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Multidimensional coculture system to model lung squamous carcinoma progression

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Corresponding Author:	Ken Geles, Ph.D. Pfizer Pearl River, NY UNITED STATES
Corresponding Author's Institution:	Pfizer
Corresponding Author E-Mail:	Ken.Geles@pfizer.com
Order of Authors:	Ken Geles, Ph.D.
	Shuang Chen
	Andreas Giannakou
	Jonathon Golas
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Kenneth Geles, Ph.D.
Senior Director, Targeted Therapeutics Discovery Unit
Oncology Research and Development
Pfizer Worldwide Research & Development
401 North Middletown Road, Building 200-4502B
Pearl River, NY 10965
Tel. +1 (845) 6028233
Ken.geles@pfizer.com

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Dear Editor:

I am pleased to submit the enclosed manuscript, "Multidimensional coculture system to model lung squamous carcinoma progression", for your consideration for publication in *JOVE*. The manuscript provides a detailed protocol for establishing a novel three-dimensional (3D) coculture system that enables mechanistic study of tumor-stromal interactions in regulation lung cancer progression. To our knowledge, this 3D model system represents the first reported evidence in lung squamous carcinoma that components in the tumor microenvironment could override cell intrinsic oncogenic changes in determining the tumor phenotype. This in vitro model system served as the foundation for those important discoveries and was recently published in *PNAS*[1].

The critical roles of tumor-stromal interaction in driving tumorigenesis have been increasingly appreciated in recent years. However, in vitro models that could faithfully recapitulate the complex and dynamic interactions between tumor and stromal microenvironment have not been readily forthcoming, especially for lung cancer. Therefore, several highlights of our protocol may appeal to your readership and production of video methods will benefit readers by visually detailing the complex aspects of performing these types of experiments. This includes:

- A novel system that captures the dynamic tissue architectural changes observed in human lung tumors in vivo as well as the complex interplay of tumor cells with key components in the tumor microenvironment.
- The system permits experimental manipulation on both the tumor cells and components in the TME to investigate the roles of diverse tumor cell-intrinsic and extrinsic changes during tumor progression with rich biological readouts.
- The system could be further adapted for monitoring the response of LUSC cells to drug treatment.

Together, we believe that our protocol bridges several gaps in the field and provides a valuable platform with broad application for LUSC research as well as the design of future therapies.

I have attached the abstract of the manuscript for your review. We hope you share our enthusiasm for this important work and look forward to your response.

Sincerely,

Kenneth G. Geles, Ph.D.

Reference

1 Chen, S. *et al.* Cancer-associated fibroblasts suppress SOX2-induced dysplasia in a lung squamous cancer coculture. *Proc Natl Acad Sci U S A*, doi:10.1073/pnas.1803718115 (2018).

TITLE:

Multidimensional Coculture System to Model Lung Squamous Carcinoma Progression

AUTHORS:

Shuang Chen[1], Andreas Giannakou[1], Jonathon Golas[1] and Kenneth G. Geles[1]

[1]Oncology R&D group, Pfizer Worldwide Research and Development, Pearl River, NY 10965

Corresponding Author:

Kenneth G. Geles (Ken.Geles@pfizer.com)

Email Addresses for Co-Authors:

Shuang Chen (Shuang.Chen5@pfizer.com)

Andreas Giannakou (Andreas.Giannakou@pfizer.com)

Jonathon Goals (Jonathan.Golas@pfizer.com)

KEYWORDS:

3D coculture, lung cancer, cancer-associated fibroblasts, acinar morphogenesis, tumor organoids

SUMMARY:

An in vitro model system was developed to capture tissue architectural changes during lung squamous carcinoma (LUSC) progression in a 3-dimensional (3D) co-culture with cancer-associated fibroblasts (CAFs). This organoid system provides a unique platform to investigate the roles of diverse tumor cell-intrinsic and extrinsic changes that modulate the tumor phenotype.

ABSTRACT:

Tumor–stroma interactions play a critical role in the development of lung squamous carcinoma (LUSC). However, understanding how these dynamic interactions contribute to tissue architectural changes observed during tumorigenesis remains challenging due to the lack of appropriate models. In this protocol, we describe the generation of a 3D coculture model using a LUSC primary cell culture known as TUM622. TUM622 cells were established from a LUSC patient-derived xenograft (PDX) and have the unique property to form acinar-like structures when seeded in a basement membrane matrix. We demonstrate that TUM622 acini in 3D coculture recapitulate key features of tissue architecture during LUSC progression as well as the dynamic interactions between LUSC cells and components of the tumor microenvironment (TME), including the extracellular matrix (ECM) and cancer-associated fibroblasts (CAFs). We further adapt our principal 3D culturing protocol to demonstrate how this system could be utilized for various downstream analyses. Overall, this organoid model creates a biologically rich and adaptable platform that enables one to gain insight into the cell-intrinsic and extrinsic mechanisms that promote the disruption of epithelial architectures during carcinoma progression and will aid the search for new therapeutic targets and diagnostic markers.

INTRODUCTION:

Lung cancer is the leading cause of cancer-related mortality worldwide. Lung squamous cell carcinoma (LUSC), which is the second most common type of non-small-cell lung cancer (NSCLC) and accounts for approximately 30% of all lung cancer, is often diagnosed at advanced stages and has a poor prognosis[1]. Treatment options for LUSC patients are a major unmet need that can be improved by a better understanding of the underlying cellular and molecular mechanisms that drive LUSC tumorigenesis.

As with most human cancers, the pathogenesis of LUSC is characterized by the disruption of the intact, well-ordered epithelial tissue architecture[2]. During this process, proper apical-basal cell polarity, cell-cell and cell-matrix contacts are lost, permitting uncontrolled growth and invasive behavior of the tumor cells. It is now widely appreciated that the malignant features of cancer cells cannot be manifested without an important interplay between cancer cells and their local tumor microenvironment (TME)[3]. Key components in the TME including extracellular matrix (ECM), cancer-associated fibroblasts (CAFs) as well as endothelial cells and infiltrating immune cells actively shape the TME and drives tumorigenesis[4]. Nevertheless, our current understanding of how the tumor cells and these key components in the TME interact to drive tissue architectural changes during LUSC progression is very limited.

Three-dimensional (3D) culture is an important tool to study the biological activities of cell-intrinsic and extrinsic changes in regulating tissue architectural changes in both normal and diseased tissues[5]. 3D cultures provide the appropriate structural and functional context that is usually lacking in traditional two-dimensional (2D) cultures. The added dimensions of such systems more closely mimic tissue in vivo in many aspects of cell physiology and cellular behaviors, including proliferation, differentiation, migration, protein expression and response to drug treatment. In recent years, efforts from various labs have led to the development of in vitro 3D models for both the normal lung as well as NSCLC[6-8]. However, a model for lung squamous carcinoma that can recapitulate both the dynamic tissue architectural changes during tumorigenesis as well as incorporate key stromal components was unavailable.

Here, we describe the methods for establishing a novel 3-dimensional (3D) coculture system using primary PDX-derived LUSC cells (termed TUM622) and CAFs[9,10]. Both TUM622 and CAFs are derived from NSCLC patient with poorly differentiated tumors[10]. When embedded as single cells in ECM, a rare subpopulation of TUM622 cells have the capacity to form organoids with acinar-like structures that display proper apical-basal cell polarity. These acinar-like structures are hyperplastic, display heterogeneous expression of stem-like and differentiation markers similar to the original tumor while remaining non-invasive, and thus mimic the earliest stage of LUSC development. Importantly, we showed that the tissue architecture of the acinar-like structures could be altered by inhibition of cell-intrinsic signaling pathways with small molecule inhibitors or addition of key components in the ECM such as CAFs, the latter of which enhances acini formation and further provokes the acini to become invasive when in close proximity. Together, these data suggest that this 3D co-culture system of LUSC organoids provides a valuable platform for the investigation of the dynamic reciprocity between LUSC cells and the TME and could be adapted for monitoring the response of LUSC cells to drug treatment[11].

