which are the precursor of the ciliated epithelial cells, goblet cells, and basal cells. The studies reported that during this stage, around 20 generations of future airways are formed in humans[8]. After that in the canalicular stage, Canaliculi branch derived from the terminal bronchioles, changed their shape and grow in width and length to become future alveolar ducts. The gas exchanging portion of lung, which called acinus, is vascularized and formed. Further, the type I alveolar cells (ATI), which are responsible for gas exchange due to very thin membrane, and type II alveolar cells (ATII), which are responsible for secretion of surfactant, are start to differentiate and lining along alveolus. Then, at saccular stage, the terminal airway is growing in length and width forming larger airspaces. The primary septa are formed at the location between 2 two airspaces. The surface of primary septa mostly is covered by type I alveolar cells while remaining is filled in by type II alveolar cells, which also acted as a progenitor for type I alveolar cells[9]. Finally, the alveolar stage, which new formed septa divided the existing airspace. Those upfolding could ne seen and those newly formed septa are called secondary septa in order to distinguish from the primary septa that formed during branching morphogenesis [9].

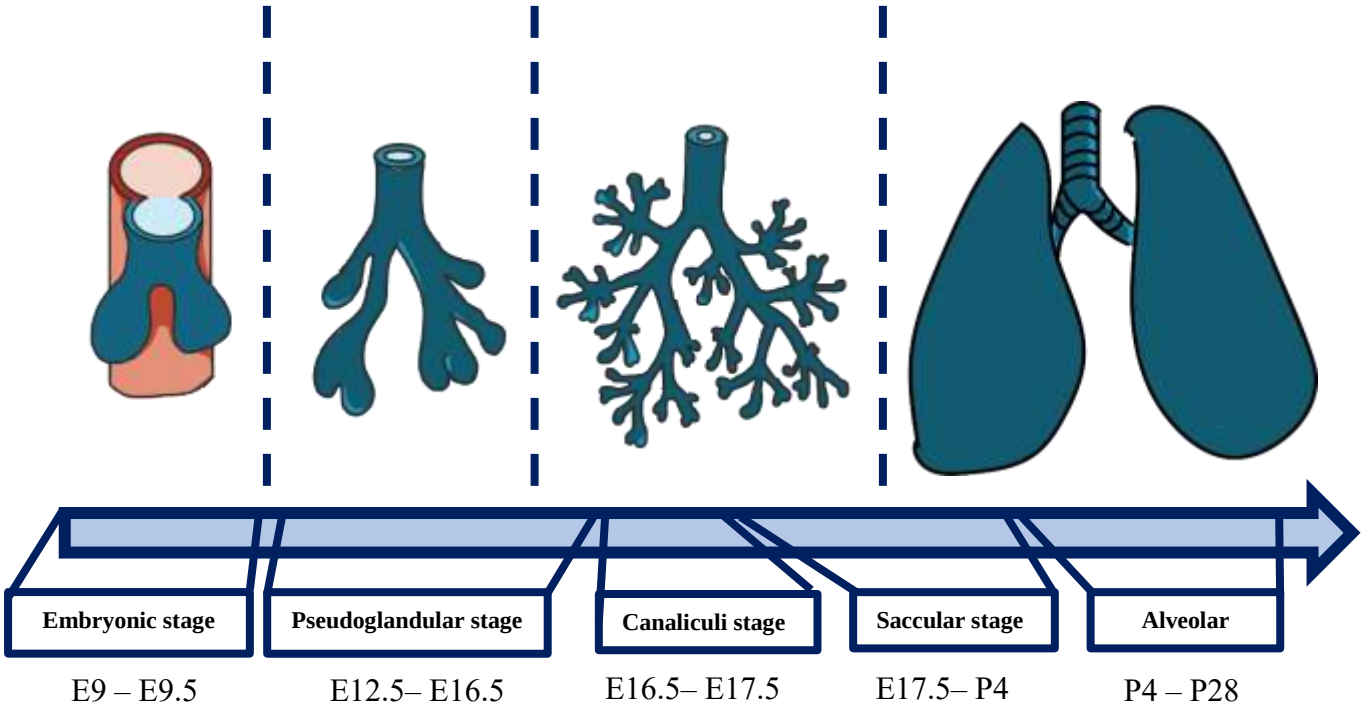


Figure 2: Mouse lung development overview, starting with embryonic stage, which small lung buds protruded from the foregut endoderm (E9-E9.5). Then, Pseudoglandular stage, which bronchiole tree are developed. Following with Canalicular stage, which terminal bronchiole and future alveolar ducts started to form. Saccular stage, which terminal airways are expanding into larger sizes. Finally, alveolar stage, which growing of alveolar unit and forming of secondary septa.

Lung regeneration

Lung stem progenitor cells are important regenerative cell population to maintain tissue homeostasis and tissue after injury. Tissue-specific stem cells remain quiescent under the normal conditions and proliferate after injury[10]. In the proximal airway, basal cells are reported to be the important stem cells for bronchiolar epithelial cells, which can give rise to ciliated cells in both homeostasis conditions and after injury[11]. In the bronchioles of mouse lungs, two distinct type of cells are reported as the lung stem and progenitor cells. One of them is Clara cells (or club cells), which have been reported to have self-renewal ability and can also give rise to ciliated cells during homeostasis[12]. Another one is bronchioalveolar stem cells (BASCs), which are the rare population of epithelial cells that have co-expression of Clara cell markers (CCSP and Scgb1a1) and alveolar type II marker (Sp-C). BASCs are reported to be the stem cells population that maintain the bronchiolar Clara cells and alveolar cells of the distal lung. For the distal lung, ATII cells have long been reported to be the progenitor for the alveolar epithelial cells, which are proliferate and differentiate into ATII and ATI cells[9, 13].

Induced-pluripotent stem cells and direct-reprogramed cells

Several treatments have been applied to treat the COPD such as medication by using Bronchodilators, which help in relax airway muscle and widening the airways, and Corticosteroid, which used as anti-inflammatory therapy for the COPD treatment[14]. Previously, organ transplantation is also one of the best methods to treat the damaged lung tissue of COPD patients. For the lung transplantation, there are around 2,000 patients remained on the waiting list for transplantation in 1998-2000. In 2005, there was improved system, called 'Lung Allocation System', which decreases the patient in the waiting list to around 1,000 people[15]. However, organ transplantation is still a dangerous and unstable method due to the immunological rejection between the donor's lung and patient's bodies. To prevent the immunological rejection, the use of engineered lung organ for transplantation is one of the novel methods for COPD treatment in the future [16]. By using the induced pluripotent stem cells (iPSCs) from patients themselves, the immunological rejection from the patients can be eliminated. IPSCs have been established by the reprogramming of adult somatic cells into embryonic stem cells by using expression of 4 factors, which are OCT4, SOX2, NANOG, and LIN28, or Oct3/4, Sox2, c-MYC, and Klf4, under ES cell culture conditions[17, 18]. Introduction of these transcriptional factors are enough to reprogram human somatic cell and mouse somatic cells into pluripotent stem cells that showing characters of embryonic stem cells. Therefore, by using iPSCs derived from patients, the recovery of damaged respiratory system can be done by eliminating risk of the immune rejection. However, iPSCs also have several problems for real application. Firstly, iPSCs has risk to generate to tumors due to the use of oncogene in the reprogramming process[19]. The viral vector systems that are used in this process can also integrate into the host DNA. Therefore, direct reprogramming is a new approach in order to overcome those problems, which will reprogram the somatic cell into desired specific cells directly without passing the pluripotent stage that reduce the risk of tumor formation[20].

Differentiation of pluripotent stem cells

The use of stem cells or iPSCs for regenerative medicine is promising, and some differentiation methods have been published. By using 3D Matrigel culture, several studies have reported the differentiation of the lung progenitor cells derived from the lung buds, embryonic lung or iPSCs [21-24]. They reported that 3D microenvironment is crucial for the maintenance of the progenitor cells, and also with the designed differentiation medium, the differentiated lung epithelial cells such as ATI cells (Aqp5, Pdpn, Hopx), ATII cells (Sftpc, Sftpb), basal cells (Krt5), and goblet cells (Muc5ac) were observed in the alveolospheres and bronchospheres. Other than this, air liquid interface (ALI) culture are also reported to differentiate lung stem cells into lung epithelial cells, especially ciliated cells [25-27]. Another effective method that have been reported for differentiation of lung epithelial cells is differentiation by using decellularized lung extracellular matrix [28-30]. Induction of NKX2.1 and SOX2 were detected after seeding lung epithelial progenitor cells in the ECM for short term. For the long-term culture, induction of ciliated cells, club cells, and basal cells were induced in the ECM culture.

Materials and Methods

Isolation of mouse embryonic lung progenitor cells

All animal experiment plans were approved by the institutional animal experiment committee and the experiments were performed in accordance with the guidelines of the animal center of NAIST. Embryonic mouse embryos were collected from ICR or CAG-EGFP C57BL6N pregnant female mice. The embryonic lungs were carefully dissected with small forceps under stereoscopic microscopes. The heart was also removed. Embryonic lungs were pooled in 6 mL of PBS on ice before mixed with 4 mL of filtered Collagenase A (2 mg/ml)/PBS solution, and incubated in the 37℃-water bath for 1 hours. Then, 400 µL of 10 µg/mL DNase I was added in the solution. Then, embryonic lungs were pipetted with the 5 mL pipette in every 15 minutes to increase the dissociation of the cells. The cells were centrifuged for 5 minutes at 1,000 rpm at room temperature. The pellet was re-suspended with the hemolysis buffer, and incubate for 5 minutes at room temperature. Then, cells were centrifuged again for 5 minutes at 1,000 rpm. Pellets were re-suspended with Advance RPMI (Gibco) + 0.2% FBS (Gibco) + P/S, and incubated in the 37℃-water bath for 2-3 hours. Next, the cells floating in the medium were centrifuged for 5 minutes at 1,000 rpm. The pellets were re-suspended with the lung progenitor cells medium, dispensed into 1:40 diluted Matrigel-coated plate, and cultured in the humidified incubator at 37℃ and 5% CO_2.

Mouse embryonic lung progenitor cells culture and expansion

Embryonic lung progenitor cells were seeded on the 1:40 diluted Matrigel-coated 60mm cell culture dished, and cultured with the lung progenitor cell mediums, which are ‘eLife medium’[24] (Table 1) and ‘MAS medium’[23] (Table 2). The mediums were changed every 2 days. The cells were passaged when they reached confluent with the 1:3 ratio. For passaging, the cells were washed with the 4 mL PBS, before added 0.5 mL TrypLE Express (Gibco), and incubated in the humidified incubator at 37℃ for 10 – 30 minutes. Then, the cells were mixed with cultured medium and centrifuged for 5 minutes at 1,000 rpm. The supernatant was removed, and cells were re-suspended and dispensed with the lung progenitor cell mediums. Re-cultured cells were incubated in humidified incubator at 37℃ and 5% CO_2.

3D-culture of mouse embryonic lung progenitor cells

Mouse embryonic lung progenitor cells cultured in 60 mm dishes were washed with PBS once, treated with the TrypLE Express (Gibco) solution, and incubated at 37℃ in a CO_2 incubator for 10 – 15 minutes. After incubation, the cells were mixed with lung progenitor cell medium (eLife or MAS) and centrifuged at room temperature at 1,000 rpm for 5 minutes. The supernatant was then removed, and the cells were re-suspended with Matrigel on ice. Fifty µL of mixture was spitted into the center of 24-well plate and incubated in a CO_2 incubator for 10 minutes. After that the plate was turned upside down and further incubated again for 20 minutes. After

incubation, 0.5 mL lung progenitor cell medium (eLife or MAS) was added into each well, and incubated in a CO_2 incubator at 37℃ for 7 days before the passaging of the cells.

Air liquid interface (ALI) culture of mouse embryonic lung progenitor cells

Mouse embryonic lung progenitor cells were washed with the PBS before treated with the TrypLE Express solution and incubated in a CO_2 incubator at 37℃ for 10 – 15 minutes. After incubation, the cells were mixed with lung progenitor cells medium (MAS) and centrifuged at room temperature with 1,000 rpm for 5 minutes. Then, the supernatant was removed, the cell pellet was re-suspended with the lung progenitor cell medium (MAS) and seeded into 12-well plate transwell (Greiner bio-one). The medium was also added outside of the transwell. The cells were cultured in a CO_2 incubator at 37℃ for 3 – 4 days until the cells were confluent. Then, the leftover medium in the transwell was removed and the medium around the transwells were changed into differentiation medium (Table3). The cells were cultured for 28 days, and the medium were renewed every 3 days.

Isolation of mouse dermal fibroblast

Wild type mouse ears were obtained from C57BL6J mice, and GFP^+ mouse ears were collected from C57BL6N (CAG-EGFP) mice. The ears were shaved and minced into small pieces with the single edge blades. Minced ears were collected into 15 mL tube with HBSS, and centrifuged for 5 minutes at 1,000 rpm. The supernatant was removed and minced ears were mixed with the 5 mL Collagenase IV / Trypsin solution (4 mg/mL Collagenase IV (Gibco), 2.5% (w/v) Trypsin (Gibco), 3 mM $CaCl_2$, and incubated in 37℃ water baths for 1 hour. Then, minced ears were pipetted with a 5 mL pipette in every 30 minutes until the ears were dissociated. The minced tissue was filtered through 100 μm filter (Millex®), and centrifuged at 1,000 rpm for 5 minutes at room temperature. Supernatant were removed, and the pellet was re-suspended with MEF medium (DEME low glucose (Nacalai tesque), 12.5% (w/v) FBS (Gibco), 1% Penicillin/Streptomycin) and centrifuged again at 1,000 rpm for 5 minutes. After that, supernatant was removed and the cells were re-suspended with 4 mL MEF medium, seeded on the 0.1% (w/v) gelatin-coated 60 mm dish, and incubated a CO_2 incubator at 37℃.

Propagation of 5 factors plasmid preparation

Glycerol stock of DH5α transformed with the 5 factor plasmids, pMYs-hFOXA2, pMYs-hGATA6, pMYs-NMYC, pMYs-hSOX2, pMYs-hIRX3, and a green fluorescent expressing plasmid, pMYs-mTFP1 were kept on ICE for 30 minutes following with heat shock at 44℃ for 1 minute. After that, solutions were kept on ICR for 2 minutes following addition of 900 μL of SOC medium. Solutions were kept at 37℃ for 90 minutes in the incubator. Then, solutions were centrifuged at 7000 rpm at room temperature for 1 minutes and resuspended on LB agarose plate overnight at 37℃. On the next day, 1 E. coli colony were picked up by the sterile toothpick. The

toothpicks were added into the tube following with 1 mL of LB medium supplemented with 100 µg/mL of ampicillin. Then, tubes were put in the shaking incubator with 200 rpm for 5 to 6 hours. After that solution were re-cultured with 100 mL of LB medium supplemented with 100 µg/mL of ampicillin in flask and kept in rotating incubator at 37℃ with 200 rpm for overnight. On the next day, solutions were transferred to 50 mL tube and centrifuged at 4℃ with 6000 rpm for 10 minutes. The supernatants were removed and white pellet were kept in the refrigerator at -30℃ until the plasmid extraction. By using the NucleoBond Xtra Midi method, the plasmid DNA of 5 factors gene and green fluorescent plasmid were purified. The concentration of plasmid DNA were adjusted to 1000 ng/µL.

Retrovirus transfection

Twenty four of each 5 factor expressing retroviral vector (1 µg/µL), which contain either FOXA2, GATA2, N-MYC, SOX2, or IRX3 was mixed with 120 µL of PEI-MAX (1 mg/mL), and 500 µL of DMEM (Low glucose) in eppendorf tubes. Solutions were vortexed vigorously and kept at room temperature for 20 minutes. Then, sub-confluent Plat E cells cultured in 10 cm cell-culture dishes with DMEM low glucose (Nacalai tesque) supplemented with 10% FBS (Gibco) and Penicillin / Streptomycin) were transfected with the DNA-PEI complex. The transfected cells were incubated in a CO_2 incubator at 37℃ overnight. On the next day, the medium was renewed and the cells were cultured for another 48 hours.

Purification of retrovirus containing 5 factors

The cultured mediums obtained from plasmid transfected Plat E cells were harvested and filtered through the 0.45 µm filter (Millex®) into 15 mL tubes. Then, the mediums were mixed with the 1/5 volume of 50% PEG8000, and 1/25 volume of 5 M NaCl. The solutions were mixed by inverting the tubes several times and stored at 4℃ overnight. These virus tubes were centrifuged 3,600 RPM 4℃ for 60 minutes. Then, the supernatant was removed and virus pellet were re-suspended with MEF medium, which is ready for infection.

