eISSN 2005-5447
International Journal of Stem Cells Vol. 17, No. 2, 2024 https://doi.org/10.15283/ijsc24041

SPECIAL REPORT

Guidelines for Manufacturing and Application of Organoids: Lung

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The objective of standard guideline for utilization of human lung organoids is to provide the basic guidelines required for the manufacture, culture, and quality control of the lung organoids for use in non-clinical efficacy and inhalation toxicity assessments of the respiratory system. As a first step towards the utilization of human lung organoids, the current guideline provides basic, minimal standards that can promote development of alternative testing methods, and can be referenced not only for research, clinical, or commercial uses, but also by experts and researchers at regulatory institutions when assessing safety and efficacy.

Keywords: Organoid Standards Initiative, Human lung, Lung organoid

Received: April 8, 2024, Revised: May 3, 2024, Accepted: May 7, 2024, Published online: May 23, 2024

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Introduction

As the usage of lung organoid has widely expanded globally, associated technologies have been intensively developed for manufacturing advanced human lung biomimetics - materials or systems that mimic the structure or function of the human lung. Nevertheless, to accurately assess the toxicity and efficacy of drugs, there is an urgent need to prepare more efficient, transferable, reproducible manufacturing methods and quality control standards for the lung organoids.

Pulmonary inhalation toxicity has been mostly assessed by testing methods that are developed based on animal experiments. However, the respiratory systems of human and rodent show considerable differences in terms of not only the overall anatomical structure, such as length or surface area of airway, but also cellular composition and physiological function at the level of the terminal bronchioles (1-6). Hence, limitations remain when assessing inhalation toxicity based only on laboratory animals. Due to a recent change in policies such as a ban on the use of animal models for development of cosmetics in Europe, U.S. FDA Modernization Act, and legislation of the Animal Replacement Test Act in Korea, there is a need to develop standardized human lung organoid-based toxicity assessment methods to replace animal tests.

Humans and primates are known to have self-renewing stem/progenitor cells residing in the terminal bronchioles, which are not found in laboratory rodents (7, 8). This suggests that there must be differences in regeneration and mechanisms following lung injury between human and rodents. By using lung organoids derived from human-derived adult stem cells (ASCs) or pluripotent stem cells, it would lead to obtain efficient, transferable, reproducible, and reliable toxicity test results that are much closer to human (9-12). However, due to differences among batches and culture methods used in various research groups, although their high usage of the human lung organoid system has been previously suggested, there have been limitations in their use for clinical trials and commercial purposes. Therefore, for the rapid commercialization and clinical utilization of the human lung organoid techniques (13), it is essential to establish common quality control protocols for the standardization of lung organoids sourced from diverse origins. Against this backdrop, these guidelines aim to outline the minimum criteria for ensuring the quality of lung organoids, drawing from the current technical standards. These guidelines can be improved and developed through further technical advances and comparative studies.

Considerations and Scope of Applications

General principles (general considerations)

This guideline is intended to present standardized assessment criteria or recommendations for each step of the standardization, including sourcing and origins of the initial cells, manufacture and quality control, and testing methods using the human organoids. At the end, we will have completed the detailed guidelines for the manufacture and assessment of the human lung organoids for the purpose of respiratory inhalation toxicity testing, and proposed a standardized draft that can be referenced during the assessment of lung organoid quality and function.

Scope

This guideline provides criteria to assess lung organoids that have been manufactured, using human ASCs or pluripotent stem cells, to a level equivalent to or consistent with cells from actual lung tissue, through flexibly differentiation culture conditions and a series of culture stages. The ultimate goal is to propose the guideline for the manufacture and assessment of the human lung organoids that are suitable for conducting respiratory inhalation toxicity tests in the future. We have excluded traditional two-dimensional cell culture methods, which were previously adopted often for the culture of lung cancer cell lines, such as CA549 or CALU-1, 3 or similar immortalized cell lines.

Any approaches that increase the diversity of cell types and enhance functional maturity by applying specific engineering techniques, such as lung organoid-based tissue composites, including devices and substances other than cells and supporting extracellular matrix (ECM), such as organ-on-a-chip or three-dimensional (3D) bio-printed tissue composites, have been omitted from these guidelines.

Definition of Terms

-Embryonic stem cells (ESCs): Cells derived from embryos around 5 days after fertilization, immediately before implantation, that can develop into all three germ layers, and are even capable of proliferating even in an undifferentiated state.

