Review Article on New Developments in Lung Cancer Diagnosis and Pathological Patient Management Strategies

Patient-derived cell line, xenograft and organoid models in lung cancer therapy

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Contributions: (I) Conception and design: All authors; (II) Administrative support: MS Tsao; (III) Provision of study materials or patients: None; (IV) Collection and assembly of data: K Huo, E D'Arcangelo; (V) Data analysis and interpretation: K Huo, E D'Arcangelo; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

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Abstract: Lung cancer accounts for most cancer-related deaths worldwide and has an overall 5-year survival rate of ~15%. Cell lines have played important roles in the study of cancer biology and potential therapeutic targets, as well as pre-clinical testing of novel drugs. However, most experimental therapies that have cleared preclinical testing using established cell lines have failed phase III clinical trials. This suggests that such models may not adequately recapitulate patient tumor biology and clinical outcome predictions. Here, we discuss and compare different pre-clinical lung cancer models, including established cell lines, patient-derived cell lines, xenografts and organoids, summarize the methodology for generating these models, and review their relative advantages and limitations in different oncologic research applications. We further discuss additional gaps in patient-derived pre-clinical models to better recapitulate tumor biology and improve their clinical predictive power.

Keywords: Lung cancer; preclinical models; organoid; xenograft; cell line; patient-derived models; 3D culture

Submitted Jan 15, 2020. Accepted for publication Apr 21, 2020.

doi: 10.21037/tlcr-20-154

View this article at: http://dx.doi.org/10.21037/tlcr-20-154

Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide, accounting for 22% or 2.09 million deaths in 2018 (The World Health Organization: https://www.who.int/news-room/fact-sheets/detail/cancer, accessed on Jan 04, 2020). The molecular basis of the highly aggressive biology of lung cancers remains largely unclear, despite efforts to identify complex genomic aberrations occurring in various histological subtypes of this disease (1,2). Treatments of lung cancer patients are determined by multiple factors including tumor histology, stage and biomarker status, the latter include actionable driver mutations and the expression level of immune checkpoint programmed death-ligand 1 (PD-L1) (3). For patients with actionable driver mutations, there are currently many clinically approved targeted therapies, most of which target the protein product of mutated oncogenes. Activating mutations in the tyrosine kinase domain of the *epidermal growth factor receptor* (*EGFR*) gene were the first genomic alterations in non-small cell lung cancer (NSCLC) to be successfully targeted (4-8). Since then, many kinase inhibitors have been developed and used as first-line treatment for patients with mutations in other actionable drivers, including *ALK*, *ROS1*, *NTRK*, *RET*, *BRAF* and *MET*, and have shown significant improvement in patient survival (9). Similarly, for patients with high levels of immune checkpoint PD-L1, immunotherapy has also prolonged survival compared to chemotherapies (10,11).

However, the initial responses to therapies almost always result in eventual therapeutic resistance, due to development of additional mutations or yet unidentified factors. Against this backdrop of poor disease prognosis with current approved treatment options, patient-derived lung cancer models continue to be developed for gaining better understanding of the molecular pathobiology of lung cancers, identification of novel therapeutic targets which may also serve as disease or treatment biomarkers, as well as testing of novel agents as new therapies, thus contribute to further improvement of precision medicine. This review will discuss and compare current patient-derived lung cancer models and their limitations, including cell lines, patient-derived xenografts (PDXs) and patient-derived lung cancer organoids (LCOs).

Established cancer cell line models

Cancer cell lines have traditionally been the most commonly used models to study tumor biology and pharmacogenomics. The National Cancer Institute (NCI) and the Hamon Cancer Center (HCC) lines are the two largest series of lung cancer cell lines that have been established; most of these are available to researchers worldwide through the American Type Culture Collection (ATCC, Manassas, VA, USA). Additional cell line repositories have been established in various countries, e.g., CellBank Australia, European Collection of Authenticated Cell Cultures (United Kingdom), Japanese Collection of Research Bioresources Cell Bank, Deutsche Sammlung von Mikroorganismen und Zellkulturen and the Riken BioResource Center Cell Bank (Japan).

Cell line models in lung cancer research

Due to their relative ease of handling and ready availability, cell lines have been widely used in numerous studies in lung cancer. Drug sensitivity observed in cell lines may mimic clinical drug response. For example, cell lines with *EGFR* aberrations (PC-9 cells with exon 19 deletion and H3255 cells with L858R mutation) showed sensitivity to the EGFR TKIs erlotinib, afatinib, rociletinib and osimertinib. In contrast, H1975 cells, which bear both L858R and T790M mutations, responded only to the new-generation TKIs afatinib, rociletinib and osimertinib (12), although it has been reported that T790M mutation may also confer resistance to afatinib in the clinic (13). Another well-studied *EGFR*-mutated cell line, HCC827, was also shown to respond to the monoclonal antibody cetuximab (14). Multiple groups have reported that the RAF-inhibitors dabrafenib and vemurafenib, as well as the MEK-inhibitor trametinib, were able to inhibit the growth of cell lines with activating *BRAF* mutations including V600E (15,16). Similarly, the growth of cell lines bearing *ALK* fusions could be inhibited by ALK inhibitors (17,18). However, coactivation of EGFR and HER2 could confer resistance to ALK inhibitors in one of these cell lines (17).

