# MITOCHONDRIAL ADAPTATION DURING ACINI FORMATION

An Undergraduate Research Scholars Thesis

by

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# ABSTRACT

Mitochondrial Adaptation During Acini Formation

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The role mitochondria play in mammary gland development is not fully understood and therefore provides an interesting opportunity to study novel interactions. The purpose of this project is to elucidate unknown cellular mechanisms in normal mammary epithelial cell differentiation. To gain a better understanding of the processes involved in mammary epithelial cell differentiation, we differentiated EpH4 mouse mammary epithelial cells in a three-dimensional, acini model. Utilizing a three-dimensional model more accurately recapitulates the structure of ducts in the mammary gland. Initially, we sought to further understand the role of *Sim2s* in mammary gland ductal development by manipulating *Sim2s* expression in EpH4 cells. We were able to confirm the role *Sim2s* plays in mammary ductal development. We then employed the use of *pMitoTimer* to trace mitochondrial networks and turnover during acini formation. By creating a new cell line with a tetracycline-inducible construct of *MitoTimer*, we established a new way to observe mitochondrial turnover over a longer period. We discovered that mitochondria turned over during the course of the acini formation. To understand the role of mitochondrial fusion, we silenced the expression of the key mediator of inner mitochondrial membrane fusion, *Opa1*, during acini formation. We found that *Opa1* expression was necessary

for acini formation. To understand the role of autophagy in acini formation, we pharmacologically inhibited autophagosome degradation with Bafilomycin A1. We have previously found that loss of autophagic flux inhibited differentiation-dependent gene expression in two-dimensional differentiation of mammary epithelial cells. In conclusion, we have shown that mitochondria do in fact adapt in a three-dimensional model of mammary epithelial cell differentiation, and this adaptation is required to reach a fully functional differentiation state.

# ACKNOWLEDGEMENTS

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# CHAPTER I

# INTRODUCTION

Our lab has found that Singleminded-2s (Sim2s, bHLH/PAS transcription factor) plays an essential role in mammary epithelial cell differentiation and is developmentally regulated (1). Based on previous research conducted in our lab, we sought to further study mouse mammary epithelial cell lines. Our studies in two-dimensional cell culture models prompted us to manipulate mitochondrial dynamics to further understand mechanisms of normal development. A three-dimensional cell culture model will be used to emulate past research to observe how mitochondria adapt in an environment similar to that of normal biology. This research is clinically relevant because it is known that disruption of the mitochondria is associated with neurodegenerative and metabolic diseases such as Parkinson's disease, Alzheimer's, and cancer (2). Furthering our understanding of these mechanisms will overall contribute to the current studies in our lab and beyond in future medical research. In developing our questions for this project, previous research from our lab has shown that mitochondrial adaptation can be observed in a two-dimensional cell culture model.

## *Sim2s*

Previous research in our lab has revealed that *Sim2s* plays a role in normal mouse mammary gland development. Figure 1 depicts the relative expression of *Sim2s* in HC11 mouse mammary epithelial cells that were induced to differentiate (1). Compared to undifferentiated cells, cells that were induced with prolactin and glucocorticoid to differentiate showed high levels of *Sim2s* expression (1). This suggests that *Sim2s* plays a role in differentiation as its

expression progressively increases during differentiation in mammary epithelial cells.

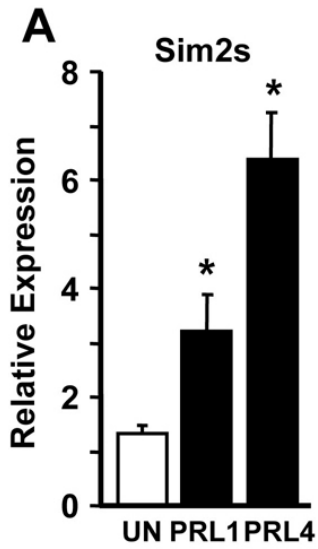


Figure 1. *Sim2s* expression in HC11 cells grown in two-dimensional culture (1).