PROTOCOL:

1. Passaging and culturing TUM622 cells and CAFs in 2D cultures

1.1) Passaging and culturing TUM622 cells

1.1.1) Warm 3D culture medium and cell dissociation reagents (see **Table of Materials**) for TUM622 cells at 37 °C.

1.1.2) Passage TUM622 cells at 80% confluency in 2D flasks. Usually, this occurs 1 week after passaging.

1.1.3) Discard old medium from a T75 flask and wash once with 6 mL of HEPES buffer. Avoid pipetting directly onto the cells.

1.1.4) Aspirate the HEPES buffer. Add 4 mL of trypsin/EDTA (0.25 mg/mL, see **Table of Materials**) for a quick rinse and discard the trypsin/EDTA.

1.1.5) Add 2 mL of trypsin/EDTA and incubate at 37 °C for 5 min. Remove flasks from the incubator and tap the flasks to loosen the cells without creating air bubbles and return flasks to the incubator for an additional 5 min.

NOTE: Prolonged exposure to trypsin will irreversibly damage the cells and alter their phenotype, thus it is recommended to limit the time cells are exposed to trypsin.

1.1.6) Confirm cells have detached and dissociated under a light microscope (4x or 10x). Add 4 mL of neutralization buffer (TNS buffer) (see subculture reagent information in the **Table of Materials**) followed by 10 mL of 3D culture medium (see **Table of Material**).

1.1.7) Pipette up-and-down gently to further dissociate the cells using a 10 mL pipette. Transfer the suspension through a 40 µm cell strainer into a 50 mL conical tube.

1.1.8) Count cell numbers using a hemocytometer or automated cell counter.

1.1.9) Seed 0.8 x 10^6 cells/T75 flask in 20 mL of 3D culture medium (see **Table of Materials**).

1.1.10) Feed the cells every other day by replacing half of the spent medium with fresh medium.

1.2) Passaging and culturing CAFs

1.2.1) Passage CAFs when cells reach confluency. Usually, this occurs after 5 days of culturing from a 1:2 split.

1.2.2) Prepare CAF medium using RPMI basal medium with 20% heat-inactivated fetal bovine serum, 1% L-Glutamine and 1% Penicillin/Streptomycin. Warm the medium to 37 °C.

1.2.3) In a T75 flask, rinse CAFs with phosphate-buffered saline (PBS) once then add 2 mL of trypsin/EDTA and incubate at 37 °C for 5 min.

1.2.4) Observe under a light microscope to ensure cells have dissociated in the flask (4X or 10X). If not, extend the incubation for another 2-3 min.

1.2.5) Once cells have detached and dissociated, add 10 mL of 3D culture medium to neutralize the trypsin/EDTA and pipette up and down several times to further dissociate the CAFs.

1.2.6) Transfer the cell suspension into a 50 mL conical tube and spin down at 300 x *g* for 5 min at room temperature.

1.2.7) Discard the supernatant and resuspend the pellet in an appropriate volume of 3D culture medium (see **Table of Materials**) and passage into two new T75 flasks.

2. Plating TUM622 cells in the extracellular matrix for 3D culturing

2.1) The day before the experiment, thaw vials of basement membrane matrix in a 4°C refrigerator overnight. Cooldown plastic pipettes (2 mL) and tips at -20 °C overnight.

NOTE: Not all lots of basement membrane matrix have the same capacity to support the 3D growth of TUM622 cells. Therefore, it is necessary to acquire and test multiple lots of basement membrane matrix to identify those that support robust acini formation. Usually, this requires a higher protein concentration (16–18 mg/mL) in the matrix.

2.2) On the day of the experiment, warm 3D culture medium, HEPES buffer, trypsin/EDTA and trypsin neutralization buffer (TNS) in a 37 °C water bath. Immediately before setting up the culture, take the thawed basement membrane matrix out of the fridge and put the vial on ice.

2.3) Cooldown the tissue culture plates on a metal platform cooler placed on ice. Place centrifuge tubes on a metal cooling rack on ice.

2.4) Using TUM622 cells obtained from step 1.1.7, calculate the desired number of cells needed for plating. Typically, 15,000–30,0000 cells are needed per well of a 24-well plate. Lower density is more suited for imaging and quantification, while higher density is preferred when collecting cells for RNA extraction or western blotting.

2.5) Transfer cell suspension into a cooled centrifuge tube (each tube containing cells for triplicate plating) and spin down at 300 x *g* in a hanging bucket centrifuge at 4 °C for 5 min.

2.6) Aspirate the supernatant carefully with an aspirating pipette attached to an unfiltered tip (20 µL), leaving approximately 100 µL of the medium in the tube (use markings on the tube as a guide).

2.7) Gently tap on the side of the tube to dislodge and dissociate the pellet before returning it to the cooling rack.

2.8) Using the 2 mL pre-cooled pipettes, gently mix the matrix by pipetting up and down a few times while keeping the vial in contact with the ice. Pipette at an even and moderate speed so that no bubbles are introduced into the matrix during this procedure.

2.9) Transfer the appropriate volume of the matrix into each centrifuge tube. For plating triplicates in a 24-well plate, add 1.1 mL of basement membrane matrix to each tube.

2.10) Using pre-cooled tips, pipette the matrix in each tube up and down about 10 times to make a uniform cell suspension.

2.11) Transfer 310 µL of cell/matrix suspension into each well of a pre-cooled 24-well plate. The pipette is placed at a 90° angle to the plate surface and the suspension added to the center of the well. The suspension should spread and cover the entire well without needing to tilt the plate.

2.12) To facilitate downstream immunofluorescence analysis, plate the cell/matrix suspension in parallel into 2-well chamber slides. Transfer 100 µL of cell/matrix suspension into the center of a well of 2-well chamber slide (see **Table of Materials**). This allows the matrix to form a dome-like structure with much smaller volume.

2.13) Return the plate and the chamber slide back into a tissue culture incubator and incubate for 30 min to allow the matrix to solidify. Examine the plate/slide under a light microscope to ensure that single cells are evenly distributed within the matrix (4x or 10x).

2.14) Add 1 mL of pre-warmed 3D culture complete medium into each well and 1.5 mL of 3D culture medium to each well of the chamber slide then return them to the incubator.

3. 3D coculturing of TUM622 cells and CAFs in the extracellular matrix

3.1) Prepare cell suspensions of TUM622 and CAFs according to section 2.

3.2) Count the CAF cell density by taking 10 µL of cell suspension and mixing it with 10 µL of trypan blue.

3.3) Add 10 µL of the mixture to each of the two chambers on a hemacytometer to count and calculate cell density.

NOTE: CAFs have irregular shapes and may not be accurately counted on an automatic cell counter.

3.4) Co-embedding TUM622 cells and CAFs in basement membrane matrix

3.4.1) Based on the cell density information, calculate the desired number of cells used for plating. CAFs are seeded at a 2:1 ratio of TUM622 cells. For example, for 30,000 TUM622 cells seeded, 60,000 CAFs are co-embedded.