The generally simple, low cost and rapid culture of cell lines is practical for large-scale studies, including pharmacogenomics projects such as the Cancer Cell Line Encyclopedia (CCLE) (19), the Genomics of Drug Sensitivity in Cancer (GDSC) (20-22), the Cancer Therapeutics Response Portal (CTRP) (23) and the Genentech Cell Line Screening Initiative (gCSI) (24,25). These projects combine massively parallel-omic profiling including genomics, copy number variation (CNV) analysis and transcriptomics, with screening of drug responses to more than 100 drugs on hundreds of cancer cell lines (including over 100 lung cancer lines), in order to identify associations between molecular markers and drug sensitivity. These valuable databases allow researchers worldwide to identify genes and drug responses in specific cell lines of interest. Machine learning models have also been applied to interrogate these pharmacogenomics datasets based on cancer subtypes or molecular markers (26,27). Another large-scale screening project using cancer cell lines is the Connectivity Map (CMap), which explores gene expression changes after drug treatments (28,29). The CMap study generated over a million gene expression profiles in 3 to 77 cell lines before and after treatments with 42,080 perturbagens, including 19,811 small molecule drugs, 18,493 shRNAs, 3,462 cDNAs and 314 biologics. Their data provide information on drug responses in cancer cell lines on the RNA level, from which researchers have identified drugs that can potentially be repositioned to treat cancer. Using the CMap data, Jahchan *et al.* (30) reported that the tricyclic antidepressant imipramine, the Histamine H1 receptor antagonist promethazine and the calcium channel blocker bepridil can reverse the small cell lung cancer (SCLC)-associated gene signatures, and showed anti-tumor effects in both *in vitro* and *in vivo* SCLC models.

Cell lines are also relatively easy to work with for genetic manipulations, as compared to other models. For instance, a loss-of-function study using the CRISPR-Cas9 system would be technically more challenging if carried out in organoid or PDX models (see next sections). Moreover, cell lines enable clonal selection and expansion to validate and select for positive knock-out cells (*Figure 1*). CRISPR-Cas9 loss-of-function studies enable us to identify essential genes in different cancer subtypes and test for synthetic lethality relationships between specific genes and driver mutations. For example, CRISPR/Cas9 knockout

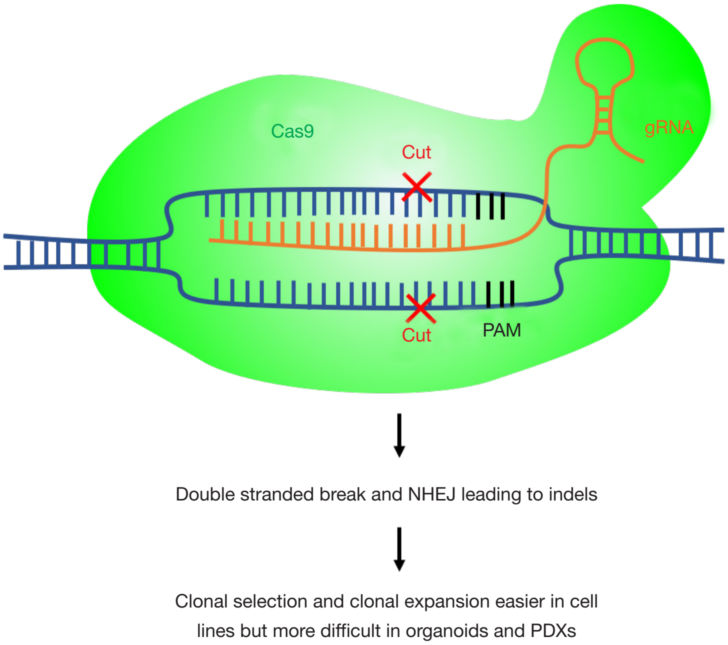

Figure 1 Schematic of CRISPR-Cas9 knockout system. PAM, protospacer adjacent motif; NHEJ, non-homologous end joining; PDX, patient-derived xenograft.

of focal adhesion kinase (*FAK*) in NSCLC cells with mutant KRAS showed sensitization to radiotherapy (31). In a further study, mutant *EGFR*-specific knockout by CRISPR/Cas9 was shown to inhibit the proliferation of the *EGFR*-mutated lung cancer cell line H1975 and to reduce tumor volumes of xenografts implanted with H1975; this was not observed when using A549 lung cancer cells that carry wild-type *EGFR* alleles (32). As discussed previously, cell line models facilitate large-scale studies: The Achilles project performed a genome-wide scale CRISPR-Cas9 knockout screen to identify essential genes in 689 lines of different cancer types, including 97 lung cancer cell lines, in the 19Q4 release (33-35). Although still in its infancy, CRISPR-Cas9 cell engineering may hold great promise in future cell therapy.

Limitations of cell line models

Important limitations of these models must be considered. Cell lines likely represent a subpopulation of the original tumor and are largely homogenous, due to the selective survival pressures present in culture conditions devoid of the original microenvironment involving interactions with stromal, immune and inflammatory cells (36,37). As a result, there are genetic and epigenetic differences between the cell lines and the original tumors, which make it difficult to evaluate how much of the original tumor biology is retained in established cell line models that have been maintained long-term *in vitro* (38). The lack of cell-cell interactions within a three-dimensional (3D) environment also limits the translational potential of cell line studies. However, in an effort to elevate the relevance of two-dimensional (2D) cell cultures for modelling patient disease, lung cell lines have been utilized in a variety of studies to develop engineered microenvironments (so-called 'on-chip' culture approaches) to allow for growth in 3D, as well as growth in co-cultures with stromal cells (endothelial cells, immune cells and fibroblasts) (39-41). Such 3D on-chip cultures have shown superiority for modeling drug sensitivity over traditional 2D culture (42).

Differences between the 2D and 3D culture conditions may become apparent when examining surface marker expression: for example, expression of TTF1 (a marker for lung adenocarcinoma) and TP63 (a marker for squamous cell carcinoma) was found to be similar between the adenocarcinoma and squamous cell lines included in the CCLE project, with more than half of the squamous cell lines having very low TP63 expression (*Figure 2*, Raw data obtained from https://portals.broadinstitute.org/ccle, accessed on Sep 3rd, 2019). This contradicts

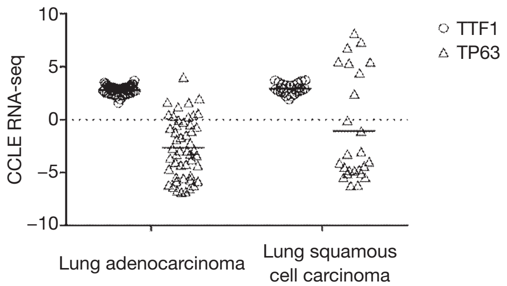

Figure 2 CCLE RNA-seq data on TTF1 and TP63 expression in lung adenocarcinoma and lung squamous cell carcinoma. TTF1, Transcription Termination Factor 1; TP63, Tumor Protein P63. Raw data obtained from https://portals.broadinstitute.org/ccle, assessed on Sep 3rd, 2019.

the clinical histopathology finding that 70–80% of lung adenocarcinoma are positive for TTF1, while lung squamous cells are negative for TTF1 and 100% positive for TP63 (43,44). While these discrepancies might be attributed to differences between the output of RNA-seq and immunohistochemistry (IHC) assays, others have reported no significant difference between TTF1 mRNA and IHC protein expression (45). These contradictions highlight the importance of ascertaining whether cell lines of interest in fact retain certain key aspects of tumor biology, prior to their use in specific studies.

