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Selection of the Most Suitable Culture Medium for Patient-Derived Lung Cancer Organoids

Melissa Acosta-Plasencia[a] Yangyi He[a, b] Daniel Martínez[c, d, e]
Juan Pablo Orozco[f] Antonio Carrasco[c] Antonio Altuna-Coy[a]
Tianmiao Yang[a] Tania Díaz[a] Laureano Molins[d, e, g] Ricard Ramos[d, e, g]
Ramón M. Marrades[d, e, f, h] Alfons Navarro[a, d, e]

[a]Molecular Oncology and Embryology Laboratory, Department of Surgery and Medical Specializations, Human Anatomy and Embryology Unit, Faculty of Medicine and Health Sciences, Universitat de Barcelona (UB), Barcelona, Spain; [b]School of Basic Medical Sciences, Chengdu University, Chengdu, China; [c]Department of Pathology, Hospital Clínic de Barcelona, University of Barcelona, Barcelona, Spain; [d]Thoracic Oncology Unit, Hospital Clínic, Barcelona, Spain; [e]Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain; [f]Department of Pneumology, Institut Clínic Respiratori (ICR), Hospital Clínic de Barcelona, University of Barcelona, Barcelona, Spain; [g]Department of Thoracic Surgery, Hospital Clínic de Barcelona, University of Barcelona, Barcelona, Spain; [h]Centro de Investigación Biomédica en Red de Enfermedades Respiratorias (CIBERES), Instituto de Salud Carlos III, Madrid, Spain

Keywords
Lung organoids · Lung cancer organoids · Culture medium · Non-small cell lung cancer · Alveolospheres · Bronchospheres

Abstract
Introduction: Patient-derived organoids have emerged as a promising in vitro model for precision medicine, particularly in cancer, but also in noncancer-related diseases. However, the optimal culture medium for culturing patient-derived lung organoids has not yet been agreed upon. This study aimed to shed light on the optimal selection of a culture media for developing studies using patient-derived lung organoids. ***Methods:*** Tumor and normal paired tissue from 71 resected non-small cell lung cancer patients were processed for organoid culture. Lung cancer organoids (LCOs) were derived from tumor tissue and normal lung organoids (LNOs) from nonneoplastic lung tissue. Three different culture media were compared: permissive culture medium (PCM), limited culture medium (LCM), and minimum basal medium (MBM). We assessed their effectiveness in establishing organoid cultures, promoting organoid growth and viability, and compared their differential phenotypic characteristics. ***Results:*** While PCM was associated with the highest success rate and useful for long-term expansion, MBM was the best option to avoid normal organoid overgrowth in the organoid culture. The density, size, and viability of LNOs were reduced using LCM and severely affected with MBM. LNOs cultured in PCM tend to differentiate to bronchospheres, while alveolosphere differentiation can be observed in those cultured with LCM. The morphological

karger@karger.com
www.karger.com/cto

Correspondence to:
Alfons Navarro, anavarroponz@ub.edu

phenotype of LCO was influenced by the culture media of election. Mesenchymal cell overgrowth was observed when LCM was used. ***Conclusion:*** This work highlights the importance of considering the research objectives when selecting the most suitable culture medium for growing patient-derived lung organoids.

Introduction

To date, lung cancer remains one of the most prevalent cancers worldwide and is the leading cause of cancer-related mortality, with approximately 1.8 million deaths per year [1]. This heterogeneous disease is classified into two main types: small cell lung cancer and non-small cell lung cancer (NSCLC). NSCLC is the most frequent type (80–85% of cases) and can be further classified into different histological subtypes where adenocarcinoma (ADK), squamous cell carcinoma (SCC), and large cell carcinoma are the most frequent ones. In early-stage NSCLC, surgical resection is considered the preferred and potentially curative therapeutic approach followed by adjuvant therapy in some patients (mainly stage II patients). Cisplatin-based adjuvant treatment is the most frequent option, but osimertinib can also be used in patients harboring EGFR mutations [2–4]. Recently, immunotherapy followed by surgical resection of the tumor has also begun to be considered a neoadjuvant treatment since good results have been obtained in clinical trials including early-stage patients [5]. However, with current treatment approaches, around 30% of resected patients will experience disease relapse, even after receiving adjuvant treatment [6]. After relapse, surgical patients can be treated with targeted therapy, depending on the mutational profile of the tumor, or even with immunotherapy. Therefore, the tumoral tissue obtained during the resection is used to generate in vitro models where test drug responses could permit the prediction of treatment outcomes or even help with personalized treatment decisions.

In recent decades, significant progress has been made in developing preclinical in vitro models for NSCLC, with cancer cell lines being the most widely used. However, this two-dimensional cell culture model does not faithfully replicate the complexity and heterogeneity of the original tumor and may lose key mutational features during long-term culture [7]. Patient-derived xenografts, on the other hand, exhibit a higher level of fidelity to patient tumors, but their establishment and utilization are time-consuming, expensive, and labor-intensive [8]. Recently, an in vitro model generated directly from adult stem cells, known as patient-derived organoids, has gained popularity in precision medicine. Lung cancer organoids (LCOs) are three-dimensional (3D) structures that mimic each patient's tumor's genetic and molecular characteristics, even recapitulating the cellular diversity and tumor microenvironment [9]. Although LCOs generated from resected tumor tissue can be a valuable tool for personalized treatment, the methodology for its culture is still in a phase of ongoing development and continuous optimization without a unique solution for its establishment and culture. To ensure the optimal growth and development of LCOs, the presence of an extracellular matrix providing a 3D scaffold for organoids and facilitating crucial cell-ECM interactions is indispensable. This can be from different sources being Matrigel and collagen the most frequent ones [10]. Furthermore, for the stem cells responsible for generating LCOs to proliferate and differentiate into cell lineages that self-organize, providing an organoid culture medium with specific supplements is crucial [11], but each research group uses different media [12–20], which can affect the reproducibility of the organoid studies. Overall, the distinct culture media described in the literature can be categorized into two primary types: permissive culture medium (PCM) [13] and minimum basal medium (MBM) [14]. While PCM enhances the viability and long-term expansion of LCOs, it may lead to excessive proliferation of normal lung organoids (LNOs) [12, 13]. In contrast, MBM allows the obtention of a more enriched LCOs culture but considerably reduces the LCO expansion [14, 15]. Additionally, there are intermediate options, referred to by the own authors [15] as limited culture medium (LCM), which do not contain most of the growth factors necessary for the growth of LNOs such as FGF7, FGF10, R-spondin, and Noggin included in the PCM, but it contains other factors such as dexamethasone and forskolin not contained in MBM. Currently, there is no consensus on the optimal culture medium for growing patient-derived LCOs for personalized medicine applications [10].

In this study, we compared three of the most used LCO culture media [12, 14, 15] and evaluated the success rate of each one in establishing both LCO and LNO cultures, the viability of the organoids obtained, and described their main phenotypic characteristics in correlation with the clinical-pathological characteristics of the patient of origin. The medium described by Sachs et al. [12], a PCM, has been compared against the LCM reported by Hu et al. [15] and the MBM from Kim et al. [14]. The results of the present work confirm that important differences exist between organoid cultures obtained with each medium and aim to help in the decision of the best medium selection for each scientific purpose.