To understand the effect of *Sim2s* on development, we sought to knock out and overexpress *Sim2s* in normal mouse mammary epithelial cells. We used a cell line denoted as EpH4 from mid-pregnant BALB/c mice that are spontaneously immortalized and nontumorigenic (9). By stably transfecting a short hairpin RNA, denoted as *shSim2s,* into our cells, we will knock down *Sim2s* expression by degrading mRNA associated with *Sim2s*. We will employ a control for this knock down by stably transfecting a plasmid with the same sequence but scrambled. To overexpress *Sim2s*, we will stably transfect a *Sim2s* plasmid that will allow us to observe changes in development. The control for overexpression will be the transfection of an empty plasmid.

**Mitochondrial Turnover**

HC11 normal mouse mammary epithelial cells transfected with *pMitoTimer* allowed us to visualize the phenomenon of mitochondrial turnover. These cells were allowed to differentiate over a 48 hour time period and imaged at various time points to observe fluorescence. Figure 2

shows that transfection of *pMitoTimer* into the HC11 cells was successful because they fluoresced red and green. Images taken at hour 0 through hour 24 show that mitochondria will turnover, meaning the mitochondria are being oxidized and targeted for autophagy (3). When the cells fluoresce green, this is indicative of newly synthesized cytochrome c oxidase subunit VIII, the terminal enzyme in mitochondrial respiration (3). When the cells fluoresce red, cytochrome c oxidase subunit VIII is oxidized (3).