-ASCs: Stem/progenitor cells residing in multiple organs or differentiated tissues with a limited capacity to self-replicate or differentiate to downstream cell types.

-Lung progenitor cells: progenitor cells capable of differentiating into various epithelial cells that comprise airway and alveoli in a lung.

-Alveolar type 2 (AT2) cells: Cuboidal epithelial cells with lamellar bodies in their cytoplasm and microvilli

on their apical cell membrane, located in distal lung alveoli that secrete surfactant to reduce surface tension and promote alveolar expansion.

-Airway epithelial cells: Diverse types of airway epithelial cells are found throughout trachea, bronchi, and bronchioles, including basal cells and secretory cells. These cells generally maintain an appropriate level of moisture at the air-liquid interface (ALI), remove inhaled particles through ciliary movement, and secrete mucus to form a mucociliary barrier to pathogens and other exposed substances. Some airway cells differentiate into several other types of airway cell to assist regeneration.

-Air liquid interface (ALI) culture: A culture system in which cells or tissues are attached on top of an insert membrane allowing them to directly contact the air, while interacting with the culture medium through the membrane (14, 15).

General Articles

Cell source

ASCs:

a. Cell types and characteristics

In human, adult lung stem/progenitor cells can be classified based on their location within lungs, either in the distal or proximal regions. From developmental and anatomical perspectives, the distal area of the human lungs comprises alveolar cells–especially AT1 and AT2 cells,