Methods

Patient Samples

Tumor and normal paired tissue were prospectively collected from 71 adult patients diagnosed with NSCLC who underwent complete surgical resection at the Hospital Clínic of Barcelona (Spain) from January 2021 to July 2023 and who consented to participate in the study. Samples were obtained within 6 h of surgery, submerged in phosphate-buffered saline, and transported on ice to the laboratory to establish organoid cultures (Fig. 1a). The main clinical and histological characteristics of the patients used for organoid culture establishment are presented in online supplementary Table 1 (for all online suppl. material, see https://doi.org/10.1159/000541274). The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Clinical Research Ethics Committee of the Hospital Clínic of Barcelona (project approval number HCB/2021/0711).

Organoid Establishment, Culture, and Passage

Tissue biopsies were mechanically dissociated into smaller pieces with a sterile scalpel and then enzymatically dissociated by incubation with 1 mg/mL collagenase type II (Merck, Darmstadt, Germany) and 100 μg/mL DNase I (Merck) in DMEM/F12 (Thermo Fisher Scientific, Waltham, MA, USA) with intermittent agitation on an orbital shaker at 37°C for 2 h. After enzymatic digestion, the suspensions were passed through a 100-μm cell strainer and centrifuged at 400 *g* for 5 min. If a red pellet was observed, 1 mL red blood cell lysis buffer (Sigma-Aldrich, Burlington, MA, USA) was added to lyse the erythrocytes for 5 min at room temperature. The resulting cell clusters were resuspended in cold Matrigel (Corning, NY, USA) and 30 μL drops were seeded into prewarmed 48-well culture plates (Greiner Bio-One, Frickenhausen, Germany) at 37°C for 10–20 min. After solidification, 400 μL of organoid culture media was added to each well, and plates were placed into incubators in a humidified atmosphere at 37°C and 5% CO_2. This was considered passage 0. The culture medium was changed every 3 days and at 2 weeks organoids were passaged as previously described [21]. Briefly, organoid cultures were resuspended in 10 mL cold DMEM/F12, enzymatically dissociated in 2 mL TrypLE Express (Gibco, Thermo Fisher) for 5 min at 37°C, and mechanically dissociated with flamed glass Pasteur pipettes. After centrifugation at 400 *g* for 5 min, the fragmented organoid suspension was mixed with cold Matrigel and reseeded at a 1:4 ratio, allowing new organoids to form. This was considered passage 1 and so on.

Organoid Culture Media

The three different self-renewing culture media (PCM, LCM, and MBM) used for LNOs and LCOs are described in Table 1. Briefly, PCM [12] contained Advanced DMEM/F12 supplemented with 100 μg/mL penicillin/streptomycin (Gibco), 2 mM GlutaMax 100X (Gibco), 10 mM HEPES (Gibco), 1:50 B-27 (Gibco), 5 mM nicotinamide (Sigma Aldrich), 1.25 mM N-acetylcysteine (Sigma Aldrich), 500 ng/mL R-spondin 1 (PeproTech), 25 ng/mL FGF-7 (StemCell), 100 ng/mL FGF-10 (StemCell), 100 ng/mL Noggin (PeproTech), 5 μM Y-27632 (StemCell), 500 nM A83-01 (Sigma Aldrich), and 500 nM SB202190 (Sigma Aldrich). LCM [15] included Advanced DMEM/F12 supplemented with 100 μg/mL penicillin/streptomycin (Gibco), 2 mM GlutaMax 100X (Gibco), 10 mM HEPES (Gibco), 1:50 B-27 (Gibco), 5 mM Nicotinamide (Sigma Aldrich), 1:100 N-2 supplement (Gibco), 1.25 mM N-Acetylcysteine (Sigma Aldrich), 50 ng/mL EGF (StemCell), 10 μM Y-27632 (StemCell), 5 μM A83-01 (Sigma Aldrich), 3 μM SB202190 (Sigma Aldrich), 10 μM Forskolin (Selleckchem), and 3 nM dexamethasone (Selleckchem). MBM [14] consisted of Advanced DMEM/F12 supplemented with 100 μg/mL penicillin/streptomycin (Gibco), 1:50 B-27 (Gibco), 1:100 N-2 supplement (Gibco), 50 ng/mL EGF (StemCell), 20 ng/mL bFGF (Gibco), and 10 μM Y-27632 (StemCell).

Organoid Visualization and Area Measurement

Images of the LNOs and LCOs were taken every 3–4 days with the Olympus IX53 inverted microscope. The area of each organoid was measured using the Fiji plugin of ImageJ version 2.14.0/1.54f.

Organoid Viability Analysis

After passage was performed as described above, 1×10^3 cells were resuspended in Matrigel, and 10 μL drops were seeded in a white, opaque-walled 96-well plate (Greiner Bio-One). Upon Matrigel solidification, 100 μL of culture medium was added. Viability measurements were performed on 0 h, 72 h, and 144 h using CellTiter-Glo 3D Viability Assay (Promega, Madison, WI, USA) as per manufacturer's instructions. Briefly, after 30 min of plate equilibration to room temperature, CellTiter-Glo 3D (Promega) reagent was added to each well in a volume of 1:1. The plate was shaken at 1,440 rpm for 5 min and incubated for 25 min at room temperature. The luminescence was measured in an Onion II Microplate Luminometer with an integration time of 1 s per well.

Immunofluorescence Analysis of Organoids

Prior to fixation, organoids were removed from Matrigel using Corning Cell Recovery Solution (Corning, 354253). The organoids were fixed in 4% paraformaldehyde,

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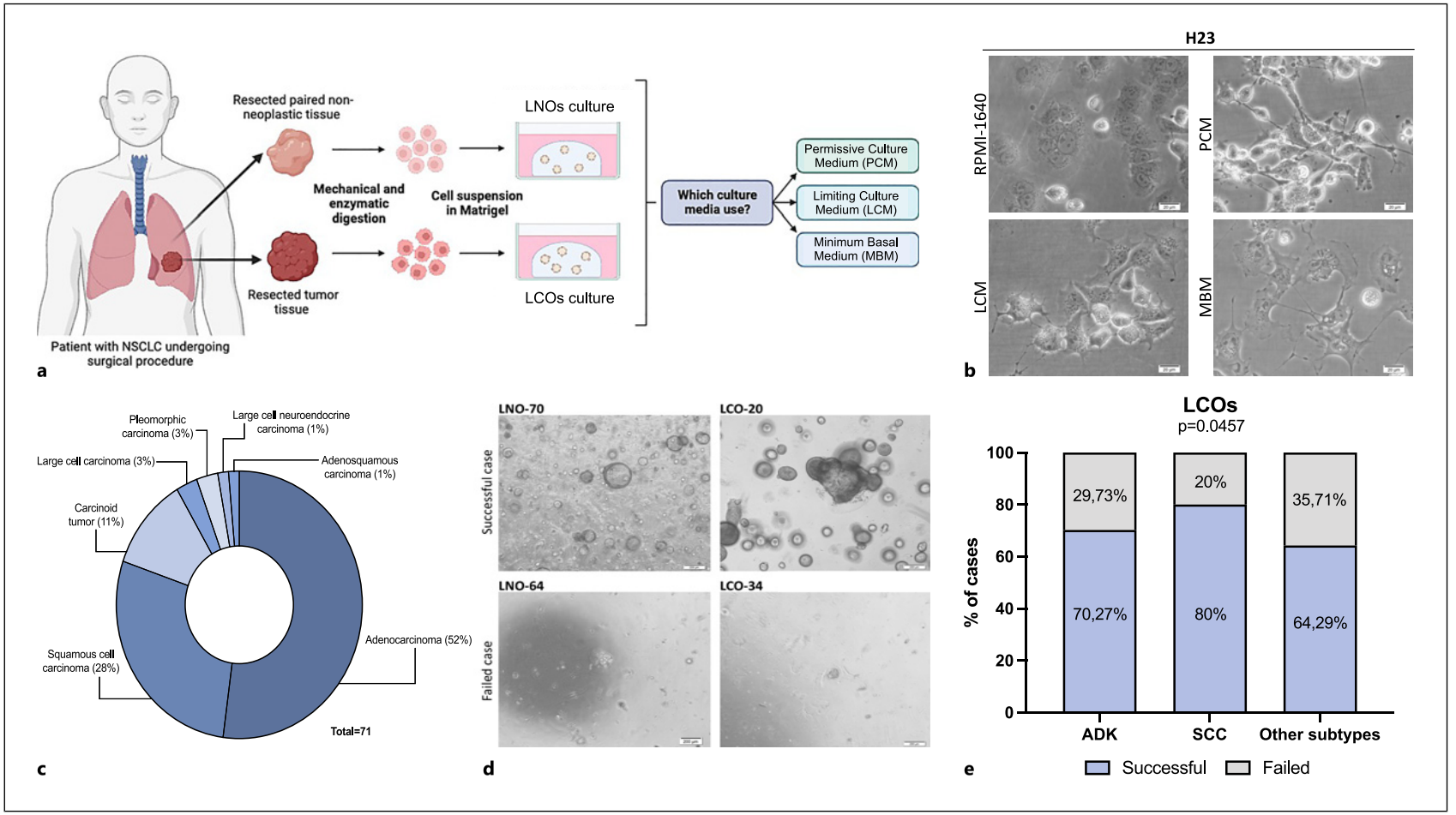

