Organoid Cultures as Preclinical Models of Non–Small Cell Lung Cancer

Ruoshi Shi1,2, Nikola Radulovich1, Christine Ng1, Ni Liu1, Hirotugu Notsuda1, Michael Cabanero1, Sebastiao N. Martins-Filho1, Vibha Raghavan1, Quan Li1, Arvind Singh Mehta1, Joshua C. Rosen1,3, Ming Li1, Yu-Hui Wang1, Laura Tamblyn1, Nhu-An Pham1, Benjamin Haibe-Kains1,2,4,5, Geoffrey Liu1,2,7, Nadeem Moghal1,2, and Ming-Sound Tsao1,2,3

Introduction

Non–small cell lung cancer (NSCLC) is the leading cause of cancer-related death worldwide with a 5-year overall survival rate of 15% (1). Over the last decades, there has been tremendous effort in developing preclinical models of NSCLC, including two-dimensional (2D) cell lines, air–liquid interface cultures, genetically engineered mouse models (GEMM), and patient-derived xenografts (PDX; refs. 2–4). These models have been used to accelerate our understanding of NSCLC biology and pathogenesis. Although cell lines are still widely used in preclinical studies, they often do not reflect the biology of their parental tumors or drug sensitivity to targeted therapeutics of their patient tumors (5). In addition, although GEMMs and clinically relevant PDXs may be closer to the ideal models to study drug response in patients, studies using these models are labor intensive, costly, and time consuming (6). Thus, research efforts are underway to develop novel preclinical models derived from patient with NSCLC and PDX tissue that are economical, rapid to use, and accurately reflect the biology of the disease.

Over the past few years, organoid cultures derived from primary patient tumors and PDXs of various cancers including the colon, pancreas, prostate, liver, and breast have been described (7–16). These cancer organoids have been utilized for numerous applications, such as drug screening and biomarker identification (17–20). They have been proposed to be better in vitro models than 2D cell lines due to higher rates of preservation of key histologic and molecular traits of their parental tumors (14, 15). In addition, drug screening in patient-derived organoids (PDO) has shown high concordance with that of the matched patient tumor (14, 18). Some reports have demonstrated the ability to generate normal lung organoids composed of airway cell lineages (21, 22). These models were primarily generated from normal mouse and human airways to understand normal lung development and function. In addition, methods to generate lung organoids from pluripotent stem cells have been reported to aid in the study of genetic pulmonary diseases such as cystic fibrosis (21, 23). A major advance was outlined in recent reports describing protocols for the development of NSCLC organoids (24–26). However, although many of the models reported in these studies were cultured short-term and were useful for acute studies, lack of systematic documentation of organoid tumor cell purity was a significant issue and specific details regarding long-term growth of the models were not provided (25, 26).

Results: We have identified cell culture conditions favoring the establishment of short-term and long-term expansion of NSCLC organoids derived from primary lung patient and PDX tumor tissue. The NSCLC organoids recapitulated the histology of the patient and PDX tumor. They also retained tumorigenicity, as evidenced by cytogentic features of malignancy, xenograft formation, preservation of mutations, copy number aberrations, and gene expression profiles between the organoid and matched parental tumor tissue by whole-exome and RNA sequencing. NSCLC organoid models also preserved the sensitivity of the matched parental tumor to targeted therapeutics, and could be used to validate or discover biomarker–drug combinations.

Conclusions: Our panel of NSCLC organoids closely recapitulates the genomics and biology of patient tumors, and is a potential platform for drug testing and biomarker validation.
Translational Relevance

Currently, there is an urgent need for clinically relevant preclinical models of non–small cell lung cancer (NSCLC) for biomarker and drug discovery due to the lack of preclinical models that recapitulate the biology of the patient tumor. Three-dimensional (3D) organoids have become valuable preclinical models to study disease pathogenesis and identify novel drug targets. We have established a protocol for the development of NSCLC organoids from patient tumor and patient-derived xenograft models. This protocol allowed for the efficient generation of organoids for multiple potential applications. Importantly, we showed that these organoids retained the histologic and molecular features of their parental tumors and demonstrated their utility for drug testing. Our organoid platform provides additional preclinical models of NSCLC and may be useful for future drug screening and biomarker identification.

Furthermore, there still remains a great need to develop a NSCLC organoid platform suitable for drug screening and biomarker identification in lung cancer.

Here, we describe a culturing protocol that enables generation of short-term (1–3 months, 1–9 passages) and long-term (>3 months, >10 passages) NSCLC organoids from most and a subset of primary lung patient tumors and PDXs, respectively. These models were able to initiate from tumor tissues with 88% (57/65) success rate. Specifically, 72% (47/65) of the organoids were maintained in culture short-term, whereas 13% (10/65) were maintained in culture long-term. We demonstrated that both short-term and long-term established NSCLC organoids grown in vitro and as xenografts recapitulated the histologic features and tumorigenicity of their matched tumor tissue. Whole-exome sequencing (WES) and RNA-sequencing revealed that the long-term NSCLC organoids, despite having been grown in in vitro environments with multiple passaging, preserved the mutation, copy number, and gene expression profiles of their parental tumors. Finally, we demonstrate that these biologically relevant models of NSCLC can be used for drug testing, supporting their application for both disease modeling and therapeutic testing.

