3D ACINAR CULTURE FOR IMAGING ACINAR DYNAMICS

An Undergraduate Research Scholars Thesis

by

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I, Kathryn Adele Leach, certify that all research compliance requirements related to this Undergraduate Research Scholars thesis have been addressed with my Faculty Research Advisor prior to the collection of any data used in this final thesis submission.

This project did not require approval from the Texas A&M University Research Compliance & Biosafety office.

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ABSTRACT

3D Acinar Culture for Imaging Acinar Dynamics

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Epithelial cells line surfaces of the human body. Since they define the boundary between internal and external environments, these cells maintain a polarity, created by localizing proteins at opposite ends of each cell, in order to facilitate transport of substances between these environments. Basal proteins, which face the external environment, and apical proteins, which face the internal environment, define this polarity. When grown *in vitro* using a 3D culture, epithelial cells group together to form hollow, fluid-filled spheroids of cells called acini. These acini reflect *in vivo* polarity with basal proteins facing the 3D matrix and the apical proteins facing the fluid-filled interior. In some cancers, the polarity of epithelial cells becomes inverted which may play a role in cancer metastasis. Previous research projects investigated the process responsible for everting the acini and found a variety of mechanisms. This research project builds on past discovery of using RhoA activation to initiate acinar eversion. Activating RhoA increases the activity of myosin, resulting in higher contractility in the cell. This higher contractility was found to disrupt the mechanical equilibrium of the acini, resulting in breakage, breach, and eversion of acini's polarity as they collapse and flip inside-out. To further investigate

this mechanism involving RhoA, this project documents the procedures to form and image acini using previously untested cell lines made from cancerous lung epithelial cells: 344SQ_shCTRL, 344SQ_shZEB1, and 393P_ZEB1. It was found that 344SQ_shCTRL and 344SQ_ZEB1 acini formed lumens. In making the acini, changes to the protocol included adjusting methods for avoiding imaging complications with thick Matrigel layers, shortening the time period allotted for lumen development, and increasing the concentration of RhoA activator used in experimentation. Initial experimental and fixed staining results collected demonstrate the exciting opportunity for many future research projects utilizing acini such as investigating lumen nuclei orientation, quantifying the factors impacting lumen development, and continuing investigation with acinar eversion.

DEDICATION

To my family for their unwavering support and encouragement throughout this project.

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I would like to thank my faculty advisor, Dr. Lele, and my kind graduate student, Hailee Patel, for their guidance and support throughout the course of this research.

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All other work conducted for the thesis was completed by the student independently.

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NOMENCLATURE

ECM	Extracellular Matrix
EDTA	Disodium Ethylenediaminetetraacetic Acid
DAPI	4′,6-diamidino-2-phenylindole
DMSO	Dimethyl Sulfoxide
FBS	Fetal Bovine Serum
MDCK	Madi-Darby canine kidney
PBS	Phosphate-buffered Saline
PFA	Paraformaldehyde
RhoA	Rho Activator
RMP	Rotations-per-Minute
ZDC	Z-Drift Compensator

1. INTRODUCTION

1.1 Epithelial Polarity

The human body is composed of four main cell types: epithelial cells, nerve cells, muscle cells, and connective tissue cells. Epithelial cells cover the interior and exterior surfaces in the body such as the linings of organs or vasculature. Depending on its location within the body, the function of epithelial cells varies as reflected in their variety of structures. However, since epithelial cells form the boundary between external and internal environments, one common function of these cells includes transporting substances, such as the hormonal excretions from an organ, nutrients, or waste products, between these environments [1].

To accomplish this function, most epithelial cells utilize a polarity created by compartmentalizing specific proteins to opposite ends of the cell. Apical proteins, such as podocalyxin and ezrin, localize at the side of the cell facing the lumen while basal proteins, such as β1-integrins and β-catenin localize at the side of the cell facing the external environment [2]. Tight junctions help enforce this organization by restricting the lateral diffusion of these proteins between the apical and basolateral membrane compartments.

When grown in a three-dimensional (3D) environment *in vitro*, previous research found certain epithelial cells group together into spheroids of cells and establish a lumen using similar apical-basal polarity found *in vivo* with the basal side forming the exterior of the ball of cells and the apical side facing the interior lumen [3]. Recently, researchers began investigating these fluid-filled spheroid of epithelial cells, or acini, further, specifically looking at potential mechanisms for flipping their polarity from basal-out to apical-out. This project documents acini procedures, including culturing, lumen development, and imaging, throughout a long-term

research project investigating acinar dynamics for three untested cell lines and their relevance in cancer metastasis.

1.2 Previous Research

1.2.1 Inverted Polarity for Use as Experimental Models

Research conducted by Co et al pursued apical-out acini due to their potential for use as experimental models for pathogen-epithelial interactions. Their study used human enteriods, epithelial spheroids made from gastrointestinal tissue, and found that the apical-out enteriods continued to function, completing polarized absorption of nutrients. Mechanisms they tested for creating the inverted polarity included transferring enteroids made in Matrigel to suspension culture and treating enteroids in Matrigel with β1-integrin function-blocking antibody. After creating the apical-out enteriods, the study used the inverted spheres to investigate epithelial cell interactions with two bacterial pathogens: Salmonella enterica serovar Typhimurium, which invade epithelial cells from the apical side, and Listeria monocytogenes, which interacts with epithelial cells from the basal side. Both bacteria interacted with non-inverted or inverted enteroids as expected, leading the study to argue that apical-out enteroids provide a more accurate model and are easier to work with than past techniques of microinjection [3].

1.2.2 Inverted Polarity as Potential Mechanism for Cancer Metastasis

Another study by Narayanan et al investigated acinar polarity eversion due to its relevance in cancer research. In several glandular cancers, epithelial cells invert to apical-out polarity and potentially use that inversion in the metastasis process. Knowing cancer cells sometimes exhibit an upregulation of RhoA, the study first investigated direct and indirect pathways for activating RhoA in the acini. The study used pre-formed, 10-day old acini made from Madi-Darby canine kidney cells (MDCK). From the experiments that directly added RhoA

activator to the acini, they found inversion occurs with the cells moving en masse and that the effect of RhoA activation depends on the dosage, with higher dosages resulting in a loss of E-cadherin linkages which break down the diffusive barrier between cells and disrupts the inverting process. Additionally, the indirect pathways of inversion tested by the study included treatment of acini with doxycycline, which disrupts the LINC complex, and femtosecond laser ablation. The study concluded that disruption to mechanical homeostasis acts as the driving force for eversion. RhoA increases contractile forces, decreasing the inner radius of the acini faster than the outer radius. This change in shape increases the difference in surface energy between the apical and basal sides of the acini, increasing the strain on the cells which leads to weakening on one side and local breaches in the acinar wall. Mathematically, the Law of LaPlace described this relation between tension, curvature, and pressure gradient. When the acinar wall breaks open, the fluid inside the lumen rushes out, resulting in collapse and inversion of the acini, and the lowered energy state of the acini makes it unlikely to revert back to basal-out polarity. However, compared to past research, this study's conclusions that inverted polarity resulted from a mechanical eversion contrasted with prior research's conclusions that protein trafficking establishes the apical-out polarity, suggesting that different mechanisms for inversion may exist depending on the level of acinar development [2].

