EXAMINING GENETIC PROPERTIES AND CELLULAR STRUCTURES OF CLONAL 3D CULTURES CONSTRUCTED FROM PATIENT- DERIVED ORGANOIDS

by

Anna Wade

B.S. (University of Illinois) 2014

MOCK GRANT PROPOSAL

Submitted in partial satisfaction of the requirements

for the degree of

MASTER OF SCIENCE

in

BIOMEDICAL SCIENCE

in the

GRADUATE SCHOOL

of

HOOD COLLEGE

January 2021

Accepted:

_____	_____
Oney P. Smith, Ph.D. Committee Member	Ann L. Boyd, Ph.D. Director, Biomedical Science Program
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Craig Laufer, Ph.D. Committee Member	
_____	_____
Ann L. Boyd, Ph.D. Project Adviser	April M. Boulton, Ph.D. Dean of the Graduate School

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DEDICATION

I would like to dedicate this work to my family.

ACKNOWLEDGEMENTS

I would like to thank my committee members for their guidance and patience during the preparation of this project. I would like to thank Leidos Biomedical Research for the financial help through their educational assistance program. Most of all, I would like to thank my family and friends for their continued support.

Mock Grant Application

Modeled after Department of Health and Human Services
Public Health Services
(based on Form PHS 398)

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Type	Activity	Version – HCBMS.011712
Review Group		Formerly
Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT *(Do not exceed 81 characters, including spaces and punctuation.)*
EXAMINING GENETIC PROPERTIES AND CELLULAR STRUCTURES OF CLONAL 3D CULTURES CONSTRUCTED FROM

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☒ NO ☐ YES
(If "Yes," state number and title)
Number: Title:

3. PROGRAM DIRECTOR/PRINCIPAL INVESTIGATOR — **New Investigator** ☐ **No** ☒ **Yes**

3a. NAME (Last, first, middle): Wade, Anna

3b. DEGREE(S): B.S.

3h. eRA Commons User Name: N/A

3c. POSITION TITLE: Graduate Student

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3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT: Department of Biology

3f. MAJOR SUBDIVISION: Biomedical Science Program

3g. TELEPHONE AND FAX *(Area code, number and extension)*
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4. HUMAN SUBJECTS RESEARCH ☐ No ☒ Yes

4a. Research Exempt: N/A

4b. Federal-Wide Assurance No.: N/A

4c. Clinical Trial ☒ No ☐ Yes

4d. NIH-defined Phase III Clinical Trial ☒ No ☐ Yes

5. VERTEBRATE ANIMALS ☒ No ☐ Yes

5a. Animal Welfare Assurance No.: N/A

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)

9. APPLICANT ORGANIZATION
Name
Address: Department of Biology, Hood College, 401 Rosemont Ave, Frederick, MD 21701

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☐ State ☐ Local
Private: → ☒ Private Nonprofit
For-profit: → ☐ General ☐ Small Business
☐ Woman-owned ☐ Socially and Economically Disadvantaged

11. ENTITY IDENTIFICATION NUMBER
DUNS NO. N/A Cong. District N/A

12. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
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14. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 13. *(In ink. "Per" signature not acceptable.)*: Anna Wade

DATE: 11/16/2020

PROJECT SUMMARY (See instructions):

Current anti-cancer drug development studies are progressing towards the use of 3D organoid cell line cultures generated from cancer patient tumors for in vitro models. Establishing clonal 3D culture cell lines in vitro from a patient's tumor will supply drug trials the type of model containing their target without the additional cells- that differ and may interfere with the results of the drug study. This would precede the tested drug being combined with others for personalized cancer medicine for the patient. Lung cancer, similar to other cancer types, may result from more than one cellular mutation and be a heterogeneous collection of cells. Targeting different cell subpopulations of the tumor is a solution to eradicate the entire cancer. This project will identify the benefits presented by using clonal cancer cell lines generated in 3D culture compared to clones grown in 2D culture through establishing lung cancer clonal 3D cultures in optimal conditions, isolating cell populations found in lung cancer, and testing anti-cancer drug efficacy on 2D and 3D cultures.

RELEVANCE (See instructions):

Lung cancer is responsible for one-quarter of cancer-related deaths (Siegel, 2019). Lung cancer contains cellular heterogeneity and various genetic mutations (Chen, 2014). Supplying drug development studies clonal 3D cultures allows testing the efficacy of their drug on the expected target. Identifying different anti-cancer drugs for corresponding cancer mutations is a step towards personalized cancer medicine.

PROJECT/PERFORMANCE SITE(S) (if additional space is needed, use Project/Performance Site Format Page)

Project/Performance Site Primary Location			
Organizational Name: Department of Biology, Hood College			
DUNS:			
Street 1: 401 Rosemont Ave		Street 2:	
City: Frederick	County: Frederick		State: MD
Province:	Country: United States		Zip/Postal Code: 21702
Project/Performance Site Congressional Districts: 6th Congressional District			

Additional Project/Performance Site Location			
Organizational Name:			
DUNS:			
Street 1:		Street 2:	
City:	County:		State:
Province:	Country:		Zip/Postal Code:
Project/Performance Site Congressional Districts:			

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Anna Wade	Graduate Student
eRA COMMONS USER NAME (credential, e.g., agency login)	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Illinois, Urbana-Champaign, IL	B.S.	2014	Molecular and Cellular Biology
Hood College, Frederick, MD	M.S.	2021 (anticipated)	Biomedical Science

A. Positions and Honors

2019 – present	Research Associate I (Leidos Biomedical Research Inc.)
2016 – 2019	Research Technician (Leidos Biomedical Research Inc.)
2015 – 2016	Lab Technician (Cumberland Valley Analytical Services)
2012 – 2014	Intern (SmileHealthy)

B. Selected peer-reviewed publications (in chronological order)

N/A

C. Research Support

N/A

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the project/performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. If research involving Select Agent(s) will occur at any performance site(s), the biocontainment resources available at each site should be described. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:

Biosafety Level 2

Clinical:

N/A

Animal:

N/A

Computer:

N/A

Office:

N/A

Other:

N/A

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

The following major equipment is available on site:

- Centrifuges – collections of cells
- Biosafety Level 2 Cabinet
- BD FACSCelesta™ (BD Biosciences)

The following consumables will be purchased with grant funding:

- Petri Dishes & scalpels – chopping tumor fragments
- 50 mL conical tubes
- 10 mL, 25 mL, 50 mL pipette tips
- 20 µL, 200 µL, 1 mL pipettes
- 20 µL, 200 µL, 1 mL pipette tips
- Pipette man
- Collagenase II and Dispase
- Advanced DMEM
- LIVE/DEAD™ Viability/Cytotoxicity Kit, for mammalian cells (Thermo Fisher Scientific)
- Media reagents listed in Research and Design Section
- Qiagen DNeasy Blood & Tissue Kit (cat. 69506)

SPECIFIC AIMS

The goal of this research is to provide viable clonal 3D cultures derived from Non-small-cell lung carcinoma (NSCLC) patients' tumor fragments to help predict tumor drug sensitivity. Pasch et al. (2019) demonstrated that patient-derived cancer organoids maintain molecular and histological characteristics similar to the primary tumor. Xie et al. (2017) identified a side population of cells in a lung cancer cell line (A549) that carried different characteristics from the main population; that included an increased ability in tumorigenicity (replication rate), invasiveness, and chemoresistance. Dissecting a tumor into discrete cell populations based on somatic mutations allows more precise drug-screening. Categorizing patient-derived cancer organoids based on clonal population may provide an advanced preclinical tool to represent the patients' tumor in personalized treatment.