Infection of fibroblast with the retrovirus expressing 5 factors for reprogramming

Mouse dermal fibroblasts (MDFs) were passaged from 60 mm dish to a 12-well plate one day before infection. On the day of infection, the medium of MDF was renewed and supplemented with 0.5 µL of 8 mg/mL Polybrene. The viruses that was re-suspended with MEF medium were added into MDF cells cultured in a 12-well plate. The cells were centrifuged at 35℃ with 1,800 rpm for 60 minutes. After centrifugation, cells were incubated in a CO_2 incubator at 37℃ overnight. On the next day, the medium was renewed. Then, the cells were cultured with MEF medium for 7 days. Then, the cells were passaged into 3D-Matrigel culture for generation of organoids in the lung progenitor cell medium (MAS medium) until day 21. The medium was renewed in every 3 days.

3D-matrigel culture of the reprogrammed cells

The reprogrammed cells cultured for 21 days with the lung progenitor cell medium (MAS) were passaged with the 1:4 ratio from 12-well plate to 24-well plate. The cells were washed once with PBS, and treated with the TrypLE Express solution, and incubated at 37℃, a CO_2 incubator for 10 – 15 minutes. The supernatants were collected with MEF medium and centrifuged at room temperature at 1,000 rpm for 5 minutes. After centrifugation, supernatant was removed and cell pellet was re-suspended with the homemade Matrigel solution on ice. Fifty µL of cell-Matrigel mixture was spitted into the center of 24-well plate and incubated at 37℃ in a CO_2 incubator for 10 minutes. The plate was turned the plate upside down and further incubated for 20 minutes. After the incubation, 0.5 mL of lung progenitor cells medium (MAS) was added into each well, and cells were cultured at 37℃, in a CO_2 incubator for 21 days. The medium was renewed in every 3 days.

Air liquid interface (ALI) culture of the reprogrammed lung progenitor cells

The organoids generated from the reprogrammed cells cultured in 3D culture were washed with PBS once and dissociated by pipetting. The dissociated organoids were centrifuged at 1,000 rpm room temperature for 5 minutes. The supernatant was then removed, and the cells were treated with the TrypLE Express solution at 37℃ in a CO_2 incubator for 10 – 15 minutes. Then, the cells were centrifuged again at 1,000 rpm for 5 minutes, and the supernatant were removed. The cell pellet was re-suspended with the lung progenitor cells medium (MAS) and seeded into 12-well plate transwells. The lung progenitor cells medium was also added outside of the transwell. The cells were cultured in a CO_2 incubator for 3 – 4 days until the cells were confluent. Then, the leftover medium in the transwell was removed, and the medium around the transwell were changed into differentiation medium. Then, the cells were cultured at 37℃ in the CO_2 incubator for 28 days. The medium was renewed every 3 days.

Preparation of mouse lung extra-cellular matrix (ECM)

C57BL/6J wild type mouse was injected with the Anesthetic drug (100 µL / 10 g of mouse weight). While waiting for the mouse to anesthetize, 50 mL of perfusion mixture solution were prepared by mixed 246 mg of CHAPS detergent powder, 2.5 mL of 0.5 M EDTA (pH 8), 10 mL of 5 M NaCl, 2.5 mL of 20x PBS and added Deionized water to make exactly 50 mL. After the mouse anesthetized, the mouse was clamped to the plate with pins and the skin of the mouse was sprayed with 70% EtOH. Then, the mouse was laparotomized from the stomach up to chest. After cut the diaphragm, quickly cut both side ribs. Then, open them, held the chest cavity using the mosquito forceps. Then, left ventricle of the heart was pieced with a blunt needle connected to prewarmed PBS (50 mL), and also cut at the right article of the heart, then opened the valve to flow PBS to perfused the organs through the heart and blood vessel. After the PBS had run out, perfused the organs again using the perfusion mixture solution through heart and blood vessel

using 50 mL syringe with blunt end needle. Finally, perfused the organs again with 50 mL of PBS through the heart and blood vessel. The perfused lung extracellular matrix (ECM) were excised with the trachea and soaked in the Benzonase solution (8 µL of Benzonase nuclease, 200 µL of Tris buffer (pH = 8) in 2 mL PBS) for 2 days at room temperature. Then, the lung ECM were treated with 70% EtOH at 4℃ overnight. After that the lung ECM was washed and kept in 2 mL PBS at 4℃ for the experiment.

Mouse lung extra-cellular matrix (ECM) culture with mouse embryonic lung progenitor cells

Mouse embryonic lung progenitor cells cultured in 60 mm dish were washed with PBS once, and treated with 0.5 mL of TrypLE Express solution in a CO_2 incubator at 37℃ for 10 – 15 minutes. After incubation, cells were mixed with lung progenitor cells medium (MAS) and centrifuged at room temperature at 1,000 rpm for 5 minutes. The supernatant was removed, and cell pellets was re-suspended with 2 mL of Matrigel. Cell-Matrigel mixture was injected into Lung ECM through trachea into each lobe of the lung ECM. Cell-Matrigel injected ECM were incubated in a CO_2 incubator at 37℃ for 20 minutes before cut into thin section using a single edge blade and cultured each piece in 24-well plate with lung progenitor cell medium (MAS) for 7 days before changed to lung differentiation medium.

Table 1; Lung progenitor cells maintenance medium (eLife)

Reagent	Company	Final concentration
Advance DMEM	Gibco	1x
Penicillin / Streptomycin	Nacalai tesque	1:100
HEPES	Dojindo	1:100
Glutamax	Gibco	1:100
N2	WAKO	1:100
B22	Miltenyi Biotech	1:50
N – acetylcrystein	SIGMA life science	1.25 mM
FGF10	Peprotech	100 ng/mL
FGF7	Peprotech	100 ng/mL
EGF	Peprotech	50 ng/mL
R-spondin	Homemade	5% v/v
LDN193189	Axon medchem	0.1 µM
CHIR99021	LC Laboratories	3 µM
SB431542	Cayman CHEMICAL	10 µM

Table 2; Lung progenitor cells maintenance medium (MAS)

Reagent	Company	Final concentration
Advance DMEM/F12	Gibco	1x
Penicillin / Streptomycin	Nacalai tesque	1:100
L-Glutamine	Gibco	1:100
Heparin	Wako	5 µg/mL
Insulin	Nacalai tesque	10 µg/mL
Transferrin	Nacalai tesque	15 µg/mL
FGF10	Peprotech / Homemade	50 ng/mL
FGF9	Peprotech / Homemade	50 ng/mL
EGF	Peprotech	50 ng/mL
BIRB796	BLD pharm	1 µM
A83-01	Focus Biomolecules	1 µM
CHIR99021	LC Laboratories	3 µM
Y27632	BLD pharm	10 µM

Table 3; Lung Differentiation medium

Reagent	Company	Final concentration
DMEM/F12	Nacalai tesque	1x
HEPES	Dojindo	1:66
$NaHCO_3$	-	0.03% (w/v)
Insulin	Nacalai tesque	2 mg/mL
Transferrin	Nacalai tesque	5 mg/ mL
Cholera toxin	Bioacademia	25 ng/ mL
EGF	Peprotech	5 ng/ mL
BPE (Bovine pituitary extract)	-	0.03 mg/ mL
BSA (Bovine serum albumin)	SIGMA Life science	0.1 % (w/v)

RNA Isolation

Cells cultured on a plate were washed with PBS once before addition of 1 mL ISOGEN. The cells were lysed by extensive pipetting and transferred to a 1.5 mL Eppendorf tube. For the ALI cultured cells and ECM cultured tissues, after samples were collected in 1 mL ISOGEN in Eppendorf tube, samples were homogenized into small pieces using a homogenizer. All samples were pipetted around 20 times and incubated in room temperature for 5 minutes. Then, samples were supplemented with 0.5 µL of Ethachinmate and 0.2 mL of Chloroform and vigorously vortexed for 15 seconds. The mixtures were stood at room temperature for 2 – 3 minutes and centrifuged at 4℃ at 12,000 rpm for 15 minutes. After centrifugation, the aqueous phase of the samples was transferred into a new 1.5 mL Eppendorf tube, and 0.5 mL of Isopropanol was added. The mixture was inverted upside down and stood at room temperature for 10 minutes before centrifuged at 4℃ at 12,000 rpm for 10 minutes. After centrifugation, the supernatant was removed and 70% EtOH were added to the white pellet, vortexed and centrifuged at 7,500 rpm 4℃ for 5 minutes. Finally, 70%EtOH were removed, and white pellets was dried by air for 5 – 10 minutes. The total RNA was dissolved in 10 µL of MilliQ water. The concentration of RNA was measured by using NanoDrop machine (Thermo Fisher Scientific) by absorbance at 230 nm, 260 nm, and 280 nm. After that, RNA samples were kept at -80 ℃ deep freezer.

cDNA Synthesis

RNA samples (0.5 µg) were mixed with the deionized water up to 7 µL in PCR tube. Then, mixtures were incubated at 65℃ for 5 minutes. Then, 1 µL of homemade 10xDNase buffer, 1 µL of homemade MRI (RNase Inhibitor), and 1 µL of homemade DNase I were added to the mixture on ice and further incubated at 37℃ for 15 minutes. Finally, mixtures were mixed with 1 µL of reverse transcriptase (RT), and 9 µL of reverse transcriptase buffer (RT buffer) and incubated at 25℃ for 10 minutes, 42℃ for 45 minutes, and 70℃ for 15 minutes sequentially. cDNA samples were kept in deep freezer at -20℃ for the qRT-PCR analysis.

qRT-PCR

cDNA samples (1 µL) were mixed with 5 µL of 2x SYBR qPCR mix, 1 µL of 2 mM dNTP, 2.1 µL of MilliQ water, 0.3 µL of homemade Taq, 0.3 µl of 10 µM primer forward, and 0.3 µL of 10 µM primer reverse in the qRT-PCR tubes. qRT-PCR were performed by using CFX96™ Real-Time System C1000™ Thermal Cycler (BIORAD) with the condition of 95℃ for 1 minutes, then 95℃ for 15 seconds and 60℃ for 30 seconds which repeated for 40 cycles. Then, reacted at 65℃ for 5 seconds before raise to 95℃.

qPCR primers that are used;

mGapdh

primer Fw : 5’- ACCCAGAAGACTGTGGATGG -3’

primer Rv : 5’- CACATTGGGGGTAGGAACAC -3’

mSp-B

primer Fw : 5’- AGCTAGTCTGTGGCCTTGTCC -3’

primer Rv : 5’- CACAGACTTGCAGAAATGGC -3’

mSp-C

primer Fw : 5’- GGCATCGTTGTGTATGACTACC -3’

primer Rv : 5’- CTTGGCCCTGAAGTTCTGGA -3’

mHopx

primer Fw : 5’- GGAGGAGCAGACGCAGAAAT -3’

primer Rv : 5’- TCCAAGAGCAAGCTCAAGGG -3’

mAqp5

primer Fw : 5’- CATCTTGTGGGGATCTACTTC -3’

primer Rv : 5’- TACCCAGAAGACCCAGTGAG -3’

mScgb1a1

primer Fw : 5’- ATGAAGATCGCCATCACAATCAC -3’

primer Rv : 5’- TACCCAGAAGACCCAGTGAG -3’

mScgb3a1

primer Fw : 5’- ATGAAGATCGCCATCACAATCAC -3’

primer Rv : 5’- TACCCAGAAGACCCAGTGAG -3’

mFoxJ1

primer Fw : 5’- GAAATGGTCTCTAAGCCCGC -3’

primer Rv : 5’- AAGTCAGGCTGGAAGGTTTG -3’

mβ-Tubulin

primer Fw : 5’- GAAATGGTCTCTAAGCCCGC -3’

primer Rv : 5’- AAGTCAGGCTGGAAGGTTTG -3’

mMuc5ac

primer Fw : 5’- AATGTGTTTCCTCCAAGACTG -3’

primer Rv : 5’- CACAAGATGGTTCATTCAGC -3’

mMuc5b

primer Fw : 5’- AATGTGTTTCCTCCAAGACTG -3’

primer Rv : 5’- CACAAGATGGTTCATTCAGC -3’

mP63

primer Fw : 5’- AGCTTCTTCAGTTCGGTGGA -3’

primer Rv : 5’- CCTCCAACACAGATTACCCG -3’

mKrt5

primer Fw : 5’- ACCTTCGAAACACCAAGCAC -3’

primer Rv : 5’- TTGGCACACTGCTTCTTGAC -3’

mNkx2.1

primer Fw : 5’- ATCTGGCCAGCATGATTCACC -3’

primer Rv : 5’- GCTGCGCCGCCTTGTCCTTA -3’

mFoxp2

primer Fw : 5’- CCACCATCACACATCATTC -3’

primer Rv : 5’- TGCGCCATAGAGAGTGTGCGA -3’

mE-cadherin

primer Fw : 5’- ACCGATTCAAGAAGCTGGC -3’

primer Rv : 5’- ACCATCCTAACACAGACAGTCC -3’

mEpCAM

primer Fw : 5’- TTGCTCCAAACTGGCGTCTA -3’

primer Rv : 5’- ACGTGATCTCCGTGTCCTTGT -3’

mThy1

primer Fw : 5’- TGAGGGCGACTACTTTTGTG -3’

primer Rv : 5’- CTTATGCCGCCACACTTGAC -3’

mSma

primer Fw : 5’- CCCAGACATCAGGGAGTAATGG -3’

primer Rv : 5’- TCTATCGGATACTTCAGCGTCA -3’

Results

Generation of lung organoids by using embryonic mouse lung progenitor cells with lung progenitor cells maintenance medium

Embryonic lung progenitor cells derived from the E13.5-E15.5 ICR mouse were firstly cultured in adherent culture in 1:40 Matrigel-coated 60 mm cell-culture dishes with 2 candidate lung progenitor maintenance mediums, which are 'eLife medium' (Table 1), and 'MAS medium' (Table 2) for several days until they reached confluent. Then, the cells were passaged to 3D Matrigel culture with both 2 candidate mediums for 7 days before collected for the qRT-PCR. Morphology of the cells were also observed on day 3 and day 6, which the cells that cultured with the MAS medium generated several organoids while the cells that cultured with eLife medium have failed to generate organoids (Figure3). Based on the qRT-PCR result, the expression of lung progenitor cell markers (*Nkx2.1, Foxp2*), basal cell markers (*P63, Krt5*), and epithelial cell markers (*E-cadherin, Epcam*) were highly observed in the cells that were cultured with the MAS medium (Figure 4).

Generation of lung organoids from reprogrammed lung progenitor cells with lung progenitor cells maintenance medium

Reprogrammed lung progenitor cells were generated from mouse dermal fibroblast (MDF) infected with 5 factors gene (SOX21, FOXA2, IRX3, GATA6, N-MYC). The inflected cells were cultured with MEF medium in adherent culture for 7 days, passaged to 3D Matrigel culture, and further culture for 3 weeks with lung progenitor cells mediums (MAS medium and eLife medium). The reprogrammed cells cultured with both medium showed the same morphology after 2 weeks cultured (Figure 5). On day 26^{th} after passaging to 3D, the reprogrammed cells cultured with MAS medium could generated small organoids, and those organoids grew bigger when cultured for longer time. However, the reprogrammed cells cultured with eLife medium failed to generate the organoids, which were similar with the embryonic lung progenitor cell's result (Figure 6). As for the gene expression, the cells that cultured with MAS medium also showed the induction of lung progenitor cell markers (*Nkx2.1, Foxp2*), epithelial cell marker *(E-cadherin*), and basal cell marker (*P63*), which were higher than the cells that cultured with eLife medium. However, *Krt5*, which is one of the basal cell markers, and *Epcam*, which is also one of the epithelial cell markers, and *Sma*, which is lung mesenchymal cell marker, were highly expressed in cells that cultured with eLife medium. Based on the morphology and gene expression, MAS medium, which could form the organoids and showed higher expression of lung progenitor cells, basal cells, and epithelial cells, was suitable for the lung progenitor cell maintenance (Figure 3-6).

Differentiation abilities of mouse embryonic lung progenitor cells by air-liquid interface (ALI) culture method with differentiation medium

Embryonic lung progenitor cells were cultured in 3D Matrigel culture conditions with lung progenitor maintenance medium (MAS medium) to generate lung organoids. After that, the cells were passaged to transwell plate and firstly cultured with the lung progenitor maintenance medium (MAS medium) (Table 2) until they reached confluent. The medium inside the transwell were removed and medium outside transwell were changed to a differentiation medium (Table 3) and the cells were cultured for 4 weeks. The medium was renewed every 3 days. As for the cells cultured in ALI culture with differentiation medium, *Hopx* and *Aqp5*, markers for alveolar type I cells were highly induced comparing with cells cultured in 3D Matrigel with lung progenitor cell maintenance medium (Figure 7). However, *Sp-B* and *Sp-C*, markers for alveolar type II cells were not be observed. Club cell markers, *Scgb1a1* and *Scgb3a1*, basal cell markers, *P63* and *Krt5*, and epithelial cell markers, *E-cadherin* and *Epcam* were extremely induced in the ALI culture condition comparing to 3D Matrigel culture. *Muc5ac* and *Muc5b*, markers for goblet cell markers, and *Thy1* and *Sma*, markers for lung mesenchymal cells, were moderately induced. As For the ciliated cells, *Foxj1* and *β-tubulinIV* were significantly induced in this condition. The lung progenitor cell markers, *Nkx2.1* and *Foxp2* were expressed at the similar expression level (Figure 7). Thus, ALI culture seem to be one of the good candidates to induce the differentiation which can increase almost all type of the lung tissue cells except the alveolar type II cells.