1.3 Project Outline

1.3.1 Research Objective

To build off those findings, this project aimed to record experimental procedures with several untested epithelial cell lines with varying degrees of natural RhoA expression, reflected in the varied ZEB1 gene. These procedures included 2D culturing of the cells, 3D culturing of the acini, observations regarding lumen development, ideal acini growth duration, confocal

imaging, RhoA activator concentration, and staining. Throughout this process, two main questions were also answered: did the epithelial cells form acini, and if so, did those acini evert when directly exposed to RhoA activator? The starting methods of this study parallel the methods used by Narayana et all.

1.3.2 Overview of Materials and Methods

The cell lines tested during this project included 344SQ_shCTRL, 344SQ_shZEB1, and 393P_ZEB1. Both cell lines, 344SQ and 393P, are mouse models of human lung adenocarcinoma [4],[5]. ZEB1 is a gene that many human cancers overexpress, potentially contributing to the metastasis process. The variance in ZEB1 expression alters the cell's natural RhoA regulation, giving insight into how initial RhoA regulation impacted acini appearance, lumen development, and eversion success.

For each cell line, first the cells were expanded in 2D media. After sufficiently expanding the cell lines, 70-80% confluent cells cultures were passaged and used to form the acini by transferring them to 3D media, made from mixing the 2D media with Matrigel, in Matrigel-lined live and fixed well plates. The cells were monitored and fed with fresh 3D media every 2-3 days for 14 days, monitoring the lumen development daily. Then, the cell lines that formed lumens were either treated with RhoA activator or fixed and stained before imaging.

1.3.3 Significance

Results from this experiment provide improvements to acinar handling techniques as well as further insight into mechanisms impacting epithelial cell polarity. These procedures and mechanisms are important for both advancing techniques available for future research projects as well as gaining a deeper understanding of cancer metastasis. For many cancer patients, cancer metastasis marks a turning point for the likelihood of remission and an unfortunate decrease in

life expectancy. Therefore, any information related to the mechanisms of cancer metastasis, and by extension any improvements to acinar experimentation procedures, may prove helpful in combating cancer more effectively.

2. METHODS

2.1 Cell Culture

2.1.1 Growth Media

Growth media is the solution the cells are placed into when they grow. To promote growth, growth media contains a mixture of chemicals to feed, stabilize, and protect the cells from infection. The composition of this mixture depends on the individual cell line and whether the cells will be grown in a 2D or 3D environment.

In this lab, all cell lines used the same composition of media: 89% RPMI 1640, 10% fetal bovine serum (FBS), and 1% Penicillin-Streptomycin (PenStrep). Manufactured by ThermoFisher, RPMI 1640 contains the reducing agent glutathione, biotin, vitamin B12, PABA, and high concentrations of inositol and choline to help maintain physiological pH in a 5-10% CO_2 environment. The addition of FBS supplements for the lack of proteins, lipids, or growth factors in RPMI 1640. Lastly, PenStrep prevents bacterial contamination. When making the media, a filtration system in the biohood was used to create a sterile solution. To optimize freshness, typically the 2D media was produced in 561.6mL batches, consisting of 500 mL of RPMI, 56 mL of FBS, and 5.6mL of PenStrep. Depending on the amount of cultures grown simultaneously, this amount lasted for about six weeks. The media was stored in the 4^{o} C, and as the media ages, the color shifted from orange to pink. All 2D RPMI media was heated in a 39^{o}C water bath prior to using it on any cells.

2.1.2 Thawing

Since the purchased cell lines arrived frozen, the first step to begin processing the cells involved thawing the cells and plating them into a T-75 flask. When freezing cells, the freezing

medium contains dimethyl sulfoxide (DMSO) as a cryoprotective agent. Also from ThermoFisher, DMSO reduces the risk of ice crystal formation by lowering the freezing point of the medium, thus slowing its cooling rate. However, exposure to DMSO at room temperature risks harming the cells. Therefore, once the frozen cell mixture becomes a solid, it is important to remove the cells from the liquid freezing medium as soon as possible.

To begin the thawing process, with 15mL of 2D RPMI media preheated, first the small cell vial was located inside the liquid nitrogen basin. Next, the vial was briefly swirled in the 39^{o}C hot water bath until the mixture inside just started transitioning from a frozen to a liquid. Once the solution in the vial became a liquid, the solution was immediately transferred into a 15mL test tube and spun in the centrifuge for five minutes at 1000 rotations-per-minute (RPM). Finally, the freezing medium was aspirated, and the 15mL of 2D RPMI media was used to resuspend and plate the cells into a labeled T-75 flask. One T-75 flask was used per 1 million cells.

2.1.3 2D Growth

When growing the cells to expand the cell line or use in making acini, the cells lines grew in T-75 flasks using 2D RPMI media. The cells adhered to the bottom surface of the flask and grew in the sterile incubator environment. The incubator settings maintained a temperature of 37^{o}C and a CO_2 percentage of 5%. The term “confluent” describes the degree of adhesion and growth within a flask. A microscope was used to routinely monitor the cells’ growth. Once cells became 80-90% confluent, it was important to passage them into new flasks or use them in an experiment. Overly confluent cells resulted in cell death, reflected in dead cells seen floating in the 2D RPMI media, since the surface area of the flask was completely full. In addition to confluency, the appearance of the media also faced routine inspection. Clear, colorless media

indicated that the cells were “hungry” and needed a new addition of fresh media while cloudy media indicated potential infection.

2.1.4 Passaging

Passaging cells was the process of removing cells from their current 2D culture. This process occurred prior to using the cells in any experiment or maintenance tasks. For example, one application of passaging was expanding newly purchased cell lines to provide backup cells. Expanding a cell line involved passaging the cells from one T-75 flask to multiple T-75 flasks depending on the number of cells available. Other applications of passaging included transferring cells into cold storage or making the acini out of the cells.