Theoretically, experimental results generated using the same line in different laboratories should be directly comparable. However, in practice, this may not be the case. Haibe-Kains *et al.* reported a poor concordance between the CCLE and the GDSC drug response data (46), and although improved consistency was obtained using a different statistical approach, poor correlation was still observed in some cases, especially in cell lines with poor drug sensitivity (47). It should be noted that even when comparing drug response of the same drug and the same cell line, results may be affected by differences in experimental protocols. Standardization of methodology would certainly help to improve the consistency of cell line experimental results across different laboratories.

Primary patient-derived cell culture models

As established cell lines have undergone unspecified long-term passaging *in vitro*, they may no longer faithfully represent the molecular heterogeneity of primary patient tumors. Comparison of transcriptomic and/or proteomic profiles of patient NSCLC tumors and established cell lines have demonstrated significant divergence (48,49). More recently, a new technique involving Rho-associated kinase inhibitor-induced conditional programming to develop primary lung cancer cell cultures derived from patient biopsy samples has been reported with a ~50% success rate (50,51). These models might better retain the molecular characteristics of patient tumors and be a better alternative to study cancer biology and drug sensitivity. The generation of these primary cell cultures is of particular importance for studies aiming at guiding decision making in the clinic, due to the speed at which they can be established, as well as the concordance of drug responses between the patients and the respective cell lines (52).

Patient-derived tumor xenograft (PDX) models

PDXs are cancer models established by engrafting and growing human tumor tissue/cells in animal host, most commonly immunodeficient or humanized mice (53,54). Unlike cell lines that grow *in vitro* under non-physiological conditions, PDX models grow in a 3D microenvironment, which includes vasculature that provides *in vivo* delivery of nutrients and oxygen, and host stromal and immune cells interact and communicate with the tumor cells. PDXs mostly retain the genomic and phenotypic profiles of the original patient tumors, thus may better reproduce the clinically observed drug response (49,55-57). Thus, PDXs hold promise as better pre-clinical models in personalized medicine, for which they may be used to predict drug-responses and potentially guide patient therapies.

Methodology of generating PDX models

PDX tumors have primarily been established from solid tumors and most published lung cancer PDX models were established using surgically resected tumors. However, successful engraftment can also be achieved using smaller tissue samples, such as computed-tomography (CT)- and endobronchial-ultrasound (EBUS)-guided biopsies (58-60). The latter approaches have been particularly useful in generating SCLC PDXs, since these patients seldom undergo surgical resection of the tumor (61,62). Different types of tumor histologies and collection methods may impact on the success rate of engraftment. In NSCLCs, the engraftment rates range from 25–60% (43,49,50,54,57-63) (*Table 1*), with squamous cell carcinoma having a higher

Table 1 Representative large studies of Lung PDX model development

Publication	Patients	Tissue collection	Mouse strain	Engraftment rate	Engrafted tumor histology [n]
Fichtner *et al.* (55)	NSCLC	Surgical resection	NOD/SCID	25/102 (24.5%)	Adenocarcinoma [6]
					Squamous [12]
					Others [7]
Wang *et al.* (49)	NSCLC	Surgical resection	NOD/SCID	127/441 (28.8%)	Adenocarcinoma [52]
					Squamous [62]
					Others [13]
Hao *et al.* (64)	NSCLC	Surgical resection	NOD/SCID	23/88 (26.1%)	Adenocarcinoma [11]
					Squamous [9]
					Others [3]
John *et al.* (63)	NSCLC	Surgical resection	NOD/SCID	Adenocarcinoma 30/96 (31.1%)	Adenocarcinoma [30]
				Squamous 29/45 (64.4%)	Squamous [29]
					Others [4]
Nakajima *et al.* (60)	NSCLC& SCLC	EBUS-guided biopsy	NSG	Adenocarcinoma 3/12 (25%)	Adenocarcinoma [3]
				Squamous 2/3 (66.7%)	Squamous [2]
				Small cell carcinoma 2/3 (66.7%)	Small cell carcinoma [2]
				Large cell carcinoma 1/1 (100%)	Large cell carcinoma [1]
Fang *et al.* (65)	NSCLC& SCLC	Surgical resection	BALB/c nude mice	N/a	Adenocarcinoma [15]
					Squamous [68]
					Others [16]
Kang *et al.* (66)	NSCLC	Surgical resection & biopsy	NOD and nude	Adenocarcinoma 30/92 (32.6%)	Adenocarcinoma [30]
				Squamous 11/30 (36.7%)	Squamous [11]
Kita *et al.* (67)	NSCLC	Surgical resection	NSG & SHO	Adenocarcinoma 13/48 (27.1%)	Adenocarcinoma [13]
				Squamous 6/10 (60%)	Squamous [6]
				Large cell carcinoma 1/2 (50%)	Large cell carcinoma [1]
Cuenca *et al.* (68)	NSCLC	Surgical resection	SCID	Adenocarcinoma 9/28 (32.1%)	Adenocarcinoma [9]
				Squamous 6/19 (31.5%)	Squamous [6]
					Adenosquamous [1]
Drapkin *et al.* (56)	SCLC	CTC	NSG	16/42 (38.1%)	Small cell carcinoma [16]
Hodgkinson *et al.* (69)	SCLC	CTC	NSG	4/6 (66.7%)	Small cell carcinoma [4]

NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; CTC, circulating tumor cell; NOD, nonobese diabetic; SCID, severely compromised immune deficient; NSG, NOD-SCID gamma.

take rate than adenocarcinoma (60,63,67). The ability of resected NSCLC to engraft and form PDX is strongly associated with poorer patient prognosis (49). In SCLCs, EBUS-guided biopsies have a 67–83% success rate in PDX establishment (61,62).