Fig. 1. LNOs were established from patients diagnosed with non-small cell lung cancer. **a** Illustration of the procedure used to culture LNOs and LCOs. Paired nonneoplastic and tumor tissues were obtained by surgery, digested, and resuspended in Matrigel to generate LNOs and LCOs, respectively. **b** H23 cell line was cultured using RPMI-1640 medium, PCM, LCM, and MBM. Scale bar: 20 μm. **c** Pie chart showing the subtypes of the tumor tissues used to generate the 71 LCOs included in this work. **d** Bright-field microscopy images of successful and failed cases of LNOs and LCOs. Scale bar, 200 μm. **e** Bar graph comparing the percentage success rate of LCOs derived from ADK (n = 37 cases), SCC (n = 20 cases), and other tumor tissues (n = 14 cases).

Table 1. The three different self-renewing culture media for patient-derived LNOs

Reagents	Functions	Source	Final concentration		
			permissive culture medium	limiting culture medium	minimum basal medium
			Sachs et al. [12] (2019)	Hu et al. [15] (2021)	Kim et al. [14] (2019)
Advanced DMEM/F12	Base medium	Thermo Fisher Scientific	**1x**	**1x**	**1x**
Penicillin/ streptomycin	Antibiotics	Gibco	**100 µg/mL**	**100 µg/mL**	**100 µg/mL**
GlutaMax 100x	Nutrient	Gibco	**2 mM**	**2 mM**	
HEPES	Buffer	Gibco	**10 mM**	**10 mM**	
B-27	Cell proliferation and tumor spheres formation	Gibco	**1:50**	**1:50**	**1:50**
Nicotinamide	Cell death prevention	Sigma Aldrich	**5 mM**	**5 mM**	
N-2 supplement	Co-enzyme precursor	Gibco		**1:100**	**1:100**
N-Acetylcysteine	Oxidative stress protection	Sigma Aldrich	**1.25 mM**	**1.25 mM**	
R-spondin 1	Stem cell proliferation and self-renewal	PeproTech	**500 ng/mL**		
FGF-7	Epithelial stem cell maintenance	StemCell	**25 ng/mL**		
FGF-10	Lung stem cell differentiation and organoid branching	StemCell	**100 ng/mL**		
EGF	Epithelial cell growth and differentiation	StemCell		**50 ng/mL**	**50 ng/mL**
bFGF	Stem cell capacity and survival	StemCell			**20 ng/mL**
Noggin	Epithelial stem cell maintenance	PeproTech	**100 ng/mL**		
Y-27632	Cell death prevention	StemCell	**5 µM**	**10 µM**	**10 µM**
A83-01	Cell growth and proliferation	Sigma Aldrich	**500 nM**	**5 µM**	
SB202190	Cell death prevention	Sigma Aldrich	**500 nM**	**3 µM**	
Forskolin	LNO survival	Selleckchem		**10 µM**	
Dexamethasone	Alveolar maturation	Selleckchem		**3 nM**	

Entries in bold denote the reagents present in the medium.

included in Histogel (Fisher Scientific, 12006679), and subsequently embedded in paraffin. Immunofluorescence procedures were performed as previously described [22]. For immunostaining, the following primary antibodies were utilized: rabbit anti-p63 (Proteintech, 12143-1-AP), mouse anti-CC10 (Santa Cruz, sc-365992), mouse anti-AQP5 (Santa Cruz, sc-514022), and rabbit anti-SFTPC (Proteintech, 10774-1-AP). Secondary antibodies included Alexa 488-anti-mouse donkey IgG antibody (Thermo Fisher Scientific, A28175) and Alexa 568 anti-rabbit donkey IgG antibody (Thermo Fisher Scientific, A10042).

Cell Lines

Cryopreserved samples of the lung cancer cell line H23 (CRL-5800, ATTC, Manassas, VA, USA) were received in our laboratory and passaged for less than 6

months. H23 was cultured in RPMI 1640 (catalog number 21875091, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (catalog number 16000044, 1:10, Thermo Fisher Scientific) or with organoid culture media used in the study. The cell line was grown at 37°C in 5% CO_2 and 95% relative humidity.

Statistical Analysis

T-tests or U-Mann Whitney test, when appropriate, were used for comparisons between two groups of samples, and ANOVA or Kruskal-Wallis test was used for comparisons between more than two groups. Fisher's exact probability test or χ^2 test when appropriate was used to compare differences in frequency distributions between groups. At least three technical and three biological replicates have been performed in each in vitro experiment. All statistical analyses were performed using GraphPad Prism v. 10.1.1 (La Jolla, CA, USA).

Results

Effect of Organoid Culture Media Selection on an Established Lung Cancer Cell Line

After tissue obtention and processing from surgical patients for organoid culture preparation (illustrated in Fig. 1a), we must choose the appropriate culture medium to establish them. This election is significant because the different culture media used have a different effect not only on organoids' growth but also on their intrinsic characteristics. To highlight this, we cultured the H23, a well-characterized lung cancer cell line, with its regular medium or with the 3 organoid culture media used in the present study (Fig. 1b). Different morphologies can be appreciated in the 2D culture of the H23 cell line when cultured with its regular medium (RPMI-1640) or with the different organoid culture media analyzed in the present study. When cultured in RPMI-1640 medium, the H23 cells exhibited robust growth and maintained high viability across multiple passages. Morphologically, these cells predominantly exhibited a rectangular shape, with only a minority showing branching or extensions. In contrast, when cultured with the PCM, LCM, and MBM, the H23 cells failed to establish successfully across consecutive passages. The remaining cells underwent significant morphological changes, with some adopting a rounded morphology and the majority showing an elongated shape with several ramifications and slight differences between the three media. Clear phenotypic differences can be observed depending on the culture media used (Fig. 1b).

