

# Therapeutic applications of three-dimensional organoid models in lung cancer

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## Introduction

Lung cancer is a major public health problem and the most common cause of cancer death worldwide [1]. About 80% to 85% of lung cancers are non-small cell lung cancer (NSCLC). The main subtypes of NSCLC are adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. The discovery of treatable

Lung cancer, which remains a major cause of mortality worldwide, is a histologically diverse condition and demonstrates substantial phenotypic and genomic diversity among individual patients, manifesting as both intertumoral and intratumoral heterogeneity. This heterogeneity has made it difficult to develop lung cancer models. Two-dimensional (2D) cancer cell lines have been used to study genetic and molecular alterations in lung cancer. However, cancer cell lines have several disadvantages, including random genetic drift caused by long-term culture, a lack of annotated clinical data, and most importantly, the fact that only a subset of tumors shows 2D growth on plastic. Three-dimensional models of cancer have the potential to improve cancer research and drug development because they are more representative of cancer biology and its diverse pathophysiology. Herein, we present an integrated review of current information on preclinical lung cancer models and their limitations, including cancer cell line models, patient-derived xenografts, and lung cancer organoids, and discuss their possible therapeutic applications for drug discovery and screening to guide precision medicine in lung cancer research. Altogether, the success rate of generating lung cancer organoids must be improved, and a lung cancer organoid culture system is necessary to achieve the goal of designing an individualized therapeutic strategy for each lung cancer patient.

**Keywords:** Lung cancer; Organoid

oncogenic alterations led to the recommendation to include molecular testing in the standard approach [2]. This includes testing for mutations in the gene encoding epidermal growth factor receptor (EGFR), the B-Raf proto-oncogene, and serine/threonine kinase V600E, as well as searching for translocation in the genes encoding anaplastic lymphoma kinase and proto-oncogene 1 (ROS1). Targeted therapy against those driver mutations led

to longer survival in patients with oncogenic driver mutations who received targeted therapies than among either patients with driver mutations who did not receive targeted therapies or patients without driver mutations [3]. However, platinum-based chemotherapy remains the cornerstone of treatment in patients who have advanced NSCLC without treatable oncogenic alterations. The response rate is approximately 25% to 30%, the median survival is 8 to 12 months, and the 1-year survival rate is 30% to 40% in these patients [4]. This field is rapidly evolving, with multiple nuances deserving thorough discussion.

In recent years, the treatment and prognosis for patients with metastatic lung cancer have profoundly changed due to the introduction of immune checkpoint inhibitors [5]. Inhibition of programmed cell death 1 (PD-1) and programmed death ligand 1 (PD-L1) interactions can lead to restored T-cell function and antitumor activity [6]. The PD-L1 tumor proportion score is now routinely used to predict whether patients will benefit from anti-PD-1 agents and select therapy in advanced NSCLC. In addition, the tumor mutation burden has also been proposed as a potential predictive biomarker of benefit from anti-PD-1 therapy [7]. Therefore, understanding genetic alterations and the tumor immune microenvironment associated with therapeutic screening is important.

NSCLC is histologically diverse and demonstrates substantial phenotypic and genomic diversity among individual patients, manifesting as both intertumoral and intratumoral heterogeneity; this heterogeneity makes it difficult to create animal models [8]. Each tumor harbors a different mutational pattern and even tumors with the same histological appearance exhibit molecular diversity [9]. Tumor heterogeneity results in significant differences in the tumor growth rate, invasion ability, drug sensitivity, and prognosis [10]. However, a major challenge in studying NSCLC is the low quantity of cells that can be isolated from lung tissue biopsies [11]. Patient-derived lung cancer models continue to be developed to gain a better understanding of molecular pathogenesis, to identify novel therapeutic targets that may also serve as promising biomarkers, and to test novel therapeutic agents, thereby allowing personalized anti-cancer therapy in clinical settings [12,13]. As the establishment of a high-fidelity preclinical cancer model is urgently needed [13], cancer cell lines and patient-derived xenograft (PDX) models have been used to study genetic and molecular alteration. However, these models of human lung cancer have many limitations [14].