Circulating tumor cells (CTCs) can be non-invasively collected from blood, and these cells generally have acquired greater metastatic potential (70). Given the low number of CTCs in NSCLC patients, generating NSCLC PDXs from CTCs has been technically challenging (71), with only one case study found in the literature (72). In contrast, most SCLC patients with advanced stage disease release high numbers of CTCs (73,74), and since surgical resection is rarely performed in SCLC patients, CTCs have become an important material source for SCLC PDXs establishment. Reported engraftment rate for CTC-derived SCLC PDXs ranged between 38% (16/42) to 67% (4/6), depending on the CTC counts in patient blood samples (56,69).

In most studies, immunodeficient mice are used as the animal host to generate PDX models. One of the most commonly used immunodeficient mice is the non-obese diabetic (NOD)-severely compromised immune deficient (SCID) mouse, which lacks mature T and B cells and has deficient natural killer cell function (57,75). To enhance engraftment rates, NOD-SCID gamma (NOD. Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) mice, also known as NSG mice, have been developed from a NOD-SCID background with additional interleukin 2 receptor gamma chain impairment. This causes deficient cytokine signaling and consequently enhanced immunodeficiency. NSG mice also have longer lifespans compared to NOD-SCID mice, which appear to have higher rates of developing thymic lymphomas (76,77). However, PDXs generated in immunodeficient mice cannot efficiently be used to study immunotherapies, since an intact immune system, and preferably one of human origin, is required. For this purpose, humanized mice have been developed by engrafting human leukocytes and purified CD34+ hematopoietic stem cells into immunodeficient mice. Hematopoietic stem cells can be obtained from different tissue sources, including umbilical cord blood, bone marrow, fetal liver and neonatal thymus (78-81). Rosato *et al.* demonstrated increased human cytokine levels in not only the plasma, but also the tumors of humanized NSG mice compared to non-humanized NSG mice (82). Further, it has also been shown that the growth of human stromal and immune cells can be maintained in humanized mice, therefore better mimicking the original tumor microenvironment (83).

Few technical considerations may help to enhance the success rate of PDX tumors establishment with a higher degree of patient fidelity. For example, solid tumors can be first dissociated and injected into host animals as single-cell suspension; this may potentially avoid selection of clonal subpopulation of the tumor for engraftment (77). This approach also allows genetic manipulations of the tumor cells prior to engrafting. Dissociated tumor cells can be mixed with matrix protein cocktail Matrigel to increase the rate of successful engraftment and growth of tumor cells (84). Patient tumors can be implanted heterotopically or orthotopically into the host animals. Most studies on lung cancer PDX models use heterotopic implantation, which provides the advantages of an easy procedure and accurate tumor size measurements. On the other hand, orthotopic implantation should better recapitulate the native tumor environment of lung cancer and enable the study of metastasis, but this is technically challenging (85). Only few labs have used this approach to make lung cancer PDX models, where a tumor fragment is sewn into the left lung or placed into the left thoracic cavity in a thoracotomy procedure (68,86). Cuenca *et al.* reported a 30.8% (16/52) engraftment rate in NSCLC models using orthotopic implantation (68).

PDX models in lung cancer research

Our lab has demonstrated that NSCLC PDXs with *EGFR* exon 19 deletion and L858R mutations responded to first/second generation EGFR TKIs (gefitinib, erlotinib, dacomitinib and afatinib), consistent with clinical observations (87). In contrast, a PDX model with both *EGFR* exon 19 deletion and T790M mutations is resistant to gefitinib/erlotinib, but responded to cetuximab (87,88). Zhang *et al.* have reported that a lung PDX model with *KRAS* mutation was resistant to gefitinib (89), agreeing with clinical data (90,91). We have also shown that lung squamous carcinoma models bearing *PIK3CA* E542K mutations are sensitive to PI3K inhibitors, and CDK4/6 inhibitors conferred a synergistic anti-tumor activity when used in combination with PI3K inhibitors (92). Several groups have demonstrated sensitivity of PDXs established from lung cancer patients with *ALK* gene rearrangement to ALK inhibitors (65,66). In contrast, a PDX model established from a patient who developed resistance to multiple ALK inhibitors demonstrated a similar response profile as observed clinically (93). Ambrogio *et al.* demonstrated that dual inhibition of DDR1 and the

Notch signaling pathway induced tumor regression in *KRAS*-mutated PDXs, opening up the possibility of using the combination of dasatinib and Notch inhibitor (e.g., demcizumab) to treat patients with *KRAS* mutations (94,95), for whom there currently is no targeted therapy available. Both NSCLC (55) and SCLC (96) PDX models show responses to chemotherapies similar to those observed in clinical settings. In SCLC, EZH2 inhibition provides synergistic tumor suppressive effect when combined with chemotherapies, even in chemoresistant PDX models (97). Nevertheless, PDXs do not always accurately predict patient response. For example, although it has been reported that the ERK1/2 inhibitor CC-90003 (98) and the XPO-1 inhibitor KPT-330 (99) were able to repress tumor growth in PDXs, neither showed encouraging results in clinical trials.

Immunotherapies have revolutionized cancer treatment and have led to promising clinical outcomes in various types of advanced cancers, including lung cancer (3). There is increasing interest in testing immunotherapy responses using PDX models established in humanized mice. Hu *et al.* observed an anti-tumor response upon adoptively transferring human T cells that express melanoma antigen MART-1-specific T cell receptors to their humanized melanoma PDXs (100). Anti-PD-1 antibodies have also shown anti-tumor effects in humanized triple-negative breast cancer (82), osteosarcoma (101) and lung cancer (102) models. In lung cancer specifically, anti-PD-1 checkpoint inhibitors were shown to inhibit tumor growth in humanized mice engrafted with A549 lung tumor cells (102).