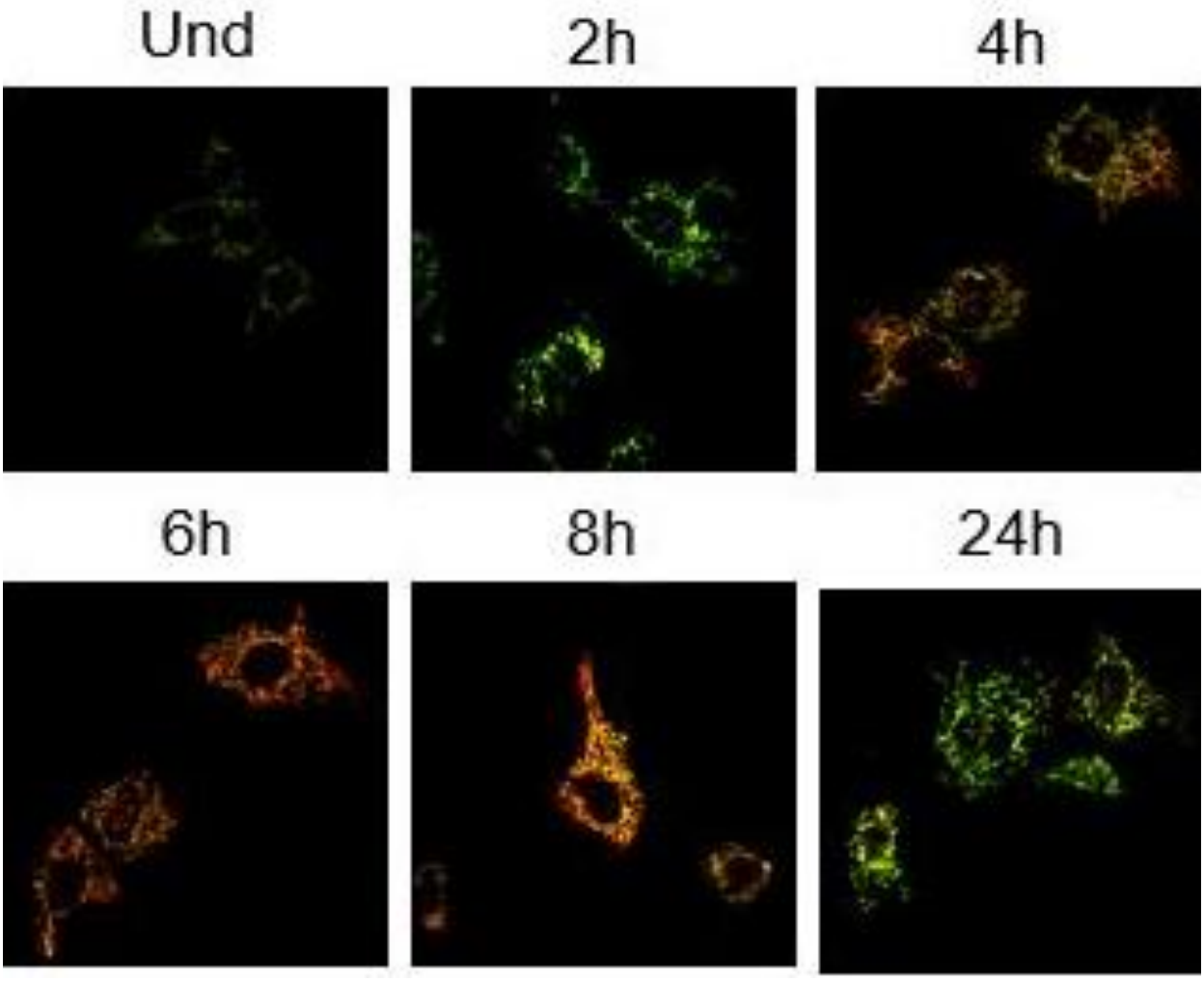


Figure 2. HC11 cells time course with *pMitoTimer*.

To understand what mechanisms are at work during mitochondrial differentiation in the three-dimensional model, a fluorescent probe called *pMitoTimer* will be used. The *pMitoTimer*

incorporated into the cells will fluoresce either red or green depending on mitochondrial conditions, and this will provide information in regards to overall mitochondrial health (3). This technique will allow us to visualize changes during differentiation and acini formation.

**Mitochondrial Fusion**

Additionally, our lab has studied the process of mitochondrial fusion in an HC11 two-dimensional cell culture model. Beta-casein (*Csn2*) expression is associated with differentiation, and detection of *Csn2* expression can be used as an indicator of differentiation status in our model of mammary epithelial cell differentiation (10). The gene expression of *Csn2* gradually increases over the course of cellular differentiation and then decreases as cells undergo apoptosis (Figure 3).

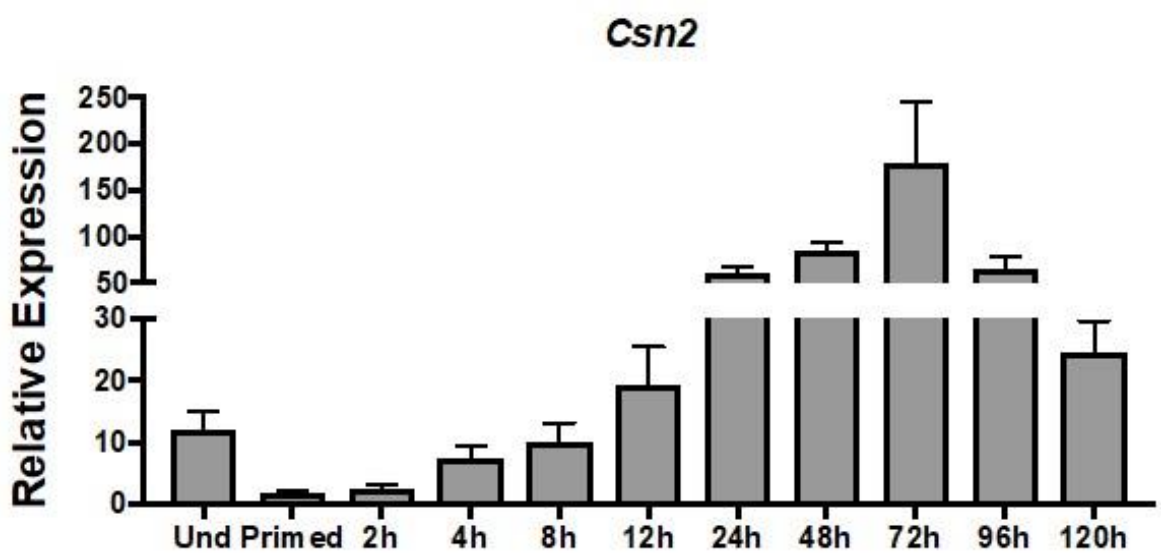


Figure 3. *Csn2* expression in differentiating HC11 cells.