Differentiation abilities of reprogrammed lung progenitor cells in ALI culture condition with differentiation medium

To confirm the differentiation potential of reprogrammed lung progenitor cells in ALI culture conditions, the same conditions for embryonic lung progenitor cells were used. The MDF were cultured in adherent culture for 7 days after infection with retrovirus transducing 5 factors. Then, the cells were passaged to 3D Matrigel culture to generate organoids. After culturing in 3D Matrigel for 3 weeks, the cells were passaged again to transwell and cultured with lung progenitor cell maintenance medium (Table 2) until they reached confluent. Then, the medium was changed to differentiation medium (Table 3) and cultured for 4 weeks. As the result, alveolar type I cell markers (*Hopx, Aqp5*), alveolar type II cell markers (*Sp-B, Sp-C*), goblet cell markers (*Muc5ac, Muc5b*), and epithelial cell markers (*E-cadherin*) were reduced after differentiation process (Figure 8). However, club cell markers (*Scgb1a1, Scgb3a1*), basal cell marker (P63, Krt5), and lung mesenchymal cell markers (*Thy1, Sma*) were induced after the differentiation process (Figure 7-8). As conclusion, ALI culture for reprogrammed lung progenitor cells could induce the expression of club cells, and basal cells. Despite, they failed to induce the differentiation toward alveolar epithelial cells, and goblet cells (Figure8).

Differentiation abilities of mouse embryonic lung progenitor cells in 3D Matrigel culture condition with differentiation medium

To confirm the differentiation potential of embryonic lung progenitor cells in 3D Matrigel culture conditions with differentiation medium, embryonic lung progenitor cells were cultured in 3D Matrigel condition with lung progenitor cell maintenance medium (Table 2) for 3 days. Then, the medium was changed to differentiation medium (Table 3) and cultured for 7 days. For the gene expression, alveolar type II cell markers (*Sp-C*), alveolar type I cell markers (*Aqp5*), club cell markers (*Scgb1a1*), lung mesenchymal cell markers (*Sma, Thy1*), and basal cell markers (*P63, Krt5*) were induced after cultured in differentiation condition for 7 days. However, goblet cell markers (*Muc5ac, Muc5b*), and lung progenitor cell markers (*Nkx2.1, Foxp2*) were reduced after the differentiation condition (Figure 9). This result suggested that 3D Matrigel culture could induce the differentiation of the alveolar type cells, basal cells, ciliated cells, and lung mesenchymal cells, but not for goblet cells, club cells.

Analysis of differentiation abilities of reprogrammed lung progenitor cells in 3D Matrigel culture condition with differentiation medium

To confirm the differentiation potential of reprogrammed lung progenitor cells in 3D Matrigel culture condition with differentiation medium, the reprogrammed lung progenitor cells were cultured in 3D Matrigel condition with lung progenitor cell maintenance medium for 7 days before changed to differentiation medium (Table 3) and cultured for 14 days. As for the morphological changes, cells cultured in the differentiation medium, showed almost the same in size of the organoids comparing to the cells that continuously cultured in lung progenitor cell maintenance medium (Table 2). As for the gene expression, the several lung tissue cells including, alveolar type I cells (*Hopx, Aqp5*), ciliated cells (*Foxj1*), goblet cells (*Muc5ac, Muc5b*), club cells (*Scgb1a1*), and basal cells (*P63*) differentiation were induced. (Figure10).

Analysis of differentiation abilities of mouse embryonic lung progenitor cells and reprogrammed lung progenitor cells in decellularized lung extracellular matrix (ECM) with differentiation medium

To confirm the differentiation potential of embryonic lung progenitor cells using the decellularized lung extracellular matrix (ECM), GFP^+ embryonic lung progenitor cells prepared from CAG-EGFP mouse E13.5 embryos were cultured in the 3D Matrigel in the maintenance medium (Table 2) until they generated several lung organoids. Then, the cells were passaged and mixed with the 2 mL Matrigel and injected into decellularized lung matrix through the trachea with a syringe. The ECM injected with organoids and Matrigel were cut into small pieces with single edge blade and cultured in a 24 well-plate, 1 piece per 1 well, with lung progenitor cells maintenance medium (Table 2) for 7 days. The medium was changed to differentiation medium and further cultured for 3 weeks. During cultivation, cell proliferation was monitored based on the GFP fluorescence of injected GFP^+ embryonic lung progenitor cells. The GFP signal was observed on day 3, 4, 14, and 22. However, the GFP signal was gradually decreased when cultured for long longer time (Figure11). As for the gene expression of differentiation marker genes, alveolar cell markers (*Sp-B, Sp-C, Hopx, Aqp5*), club cell markers (*Scgb1a1, Scgb3a1*),

goblet cell markers (*Muc5ac, Muc5b*), and lung progenitor cell markers (*Nkx2.1, Foxp2*) expression were drastically decreased after cultured in differentiation conditions. However, lung mesenchymal cell marker (*Sma*), epithelial cell markers (*E-cad, Epcam*), and ciliated cell markers *(FoxJ1, β-tubulin*) expression were almost the same level comparing to undifferentiated conditions. Only basal cell markers (*P63, Krt5*) were induced after cultured in this differentiation conditions (Figure12). For the reprogrammed lung progenitor cells, GFP^+ MDF were reprogrammed and passaged into 3D culture and cultured with lung progenitor cell maintenance medium until they generated the organoids. The cells were mixed with 2 mL Matrigel and injected into decellularized lung extracellular matrix with the same methods as embryonic lung progenitor cells. ECM injected with the cells amd Matrigel mixture were cut into small pieces with single edge blades and cultured in 24 well-plate, 1 piece per 1 well, with lung progenitor cell maintenance medium for 7 days, before changed to differentiation medium and further cultured for 3 weeks. During cultivation, unlike embryonic lung progenitor cells, reprogrammed lung progenitor cells injected ECM showed the same level of GFP^+signal from the first day of cultivation until the collection day indicating no proliferation of the cells inside the ECM (Figure11). Samples were collected for performing qRT-PCR. However, the RNA concentration of samples was too low to perform the qRT-PCR.

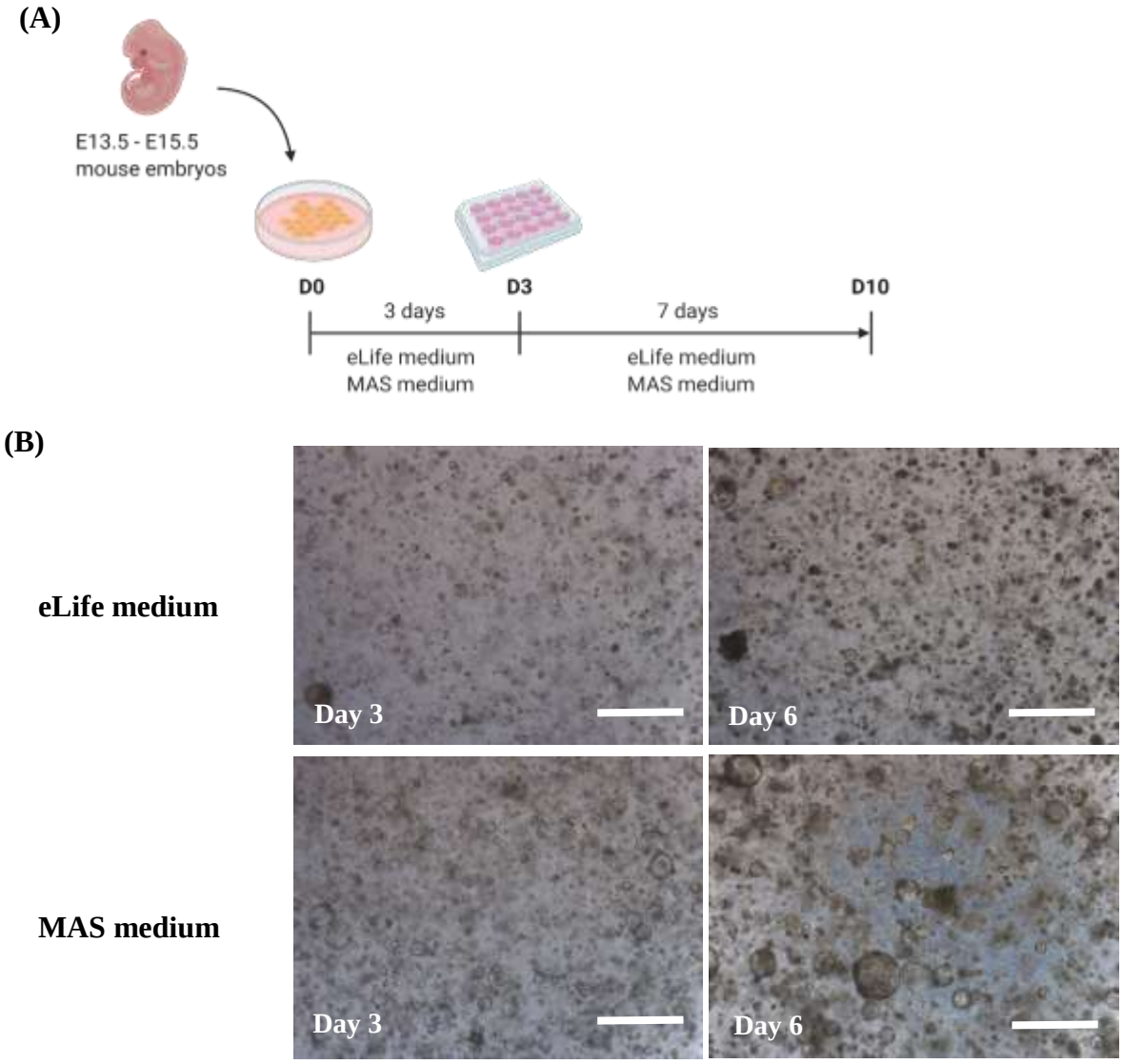
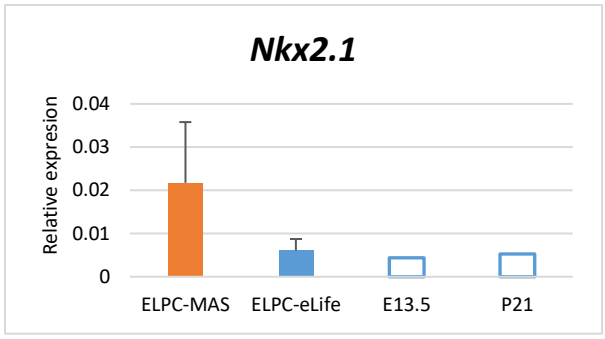
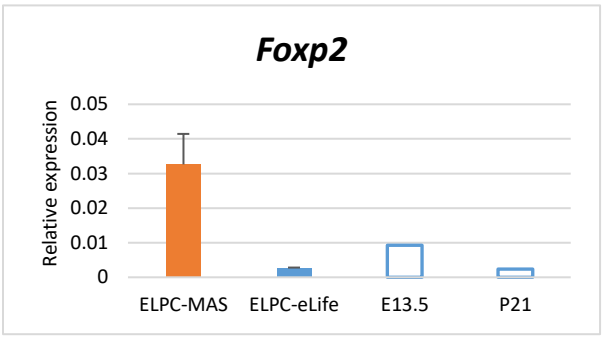
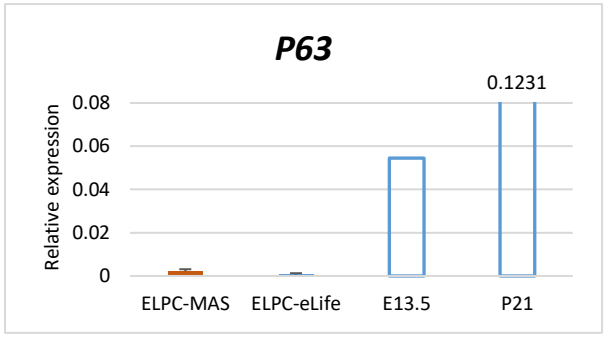
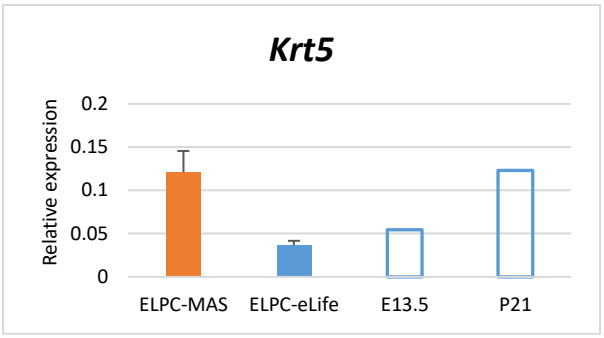


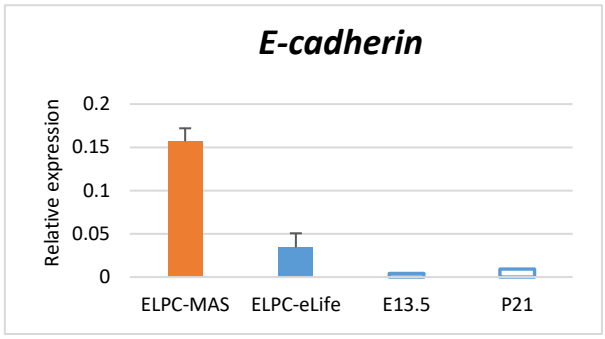
Figure3: Generation of lung organoids using mouse embryonic lung progenitor cells with lung progenitor maintenance medium. (A) A schematic of the experiment for the preparation of the organoids. Mouse embryonic lung progenitor cells (E13.5-E15.5). The cells were isolated from embryos and cultured in adherent cultured for 3 days, until they reached confluent. Then, the cells were passaged into 3D Matrigel culture with eLife medium or MAS medium for 7 days. The cells were subjected for qRT-PCR. (B) Morphological images of embryonic lung progenitor organoids that was observed on day 3, and day 6 cultured with both candidate lung progenitor maintenance mediums, eLife medium and MAS medium. Scale bar = 5 mm.

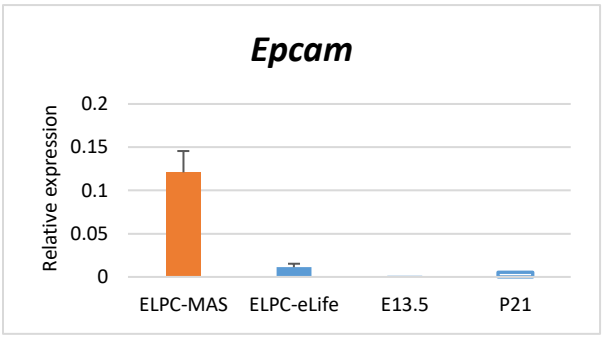












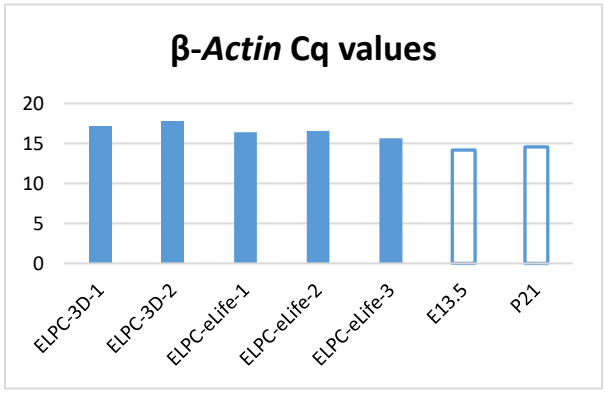


Figure4: qRT-PCR gene expression analysis of mouse embryonic lung progenitor cells cultured in MAS medium and eLife medium in 3D culture including positive controls, which are the embryonic lung tissue (E13.5) and adult lung tissue (Postnatal 21 days, P21). The gene expressions include lung progenitor cell markers (*Nkx2.1*, *Foxp2*), basal cell markers (*P63, Krt5*), and epithelial cell markers (*E-cadherin*, *Epcam*), were normalized with a housekeeping gene (*β-actin*) (n=2).