In contrast to *in vitro* cell line assays, large-scale experiments are more difficult to perform in PDXs. Nevertheless, efforts in this direction are underway and Gao *et al.* (103), for example, recently published a high-throughput drug screening project using PDXs of various cancer types with different driver mutations, allowing discoveries of associations between biomarkers, pathway activation and drug sensitivity in different cancer subtypes.

Limitations of PDX models

Although PDXs retain tumor heterogeneity and potentially better mimic tumor biology and microenvironment compared to cell lines, several limitations and challenges apply to this type of models. Compared to cell lines and 3D organoids (see next section), the establishment of PDX models is more time-consuming and costly, especially when using humanized mice. Concerns have been raised regarding potential genetic drift of tumor cells in xenografts of late passages, though several studies have shown that no major genetic difference could be found through at least 10 passages. Nevertheless, it is common practice to limit PDX experiments to less than 10 passages (104-106). When non-humanized mice are used, 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 (55,107). Overall, the methodology of PDX establishment remains suboptimal and current efforts in this field are devoted to better recapitulate the patient tumor and microenvironment, and to increase engraftment rate in order to lower the cost.

Patient-derived LCOs

Because 2D cell cultures retain only limited analogy to their parent tissue (108), scientific focus has shifted to 3D cell culture strategies (109). Specifically, patient-derived cell populations can be grown in a 3D extracellular matrix, giving rise to structures termed organoids, which are characterized by their capacity to self-organize into structures that are reflective of the tissue they are derived from (110). Tumor organoids have been generated from a large number of tissues, most recently also from lung.

Because of the genotype-phenotype similarity to their parent tumors, as well as the possibility of rapid expansion for drug sensitivity assays, tumor organoids represent preclinical tools of great relevance to patient disease. For example, a recent study comparing DNA copy numbers in breast tumor organoids and 2D cell lines to patient tumors found greater concordance between tumor samples and organoids over 2D cell lines (111), indicating that organoids may be a more apt culture system to model patient tissue heterogeneity (112,113).

Methodology of generating LCO models

Patient-derived LCOs require growth within a supporting 3D matrix, most commonly Matrigel (114,115), as well as a defined cocktail of growth factors and inhibitors. The formulation of this growth media varies between laboratories (113,116-119) (*Table 2*), but all formulations contain factors for lung stem cell maintenance. While the only factor common to all reported LCO-specific media formulations is the B27 supplement, an optimized serum substitute initially utilized for neuronal cell cultures (120), growth factors included are either epidermal growth factor (EGF) or members of the fibroblast growth factor (FGF)

Table 2 Summary of LCO culture strategies

Publication	Media composition	ECM composition	Culture setup	Reported successful culture time
Neal *et al.* (116)	ADMEM/F12 + HEPES, Glutamax, Pen-Strep, NALC, B-27 (- vit. A), R-spondin 1, Noggin, Nicotinamide, A 83-01, SB-202190, Wnt3a, Gastrin, EGF	Rat tail type 1 collagen	Tumor pieces in ALI culture	Up to 100 days in culture
Tamura *et al.* (117)	FBIM002 medium	NA	Tumor pieces in suspension culture	Over 6 months
Sachs *et al.* (118)	ADMEM/F12 + NALC, B-27, R-spondin, Noggin, Nicotinamide, A 83-01, SB202190, Y-27632, FGF7, FGF10	BME type 2	Dome culture	NA
Kim *et al.* (119)	DMEM/F12 + Pen-Strep, B-27, Y27, bFGF, EGF, N2	GFR MG	Dome culture	Over 6 months
Shi *et al.* (113)	ADMEM/F12 + HEPES, Glutamax, Pen-Strep, NALC, B-27, Noggin, A 83-01, Y-27632, FGF10, FGF4, EGF, CHIR 99021, SAG	GFR MG	Dome culture	Over 10 passages, over 3 months in culture

ADMEM, advanced DMEM; Pen-Strep, penicillin/streptomycin; EGF, epidermal growth factor; FGF, fibroblast growth factor; SAG, smoothened agonist; NALC, N-Acetyl-L-cysteine; FBIM002 - media pertaining the Fukushima Translational Research Project; NA, not applicable; ALI, air-liquid interface; BME, basement membrane extract; GFR MG, growth factor reduced Matrigel.

family, and inhibitors/activators of specific pathways, namely TGF-β and Rho-associated protein kinase (inhibited) and Wnt (activated). LCOs may be generated from tumor biopsies or resections and can be maintained in both short- and long-term (> passage 10) cultures, with reported high overall success rates of culture initiation (approx. 90%) (113,118). Specifically, tumor tissue is minced and dissociated enzymatically to obtain single cells, which are then typically mixed with Matrigel, deposited in so-called domes (i.e., large droplets) in 24- or 48-well plates, and finally topped with the appropriate growth medium. Whether or not this procedure yields viable and proliferating LCOs depends on a number of factors, including cell viability after tissue digestion and cellularity of the tumor tissue processed. Alternatively, LCOs can be established form CTCs isolated from blood (73,121).

Contamination of cultures by normal lung epithelium was identified in a number of studies attempting to grow LCOs (118,119,122), whereby normal cell clusters tend to outgrow LCOs in these cultures (113). This is possibly due to the genetic instability and consequently increased cell death in LCOs (123) or because all published LCO media are based on stem cell media formulations supportive of normal epithelial growth. Besides manually separating LCOs from normal lung organoids based on their morphological appearance (the feasibility of this approach remains unclear), Sachs *et al.* achieved pure LCO cultures through the addition of Nutlin-3A, which specifically inhibits the growth of *TP53* wild type cells and thus, when added to *TP53* mutant LCOs, will lead to drop-out of normal epithelium from cultures (118). Alternatively, the use of a suboptimal medium for LCO culture was most recently described by Kim *et al.*, which did not support normal cell growth (119).