Establishment of Patient-Derived LNOs and LCOs from Patient Samples

We cultured with the aim to establish new organoid cell lines 142 tissue samples, 71 from resected tumor tissue and 71 from the paired nonneoplastic tissue (online suppl. Table 1). Most of the tissues were obtained from ADK patients in 52% of cases and from SCC patients in 28% of cases. Less frequent histological types of the tumor of origin included large cell carcinoma (3%), pleomorphic carcinoma (3%), large cell neuroendocrine carcinoma (1%), or adenosquamous carcinoma (1%) (Fig. 1c). To assess the effectiveness of organoid generation from resected lung tissues, we considered a successful case when organoid formation was observed from passage 1 to passage 2. Figure 1d shows images of successful and failed cases of LNOs and LCOs. Independently of the media used, we observed significant differences in the LCO establishment ratio depending on the histology of the tumor of origin ($p = 0.0457$, Fig. 1e). LCOs derived from SCC samples showed an 80% success rate, followed by those derived from ADK with 70% and from other lung cancer subtypes with 64.29%.

Effectiveness of Each Culture Medium for Patient-Derived LNOs and LCOs Establishment

When LNOs and LCOs were cultured, variations in success rates were noted between passages 1 and 2 among the three different self-renewing culture media (Fig. 2, $p < 0.001$). The highest success rate in patient-derived LNO establishment was achieved with the PCM. Using PCM, culture success rates were notably higher in both types of LNOs compared to other media, reaching 73% for LNOs and 81% for LCOs. The LCM yielded a slightly higher success rate for LCOs at 63%, while it was 50% for LNOs. The medium associated with the highest failure rates was the MBM, with a success rate of only 29% for LNOs and 35% for LCOs (Fig. 2a). This is evidenced by the bright-field images of cases LNO-69 and LCO-69, illustrating a decrease in organoid establishment between passages 1 and 2 when cultured with the LCM or MBM instead of PCM (Fig. 2b).

Growth Rate and Cell Viability of Patient-Derived Organoids with Each Culture Medium

Organoid size and growth were also affected by the culture media, and notable differences were identified mainly between the PCM and MBM (144 h, $p = 0.0004$). LCM occupied an intermediate position (Fig. 3a, b). The mean organoid area (mm^2) after 144 h of culture with PCM was 19.5×10^{-3}, representing 178.5% of growth from 0 h. In comparison, organoids cultured with LCM

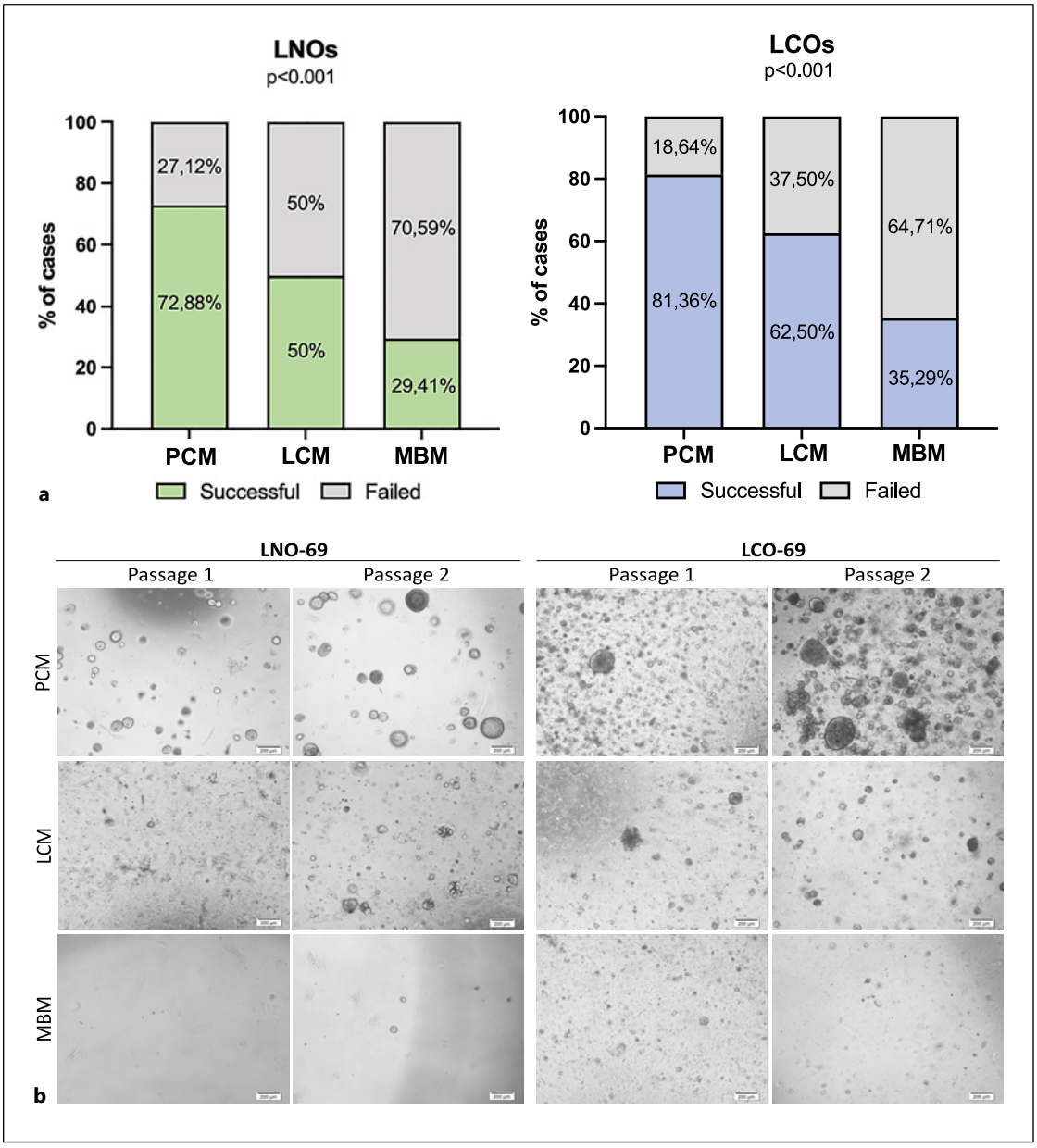

Fig. 2. Culture success rates of LNOs and LCOs using the three-culture media. **a** Bar graph illustrating the percentage success rates of LNOs and LCOs cultured in PCM (n = 118 cases), LCM (n = 16 cases), and MBM (n = 34 cases). **b** Bright-field microscopy images of LNO-69 (originated from the paired nonneoplastic tissue of a lung SCC patient) and LCO-69 (originated from a cancerous tissue of a lung SCC patient) lines cultured using PCM, LCM, and MBM across passages 1 and 2. Scale bar, 200 μm.

exhibited an area of 9.4×10^{-3} mm^2 (143% growth), while those cultured with MBM showed an area of 4.1×10^{-3} mm^2 (103% growth). Moreover, we assessed whether the choice of the culture media influenced the number of metabolically active cells by measuring ATP levels (as an indirect measure of the number of viable cells). This analysis (Fig. 3c) showed significant differences at 144 h between PCM and either LCM (p = 0.0004) or MBM (p = 0.0005). In the bright-field images, it becomes apparent that, generally, there is a higher quantity of LNOs cultured in the PCM, and they had a major increase in size over the days in comparison to LCM and MBM. It is important to highlight that the density of LNOs was highly compromised when grown with MBM (Fig. 3b).