To passage cells, first the cells were inspected with a microscope to evaluate their confluency. Then, the 2D RPMI media was aspirated, and 5mL of Phosphate-buffered saline (PBS) was added. The PBS used in passaging was PBS-- which disrupts the positive cation interactions between the cells to help the cells successfully lift from the surface of the flask. The flask was tilted back and forth to wash the bottom surface containing the cells thoroughly with PBS. After aspirating the PBS, 2mL of warmed 0.25% Trypsin with disodium ethylenediaminetetraacetic acid (EDTA) was added to the flask, which was again tilted to thoroughly wash the surface of the cells, and then the flask was returned to incubator. Trypsin breaks down the proteins the cells use to adhere to the bottom surface of the flask. The addition of EDTA chelates the divalent cations of Ca^{2+} and Mg^{2+}. Extended exposure to Trypsin damages cells, so the Trypsin wash was limited to roughly five minutes. Once the five minutes elapsed, the cells were examined to confirm they successfully lifted off the bottom of the surface, appearing as floating in the media and making it cloudy. Slight tapping of the flask helped the cells life. Then, 2 mL of 2D RPMI media was added to the flask to neutralize the Trypsin. This

solution of cells and equal parts 2D RMPI media and Trypsin was pipetted into a 15mL flask and centrifuged for five minutes at 1000 RPM. Lastly, the media and Trypsin solution was carefully aspirated off the cell pallet at the bottom of the flask, and the cells were resuspended in at least 1mL of media. From this point, this cell solution was utilized as needed.

2.1.5 Cell Counting

Cell counting was used prior to tasks requiring a specific number of cells. For example, making acini or freezing cells requires cell counting. To count the cells, first they were passaged from their flask and resuspended into 1 mL of 2D RPMI media, miking thoroughly to disperse cell clusters and evenly disperse the cells throughout the solution to ensure accurate cell count results. Into a separate small vial, 12uL of Trypan Blue stain was pipetted. Trypan Blue uses the methodology that non-living cells absorb the blue dye while living cells do not. It also has a greater affinity for serum proteins than cellular proteins. In a separate small container, an approximate 100uL to 200uL of the 1mL cell solution was added. After mixing this solution again thoroughly, 12uL were added into the small container and mixed with the 12uL of Trypan Blue. Lastly, 10uL of this mixture were added to each well of the cell counter slide which was then inputted into the cell counting machine. The focus on the machine was adjusted to a setting where each cell included well defined edges and a small white spot in their center. The average of the two cell counts, one from each side of the slide, was used to determine the volume of the 1mL cell solution needed to create a solution with a desired concentration of cells.

2.1.6 Freezing

For long term storage, cells were kept in liquid nitrogen storage. Freezing cells conserves materials by reducing the number of cultures growing in the incubator simultaneously, resulting in reduced waste of cells and chemicals. The process of freezing cells began with labeling

several cryovial with a cryogenic marker, including the name of the cell line, date of its freezing, number of cells per vial, and initials of the researcher. Next, the cells were passaged and counted. The total amount of cells determined the number of vials as well as the total volume needed since each vial received 1mL of solution containing 1 million cells. To protect the cells during the freezing process, 5% of the total solution contained DMSO. However, the DMSO was only added to the cells once the vials were ready to fill. Once the DMSO was added to the solution, the vials were quickly filled with 1mL each and promptly moved into -80^{o}C storage to avoid any negative effects of DMSO exposure at room temperature. For this initial freeze, the vials were placed into a Mr. Frosty container which assists in creating a uniform preliminary freeze. The cells remain in the -80^{o}C storage for at least 24 hours. Afterwards, the vials were moved into the liquid nitrogen storage, using colored tabs on each storage cane to make finding the cells in the future easier.

2.2 Acini Culture

2.2.1 3D Acini Culture Protocol

To form the acini, the epithelial cells require a 3D Matrix environment, created by using Matrigel: a commercially available matrix derived from mouse tumors containing laminin, collagen, and other extracellular matrix (ECM) proteins. Once the cells were transferred to this 3D Matrix, given the correct circumstances, they naturally group into acini and potentially develop lumens by Day 14.

Since the goal was to conduct live and fixed imaging on the acini, 8-well imaging slides were used to house the growing acini. First, the slides were prepped in the biohood by coating the bottom of each slide with 40uL of Matrigel. However, Matrigel solidifies into an unusable gel at room temperature. A container of ice was used to hold the Matrigel vial during this

process, and initially, solely an icepack was placed under the imaging slides to keep the Matrigel cold, but issues were still encountered with the Matrigel coating's thickness. Thus, the procedures were adapted to include chilling the imaging slides as well as the pipet tips beforehand. This adjustment aided in Matrigel spreading and resulted in thinner coatings that fulfilled imaging requirements. Reverse pipetting was used to place and slightly spread 40uL of Matrigel into the center of each well. Overspread wells resulted in majority of the coating adhering to the sides of the chamber while under spreading created a coating too tall to image correctly. After finishing with the coatings, the slide was placed into the incubator for at least one hour. Ideally, the acini were made on the same day as when the slides were prepared, but if needed, the slides were prepped a day in advance.

Next, the cell solution to add to each well was prepared consisting of 400uL of 3D RPMI media with roughly 5000 cells per well. 3D media was created by adding 2% Matrigel to 2D RPMI media. For a single eight wells, 3.2mL of 2D RMPI was needed for each well to have 400uL of total solution. Therefore, 64uL of Matrigel was added to the 3.2mL of 2D RPMI. Once the 3D media was prepped, the cells were examined to ensure they met the ideal level of confluence of 70-80%. If the flask was more confluent than 80%, the cells were passaged instead and allowed to regrow back to the ideal confluence level. For cultures within the ideal range, the cells were passaged, resuspended into 1mL of 2D RPMI media, and counted. The average cell count value was used to calculate how much of the cell solution was added to the prepped 3D media. Lastly, 400uL of the total solution, made from 2D RPMI, Matrigel, and a portion of the resuspended cells, was pipetted into each well, and the completed slides were placed into the incubator. Any leftover cells from the cell solution were either discarded or replated into a new T-75 flask. Over the next two weeks, the acini were monitored daily and fed every three days.

2.2.2 Feeding Acini

At least every three days, the acini required feeding. As the acini grew larger, towards Day 10 and later, they consumed the media at a faster rate.

The acini were fed with 3D media. Similar to the process of creating the acini, the 3D media was made by adding 2% Matrigel to the total volume required. After removing the acini from the incubator, the old 3D media solution was aspirated off the top of each well, taking care not to disturb the bottom Matrigel coating where the acini grow. The slide was tilted slightly so that the media was gently removed from a side corner of each well. It was important to change tips if working with multiple acini cell lines simultaneously. Soon after the media was removed, 400uL of the 3D media were added to each well, and the slides were returned to the incubator.

2.3 Experimentation

2.3.1 RhoA Activation

Experimentation with RhoA activation used RhoA activator doses 1 ug/mL and 3 ug/mL. Treatment was administered by adding the RhoA activator into 3D media and pipetting 400 uL of total solution per well immediately prior to starting the live imaging.

2.3.2 Confocal Imaging

Both the fixed and live imaging were conducted using an Olympus FV3000 with UPLXAPO20X and UPLZAPO60XOHR. For the live cell imaging, an environmental chamber was used to maintain ideal conditions for the acini: 37^{o}C and 5% CO_2.