Materials and Methods

Tumor tissue processing and organoid establishment

The collection of surgically resected primary tumors from patients with early-stage NSCLC and the development of PDXs were approved by the University Health Network Research Ethics Board (REB: 17-558) and Animal Care Committee (AUP: 5555). Informed written consent was received from all patients. All studies were performed in accordance with TRI-Council Policy Statement: Ethical Conduct for Research Involving Humans. Clinical diagnosis of NSCLC subtypes was validated by pathologic review. The protocol for establishing NSCLC PDXs was previously described (4, 27, 28). For organoid establishment from tumor tissues with 88% (57/65) success rate, specimens were dissected and homogenized in Advanced DMEM/F12 (GIBCO) with Liberase TM (Sigma) for 30 min at 37°C with rotation. Tumor pieces were dissociated into single cells in tris-buffered saline solution with 10% SDS, resuspended in 100% growth factor reduced Matrigel (VWR), plated on 24-well tissue culture plates as Matrigel domes and maintained in 5% CO2 with media overlaying the Matrigel dome. Media used for this algorithm were a combination of SureSelect Human All Exome (Agilent) and proprietary solutions provided by a DNA Extraction Kit (Norgen Biotek). DNA quality was assessed using Bioanalyzer, Tapestation, and QPCR. One-hundred to 200 ng of genomic DNA was used for library preparation (Agilent SureSelect Human All Exon v5 Capture Kit). DNA was sequenced using 125-cycle paired-end protocol and multiplexing to obtain 150× coverage on Illumina Hiseq2500 sequencer. DNA calling was further processed for quality control using the standard GATK pipeline, including Picard v1.140 (31), Mutect v1.1.5 (32), and Varscan v2.3.8 (33) were used for mutation calling, whereas dbSNP (34), ExAC (35), and ESP (36) were used as filters for samples without matched normal tissue. Annovar (37), vcf2maf v1.6.14, and Variant Effect Predictor v87 (38) were used to annotate final mutation calls, following which, the R package “ComplexHeatmap” (39) was used to generate oncoarrays and visualize the data. CNV kit (40) was used to infer copy number from exome-sequenced samples by applying circular binary segmentation (CBS; ref: 41) to make calls in both targeted regions and nontargeted regions. The targeted regions used for this algorithm were a combination of SureSelect Human All Exon V4 and V5 regions. A panel of normal lung tissue was used for samples lacking a matched normal. Subsequently, GISTIC2.0 (42) was run to identify genes affected by copy number alterations, while also taking into account the frequency and amplitude of the events. WES data were deposited in the sequence read archive (SRA; accession nos.: SRP158596).

RNA extraction and RNA-sequencing analysis

Organoids were extracted from Matrigel using Cell Recovery Solution (Corning) on ice for 1 hour. Total RNA from homogenized samples was extracted using TRIzol (Invitrogen) in 37°C with gentle shaking. Mouse cell depletion in PDX samples was performed after tissue dissociation using H-2Kb/ H-2Db antibody (#MAS-17998; Invitrogen) labeling and Streptavidin (BD Biosciences) bead magnetic separation. Cells were counted and resuspended in 100% growth factor–reduced Matrigel (VWR), plated in 24-well tissue culture plates as Matrigel domes and maintained in 37°C 5% CO2 with media overlaying the Matrigel dome. Organoid growth was monitored weekly for the detection of initiated organoids, and organoids were kept in the same passage for no longer than four weeks. The identity of PDX and organoids were authenticated by short tandem repeat (STR) analysis and matched to patient tissue. Organoid cultures were tested routinely for mycoplasma. Additional methods can be found in Supplementary Materials and Methods.
tumor tissue and pelleted organoids were extracted using TRIzol (Invitrogen) method, followed by isolation and precipitation in chloroform and 70% ethanol. DNA cleanup was performed using DNA Cleanup Kit (Invitrogen). Total RNA was quality checked via BioAnalyzer (Agilent), Tapestation, and qPCR. Library preparation was performed using Illumina TruSeq Stranded mRNA Sample Preparation Kit (Illumina). RNA was sequenced using HiSeq 2000 sequencer with 75-cycle paired-end protocol and multiplexing to obtain 40–80 million reads/sample. Xenome (version 1.0.1 with standard parameters; ref. 29) was used to filter mouse reads from human reads. For transcript quantification, Salmon (version 0.8.2 with default parameters; ref. 43) with quasi-mapping was applied to assign reads directly to transcripts to obtain transcripts per million (TPM) values. The log₂(TPM) values were included in this study: pFGFR (Y653/654; #3471), FGFR1 (#9740), pErk (T202/Y204; #9101), Erk (#9102), pAkt (S473; #9271), and Akt (#9272) were obtained from Cell Signaling Technology. β-Actin antibody (A1978) was obtained from Sigma.