3.4.2) Transfer the appropriate volume of TUM622 as well as CAFs cell suspension into the same centrifuge tube and follow steps 2.5–2.11 for plating into 24-well plates. For immunofluorescence, transfer 60 µL of TUM622/CAFs mix to chamber slides as described in step 2.12).

3.5) Coculturing TUM622 with overlaid CAFs in basement membrane matrix (see **Table of Materials**)

3.5.1) Set up TUM622 mono-culture according to steps 2.5–2.13.

3.5.2) Transfer twice the number of CAFs suspension (compared to the number of TUM622 cells seeded) into a centrifuge tube and spin down at 300 x *g* for 5 min at room temperature.

3.5.3) Aspirate the supernatant and resuspend the CAFs in 1 mL of 3D culture medium.

3.5.4) Transfer the 1 mL of CAFs suspension to the well containing the embedded TUM622 cells.

4. Harvesting TUM622 acini for RNA/protein extraction and fluorescence-activated cell sorting (FACS)

4.1) Prepare wash buffer and cell harvesting buffer according to the 3D cell harvesting kit protocol the previous day and chill overnight at 4 °C.

4.2) Keep plates on a plate cooler and other reagents on ice before starting the extraction process.

4.3) Aspirate media from 3D culture wells without touching the matrix and gently wash the well 3 times with 1 mL of wash buffer.

4.4) Aspirate the final wash and add 1 mL of cell harvesting buffer to each well.

4.5) Use a p1000 pipette tip to scrape the matrix off of each well.

4.6) Pipette up and down to further dissociate the matrix.

4.7) Transfer 1 mL of the mix to a pre-chilled 15 mL conical tube. Add another 1 mL of harvesting buffer to the same well.

4.8) Repeat steps 4.5–4.7, and transfer all mix of the same well into one 15 mL conical tube.

4.9) Cap the tubes and rock at 4 °C for 30 min.

4.10) Fill each tube with ice-cold PBS up to 10 mL and then centrifuge at 300 x *g* for 5 min at 4 °C.

4.11) Aspirate the supernatant without touching the pellet. The supernatant should contain matrix fragments, but the spheroids should all be collected at the bottom of the tube.

4.12) Add ice-cold PBS for a second wash. Invert the tube a few times to dissociate the pellet. Spin down at 300 x *g* for 5 min.

4.13) While spinning, prepare lysis buffer for protein and RNA collection.

4.14) Carefully aspirate the supernatant and add lysis buffer for downstream processing to collect protein or RNA. Alternatively, cells could be resuspended for flow analysis/FACS sorting or serial passaging.

5. Immunofluorescence of TUM622 acini

5.1) Prepare immunofluorescence buffer (IF buffer: PBS with 0.1% bovine serum albumin (BSA), 0.2% Triton X-100 and 0.05% Tween-20), primary blocking buffer (IF buffer with 10% goat serum), secondary blocking buffer (primary blocking buffer with 20 µg/mL goat anti-mouse $F(ab')_2$)

5.2) Aspirate medium from 2-well chamber slides, rinse once with PBS and set the slide on metal plate cooler on ice. The chamber slide should remain on the metal plate cooler for the remainder of the protocol.

5.3) Add pre-chilled 4% PFA to fix the acini and incubate on ice for 20 min.

5.4) Remove 4% PFA and wash three times with 2 mL of pre-chilled PBS each for 5 min with gentle rocking on a rocker.

5.5) Aspirate PBS and permeabilize with 1.5 mL of 0.5% Triton X-100 in PBS (pre-chilled) for 20 min. By the end of this procedure, the dome-like structure will become loose.

5.6) Gently aspirate the permeabilization buffer from the chamber slide to avoid sample loss. This is achieved by adding a fine tip (20 µL) to the aspirating pipette and pressing the tip towards the corner of the chamber.

5.7) Wash three times with 2 mL of pre-chilled PBS each for 5 min with gentle rocking on a rocker.

5.8) Block the sample with the primary blocking buffer on ice for 1 h.

5.9) Remove primary blocking buffer and add secondary blocking buffer and block for 30 min.

5.10) Add primary antibodies in primary blocking buffer and incubate overnight at 4 °C.

NOTE: The concentration of the antibodies used here should be higher than normally used for staining cells in 2D culture. Most of the primary antibodies used in this study are diluted at 1:100 dilution (see **Tables of Materials**).

5.11) Remove primary antibodies and wash the sample 3 times with 2 mL of cold IF buffer.

NOTE: The samples could be loose, take extra caution when aspirating.

5.12) Incubate the samples in secondary antibodies diluted in primary blocking buffer for 1 h at RT. The preferred secondary antibodies should be highly cross-adsorbed to reduce background staining. Most secondary antibodies used in this study are diluted at 1:200 dilution.

5.13) Remove secondary antibodies and wash the sample 3 times with 2 mL of cold IF buffer.

NOTE: The samples could be loose, take extra caution when aspirating.

5.14) Add PBS with DAPI (1:1000 dilution) during the last wash to stain the nucleus. The perform another 2 washes in PBS.

5.15) Image the samples on a confocal microscope within 3 days.

NOTE: Due to the size of the organoids and limits in the objective's working distance, samples are usually imaged at 10x or 20x magnification.

6. Preparing 3D culture samples for immunohistochemistry

6.1) Aspirate medium from 2-well chamber slides and rinse once with PBS.

6.2) Fix 3D cultures in 4% PFA at 37 °C overnight.

6.2) Remove 4% PFA, surround cultures with 2.5 mL of histology sample gel (see **Table of Materials**) and place the slide at 4 °C to solidify for at least 1 h.

6.3) Transfer samples surrounded with histological sample gel to tissue cassettes and processed in an automated tissue sample processor overnight.

6.4) Embed samples in paraffin wax and prepare for sectioning[12].

7. 3D cytotoxicity assay for compound screening (example for one 96-well plate)

7.1) Set a 96-well plate on a plate cooler, a 25 mL reservoir on a reservoir cooler and a 15 mL conical tubes on ice before starting the experiment.

7.2) Prepare cell matrix suspensions of TUM622 cells in a pre-chilled 15 mL polypropylene conical tube by adding the appropriate volume of basement membrane matrix to cells. The desired density for TUM622 cells is 10,000 cells per 70–75 µL of basement membrane matrix. Pipette up and down a few times to allow even mixing of cells within the matrix.

7.2) Move the plate with plate cooler and reservoir with a reservoir cooler away from the ice to a dry surface to avoid contact of basement membrane matrix with ice during transfer.

7.3) Transfer the matrix cell mixture to the cooling reservoir without creating bubbles.

7.4) Using a mechanical multichannel pipette (10–300 µL), transfer 70–75 µL of the mix cells into each appropriate well of a 96-well plate.

7.6) Incubate plate at 37 °C and 5% CO_2 for 30 min for the basement membrane matrix to solidify.

7.7) Add 100 µL of media in all rows and return the plate to the incubator.

7.8) Start compound dosing the next day or later depending on the goal of the experiment.

7.9) Spheroids can be re-fed and re-dosed every 2–3 days for up to 10 days, by removing spent media with an 8- or 12-well vacuum manifold and replacing with fresh media with or without desired compounds.

7.10) The number of TUM622 spheroids could be quantified using 3D imager according to the manufacturer's protocol.