To this date, only a handful of studies have documented the establishment of patient-derived LCOs (113,116-119,122,124,125), whereby the success rates of long-term culture establishment vary substantially (*Table 2*), and detailed descriptions of the number of passages achieved and split ratios used were typically not reported. An exception to this is the distinction between long and short-term models described by our lab (113), where long-term LCO cultures were defined as achieving passage 10 (and a corresponding culture time of over 3 months), while the rest were deemed short-term cultures. The efficiencies of culture establishment for these models were 15% and 72%, for long- and short-term, respectively. The following sections largely focus on insights from the few available LCO studies to date, as well as from pertinent papers investigating tumor organoids in other tissues.

LCO resources and characterization

Large collections of patient-derived LCOs, as well as normal lung organoids, are being established and biobanked (123,126). At the basis of such efforts lies the observation that LCOs closely resemble the genotypic and histological heterogeneity of their parental tumors. Immunohistochemical analyses of LCOs saw major lung tumor subtypes reflected in LCO cultures, using markers such as TTF-1, cytokeratin 5 and synaptophysin for adenocarcinoma, squamous cell carcinoma and small cell carcinoma, respectively (113,116,119). Implantation of patient-derived LCOs into mice resulted in growth of tumor xenografts at variable success rates (30–100%) and with preserved parental tumor histology, proving LCO tumorgenicity (113,118,119).

LCOs further maintained defining genetic characteristics from their parental tumors. Next generation sequencing of LCOs and tumors revealed the presence of matching somatic mutations, such as *TP53*, *KRAS*, *EGFR* and others, which are commonly associated with NSCLC (1,127); no discordant driver mutations were observed. Analyses including SNP genotyping, variant allele frequency distribution analysis and CNV profiling similarly showed that concordance could generally be maintained between LCOs and their parental tissues during long-term culture, highlighting that clonal stability in LCOs is, at least for a certain number of passages, preserved (113,118,119).

Gene-drug correlation studies

Cancer organoids can be used to model human diseases and as a platform for drug screening (128). Across tumor types, studies correlating drug sensitivity and genotype data, have demonstrated that drug responses generated in patient-derived organoids are in line with the molecular profiles of their parental tumors (126). For example, erlotinib was effective against the growth of LCOs with *EGFR* exon 19 deletions (113). Kim *et al.* (119) observed resistance to erlotinib in one LCO line, despite the presence of an *EGFR* mutation, but genomic analysis revealed the presence of *MET* amplification, which is associated with EGFR TKI resistance. Our lab further demonstrated the use of LCOs to identify potential combination treatments (113). Using an *FGFR1*-mutated LCO line insensitive to FGFR1 inhibitor BGJ398, we examined the efficacy of BGJ398 in combination with either a MEK inhibitor (trametinib) or a PI3K inhibitor (BKM120), both of which have been reported to decrease the growth of LUSC in cell line studies (129,130), and found a synergistic effect of the former combination in LCO cultures. This finding was then validated in the parental PDX model.

LCOs can also be generated from tumors further along the metastatic cascade. For example, SCLC CTCs spontaneously formed ‘tumorospheres’ in culture, even without the need for lung stem cell media (74). These CTC-derived tumorospheres expressed all typical SCLC markers and showed reduced chemosensitivity, compared to CTC single cells. This may possibly suggest that the growth of CTCs as clusters, rather than as single cells, could explain the commonly observed chemoresistance in relapsed SCLC patients.

Exploring different treatment strategies in patient-derived LCOs during the patients’ lifetime would represent a powerful approach for informing personalized care. Such efforts are underway: for NSCLC specifically, patient-derived LCOs are being screened with 8 different therapeutic agents at The Netherlands Cancer Institute, with the aim of identifying effective drugs, in order to offer patients the option to adjust their treatment accordingly (126).

Screening for drug target discovery

One of the most cited reasons for the low success rate in clinical trials is the utilization of 2D cell cultures as pre-clinical disease model systems (131). Tumor organoids may represent an improved tool for drug screens aiming to identify novel actionable molecular targets. While no large-scale drug screen has been attempted using LCOs yet, the overall approach shows promise in other tumor types, including colorectal cancer (132) and ovarian cancer (133). Pauli *et al.* (134) described high-throughput dose response screening on four tumor organoid lines against 160 compounds (FDA approved and under development) in mono- and combination treatment. Subsequent to hit validation in PDXs, this study was able to identify effective treatment strategies for two endometrial carcinoma lines, for which there are no currently approved targeted therapies, and two colorectal carcinoma lines.

Studies have found that different media formulations (see *Table 2*) are supportive of growth of tumor cells that carry genetic markers (e.g., *EGFR*), as well as protein markers (e.g., TTF-1, p63, cytokeratin 5) of interest, confirming that the *in vitro* cultures generated are, as far as these markers are concerned, representative of patient material. Beyond this, it remains unclear how the presence of different growth factors in media formulations affects marker expression at the protein level and the response to different drugs. To our knowledge, to date there has been no comparative study of organoid marker expression or drug responses in the different published media formulations.

Modeling of the tumor microenvironment

As the presence and heterogeneity of stromal cells within a tumor mass may alter treatment response, LCO cultures will need to include stromal cell types, in order to model disease in a more comprehensive way (135). Given the very encouraging successes in immunotherapy, the first studies investigating tumor-stroma relationships using LCOs have focused on incorporating immune cells into LCO cultures; to this date, studies specifically exploring fibroblast- LCO or endothelial cell-LCO relationships are still lacking.

While defined media are successful for growing LCOs, the same media also lead to the loss of stromal cell fractions during LCO establishment. This is because certain factors are specifically inhibitory to fibroblast proliferation, [e.g., Noggin, B27 supplement and various TGBβ inhibitors (136)], and immune and endothelial cells drop out from cultures over time (116). To include stromal populations into LCO cultures, two approaches have been reported. Neal *et al.* have generated 20 *en-bloc* LCO cultures that maintain their original stromal cell fraction for several passages by culturing minced tumor tissues in air-liquid interphase (ALI) culture (116), which recapitulated the tumor T- and B-cell repertoire. Anti-PD-1 antibody induced TIL-mediated check-point inhibition in one out of three NSCLC LCO lines examined and tumor cell killing was further assessed in tumor organoids from various tissues. Overall, this study demonstrated the possibility of culturing *en-bloc* LCOs for short term studies, which preserve a functional immune component.