The main objectives of this study are:

1. Establish patient-derived clonal 3D cultures through finding minimum optimal media conditions for Non-small-cell lung carcinoma cell line viability in 3D culture.

2. Identify different cell types and characteristics of the isolated subpopulations in Non-small-cell lung carcinoma organoids.

3. Test five anti-cancer drugs for NSCLC cancer, that are FDA approved and widely used, to compare drug efficacy against clones in 3D culture, clones in 2D culture, and the heterogenous organoids.

BACKGROUND AND SIGNIFICANCE

Organoids are three- dimensional *in vitro* culture models. An organoid is a mass of cells that resembles an organ and mimics the structure and function of the organ (Luca et al. 2013). Organoids may develop from isolated cancer patient tumor samples that are combined with a proper extracellular matrix support and serum-free medium with growth factors to encourage growth of organoids. Attractive attributes of organoids as models for drug discoveries include shorter time periods for growth and lower maintenance costs than patient derived xenografts and more genetic stability than 2D cell culture (Li, 2020). Organoids have shown similar morphologies and drug sensitivity as the primary patient tumor (Li, 2020).

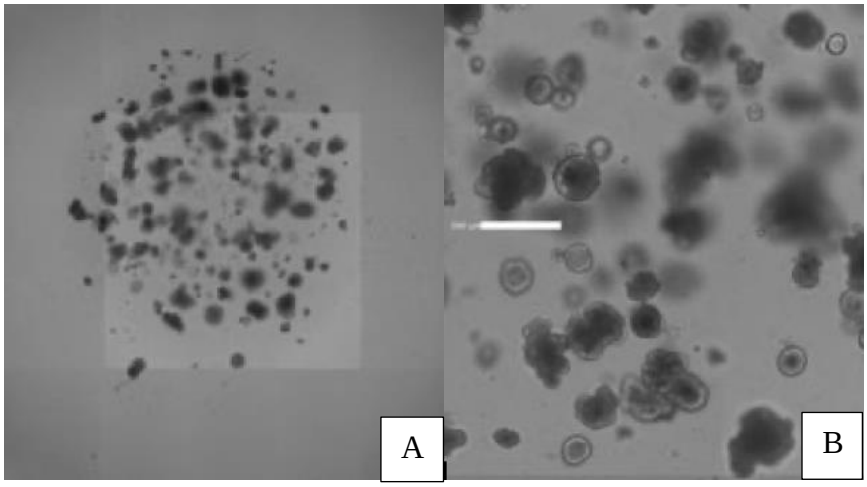

Figure 1. A. Picture taken with Celigo, viewpoint is from looking down at a single 24-well plate dome containing organoids. B. Picture of A. at a higher magnification, measure of reference is a 500 µm scale bar. (NCI Patient-Derived Models Repository, 2019).

Non-small Cell Lung Cancer

Cancer is responsible for one in six deaths globally as of 2015 (World Health Organization). Also, cancer creates a significant burden on the global economy. Cancer affects all people, including low and high- income populations. Cancer is a disease in which abnormally mutated cells replicate rapidly and damage surrounding body tissue. Cancer is a complex disease in which there are various factors- genetic and environmental- that increase the risk of incidence. Lung cancer is responsible for 25 percent of all cancer related deaths. Those diagnosed with lung and bronchus cancer have only an eighteen percent chance of surviving five years (National Cancer Institute, 2019). More than 80 percent of lung cancers are Non-small cell lung cancer (NSCLC) (American Cancer Society, 2019). NSCLC is comprised of Adenocarcinomas, Squamous cell carcinomas, and large cell carcinomas. These types of cancers may arise from the following types of cells; basal cells, bronchiolar progenitor cells, bronchioalveolar stem cells (BASCs), and stem cells (Chen, 2014). There are several studies focusing on the disease pathogenesis and creating a platform for cellular pharmacology. Different genetic alterations may cause the onset of lung cancer; this includes somatic mutations in the TP53 gene (present in half of lung cancers), EGFR gene (occurs 30 to 40 percent in Asian population with Adenocarcinomas),and KRAS gene (occur in 15 to 25 percent of lung cancer cases) (Genetics Home Reference, 2019).

Figure 2. Picture of an organoid derived from a lung cancer cell line at 40X magnification. (Stockwin et. al, 2017).

Current Culture Conditions for Cancer Clones

Current 2D cell cultures of cancer cell lines have limitations in fully representing the cancer sample (Jin, 2018). With 2D cell cultures, the cells in the sample come into contact with growth substratum such as a plastic flask. This attachment is believed to interfere with the heterogeneity of the *in vitro* model, disrupting growth of certain types of cells such as nonadherent cells within the tumor. This may be a result of cells not favoring the environment of the plastic, or cell outgrowth from advantages that come with direct contact with plastic. Organoids maintain greater heterogeneity of the cancer in the culture since growth is not dependent on adherence to the plate. Pampaloni concluded that 3D culture is a more accurate representation of the cell-cell and cell-matrix interactions than 2D cell cultures (Pampaloni et.al, 2007). Based on the findings from Luca, when comparing 2D and 3D cell culture models, the 3D culture offers the opportunity to investigate an unlimited supply of cells with similar genotypes and phenotypes under more physiological

conditions (Luca et.al.,2013). Van de Wetering found that organoids are suitable for biobanking and high-throughput screens due to their amendable characteristic and ability to extend expansion in culture while maintaining a greater genome stability (van de Wetering, 2015). Therefore, organoids will offer a greater diversity of resulting clones than the 2D cell line. Thus organoid cultures can be used to screen for drug inhibition of cell proliferation within a cluster of cells having differing phenotypes and presumably different genotypes.

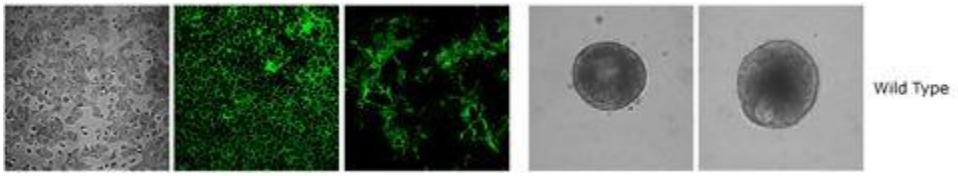

Figure 3. All images are of the same cell line from a Lung Cancer. Left three images are of 2D cultures/ monolayer. Right two images are of 3D culture/ organoids. (Jacobi et. al, 2017)

Clones are a group of identical cells that are derived from a single cell. Former methods of establishing cell lines from tumors used disaggregated cells and the cells that grew in specific culture conditions may or may not reflect the full differentiation of cells in the original tumor sample. The heterogenic cells express different proteins, survive in different environments, and may express different signal transduction pathways.