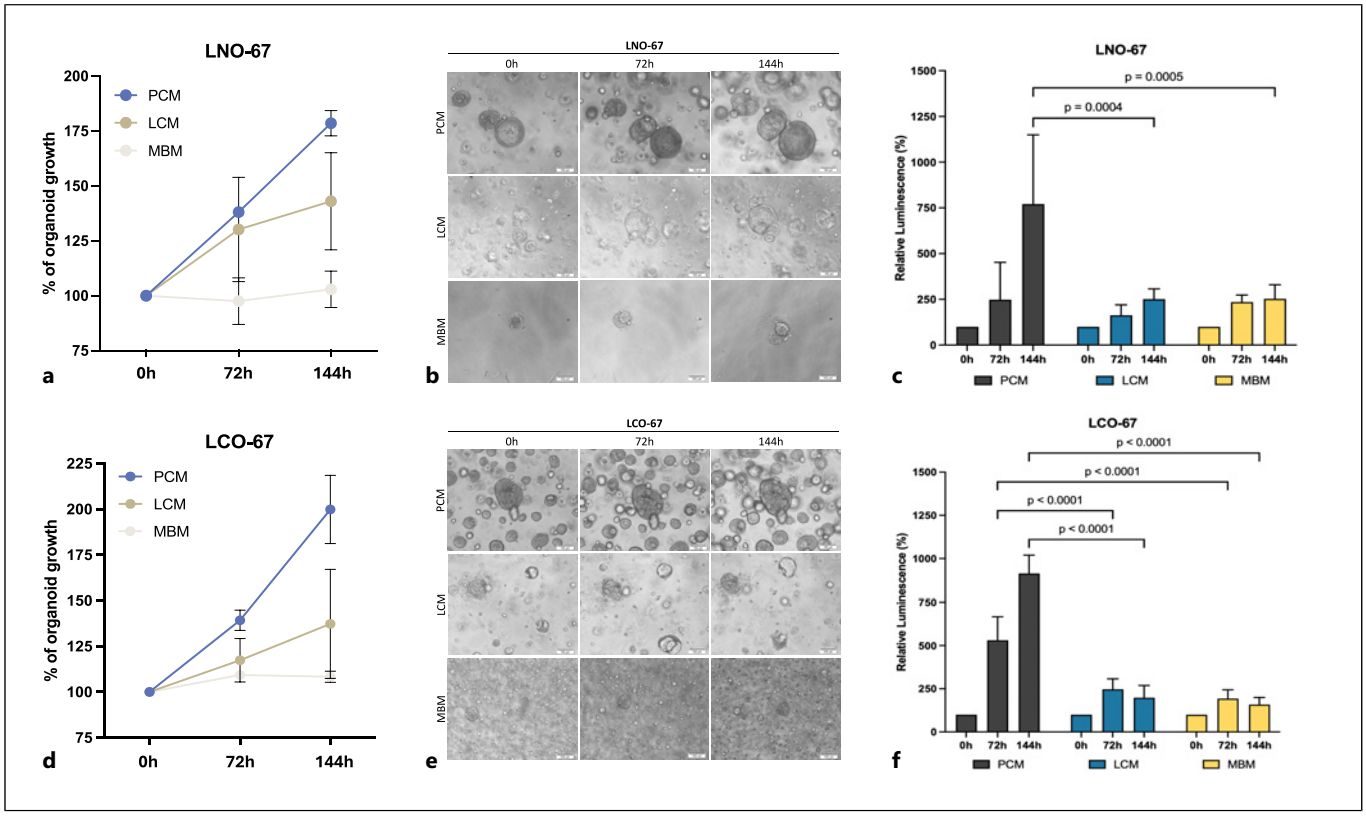

Fig. 3. Representation of growth rate and quantification of metabolically active cells (ATP levels) among patient-derived organoids when cultured with each culture media. **a** The area of organoids from the LNO-67 case (originated from the paired nonneoplastic tissue of a lung ADK patient) cultured in PCM (n = 7 individual organoids), LCM (n = 5 individual organoids), and MBM (n = 3 individual organoids) was measured, and their growth rates over 144 h days were plotted. The growth rate was calculated by tracking each organoid and dividing the area on the final time by that on 0 h (day 3 of passage 3). **b** Representative images of LNO-67 case organoids cultured in PCM, LCM, and MBM, illustrating their changes on 0 h, 72 h, and 144 h. Scale bar, 100 µm. **c** Quantification of metabolically active cells by measuring ATP levels in the culture of LNO-67 line cultured with PCM, LCM, and MBM media for 144 h days. The graph shows % of 0 h (day 0 after passage 3) of relative luminescence. **d** Growth rates during 144 h of LCO-67 case (originated from cancerous tissue of a lung ADK patient) cultured with PCM (n = 7 individual organoids), LCM (n = 5 individual organoids), and MBM (n = 3 individual organoids). **e** Bright-field microscopy images capturing the growth progression of LCO-67 case organoids cultured in PCM, LCM, and MBM on 0 h (day 3 of passage 3), 72 h, and 144 h. Scale bar, 100 µm. **f** Bar graph comparing the use of PCM, LCM, and MBM in relation to the quantity of metabolically active cells by measuring ATP levels in the culture of LCO-67 case over 144 h. The graph shows % of 0 h (day 0 after passage 3) of relative luminescence. Data are represented as mean ± SEM.

In the case of LCOs (Fig. 3e, f), those cultured with PCM also exhibited the highest growth during the 144 h. Significant differences in growth in PCM versus LCM (p = 0.003) and PCM versus MBM (p < 0.0001) were observed (Fig. 3d). LCOs cultured with PCM exhibited an area of 15.2×10^{-3} mm^2 (200% growth), while organoids cultured with LCM showed an area of 7×10^{-3} mm^2 (136% growth), and those cultured with MBM showed an area of 3.9×10^{-3} mm^2 (108% growth). Moreover, the quantification of the metabolically active cells (Fig. 3f) showed significant differences at 72 h and 144 h between PCM and either LCM (p < 0.0001) or MBM (p < 0.0001). Bright-field images substantiate these findings, clearly illustrating a higher number of LCOs and enhanced growth when cultured with PCM compared to the other media (Fig. 3e).

Lung Organoid Cultures Exhibit Evident Differences in Their Morphological Characteristics when Grown with Each Culture Medium

We observed that the selection of the culture medium impacted the phenotypic features of patient-derived LNO cultures, from their generation and establishment to the subsequent passages. It has been reported before that