A second approach for establishing tumor-stroma LCOs is to recombine stromal and parenchymal tumor fractions after culturing them separately. For example, the interaction between patient-derived LCOs and infiltrating T cells was investigated by co-culturing tumor epithelial cells and autologous blood lymphocytes. In this system, tumor cell killing by reactive T-cells occurred, indicating the utility of this culture method for assessing patient sensitivity to T-cell based therapies (122).

This re-combination approach has also been utilized for co-culturing tumor organoids and activated fibroblasts in pancreatic cancer studies. To model the functions of carcinoma associated fibroblasts (CAFs) present in desmoplastic PDAC stroma, PDAC organoids were established and co-cultured with murine fibroblast progenitors (136). Upon activation by tumor organoids, CAFs adopted one of two identified phenotypes: CAFs in direct contact with organoids upregulated α-smooth muscle actin (α-sma) expression and TGFb response genes, while CAFs located further away from organoids secreted IL-6, a potent pro-survival signal for PDAC organoids. Separately, in a library of 39 PDAC organoids, CAFs were found to provide a Wnt niche required for maintenance of tumor stem cells, thereby supporting early tumor development (137). Tsai *et al.* also established co-culture pancreatic tumor organoids to study pancreatic cancer progression and fibroblasts activation, as well as tumor-dependent lymphocyte infiltration (138). Such studies particularly apply to LCOs, as desmoplasia commonly occurs in lung cancers and CAF-mediated effects on tumor growth and drug sensitivity have been amply reported (139,140). For example, one study using lung tumor spheroids formed by the PC-9 cells in 3D co-culture with CAFs identified a pro-proliferative role of specifically podoplanin-positive CAFs on lung tumor cells (141), further demonstrating the heterogeneous effects of CAF subpopulations on disease progression.

Overall, the field of tumor organoid co-cultures is in its infancy, with disease modelling being the primary goal of current studies. Particularly for LCOs, no methods for stable, long-term cultures of stromal-parenchymal organoids have been identified, nor have media formulations across laboratories been standardized. However, even initial results, particularly with respect to immune cell-tumor cell interactions, indicate that the use of stroma-parenchymal LCOs for biomarker identification, drug screening and modeling of therapy resistance lies in the near future.

Drawing inspiration from the established on-chip models initially generated for use with cell lines, efforts are underway for using LCOs in microfluidic or otherwise engineered microenvironments. On-chip culture platforms have been used to model various tumors using organoids and other cell types (including endothelial cells for neovascularization), and the reader is referred to expert reviews describing the different on-chip setups, their uses and limitations (142-145). Because culture media for LCO growth have only recently emerged, to the best of our knowledge only one study has described the use of LCOs in an on-chip application (125). Jung *et al.* used a microfluidic setup with continuous media flow for generating small cell LCOs for sensitivity testing of the drugs cisplatin and etoposide; LCO death was identified in a dose-dependent manner. Microfluidic devices have further been used to

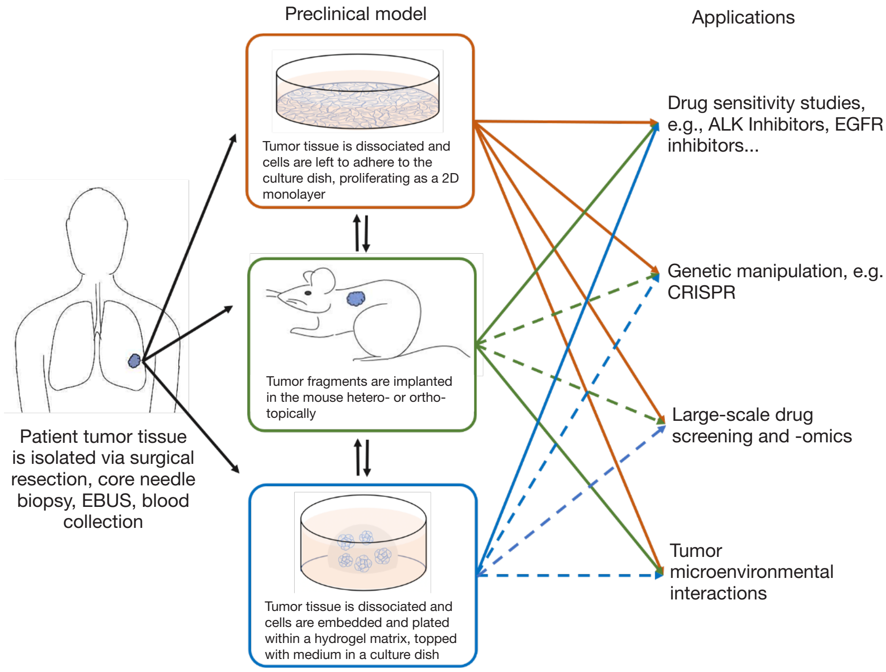

Figure 3 Methodology and applications of preclinical patient-derived lung cancer models. Solid lines represent greater compatibility between models and applications. Dashed lines represent lesser compatibility between models and applications. EBUS, endobronchial ultrasound.

isolate and expand lung CTCs and patient immune cells from liquid biopsies (146), by using laser ablation to generate an array of microwells, and seeding CTCs and white blood cells into these to allow cluster formation. This platform can then be used to assess CTC drug response.

Limitations of LCOs

Patient-derived LCOs have to date effectively been used for disease modeling, genotype-phenotype studies and drug sensitivity testing, and have already proven to be exceptionally useful models for evaluating targeted therapeutics in a patient-specific fashion. However, certain aspects of current LCO culture methods represent hurdles that hamper the use of this model for different applications.