REPRESENTATIVE RESULTS:

TUM622 and CAFs in 2D culture

Figure 1 presents the typical morphology of TUM622 cells and CAFs in 2D culture. TUM622 cells are rounded with large nuclei while CAFs are flat and elongated. TUM622 cells can reach 80%–90% confluency in culture. Further proliferation leads to more, but smaller cells aggregated in colonies that do not come into direct contact. In contrast, CAFs prefer to grow at higher cell density and will keep proliferating at full confluency if sufficient nutrients are provided.

Growth and morphology of TUM622 acini in 3D ECM

Figure 2 presents a time-course experiment of TUM622 cells seeded in 3D culture. Data show that single TUM622 cells are capable of forming organoids with acinar-like morphologies when

embedded. Between days 5 and 7, a lumen becomes apparent in the acinar-like structures and remains hollow thereafter (**Figure 2A**). Each acinus, composed of a monolayer of cells surrounding the hollow lumen, displays proper apical-basal polarity similar to that of lung epithelium in vivo (**Figure 2B**). These acinar-like structures are hyperplastic and continue to grow as long as sufficient nutrients are provided. The culture can be maintained for up to 24 days before the ECM completely disintegrates (**Figure 2C**). Through limiting dilution assay (LDA) (data not shown), it is estimated that only a rare subpopulation of TUM622 cells (<0.02%) have the capacity to form acinar-like structures[9].

Growth and morphology of TUM622-CAFs coculture

Figure 3 depicts the setup and representative results of TUM622-CAF cocultures. CAFs could be integrated into the coculture by either overlaying on top of the matrix or co-embedded with the TUM622 cells. Regardless of the setup, the presence of CAFs greatly enhanced the number and size of the spheroids formed (**Figure 3B**). Interestingly, when TUM622 acini come into close proximity with CAFs, they induce the acini to become invasive and migrate towards the CAFs, forming "tear-drop" like structures (**Figure 3C**). Note that TUM622 acini in monoculture do not display invasive behavior and only form “tear-drop” like structures when close to CAFs.

Representative immunofluorescent and immunohistochemistry staining results

Figure 4 shows representative results from immunofluorescence and immunohistochemistry staining of TUM622 acini after 10 days of culturing. Confocal images were taken at the equatorial plane of immunofluorescently stained TUM622 acini (**Figure 4A**). In contrast, each section from the immunohistochemistry sample may capture acini at different planes (**Figure 4B**). Both results showed heterogeneous expression of stem-like and differentiated cells within each acinus.

TUM622 3D cytotoxicity assay using a Wnt pathway inhibitor

Figure 5 shows the dose-response of TUM622 acini treated with XAV939, a tankyrase inhibitor (**Figure 5A,B**). XAV939 was added to the culture 1 day after plating and refreshed every 2 days for a total of 10 days. At the end of the experiment, the number of acini was quantified by an imager. Brightfield images at higher magnification were also acquired to capture the morphology of spheroids in control versus XAV939-treated wells (**Figure 5C**). Overall, XAV939 displays dose-dependent inhibition on acini formation and alters the tissue architecture of the spheroids formed. These results suggest that activation of the canonical Wnt pathway is required during TUM622 acinar morphogenesis.

FIGURES LEGENDS:

Figure 1: **TUM622 and CAFs in 2D culture.** Representative bright-field images of TUM622 cells and CAFs cultured in 2D. Scale bar = 100 µm. This figure has been modified from Chen et al.[9] and used with permission.

Figure 2: Growth and morphology of TUM622 acini in 3D ECM. (**A**) Time course images of TUM622 cells cultured in basement membrane matrix over a 10-day period. Scale bar = 100 µm. (**B**) Immunofluorescence of TUM622 acini stained with apical-basal cell polarity markers, Golgi-

enzyme (GM-130, green, apical) and Integrin alpha 6 (CD49f, basal, red). (**C**) Quantification of acini number (right y-axis, red) and the average size of acini (left y-axis, blue) plated in triplicate in a 24-well plate over 24 days in culture. Error bars represent SD. This figure has been modified from Chen et al.[9] and used with permission.

Figure 3: Growth and morphology of TUM622-CAFs coculture. (**A**) Schematic drawing of the setup of TUM622-CAFs coculture. CAFs are overlaid or co-embedded with TUM62 cells in ECM. After 6–12 days in coculture, TUM622 cells are able to form more and larger acini compared with mono-culture and invade the ECM when in close proximity and direct contact with CAFs. Note that the invasive phenotype could only be observed in the co-culture. (**B**) Brightfield image of TUM622 3D cultures in the presence or absence of overlaid CAFs after 8 days. Scale bars = 200 µm. (**C**) Brightfield images showing tear-drop shaped acini forming in cocultures regardless of CAFs are overlaid or co-embedded in the ECM. Scale bars = 200 µm. This figure has been modified from Chen et al.[9] and used with permission.

Figure 4: Representative immunofluorescent and immunohistochemistry staining results. (**A**) Antibody staining of acini with markers of stem/progenitor cells (CXCR4 and SOX2), mesenchyme (Vimentin), epithelial differentiation (Involucrin), apoptosis (Cleaved-Caspase-3) and proliferation (Ki67) in green, DAPI in blue, E-cadherin and Phalloidin in red. Scale bar = 50 µm. (**B**) Immunohistochemistry on FFPE sections of TUM622 acini. Scale bar = 100 µm (top) and 50 µm (bottom). This figure has been modified from Chen et al.[9] and used with permission.

Figure 5: TUM622 3D cytotoxicity assay using a Wnt pathway inhibitor. (**A**) Quantification of spheroid numbers in a 96-well plate where TUM622 cells were treated with dimethyl sulfoxide (Control) or XAV939. Each condition is assayed in triplicates. Error bars represent SD. (**B**) Whole well images from a 24-well plate taken with an imager showing the inhibitory effects of XAV939 on acini formation. (**C**) Representative brightfield images from the control vs. treated wells demonstrating the morphological changes caused by XAV939 treatment. Scale bars = 100 µm. This figure has been modified from Chen et al.[9] and used with permission.

DISCUSSION:

Tumors are heterogeneous tissues composed of cancer cells coexisting side-by-side with stromal cells such as cancer-associated fibroblasts, endothelial cells and immune cells within the ECM. Together, these diverse components cross-talk and influence the tumor microenvironment, playing an active role in driving tumorigenesis, a process that involves progressive changes in tumor architecture. Ideally, an in vitro model of tumor development should be able to capture the dynamic tissue architectural changes observed in human tumors in vivo, the complex interplay of diverse cell types within the tumor microenvironment and at the same time permit experimental manipulation on both the tumor cells and components in the TME. Although much progress has been made in 3D cancer models in recent years, such models have not been readily forthcoming for LUSC. Most models reported to date only incorporate a few aspects of these important features. Here we report the methods for a 3D coculture system of LUSC that simultaneously captures key tissue architectural changes observed during LUSC development as

well as dynamic interactions between tumor cells and major components of the TME, including the ECM and CAFs.

The ability of this system to more accurately model tissue architectural changes is based on the unique property of TUM622 cells in forming organoids with acinar-like morphologies when embedded in 3D EC. Formed from a self-renewing single cell, each acinus is composed of a monolayer of cells surrounding a hollow lumen. This monolayer of cells exhibits apical-basal cell polarity and remains non-invasive, resembling the tissue architecture of the lung epithelium. While TUM622 as a 3D mono-culture display hyperplastic growth, the addition of CAFs further enhances acinar morphogenesis and induces more and larger acini to form. Importantly, CAFs invoke dynamic tissue architectural changes in TUM622 cells when the two cell types come into close proximity, allowing the TUM622 cells to lose their apical-basal polarity and invade the matrix toward the CAFs. These phenotypic changes recapitulate both early hyperplasia as well as late invasive stages of LUSC.