The term “organoid,” which means “resembling an organ,” was used as early as 1946, and organoids now refer to three-dimensional (3D) structures composed of multiple cell types of their *in vivo* counterparts, similar to primary tissue and specific to

the parent organ [14,15]. Although major advances were outlined in recent reports describing protocols for the development of lung cancer organoids (LCOs) [16-18], the establishment of pure LCOs is challenging [19]. Herein, we describe current information on preclinical lung cancer models and their limitations, including cancer cell line models, PDXs, and LCOs, and discuss their possible therapeutic applications for drug discovery and screening to guide precision medicine in lung cancer research.

## Preclinical models

### 1. Cancer cell line models

Over the last decades, tremendous efforts have been made to develop preclinical models of NSCLC, including 2-dimensional (2D) cell lines and air-liquid interface cultures [20]. Cancer cell lines, which are characterized by low cost and ease of use, have been broadly employed for high-throughput screening of drug candidates and cancer biomarkers [13]. The US National Cancer Institute (NCI) and the Human Cancer Center lines are the 2 largest series of lung cancer cell lines that have been established, containing more than 200 lung cancer cell lines, of which perhaps 150 are well characterized [20]. Cultured tumor cells accurately represent tumor cells *in vivo* without the complex *in vivo* environment and are basically populations of pure tumor cells without admixed stromal or inflammatory cells [21]. The lack of stromal and inflammatory cells is practical for large-scale pharmacogenomic studies [12]. These projects include genomics, copy number variation analyses, transcriptomics, and screening for drug response in more than 100 lung cancer cell lines, as well as studies investigating associations between predictive biomarkers and drug sensitivity [22-25]. For example, EGFR tyrosine kinase inhibitors (EGFR-TKIs) have been developed to target activating EGFR mutations based on an *in vitro* model of mutation specificity created by calculating the ratio of IC50 values between mutated and wild-type EGFR [26]. Based on these pharmacogenomic and drug sensitivity models, we have multiple EGFR-TKI options to treat patients with lung cancer harboring activating EGFR mutations [27]. Moreover, cancer cell line models are also relatively easy to work with for genetic manipulation. CRISPR-Cas9 is a versatile genomic editing technology used to study the functions of genetic elements that accelerates the study of multigenic processes, such as the role of mutation combinations in tumor evolution [28]. Cas9 has been broadly applied in a variety of cell line- and embryo-based experiments [29,30]; however, the *in vivo* applications of Cas9 in somatic tissue remain more challenging due to a combination of factors, such as its large transgene size [28]. In addition, cell lines enable clonal selection and expansion to

validate and select for positive knock-out cells [12].

Although cell lines are commonly used in preclinical models, they have important limitations that should be considered [31]. First, contamination of long-term cultured cells represents a major problem [20]. Second, genetic and mRNA changes have been reported in cell lines [32]. Several studies showed poorly concordant drug sensitivity results in the same cell lines with different experimental protocols [33,34]. Third, cancer cells no longer retain the tumor heterogeneity present in the primary cancer [31]. Cell lines are likely to represent a subpopulation of the original tumor and are largely homogeneous due to the selective survival pressures present in culture conditions [35]. Fourth, cell lines do not contain the relevant components of the tumor microenvironment [31]. The lack of interaction with stromal, immune, and inflammatory cells limits translational cancer cell line-based studies, especially immunologic research [20]. In recent years, patient-derived cancer cell culture models from tumor biopsies were established, and their immunofluorescence-based functional assays are promising in response to targeted therapy [36]. Further studies elucidating the concordance of drug responses between patients and the respective cell lines are needed.

## 2. Patient-derived tumor xenograft models

PDXs are models involving the implantation of patient-derived tumor tissue into immunodeficient mice [37]. Compared with conventional models, PDXs are characterized by the preservation of tumor heterogeneity and the tumor microenvironment (including stroma/vasculature), which are expected to enable a high ability to predict therapeutic efficacy [37,38]. Based on recent advances, the US NCI announced plans to switch its anti-cancer drug screening system to PDX-based models [39]. The engraftment rate varies greatly depending on the type of tumor. High graft survival rates (80% or higher) have been reported for melanoma and colorectal cancer, whereas the rate is as low as approximately 30% for breast cancer [40,41].