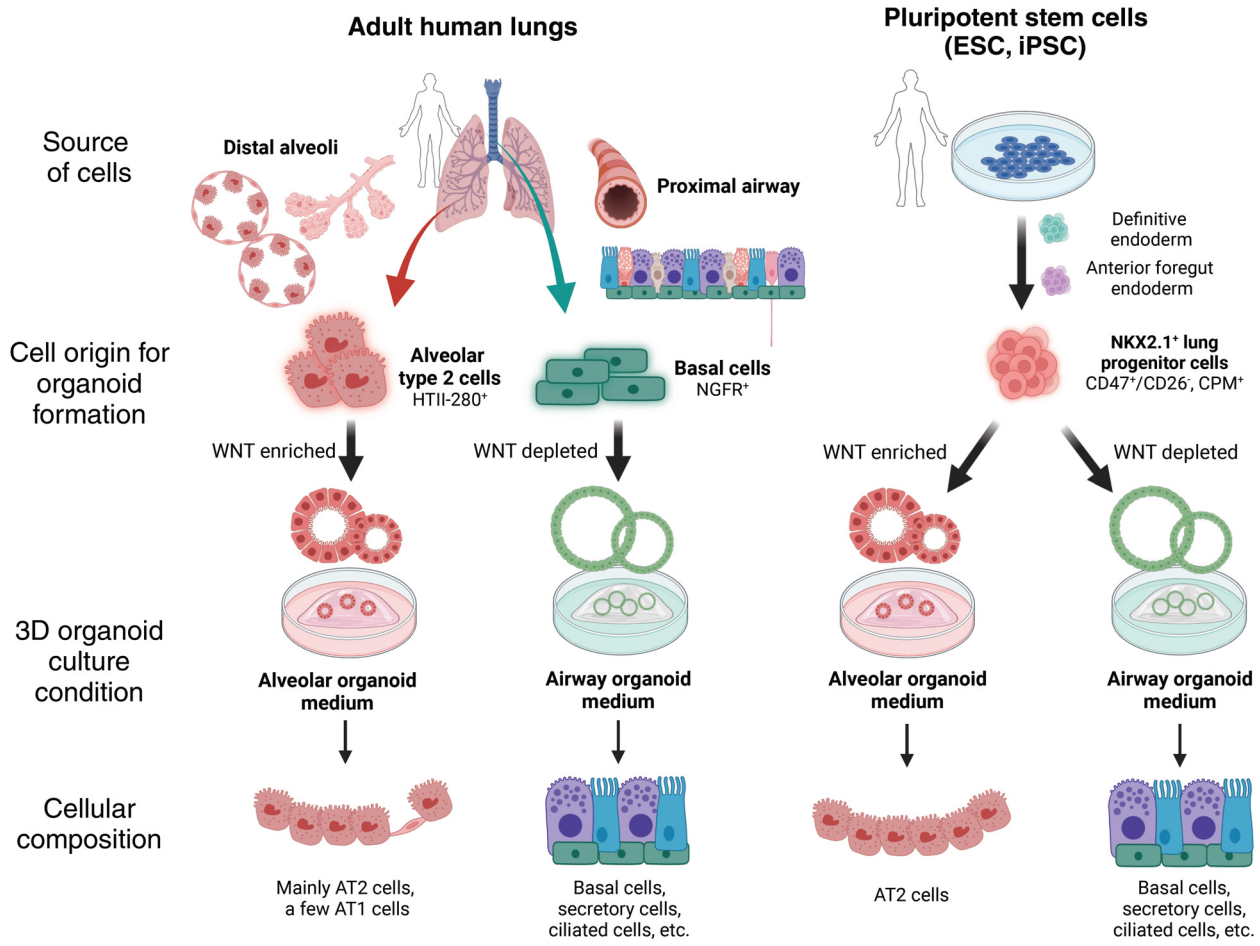

Fig. 1. A schematic diagram outlining the workflow for producing human lung organoids from adult lungs and pluripotent stem cells. In this process, alveolar type 2 (AT2) cells and basal cells from adult human lungs serve as sources of stem/progenitor cells capable of forming alveolar and airway lineage organoids, demonstrating differentiation potential towards their respective downstream cell types. Pluripotent stem cells also can be utilized as a source of cells, giving rise to lung lineage cell type via definitive endoderm, anterior foregut endoderm, and essentially $NKX2.1^{+}$ lung progenitor cells. Under suitable culture condition, the $NKX2.1^{+}$ lung progenitor cells can differentiate to alveolar, or airway lineage committed organoids. BioRender.com was used for creating this diagram. NGFR: nerve growth factor receptor, ESC: embryonic stem cell, iPSC: induced pluripotent stem cell.

which originate from fetal lung progenitor cells during development (1). Of these, the AT2 cells are known to mostly function as facultative lung stem cells, showing the ability to proliferate and differentiate to the AT1 cells and playing a key role in alveolar homeostasis and regeneration (4, 16-18).

The proximal area of the lungs contains a variety of mature airway epithelial cell types that originate from fetal airway progenitor cells (1, 4, 17), which are already fully differentiated into cells with diverse physiological functions, including secretory cells, ciliated cells, goblet cells, basal cells, and neuroendocrine cells. In general, basal cells at the base of the trachea show the ability to differentiate into various types of lower airway cells, and play central roles in airway homeostasis and regeneration (19, 20).

1) AT2 cells: The AT2 cells, found in the distal part of the adult lungs, including alveoli, function as a facultative stem cell. Particularly, when the alveoli are injured through infection or inhalation of harmful substances, the AT2 cells are thought to directly involved in the regeneration of respiratory function, sequentially proliferating and differentiating into AT1 cells (16, 18, 21-25). Thus, alveolar organoids can be derived from the AT2 cells, due to their ability to self-replicate or differentiate into the AT1 cells (Fig. 1). On the other hand, AT1 cells in the distal area have a highly limited potential for the formation of 3D organoids since their restricted capacity to proliferation, making subculture challenging to date - although recently it is reported environmental cues to differentiate pluripotent stem cells to AT1-like cells (26, 27).
2) Basal cells: Since various types of epithelial cells reside along both the upper and lower airway tracts, the cellular characteristics of cultured epithelial organoids depends on the specific cell types from which they originate. Basal cells, located on the basal lamina of the airway epithelial layer, play a crucial role as progenitor cells in replenishing damaged areas of the airway during an injury repair. Therefore, in a specific culture condition mimicking their physiological regeneration condition, proliferating airway epithelial organoids can be readily formed from the basal cell population, which has the capability to differentiate into major airway cells with distinct shapes and characteristics, including secretory cells, ciliated cells, and goblet cells (Fig. 1) (25, 28, 29).