After following manufacture guidelines for powering on the microscope and focusing the objective, three to four viable acini were found per well. Then, they were centered, the gain and focus were adjusted to ensure each lumen was well defined, and their z-drift compensator (ZDC) positions were registered. Thinner Matrigel coatings in the wells helped avoid issues with acini

being located outside of the z-axis range. Images were taken line-by-line with a minimum of four times per line with a minimum image size of 1024x1024.

2.3.3 Fixing

Fixing acini freezes the acini in time, meaning they are no longer alive but protected against decomposing. This process was useful for acini that were not live imaged since it allowed for consistent growth durations. While fixed samples were unable to complete any dynamic biological processes, they still interacted with staining. Thus, fixation of acini was used either after the live experiments or on samples that were used to make visual observations on acini structure and abundance.

The process of fixation started by warming PBS and diluting paraformaldehyde (PFA) to a concentration of 2% with PBS. Then, the samples were washed with PBS. The 3D media was removed from each well and 200uL of PBS was added. After 5 minutes, the PBS was removed, and, working in the fume hood, 100uL of 2% PFA were added into each well. After 15 minutes, the PFA was removed and three additional washes with 200 uL of PBS per well were conducted. Each wash sat for five minutes. Finally, roughly 200uL of PBS were added to each well prevent the fixed samples from drying out in storage, and the slide lids were wrapped in parafilm and placed into 4^{o}C storage.

It was discovered that washes with PBS-- frequently resulted in loss of samples due to the Matrigel coating lifting from the bottom of the well. Later into the project, PBS++ was used instead to prevent the Matrigel from lifting. Additionally, when conducting the washes, the samples were handled gently by using smaller pipet tips and removing the washes at the side corners of the well.

2.3.4 Staining

Fixed samples were stained with 4′,6-diamidino-2-phenylindole (DAPI) and 647 Phalloidin. DAPI stained the nuclei of cells blue while 647 Phalloidin stained F-actin red. Since these fluorescents were light sensitive, staining was conducted in a darkened room. First, the staining solution was mixed by performing a 1:400 dilution of 647 Phalloidin and a 1:2000 dilution of DAPI given that total solution consisted of 100uL of PBS++ per well plus an addition 10% PBS to account for error. Then, current PBS++ in each well was aspirated and 100uL of the 647 Phalloidin, DAPI, and PBS++ mixture was added to each well. After 20-30 minutes, the staining solution was aspirated off and three washes with 200mL of PBS++ were conducted with five-minute intervals. For live slides, the samples were stored by adding 200uL of PBS++, wrapping in foil, and placing in 4^{o}C storage. For fixed slides, the chamber of the slide was removed and approximately 50uL of Vectashield prior to placing a cover slip on the samples. In a dark box or wrapped in foil, these samples were placed into the 4^{o}C storage and the Vectashield was allowed to dry before collecting images.

3. RESULTS

3.1 General Observations

3.1.1 Growth and Lumen Development

Throughout the process of caring for the various cell lines, several qualitative observations were made regarding the visual appearance of the cells and acini during their growth periods.

3.1.1.1 344SQ_shCTRL

The 344SQ_shCTRL cells grow as patches in the 2D RPMI media. These patches made judging confluency difficult since the larger patches created empty gaps across the flask. However, it was found that the confluency was already too high for making acini once the cells grew to where these large patches formed. **Figure 1** shows how the cells appeared with an average cell count of $1.29x10^7$ cells/mL.

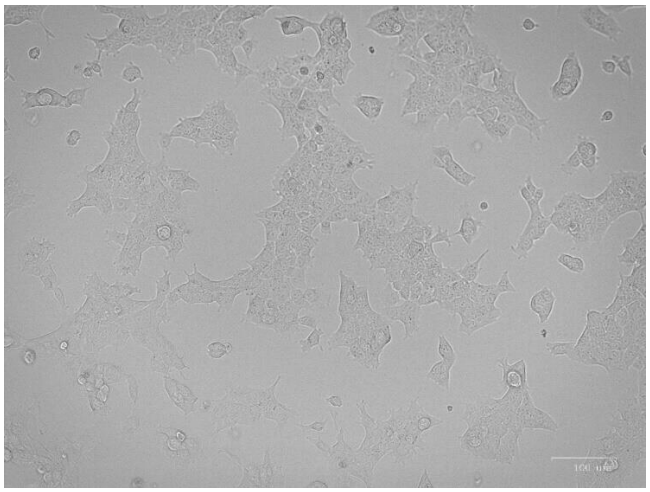

Figure 1: 344SQ_shCTRL cells with cell count of $1.29x10^7$ cells/mL

Figure 2 exemplifies cells that were significantly confluent and needed passaging.

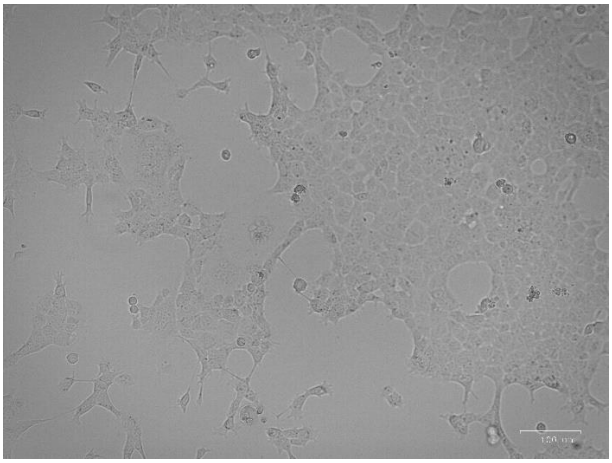

Figure 2: 344SQ_shCTRL cells with 90-100% confluency

The cells in **Figure 1** were used to make acini, but the resulting acini formed fewer lumens than expected. Therefore, these cells will be passaged instead in future experiments. Due to the fast growth of the 344SQ cell line, it was found that the cells needed less than 24 hours of growth before reaching the ideal 70-80% confluency. The ideal appearance of the cells was several small, separate groups of cells with space in between.

Once in 3D media, the 344SQ_shCTRL cells both formed acini and lumens as expected with their normal levels of RhoA regulation. While monitoring the acini growth, it was noticed that the number of lumens seemed to decrease once growth reached Day 14 and later. Therefore, with the goal of determining the ideal time to begin RhoA experimentation and live imaging, the lumen development was monitored during the growth cycle of a 344SQ_shCTRL acini sample made on February 20, 2023 from an average cell count of 7.89×10^6 cells/mL.

With the acini made on Day 0, Day 1 through Day 6 consisted of the acini forming and growing larger. The acini start on Day 1 as small, loosely scattered, and mostly spherical groups of cells. In general, as time progressed, more acini became irregularly shaped. However, for the

344SQ cells, majority of the acini remained roughly spherical with smooth edges. **Figure 3** shows an image of the acini at Day 3 taken with the digital microscope. The acini are the dark spheres in the image while the gray background is the Matrigel coating.