**In vivo organoid implantations**

Dissociated organoids were isolated from growth factor–reduced Matrigel using Cell Recovery Solution (Corning) for 1 hour on ice. Organoids were resuspended with 500,000 cells in 200 μL M26 media prior to injection in the subcutaneous flank of 4- to 6-week-old NOD/SCID mice. Tumor growth was monitored once or twice weekly by caliper measurement. Tumors were harvested, formalin-fixed paraffin embedded for histologic analysis, and snap frozen for DNA/RNA/protein isolation.

**In vivo therapeutic studies**

Cryopreserved PDX tissue (below passage 10) was thawed and implanted into the subcutaneous flank of NOD/SCID mice. The tumor was harvested and cut into 4-mm–diameter pieces at endpoint and expanded into experimental arms for drug testing when the average size reached 150 to 200 mm³. Trametinib (1 mg/kg) and BGJ398 (25 mg/kg) were dissolved in 0.5% hydroxyethyl cellulose with 0.2% Tween80 in sterile H₂O and 10% Tween80, respectively. Compounds were delivered once daily via oral gavage for 21 to 28 days. Tumor size was monitored twice weekly by caliper measurement.

**Results**

**Organoid establishment from NSCLC patient tumor and PDXs**

From December 2015 to 2017, 19 surgically resected lung adenocarcinomas (LUAD), 15 lung squamous cell carcinomas (LUSC), 16 LUAD PDXs, and 26 LUSC PDXs were processed for organoid establishment (Fig. 1A and B; Supplementary Table S1). In vivo organoid formation upon plating in passage zero (Supplementary Table S2). Seventy-two percent (47/65) of the models exhibited a range of short-term growth (passage 1–9, 1–3 months), providing opportunities for most tissues to be used in acute studies (Fig. 1B; Supplementary Tables S1 and S2). In addition, 15% (10/65) of the models achieved long-term growth (Fig. 1B; Supplementary Tables S1 and S2).
Figure 1.

Establishment of NSCLC-derived organoids and characterization of short-term organoid cultures. **A**, Schematic of NSCLC organoid development from surgically resected tumors or PDX. Models propagated below 10 passages and under 3 months were considered to be short-term cultures, whereas models propagated beyond 10 passages and over 3 months were considered to be long-term cultures. **B**, Maximum number of days in culture of all models attempted visualized on a swimmer’s plot. Models contaminated with mouse or normal cells or derived from metastasis were excluded. **C**, Selected short-term NSCLC organoid histology and IHC staining. Note that LPTO126 patient tumor was both TTF-1 and TP63 negative, whereas PDXO137 was TTF-1 positive and TP63 negative. The organoids reflected the TTF-1 and TP63 staining of their parental tumors. Scale bar, 100 μm. **D**, Organoid cell growth of short-term organoid cultures. Each point on the graph represents a passage. Growth was calculated by plotting the time to passaging and the cumulative sum of the number of wells plated. **E**, Erlotinib testing in short-term organoid models.
Tumor purity of short-term organoid cultures
A recurrent issue highlighted by previous work is the outgrowth of normal epithelial cells of organoid cultures derived from primary patient tumor (24, 26). Consistent with previous reports of NSCLC organoids, we observed the outgrowth of normal epithelial cells in 58% (7/12) of our short-term organoid cultures derived from patient tumor and PDX (Supplementary Tables S1 and S2). Because surgically resected lung tumors or biopsies may contain entrapped normal lung airway/alveolar epithelial cells, we speculate that these normal organoids derived from patient tissue likely arose from this region. In contrast, normal organoids arising from subcutaneously implanted PDX could have arisen from entrapped murine breast/sweat gland tissues at the implantation site. To determine tumor purity in PDOs, we performed cytologic evaluation by H&E and IHC for the lung markers TTF-1 and TP63 of the cultured cells and the original patient tumor. We observed that the normal-like organoid models do not reflect the IHC results of their parental tumor (Supplementary Fig. S1; Supplementary Table S1). For example, the LPTO124 patient tumor is an adenocarcinoma that stains negative for both TTF-1 and TP63, but the matched organoid stains positive for TP63 and negative for TTF-1. Because TP63 is a marker for lung basal cells, we speculate that the organoids derived from the LPTO124 patient tumor reflects a cell population growing from normal cells of basal cell origin. To assess the percentage of tumor cells versus mouse cells in the PDX-derived organoids (XDO), EpCAM+ human epithelial cells) and H2K+ (mouse cells) cell populations were characterized by flow cytometry analysis. Overall, for short-term cultures, 75% (3/4) of the evaluable PDO models were contaminated with this normal cell population, whereas 50% (4/8) of the evaluable XDOs were contaminated with mouse cells (<2% human EpCAM, >60% H2K; Supplementary Tables S1 and S2). PDO and XDO models that were deemed to not be largely contaminated with normal cell populations exhibited 75%–97% and 50%–90% tumor cell populations, respectively (Supplementary Table S1). Finally, we were not able to detect the presence of fibroblasts and immune cells in the short-term organoid cultures by histologic assessment.