In NSCLC, the engraftment rates range from 25% to 60%, depending on 3 essential elements of PDX models: (1) the tumor properties; (2) the recipient mice; and (3) the recipient site [42-44]. Surgically resected tumors are usually established in PDX models; however, PDXs can also be created using biopsy specimens or circulating tumor cells collected from the blood, since advanced lung cancer patients seldom undergo surgical resection [42,45]. Moreover, the probability of the successful engraftment of PDX lesions is higher for tumors from metastatic foci or with greater malignancy potential [46,47]. To improve the efficacy engraftment rates, severe combined immune deficiency (SCID) mice were crossbred with non-obese diabetic (NOD) mice,

yielding NOD/SCID mice with composite immunodeficiency [48,49]. However, NOD/SCID mice have a short lifespan and a high incidence of thymoma, and lack mature T-cell graft survival [50]. In 2002, NOD/SCID gamma (NSG) mice were developed from a NOD/SCID background with additional interleukin-2 receptor gamma chain impairment [51]. NSG mice are considered to be the best immunodeficient animal for human graft transplantation [52]. Regarding the recipient site, heterotopic implantation (e.g., subcutaneous grafting) provides the advantages of a simple procedure and accurate tumor size measurements [53]. However, orthotopic implantation provides a native tumor and metastasis environment, although it is technically challenging [54].

Although PDX models are considered promising tools for individualized cancer therapy, drug development and coclinical trials [38], several important limitations of PDX models should be considered. First, the tumor microenvironment is virtually non-existent due to the lack of stromal cells and degradation of tissue architecture [38]. The original human stromal and immune cells are replaced by mouse stromal cells after serial passages, thereby losing the contribution of human stromal cells to the original tumor biology. Immunotherapy has recently revolutionized cancer treatment; however, PDXs generated in non-humanized immunodeficient mice cannot be efficiently used to study immunotherapy. Humanized mice have been developed by intravenous injections of CD34<sup>+</sup> cells isolated from the blood of patients into mice to reconstruct a functional system in murine models that would mimic that of patients [55,56]. Second, large-scale experiments are difficult to perform in PDXs; in addition, genetic drift may occur after numerous passages. It is common practice to limit PDX experiments to fewer than 10 passages [57]. Third, less aggressive tumors exhibit decreased implantation rates and more aggressive tumors exhibit increased formation rates. Therefore, improvement of the implantation rate is urgently required. Fourth, substantial resources (e.g., money, time, and labor) are needed to create PDX models [37]. For example, typically, at least 3 months are required to develop PDXs that may be used for preclinical studies, and not all patients may be able to afford the high costs of PDX models, especially when using humanized mice [12].

## Three-dimensional lung organoids

### 1. General concepts of organoids

Organoids are defined as 3D structures derived from organ-specific stem cells that self-organize through cell sorting and spatially restricted lineage commitment in a manner reminiscent of the native organ with some degree of organ functionality [58,59].

Organoid cultures can be established from embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and adult stem cells (ASCs) [60]. ASC-derived organoids have been used to study models of infectious, hereditary, and oncological diseases that recapitulate the essential features of *in vivo* disease [60]. Although organoids were first successfully derived from the mouse small intestine using single Lgr5<sup>+</sup> stem cells [61], organoids have been cultured from multiple endoderm-derived organs, including the human colon, prostate, and intestine [62-64]. Organoid growth requires the initiating stem cell population to self-renew, increase the organoid size, and differentiate [65].

Attempts have been made to develop artificial counterparts of *in vivo* organs from their tissues or cells, and organoid technology as a technological field emerged in tissue engineering [66]. Schwank et al. [67] reported functional repair of the cystic fibrosis transmembrane conductor receptor by CRISPR/Cas9 in cultured intestinal stem cell organoids from cystic fibrosis patients. These organoids provide a powerful platform for elucidating disease development mechanisms, modeling diseases, and screening drug candidates for genetic, infectious, and malignant diseases [68]. Organoid cultures will be facilitated by the application of 3D printing as a new and potentially promising technology [69]. For example, organoids have been used as a promising platform to research how coronavirus disease 2019 affects humans and causes damage and to identify possible drug targets [70]. In the field of regenerative medicine, there is still a long way to go for the transplantation of organoids as therapy [71,72]. Below, we discuss the present limitations and potential future applications of organoids for research on malignancies, especially lung cancer.