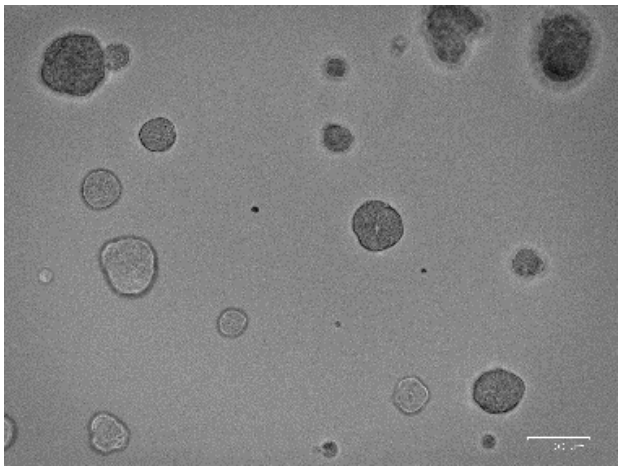

Figure 3: Day 3 344SQ_shCTRL Acini

Lumens were first observed on Day 7. Generally, these initial lumens formed in the smaller acini and had thicker cell walls. Approximately 5 lumens were found per well. Days 8 through 14 consisted of overall growth of the acini with an increasing number of irregularly shaped acini as well as an increasing number of lumens. The lumens that formed earlier became more defined with thinner cell walls. Larger acini and irregularly shaped acini formed lumens at a lesser frequency. **Figures 4**, **5**, and **6** demonstrate these observations across Days 7, 8, and 9. The lumens appear as hollow, round cutouts of the dark acini.

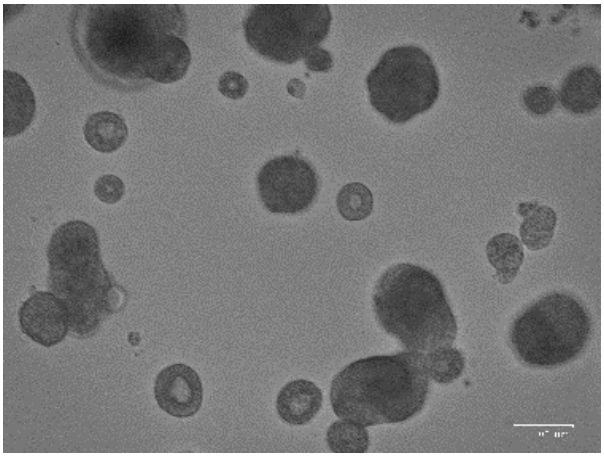

Figure 4: Day 7 344SQ_shCTRL Acini

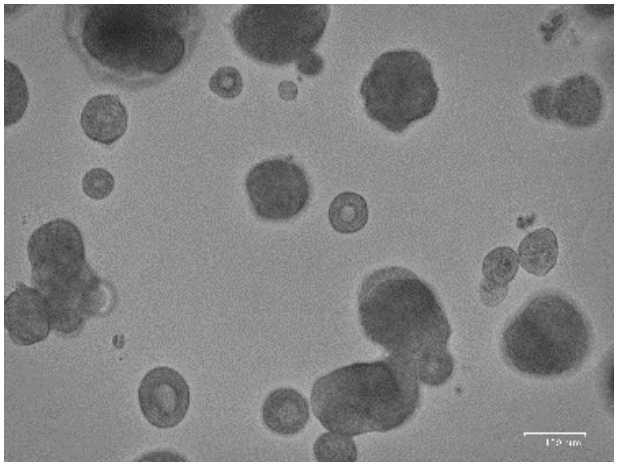

Figure 5: Day 8 3344SQ_shCTRL Acini

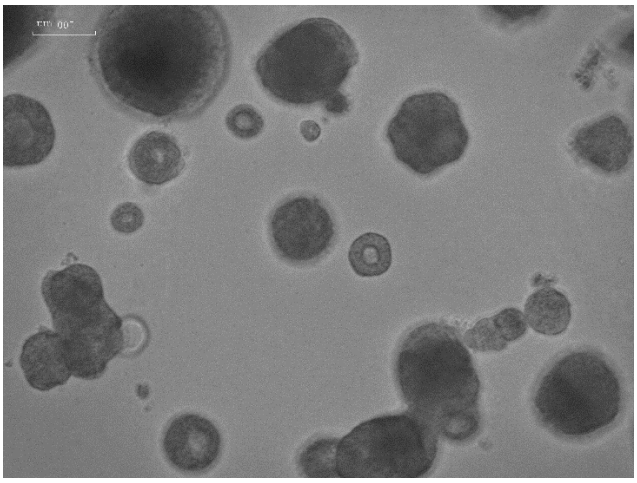

Figure 6: Day 9 3344SQ_shCTRL Acini

However, it was noticed that while some lumens continued to develop into the ideal dimensions for experimentation, the total amount of viable lumens seemed to decrease across Days 10 through 14. One potential cause for this loss of lumens was acini with lumens commonly merged into the larger, irregular acini without lumens. Figures 7 and 8 show examples of this behavior with well-developed lumens from Days 10 and 11 respectively.

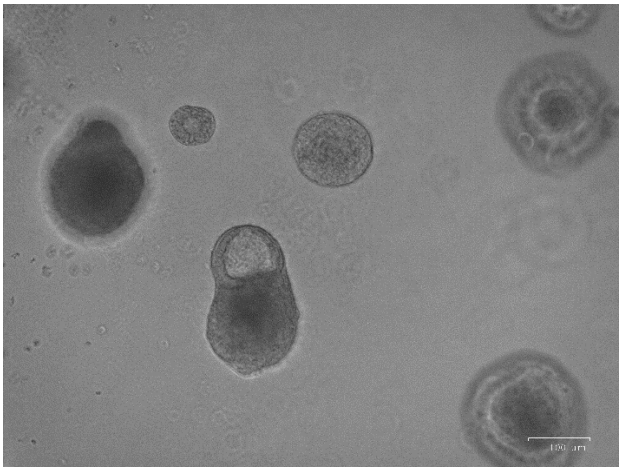

Figure 7: Day 10 344SQ_shCTRL Acini

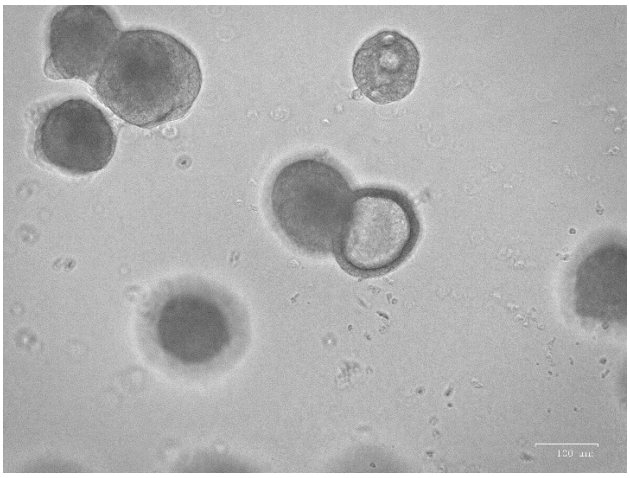

Figure 8: Day 11 344SQ_shCTRL Acini

3.1.1.2 344SQ_ZEB1

When grown in 2D culture, the 344SQ_ZEB1 cells also grew in patches similar to the 344SQ_shCTRL cells. Like the 344SQ_shCTRL, the cells quickly reach the ideal confluency for making acini, requiring less than 24 hours.