One clone may be more detrimental to the health of the patient than another. In a 2017 experiment, a subpopulation of cells in a tumor was found to account for resistance to chemotherapy, tumor growth, invasion, and metastasis (Xie, 2017). Therefore, a pharmaceutical treatment that may interact with a specific pathway may only influence

those specific type of cells. There is value in examining the different cells contained in one culture from one tumor sample in separate experiments. This allows the research to validate their findings; instead of the drug only eliminating few cells in a cell line containing all variants. Constructing a sample of organoids created from one clone from a patient-derived cell line will help provide the model of interest and eliminate variables that are attached to models containing diverse cell populations. In the present experiment, donated tissue from patients will generate *in vitro* models from NSCLC cancers- lung and bronchus cancers. Constructing a sample of organoids created from one clone from a patient-derived cell line will help provide the model of interest and eliminate variables that are attached to models containing diverse cell populations. Having the clones constructed into organoids allows the development of samples that will represent cellular pathways individually and provide a model to test the drug reaching cells within the structure as opposed to just the cells on the surface.

Drug penetration into a tumor may be incomplete and lead all cells to escape the anticancer drug. Creating 3D cultures should allow characterization of whether an anticancer drug compound can penetrate through the cells surrounding the exterior of a tumor and reach the cells in the center of the tumor mass and still be effective. High-throughput screening of cancer cell lines have been used to determine drug-sensitivity patterns in relationship with genomic alterations of the cancer cells. In 2015, van de Wetering was able to identify certain chemotherapeutic drugs toxicity that correlated with specific genetic mutations using organoids (van de Wetering et.al, 2015). Potentially, if validated as quantitatively reliable, organoids could become the standard of evaluation of

a patient's tumor in order to select the most efficacious anticancer drug. Precision medicine- medicine that is designed to provide optimum efficiency that is tailored to the individual- for cancer patients will become possible with accurate representation of the patient's tumor *ex vivo*.

For cancer drug discoveries, research groups typically test a drug on one specific type of cancer cell. The drug targets a specific tumorigenic pathway, but the actual tumor may contain cells resistant to the drug due to differing mutations. 2D cultures may not be able to host that specific cell population that is resistant to the treatment. Currently cancer cell line clones are being expanded in 2D cultures (t25 flasks).

Mammary epithelial cells (MECs) were separated into single cell clones using flow cytometry to isolate basal and luminal cells (Jamieson, 2017). In the proposed study we intend to culture cells from the tumor resection as organoids, then isolate single cells to grow clonal populations that can be compared for tumorigenic properties. Each isolated clone will be amplified using a basement membrane extract (BME) and medium containing growth factors typical of the cancer location in the patient. A shortcoming of organoids is the lack of vasculature and no systemic immune responsive cell interactions. Once the organoids reach a few million cells, cells will be examined morphologically with hematoxylin-eosin (H&E) staining, Cells will be compared to cells in the original tumor resection also using H&E staining.

Identifiler analysis to authenticate clones to original patient tumor fragment, drug screen with five different anticancer drugs, whole-exome sequencing (WES) for genomic stability, and RNA-seq for gene expression are techniques that will be used to compare the

clones. Each clonal population of cells derived from the organoid grown from the tumor sample will be cryopreserved for future experiments. Samples of cells will be treated with five chemotherapy agents and assayed for cytotoxicity using Live/ Dead assay. Whole exome sequencing on each clonal population of cells will be used to determine mutations against the primary resection and 1000 Genomes Project.

Conclusions from these analyses will determine if 3D clones are more accurate models than 2D current clone models.

PRELIMINARY REPORT / PROGRESS REPORT

Organoids are being used to advance research for several human diseases. We hypothesize that using 3D culture to further research for high throughput drug screening and discovery for lung cancer is more valid than using 2D culture. Tiriac and Van de Wetering showed that it is possible to establish organoids from fresh surgical resections from primary tumors and metastases in human patients (Tiriac, 2018; van de Wetering, 2015). This provides the rational for establishing lung cancer organoids for drug discoveries. Constructing organoid clones are viable.

Supplements for Production of Clonal 3D cutlures

The extracellular matrix support that the present experiment will use is Basement Membrane Extract (BME). BME has been shown to sustain expanding organoids in the van de Wetering research (van de Wetering, 2015). The BME culture condition allows the organoids to display phenotypes similar to the original tumor based on H&E staining. However, the extracellular matrix may not be able to represent all important physiological processes in a tumor, such as vasculature. The organoids are still semiphysiologic, where the cells expanded in 2D culture are not. The matrix provides xenobiotics and tissue organization. Phenotypic changes to the organoids may be a result of genetic mutations, but also a result of epigenetic modifications after isolation (Lee et al., 2018). Therefore, when choosing the composition of the medium, for the organoids the present experiment must expect to isolate potentially different clones in different media. As seen in previous experiments, key phenotypic characteristics of cancer cells are maintained across passages when media conditions are the same (Pasch, 2019). The first objective is to construct the

optimal conditions for viability of the clones in 3D culture, in order to obtain samples representing distinct cellular pathways and provide an in vitro model to test the efficiency of anticancer drug reaching cells within a tumor.

A drug may only contact/ affect the cells on the peripheral of an organoid. It was documented that cells in the center of lung organoids survived drug screens while cells on the outside were killed (Jung, 2019). Cytotoxicity in the current study will use Live/Dead assay from Thermofisher to observe the conditions of cells in different parts of the organoid- including center- after drug treatment (Pauli, 2017). The Live/ Dead assay uses cell imaging and fluorescence to highlight individual cells. To indicate that the drug is penetrating the organoid and reaching cells in the center, there will be a result of red fluorescence cells in the center. Red-fluorescent ethidium homodimer-1 indicates death by loss of plasma membrane.

Primocin, an antibiotic, is in the medium to guard against bacterial or yeast contamination. Three different media will be tested. Two media differ based on the use of conditioned medium. A third medium differing from the other two based on including conditioned medium and the use of added reagents. These reagents include: EGF Recombinant Human Protein (hEGF), Recombinant Human FGF-10 (hFGF-10), Recombinant Human FGF-basic (hFGF-2), Prostaglandin E (PGE2). The media recipes come from the NCI repository. Reagents used in each media and further description is provided in the Research and Design.

Biomarkers for NSCLC clonal 3D culture

CD133, p63, keratin 5, TTF1, and keratin 7 are the five biomarkers selected for this study. CD133 is known to be a common stem cell expressed marker (Jung, 2019). P63 and keratin 5 are usually expressed within the basal cells of lung cancer. Basal cells have been proposed as the cell of origin for lung squamous cell carcinoma of NSCLC.

TTF1 (thyroid transcription factor 1) and keratin 7 are usually expressed in epithelial cells of lung cancer and have been associated with adenocarcinoma of NSCLC. The identification of cell types is based on a range of tumors. A single tumor resection may contain all of the five biomarkers selected for this experiment or may contain a few or none.