Unlike many tumor spheroid models where each spheroid is formed by aggregation of many cells, each TUM622 acinus is derived from a single cell[9]. By in vitro LDA, it is estimated that only a minor subpopulation (≤0.02%) of TUM622 cells have such capacity[9]. Although rare, these cells could self-renew as evidenced by their capacity to undergo serial passaging in 3D as well as differentiate into a heterogeneous population of cells similar to that of the original tumor. Due to this unique feature of TUM622 cells, it is critical to ensure even distribution of single TUM622 cells within the ECM at the time of plating for successful culturing and downstream analysis. To achieve this goal, several key points need to be followed carefully in the protocol, including the determination of appropriate seeding density, keeping all tools and reagents cool during the mixing of cells and matrix to prevent premature solidification, avoiding the introduction of bubbles during the mixing process and allowing sufficient time for matrix to fully solidify before adding culture medium. Together, these precautions will help to achieve a more uniform matrix substrate and culture condition for all embedded cells.

Once successfully established, this culture can be used for a variety of downstream analyses to dissect the cell and biochemical process that regulate tumorigenesis. The number and size of acini formed in each well can be monitored over time with bright field imagers and used as a readout for the proliferative and self-renewal capacity of TUM622 cells. More detailed dynamics in the morphogenesis of each acinus could be observed with live-imaging on a confocal microscope, with or without various labeling dyes. The conditioned medium can be collected at multiple time points during the culture period for analyzing soluble factors that may mediate cell-cell or cell-matrix cross-talks. TUM622 cells extracted directly from the ECM using protocol 4 are suitable for RNA and protein extraction for gene expression analysis, flow cytometry quantification or FACS sorting based on cell surface markers. Alternatively, the cultures could be fixed for in situ immunofluorescence or immunohistochemistry studies to understand the spatial-temporal distributions of various markers. Although similar, immunohistochemistry complements immunofluorescence methods in that it allows the sampling of entire acini that may not be possible due to the limiting imaging-depth of the confocal objectives. For both of these methods, the time and temperature at which fixation and permeabilization are performed

are critical, especially given that TUM622 cells are embedded in a dense matrix (>90% basement membrane matrix) in contrast to many other 3D cultures where matrix density is much lower. Therefore, attention to standardized and consistent fixation and processing is necessary to obtain replicative results.

Using this system as a platform, one can then investigate how cell-intrinsic changes in the tumor cells, as well as cell-extrinsic changes in the tumor microenvironment, influence epithelial architecture and model early events involved in carcinoma formation. For example, the roles of oncogenes or tumor suppressor genes in regulating tumor tissue architecture could be studied by gain- or loss-of-function experiment targeting the gene of interest in the tumor cells. Indeed, we demonstrated that over-expression of SOX2, which is commonly observed in LUSC, alters the phenotype of TUM622 cells as evidenced by a loss of hyperplasia in 3D and progression towards dysplastic growth[9]. On the other hand, one could compare normal versus cancer-associated fibroblasts in coculture settings, determine how matrix components or its stiffness impact acini growth/morphology/invasion, and if blocking certain cytokines could interfere with cell-cell communication and in turn affect tissue architecture and tumor progression. Importantly, all these assays could be performed in the presence or absence of certain therapeutic agents and be used as a tool to determine the drug response of LUSC cells with a multidimensional readout[11]. It is also important to note that this system is limited in regards to the pathways it could be used to interrogate, as only some but not all major signaling pathways regulate the growth and morphology of TUM622 organoids in culture (i.e., inhibition of Wnt but not Notch signaling affects acinar mophogenesis of TUM622 cells)[9].

In summary, we demonstrate that this organoid system provides a unique platform for generating new insights into the dynamic interplay between LUSC cells and the tumor microenvironment during tumor progression. We anticipate that our model system will be a valuable platform for drug discovery and development. In this respect, screening novel anti-cancer therapeutics in a native tumor tissue context should aid in the selection and development of more effective therapeutics targeting LUSC.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors are employees and shareholders of Pfizer Inc.

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Figure 1

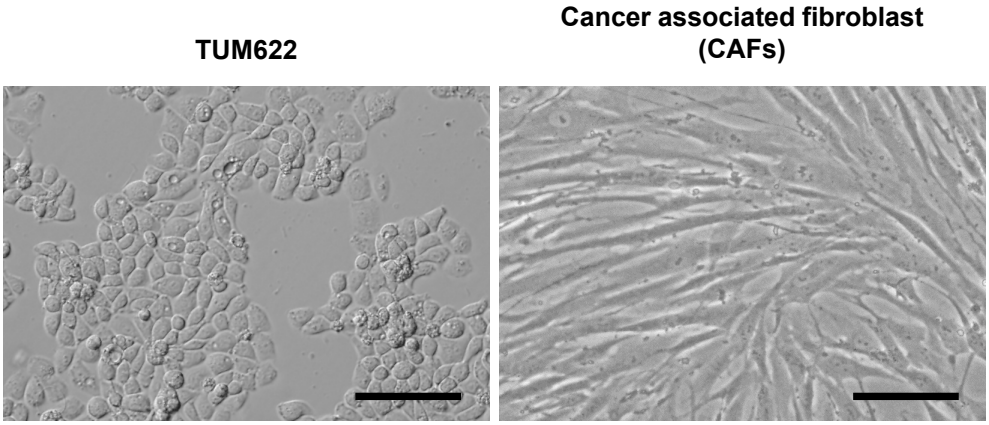


Figure 2

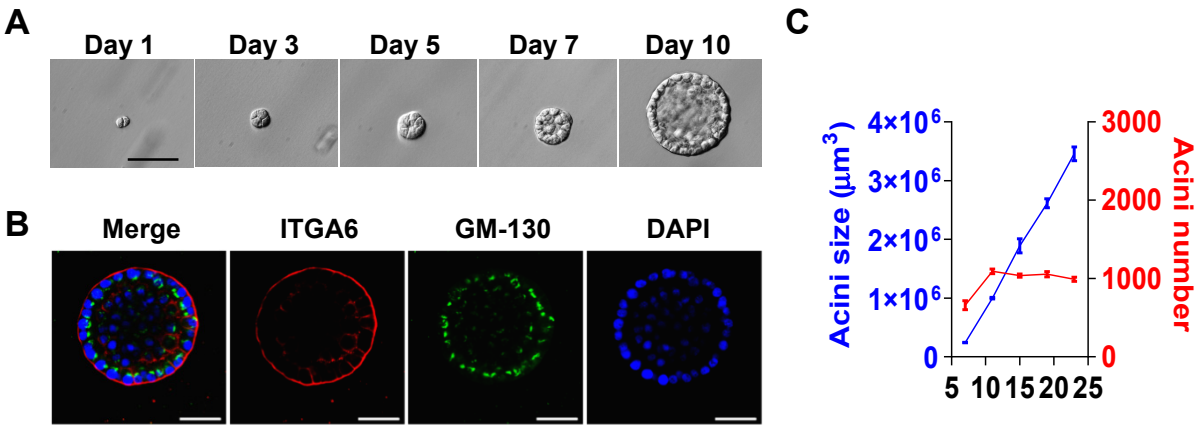
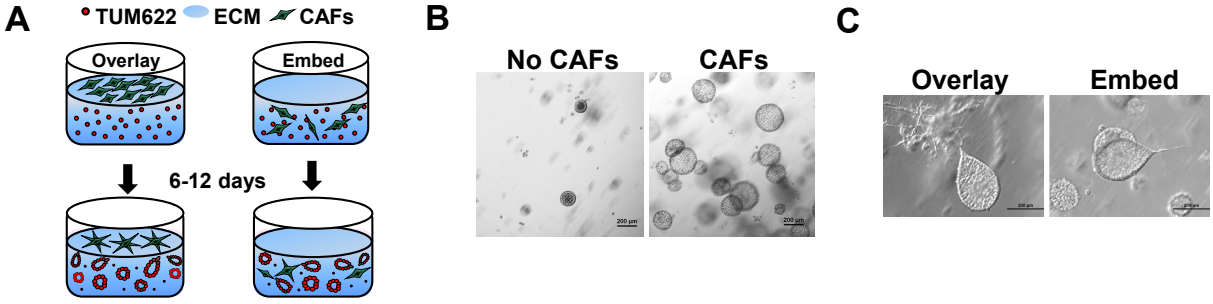