Recapitulation of histologic and lineage features of parental tumors by short-term NSCLC organoid cultures
To assess the quality of our short-term NSCLC organoids for downstream applications, we assessed the organoid models by cytology/histology. Note that histologic and tumor purity assessment were performed in the short-term cultured LPTO126 and PDXO137 organoids before they were later established as long-term models. Histologic evaluation of the short-term models revealed LUAD and LUSC representing various histologic subtypes such as mucinous (LPTO126) and acinar (PDXO137) morphology in LUAD, and moderate differentiation (PDXO321) in LUSC (Fig. 1C). The organoids also reflected the TTF-1 and TP63 staining pattern of their parental tumors, suggesting that they recapitulate the histology of their parental tumors (Fig. 1C).

To demonstrate the utility of short-term organoid models for drug testing, we first determined whether there was sufficient number and growth of cells for these experiments. The four short-term models used in our drug test were propagable in the first few passages and contained enough cells for plating (Fig. 1D). We evaluated the efficacy of clinically approved EGFR-targeted therapy in NSCLC in four short-term organoid models. Although some of the organoids later on became long-term models, the drug test was performed in early passages (P1) of those organoids for the purpose of assessing the ability of short-term models for drug testing. We evaluated the EGFR inhibitor erlotinib in three models with wild-type EGFR and one model with EGFR exon 19 deletion. The EGFR exon 19 deleted organoid model PDXO137 was the most sensitive to erlotinib, whereas the EGFR wild-type models were less sensitive (Fig. 1E). The parental PDX of PDXO137 has also been previously shown to respond to erlotinib (27), demonstrating that organoid drug responses reflect those of its parental tumor. Therefore, we demonstrated as a proof-of-principle that short-term organoids contain sufficient cell numbers for drug testing and may be used as preclinical models for biomarker validation.

Characterization of long-term NSCLC organoid cultures (growth, purity, histologic/lineage marker)
Fifteen percent of NSCLC organoid models became long-term cultures, as defined by continuous cell growth that maintained the same split ratios in late passages (beyond 10 passages, over 3 months) and retained a high percentage of tumor cells (Fig. 1B; Supplementary Figs. S1 and S3). These cultures could be propagated beyond 10 passages for over 1 year in culture with a splitting ratio of at least 1:3, and without a decline in proliferation as the passage number increased (Fig. 2A). They were also recoverable from >1 year of cryopreservation and could be expanded in culture after thawing.

Using the same method to assess tumor purity as described for short-term, in long-term cultures, none were contaminated with normal or nonhuman cells. PDOs consisted of over 85% of tumor cells and the majority of the XDOs contained over 75% of EpCAM+ cells (Fig. 2B; Supplementary Table S2), with <8% of H2K-positive cells in all of the long-term organoid models.

Long-term established NSCLC organoids also retained the histologic features of their parental tumors. LUAD tumors can be classified into multiple histologic subtypes, which include acinar, lepidic, solid, papillary, and mixed histology. Four patient with LUAD and PDX tumors (LPTO54 tumor, LPTO85 tumor, PDXO426 PDX, and PDXO4065 PDX) collectively represented three histologic subtypes of LUAD: acinar predominant, mucinous, and solid predominant (poorly differentiated; Fig. 2C; Supplementary Fig. S2A). These histologic subtype patterns were reflected in the matched organoids of the tumor samples. In addition, expression of LUAD lineage markers such as TTF-1 was preserved. LPTO54 tumor and PDXO4065 PDX, as well as their matched organoids were positive for TTF-1, whereas LPTO85 tumor and PDXO426 PDX, along with their respective organoids, were TTF-1 negative (Fig. 2C; Supplementary Fig. S2A).

All three LUAD PDX models exhibited features of moderately differentiated LUSC. Likewise, our long-term LUSC PDXs and matched organoids were moderately differentiated and nonkeratinizing squamous cell carcinomas (PDXO274 and PDXO377), except for PDXO149 which was a keratinizing LUSC (Fig. 2D; Supplementary Fig. S3A and S3B). The LUAD organoids preserved the histology of their matched PDX models. LUSC organoids were positive for TP63 and CK5, and negative for TTF-1 and CK7, which is characteristic of LUSC (Supplementary Fig. S3C).