b. Quality requirements for (source) cells

1) Quality requirements for AT2 cells: Flow cytometric sorting strategy using HTII-280 antibody has been widely used for purification of AT2 cells from adult human lung tissue (30). The AT2 cells exhibit apical-basal polarity in their morphology and express key AT2 marker genes at both transcriptome and protein levels (4, 31, 32). Moreover, they demonstrate the capacity for synthesis, transport, secretion, and recycling of mature surfactant protein at the physiological level (18, 33, 34).
   (1) Morphological characteristics: apical-basal polarity, immunofluorescence staining, etc.
   (2) Physiological functions: synthesis, transport, and secretion of mature surfactant protein; immunofluorescence staining, western blot, transmission electron microscopy, etc.
   (3) Transcriptome expression: transcriptome analysis using quantitative real-time polymerase chain reaction (qRT-PCR), RNA sequencing, etc.
2) Quality requirements for basal cells: As mentioned previously, the airway contains a mixture of epithelial cell types with diverse functions, including basal cells, secretory cells, ciliated cells, and goblet cells. Of these, basal cells are a key source of cells for the formation of 3D lung airway organoids. To isolate basal cells using flow cytometry, nerve growth factor receptor (NGFR) or integrin alpha chain 6 (ITGA6) antibodies can be used (19, 35, 36). After isolation of $NGFR^+$ and/or $ITGA6^+$ basal cells, it is important to ensure cellular heterogeneity within the airway organoid during culture. Following differentiation, each cell type should show its specific mature phenotype, including morphological characteristics and physiological functions.
   (1) Morphological characteristics: apical-basal polarity, immunofluorescence staining, etc.
   (2) Physiological functions: marker expression of basal cells and other cell types following differentiation; immunofluorescence staining, western blot, transmission electron microscopy, etc.
   (3) Transcriptome expression: transcriptome analysis using qRT-PCR, RNA sequencing etc.

c. Cell suppliers

1) AT2 cells can be isolated from the healthy parts of tissues acquired from surgical removal or biopsy from lung disease patients, including lung fibrosis or cancers.
2) Healthy tissues can also be acquired from the tissue of donors for lung transplant.

Pluripotent stem cell-derived lung progenitor cells:

a. Cell types and characteristics

Human pluripotent stem cells (hPSCs) including ESCs and induced pluripotent stem cells (iPSCs) can used as an alternative cell source to generate lung cells by mimick-

ing fetal lung developmental process. During development, all epithelial cells in the lungs originate from lung progenitor cells expressing a core transcription factor NKX2.1, which is well-known to be involved in lung cell fate-specification (32, 37-40). Therefore, $NKX2.1^{+}$ lung progenitor cells should be essentially obtained for further differentiating it to alveolar and airway cell types (Fig. 1). Indeed, Lung progenitor cells can be differentiated from pluripotent stem cells following a method of sequential differentiation that mimics differentiation in the lungs. The specific duration and methods of differentiation may differ depending on the protocol, but all lung progenitor cells should expression NKX2.1 transcription factor, and should be capable of proliferating and differentiating into airway and alveolar epithelial cells (32, 37-39).

After differentiation of pluripotent stem cells into definitive endoderm, $NKX2.1^{+}$ lung progenitor cells can be induced via an intermediate stage, anterior foregut endoderm. However, simultaneous differentiation into other endodermal epithelial cells, such as stomach or liver cells, has been reported during differentiation of lung progenitor cells. Hence, it is strongly advised to prioritize the high purity of lung progenitor cells through the isolation of $CD47^{high}/CD26^{neg}$ or CPM^{+} populations (Fig. 1) (37, 38, 41, 42). Additionally, when utilizing NKX2.1-GFP reporter knock-in pluripotent stem cells, fluorescent GFP-positive cells can be easily purified as $NKX2.1^{+}$ lung progenitor cells (37, 41).

b. Quality requirements for cells

It is critical to control the quality of pluripotent stem cell-derived $NKX2.1^{+}$ lung progenitor cells, which are the cell source for lung organoid formation, because the quality of the cells ultimately affects the organoid forming efficiency and quality of the airway or alveolar organoids. Thus, we recommend that the following quality requirements should be satisfied.