Techniques for Verification of Genomic Stability in Clonal 3D culture

The established clonal cell lines will be subjected to short tandem repeat (STR) with the use of AmpFLSTR Identifiler PCR Amplification Kit from Thermo Fisher Scientific. Short tandem repeats are informative because they are a result of polymorphism and a source of a genetic disease. STR analysis is used to compare allele repeats at specific locations in DNA between two or more samples. The 2D culture clones, 3D culture clones, and organoid cultures will be compared to the original tumor tissue from the patient for authentication of identity. This is good cell culture practice.

The clonal cell lines will also be stained with hematoxylin-eosin (H&E) to visually show differences in morphology between 2D and 3D cultures of the clonal lung cancer organoids. The substratum- the environment- may influence the cell populations growth or expression of proteins therefore affecting the architecture of the cells.

The cells will be cryopreserved for the intent of future testing. A whole exome sequencing (WES) array will be performed on the original patient tumor tissue, organoid,

2D culture, and 3D culture by using Illumina. Qiagen DNeasy Blood &Tissue Kit (cat. 69506) will be used to isolate the DNA. The sequenced DNA of all of the samples will aligned to the human reference genome. WES is used to compare the somatic mutation and copy number variation. All of the samples will be compared to see any genomic drift and compare genomic stability between the different cultures. 2D cultures may prevent the growth of certain cell populations due to the substratum. This would cause 2D cultures to lose genome stability.

RNA-sequencing analysis will be performed using the Illumina Truseq Stranded mRNA Sample Preparation Kit (Illumina) for sample preparation. HiSeq 2000 sequencer will be used to sequence the RNA (this would be done outside of the lab). The purpose of RNA-sequencing is to view preservation of gene expression profiles.

3D culture clones compared with primary tumor to validate the use of 3D cultures for drug discoveries because they show resemblance. All of these techniques will be used to view any divergence from the primary tumor (Shi et. al, 2019).

3D culture cancer models have shown great accuracy in predicting clinical outcomes (Jacobi et. al, 2017). The drug resistance observed in the 3D cultures resembles the patient treatment response to the same drug (Vlachogiannis, 2018). Therefore, clonal 3D cultures should help identify new drugs targeting specific pathways.

Based on the research performed by Jacobi et al., (2017) 3D cultures and 2D cultures show variable results in drug screens. We predict that the proposed comparison of clonal 3D vs clonal 2D cultures from a tumor will show different sensitivity to five selected anticancer drugs based on drug penetrance of the 3D cell population and tumor specific

mutations in individual clones. The drugs to be tested are five Food and Drug Administration (FDA) approved anticancer drug treatments that are currently used in treating patients with lung cancer: Crizotinib (targets ALK, HGFR), Erlotinib (targets EGFR), Cisplatin (targets DNA- small molecule inhibitor), Sunitinib (multiple target tyrosine kinase inhibitor), and Paclitaxel (targets tubulin- small molecule inhibitor) (Tamura, 2018). Jacobi et al., (2017) did not observe any differences in cisplatin sensitivity between the 2D and 3D cultures. When treating the cells with paclitaxel, the cell lines reacted differently. Paclitaxel was found to be toxic for cells cultured in 2D. The same cells were found to be insensitive to paclitaxel in the 3D culture (Jacobi et.al, 2017). Because 2D and 3D cultures react differently to Paclitaxel, it is expected that 3D culture more closely reflects in vivo tumor responsiveness to chemotherapy.

RESEACH DESIGN / METHODS

The procedure will be done according to Biosafety Level 2 Standards. A Biological Safety cabinet will be used for cultivation of tumor cells. Personal Protective Equipment- such as lab coat, appropriate gloves, face mask, etc.- are worn to prevent any hazard to the personal, from unidentified virus or other infectious agents in the patient sample.

Aim 1: Establish patient- derived organoids and clones (Months 01-06)

Donated tissue from 10 patients with lung cancer that have signed an informed consent will provide material to generate in vitro models. No benign cancer tumors will be accepted. Tumors will be excised from the patient before the patient begins any treatment. The excised fragment of the patient's tumor will be packaged and transported in a sterile conical tube, from the hospital to the laboratory in a transport medium (CO_2 Independent Medium containing primocin 100 µg/mL). The tumor sample will be dissociated using Collegenase/Dispase into single cell suspension. The cells will be washed with complete medium with Fetal Bovine Serum to neutralize the collagenase. Single cells will be introduced into a 3D culture. Clonal growth will be compared using the following three medium compositions in table 1. Two media differ based on the use of conditioned medium. A third medium differing from the other two based on the use of added reagents.

3D culture preparation:

The cells will be centrifuged at 1100 rpm to concentrate cells, and resuspended with basement membrane extract and plated onto 24- well plate. An appropriate amount of the designated complete, serum-free medium will be added to each well to cover the matrix

containing the cells. These cells will later be single cell cloned. The plates will be incubated at 37°C in 5% Carbon dioxide humidified incubator. The plate will be monitored twice every week and medium replaced every seven days.

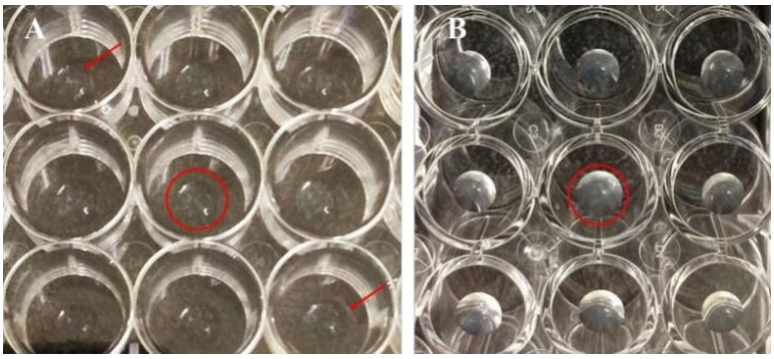

Figure 4. Pictures of the 24-well plate containing plated cells within BME before medium is added to the wells. A. Viewpoint from looking down at 24-well plate. B. Viewpoint from below 24-well plate looking up. (NCI Patient-Derived Models Repository, 2019).

Figure 5. Picture of each well's matrix confluent with organoids (before medium added).

Item:	Medium 1	Medium 2	Medium 3
Advanced DMEM/F12 (1X)	X	X	X
HEPES (1M)	X	X	X
GlutaMax Supplement (100X)	X	X	X
Primocin (50mg/mL)	X	X	X
N-acetylcysteine	X	X	X
L-WRN Conditioned Medium	X		X
Nicotinamide	X	X	X
N21-MAX Medium Supplement (50X)	X	X	X
N-2 MAX Medium Supplement (100X)	X	X	X
Y- 27632 dihydrochloride	X	X	X
EGF Recombinant Human Protein (hEGF)			X
Recombinant Human FGF-10 (hFGF-10)			X
Recombinant Human FGF-basic (hFGF-2)			X
Prostaglandin E (PGE2)			X

Table1. Includes what each medium type contains. Media must be prepared fresh each week- certain reagents have a short half-life. (NCI Patient- Derived Models Repository).