Overall, the differences in appearance between the 344SQ_shCTRL acini and 344SQ_ZEB1 acini were small. However, the lower RhoA regulation from the genetic ZEB1 modification created some minor differences. This lower RhoA resulted in fewer irregularly shaped acini than the shCTRL cell line. Although, like the shCTRL cells, the number of irregularly shaped acini increased over time. The ZEB1 cell line also seemed to produce fewer lumens overall but no quantitative evidence was collected.

Figure 9 exemplifies these similarities with Day 14 344SQ_ZEB1 acini made from an average cell count of 1.02×10^7 cells/mL.

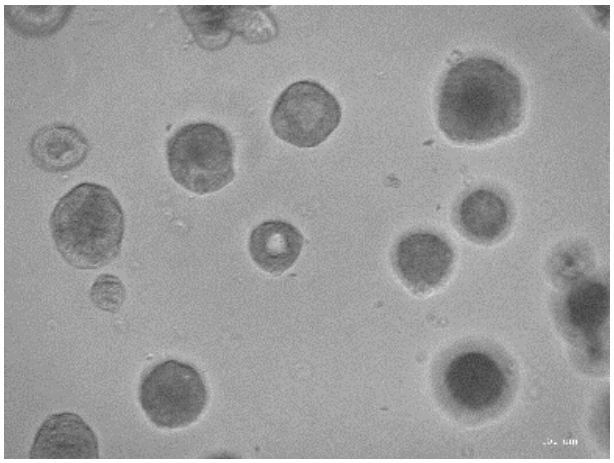

Figure 8: Day 14 344SQ_ZEB1 Acini

Despite reaching Day 14 of growth, majority of these 344SQ_ZEB1 acini kept their round shapes and fewer merged into large, irregularly shaped acini.

3.1.1.3 393P_ZEB1

Unlike the 344SQ cell line, the 393P_ZEB1 cells grew in 2D culture as small, distinct cells rather than in patches.

Additionally, the 393P_ZEB1 acini looked vastly different than the 344SQ acini. Due to the upregulation of RhoA in the 393P_ZEB1 cells, the acini formed large, irregular, and uneven shapes with rough boarders. The increased RhoA also prevented lumen formation. Thus, the 393P_ZEB1 cells were not used in any further experimentation since they had no lumens to attempt to evert. **Figure 10** shows Day 10 393P_ZEB1 acini made with an average cell count of $4.735x10^6$ cells/mL.

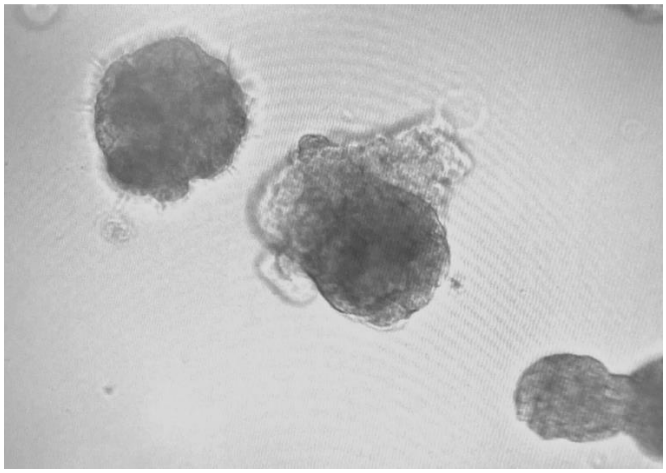

Figure 10: Day 10 393P_ZEB1 Acini

3.2 Confocal Imaging

3.2.1 Live Imaging of RhoA Experimentation

No cell lines everted during this research project nor showed significant changes in mobility. Testing was conducted only with the cell lines that formed lumens: 344SQ_ZEB1 and 344SQ_shCTRL. The initial treatment with 1 ug/mL did initiate a gradual collapse of the lumens but lacked the force required to mechanically evert the acini. In an attempt to increase these forces, the treatment was increased to 3 ug/mL, but the experiment resulted in similar outcomes.

3.2.1.1 344SQ_shCTRL

Figure 11 shows frames from the video created after live imaging for 48 hours that demonstrates the collapse of the lumen after the 1 ug/mL treatment. The lumen disappeared between 14 and 15 hours after adding the RhoA activator, but no eversion occurred. Afterwards, the appearance and position of the treated acinus remained constant.

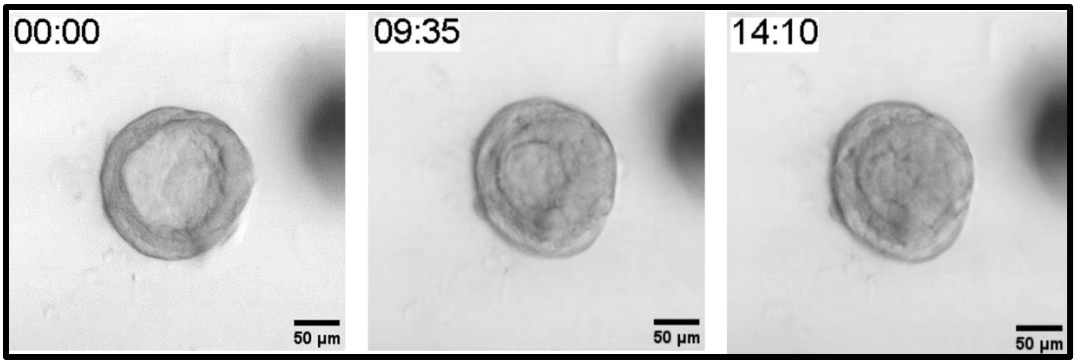

Figure 11: 1ug/mL RhoA Activator Treatment on Day 14 344SQ_shCTRL

The control acini during the experiments that did not receive any treatment remained constant throughout the experiment with both their lumen structures and their position.

3.2.1.2 344SQ_ZEB1

Figures 12 and **13** show results from the 1 ug/mL treatment conducted over 19 hours and 3 ug/mL treatment conducted over 48 hours of the 344SQ_ZEB1 acini respectively.