Firstly, robust strategies for parenchymal-stromal co-cultures are lacking, thereby impeding long-term studies of stromal involvement in tumor growth, drug sensitivity and resistance (147). Secondly, factors present in LCO medium, such as small molecule inhibitors, may alter drug responses. A second issue with regard to media formulations is that it remains unclear to what extent tissue ‘maturation’ is inhibited in LCO cultures, i.e., to what extent media factors inhibit cellular differentiation (148). Similarly, it is known that Matrigel, the most common matrix environment for LCO growth, is an abundant source of growth factors and plays a role in suppressing differentiation. In fact, a number of alternative, natural and synthetic, polymers are being explored for organoid growth (148). Further, culture scalability represents a major bottleneck, especially for high-throughput drug screening applications, as LCOs vary wildly in growth dynamics (149). Finally, due to the lack of viable blood supply and waste removal, LCOs suffer from size-dependent cell death; incorporation of endothelial cells or appropriate use of engineered culture technologies may eliminate this phenomenon (150).

Conclusions

Patient-derived models help researchers better understand cancer biology and genetics, allowing identification of biomarkers for clinical diagnosis and prediction of drug response. Some models, i.e., cell lines, have been used extensively in lung cancer research, while others have recently emerged, such as organoids, and their exploitation remains in infancy. This review has discussed the

Table 3 Summary of the readouts, advantages and limitations of the available lung cancer preclinical models

Lung cancer preclinical model	Readouts	Advantage	Disadvantage
Cell lines	Growth/metabolic assays	Ease and low cost of culture and scale-up	Lack of clonal heterogeneity
	2D Migration	Compatible with screening applications	Poor representation of cell-cell and cell-substrate interactions in patient tumor
	Marker expression	Unlimited passaging	Possible changes in marker expression after long-term culture
	Multi-omics profiling	Clonal homogeneity	
	Tumor cell killing	Well characterized	
	3D invasion	Easy transferability among laboratories	
		Ease of genetic manipulation (e.g., CRISPR)	
		Co-cultures of specific cell types of interest possible	
PDXs	Tumor volume	*In vivo* drug response assessment mimicking clinical situation	Long-term lines
	Metastases	Mouse or humanized tumor microenvironment	Labor intensive and costly to use or maintain
	Histology		Humanized mice difficult to establish
	Omics		Difficulty to scale up (low throughput)
			Possible genetic drift after numerous passages
LCOs	Growth/metabolic assays	Maintenance of clonal heterogeneity	Difficulties with culture initiation and long-term maintenance
	Histology	Model closest to patient tumor	Scale-up more cumbersome than cell lines
	Multi-omics profiling	Genetic manipulations are possible	Lack of robust co-culture strategies for tumor epithelial and stromal cells
	Tumor cell killing	Large number of passages (case dependent)	Requirement for costly and non-standardized culture ingredients
	3D invasion	Co-cultures between specific cell types of interest possible	
	Immune cell recruitment		

methodology and applications, as well as the advantages and limitations specific to each preclinical model and provides an overall summary of these observations (*Figure 3* and *Table 3*).

Cell lines are easy and fast to culture, making them the first choice for large-scale studies, but the lack of heterogeneity and cell-cell interactions within a 3D environment limits the translational potential of findings from cell line studies. This limitation has been in part offset by culturing cells as 3D spheroids or in 3D on-chip environments (40). Compared to cell lines, PDX models retain tumor heterogeneity and the *in-situ* tumor microenvironment, but are more time-consuming and expensive, therefore hampering large-scale experiments. Organoids on the other hand, are established and grown in a 3D environment within a comparatively short time frame and have the potential to be co-cultured with other cell types of interest. While it takes 3–6 months to establish a PDX model, LCO generation form the same tumor specimen can be accomplished in as little as 4 weeks (118,119,123), and may therefore have the potential to inform clinical decisions during the patient's lifetime. Moreover, cell lines and organoids allow gain- and loss-of-function genetic manipulations to study genes of interest, which are technically much more challenging to perform in PDXs. However, among all the patient-derived cancer models, PDXs may best recapitulate tumor biology and microenvironment, especially when using orthotopic implantation or humanized mice for engraftment. PDXs also have the advantage over other models in drug response studies, as they allow tumor size measurements and toxicity examinations in animals. Patient-matched PDXs with co-engrafted stromal and immune cells from the tumor donors may provide a better option for clinical decision making in precision medicine.

While there is no ideal model for lung cancer research, study design must consider all the advantages and disadvantages of different models, as well as the compatibility with different experiments, before selecting one to answer relevant biological questions.

Acknowledgments

We would like to thank Drs. Nadeem Moghal and Sebastiao N. Martins-Filho for helpful scientific discussion.
Funding: This work is supported by the Canadian Institute of Health Research (CIHR) Foundation grant FDN-148395 (MST). D'Arcangelo is supported by the Princess Margaret Post-doctoral Fellowship.

Footnote

Provenance and Peer Review: This article was commissioned by the Guest Editor (Helmut H. Popper) for the series "New Developments in Lung Cancer Diagnosis and Pathological Patient Management Strategies" published in *Translational Lung Cancer Research*. The article was sent for external peer review organized by the Guest Editor and the editorial office.

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tlcr-20-154). The series "New Developments in Lung Cancer Diagnosis and Pathological Patient Management Strategies" was commissioned by the editorial office without any funding or sponsorship. MST reports grants and personal fees from AstraZeneca, personal fees from BMS, grants and personal fees from Bayer, personal fees from Hoffmann La Roche, personal fees from Amgen, personal fees from Pfizer, personal fees from Takeda, outside the submitted work. MST serves as an unpaid editorial board member of *Translational Lung Cancer Research* from Jul 2014 to Jul 2021. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Cite this article as: Huo KG, D'Arcangelo E, Tsao MS. Patient-derived cell line, xenograft and organoid models in lung cancer therapy. Transl Lung Cancer Res 2020;9(5):2214-2232. doi: 10.21037/tlcr-20-154