When the matrix in each well become confluent with organoids, the 3D culture will be subcultured to amplify the clone and preserved by cryopreservation. To break down the matrix and release the organoids, complete medium with fetal bovine serum and Dispase will be added to each well. Plate will be returned to incubate at 37°C in 5% Carbon

dioxide humidified incubator. After enough time (one and a half hours), all of the contents in the wells will be transferred into a 50 mL conical tube. The cells will be pelleted by centrifugation at 1100 rpm, medium will be removed and fresh complete medium with fetal bovine serum will be added to the cells to ensure removal of all Dispase. Cells will be centrifuged again to allow removal of all media and transferred into a 1.5 mL starstedt tube. Appropriate amount of Basement Membrane Extract will be added to the cells to provide an extracellular matrix support. 30 µl of the cells and matrix mixture will be added to the appropriate number of cells. Plate will be inverted and placed in incubator for 25 minutes. Plate will be inverted back to upright before appropriate amount of designated medium is added to each well to cover the cells and matrix. Cells will be monitored every other day. When matrix is confluent with cells, cell line will be ready for expansion. If cells are not ready for expansion, culture medium will be replaced with fresh medium without disturbing the matrix of cells. The cell line will be passaged until the cell line contains a cell count of roughly five million cells (averaging two million cells per confluent 24-well plate).

When the cell line sample has an adequate number of cells, it will be ready to be picked from for preparation of clonal cell culture. The organoids will be harvested by adding medium containing Dispase to degrade the BME. The cells will be washed with DPBS (1x). To help dissociate the organoids into single cells, the cells will be added to Accutase (a dissociation agent). Cells will be washed again with DPBS (1x). The single cells will be plated into soft agar onto 6-well plates. Within the week, cells from the soft agar will be individually picked to establish a clonal cell culture in BME for 3D culture

and cells picked for 2D culture. Organoids will be developed from those clones in BME. A sample (roughly one million cells) from each clonal 3D culture cell line will be subjected to flow cytometry to confirm the possession of at least one of the biomarkers expected for lung cancer cells. If negative, then the cell line will be cryopreserved for future use in other experiments. Those clones will not carry through this experiment. Having the WES analysis already for the primary tumor will allow the knowledge of what mutations that cell line contains and how that cell line may be applied in future use.

Soft Agar:

A 6-well Corning plate will be prepared with a layer of 1% agarose in each well. Soft Agar will be used to isolate the clonal cells. The organoids will be harvested by introducing Dispase to the well containing the domes. Once the BME is completely degraded, the organoids can be harvested. After washes, the cells will be introduced to Accutase to dissociate the cells into single cells. A cell count will be taken with Nexelcom. The cells will be diluted in 0.5% agarose to dilute the cells to be plated 5,000 cells per well. The agarose and cell mixture will be added to surface of the 1% agarose layer in the well. Medium will be added above the layer of agarose and cells. After sufficient time for colonies to form from one cell (typical doubling time is 18 to 30 hours)(Li, 2018), the colony will be mechanically excised from the agar and plated into a 48-well plate well with growth medium, aiming to have one colony per well. There will be agarose layer at the bottom of the well to prevent contact between cells and plastic. The colony will be expanded in the well and harvested one there is a confluency of at least 80 percent in the well. Cells harvested will be subcultured and introduced to BME

and plated in wells on a 24-well plate for 3D culture as well as a T25 flask for 2D culture. The clones in 3D culture will be passaged the same as the organoids.

2D culture preparation:

Cells will be introduced to 10-mL of complete medium that includes 10% Fetal Bovine Serum and plated into a T25 flask. The flask will be incubated at 37°C in 5% Carbon dioxide humidified incubator. The flask will be monitored twice every week and medium replaced every seven days. The 2D culture will contain the same feeding medium for the corresponding organoid culture but with added 10% Fetal Bovine Serum.

After expanding cell line to the same number of passages as the organoid cell culture, cells will be harvested. Cells will be subcultured using standard methods: removal of medium, DPBS rinse, and dislodged from substratum using 0.25% Trypsin. Suspended cells are mixed with growth medium, transferred to a new T25 flasks and incubated at 37°C in 5% Carbon dioxide humidified incubator

It is expected that the 3D culture will establish cell lines from patient- derived tumor material. Medium 3 will most likely provide the optimal conditions for the 3D cultures due to it containing the most reagents. 3D culture will expand more quickly than 2D culture.

Aim 2: Identify characteristics of subpopulations in organoids (Months 06-12)

For each organoid cell line, roughly one million of the cells will be taken for Fluorescence-activated cell sorting (FACs) by BD FACSCelesta. The organoids will be harvested by adding medium containing Dispase to degrade the BME. The cells will be washed with DPBS (1x). To help dissociate the organoids into single cells, the cells will

be added to Accutase (a dissociation agent). Cells will be washed again with DPBS (1x). The cells will be categorized based on the following cell-specific antibodies targeting: CD133, p63, keratin 5, TTF-1, and keratin 7. A sample from the organoid before being broken down into clonal populations will be used to identify collectively the biomarkers present in the organoid. After clonal populations are established, another sample will be taken for FACs to confirm the properties of that clone. The clonal 3D culture clone will be prepared the same as the organoid for FACs.

3D culture and 2D culture clones will be harvested and broken down into single cells with use of Accutase (from above). Cells will be counted with use of Nexelcom- roughly 500,000 cells will be placed in one 1.5mL tube with complete medium. Those organoids will be sent to a lab for histology reports. From hematoxylin-eosin (H&E) staining, cells will be differentiated based on visual characteristics/ morphologies. We will compare morphologies between clones from organoids and 2D cultures that share similar biomarkers from the FACS analysis. Also, compare all clones to the primary tumor sample.

Roughly 500,000 cells will be provided for short tandem repeat analysis to ensure the 2D and 3D culture are in fact from the same patient sample. This is to confirm that there were no mistakes during the processing of the samples, that there was no contamination or switches.

Whole exome sequencing using isolated DNA from a sample of the original tumor specimen, mixed cell culture 3D, and single cell clones 2D and 3D sequenced on the Illumina platform (by core sequencing facility). Based on WES analysis, using 1000

Genomes Project, mutations will be compared to those previously identified in lung cancers as well as comparison between the different cultures from the same patient tissue. WES will show any genetic drift that may have occurred during the passaging of the clonal populations. We anticipate single cell clones will share some mutations and possess different cancer-related mutations. We expect the 3D culture clones to have greater genomic stability than the 2D culture clones.

RNA- sequencing will be used to identify mutations expected in lung tumor cells, e.g. TP53, EGFR, KRAS genes, and examine if gene expression was preserved through the cultures. The organoids will be compared to the original patient tissue. The 3D culture clones will be compared the original patient tissue, organoid, and 2D culture clones.