In studying mitochondrial fusion, our lab has shown that optic atrophy 1 (*Opa1*) is involved in differentiation. By silencing *Opa1* expression in HC11 cells, we found that *Csn2* expression was decreased at the time of peak differentiation. Figure 4A showed that silencing *Opa1* was effective and in Figure 4B, silencing *Opa1* affected the relative expression of *Csn2*.

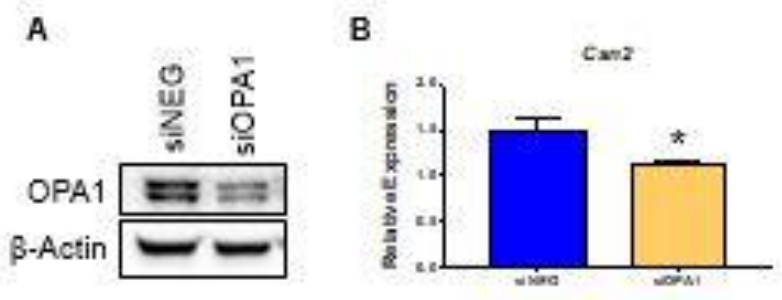


Figure 4. (A) Western blot analysis. (B) Expression of *Csn2* in relation to presence of *siOPA1*.

To observe the role of mitochondrial fusion in a three-dimensional model, a silencing RNA targeted to *Opa1* will be used. *Opa1* is a known mediator of fusion of the inner mitochondrial membrane and promotes electron transport chain activity by association of cristae structures (4). We aim to see the effects on differentiation in a three-dimensional model after silencing the expression of this gene.

**Autophagy**

Autophagy is an essential intracellular recycling system that works to sustain cellular metabolism and maintain mitochondrial homeostasis (5). Previous research has demonstrated that autophagy is required for functional differentiation; therefore, we aim to further understand the mechanism of autophagy in differentiation (7-8). To observe the role of autophagy in differentiation, we will pharmacologically inhibit autophagy with Bafilomycin A1.

**Objectives**

The project aims to answer the following objectives to contribute to known information about differentiation in mammary gland development.

- To observe the effect *Sim2s* has on mammary epithelial cell differentiation.
- To use a fluorescent reporter probe to observe possible intracellular changes in mitochondria.

- To discover if mitochondrial fusion is required for differentiation by silencing the gene associated with fusion control.
- To discover if autophagy is required for differentiation by pharmacologically inhibiting autophagic flux.

The results from each objective will contribute to the known mitochondrial mechanisms we understand and will provide new information regarding the mechanisms in a model similar to that of normal gland biology.

# CHAPTER II

# MATERIALS AND METHODS

## Cell Lines

In the following protocols, the EpH4 cell line of normal mouse mammary epithelial cells will be used. These cells are gift from Margret Neville at the University of Colorado Health Science Center in Denver. The cells are maintained in a normal growth media consisting of DMEM F12, fetal bovine serum, gentamycin, insulin, and mouse epidermal growth factor (EGF).

For retroviral transfection, Phoenix-AMPHO 293T cells were purchased from ATCC. The cells were maintained as recommended in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

## Acini Formation Protocol

The objectives will be answered by employing a three-dimensional cell culture model using EpH4 cells. This protocol is adapted from a paper that studied MCF10A human mammary epithelial cells. This protocol is advantageous because it allows for easier observation of the acini structures and provides a suitable environment for larger acini structures to grow (6). The basic structure for the acini formation protocol will primarily follow the recommendations for human mammary epithelial cells and will take place over the course of 10 days (6).