Figure 3



Figure

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Figure 4

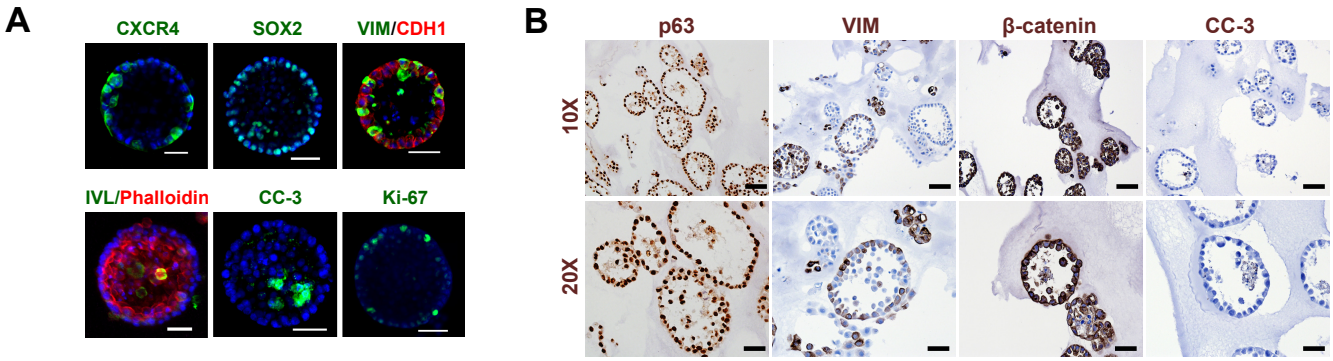

Figure 5

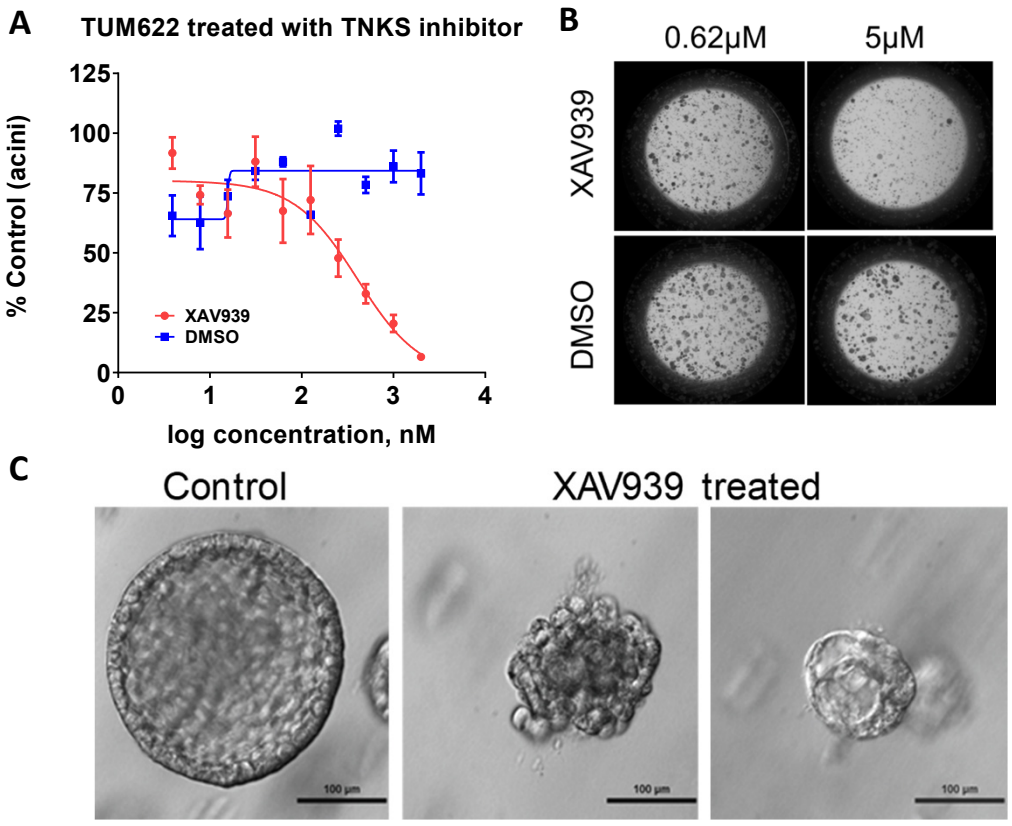

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials (1).xlsx

Names	Company	Cat. No.
Bronchial Epithelial Growth Medium	Lonza	CC-3170
Cell Strainer 40um	ThermoFisher	352340
Cleaved Caspase 3 antibody	Cell Signaling Technology	9661 (RRID:AB_2341188)
CoolRack CFT30	Biocision	BCS-138
CoolSink XT96F	Biocision	BCS-536
Cultrex 3D Cell Harvesting Kit	Bio-Techne	3448-020-K
Cultrex (preferred for co-culture)	Bio-Techne	3443-005-01
CXCR4 antibody	Abcam	Ab124824 (RRID:AB_10975635)
E-cadherin antibody	BD Biosciences	610182 (RRID:AB_397581)
GelCount	Oxford Optronix	
GM130 antibody	BD Biosciences	610822 (RRID:AB_398141)
Goat Serum	Vector Labs	S1000 (RRID:AB_2336615)
Heat-inactivated FBS	Gibco	10082-147
Histology sample gel	Richard Allan Scientific	HG-4000-012
Integrin alpha 6 antibody	Millipore Sigma	Mab1378 (RRID:AB_2128317)
Involucrin antibody	Abcam	Ab68 (RRID:AB_305656)
Ki67 antibody	Abcam	Ab15580 (RRID:AB_443209)
Lab-Tec II chambered #1.5 German Coverglass System	Nalge Nunc International	155379 (2)
Lab-Tec II chambered #1.5 German Coverglass System	Nalge Nunc International	155409(8)
L-Glutamine	Gibco	25030-081
Matrigel (preferred for mono-culture)	Corning	356231
p63 antibody	Cell Signaling Technology	13109 (SRRID:AB_2637091)
Pen/Strep	Gibco	15140-122
ReagentPack Subculture Reagents	Lonza	CC-5034
RPMI	ThermoFisher	11875-093

Sox2 antibody	Cell Signaling Technology	3579 (RRID:AB_2195767)
TrypLE Express	Gibco	12604-021
Vi-Cell	Bechman Coulter	
Vimentin antibody	Abcam	Ab92547 (RRID:AB_10562134)
β-catenin antibody	Cell Signaling Technology	2677s (RRID:AB_1030943)