To determine whether the organoid culture conditions preserved the tumorigenic properties of the cancer cells, organoids derived from patient tissue and PDX were implanted into immunocompromised NOD/SCID mice. The NSCLC organoid models formed tumor xenografts that histologically recapitulated their parental tumors (Supplementary Fig. S4A–S4D). Among LUAD models, the PDXO4065 xenograft formed a solid LUAD positive for TTF-1 while the LPTO85 xenograft exhibited features of a mucinous LUAD negative for TTF-1, which is typical of mucinous LUAD (46). Among LUSC models, both PDXO274 and PDXO149 organoid xenografts
formed LUSC expressing the LUSC markers CK5 and TP63 (Supplementary Fig. S4A–S4D). Overall, our data indicate that even over long-term, our organoid culture conditions allow the cancer cells to retain key biological properties observed in the patient tumors, including histologic differentiation and tumorigenicity.

**NSCLC organoids preserve the mutation and copy number landscape of their parental tumors**

To evaluate the genome profile concordance of organoid cultures to their source, we compared the spectrum of somatic mutation and copy number aberrations between nine long-term organoid cultures and their parental tumors by WES. These samples included three patient–organoid pair (LPTO54, LPTO85, LPTO126), one PDX–organoid pair (PDXO4056), and five patient–PDX–organoid groupings (PDXO426, PDXO44, PDXO137, PDXO149, PDXO274). The spectrum of mutations was highly concordant between the organoid and their matched patient tumor and/or PDX tissue (Fig. 3A; Supplementary Table S3). Furthermore, the mutation burden in the five long-term established organoids was also similar to that of their parental patient/PDX tumors (Fig. 3A), indicating that the culture conditions do not destabilize the cancer genomes. The WES data further revealed that our organoid models harbored common mutations that were previously identified in...
Figure 3.
Mutation, copy number, and transcriptomic landscape of organoids and matched patient tumor/PDX. **A**, Mutational concordance and mutation burden between patient tumor and respective PDX and organoids. Heatmap represents the fraction of concordant mutations between corresponding samples. **B**, Copy number concordance heatmap on the global gene level. Pearson correlation of gene copy number was computed per sample. A panel of normal tissues was used for copy number calling for samples without matched normal tissue (model 274, 54, 4056, 426, 126, 85, 344, 137). **C**, Gene expression Pearson correlation heatmap (95% confidence interval) showing gene expression clustering using 893 genes differentially expressed between LUAD and LUSC PDX models in 9 patient/PDX-organoid models (total of 23 samples). P, patient; X, patient-derived xenograft; O, organoid.
independent NSCLC patient profiling studies (47–49). The affected genes included TP53, DDR2, KRAS, KEAP1, CUL3, NOTCH, etc. (Supplementary Table S3).

Copy number variation (CNV) analysis also supported the tumor origin of the organoids and indicated that CNV profiles of the parental tumors were largely preserved during organoid culture (Fig. 3B). Notably, major chromosomal copy number changes associated with LUAD and LUSC including, chr.1q and 3q amplifications, respectively, were detected in our patient/PDX/organoid cohorts (Supplementary Table S4). LPTO85 and LPTO344 patient tumors appeared to be highly correlated with one another by CNV. We confirmed the distinct identities of these samples by STR profiling. However, more detailed analysis of their genomes revealed that they were both close to copy number neutral, suggesting that this similarity largely accounted for their close correlation by CNV analysis. At the gene level, we also detected amplification in KRAS and deletion of CDKN2A in PDXO246, which are frequent occurrences in LUAD (47), as well as FGFR1 amplification in PDXO274, which is commonly observed in LUSC (48). Overall, the cancer mutations and CNV detected in our study support the tumor origin of the organoids and indicate that they retain the genomic aberrations and potentially other key cancer properties of their parental tumors.

Gene expression profiles are similar between NSCLC organoids and parental tumors

To determine whether gene expression profiles are preserved in the organoids, we used RNA-seq to analyze gene expression of nine matched organoid–patient and/or PDX tumor pairs described in the genomic analysis. Because of the confounding situation of human-stromal cells uniquely contributing to gene expression in the patient samples, we sought to identify a gene set that reduced the number of stromal-specific genes and enriched for genes expressed in tumor epithelial cells. To identify such a gene set, we used gene expression profiles for primary patient LUAD and LUSC growing as PDXs that were obtained using human-specific microarray chips (4). From these gene expression profiles, we obtained a list of 893 genes that are differentially expressed between LUAD and LUSC at a level of 2-fold or more. Using this list, we determined that the overall gene expression correlation between patient tumor and organoids is 0.59, whereas PDX and organoids is 0.8 (Fig. 3C). Furthermore, gene expression between five of six XODs and one of three PODs was more highly correlated with their matched tumor tissue from which the organoid was derived, than with any other organoid model (Supplementary Table S5). In addition, gene expression correlations were also calculated with 1,492 differentially expressed genes (a cutoff of 2-fold change) between patient with LUAD and LUSC samples from the TCGA. Using this gene set, the correlation coefficient between patient organoids is 0.66, whereas PDX organoids is 0.85 (Supplementary Fig. S5), similar to what we observed using the tumor epithelial-enriched gene list. Overall, the molecular data indicate that our in vitro growth conditions allow organoid tumor cells to largely maintain key molecular properties of their parental tumors.