1) Purity: The purity of $NKX2.1^{+}$ lung progenitor cells differentiated from hPSCs should be ≥90% (37, 41, 43). The expression of NKX2.1 transcription factor can be verified by immunofluorescence staining and fluorescence-activated cell sorting (FACS).
2) Potency and proliferation ability: The lung progenitor cells should be capable of proliferating and of forming lung tissue by differentiating into the epithelial cells that constitute the airway and alveoli (32, 44).
3) Contamination prevention: The cells should avoid from contamination by various bacteria, including mycoplasma, fungi, or viruses.
4) Viability: The lung progenitor cells should survive in a healthy condition, capable of producing adenosine triphosphate. If the viability of the progenitor cells is too low, it can affect organoid productivity. Therefore, it is recommended to maintain the survival rate of ≥90%.
5) Identification and characterization: Identification and characterization should be possible using lung progenitor cell-specific markers.

c. Cell suppliers

In principle, the pluripotent stem cells include iPSCs generated by introducing reprogramming factors to somatic cells, and human ESCs isolated from the inner cell mass of blastocysts.

To ensure differentiation to the lung progenitor cells in a high quality, it is extremely important to also control the quality of the pluripotent stem cells. For matters relating to this process, please refer to documents on the quality control of pluripotent stem cells distributed by the Korea Disease Control and Prevention Agency and the International Society for Stem Cell Research. Stem cells for research purposes can be obtained from the Korea National Stem Cell Bank, and other places.

Culture

Essential elements and reagents:

a. Media components

To produce human lung organoids from adult or pluripotent stem cells, various environmental cues supporting lung differentiation and maturation, including growth factors and maturation-promoting factors, should be sequentially added to the basal medium at optimal time points. Different compositions of the factors may be adopted at each laboratory. The basal medium contains the essential components for cell growth and maintenance, such as amino acids, vitamins, glucose, salts, and buffers. One of examples include Advanced Dulbecco's modified Eagle medium/Ham's F-12 and Iscove's modified Dulbecco's medium/Ham's F-12, while serum-free replacement, bovine serum albumin, and antibiotics can be added to aid cell growth and survival.

b. Growth factors

Various growth factors can be used to generate lung organoids and maintain their growth and physiological function. The types and concentrations of essential growth factors for culturing human lung organoids may differ depending on the source of cells, or on the type of lung organoids (airway or alveolar). The list of growth factors below is a partial list of examples commonly used by the authors, and are replaceable if, in the future, a more reliable protocol for fabricating and culturing lung organoids is developed.

ASC organoids require the following essential growth factors:
-Recombinant KGF (FGF7)
-Recombinant FGF10
-Recombinant Rspondin-1
-Recombinant EGF
-Recombinant Noggin

c. Reagents
1) Reagents for airway organoid culture: inhibitors of transforming growth factor (TGF) signaling (e.g., SB431542 or A-83-01) are required.
2) Reagents for alveolar organoid culture: glucocorticoid class steroids (e.g., dexamethasone, etc.), Wnt activators/promoters (e.g., CHIR99021, Rspo1), BMP signaling inhibitors (e.g., Noggin), TGF signaling inhibitors (e.g., SB431542 or A-83-01), and Notch signaling inhibitors (e.g., gamma-secretase inhibitor) are required.

Culture process and requirements:

a. Culture protocols
Lung organoid culture protocols can include various medium compositions and culture conditions at different laboratories, depending on the differentiation strategy selected. Below, we present example protocols depending on the source cells and organoid type.
1) ASC-derived airway/alveolar organoids:
・At least 5～7 hours before culturing the cells, an ECM (e.g., Matrigel) needs to be prepared by dissolving at 4℃. Since Matrigel rapidly solidifies at temperatures of ≥10℃, it is essential to prepare the matrix on cold ice.
・1～2×10^4 cells are mixed with and embedded in 20 μL of 100% Matrigel, and then seeded in the form of a droplet onto a 48-well plate. Here, a care must be taken to prevent the inclusion of bubbles in the droplets.
・Without a delay, the plate is placed in an incubator at 37℃ and left exposed for 20～30 minutes to allow the Matrigel to solidify.
・To each well containing a solid Matrigel droplet, 250 μL of culture medium is added.
・The culture medium is changed every 2～3 days.
2) Pluripotent stem cell-derived airway organoids:
・To date, various protocols have been developed for differentiating airway organoids from pluripotent stem cells, but, broadly, the following 4-step culture process is followed for differentiation and maintenance of the organoid.
・Pluripotent stem cells are differentiated to lung progenitor cells on a two-dimensional cell culture plate, following a sequential induction procedure of definitive endoderm, anterior foregut endoderm, to lung progenitor.
・Lung progenitor cells are separated at a high purity using FACS-sorting based on surface markers, by $CD47^{high}/CD26^{neg}$ or CPM^{+}, as mentioned above.
・The isolated lung progenitor cells are embedded in 50%～80% Matrigel (or a similar ECM), and cultured in organoid differentiation medium.
・For maturation, the airway progenitor cells can be seeded onto a Transwell plate, and an ALI culture method can be used.
3) Pluripotent stem cell-derived alveolar organoids:
・Here we present an example for inducing the differentiation of pluripotent stem cells into alveolar organoids.
・High purity of lung progenitor cells can be induced using the same steps of differentiation from the pluripotent stem cells, as for airway organoids above because it shares the common lung progenitor. Then, the cells are to be embedded in 50%～80% Matrigel (or a functionally equivalent ECM material) and cultured in an alveolar organoid medium containing a WNT activator (e.g., CHIR99021) and a TGF-β inhibitor (e.g., SB431542).
・Alveolar organoids are capable of proliferation, meaning that they can be subcultured. After 1～2 weeks of culture, the alveolar organoids are dissociated to single cells by treating with accutase or dissociated into small clusters using gentle dissociation buffer, and then re-embedded in ECM for subculturing. For quality control of the lung organoid subculture, it is highly recommended to regularly test the quality of the alveolar organoids using the evaluation tools mentioned below; Quality requirements and assessment.
・Pluripotent stem cell-derived alveolar organoids consist of ≥90% AT2 cells and can also differentiate into AT1 cells via YAP activation.