Abbreviations & Comments
BEGM
For passing TUM622 cells
Rabbit
For 3D culture
For 3D culture
For 3D culture
Rabbit
Mouse
For Acini counts and measurements
Mouse
For Immunofluorescence
For CAFs
For Immunofluorescence
Rat
Mouse
Rabbit
For 3D culture
For 3D culture
For CAFs
For 3D culture
Rabbit
For CAFs
For TUM622 cell dissociation
For CAFs

Rabbit
For CAF dissociation
Automatic cell counter
Rabbit
Mouse

Dear Editor,

Thank you for the opportunity to respond to the reviewer's questions and improve our manuscript. We appreciate the reviewers' insightful comments which helped us to further clarify key points of the protocols. As recommended, we have addressed each one of the review's concerns and incorporated necessary changes to the protocol accordingly. A few of the comments suggested more in depth understanding of the underlying biological mechanisms of the co-culture system which are very interesting but nonetheless beyond the scope of this manuscript. For these questions, we have cited relevant data from our previous publication in PNAS and will be following up in future research. Please see below for our detailed responses that are highlighted in blue. We look forward to your decision.

Respectfully,

Kenneth Geles

Editorial comments:
You will find Editorial comments and Peer-Review comments listed below. Please read this entire email before making edits to your manuscript.
NOTE: Please include a line-by-line response to each of the editorial and reviewer comments in the form of a letter along with the resubmission.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

• **Protocol Language:** Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
1) 1.1,1.2: Add a step to describe culture conditions, media used etc. Corrected
2) 1.1.2: Should this step appear before 1.1.1? please ensure a logical chronological sequence as much as possible. Corrected
3) 1.1.6.: magnification? Define BEGM Corrected
4) 1.1.7: mention tip size Corrected
5) 1.2.4: magnification? Corrected
6) 1.2.5: which medium? Corrected
7) 2.13: Magnification? Corrected

8) 5.8, 5.9,5.11: What is the composition of primary blocking buffer, secondary blocking buffer, IF buffer? define IF. Defined in 5.1
9) 5.10, 5.12: mention antibodies used, and their concentrations. Any antibody could be used according to the user's interest. The antibodies we used are listed in material and reagents and are now referred to in the text.
10) 5.15: mention imaging settings including magnification, lens NA, excitation and emission settings etc. Imaging magnification information added. Lens NA, excitation and emission depends on users imaging setup and choice of antibody.
11) 6.4: cite a reference for embedding. Reference added.

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps. Steps highlighted in yellow.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

• **Figures:** Please expand the legends to adequately describe the figures/tables. Each figure or table must have an accompanying legend including a short title, followed by a short description of each panel and/or a general description. Short title added to figure legend.

• **Commercial Language:**JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Lonza BEGM bullet kit, Lonza ReagentPack, ReagentPack, (Vi-Cell, TrypLE Express, Matrigel, Eppendorf, Cultrex, Histogel Specimen Processing Gel (Richard Allan, Tissue-Tek VIP processor (Sakura), GelCount TM
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

Commercial sounding language replaced with generic names.

• **Table of Materials:** Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as cell lines, antibodies with RRIDs.

File converted to excel sheet and now includes antibodies with RRIDs.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you

must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Editorial policies of previous publisher uploaded and citation added to figure legends.

Comments from Peer-Reviewers:

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
In the manuscript entitled "Multidimensional coculture system to model lung squamous carcinoma progression," Dr. Geles et al describe a method to coculture lung squamous carcinoma cells (TUM622) in an organotypic system with the ability to introduce and interrogate the effects of the tumor microenvironment. The Reviewer appreciates the need to design easily accessible and reproducible models to study the basis of this subtype of non-small-cell-lung cancer and can envision the effect of such a platform being utilized in other diseases. Furthermore, the protocol is well-written and allows for the broad range of applications. However, there are a few concerns that the Reviewer feels are important to address.

Major Concerns:
1. Although CAFs are an important source of cell-cell communication, it is important to also seed a biologically relevant number of CAFs in a coculture system to produce a meaningful result that represents what is seen in patients. Is the 2:1 ratio of CAFs to TUM622 cells an accurate representation of the disease?

We thank the reviewer for this important comment. Based on data from the literature and our own, the stroma: tumor ratio varies widely among the LUSC patients as well as in different regions of the same tumor. Therefore, we believe there is not a single ratio between the CAFs and tumor cells that could accurately represent the entire patient population. Our ratio of 2:1 (CAFs: cancer cell) is within range of previously established co-culture assays for NSCLC as well as in vivo observations in patients, which ranges anywhere from 1:1 to 10:1 (1-3). We have also experimented with a ratio of 1:1 and 5:1. The general observation is that more CAFs promoted more and larger acinar-like structures to form. However, more CAFs also leads to a faster degradation of the basement membrane matrix and limits the duration of the co-culture. 2:1 is therefore an optimal ratio considering all these factors.

2. In Figure 3C, the authors describe that the "tear-drop" structures indicate a more metastatic and invasive phenotype compared to TUM622 acini cultured without CAFs. Are there any other markers that would support this phenomenon, such as loss of cell-cell junction protein expression? Also, how often do these structures form in comparison to non-co-cultured cells? Do all co-cultured cells become "tear-drop"-like? Some quantification of this data would be appreciated.

As the reviewer pointed out, the leading front of the “tear-drop” structure stains positive for Vimentin, but negative for CHD1 and ITGA6 indicating a more invasive phenotype (see Figure 5E in original PNAS

publication). We have also tested tight junction antibodies including ZO-1 and Claudin-1 from various vendors but did not detect distinct signal at cell-cell junctions in TUM622 3D spheroids (data not shown). This might be expected as we have demonstrated that TUM622 are highly malignant cells and their degree of polarity may not be the same as that of normal tissue. This observation is similar to what have been reported in MCF-10A, where tight junctions could only be detected in 2D but not 3D structures (4).

The formation of the tear-drop structure only occurs in the co-culture not the mono-culture setting and only those acinar-like structures that are within close-proximity to the CAFs undergo invasion (see Figure 6B in original PNAS paper). We have now emphasized this point in the representative results section as well as in the figure legend.

3.In Figure 5A, TUM622 cells were treated with XAV939 to show the ability of treating these cells in a 96-well format and illustrate the role of Wnt signaling in the survival of these organoids. However, these cells were not described to be co-cultured with CAFs, so the ability to interrogate treatment effects in response to the tumor microenvironment is not well-demonstrated in this system. Even if Wnt signaling is critical to organoid survival, what role does this pathway play in the presence of the microenvironment, which is a fundamental feature of the co-culture system? Performing validation experiments to show a (unique) effect when the co-cultured system is tested compared to TUM622 cells only, CAFs only, and DMSO-only treated cells may be one way to illustrate the versatility of the co-culture system.

We appreciate the suggestion by the reviewer and agree this would be an interesting set of studies. However, Figure 5 is only used to demonstrate that this system could be adapted to a more high-throughput format and be used for testing drug response. We felt that a comprehensive analysis of drug response in mono-culture vs. co-culture is beyond the scope of this protocol and will be explored in future studies.

Minor Concerns:
Please be consistent in including experimental information across the figures, figure legends, and results. For example, what timepoint(s) are being shown in Figure 2B? Also, the figure legend assignments of part A,B, and C are not consistent with the labeling with the Figure data. Please correct this information.

Corrected.