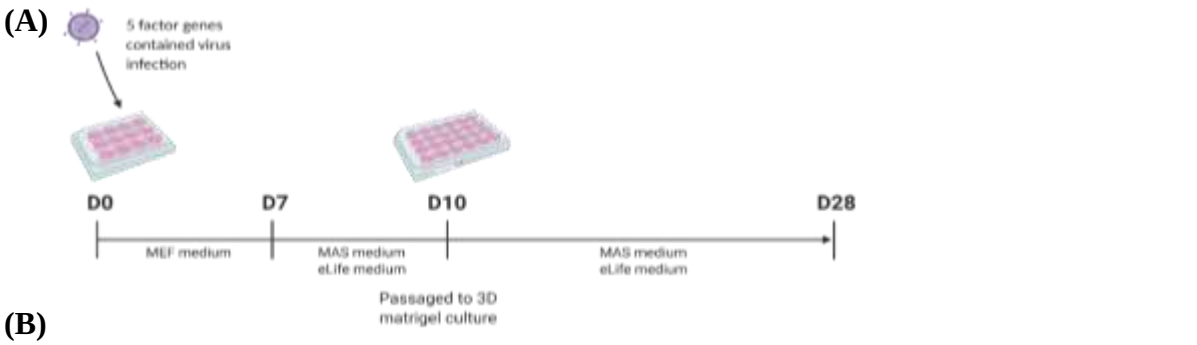


Figure5: Generation of lung organoids using reprogrammed lung progenitor cells cultured with lung progenitor cells maintenance mediums. (A) A schematic of experiment from infection of viruses expressing 5 factor genes (FOXA2, GATA6, SOX21, N-MYC, IRX3) into mouse dermal fibroblasts (MDF) in 2D culture with MEF medium. The medium was changed into lung progenitor cell maintenance mediums, eLife and MAS medium, on day 7th and the cells were passaged into 3D Matrigel and cultured with both lung progenitor cell maintenance mediums for 24 days. (B) The observation of morphological change of the cells cultured in 3D Matrigel conditions with lung progenitor cell maintenance medium on day 14, 26, and 34. Scale bar = 500 µm

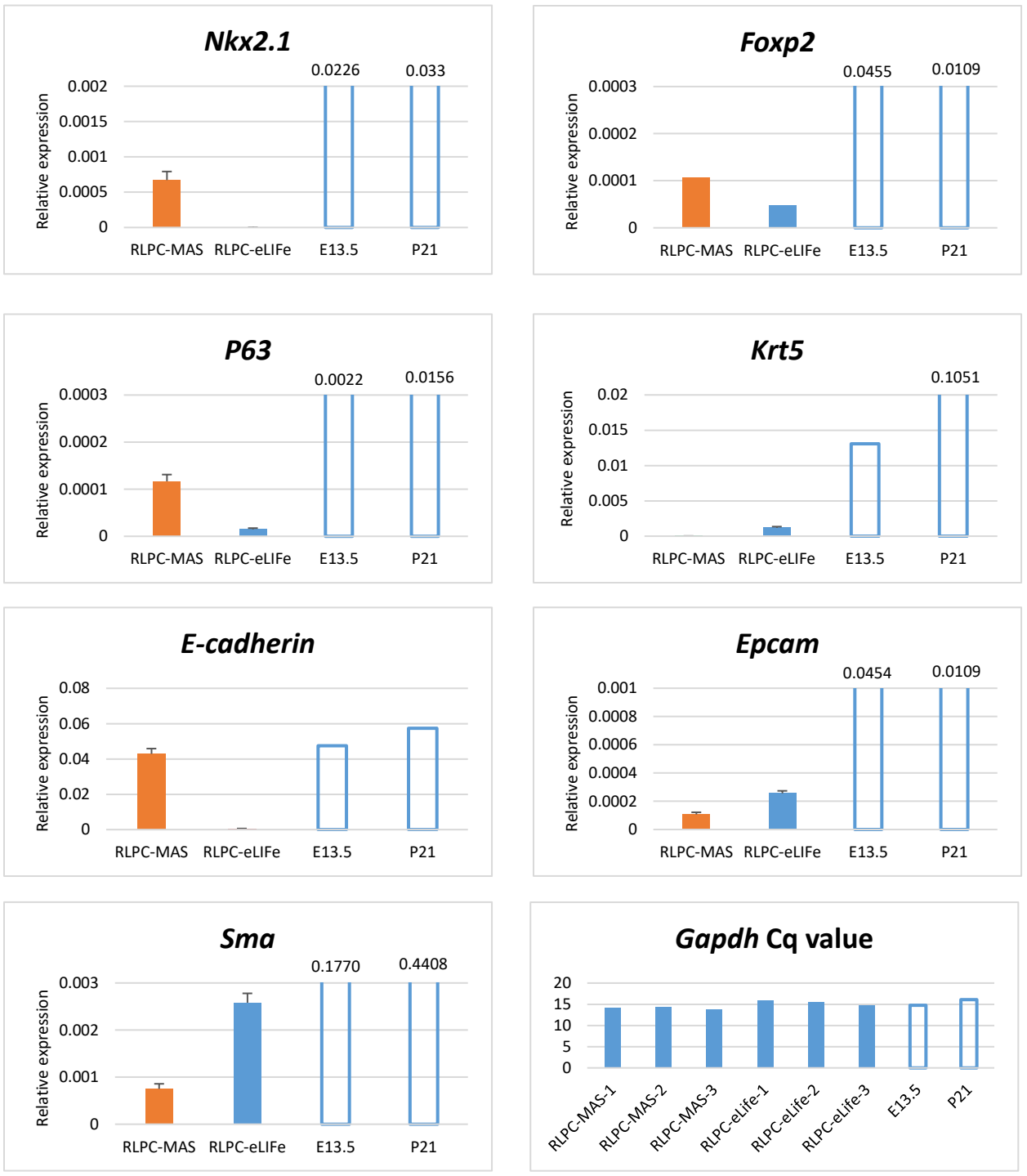
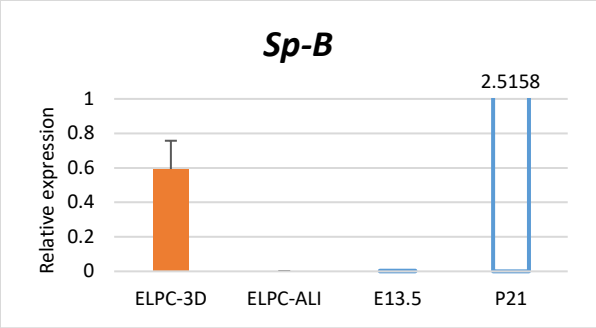
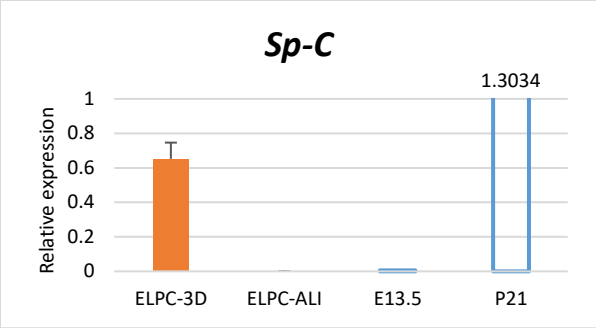
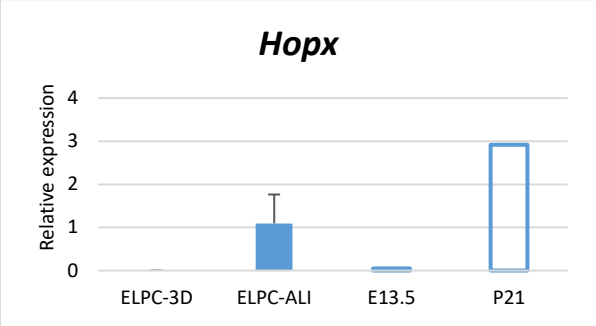
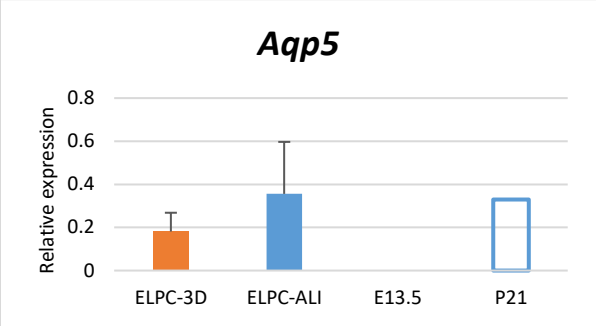


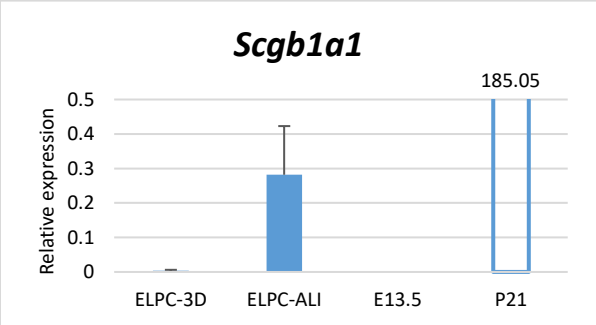
Figure6: qRT-PCR gene expression analysis of reprogrammed lung progenitor cells culturing in MAS medium or eLife medium in 3D culture including positive control, which are the embryonic lung tissue (E13.5) and adult lung tissue (Postnatal 21 days, P21). The gene markers include lung progenitor cell markers (*Nkx2.1*, *Foxp2*, *Irx2*), basal cell markers (*P63*, *Krt5*), epithelial cell markers (*E-cadherin*, *Epcam*), and lung mesenchymal cell marker (*Sma*) were analyzed. The gene expression were normalized with housekeeping gene (*Gapdh*) (n=3).

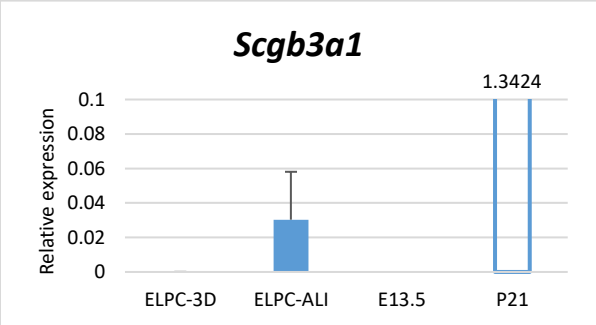

Sp-B
2.5158
Relative expression
1
0.8
0.6
0.4
0.2
0
ELPC-3D
ELPC-ALI
E13.5
P21


Sp-C
1.3034
Relative expression
1
0.8
0.6
0.4
0.2
0
ELPC-3D
ELPC-ALI
E13.5
P21


Hopx
Relative expression
4
3
2
1
0
ELPC-3D
ELPC-ALI
E13.5
P21


Aqp5
Relative expression
0.8
0.6
0.4
0.2
0
ELPC-3D
ELPC-ALI
E13.5
P21


Scgb1a1
185.05
Relative expression
0.5
0.4
0.3
0.2
0.1
0
ELPC-3D
ELPC-ALI
E13.5
P21


Scgb3a1
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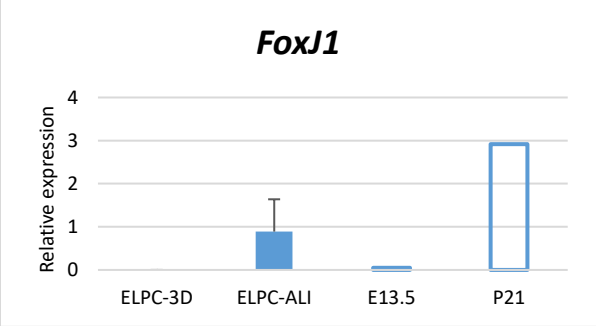
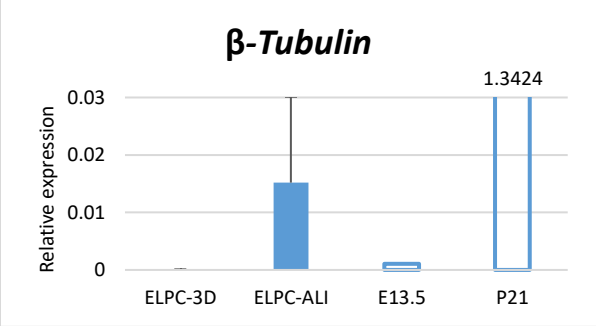
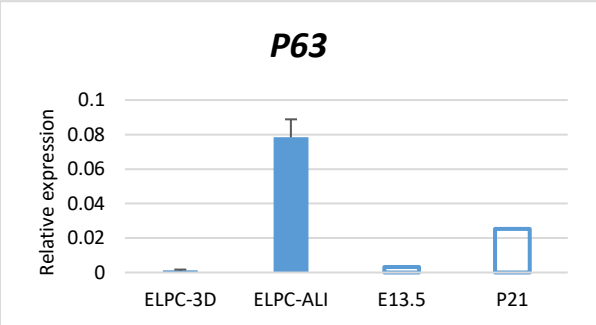
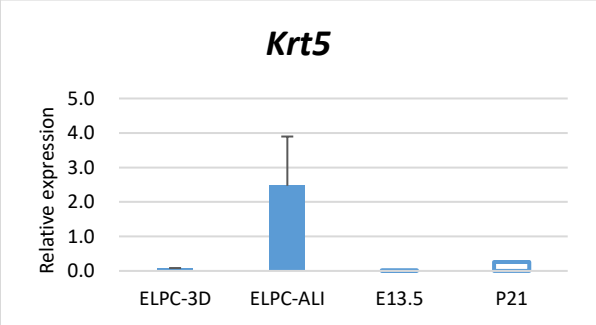
Relative expression
0.1
0.08
0.06
0.04
0.02
0
ELPC-3D
ELPC-ALI
E13.5
P21