Utility of long-term NSCLC organoids for drug testing

To explore the utility of our long-term NSCLC organoids for drug testing, we first surveyed genomic data of the five well-characterized long-term models for potential sensitizing biomarker alterations. The KRAS G13C mutation and amplification was detected in the patient, PDX, and organoid of PDXO426. Preclinical studies with KRAS mutant cell lines have suggested that such mutant tumor cells may be more sensitive to MEK inhibitors, as compared with KRAS wild-type cell lines (50, 51). To determine whether the KRAS mutation and amplification in the PDXO426 organoid confers sensitivity to the MEK inhibitor trametinib, we compared its response with the drug to that of three other organoid models without KRAS alterations. Consistent with previous studies, we found that the KRAS mutant PDXO426 was much more sensitive to the MEK inhibitor trametinib (IC₅₀ < 0.05 μmol/L) than three other organoids with wild-type KRAS (IC₅₀ > 0.5 μmol/L; Fig. 4A).

Similar results were also generated with the MEK inhibitor selumetinib (Fig. 4B). To confirm that the KRAS alterations in PDXO426 act specifically to confer sensitivity to targeted therapy, we also examined the responses of these four organoid models to the EGFR inhibitor afatinib (Fig. 4C). As expected, none of the four organoid models responded to afatinib relative to the HCC827 cell line, which has an EGFR mutation that sensitizes cells to EGFR inhibitors.

Combination therapy in NSCLC organoids

We next explored whether NSCLC organoids can also be used as discovery tools for novel biomarker and combination therapy approaches. CNV analysis revealed chromosome 8p amplification in the patient, PDX, and organoid model of PDXO274. FGFR1 amplification in this region is a common occurrence in LUSC, which occurs in 20% of LUSC cases (48). However, FGFR1 amplification by itself is not a good biomarker for FGFR inhibitor monotherapy in LUSC, as only 7%–11% of preselected patients demonstrated durable response in clinical trials (52, 53). Thus, we utilized PDXO274 to model potential combination therapies in FGFR1-amplified LUSC.

FGFR1 mRNA and protein quantification by RT-PCR and Western blot analysis revealed that PDXO274 exhibited more than a 10-fold increase in FGFR1 mRNA expression and higher phospho-FGFR1 (pFGFR1) and total FGFR1 protein expression relative to PDXO149 (FGFR1 wild-type; Fig. 5A and B). These results indicated that in the PDXO274 organoid model, FGFR1 amplification correlated with increased FGFR1 mRNA levels, protein expression, and pathway activation. However, reflective of the low response rates to FGFR inhibitors in patients, in vitro drug testing of the FGFR inhibitor BGJ398 revealed that PDXO274 was largely insensitive to FGFR inhibition (Fig. 5C). On the basis of previous cell line studies showing efficacy of the combination of MEK and PI3K inhibitors with FGFR inhibitors in FGFR-aberrant cancers (54, 55), we tested trametinib and the PI3K inhibitor BKM120 with BGJ398 in our FGFR1-amplified organoid model. Strong synergy (combination index < 0.5) was observed in the BGJ398 + trametinib combination, whereas weaker synergy (combination index > 0.5) was observed in the BGJ398 + BKM120 combination (Fig. 5D). In addition, although single-agent BGJ398 inhibited pFGFR and pAkt, and single-agent trametinib inhibited only pErk, targeted inhibition of all three phosphoproteins was achieved with the combination of the two compounds (Fig. 5E). The efficacy of the trametinib and BGJ398 combination was further verified in vivo, in the parental PDXO274 PDX (Fig. 5F), which further supports our earlier contention that organoid models can...
retain the targeted therapy sensitivity of its source tumor tissue. Our data thus support combining FGFR and MEK inhibitors in FGFR1-amplified LUSC. Collectively, our drug studies generally support the addition of organoid and PDX models to a validation/discovery pipeline from cell lines to the patient, which may vastly improve the clinical response rate.

**Discussion**

Organoid methodology has gained widespread popularity in the past few years for its utility in disease modeling and drug screening (56–59). We aimed to establish a protocol of culturing NSCLC organoids from patient tumors and PDXs, with the eventual

Figure 4.