Reviewer #2:
Manuscript Summary:
In this manuscript, Chen et al described comprehensive protocol of a 3D co-culture model utilizing established lung squamous cancer (LUSC) patient-derived xenograft and cancer-associated fibroblasts (CAFs). In this co-culture organoid system, the authors demonstrated that LUSC from an acinar-like structure that nicely recapitulates dynamic tumor-stromal interactions. Importantly, the authors provide experimental evidence that 3D culture system can be employed as a platform for drug screening and validation.

Overall, the manuscript is well written and logically presented. Only a few minor points need to be addressed:

Major Concerns:
None

Minor Concerns:
Figure 2C and Figure 5A: graphs need to be revised. Labels in the x- and y-axis are not clear.

Corrected.

Although appropriate references are cited, it would be nice if the authors provide key characteristics of TUM622 and CAFs (e.g. patient information, mutation, if available) in the Introduction.

We thank the reviewer for this comment and have now added the following information as well as relevant reference to the introduction.

"Both TUM622 and CAFs are derived from NSCLC patient with poorly differentiated tumor (5)"

Reviewer #3:
Manuscript Summary:
The main purpose of this article is to understand the role of stromal cells and their dynamic interactions with lung squamous carcinoma (LUSC) cells throughout tumorigenesis. A 3D coculture model is utilized to gain insight into both cell-intrinsic and extrinsic mechanisms that lead to the epithelial to mesenchymal transition. The protocol is very well presented and supported by morphological and immunofluorescence studies. The authors have also published studies on this model in a previous PNAS paper. However, there are serious concerns about the generalizability of these results.

We appreciate the reviewer's comments and in addition to answering each individual point below, we would like to provide a general response regarding the key attributes of our model and its generalizability.

The key feature of our culture system is its ability to model phenotypic plasticity of LUSC cells in response to tumor-stromal interactions (matrix-cell and cell-cell interactions) and how these interactions regulate tumor morphology during LUSC progression. Our goal was not to model normal lung development or a reversion of LUSC cells to a normal lung architecture (i.e. pseudostratified ciliated columnar epithelium). Instead, we are modeling how changes in the tumor microenvironment could override genetic mutations within highly malignant LUSC cells and revert them to a less aggressive phenotype. Similarly, elegant work has been done in the field of breast cancer where various labs (Mina Bissell, Joan Brugge, Senthil Muthuswamy, and others) have used a breast epithelial cell line MCF-10A to understand the dynamic reciprocity between these cells and their microenvironment in 3D culture. To our knowledge, it has not been previously shown that LUSC has this capacity and we believe our system might serve a similar purpose as MCF-10A in understanding the molecular mechanisms underlying LUSC progression. To this end, we established this current protocol and used it to answer specific questions about how particular tumor cell-intrinsic or extrinsic changes regulate tumor progression.

Previously, other labs have attempted to induce LUSC formation in 3D cultures from normal lung epithelium by introducing various genetic mutations. This has proven to be challenging due to the high intrinsic genetic complexity of LUSC (6, 7). In contrast, our approach was different in that we initiated

our studies with a malignant LUSC culture in order to study tumor cell plasticity. This was made possible by the fact that our TUM622 cells, although surprising, had the ability to restore apical-basal cell polarity and undergo acinar morphogenesis when cultured in a 3D matrix. Importantly, when we profiled the acinar-like structures and analyzed the expression profile with GSEA, two of the top enriched gene-sets are related to breast acinar morphogenesis (8), suggesting that this is indeed more broadly generalizable to acinar morphology. This finding provided the motivation to explore additional factors (such as SOX2 and CAFs) that might induce even more aggressive morphological changes in these malignant cells when cultured in 3D. Furthermore, the tumorigenic potential of cells from these acinar-like structures was supported by enrichment of a common set of genes related to poor prognosis for both breast and lung cancer, highlighting the malignant nature of these cells as well as the generalizability of this system (8). Finally, the capacity of our system to model LUSC biology in vitro while preserving the plasticity of malignant cells further distinguishes it from other in vitro LUSC models.

Major Concerns:

1) It is not clear what the authors are modeling in this manuscript. The cell line TUM622 was apparently developed in the authors' laboratory from a PDX. Even if they derive from a squamous cell carcinoma, they form acinar structures in vitro. Acinar structures are not observed in squamous cell carcinoma, but are typical of adenocarcinoma. The authors should explain or at least comment on this peculiarity.

We thank the reviewer for pointing out the lack of clarity regarding this point. The term acinar-like structure or acini in this manuscript is strictly used to describe the morphology of the tumor organoids in our 3D culture (monolayer of cells surrounding a hollow lumen) and should not be equated to the histological definition used to describe lung adenocarcinoma samples. We have now adjusted the language in the introduction to emphasize this point.

2) The authors mentioned that the organoids are formed by a rare subpopulation of the PDX-derived LUSC cells that form acinar-like structures. The acinar structures seem to be a clonal peculiarity, likely not representative of the totality of cancer stem cells present in the tumor. Therefore, it is not clear how representative these are of the whole tumor, which is not an acinar tumor but a squamous carcinoma. This raises issues of generalizability of any drug assays performed utilizing this cell line.

We appreciate the comment from the reviewer and its implications for in vitro screening. We would like to clarify that this model does not represent the whole tumor. Instead, it selects for a rare population of less differentiated cells with cancer stem cell characteristics (5). Importantly, these cells can self-renew and differentiate, recapitulating the heterogeneous population of tumor cells observed in the PDX as well as original tumor (8). Whether this is a subpopulation of all cancer stem cells needs to be prospectively identified by surface markers and is an interest of future studies. With regards to the generalizability to the drug assays that could be performed using this model, we agree that this system is only applicable to interrogating pathways that are relevant in modulating the organoid phenotype. For example, we show that the Wnt but not Notch signaling pathway is relevant in regulating the acinar-like phenotype (8). However, the system will be suitable for any general cytotoxicity assays. We have now included this comment on the limitation of the system in the discussion section.

3) The authors present these organoids as a model of hyperplasia, recapitulating the earliest stages of LUSC development. The authors state that this model recapitulates the proper apical-basal polarity of the normal lung epithelium. However, these cells derive from an invasive LUSC that was passaged in

mouse as PDX, well beyond the pre-malignant stage. Moreover, the normal lung epithelium is a pseudostratified ciliated columnar epithelium, and the pre-malignant lesions of LUSC are characterized by squamous metaplasia and dysplasia. None of these features are recapitulated in the TUM622 organoids. The utility of the presented organoids to model lung normal epithelium or pre-neoplasia is questionable. The authors should explain clearly what they are modeling with this cell line and should avoid claims that the acinar structures recapitulate features of the lung epithelium or the earliest stages of LUSC development.

Again, we agree that this is not a model for normal lung epithelium but instead a model for the phenotypic plasticity of LUSC tumor cells. See also general response.

4) The applicability of the presented protocol to other patient samples or PDX lines is unclear. Is this acinar morphology a peculiarity of this particular line? In this case, what is the utility of the presented protocol? The applicability of the protocol to other lines/PDXs should be presented or at least stated.

We thank the reviewer for this comment and would like to refer to our original PNAS publication Figure 1, to answer this question. The ability to form acinar-like structure is indeed unique to TUM622 cells, at least from the different PDX derived lines we have tested. Therefore, other patient PDX/cell lines may or may not adopt similar phenotypes like TUM622 culture and will have to be tested by individual users.

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CORRESPONDING AUTHOR

Name:	Kenneth G. Geles
Department:	Oncology R&D
Institution:	Pfizer Worldwide Research, Development and Medical
Title:	Senior Director
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