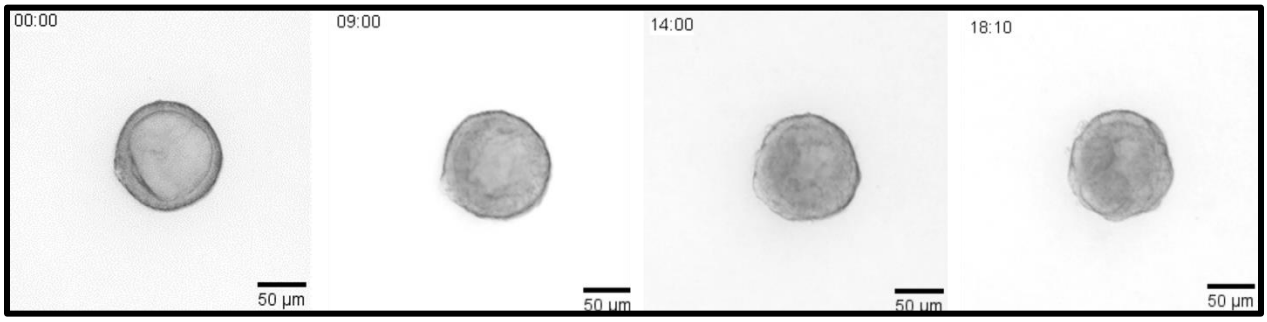

Figure 12: 1ug/mL RhoA Activator on Day 14 344SQ_ZEB1

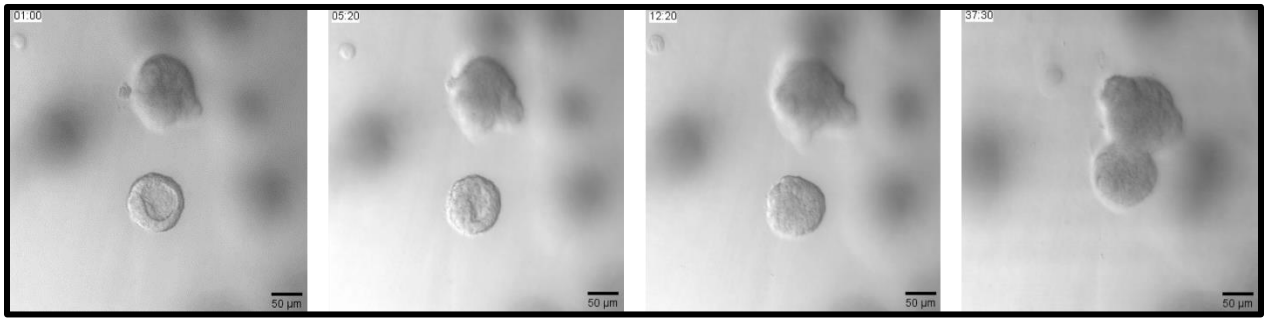

Figure 13: 3ug/mL RhoA Activator Treatment on Day 14 344SQ_ZEB1

The 1 ug/mL treatment showed complete collapse of the lumen at around 16 hours after receiving treatment and no movement at any point. Comparatively, the 3 ug/mL treatment resulted in a faster, but still gradual, lumen collapse at roughly 8 hours and the collapsed acinus merged into a nearby acinus afterwards. Neither of these lumen collapses resulted in eversion.

For the control acini in both experiments, they did not show a change in lumen nor position during the imaging period.

3.2.2 Stained Imaging

The stained images, with blue corresponding to the DAPI-stained nuclei and red corresponding to the Phalloidin-stained F-actin, provided an interesting insight into the structure of acini, specifically a radial alignment of the nuclei.

3.2.2.1 344SQ_shCTRL

Figure 14 shows the results after the immunostaining for two different untreated Day 14 344SQ_shCTRL acini samples.

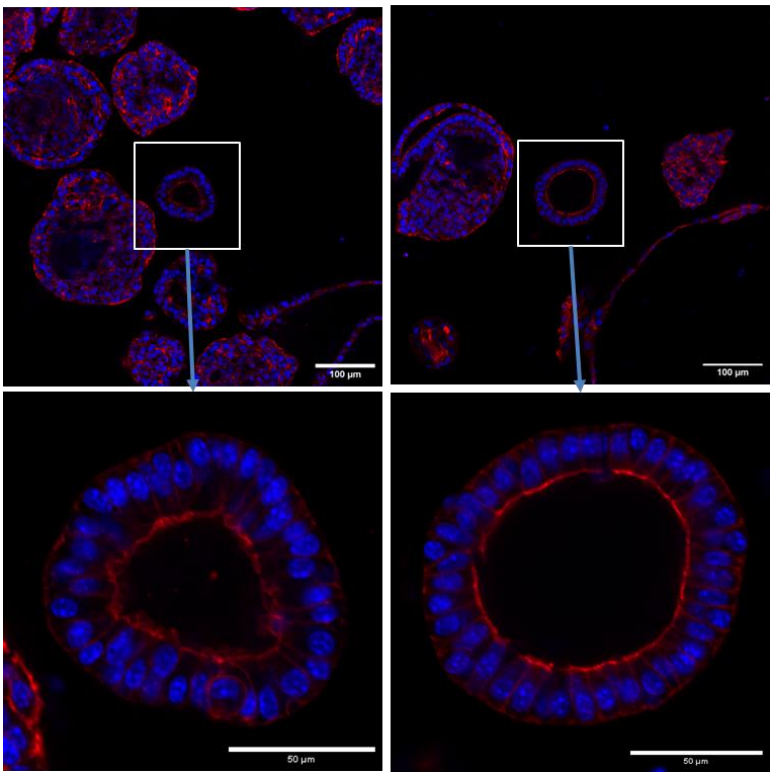

Figure 14: Immunostaining of Day 14 344SQ-shCTRL

The two images provide a comparison between an irregular acinus versus a rounder acinus and showcase the F-actin located on the apical side of the lumen as well as nuclei alignment radially.

3.2.2.2 344SQ_ZEB1

Figures 15 and **16** show the results after immunostaining two different samples of the 344SQ_ZEB1 acini.