Drug testing in long-term NSCLC organoids. Trametinib (A), selumetinib (B), and afatinib (C) drug testing in three LUAD models and one LUSC organoid model performed in technical and biological triplicates. HCC827 cell line was used as an EGFR-positive control. Error bars were determined as the SEM. Final drug curves were calculated as an average of three independent experiments. D, In vivo trametinib (1 mg/kg) sensitivity curves at experimental endpoint in PDXO426 and PDXO274 PDX. Error bars were determined as the SEM. N = 5 mice (PDXO426 PDX) and N = 6 mice (PDXO274 PDX) were used for each arm (*, P > 0.05; **, P < 0.05).
Figure 5.
Combination of FGFR1 and MEK inhibitors in LUSC organoid models. A, qRT-PCR of FGFR1 in PDXO274 PDX and organoid. FGFR1 expression was normalized to PDXO149 PDX. Error bars were determined as the SEM. B, FGFR1 protein expression in PDXO274 validated by Western blot analysis. C, in vitro screen of BGJ398 in PDXO274 and PDXO149 performed in technical and biological triplicates. Error bars were determined as the SEM. Final drug curves were calculated as an average of three independent experiments. D, Combination drug screen of BGJ398 with trametinib and BKM120 performed in technical and biological triplicates. ED50 is the drug synergy at 50% inhibition of cell viability, and ED75 is the drug synergy at 75% inhibition of cell viability. Error bars were determined as the SEM. Final drug curves were calculated as an average of three independent experiments. Combination indices were determined in CompuSyn software. E, Targeted inhibition of FGFR1 downstream proteins with single agents and combination treatment at 1, 3, and 5 μmol/L for 24 hours. F, In vivo confirmation of the trametinib (1 mg/kg) and BGJ398 (25 mg/kg) combination in PDXO274 PDX. N = 4–6 mice were used per arm. Error bars were determined as the SEM. **, \( P < 0.05 \) comparing between single agent and combination therapy using Student t test at the 38-day timepoint.
goal of establishing an improved platform for drug testing and biomarker discovery in NSCLC. Recent reports described two distinct methods for generating NSCLC organoid cultures, which harbored a mixture of normal and tumor cell populations (24, 26). In one method, the authors found it necessary to treat the organoid cultures with the MDM2 inhibitor nutlin-3a to enrich for tumor cells harboring TP53 mutation, due to the large amount of contamination by nontumor cells (26). However, long-term exposure to such chemicals may have unexpected consequences on tumor cell biology and although TP53 mutations are common in NSCLC, they do not occur in all cases (47, 48, 60), precluding this method for such tumor samples. In addition, there should be continued efforts to improve media to better enrich for tumor cells from a greater number of samples. Future development of organoid models and media formulations would benefit from a standardized set of parameters to calculate “success rates” of model establishment. Currently, different studies use different criteria for deeming their cultures to be initiated or established. The definition of established models, for example, is defined by continuous propagation for 6 months in one study (61) whereas others use 1 month (25) or ambiguous cutoffs (26) to define the longevity of their organoid cultures. Using our methods, which includes histologic and flow cytometric characterization of cultures, we were able to achieve a collective overall establishment rate of 88% for both short-term and long-term NSCLC organoids. One question that would be interesting to address in future studies is what are the differences between tumor samples that determine whether tumor cells can adapt at all versus short-term versus long-term to organoid culture. Reasons for these differences are currently unknown, although mitotic instability and lack of physical environmental support and essential media components, and the quiescent nature of lung stem cells have been speculated to account for some failures (22). We attempted to compare tumor features (histologic subtype, size) and mutation status between long-term and short-term organoid models (Supplementary Tables S6 and S7). However, these association studies are currently limited by our small sample size, which may be overcome as more organoid models become available. Interestingly, we did observe that organoids were more easily formed from PDXs than primary patient tumors. This finding suggests that a prior selection pressure in the nonorthotopic in vivo PDX environment might enrich for tumor populations that are more likely to survive in vitro. We also observed that it was more difficult to establish organoids from LUSC patient tumors than LUAD tumors. These suggest that the in vivo conditions are still not optimal for LUSC, and consequently, LUSC exhibits a marked preference for an in vivo environment, even nonorthotopic. Indeed, it has generally been more difficult to establish 2D cell lines from LUSC, as compared with LUAD, whereas LUSC patient tumors engraft much better as PDXs (60% vs. 25%, respectively; refs. 4, 28).

In addition to recapitulating the biology that drives histologic appearance of their parental tumors growing in vivo, another attractive feature of our organoid models is that they have not yet drifted on the molecular level, as many cell lines have. Previous studies have shown that organoids from other cancers can retain the molecular profiles of their parental tumors, even with subsequent passaging (15, 61–63). We examined the mutation and CNV profiles of our NSCLC organoids and identified major somatic alterations in lung cancer that were preserved in the parental tumors. We also observed that organoids of late passage (even after more than 10 passages) still retained the molecular features of their parental tumors. This suggests that mutation and CNV profiles are largely stable in organoid cultures. In addition, there have been reports of both concordance and discordance of gene expression profiles between organoids and their parental tumors (15). We showed that majority of our organoid models (>10 passages) exhibited a strong correlation in gene expression with their parental tumors. However, it did appear that the gene expression correlations were better for organoids derived from PDXs than patient tumors. This may reflect the patient tumors having the most heterogeneity among cell populations, with the PDX and organoid models potentially selecting for fewer clonal populations.