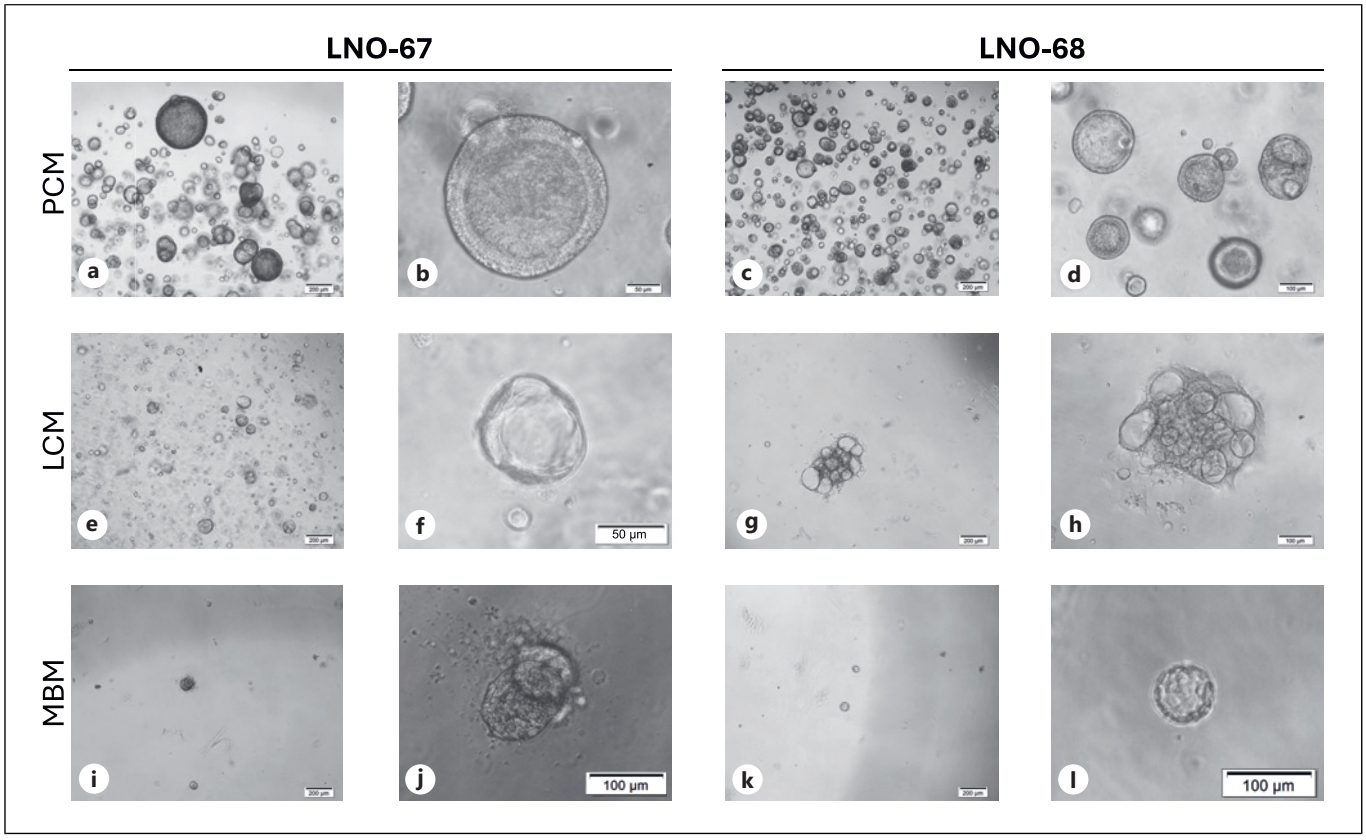

Fig. 4. Phenotypic characteristics of LNOs when cultured with the three-culture media. Comparative of LNOs derived from the paired nonneoplastic tissue of a lung ADK patient (LNO-67) and a lung SCC patient (LNO-68) when cultured in PCM, LCM, and MBM. LNO-67 (**a**, **b**) and LNO-68 (**c**, **d**) organoids cultured with PCM exhibit lumens and larger sizes, morphological characteristics of bronchospheres. LNO-67 (**e**, **f**) and LNO-68 (**g**, **h**) organoids growth with LCM resulted in organoids with lumens, but these were smaller and resembled alveolosphere-like structures. LNO-67 (**i**, **j**) and LNO-68 (**k**, **l**) organoids cultured with MBM appeared as undifferentiated structures. Scale bar: 200, 100, and 50 µm.

LNOs can become differentiated in bronchospheres [23, 24] and alveolospheres [25] resembling bronchial histology or alveolar histology, respectively. We observed that the frequency of emergence of the different types of differentiated structures varies depending on the culture media used. When LNOs were cultured with PCM, more bronchosphere-like structures could be observed independently of the histological subtype of origin. As observed in the case of LNO-67 (paired nonneoplastic tissue from an ADK patient; Fig. 4a), the culture was characterized by large-sized organoids with a multicellular peripheral layer surrounding a central lumen (Fig. 4b). The same can be observed for LNO-68 (paired nonneoplastic tissue from an SCC patient; Fig. 4c, d). To validate that the morphologies observed by brightfield correspond to bronchospheres-like structures, we embedded the LNOs cultured in PCM in paraffin and performed hematoxylin staining (Fig. 5a) and immunofluorescence analysis (Fig. 5b, c). Immunofluorescence showed that the LNOs cultured with PCM were positive for P63, a basal marker, and CC10, a club cell marker. The presence of basal and club cells is a characteristic of bronchospheres.

In contrast, most of the organoids cultured with LCM (Fig. 4e, g) appeared smaller and exhibited a single-layered external membrane of flattened cells around the central lumen, resembling alveolospheres (Fig. 4f, h). To validate that the morphologies observed by brightfield correspond to alveolosphere-like structures, we embedded the LNOs cultured in LCM in paraffin and performed hematoxylin staining (Fig. 5d) and immunofluorescence analysis (Fig. 5e, f). Immunofluorescence showed that the LNOs cultured with LCM were positive for AQP5, an AT1 cell marker, and SFTPC, an AT2 cell marker. The presence of AT2 and AT1 cells is a characteristic of alveolospheres.