## 2. Lung organoids

The lung is a complex organ comprising multiple cell types that perform a variety of vital processes, including immune defense and gas exchange [58]. The lung is comprised of 2 main compartments (airways and the alveolar space), which contain distinct stem cell populations: basal and club cells in the airway and alveolar epithelial type 2 (AT2) cells in the alveolar space [73]. Organoids providing a promising platform to investigate the function of lung epithelial and progenitor cells could be derived from basal cells, club cells, variant club cells, bronchoalveolar stem cells, and AT2 cells [14]. Human pluripotent stem cells (hPSCs), including ESCs and iPSCs, have been recently adopted for research [74]. A number of pulmonary diseases, including chronic obstructive pulmonary disease, asthma, pulmonary fibrosis, viral infectious diseases, and lung cancer, have been proposed to be associated with improper epithelial regeneration [14,75]. Epithelial damage and impaired regeneration, which result from stem cell exhaustion,

contribute to recurrent pulmonary infection and persistence of inflammation, since cells that are injured vary in different lung diseases [76,77]. Although culture models from animal lungs have been developed [78], culture from primary human cells can be hampered by logistical challenges.

The first self-organizing 3D structure of adult human airway epithelial cells cultured on collagen was described in 1993 [79]. Airway basal cells gave rise to tracheo/bronchospheres in a 3D air-liquid interface and produced branching structures with multipotent potential [80,81]. The first attempt to generate hPSC-derived organoids was reported in 2015, and showed that hPSCs differentiated into multi-lineage organoids, containing basal, ciliated, and club cells [82,83]. Lung bud organoids (LBOs), which were later induced from hPSCs, recapitulate many aspects of lung development, allowing branching morphogenesis and initial alveologenesis [84]. LBOs consist predominantly of AT2 cells that actively take up and secrete surfactant, reflecting an important function of AT2 cells. Tan et al. [85] combined human adult primary bronchial epithelial cells, lung fibroblasts, and lung microvascular epithelial cells in supportive 3D culture conditions to generate airway organoids. Mixed cell populations underwent rapid condensation to self-organize into discrete epithelial and endothelial structures that were stable for up to 4 weeks of culture. In a recent study, long-term expanding human airway organoids from bronchoalveolar resections or lavage material were established, which provided versatile models for the study of hereditary, malignant, and infectious diseases [17].

Although lung organoids currently represent the closest model to the human pulmonary system, several limitations should be considered. First of all, the absence of the immune system, circulatory system, and naïve extracellular matrix is a limitation. Current organoid matrix materials have lot-to-lot variability and spatial heterogeneity [86]. Combined with CRISPR/Cas9 technology, lung organoids can be used to model genetic diseases and test drug treatment. Most drug screening platforms require the use of the same starting material; however, organoids are self-organized tissues, and therefore are usually not uniform in size [74]. A number of outstanding challenges currently need to be addressed; however, the use of organoids combined with the novel techniques of live imaging, genetic engineering and biomaterials will hugely advance the pulmonary field.

## LCOs and translational research in the clinic

### 1. LCOs

Recent advances using 3D organoid cultures derived from patient

cells have opened the possibility to employ LCOs as tools for personalized medicine [16-19,87,88]. However, the success rates of long-term culture establishment vary substantially, and detailed descriptions of the number of passages achieved and split ratios used are typically not reported (Table 1) [16-19,87,88]. A major challenge when culturing cancer samples from primary intrapulmonary tumors is potential overgrowth by normal epithelial cells, limiting the overall establishment rate of pure LCOs to 17% [19]. One method to increase establishment is to treat organoid cultures with the MDM2 inhibitor nutlin-3a to selectively grow out p53 mutant cells, resulting in pure tumor cells harboring *TP53* mutations; this method is useful due to the large amount of contamination by nontumor cells [17]. Alternatively, the use of a suboptimal medium for LCO culture did not support normal cell growth, and use of feeder-free LCO lines could avoid fibroblast contamination, resulting in a 70% success rate [87]. In addition, an understanding of biases in fibroblast outgrowth may help formulate culture media that are more permissive [89]. Intratumoral heterogeneity should be considered because the populations of cancer cells in different parts of the same tumor may exhibit different drug sensitivity; therefore, multiple sampling may be necessary [14].