b. Culture environments
Stem cell and organoid culture processes are performed on a clean bench, and all equipment used for culture is sterilized using an autoclave or 70% ethanol. Investigators should wear complete personal protective equipment.
1) Cell incubator: Generally, cell incubators should be set to constant conditions for temperature (37℃) and CO_2 concentration (5%), and the relative humidity inside the incubator should be maintained at ≥95%.
2) Cell culture method: In general, the culture methods that can be used for the differentiation and maintenance of lung organoids include adherent culture in two-dimensional culture medium, three-dimensional culture in 50%～80% Matrigel, and ALI culture on Transwell plates. A form of adherent culture can be performed on two-dimensional culture plates coated with 1% Matrigel for the process of differentiating

pluripotent stem cells into lung progenitor cells.

3) For the differentiation of alveolar and airway organoids, three-dimensional culture is performed using Matrigel (or a functionally equivalent ECM material). Matrigel, which is a solubilized basement membrane matrix secreted by Engelbreth-Holm-Swarm mouse sarcoma cells, is the most used ECM material for lung organoid culture, but care is required when using this product, because there have been issues with different ECM concentration, composition, and endotoxin levels in each batch.
4) Since the lung is an organ that is in direct contact with external air for gas exchange, the lung organoids can be cultured under a specialized equipment that imitate the ALI environment (14, 15), such as Transwell plates, to recapitulate the environmental profile of original respiratory tissues. These ALI culture methods can efficiently induce maturation of human lung epithelial cells, and can also be used in testing methods to assess the respiratory toxicity of inhaled substances.

Quality requirements and assessment

Quality requirements for organoids:

a. Size and morphology

Given the diverse morphological profiles observed in human lung organoids, which can vary depending on the anatomical source of the cells, relying solely on size (approximately 50～200 μm) for assessing the quality of airway or alveolar organoids may be not feasible. Instead, it is more realistic to evaluate the quality of lung organoids by analyzing the morphology and physiological profile, rather than only the size. Valid methods include analysis of markers defining morphological characteristics of cells using high-performance confocal microscopy, as well as analysis of major cell organelle properties for each cell type using electron microscopy.

b. Cell composition

The quality of ASC-derived airway and alveolar organoids can be assessed by verifying whether each cell type in the *in vitro* cultured organoid shares similar morphological and physiological characteristics to the same cell or tissue type in adults *in vivo*. Organoids can be tested using single cell sequencing and staining for key proteins using immunofluorescence staining.