FoxJ1
Relative expression
4
3
2
1
0
ELPC-3D
ELPC-ALI
E13.5
P21


β-Tubulin
1.3424
Relative expression
0.03
0.02
0.01
0
ELPC-3D
ELPC-ALI
E13.5
P21


P63
Relative expression
0.1
0.08
0.06
0.04
0.02
0
ELPC-3D
ELPC-ALI
E13.5
P21


Krt5
Relative expression
5.0
4.0
3.0
2.0
1.0
0.0
ELPC-3D
ELPC-ALI
E13.5
P21

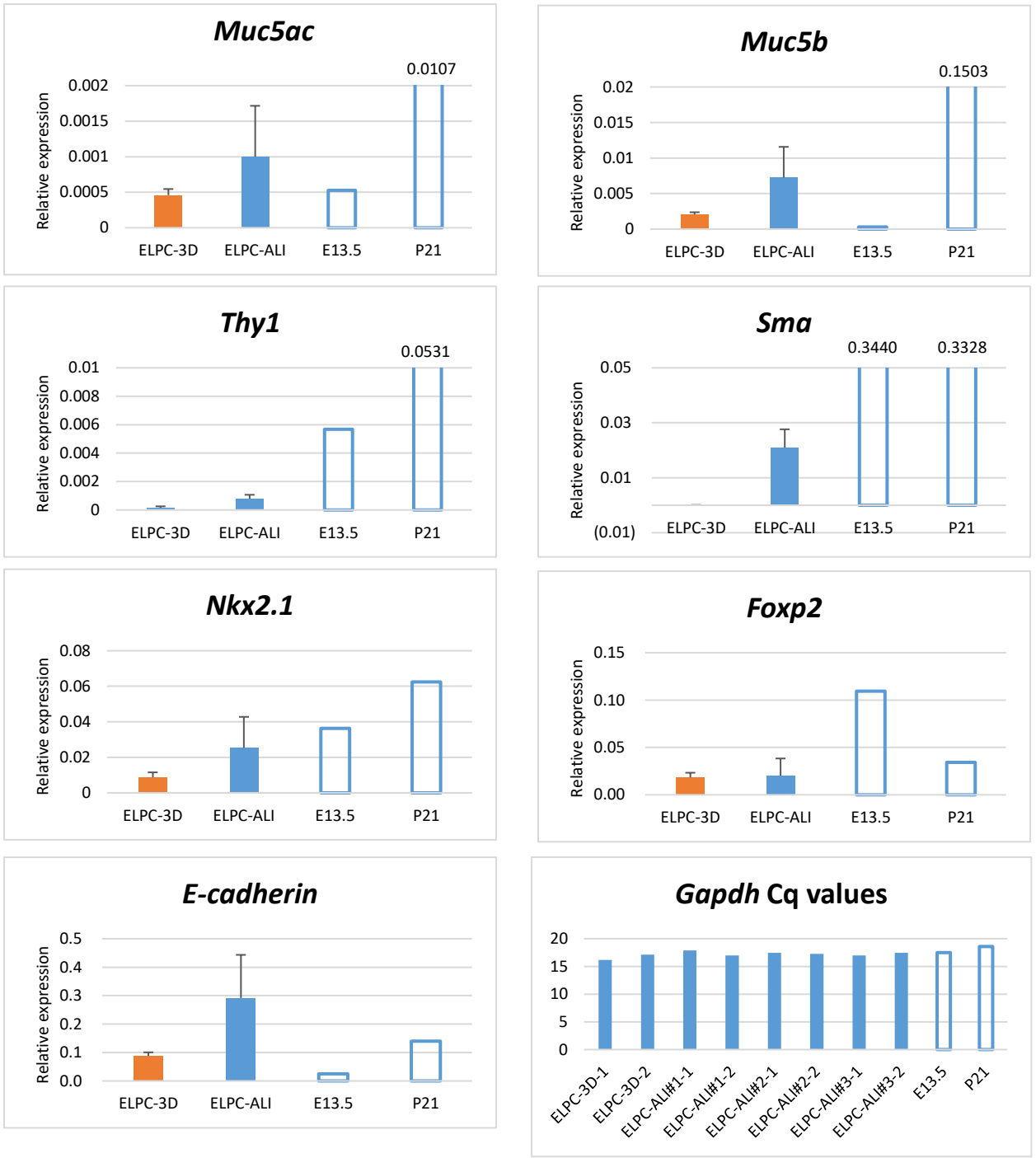
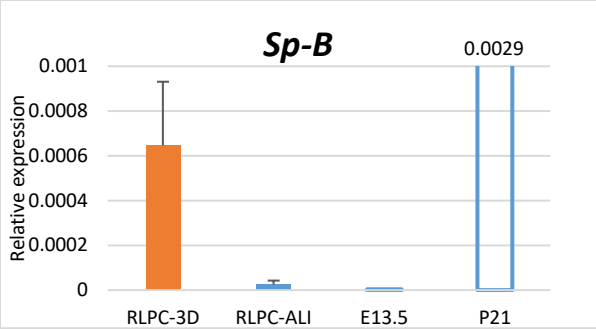
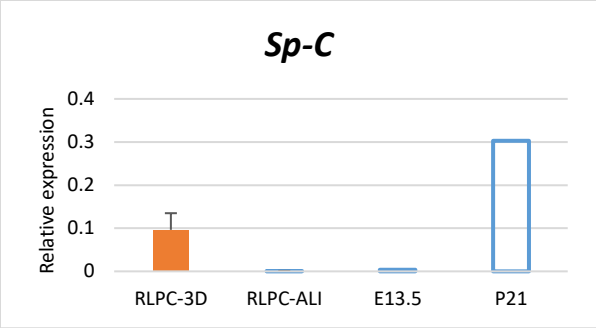
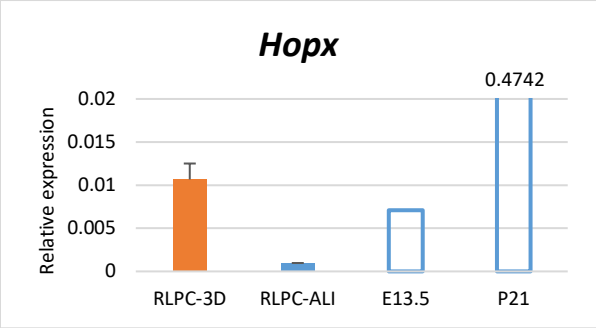
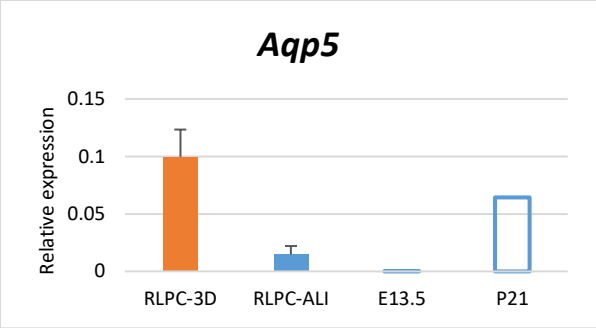


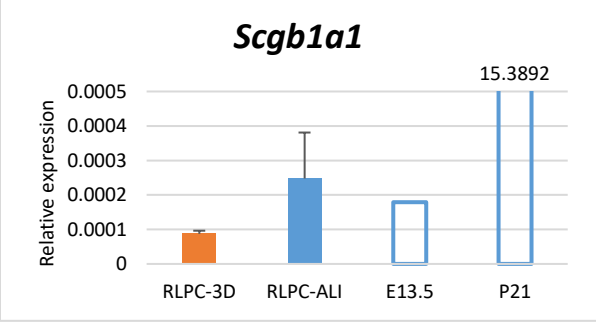
Figure7: qRT-PCR gene expression analysis of embryonic lung progenitor cells cultured (ALI) culture condition with lung progenitor cell maintenance medium (MAS) including positive control, embryonic lung tissue (E13.5), and adult lung tissue (P21). Gene marker include alveolar type II markers (*Sp-b*, *Sp-c*), alveolar type I (*Hopx*, *Aqp5*), club cell markers (*Scgb1a1*, *Scgb3a1*), ciliated cell markers (*Foxj1, β-tubulin*), goblet cell markers (*Muc5ac*, *Muc5b*), basal cell markers (*P63*, *Krt5*), lung mesenchymal cell markers (*Thy1*, *Sma*), lung progenitor cell markers (*Nkx2.1*, *Foxp2*), epithelial cell marker (*E-cadherin*) were analyzed. The gene expressions were normalized by housekeeping gene (*Gapdh*), (n=3)


Sp-B
0.0029
0.001
0.0008
0.0006
0.0004
0.0002
0
Relative expression
RLPC-3D
RLPC-ALI
E13.5
P21


Sp-C
0.4
0.3
0.2
0.1
0
Relative expression
RLPC-3D
RLPC-ALI
E13.5
P21


Hopx
0.4742
0.02
0.015
0.01
0.005
0
Relative expression
RLPC-3D
RLPC-ALI
E13.5
P21


Aqp5
0.15
0.1
0.05
0
Relative expression
RLPC-3D
RLPC-ALI
E13.5
P21


Scgb1a1
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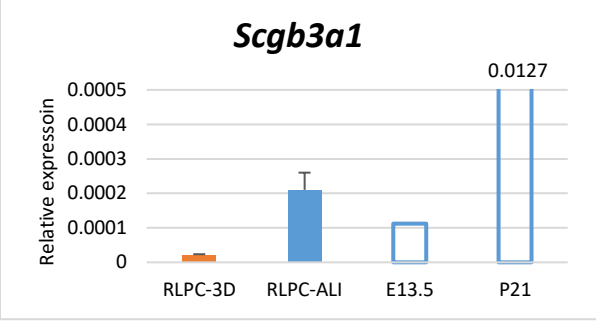
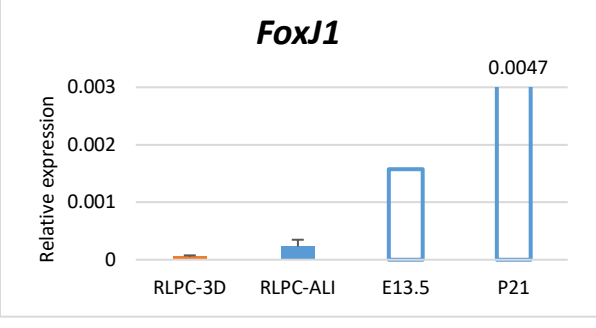
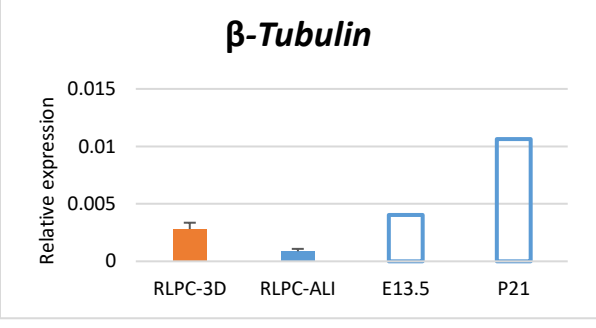
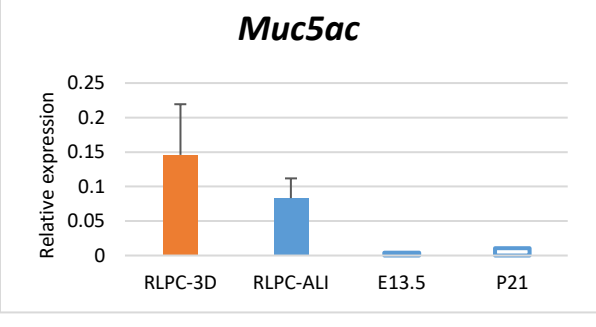
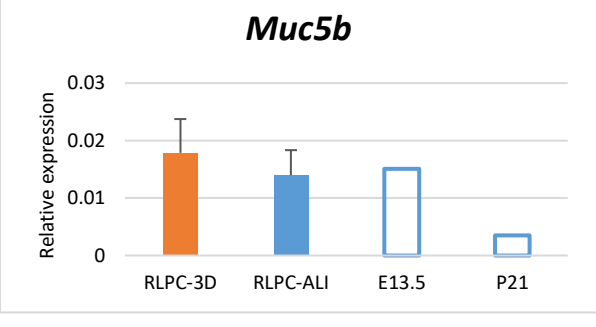
0.0004
0.0003
0.0002
0.0001
0
Relative expression
RLPC-3D
RLPC-ALI
E13.5
P21


Scgb3a1
0.0127
0.0005
0.0004
0.0003
0.0002
0.0001
0
Relative expressoin
RLPC-3D
RLPC-ALI
E13.5
P21


FoxJ1
0.0047
0.003
0.002
0.001
0
Relative expression
RLPC-3D
RLPC-ALI
E13.5
P21


β-Tubulin
0.015
0.01
0.005
0
Relative expression
RLPC-3D
RLPC-ALI
E13.5
P21


Muc5ac
0.25
0.2
0.15
0.1
0.05
0
Relative expression
RLPC-3D
RLPC-ALI
E13.5
P21


Muc5b
0.03
0.02
0.01
0
Relative expression
RLPC-3D
RLPC-ALI
E13.5
P21

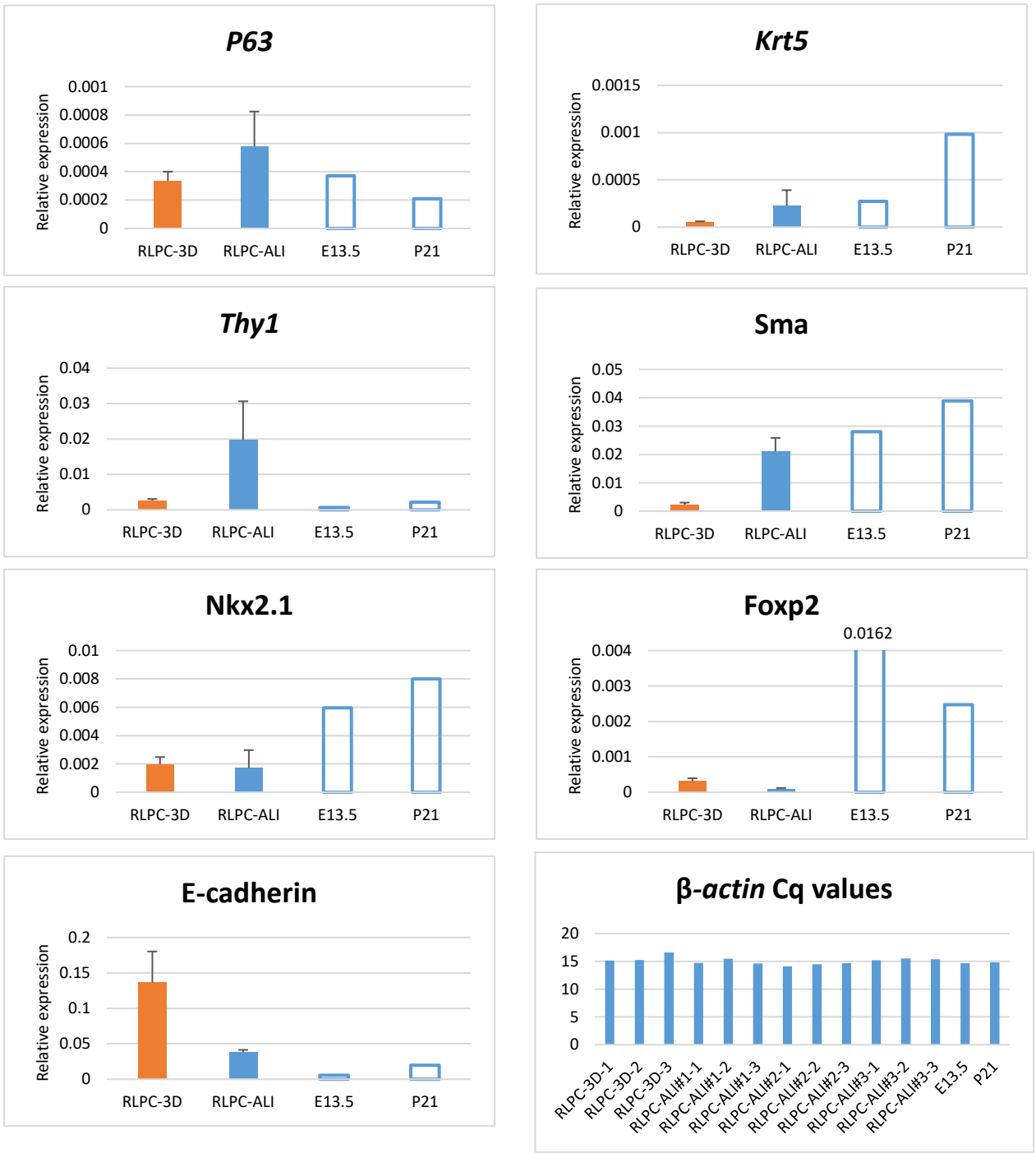
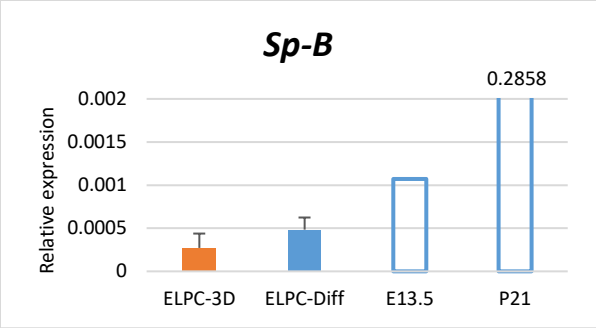
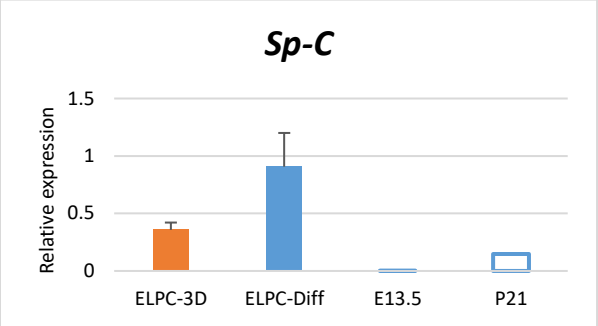
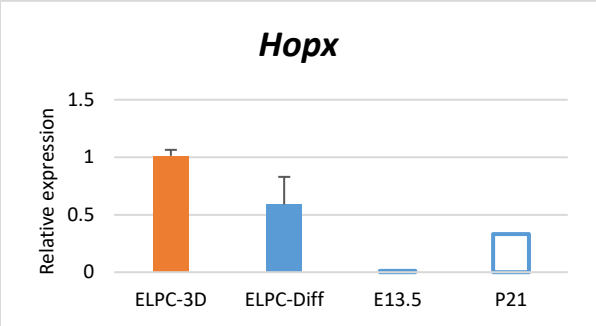
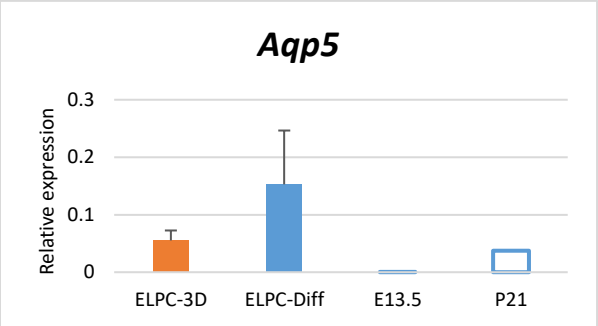