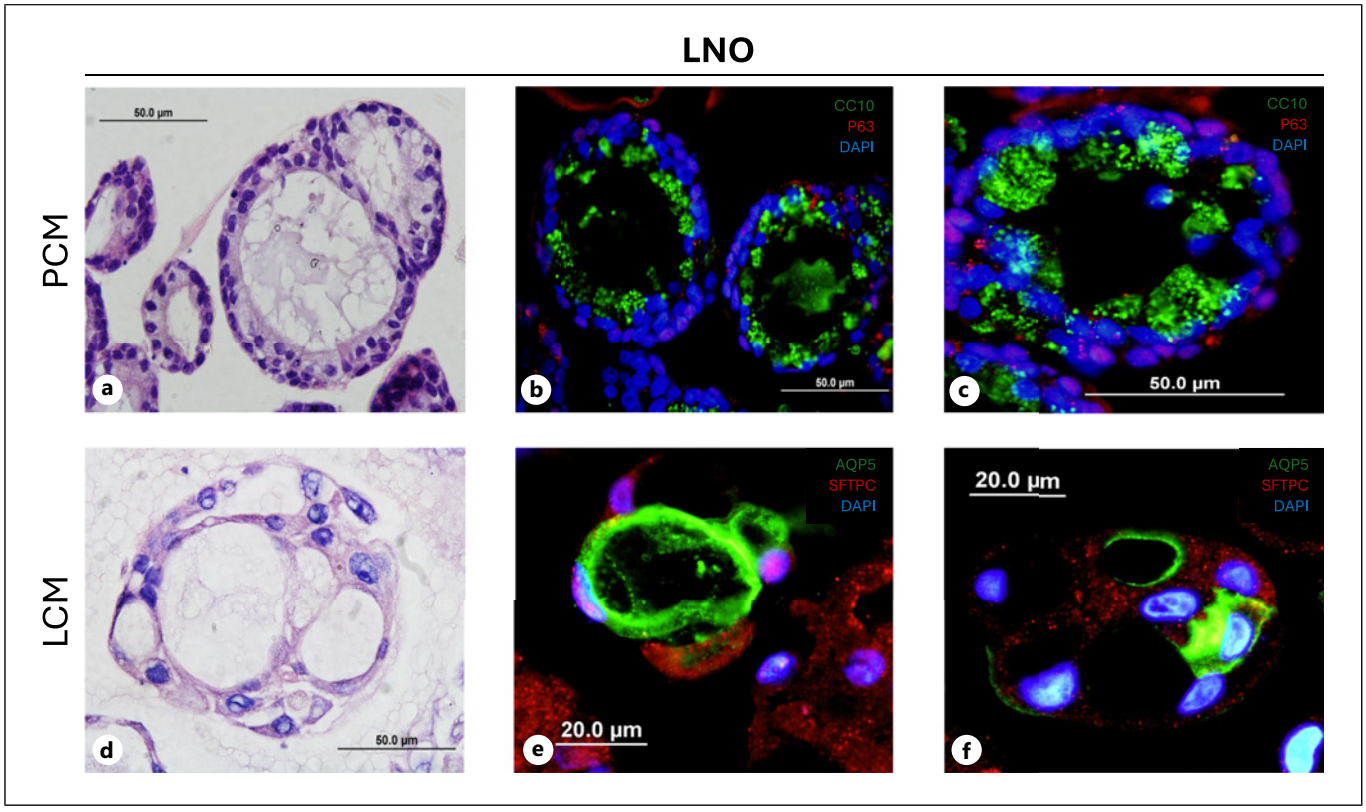

Fig. 5. Immunofluorescence analysis of LNOs. **a** Hematoxylin-eosin staining of LNOs cultured with PCM. **b**, **c** Immunofluorescence analysis of LNOs cultured with PCM. CC10 (green) was used to detect club cells, P63 (red) for basal cells, and DAPI (blue) stains all nucleus. **d** Hematoxylin-eosin staining of LNOs cultured with LCM. **e**, **f** Immunofluorescence analysis of LNOs cultured with LCM. AQP5 (green) was used to detect AT1 cells, SFTPC (red) for AT2 cells, and DAPI (blue) stains all nuclei. Scale bar: 50 and 20 µm.

While LNOs' growth was highly compromised with MBM (Fig. 4i, k), a different phenotype was observed compared to other media. LNOs cultured in MBM resembled spheroids without differentiation to either bronchosphere or alveolosphere (Fig. 4j, l).

In the case of LCOs cultured in PCM, they typically displayed disorganized growth, and an irregular architecture compared to normal organoids, with an absence of a visible lumen in most of them, as noted in the images of LCO-67 (Fig. 6a, b). However, some organoids exhibited cystic structures resembling bronchospheres, regardless of their tissue origin from ADK or SCC tissue, as observed in the case of LCO-68 (Fig. 6c, d). This could be attributed to certain reagents present in PCM, which may promote the contamination of normal organoids alongside tumor-derived ones. It should be required, when possible, to verify that the organoids reproduce the mutational status from the tumor of origin to be sure that the observed morphology corresponds to LCOs. In the case of LCO-67, there was no available information about the mutational status of the tissue of origin, and in LCO-68, the tumor of origin did not harbor point mutations that allowed this verification. In contrast, when these organoids were cultured in LCM, a variety of morphologies were observed, including lumen-less structures (Fig. 6e, f). Interestingly, numerous organoids appeared to be alveolospheres, despite the reduction in LCM of factors that typically promote normal organoid growth (Fig. 6g, h). Organoids cultured with MBM exhibited a small size and displayed a compact, disorganized cellular structure. Additionally, contamination with bronchospheres or alveolospheres was not observed (Fig. 6i–l).

Notably, some organoid cultures with LCM (both normal and tumor-derived) showed mesenchymal cell overgrowth, leading to occasional organoid clustering, as observed in the bright-field microscopy image of LNO-68 (Fig. 4g, h, 6g, h). This phenomenon was not observed in organoids cultured with either PCM or MBM. Although

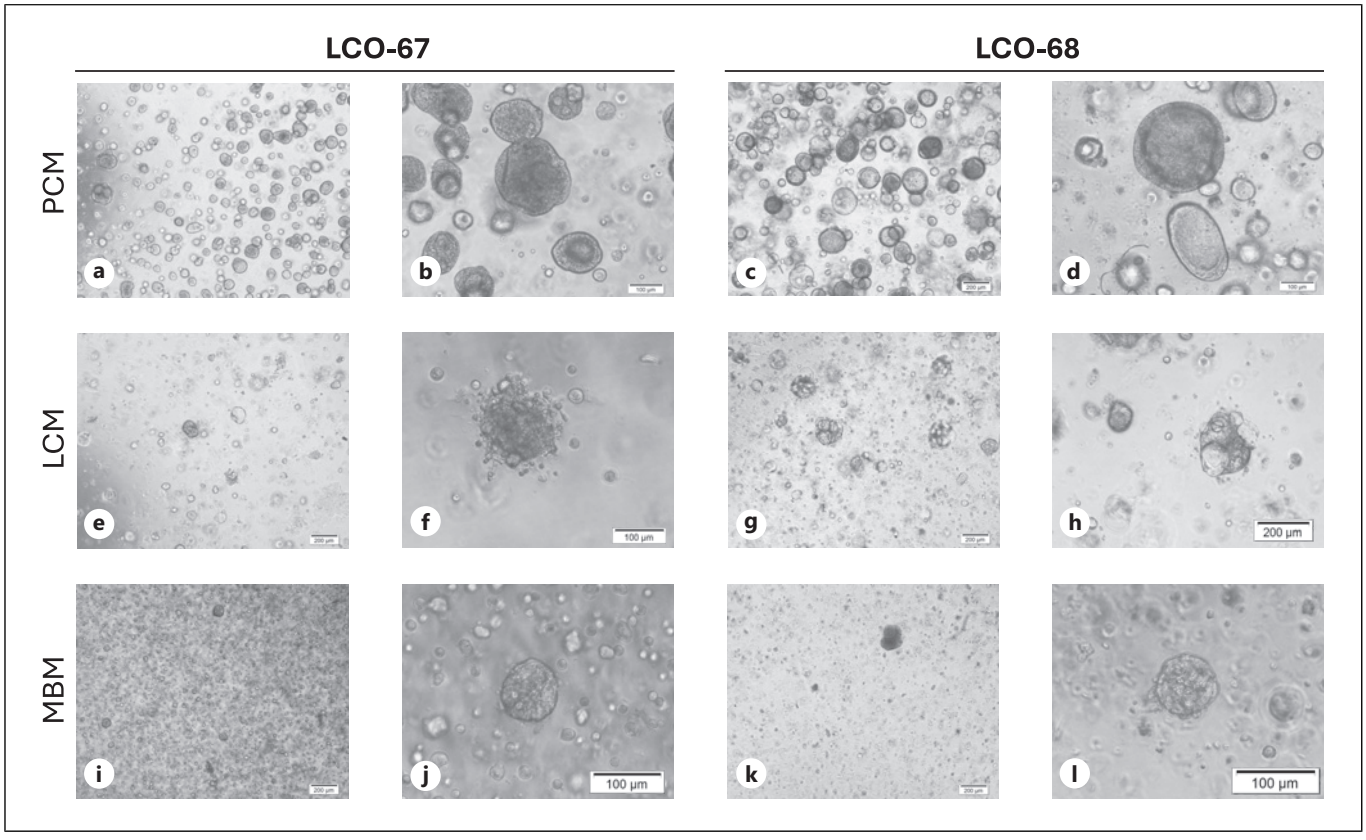

Fig. 6. Morphological features of LCOs when cultured with each culture media. Comparison of LCOs derived from lung ADK (LCO-67) and lung SCC (LCO-68) when cultured in PCM, LCM, and MBM. **a**, **b** LCO-67 organoids displayed a compact and disorganized structure, characteristic of typical tumor organoids when cultured with PCM. **c**, **d** Culture of LCO-68 in PCM showed some organoids with normal morphological characteristics, with a lumen and resembling bronchospheres. **e**, **f** LCO-67 cultured with LCM maintained a tumor-organoid structure. **g**, **h** LCO-68 organoids grown with LCM displayed alveolosphere-like structures. LNO-67 (**i**, **j**) and LNO-68 (**k**, **l**) organoids cultured with MBM showed an irregular cell architecture and absence of a visible lumen. Scale bar: 200, 100, and 50 µm.

mesenchymal cells were initially present in PCM and MBM media, their numbers gradually decreased with successive passages.