LCOs derived from primary tumors should be routinely evaluated for tumor purity using genetic methods or histomorphology combined with p63 and CK5/6 staining [19]. In mixed cultures, a small subpopulation of normal airway organoids eventually dominates the culture. Repeated evaluation of tumor purity is important for mixed tumor/normal cultures. Histomorphology combined with p63 staining can also make it easier to distinguish subtype markers for lung cancer such as

TTF-1, cytokeratin 5, and synaptophysin, thereby providing a reliable classification [16,87]. LCOs further maintain defined genetic characteristics, including copy number profiling, single-nucleotide polymorphism genotyping, and variant allele frequency distribution [19]. Next-generation sequencing of LCOs and tumors has demonstrated the presence of matching somatic mutations, such as *EGFR*, *KRAS*, and *TP53*.

Recent studies substantially overcame the limitations of LCO models, which are the lack of the complexity of the immune system and vascularization, key cell types, and high-throughput workflows. LCOs may be maintained from tumor biopsies or surgical resection in both short- and long-term (more than 10 passages) culture and show strong correlations with the parental tumor in terms of gene expression [17,88]. Studies have recently described protocols to establish organoids from various epithelial tissues and cancers, as well as protocols to test drug sensitivity in patient-derived organoids [90]. Advances in procedure to isolate circulating tumor cells or cells in aspirated pleural effusion have made it possible to establish organoids, which help to acquire relevant genetic and epigenetic information about tumors in real time, as well as to screen and test promising drugs [11,91,92]. Kim et al. [87] established a living biobank of 80 LCOs derived from major lung cancer subtypes, which predict patient-specific drug response. Last, LCO modeling of tumor immune microenvironment models with endogenous immune stroma could enable immuno-oncology investigations [16].

## 2. Drug screening applications

In the past decades, many anti-cancer drugs developed by screening traditional 2D cell cultures as preclinical disease model

**Table 1.** Summary of lung cancer organoids models: published reports

Year	Study	Success rate (%)	Source	Key feature
2018	Dijkstra et al. [18]	6/6 (100)	Resection (4/4) Biopsy (2/2)	Co-culture of peripheral blood lymphocytes and tumor organoid
2018	Neal et al. [16]	20/23 (87.0)	Resection	Success rate in adenocarcinoma (14/16), squamous cell carcinoma (6/7)
2019	Sachs et al. [17]	5/18 (27.8)	Biopsy	Use of nutlin-3a > 10 passages Neutrophil-epithelium interaction
2019	Kim et al. [87]	39/56 (69.6)	Resection	Suboptimal media Feeder-free cell lines
2020	Dijkstra et al. [19]	10/58 (17.2)	Resection (5/28) Biopsy (4/30)	Overgrowth by normal airway organoid (14/58) Success rate: extrapulmonary lesion (6/27) > intrapulmonary lesion (3/31)
2020	Shi et al. [88]	57/65 (87.7) 47/65 (72.3), short-term: 1-3 mo 10/65 (15.4), lung-term: > 3 mo	Resection	Use of nutlin-3a Whole-exome and RNA sequencing

system have proven to be ineffective in clinical studies [93]. As patient-derived lung cancer cultures enable personalized patient care [36], organoids derived from lung cancer patients can be used for further high-throughput drug screening [94]. Tumor organoid technology has the potential to predict patient response; therefore, research groups have performed drug screening on patient-derived organoids [17,87,90,95]. *In vitro* high-throughput assays using patient-derived tumor organoids were suitable for evaluating molecular-targeted drugs under conditions that better reflect pathologic conditions [96]. Recently, living biobank-based genomic alterations of the original tumors have been established; therefore, a biobanking system of tumor organoids provides promising opportunities for patient-specific drug trials [87]. Wang et al. [97] showed promising antitumor activity with pyrotinib using a patient-derived organoid model from HER2-mutant lung cancer, and they validated those preclinical findings in patients enrolled in a phase II clinical trial. Based on recent advances in microfluidic-based culture platforms that can load, expand, and identify drug responses under physiologically relevant conditions, organoid drug screening assays provide important information to guide therapeutic approaches at the preclinical level [98]. These platforms have further been used in attempts to isolate and expand lung-circulating tumor cells and patient immune cells from liquid biopsies [99]. However, there has been no comparative study of organoid marker expression or drug responses in different media formulations. In addition, robust drug screening data would be highly promising for personalized medicine.