To ensure the ability to repeat experiments cell populations will be sufficient to cryopreserve cells before proceeding with genetic analysis and flow cytometry. To cryopreserve each sample, the cells in medium will be centrifuged to allow removal of medium. Complete medium containing DMSO and a higher percentage of fetal bovine serum will be added to the cells. One million cells will be cryopreserved in each vial and placed into a Mr. Frosty and into a -80°C freezer. The following day, the vials will be transferred into liquid nitrogen tanks for storage.

It is expected that there will be a greater number of clonal subtypes available from the 3D culture compared to the 2D culture. 3D culture will provide better genomic stability as well as greater cell viability.

Aim 3: Monitor drug efficacy against clonal 3D cultures , clonal 2D cultures, and the heterogeneous organoids (Months 12-14)

Five anticancer drug treatments, currently prescribed to patients with lung cancer Crizotinib (targets ALK, HGFR), Erlotinib (targets EGFR), Cisplatin (targets DNA- small molecule inhibitor), Sunitinib (multiple target tyrosine kinase inhibitor), and Paclitaxel (targets tubulin- small molecule inhibitor), will be used on 3D original organoid mixed cell culture from tumor sample, each clonal 3D culture, and 2D culture clonal sample (if there is a 2D culture match to a 3D clonal culture) in triplicate. For a negative control, no drug treatment will be added to portion of wells for each cell line's drug screen. This will ensure that the death of cells is due to the drug treatment and not to poor environmental conditions or another variable. For a positive control DMSO will be used. We expect DMSO to kill all the cells.

The cell lines will be seeded in a 24-well plate corresponding with culture type. The designated serum-free medium will be added to cover the matrix of cells for the organoids. The plate will be incubated at 37°C in 5% Carbon dioxide humidified incubator. The therapeutic agents will be added four days later, this allows reformation of organoids (visual verification by using a microscope). Drugs will be tested in a gradient of concentration for each test: 10 μM, 20 μM, 40 μM, 80 μM, 100 μM. The cell viability will be determined each day for five days of treatment. Viability will be determined utilizing the LIVE/DEAD® Viability/ Cytoxicity Kit (Invitrogen) according to the manufacturer's instructions (Jung, 2019). For imaging with a fluorescence microscope, the cells will be stained with green-fluorescent calcein-AM (indicates intracellular esterase activity) and red-fluorescent ethidium homodimer-1 (indicates death by loss of plasma membrane) in dPBS 1x for one hour at 37 °C followed by imaging. The fluorescent imaging allows one

to monitor cell death of the cells within the core of the organoid and the cells on the periphery. The purpose is to make sure the drug treatment is penetrating the organoid.

From previous work, I expect that there will be variable results between the mixed cell culture, 2D clonal culture, and the 3D clonal culture. I would expect evidence of cell survival for most, if not all of the anti-cancer treatments used on the mixed cell organoid cultures. Due to certain populations containing chemoresistance to that specific drug. I would expect that 3D and 2D would differ in the sense that the cells in the core of organoids are not exposed to the drug directly when the drug is added to the medium/culture. Therefore, depending on the concentration of the drug, the drug may not have a strong enough effect to reach and kill the cells in the core of the organoids. Based on the findings from *Jung et. al* (2019), I believe that we will witness cell death on day two even at the concentration of 20 μM. It depends on the clonal population and the anti-cancer treatment also.

Additional research would be required to test more biomarkers to cover more somatic mutations found in NSCLC. Only five were selected in this experiment for NSCLC. Future labs may implant the clonal 3D cultures into mice to look for tumorgenicity, genome stability, or other differences due to mice containing a vasculature system, microbiomes, immune system, etc. Clones are only a small part of the tumor. To personalize a treatment for a patient, the goal is to create a cocktail of the necessary anti-cancer drugs that were found to eradicate each cell population in that person's tumor. Therefore, the cocktail of anti-cancer drugs should be tested on the mixed cell organoid culture. Some clones may not be able to be grown in vitro at all and that could cause an

issue. The side effects of combining certain drugs together would also need to be monitored before delivered to the patient.

REFERENCES

Artegiani B, Clevers H. 2018. Use and application of 3D-organoid technology. Hum Mol Genet. 27(R2):R99–R107. doi:10.1093/hmg/ddy187. https://academic.oup.com/hmg/article/27/R2/R99/5001715.

Bray, Laura J., Dietmar W. Hutmacher, and Nathalie Bock. 2019. "Addressing Patient Specificity in the Engineering of Tumor Models." *Frontiers in Bioengineering and Biotechnology* 7 (September): 217. https://doi.org/10.3389/fbioe.2019.00217.

Chen, Ya-Wen, Sarah Xuelian Huang, Ana Luisa Rodrigues Toste de Carvalho, Siu-Hong Ho, Mohammad Naimul Islam, Stefano Volpi, Luigi D. Notarangelo, et al. 2017. "A Three-Dimensional Model of Human Lung Development and Disease from Pluripotent Stem Cells." *Nature Cell Biology* 19 (5): 542–49. https://doi.org/10.1038/ncb3510.

Chen, Zhao, Christine M. Fillmore, Peter S. Hammerman, Carla F. Kim, and Kwok-Kin Wong. 2014. "Non-Small-Cell Lung Cancers: A Heterogeneous Set of Diseases." *Nature Reviews Cancer* 14 (8): 535–46. https://doi.org/10.1038/nrc3775.

Clinton, James, and Penney McWilliams-Koeppen. 2019. "Initiation, Expansion, and Cryopreservation of Human Primary Tissue-Derived Normal and Diseased Organoids in Embedded Three-Dimensional Culture." *Current Protocols in Cell Biology* 82 (1). https://doi.org/10.1002/cpcb.66.

Devarasetty M, Mazzocchi AR, Skardal A. 2018. Applications of Bioengineered 3D Tissue and Tumor Organoids in Drug Development and Precision Medicine: Current and Future. BioDrugs. 32(1):53–68.

Drost J, van Boxtel R, Blokzijl F, Mizutani T, Sasaki N, Sasselli V, de Ligt J, Behjati S, Grolleman JE, van Wezel T, et al. 2017. Use of CRISPR-modified human stem cell organoids to study the origin of mutational signatures in cancer. Science. 358(6360):234–238. doi:10.1126/science.aao3130. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6038908/.

Genetics Home Reference. 2019. Lung cancer. Genet Home Ref. https://ghr.nlm.nih.gov/condition/lung-cancer.

Huch M, Gehart H, van Boxtel R, Hamer K, Blokzijl F, Verstegen MMA, Ellis E, van Wenum M, Fuchs SA, de Ligt J, et al. 2015. Long-Term Culture of Genome-Stable Bipotent Stem Cells from Adult Human Liver. Cell. 160(1–2):299–312. doi:10.1016/j.cell.2014.11.050. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4313365/.

Jabs, Julia, Franziska M Zickgraf, Jeongbin Park, Steve Wagner, Xiaoqi Jiang, Katharina Jechow, Kortine Kleinheinz, et al. 2017. “Screening Drug Effects in Patient-derived

Cancer Cells Links Organoid Responses to Genome Alterations.” *Molecular Systems Biology* 13 (11): 955. https://doi.org/10.15252/msb.20177697.