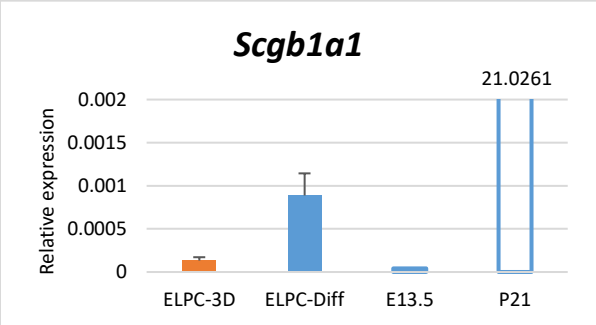
Figure8: qRT-PCR gene expression analysis of reprogrammed lung progenitor cells culturing in Air-liquid interface (ALI) culture condition with lung progenitor cell maintenance medium (MAS) including positive control, embryonic lung tissue (E13.5), and adult lung tissue (P21). Gene marker include alveolar type II markers (*Sp-b*, *Sp-c*), alveolar type I (*Hopx*, *Aqp5*), club cell markers (*Scgb1a1*, *Scgb3a1*), ciliated cell markers (*Foxj1, β-tubulin*), goblet cell markers (*Muc5ac*, *Muc5b*), basal cell markers (*P63*, *Krt5*), lung mesenchymal cell markers (*Thy1*, *Sma*), lung progenitor cell markers (*Nkx2.1*, *Foxp2*), epithelial cell marker (*E-cadherin*), which are normalized by housekeeping gene (*β-actin*) (n=3)

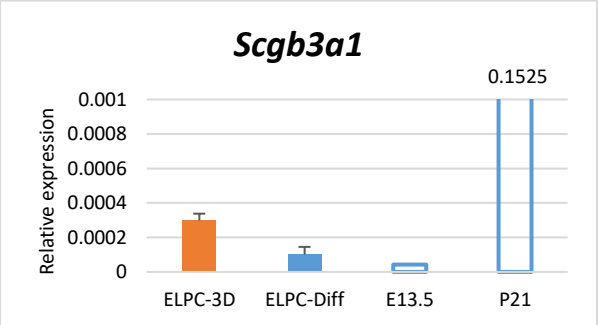

Sp-B
0.2858
0.002
0.0015
0.001
0.0005
0
Relative expression
ELPC-3D
ELPC-Diff
E13.5
P21

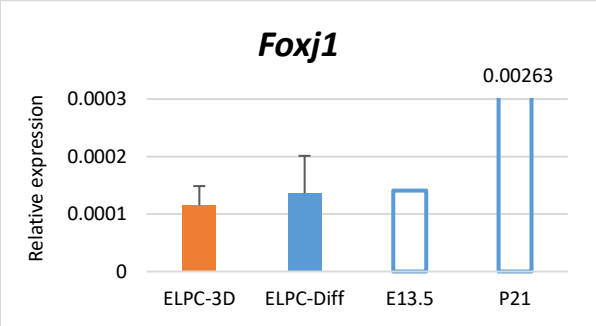

Sp-C
1.5
1
0.5
0
Relative expression
ELPC-3D
ELPC-Diff
E13.5
P21

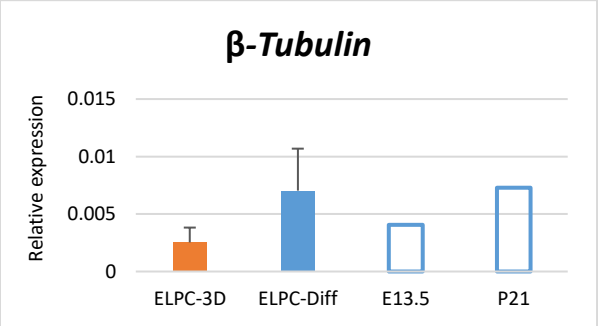

Hopx
1.5
1
0.5
0
Relative expression
ELPC-3D
ELPC-Diff
E13.5
P21

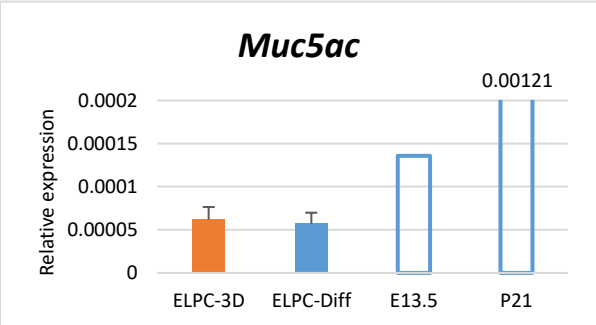

Aqp5
0.3
0.2
0.1
0
Relative expression
ELPC-3D
ELPC-Diff
E13.5
P21

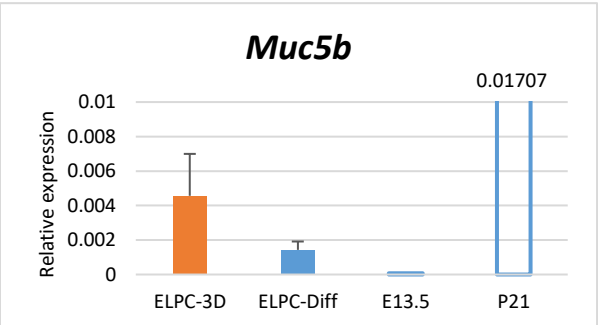

Scgb1a1
21.0261
0.002
0.0015
0.001
0.0005
0
Relative expression
ELPC-3D
ELPC-Diff
E13.5
P21


Scgb3a1
0.1525
0.001
0.0008
0.0006
0.0004
0.0002
0
Relative expression
ELPC-3D
ELPC-Diff
E13.5
P21


Foxj1
0.00263
0.0003
0.0002
0.0001
0
Relative expression
ELPC-3D
ELPC-Diff
E13.5
P21


β-Tubulin
0.015
0.01
0.005
0
Relative expression
ELPC-3D
ELPC-Diff
E13.5
P21


Muc5ac
0.00121
0.0002
0.00015
0.0001
0.00005
0
Relative expression
ELPC-3D
ELPC-Diff
E13.5
P21


Muc5b
0.01707
0.01
0.008
0.006
0.004
0.002
0
Relative expression
ELPC-3D
ELPC-Diff
E13.5
P21

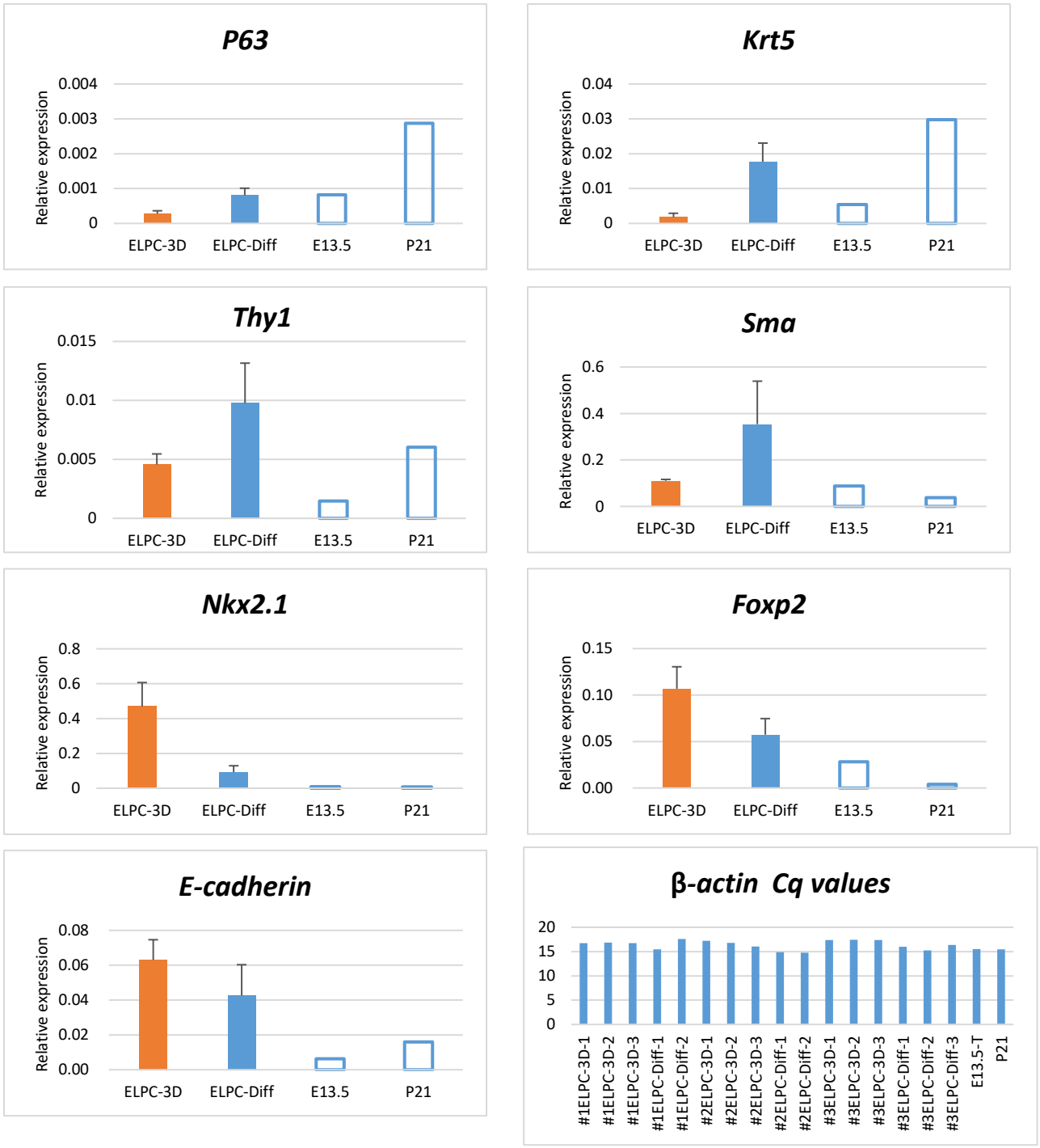
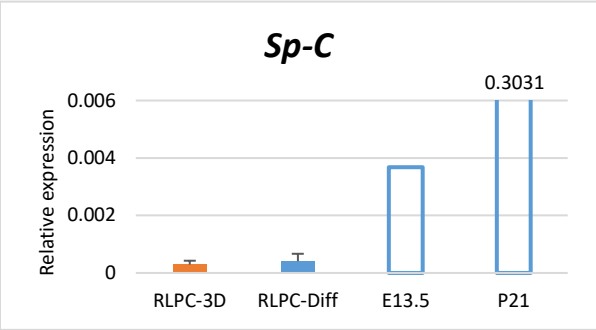
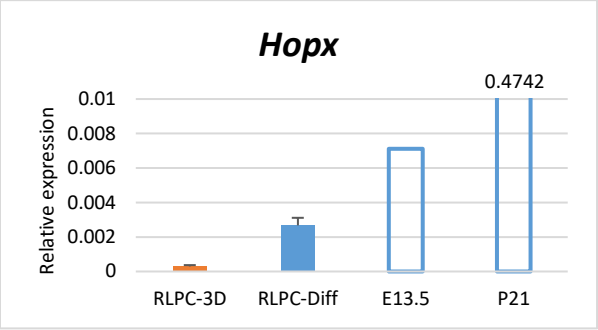
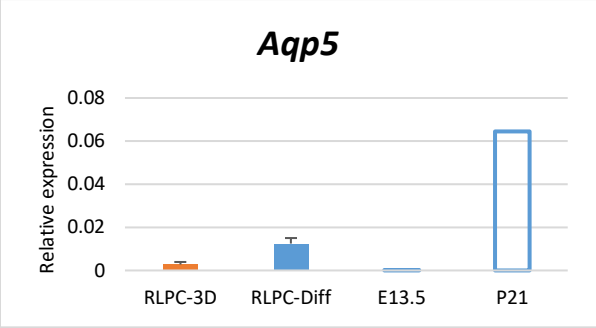
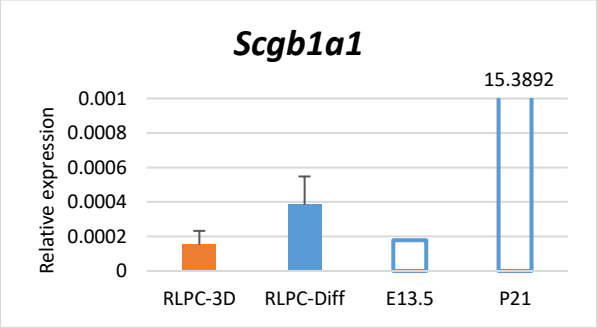


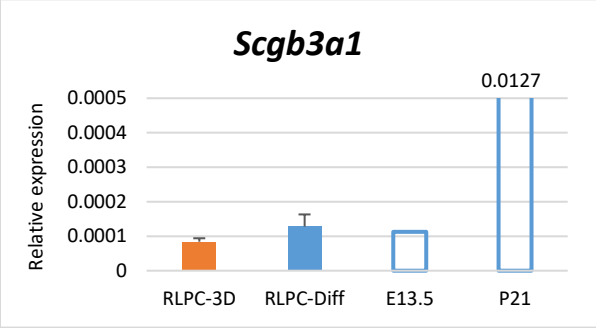
Figure9: qRT-PCR gene expression analysis of embryonic lung progenitor cells culturing in 3D Matrigel culture condition with differentiation medium. As the positive control, embryonic lung tissue (E13.5), and adult lung tissue (P21) were used. Gene marker included alveolar type II markers (*Sp-b, Sp-c*), alveolar type I (*Hopx, Aqp5*), club cell markers (*Scgb1a1, Scgb3a1*), ciliated cell markers (*Foxj1, β-tubulin*), goblet cell markers (*Muc5ac, Muc5b*), basal cell markers (*P63, Krt5*), lung mesenchymal cell markers (*Thy1, Sma*), lung progenitor cell markers (*Nkx2.1, Foxp2*), epithelial cell marker (*E-cadherin*) were analyzed. The gene expressions were normalized by housekeeping gene (*β-actin*)

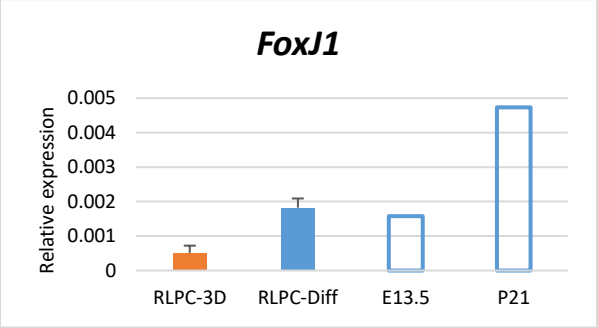

Sp-C
Relative expression
0.006
0.004
0.002
0
0.3031
RLPC-3D
RLPC-Diff
E13.5
P21

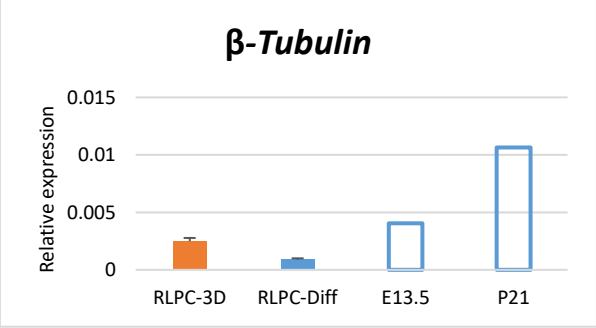

Hopx
Relative expression
0.01
0.008
0.006
0.004
0.002
0
0.4742
RLPC-3D
RLPC-Diff
E13.5
P21

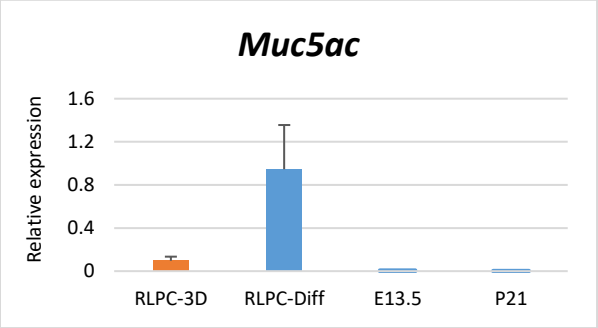

Aqp5
Relative expression
0.08
0.06
0.04
0.02
0
RLPC-3D
RLPC-Diff
E13.5
P21

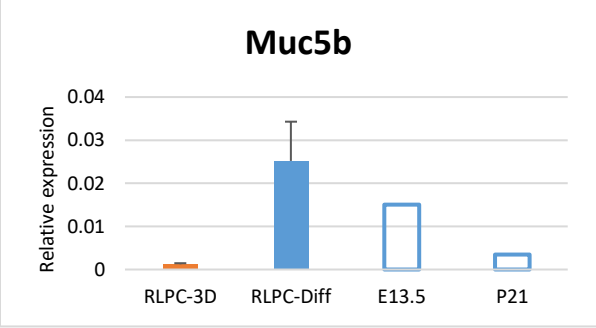

Scgb1a1
Relative expression
0.001
0.0008
0.0006
0.0004
0.0002
0
15.3892
RLPC-3D
RLPC-Diff
E13.5
P21