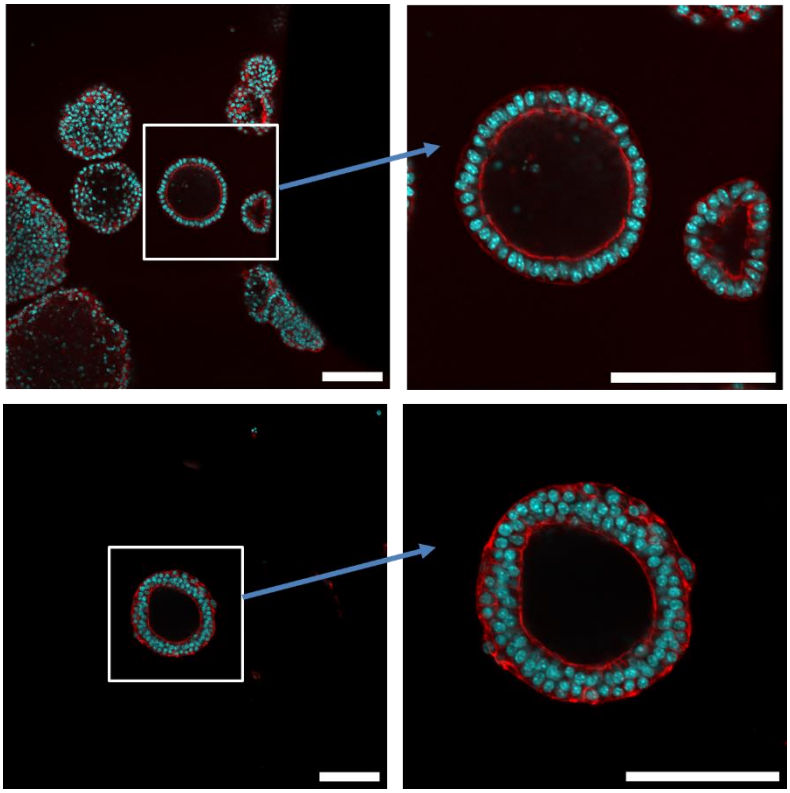

Figure 15: Immunostaining of Day 14 344SQ_ZEB1

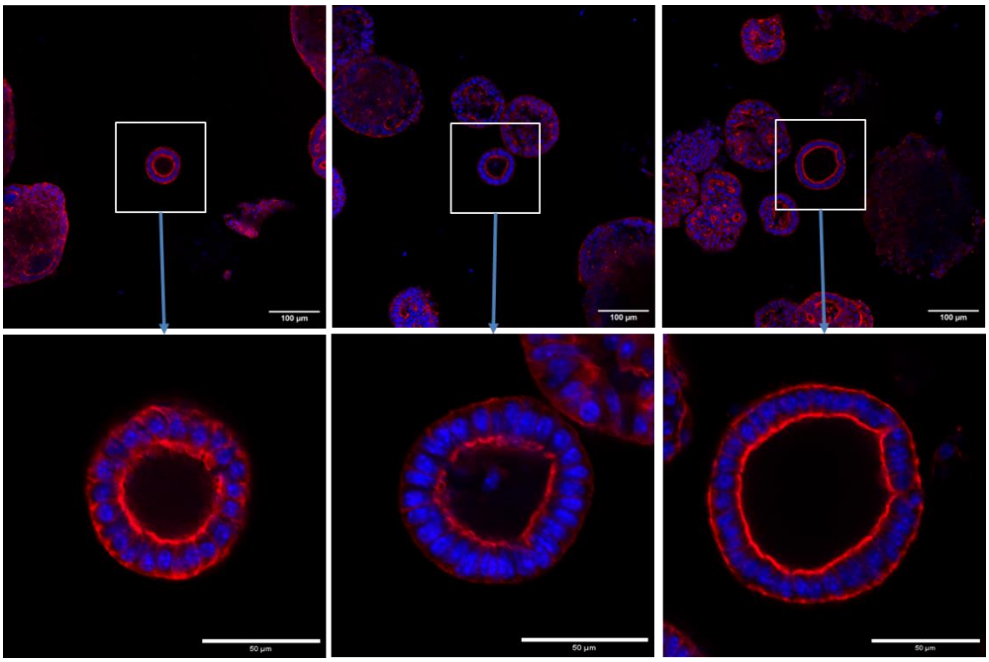

Figure 16: Immunostaining of Day 14 344SQ_ZEB1

Like the 344SQ_shCTRL stained images, these images provide insight into the structure of the acini and nuclear alignment. For the single-cell-thick lumens, the nuclei clearly showed radial alignment. For thicker lumens, some of that alignment is not as apparent.

3.3 Discussion

3.3.1 Impact of Cell's Inherent RhoA Regulation

Neither the 344SQ_ZEB1 nor the 344SQ_shCTRL acini samples everted. While additional experimentation is ongoing to evert the 344SQ_shCTRL acini, the lack of eversion with the 344SQ_ZEB1 acini may suggest that some ideal regulation of RhoA is required for eversion to occur. With high RhoA expression, as seen with the 393P_ZEB1 cells, the acini were unable to form lumens. With low RhoA expression, shown by the 344SQ_ZEB1 cells, lumens did form but RhoA activation resulted in slow lumen collapse rather than the forceful collapse required to evert the acini.

3.3.2 Proposed Improvements to Acini Procedures

Throughout the course of the experiments, changes to the protocol were made in response to difficulties encountered. One issue involved the initial Matrigel coating to prep the slides during the acini manufacturing process. The confocal microscope had z-axis range limitations, and the thickness of the Matrigel coating resulted in an inability to image a significant amount of acini with nicely developed lumens. Simply increasing the amount of physical spreading with the pipet tip resulted in the Matrigel clinging to the sides of the well. Instead, procedures were adapted to include chilling the chamber slides as well as the pipet tips in 4^{o}C storage. These chilled slides and tips helped the Matrigel disperse in a thinner layer.

Another proposed improvement to 344SQ cell line acini experiments is to shorten growth period. As shown in the 344SQ_shCTRL acini, their loss of nicely developed lumens due to their

interaction with non-lumen acini may outweigh the benefit of having additional time for the lumens to develop. Therefore, there is evidence to suggest changing the experiment protocol from a 14-day period to a 10- to 12-day period. Due to their similar growth behaviors, any changes to the timeline for the 344SQ_shCTRL cells would also be advisable for the 344SQ_ZEB1 cell line.

3.3.3 Future Directions

This investigation into acinar behavior is far from complete. Further efforts are recommended for everting the 344SQ_shCTRL acini since they exhibit regular levels of RhoA regulation. Shortening the lumen development timeline and further increasing the RhoA treatment dosage may increase the likelihood of eversion occurring. To provide quantitative reasoning for the lack of eversion with the 344SQ_ZEB1 acini, potentially the Law of LaPlace could provide more insight into relationships between RhoA regulation and forces within the acini. However, the likelihood for eversion may be a function of many other factors such as size or thickness of the lumen. Additionally, the stained images propose inquiries into acinar structure, notably the radial alignment of the nuclei in the acini having lumens with one-cell thickness. This alignment could be investigated further by creating a method to mathematically quantify this phenomenon. Other future projects include exposing the acini to other cell types, such as bacteria and neutrophils, to see how the acini respond as well as repeating the procedures in this project with additional untested cell lines to further characterize endothelial acinar behavior.

4. CONCLUSION

Cell growth, lumen development, imaging logistics, and the many potential factors impacting acinar behavior makes this investigation a long-term project. During the course of this undergraduate research, initial characterization of 344SQ_shCTRL, 344SQ_ZEB1, and 393P_ZEB1 were recorded such as growth patterns, response to RhoA experimentation, and cellular structure. From these observations and learning the experimentation process, protocols for handling these cell lines were improved. The foundational observations made from this project encourage further experimentation to address current gaps in the results regarding why these behaviors occur, successfully record an eversion occurring, and study nuclear alignment quantitatively with the ultimate goal of furthering understanding of cancer mechanisms.

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