The cells were plated in a multi-well plate. A single layer of growth factor reduced Matrigel was placed at the bottom of the well and allowed to solidify. A single-cell suspension of EpH4 cells was then placed on top of the Matrigel in an assay medium containing 2% Matrigel, 2% horse serum, 50μg/ml gentamycin, 10μg/ml insulin, 2μg/ml hydrocortisone, and 6μg/ml

prolactin. The cells were maintained in an incubator at 37°C for 10 days. The assay media was changed every 2 to 4 days. Figure 5A diagrams the process. Furthermore, in Figure 5B, the course of expected normal proliferation is diagramed. This progression will be documented.

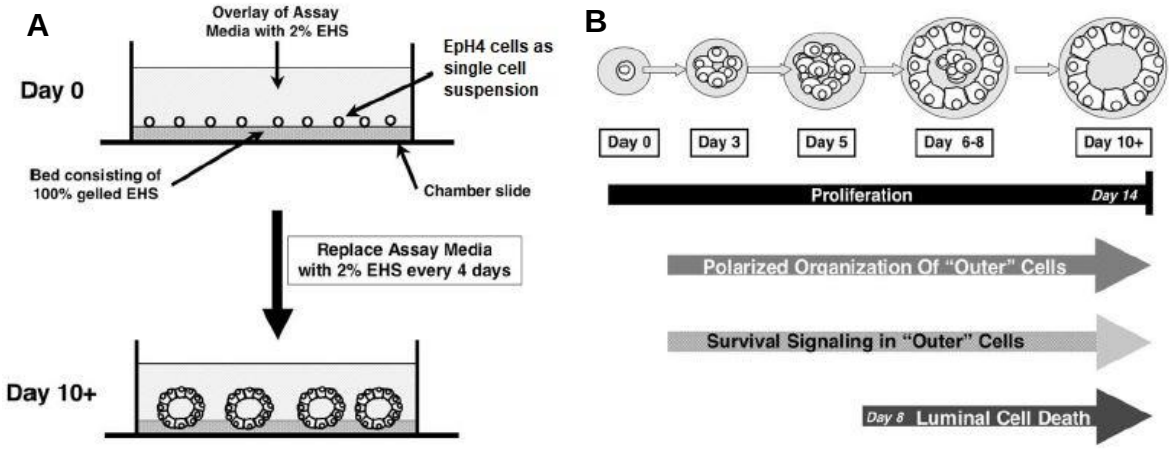


Figure 5. Growth of acini in Matrigel. Diagrams from J. Debnath et al (6).

From these basic steps, the protocols will differ regarding which objective we aim to answer. To address *Sim2s*, EpH4 cells with *Sim2s* knocked down and overexpressed will be grown for acini formation. For the next objective, the protocol will be run to observe changes in the mitochondria using a fluorescent reporter probe, *pMitoTimer*, in the acini formation. In the third objective, we will use a silencing RNA, *siOPA1*, to observe inhibition of mitochondrial fusion during acini formation. For the last objective, the protocol will undergo drug-therapy with Bafilomycin A1 to see if autophagy is required for differentiation in the cell culture model.

**Transient Transfection**

This protocol was utilized for transfecting *pMitoTimer* and *siOPA1* constructs into the EpH4 cells. A mixture of Optim-MEM, Genejuice, and *pMitoTimer* was added dropwise to the growth media on a plate of EpH4 cells. The cells were incubated at 37°C for 24 hours.

**Retroviral Transfection**

This protocol was utilized for *Sim2s* and for the tetracycline-inducible *MitoTimer* construct. A mixture of Optim-MEM, Genejuice, and desired plasmid were added to a plate of Phoenix-AMPHO 293T cells and incubated overnight. The next day this viral media was replaced with a EpH4 media-10%DMEM media mixture. On day 3, the viral media was filtered and polybrene was added. This viral media was added to the EpH4 cells and spun for an hour to enhance the success of the transfection. This process was repeated on day 4. On day 5, the media was changed on the EpH4 cells, and selection of cells with puromycin began the following day. This selection step ensures that only successfully transfected cells survive.

# CHAPTER III

# RESULTS

## EpH4 Acini Growth Progression

To observe how the EpH4 cells would adapt and grow with Matrigel, images were taken with a ZEISS Discovery microscope over the course of the 10 day protocol. These cells were transfected with *pMitoTimer*, which did not have any observed effects on the development of the acini formation. Figure 6 shows the progression from day 1 to day 9.