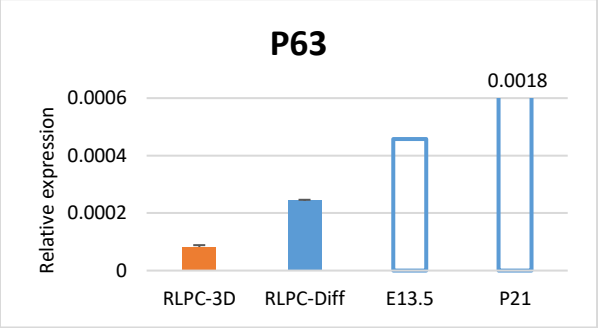

Scgb3a1
Relative expression
0.0005
0.0004
0.0003
0.0002
0.0001
0
0.0127
RLPC-3D
RLPC-Diff
E13.5
P21


FoxJ1
Relative expression
0.005
0.004
0.003
0.002
0.001
0
RLPC-3D
RLPC-Diff
E13.5
P21


β-Tubulin
Relative expression
0.015
0.01
0.005
0
RLPC-3D
RLPC-Diff
E13.5
P21


Muc5ac
Relative expression
1.6
1.2
0.8
0.4
0
RLPC-3D
RLPC-Diff
E13.5
P21


Muc5b
Relative expression
0.04
0.03
0.02
0.01
0
RLPC-3D
RLPC-Diff
E13.5
P21


P63
Relative expression
0.0006
0.0004
0.0002
0
0.0018
RLPC-3D
RLPC-Diff
E13.5
P21

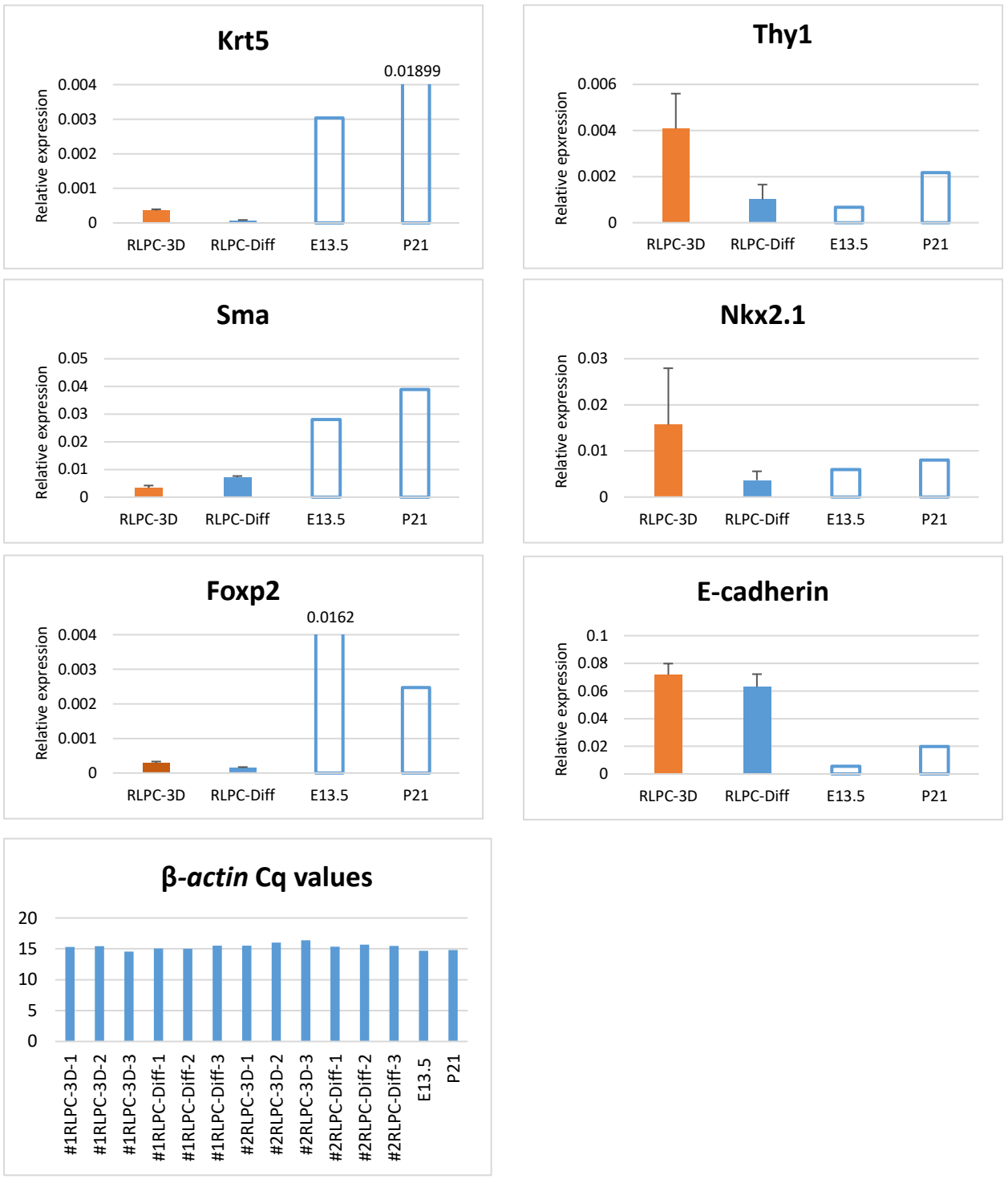
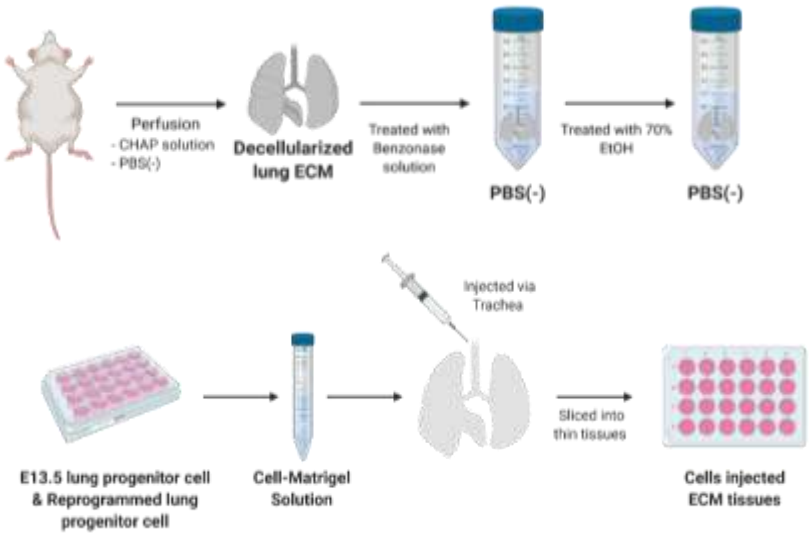


Figure10: qRT-PCR gene expression analysis of reprogrammed lung progenitor cells differentiated from the reprogrammed lung progenitor cell organoids in 3D Matrigel culture condition with differentiation medium. As for the positive control, embryonic lung tissue (E13.5), and adult lung tissue (P21) were used. Gene markers included alveolar type II markers (*Sp-b*, *Sp-c*), alveolar type I (*Hopx*, *Aqp5*), club cell markers (*Scgb1a1*, *Scgb3a1*), ciliated cell markers (*Foxj1, β-tubulin*), goblet cell markers (*Muc5ac*, *Muc5b*), basal cell markers (*P63*, *Krt5*), lung mesenchymal cell markers (*Thy1*, *Sma*), lung progenitor cell markers (*Nkx2.1*, *Foxp2*), epithelial cell marker (*E-cadherin*) were analyzed. The gene expressions were normalized by housekeeping gene (*β-actin*) (n=3)

(A)



(B)

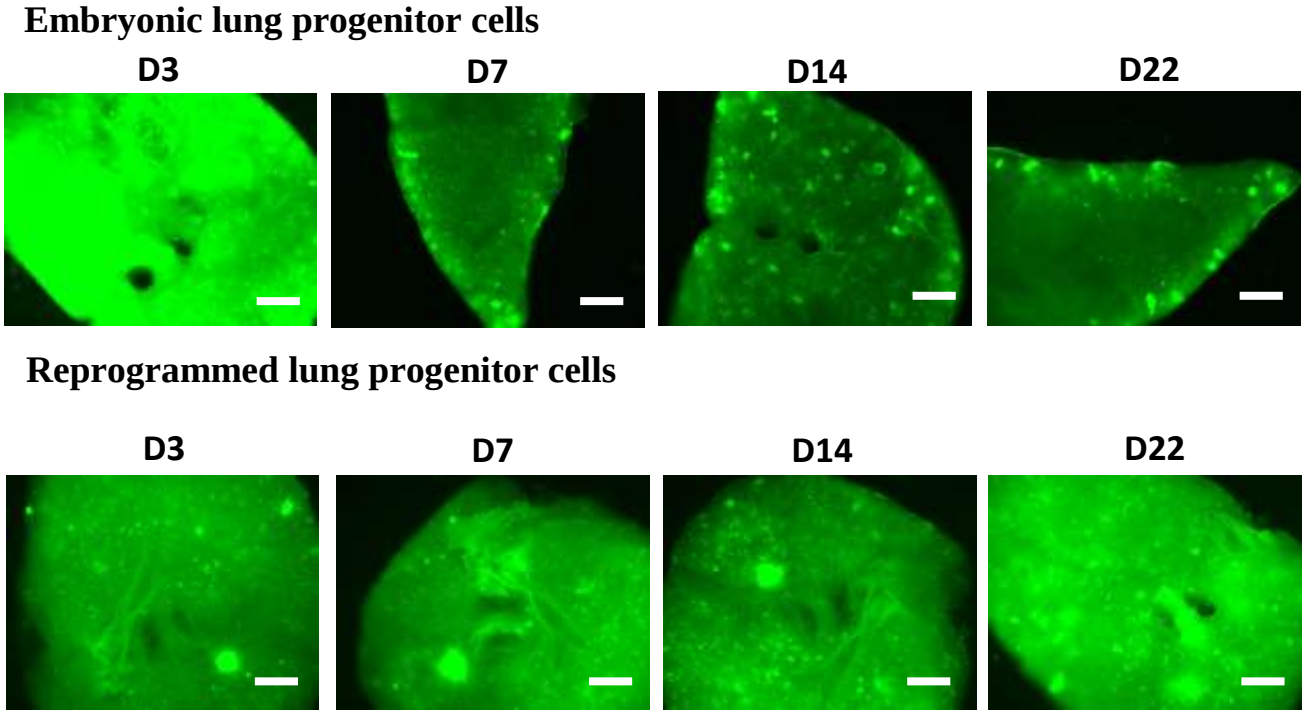
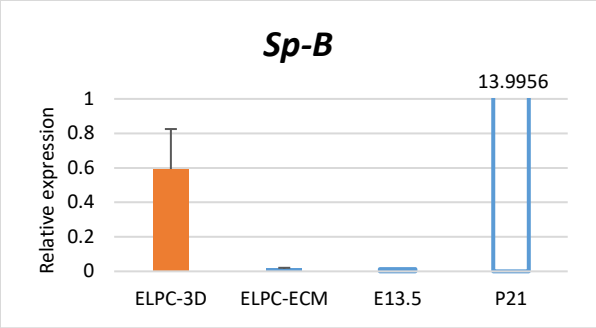
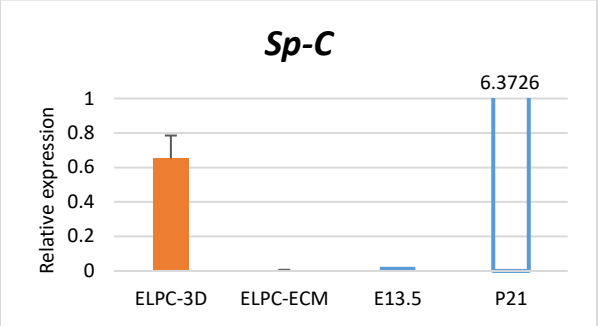
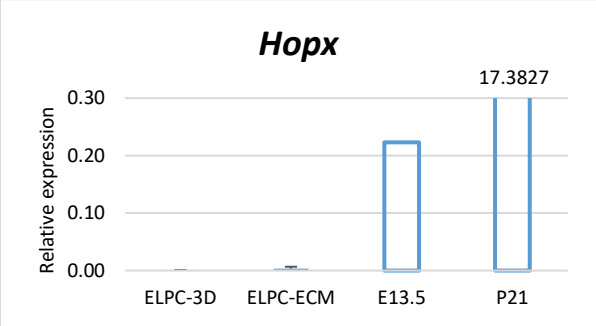
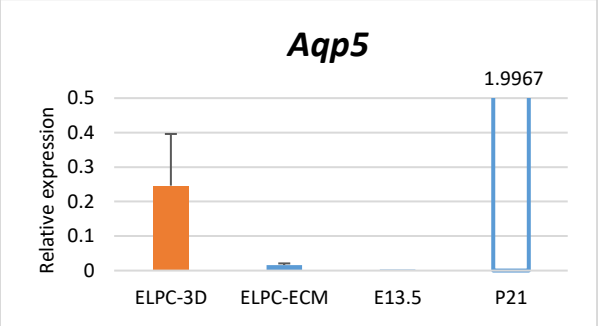


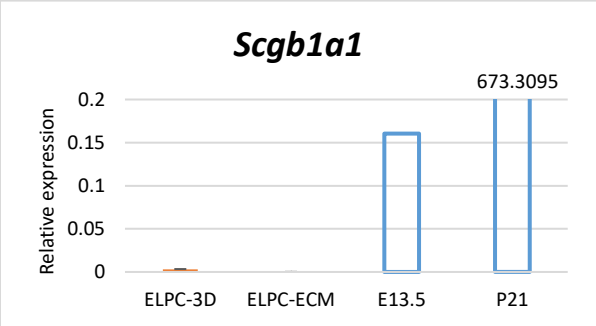
Figure11: Differentiation of embryonic lung progenitor cells and reprogrammed lung progenitor cells by using decellularized lung progenitor cells. (A) A schematic of experiment. Preparation of the decellularized lung by using CHAP detergent solution followed by Benzonase treatment. The decellularized matrix were treated sterilized with 70% EtOH before soaked in PBS. Embryonic lung progenitor cells and reprogrammed lung progenitor cells were injected into decellularized ECM through trachea and cut into small tissues section. The sections were cultured in a 24 well-plate with MAS medium for 7 days. Then, the medium was changed to differentiation medium and cultured for 3 weeks. (B) The observation of GFP^+ lung progenitor cells and GFP^+ reprogrammed lung progenitor cells injected into ECM which was cultured in differentiation medium on day3, 7 ,14, and 22. Scale bar = 500 µm