Discussion

Patient-derived organoids have emerged as valuable tools in lung cancer research, recapitulating the histological and molecular characteristics of the tissues from which they are derived. However, there are still numerous challenges to face in the culture of LNOs, including the lack of an optimal culture medium adapted to the different LNO and LCO types. Moreover, the different available culture media are complex since they include a high proportion of growth factors that as we have shown in an established lung cancer cell line (H23) and have different effects on proliferation and even in morphology of this cell line cultured in 2D. After the initial passages, we noted that growing the H23 cells with the PCM, LCM, or MBM resulted in reduced viability, with only a small percentage of cells able to establish. A subpopulation of cells had been selected, and changes in their morphology with the appearance of numerous extensions were observed. These changes could be due to certain reagents, such as FGF-7 and FGF-10, which promote differentiation toward distal progenitor lineages and, together with EGF, induce branching [26]. These results showed that the different culture media composition needs to be taken into consideration before beginning any molecular study. Therefore, a study comparing the formulations of the most used LNO culture media was necessary. This article sheds some light on the selection of the most appropriate medium, which, in our opinion, will depend on whether

we aim to work with LNOs or LCOs and on the specific experiments planned for the research, including, for instance, which pathways will be analyzed or if long-term expansion of organoids will be required. Moreover, in the case that we focus on LCOs, the presence of mutations in the tumor of origin can be useful to verify that we are growing LCOs to ensure the purity of the cancer cells present in the culture.

An LCO culture medium is considered optimal when it enables the successful establishment of organoids, long-term expansion, and maintenance of the purity of cancer cells in culture. However, none of the current media satisfy all requirements. PCM, such as the one described by Sachs et al. [12], allows viable organoids to be maintained over time but cannot prevent the overgrowth of normal organoids. Specially in this medium, it is important to verify the purity of LCOs, which can be done through verification that the mutational patterns of the tumor of origin are conserved in the generated organoid, which does not always happen [13]. In our study, only 2 of 6 (with available information) LCOs cultured with PCM (33%) retained the mutations detected in the tumor of origin. Therefore, we must consider completing the culture media with some reagents that improve the survival of mutated populations. For instance, in TP53-mutated cases we can add NUTLIN-3A in the medium, an MDM2 inhibitor that only allows the growth of TP53-mutated cells [12]. Another example is the use of the pan-ErbB inhibitor, which exploits the high prevalence of mutations in the ERBB pathway [20]. These methods are unsuitable for all cases since mutations found in the tumor of origin are diverse. Additionally, this can produce a loss of the heterogeneity found in the original tumor, where different cancer cell populations coexist even with different mutational patterns.

On the other hand, LCM or MBM media, such as those used by Hu et al. [15] and Kim et al. [14], only allow short-term viability of organoids, although they ensure reduction/inhibition of normal organoid growth. PCM had the highest success rate and was the most effective on long-term expansion of organoids since we considered that an organoid cell line was successfully established if retained viability after the second passage. In this line, PCM was the most effective in retaining viability from the second passage onward, followed by LCM and MBM. Hence, for researchers interested in long-term studies, choosing the PCM medium enables multiple passages to be conducted over an extended period. Regarding the organoids' size, growth, and viability, it was observed that the PCM medium produced significantly larger organoids with greater viability compared to the LCM and MBM media.

When we evaluated the success of LCOs establishment according to their histology using the three described media, it was noted that organoids derived from SCC exhibited the highest rate of successful establishment. In PCM-cultured organoids, this can easily be explained because it contains factors such as FGF-7, FGF-10, Noggin, and SB202190, which have been demonstrated to promote the establishment of basal stem cells [27], which are considered the cell of origin for SCC upon acquiring a malignant phenotype [28].

LNOs showed important differences in the morphological characteristics when cultured with the three different types of culture media. LNOs cultured with PCM clearly become differentiated through the passages into bronchospheres, whereas LNOs cultured with LCM also allowed alveolar differentiation. These findings can be explained by the different composition of each culture medium. LCM contains dexamethasone, which induces alveolar maturation [29], and EGF, which increases the size of alveolar organoids [30]. Regarding LNOs cultured with MBM, these did not seem to induce differentiation, probably because they presented only the minimal reagents required to allow the cells to organize into organoids for a brief period of time. In conclusion, for studies focused on the proximal airways, PCM medium is recommended to facilitate the growth of bronchospheres. On the other hand, the use of LCM medium is proposed for the study of alveolar structures.

Referring to LCO morphology, all three media resulted in the generation of compact and disorganized spheroids, typical characteristics of cancerous organoids. Notably, both PCM and LCM revealed the emergence of bronchospheres and alveolospheres, which are suspected to be derived mostly from normal stem cells. MBM was the only medium that did not show the appearance of organoids with a normal appearance in LCO cultures. Therefore, in studies aimed at obtaining a representative and specific population of cancer stem cells from the tumor, such as drug efficacy studies, the MBM medium is the preferred choice. Conversely, for investigations seeking a broader representation of the tumor microenvironment, encompassing both tumor and normal cells, the use of PCM or LCM media is recommended.

Interestingly, our work also revealed that the choice of culture medium influenced the growth of mesenchymal cells surrounding organoid cultures. Growth of mesenchymal cells was predominantly observed in

organoids cultured with LCM, often resulting in close interactions with alveolospheres, clustering them and forming structures reminiscent of alveoli. This implies that PCM and MBM may contain compounds that inhibit mesenchymal growth, such as *RSPO1*, which activates the Wnt/β-catenin pathway [31]. This singularity is relevant for research involving the coculture of tumor organoids with mesenchymal cells to study the tumor microenvironment.

In summary, our work underscores the importance of considering the research objectives when selecting an appropriate culture medium to grow patient-derived LNOs. PCM is associated with the highest success rates, increased growth, and better cell viability, making it ideal for long-term studies. However, this medium promotes contamination of bronchospheres in tumor cultures, comparable to LCM, but with the overgrowth of alveolospheres instead. The medium that best reproduces tumor cells is MBM, ideal for drug testing studies that do not need to maintain organoid growth over a long period of time. Future studies should continue to explore and optimize the components and reagents of organoid culture media to ensure the most faithful representation possible of patient tumors, ultimately leading to advances in lung cancer research.

Statement of Ethics

This study was approved by the Bioethics Commission of the University of Barcelona (Project approval no. HCB/2021/0711). All the subjects signed the informed consent. The study adhered to the ethical guidelines outlined in the Helsinki Declaration (2002).

Conflict of Interest Statement

All the authors have reviewed and approved this version of the manuscript and agree with the decision to submit it. None of the authors declare any conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Author Contributions

M.A.-P. performed the research and the statistical analysis and wrote the manuscript. Y.H., D.M., J.P.O., A.C., A.A., T.Y., T.D., L.M., R.R., and R.M.M. collaborated on the research. A.N. designed the study, wrote the manuscript, and took responsibility for the integrity of the data. All authors have read and approved the final version of the manuscript.

Data Availability Statement

All the data generated or analyzed during this study are included in this published article. Further inquiries can be directed to the corresponding author.

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