- Airway organoids: airway epithelial cells, such as basal cells, ciliated cells, and goblet/secretory cells
- Alveolar organoids: alveolar epithelial cells, such as AT1 and AT2 cells

c. Lung-specific function

1) Airway organoids: The airway contains a mixture of cell types with diverse functions, including basal cells, secretory cells, ciliated cells, and goblet cells. As such, it is important to ensure cellular heterogeneity in airway organoids during culture. After differentiation, each cell type should show its specific mature phenotype, including morphological characteristics and physiological functions.
2) Alveolar organoids: The main cell type in alveolar organoids is AT2 cells. In terms of quality requirements, these cells should polarity at the morphological level, expression of major AT2 cell markers at the transcriptome and protein expression level, and, especially, synthesis, transport, and secretion of mature surfactant protein. In addition, AT2 cells should be capable of differentiation to AT1 cells under specific conditions.

d. Genetic profile

1) Chromosomal karyotyping
2) Short tandem repeat (STR) genotyping analysis

Quality assessment items (endpoints):

a. Organoid size and morphology

Both airway and alveolar organoids share a spherical shape but, depending on the differentiation method and the type of organoids, they can form both monolayered spheres and spheres with multiple thin, overlapping, folded layers. The organoid size can vary depending on the differentiation protocol or the extent of proliferation after subculturing, potentially impacting the quality of end products. Therefore, strict management of organoid size in culture is recommended to minimize batch differences between independent samples. Additionally, for the quality control of the lung organoids, it is highly recommended to regularly evaluate the degree of differentiation, the cellular structure and morphology, and the physiological functions.

b. Cell composition

Regarding the composition of key cell types in airway and alveolar organoids, it will be useful to use either immunofluorescence staining and/or single cell sequencing to verify the presence and, if necessary, the ratio of different cell types within an organoid. It is recommended evaluating the main cell types, along with differentiating/differentiated cells depending on culture conditions, which facilitate key progenitor cell expansion and differentiation. The followings indicate the major cell types that are typically observed in organoid types.

- Airway organoids: basal cells, ciliated cells, secretory cells, and/or goblet cells
- Alveolar organoids are comprised of mainly AT2 cells with a few or rare AT1 cells.
- Other cell types (pulmonary neuroendocrine cells, ionocytes, stromal cells, endothelial cells, immune cells)

can be included in lung organoids depending on culture environments and a source of cell origin.

c. Marker expression of essential target cells and supporting cells

The differentiation quality in airway and alveolar organoids can be assessed by analyzing the expression of cell-specific markers. Expression of specific markers for each cell type can be utilized by qRT-PCR or immunostaining. Here is a list of the key markers and physiological function-related genes for cell types within airway organoids (1, 4, 17).

- Basal cell differentiation markers: TP63, KRT5, NGFR, F3
- Ciliated cell (multiciliated cell) markers: FOXJ1, RSPH1, Acetylated-Tubulin
- Secretory cell markers: SCGB1A1, SCGB3A2
- Goblet cell markers: MUC5B, MUC5AC, SPDEF

Here is a list of the key markers and physiological function-related genes for cell types within alveolar organoids (1, 4, 17).

- AT2 cell markers: SFTPC, SFTPB, SFTPA, SFTPD, LAMP3, ABCA3, NAPSA
- AT 1 cell markers: AGER, CAV1, AQP5

d. Analysis of cell morphology characteristics of organoids

To verify the maturation of essential target cells in airway and alveolar organoids, transmission electron microscope (TEM) observation can be useful for validating the specific morphological characteristics of lung epithelial cells.

1) Airway organoids
- Ciliated cells (multiciliated cells): multi-cilia
- Secretory cells: secretory vesicles
- Goblet cells: mucous granules

2) Alveolar organoids
- AT2 cells: microvilli on the apical membrane, lamellar bodies, multivesicular bodies
- AT 1 cells: thinly extended cell morphology

e. Verification of organoid function

Tests are performed to verify that the organoids can imitate the functional properties of the lungs and to assess their quality.

While gas exchange is the most important function to analyze for alveolar organoids, suitable testing methods for this purpose have not yet been developed. Therefore, an analysis for gas exchange in cultured organoids can be alternatively performed by testing marker expression and morphological features, as described above.

Ciliary movement of airway organoids can be observed by microscopy and, if necessary, can be quantified by dynamic imaging. Mucus layer formation is assessed for both airway and alveolar organoids by verifying the formation of a mucin layer via Periodic acid–Schiff (PAS) staining.

f. Organoid karyotyping and analysis of cell origin

To ensure reliability in assessing the effects of test substances on both normal lung organoids and those with genetic diseases or variants, organoid karyotyping can be used to verify chromosomal stability.