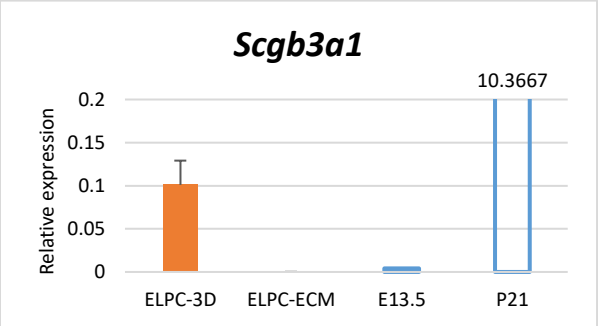

Sp-B
13.9956
Relative expression
1
0.8
0.6
0.4
0.2
0
ELPC-3D
ELPC-ECM
E13.5
P21

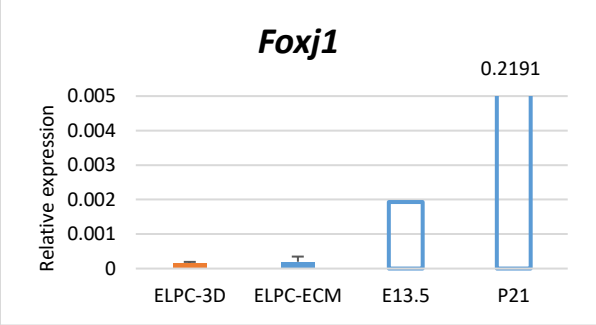

Sp-C
6.3726
Relative expression
1
0.8
0.6
0.4
0.2
0
ELPC-3D
ELPC-ECM
E13.5
P21

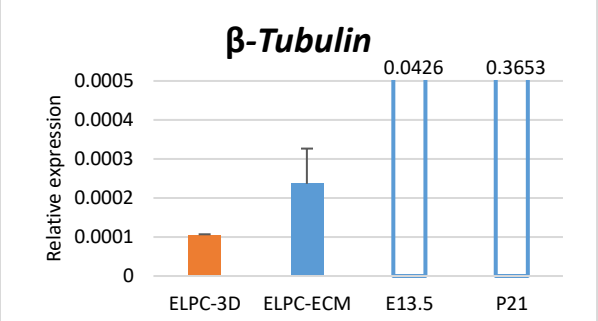

Hopx
17.3827
Relative expression
0.30
0.20
0.10
0.00
ELPC-3D
ELPC-ECM
E13.5
P21

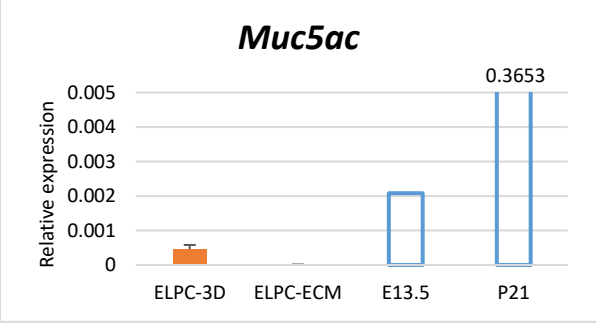

Aqp5
1.9967
Relative expression
0.5
0.4
0.3
0.2
0.1
0
ELPC-3D
ELPC-ECM
E13.5
P21

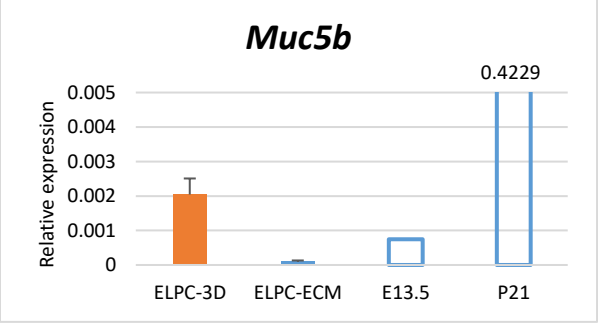

Scgb1a1
673.3095
Relative expression
0.2
0.15
0.1
0.05
0
ELPC-3D
ELPC-ECM
E13.5
P21


Scgb3a1
10.3667
Relative expression
0.2
0.15
0.1
0.05
0
ELPC-3D
ELPC-ECM
E13.5
P21


Foxj1
0.2191
Relative expression
0.005
0.004
0.003
0.002
0.001
0
ELPC-3D
ELPC-ECM
E13.5
P21


β-Tubulin
0.0426
0.3653
Relative expression
0.0005
0.0004
0.0003
0.0002
0.0001
0
ELPC-3D
ELPC-ECM
E13.5
P21


Muc5ac
0.3653
Relative expression
0.005
0.004
0.003
0.002
0.001
0
ELPC-3D
ELPC-ECM
E13.5
P21


Muc5b
0.4229
Relative expression
0.005
0.004
0.003
0.002
0.001
0
ELPC-3D
ELPC-ECM
E13.5
P21

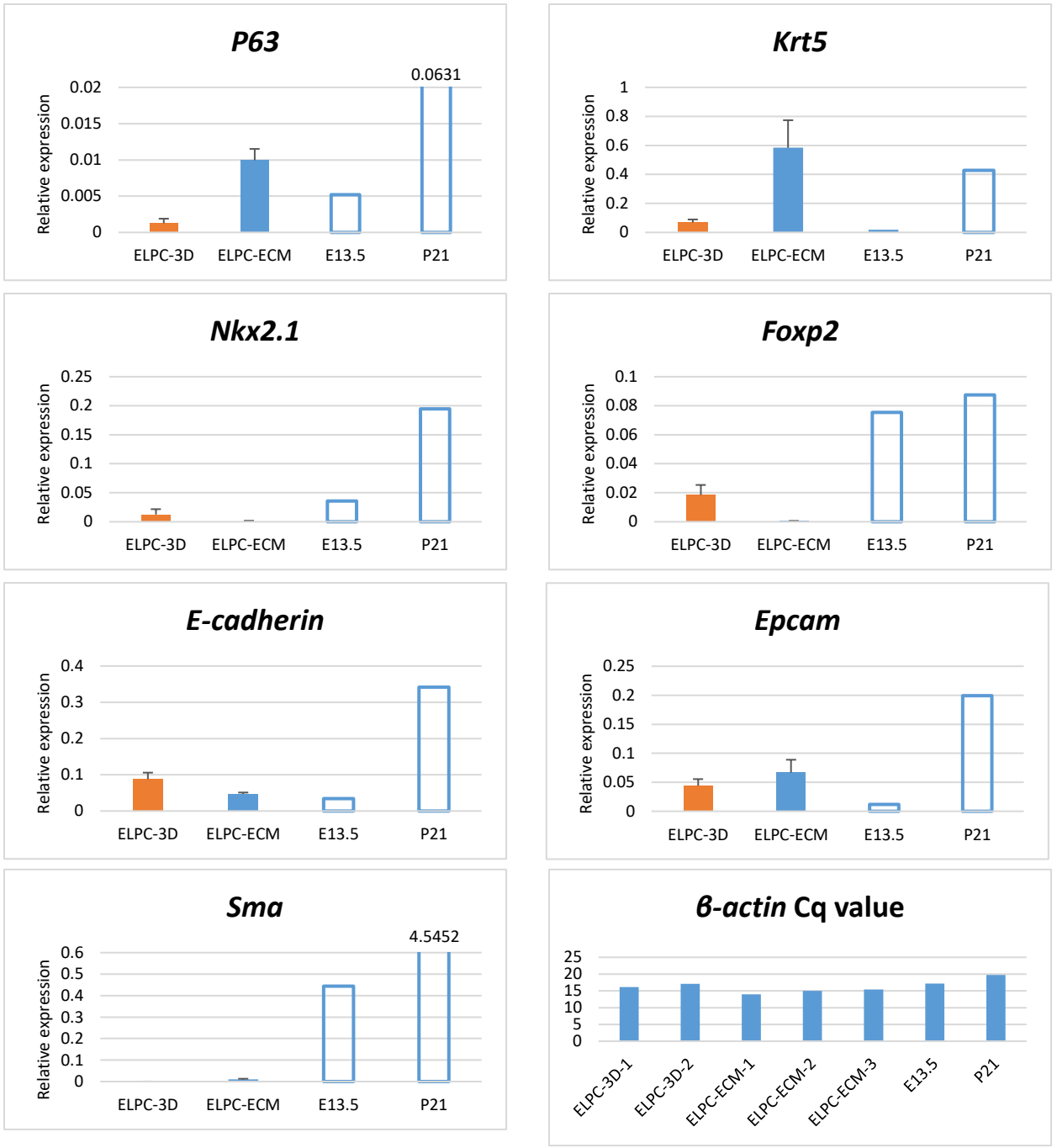


Figure12: Gene expression of embryonic lung progenitor cells differentiated in decellularized lung ECM culture condition with differentiation medium. As for positive control, embryonic lung tissue (E13.5), and adult lung tissue (P21) were used. Gene markers included alveolar type II markers (*Sp-b*, *Sp-c*), alveolar type I (*Hopx, Aqp5*), club cell markers (*Scgb1a1*, *Scgb3a1*), ciliated cell markers (*Foxj1, β-tubulin*), goblet cell markers (*Muc5ac*, *Muc5b*), basal cell markers (*P63*, *Krt5*), lung mesenchymal cell marker (*Sma*), lung progenitor cell markers (*Nkx2.1*, *Foxp2*), epithelial cell marker (*E-cadherin*, *Epcam*) were analyzed. The gene expressions were normalized by housekeeping gene (*β-actin*) (n=2)

Discussion

Based on the previous studies in our laboratory, we could generate reprogramming of lung progenitor cells from mouse dermal fibroblast (MDF) by infecting retrovirus containing 5 transcription factors (SOX21, FOXA2, IRX3, GATA6, and N-MYC). The reprogrammed lung progenitor cells were recognized by expression of lung progenitor cells specific marker, Nkx2.1. However, the expression level of the Nkx2.1 in our reprogrammed cells were considerably low reflecting the low efficiency of reprogramming process. In this study, I investigated the possibility to increase the expression of Nkx2.1 expression by modifying culture conditions for the progenitor cells. To do this, I examined two previously published candidate culture mediums developed by Nikolic et al [24], and Massimo et al [23], which I called 'eLife medium' and 'MAS medium' respectively.

I firstly examined the efficiency of both lung progenitor cells maintenance mediums by the embryonic lung progenitor cells (E13.5). These cells were cultured in 3D Matrigel conditions for 7 days. Then, the cells were passaged to second passage and further cultured for 7 days. Finally, the cells were collected for the analysis. Based on the morphology of the cells that cultured in the different medium conditions, the cells cultured in MAS medium could generate several organoids on second day and those organoids could grow bigger and the number were also increased when further culture until day seventh. On the other hand, the cells cultured with eLife medium have failed to generate organoids which was not consistent with result reported on their papers [24]. qRT-PCR results of this experiment also confirmed the morphological result in which expression of lung progenitor cell markers (Nkx2.1, Foxp2), lung epithelial cell markers (E-cadherin, Epcam), and basal cell markers (P63, Krt5) were highly increased in the cells that cultured in MAS medium around 10 times more comparing to the cells that were cultured in eLife medium.

I also examined and confirmed ability of these lung progenitor cell mediums with our reprogrammed cells with almost the same condition with the previous embryonic lung progenitor cell experiment. After cultured for 3 to 4 weeks after infection, the cells that were cultured with MAS medium could generate some small organoids and those organoids were increased in both size and number when further cultured while the cells that were cultured with eLife medium still failed to generate any organoids. The qRT-PCR results were also consistent with previous experiment which lung progenitor cell markers (Nkx2.1, Foxp2), epithelial cell markers (E-cadherin, Epcam) and basal cell marker (P63) of the cells that were cultured with MAS medium were higher around 2 to 100 times more comparing with the cells that were cultured with the eLife medium.

The reason might come from the different in component of the mediums. Even, MAS medium and eLife medium shared several same components, such as growth factors, Wnt signal activator, TGFβ inhibitor. However, the differences are the BMP signal inhibitor which was added in the eLife medium, and p38 kinase inhibitor which was added in MAS medium. Based on several published papers [32], BMP signal is required for the respiratory endoderm progenitor

fate, which is expressed in ventral mesenchyme surrounding around the anterior foregut. Loss of the BMP signal can affect the trachea agenesis and retention of branching region of the lungs *in vivo*. It was also reported that BMP signal also regulated FGF10 expression, which required for the branching of the lung.

Next, I examine the differentiation ability of the embryonic lung progenitor cells and reprogrammed lung progenitor cells by using the different culturing methods. Based on several published papers [25-27], air liquid interface (ALI) culture were frequently used to induce the differentiation of the ciliated cell, and various lung airway epithelial cells. I observed the induction of several lung tissue cell markers, which we could observe the differentiation of several genes including alveolar type I cells (*Hopx, Aqp5*), epithelial cell (*E-cadherin, Epcam*), ciliated cells (*Foxj1*, β-*TubulinIV*), goblet cells (*Muc5ac, Muc5b*), basal cells (*P63, Krt5*), lung mesenchymal cells (*Sma, Thy1*), and lung progenitor cells (*Nkx2.1, Foxp2*). However, only alveolar type II cells (*Sp-B, Sp-C*), and club cells (*Scgb1a1, Scgb3a1*) were observed at very low level in this culture conditions. These results also partially consistent with previously published paper [23, 27], which they could induced ciliated cells, goblet cells, and basal cells but not club cells and alveolar epithelial cells in ALI culture condition. When comparing these gene expression results of samples with the adult lung, I found that alveolar type I, goblet cells, epithelial cells, basal cells, lung progenitor cells, and lung mesenchymal cells were highly observed due to the 10 times difference in gene expression. I also examined the reprogrammed lung progenitor cells with the same condition. The results showed the same tendency with the embryonic lung progenitor cells. I found that our reprogrammed cells have failed to differentiate to the alveolar type II cells, club cells, and ciliated cells due to the difference in gene expression level.

I also examined the differentiation ability of those cells with the 3D Matrigel culture conditions. Previously published papers [21-24], reported that lung epithelial cell markers including alveolar epithelial cells, basal cells, goblet cells were highly induced in 3D culture conditions with the designed differentiation media. Alveolar type I and type II cells, and basal cells were differentiated but not ciliated cells, goblet cells, and club cells at very low expression level. These results were also consistent with previously published papers [31], which they induced the expression of alveolar type cells in 3D culture condition rather than club cells. As for the reprogrammed lung progenitor cells differentiated with the same 3D culture conditions, alveolar type I cell, ciliated cells but not the alveolar type II cells, club cells and basal. Based on my results, my reprogrammed cells have failed to differentiate the alveolar type II cells, club cells, and ciliated cells, which isn't consistent with the embryonic lung progenitor cells since embryonic lung progenitor cells could differentiate ciliated cells. The reasons why my reprogrammed cells couldn't differentiate into alveolar type II cells might come from the conditions of my reprogrammed cells. In this experiment for the embryonic lung progenitor cells, I confirmed that ALI culture condition could induce the differentiation of the almost all of the lung tissue cells including ciliated cells which were mostly reported. Then, in the 3D culture condition, I also confirmed that this condition could induced the differentiation of alveolar type I cell and alveolar type II cells. Despite, these results were not consistent with the embryonic lung progenitor cells result.

In this experiment for the reprogrammed cells, I used second passaged reprogrammed cells which most of the cells might already be converted into lung progenitor cells. However, there were some of the cells that still be fibroblasts. The presence of the fibroblast could affect the quality of the reprogrammed lung progenitor cells and also the differentiation efficiency. The interaction of the mesenchymal cells and alveolar type II cells regulate the alveologenesis, in which activation of the BMP4 signal or addition of BMP4 into the medium could inhibit the alveolar type II cell proliferation and promote their differentiation. Since in our medium, we didn't add BMP4 inhibitor, so this might be one of the reasons that our reprogrammed cells couldn't differentiated into alveolar type II cells in both ALI culture and 3D Matrigel culture condition.

Finally, I used the decellularized lung progenitor cells to differentiate embryonic lung progenitor cells and my reprogrammed lung progenitor cells. For the embryonic lung progenitor cells, after I injected cells into the ECM and cultured for 2-3 days, the amount of the GFP^+cells were extremely increased. However, after cultured for long period of time, the GFP^+signal were gradually decreased and almost no GFP signal left on the collection days which mean the cells inside ECM gradually died when cultured for long time under these conditions. However, for the reprogrammed lung progenitor cells, the GFP^+signal didn't increase after seeding the cells like embryonic lung progenitor cells and the signal intensity remained the same until the collection day indicating that there was almost no proliferation of the cells inside the ECM. This could happen because of two reasons. First, the number of the reprogrammed cells that injected in this experiment were quite low comparing with the amount of embryonic lung progenitor cells. Another reason was the conditions of the reprogrammed cells. I also examined the differentiation ability by using gene expression analysis by qRT-PCR. Only basal cells were observed as differentiated cells, which isn't consistent with previously published paper [28-30]. For the reprogrammed lung progenitor cells, I couldn't extract enough RNA. So, I couldn't analyze these. These results were just only the preliminary data that were done one time.

Finally, based on these results, each of the cultivation condition could induce different type of cells (Table 4). As shown in the table, air-liquid interface culture conditions could induce several airway epithelial cells but not the alveolar type II cell. On the other hand, 3D Matrigel culture method could induce the alveolar cells. These results are also consistent with the previously published papers. As for the reprogrammed cells, they gave difference results showing that our reprogrammed cells were still lacked of ability to differentiation when comparing with embryonic lung progenitor cells in all conditions. This might cause by the incomplete in reprogramming process since I used the early passage of reprogrammed cells (P2), which mean most of the fibroblasts were still presented among the cultured cells which may affect the differentiation process.

Table 4; Summary of the differentiation experiment

Type of cells	Air-Liquid Interface		3D Matrigel		Lung ECM	
	Embryonic LPC	Reprogrammed LPC	Embryonic LPC	Reprogrammed LPC	Embryonic LPC	Reprogrammed LPC
Alveolar type I cells	✓	✓	✓	✗	✗	–
Alveolar type II cells	✗	✗	✓	✓	✗	–
Club cells	✗	✗	✗	✗	✗	–
Ciliated cells	✓	✗	✗	✓	✗	–
Goblet cells	✓	✓	✗	✓	✗	–
Basal cells	✓	✓	✓	✗	✓	–
Lung mesenchymal cells	✓	✓	✓	✓	✗	–
Lung progenitor cells	✓	✓	✓	✓	✗	–

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