One of the attractive applications of our short-term cultures is that there is usually enough cell material for drug testing, which we have demonstrated for some targeted therapeutics. This may be particularly useful not only for biomarker validation studies, but also for quick assessment of which therapies would be suitable for patient use when they fail on first or second-line therapies. Recent reports have demonstrated the utility of PDXs for drug screening and personalized medicine (64). However, PDXs are limited by slow growth rate and are economically challenging to maintain. Thus, our protocol for establishing NSCLC organoids from patient tumor and PDX could potentially provide additional models of NSCLC that are biologically relevant for future drug screening studies. Indeed, we find that we are usually able to obtain enough cells even in short-term culture for drug testing, which would be particularly useful for quick assessment of alternative therapies when patients fail on first or second-line treatments. With short-term cultures, we verified a previous suggestion that MEK could be a potential clinically relevant target in some KRAS mutant LUADs (50, 51). Using our long-term organoid cultures, we also found evidence to support combining FGFR and MEK inhibitors in FGFR1-amplified LUSC, the basis for which was also initially suggested from cell line work (54). Importantly, we found that the organoid drug response is similar to that of the matched PDX, which was not adapted to ex vivo culture. Thus, organoids developed with our protocol appear to be good surrogates for clinical tissue for drug screening and biological studies.

In conclusion, our study provides a methodology of developing short-term and long-term organoid cultures from NSCLC patient and PDX tumor tissue, and demonstrates the utility of the established organoids for drug testing and biomarker validation. Our collection of NSCLC organoids is also a novel addition to existing preclinical models of NSCLC that may be useful for identifying viable therapeutic options for this disease.

Disclosure of Potential Conflicts of Interest

G. Liu is a paid consultant for AstraZeneca, Takeda, Pfizer, Novartis, Roche, Bristol-Myers Squibb, Bayer, and EMD Serono, and reports receiving commercial research grants from Takeda, AstraZeneca, and Boehringer Ingelheim. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Concept and design: R. Shi, N. Radulovich, N. Moghal, M.-S. Tsao
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Shi, N. Radulovich, C. Ng, H. Notsuda, S.N. Martins-Filho, J.C. Rosen, G. Liu, M.-S. Tsao
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Shi, N. Radulovich, C. Ng, H. Notsuda, M. Cabanero, S.N. Martins-Filho, V. Raghavan, Q. Li, A.S. Mer, B. Haibe-Kains, G. Liu, M.-S. Tsao
Writing, review, and/or revision of the manuscript: R. Shi, N. Radulovich, M. Cabanero, S.N. Martins-Filho, V. Raghavan, Q. Li, N.-A. Pham, B. Haibe-Kains, G. Liu, N. Moghal, M.-S. Tsao

1172 Clin Cancer Res; 26(5) March 1, 2020 CLINICAL CANCER RESEARCH
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Shi, N. Radulovich, C. Ng, H. Notsuda, M. Li, Y.-H. Wang, L. Tamblunny, N.-A. Pham, G. Liu, M.-S. Tsao

Study supervision: N. Radulovich, M.-S. Tsao

Acknowledgments

This work was supported by Canadian Institute of Health Research (CIHR) Foundation grant FDN-148395 (to M.-S. Tsao), Canadian Cancer Society Research Institute IMPACT grants 701595 (to M.-S. Tsao) and 703206 (to G. Liu), and the Princess Margaret Cancer Foundation (for PM Living Biobank core). R. Shi is funded by a University of Toronto Ontario Student Opportunity Trust Fund (OSOTF) and Ontario Graduate Scholarship (OGS). M. Cahanero was supported by the Terry Fox Foundation Training Program in Molecular Pathology of Cancer at CIHR (STP 53912). S.N. Martins-Filho was supported by a Training Program grant from the Terry Fox Research Institute and Princess Margaret Cancer Foundation. G. Liu is the Alan B. Brown Chair in Molecular Genetics. M.-S. Tsao is the M. Quim Chokri Chair in Lung Cancer Translational Research. The authors thank Jing Xu, Wendy So, and Jian Zhou for all ICC staining. They acknowledge the Princess Margaret Biobank for providing patient tissue samples. They thank the Princess Margaret Genomics Centre (PMGC), especially Julissa Tsao, for exome-sequencing DNA sequencing. They thank Drs. Trevor Pugh and Vi Kambal Stoburg for advice and guidance of the project.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 5, 2019, revised September 19, 2019; accepted October 30, 2019, published first November 6, 2019.

References


Organoid Cultures as Preclinical Models of Non–Small Cell Lung Cancer
Ruoshi Shi, Nikolina Radulovich, Christine Ng, et al.


Updated version
Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-19-1376

Supplementary Material
Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2019/11/06/1078-0432.CCR-19-1376.DC1

Cited articles
This article cites 63 articles, 12 of which you can access for free at: http://clincancerres.aacrjournals.org/content/26/5/1162.full#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at: http://clincancerres.aacrjournals.org/content/26/5/1162.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://clincancerres.aacrjournals.org/content/26/5/1162. Click on "Request Permissions" which will take you to the Copyright Clearance Center’s (CCC) Rightslink site.