From an administrative perspective, the cellular identity of organoids can be verified using STR analysis. It is advisable to perform regular monitoring (e.g., once per year) of the organoids to ensure that the source cells or the organoids maintain the same genotype.

Methods for each evaluation metric:

a. Observation of organoid size and morphology microscopic observation
b. Cell composition: immunofluorescence staining
c. Cell composition: single cell analysis
d. Marker expression: RT-PCR
e. Marker expression: immunoblotting
f. Cell morphology: TEM
g. Mucosal layer formation: PAS staining
h. Cell source: STR
i. Genomic stability of organoid chromosome karyotyping
j. Organoid contamination: mycoplasma test

Monitoring of quality control outcomes (per batch, per cycle): After establishing lung organoid line, the quality of the organoids in each batch can be assessed based on the assessment items mentioned above, and in the case of subcultures (e.g., once every 5 subcultures), the expression of markers for each cell type should at least be tested.

Storage and preservation

Storage protocol:

a. Freezing and thawing process
- After forming sufficient colonies, they can be separated from the Matrigel using dispase (1 mg/mL). When using Transwell plates. the insert is moved to a new well, 100 μL of dispase is added on top of the insert, and the well is left exposed for 1 hour at 37℃. When culturing on a 48-well plate, after completely removing the remaining culture medium, the wells are treated with 150 μL of dispase.
- After physically dissociating the colony in dispase by trituration with a pipette tip, the pipette tip is used to move the mixture to an e-tube.
- After briefly centrifuging the e-tube, 500 μL of Triple is added to suspend the cells. The suspension is then left exposed for 5～10 minutes at 37℃. If necessary, the mixture is stirred every 2～3 minutes by trituration.
- After completely dissociating the organoids into single cells by trituration using a pipette tip, 1 mL of co-cul-

ture medium is added to dilute and suspend the cells.
- After centrifuging for 5 minutes at 600 ×*g* to precipitate the cells, the suspension fluid is completely removed, and the remaining single cells are thoroughly mixed with around 1～2 mL of freezing medium containing dimethyl sulfoxide.
- The cells are moved to a freezing container or cryotank stored at room temperature, and then immediately stored for up to 24 hours at −70℃ to 80℃ before keeping in a liquid nitrogen container for long-term storage.

b. Essential equipment and instruments
- LN_2 tank
- Centrifuge
- Cryotank

Quality factors:

a. Post-thaw viability
After thawing, a microscope can be utilized to verify survival and organoid formation. Typically, when analyzed by FACS, a survival rate of approximately 60%～80% can be maintained.

b. Post-thaw maintenance of organoid profile
It is important to examine the maintenance of the organoid profile after freezing and thawing, by comparing the expression of target cell-specific markers from pre- and post-thaw organoid samples. Thus, it is highly recommended that the expression of target cell-specific markers and cellular characteristics remain almost identical to before freezing for normally proliferating lung organoids after thawing.

Ethical considerations

When human-derived ASCs are used as a source of cells, it is essential to obtain a consent form for the use of human-derived materials in accordance with the Bioethics and Safety Act (Enforcement Rules for the Bioethics and Safety Act, Article 34) (45). If collecting human-derived materials from minors, the research consent form for human-derived materials must be obtained from the minor and a legal proxy. Content that is not included in the form (e.g., when banking ASC-derived lung organoids or providing them to a third party, or when generating genome information from lung organoids, or providing generated information to a third party) should be written in detail on an explanation sheet, and consent for this must also be obtained.

When using ESCs as the source cell, it is essential to use registered ESCs or to register the stem cells before use, in accordance with Article 33, Paragraph 1 of the Bioethics and Safety Act, and approval for their use must be obtained from an institutional review board, in accordance with Article 32, Paragraph 2 of the Bioethics and Safety Act (45).

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Funding

This research was supported by Ministry of Food and Drug Safety in 2023 (grant number: 23212MFDS265) and Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education (grant number: 2021K1A4A7A0209775712).

Potential Conflict of Interest

There is no potential conflict of interest to declare.

Authors' Contribution

Fund acquisition: YJC, SJA. Visualization: KL. Supervision: JC, YJC, SJA. Writing – original draft: KL, MOL, JC, JHK. Writing – review and editing: EMK, CGW, CC, YHC, SHH, YJC.

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