Jacobi N, Seeboeck R, Hofmann E, Schweiger H, Smolinska V, Mohr T, Boyer A, Sommergruber W, Lechner P, Pichler-Huebschmann C, et al. 2017. Organotypic three-dimensional cancer cell cultures mirror drug responses in vivo : lessons learned from the inhibition of EGFR signaling. Oncotarget. 8(64):107423–107440. doi:10.18632/oncotarget.22475. http://www.oncotarget.com/index.php?journal=oncotarget&page=article&op=view&path[]=22475&path[]=71068.

Jamieson PR, Dekkers JF, Rios AC, Fu NY, Lindeman GJ, Visvader JE. 2017. Derivation of a robust mouse mammary organoid system for studying tissue dynamics. Development. 144(6):1065–1071. doi:10.1242/dev.145045. http://dev.biologists.org/content/144/6/1065.

Jung, Da Jung, Tae Hoon Shin, Minsuh Kim, Chang Ohk Sung, Se Jin Jang, and Gi Seok Jeong. 2019. “A One-Stop Microfluidic-Based Lung Cancer Organoid Culture Platform for Testing Drug Sensitivity.” *Lab on a Chip* 19 (17): 2854–65. https://doi.org/10.1039/C9LC00496C.

Kenny HA, Krausz T, Yamada SD, Lengyel E. 2007. Use of a novel 3D culture model to elucidate the role of mesothelial cells, fibroblasts and extra-cellular matrices on adhesion and invasion of ovarian cancer cells to the omentum. Int J Cancer. 121(7):1463–1472. doi:10.1002/ijc.22874. https://onlinelibrary.wiley.com/doi/abs/10.1002/ijc.22874.

Kim, Minsuh, Hyemin Mun, Chang Oak Sung, Eun Jeong Cho, Hye-Joon Jeon, Sung Min Chun, Da Jung Jung, et al. 2019. “Patient-Derived Lung Cancer Organoids as in Vitro Cancer Models for Therapeutic Screening.” *Nature Communications* 10 (1): 3991. https://doi.org/10.1038/s41467-019-11867-6.

Lazarus, Kyren A., Fazal Hadi, Elisabetta Zambon, Karsten Bach, Maria-Francesca Santolla, Julie K. Watson, Lucia L. Correia, et al. 2018. “BCL11A Interacts with SOX2 to Control the Expression of Epigenetic Regulators in Lung Squamous Carcinoma.” *Nature Communications* 9 (1): 3327. https://doi.org/10.1038/s41467-018-05790-5.

Lee SH, Hu W, Matulay JT, Silva MV, Owczarek TB, Kim K, Chua CW, Barlow LJ, Kandoth C, Williams AB, et al. 2018. Tumor Evolution and Drug Response in Patient-Derived Organoid Models of Bladder Cancer. Cell. 173(2):515-528.e17. doi:10.1016/j.cell.2018.03.017.

Lemjabbar-Alaoui, Hassan, Omer UI Hassan, Yi-Wei Yang, and Petra Buchanan. 2015. "Lung Cancer: Biology and Treatment Options." *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer* 1856 (2): 189–210. https://doi.org/10.1016/j.bbcan.2015.08.002.

Li M, Izpisua Belmonte JC. 2019. Organoids - Preclinical Models of Human Disease. N Engl J Med. 380(6):569–579. doi:10.1056/NEJMra1806175.

Li, Xiaodun, Hayley E. Francies, Maria Secrier, Juliane Perner, Ahmad Miremadi, Núria Galeano-Dalmau, William J. Barendt, et al. 2018. "Organoid Cultures Recapitulate Esophageal Adenocarcinoma Heterogeneity Providing a Model for Clonality Studies and Precision Therapeutics." *Nature Communications* 9 (1): 2983. https://doi.org/10.1038/s41467-018-05190-9.

Li, Y. F., Y. Gao, B. W. Liang, X. Q. Cao, Z. J. Sun, J. H. Yu, Z. D. Liu, and Y. Han. 2020. "Patient-Derived Organoids of Non-Small Cells Lung Cancer and Their Application for Drug Screening." *Neoplasma* 67 (02): 430–37. https://doi.org/10.4149/neo_2020_190417N346.

Luca AC, Mersch S, Deenen R, Schmidt S, Messner I, Schäfer K-L, Baldus SE, Huckenbeck W, Piekorz RP, Knoefel WT, et al. 2013. Impact of the 3D Microenvironment on Phenotype, Gene Expression, and EGFR Inhibition of Colorectal Cancer Cell Lines. PLoS ONE. 8(3). doi:10.1371/journal.pone.0059689. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3608563/.

Mahesh. Applications of Bioengineered 3D Tissue and Tumor Organoids in Drug Development and Precision Medicine: Current and Future - ProQuest. https://taurus.hood.edu:2803/docview/2150381680/fulltext/DDB00D09E5BA4EAFPQ/1?accountid=11467.

Meehan TF, Conte N, Goldstein T, Inghirami G, Murakami MA, Brabetz S, Gu Z, Wiser JA, Dunn P, Begley DA, et al. 2017. PDX-MI: Minimal Information for Patient-Derived Tumor Xenograft Models. Cancer Res. 77(21):e62–e66. doi:10.1158/0008-5472.CAN-17-0582. http://cancerres.aacrjournals.org/content/77/21/e62.

Ming-Zhu J, Han R-R, Qiu G-Z, Xiang-Chun J, Ge L, Wei-Lin J. 2018. Organoids: An intermediate modeling platform in precision oncology. Cancer Lett Clare. 414:174–180. doi:http://dx.doi.org/10.1016/j.canlet.2017.11.021. https://search.proquest.com/docview/1983440050/abstract/5474DBFA1C824D04PQ/1.

Mullendore M, Stockwin LH, Bonomi C, Dougherty K, Stotler H, Kimmel A, Das B, Datta V, Lih J, Williams M, et al. Clonal heterogeneity in patient-derived xenografts: The Neuroendocrine PDX model BL0479 contains stable clones with epithelial or mesenchymal characteristics and differential drug sensitivities. :1.

National Cancer Institute. 2019. Lung and Bronchus Cancer - Cancer Stat Facts. SEER. https://seer.cancer.gov/statfacts/html/lungb.html.

NHGRI. 2018. The Completion of the Sequence and Remaining Goals. Natl Hum Genome Res Inst NHGRI. https://www.genome.gov/12011241/the-completion-of-the-sequence-and-remaining-goals/.

Pasch, Cheri A., Peter F. Favreau, Alexander E. Yueh, Christopher P. Babiarz, Amani A. Gillette, Joe T. Sharick, Mohammad Rezaul Karim, et al. 2019. "Patient-Derived Cancer Organoid Cultures to Predict Sensitivity to Chemotherapy and Radiation." *Clinical Cancer Research* 25 (17): 5376–87. https://doi.org/10.1158/1078-0432.CCR-18-3590.