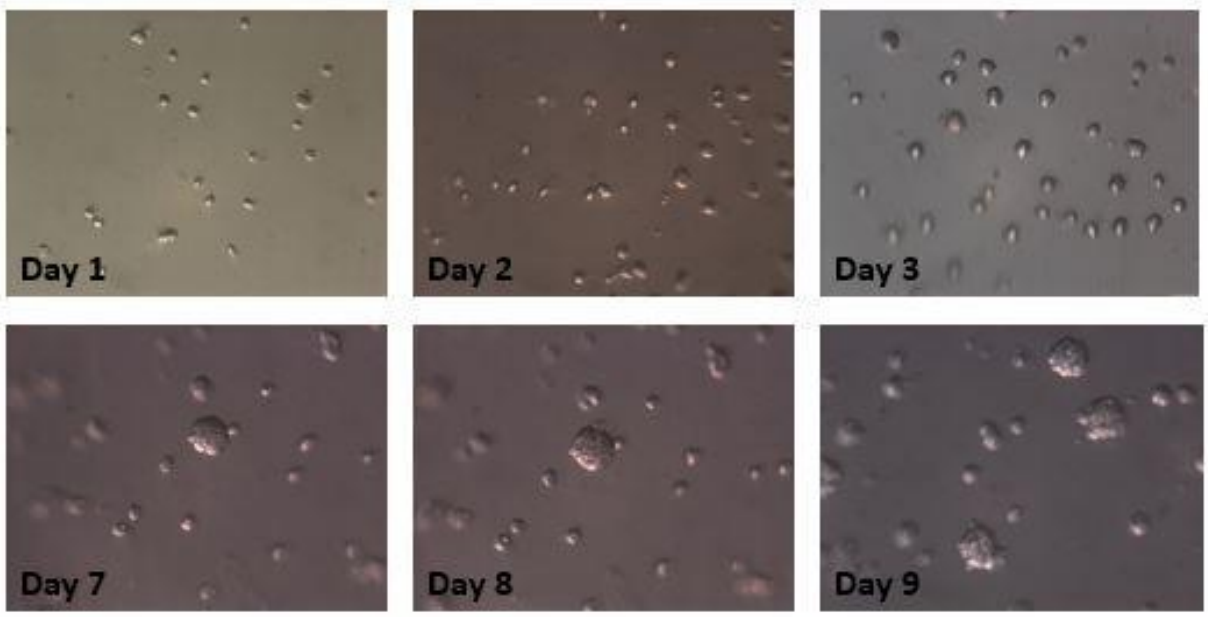


Figure 6. Progression of acini growth.

## *Sim2s* with Acini Formation

EpH4 cells were previously transfected with overexpression and knock down constructs of *Sim2s* in order to observe the effect on acini growth. On day 14 of the protocol, the images in Figure 7 were taken with a ZEISS Discovery microscope.