### 3. Research in immuno-therapy

Immune checkpoint inhibitors, such as anti-PD-1/PD-L1, have transformed the treatment landscape for lung cancer. However, preclinical models that incorporate both endogenous T cells and tumor cells are scarce. In addition, the expansion of tumor-infiltrating lymphocytes has been especially challenging for epithelial cancers [100]. While defined media led to the loss of stromal cell fraction during LCO establishment, the approach used to establish tumor-stroma organoids is to recombine stromal and parenchymal tumor fractions after culturing them separately. In 2018, Dijkstra et al. [18] showed that co-culture of autologous tumor organoids and peripheral blood lymphocytes provided a means by which to assess the sensitivity of tumor cells to T-cell mediated attack at the level of the individual patient. The procedures are as follows: (1) organoids are isolated from Geltrex (Thermo Fisher Scientific, Waltham, MA, USA) 2 days before co-culture and stimulated with interferon-gamma 1 day before co-culture; (2) on the day of co-culture, organoids are dissociated to single cells and plated together with peripheral

blood mononuclear cells (PBMCs) on an anti-CD28-coated plate, in the presence of interleukin-2 and anti-PD-1; (3) after 1 week of co-culture, PBMCs are restimulated with tumor cells; and (4) after 2 weeks of co-culture with the autologous tumor organoid, T-cell reactivity is assessed by evaluating CD107a and interferon-gamma expression or CD137 expression in the presence or absence of tumor organoids. In addition, a tumor organoid killing assay can be assessed by live-cell imaging or flow cytometry-based quantification of live tumor cells [100]. However, lung cancer is considered to comprise hypermutated tumors, whereas organoids are often derived from small biopsies, which represent only a small part of the tumor tissues and therefore might underestimate the complexity of the entire tumor tissue [101]. Furthermore, LCOs are not exposed to the external pressures that occur *in situ*, such as hypoxia or immune selection, which can influence the outgrowth of tumor clones, leading a situation in which a dominant clone *in vitro* is not as dominant *in situ* and vice versa [101]. Nevertheless, in the near future, it will be possible to model the tumor immune microenvironment using a patient-derived organoid approach that preserves the original tumor T-cell receptor spectrum and successfully models immune checkpoint inhibitors for biomarker identification, drug screening, and modeling of therapy resistance [102].

The potential use of LCOs for precision medicine is limited by their low success rate and frequent overgrowth by normal airway organoids. For all these therapeutic applications to be established, LCO success rates must be enhanced.

## Conclusion and further perspectives

The use of organoids is expanding in the field of lung cancer, and progress has been made in understanding the relationship between cancer biology and genetics. Although the use of recently emerged LCO models remains in its infancy, these models provide drug screening and applications for T-cell based immunotherapy at the level of the individual patient. With a better understanding of immuno-oncology and advanced translational research, LCOs are expected to improve the concordance between drug response and actual clinical outcomes in the future. In conclusion, our review demonstrates that the success rate of generating LCOs must be improved, and a LCO platform is necessary to achieve the goal of designing a therapeutic strategy for each lung cancer patient.

## Notes

### Conflict of interest

Seung Joon Kim has been the editor-in-chief of *Organoid* since

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### Author contributions

Conceptualization: CDY, YPY, CKP, SJK, Methodology: all authors, Data curation: CDY, YPY, CKP, SJK, Investigation: all authors, Formal analysis: CDY, YPY, Project administration: CKP, SJK, Resources: CDY, YPY, CKP, SJK, Validation: CDY, YPY, CKP, SJK, Writing—original draft: CDY, YPY, Writing—review & editing: all authors.

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