Pauli, Chantal, Benjamin D. Hopkins, Davide Prandi, Reid Shaw, Tarcisio Fedrizzi, Andrea Sboner, Verena Sailer, et al. 2017. "Personalized *In Vitro* and *In Vivo* Cancer Models to Guide Precision Medicine." *Cancer Discovery* 7 (5): 462–77. https://doi.org/10.1158/2159-8290.CD-16-1154.

Porter SN, Baker LC, Mittelman D, Porteus MH. 2014. Lentiviral and targeted cellular barcoding reveals ongoing clonal dynamics of cell lines in vitro and in vivo. Genome Biol. 15(5):R75. doi:10.1186/gb-2014-15-5-r75. https://doi.org/10.1186/gb-2014-15-5-r75.

Prestwich GD. 2008. Evaluating Drug Efficacy and Toxicology in Three Dimensions: Using Synthetic Extracellular Matrices in Drug Discovery. Acc Chem Res. 41(1):139–148. doi:10.1021/ar7000827. [accessed 2019 Apr 1]. https://pubs.acs.org/doi/10.1021/ar7000827.

Ramamoorthy, Prabhu, Sufi Mary Thomas, Gaurav Kaushik, Dharmalingam Subramaniam, Katherine M. Chastain, Animesh Dhar, Ossama Tawfik, et al. 2019. "Metastatic Tumor-in-a-Dish, a Novel Multicellular Organoid to Study Lung Colonization and Predict Therapeutic Response." *Cancer Research* 79 (7): 1681–95. https://doi.org/10.1158/0008-5472.CAN-18-2602.

Roerink, Sophie F., Nobuo Sasaki, Henry Lee-Six, Matthew D. Young, Ludmil B. Alexandrov, Sam Behjati, Thomas J. Mitchell, et al. 2018. "Intra-Tumour Diversification in Colorectal Cancer at the Single-Cell Level." *Nature* 556 (7702): 457–62. https://doi.org/10.1038/s41586-018-0024-3.

Sabaawy, Hatem. 2013. "Genetic Heterogeneity and Clonal Evolution of Tumor Cells and Their Impact on Precision Cancer Medicine." *Journal of Leukemia* 01 (04). https://doi.org/10.4172/2329-6917.1000124.

Sachs, Norman, Angelos Papaspyropoulos, Domenique D Zomer-van Ommen, Inha Heo, Lena Böttinger, Dymph Klay, Fleur Weeber, et al. 2019. "Long-term Expanding Human

Airway Organoids for Disease Modeling." *The EMBO Journal* 38 (4). https://doi.org/10.15252/embj.2018100300.

Sato T, Stange DE, Ferrante M, Vries RGJ, van Es JH, van den Brink S, van Houdt WJ, Pronk A, van Gorp J, Siersema PD, et al. 2011. Long-term Expansion of Epithelial Organoids From Human Colon, Adenoma, Adenocarcinoma, and Barrett's Epithelium. Gastroenterology. 141(5):1762–1772. doi:10.1053/j.gastro.2011.07.050. https://linkinghub.elsevier.com/retrieve/pii/S0016508511011085.

Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE, van Es JH, Abo A, Kujala P, Peters PJ, et al. 2009. Single Lgr5 stem cells build crypt–villus structures in vitro without a mesenchymal niche. Nature. 459:262.

Shi, Ruoshi, Nikolina Radulovich, Christine Ng, Ni Liu, Hirotsugu Notsuda, Michael Cabanero, Sebastiao N. Martins-Filho, et al. 2019. "Organoid Cultures as Preclinical Models of Non-Small Cell Lung Cancer." *Clinical Cancer Research*, January. https://doi.org/10.1158/1078-0432.CCR-19-1376.

Siegel, Rebecca L., Kimberly D. Miller, and Ahmedin Jemal. 2019. "Cancer Statistics, 2019." *CA: A Cancer Journal for Clinicians* 69 (1): 7–34. https://doi.org/10.3322/caac.21551.

SOP30101: Recipes for Complete Media for Patient-Derived In Vitro and Organoid Cultures. :26.

SOP30103: Initial Culture, Sub-culture, and Cryopreservation of Adherent Patient-Derived Tumor Cultures (PDCs). :11.

Stockwin LH, Moyer J, Wade A, Bonomi C, Dougherty K, Carter J, Stottlemyer J, Arthur K, Datta V, Dutko L, et al. Establishing a platform for the generation of organoids from diverse tumor types as part of the NCI patient-derived models (PDM) initiative. :1.

Takahashi, Nobuhiko, Hirotaka Hoshi, Arisa Higa, Gen Hiyama, Hirosumi Tamura, Mayu Ogawa, Kosuke Takagi, et al. 2019. "An In Vitro System for Evaluating Molecular Targeted Drugs Using Lung Patient-Derived Tumor Organoids." *Cells* 8 (5): 481. https://doi.org/10.3390/cells8050481.

Tamura, Hirosumi, Arisa Higa, Hirotaka Hoshi, Gen Hiyama, Nobuhiko Takahashi, Masae Ryufuku, Gaku Morisawa, et al. 2018. "Evaluation of Anticancer Agents Using Patient-Derived Tumor Organoids Characteristically Similar to Source Tissues." *Oncology Reports*, June. https://doi.org/10.3892/or.2018.6501.

The American Cancer Society medical and editorial content team. n.d. "What Is Lung Cancer? | Types of Lung Cancer." Accessed April 27, 2020. https://www.cancer.org/cancer/lung-cancer/about/what-is.html.

van de Wetering M, Francies HE, Francis JM, Bounova G, Iorio F, Pronk A, van Houdt W, van Gorp J, Taylor-Weiner A, Kester L, et al. 2015. Resource: Prospective Derivation of a Living Organoid Biobank of Colorectal Cancer Patients. Cell. 161:933–945.

Vlachogiannis G, Hedayat S, Vatsiou A, Jamin Y, Fernández-Mateos J, Khan K, Lampis A, Eason K, Huntingford I, Burke R, et al. 2018. Patient-derived organoids model treatment response of metastatic gastrointestinal cancers. Science. 359(6378):920–926. doi:10.1126/science.aao2774. [accessed 2019 Apr 5]. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6112415/.

Xie, Tong, Lingzhao Mo, Li Li, Naiquan Mao, Danrong Li, Deseng Liu, Chuantian Zuo, et al. 2018a. “Identification of Side Population Cells in Human Lung Adenocarcinoma A549 Cell Line and Elucidation of the Underlying Roles in Lung Cancer.” *Oncology Letters*, February. https://doi.org/10.3892/ol.2018.7956.
. 2018b. “Identification of Side Population Cells in Human Lung Adenocarcinoma A549 Cell Line and Elucidation of the Underlying Roles in Lung Cancer.” *Oncology Letters*, February. https://doi.org/10.3892/ol.2018.7956.

Zhang, De-Geng, Ai-Gui Jiang, Hui-Yu Lu, Li-Xin Zhang, and Xiao-Yan Gao. 2015. “Isolation, Cultivation and Identification of Human Lung Adenocarcinoma Stem Cells.” *Oncology Letters* 9 (1): 47–54. https://doi.org/10.3892/ol.2014.2639.