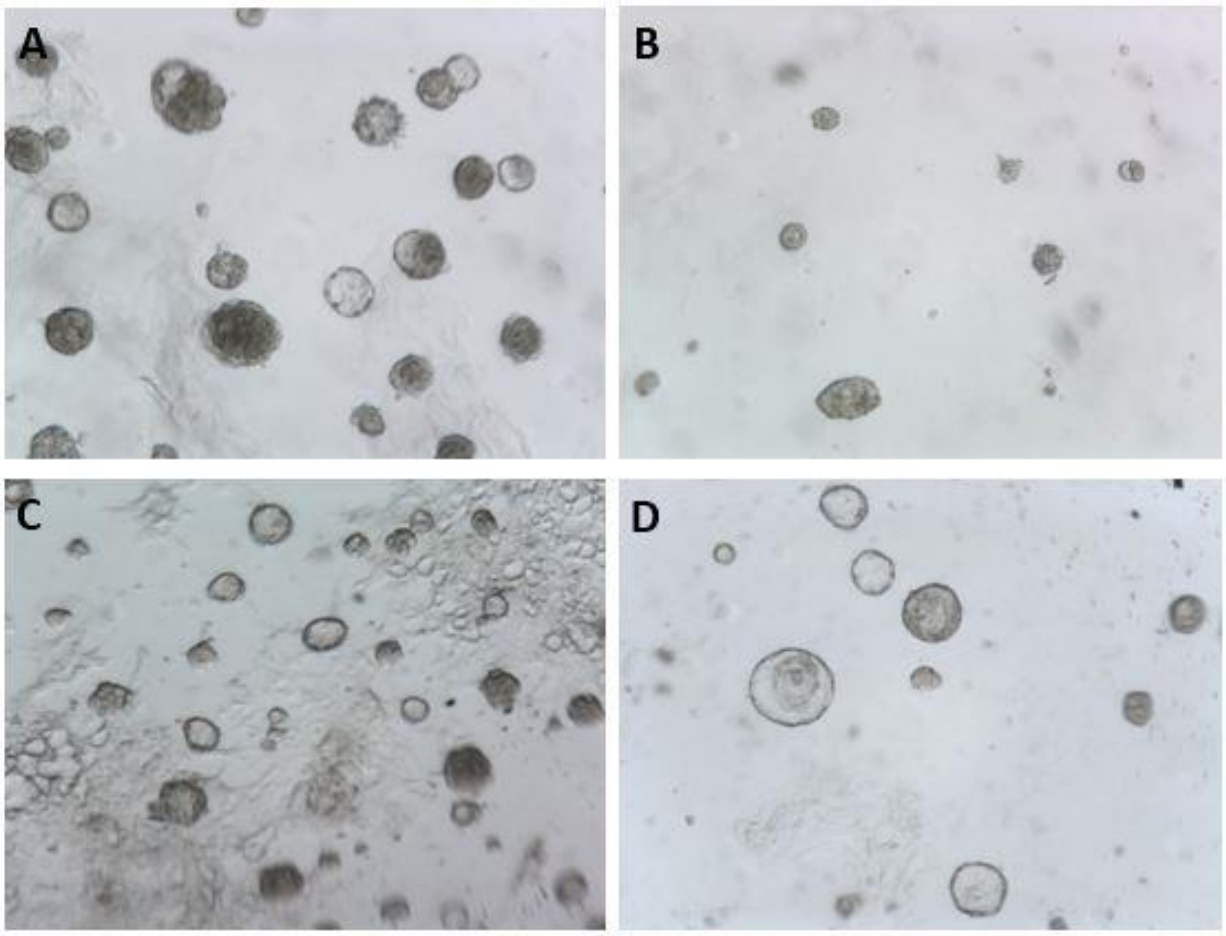


Figure 7. *Sim2s* and EpH4 acini growth. (A) *sh*Control for knock down of *Sim2s*. (B) *shSim2s* for knock down of Sim2s. (C) Control for *Sim2s* overexpression. (D) *Sim2s-flag* for *Sim2s* overexpression.

***pMitoTimer* with Acini Formation**

First, we sought to determine the longevity of the *pMitoTimer* plasmid in the EpH4 cells. For this, we selected three different time points to insert the plasmid during acini growth. This would help determine the best technique to ensure the plasmid could be observed and whether the plasmid could be inserted into the cells during acini growth. The cells underwent three time-point transfections in order to observe any differences in response. The *pMitoTimer* plasmid was transiently transfected into the cells prior to plating (pre-treatment), on day 1, and on day 4 of

acini growth. Images from each time point were collected. The cells were observed under a ZEISS Discovery microscope and images were recorded throughout the protocol.

Once the best way to transfect the MitoTimer into the cells was determined, the acini protocol was repeated with only pre-treated cells. Acini growth was recorded for day 1 through day 3. The growth and fluorescence were recorded. The data was not completely collected throughout the 10 day protocol because on day 4 the cells died. This was concluded to be due to lack of media in the wells from evaporation overnight, and unfortunately, the data was not conclusive. Additionally, during this experiment the fluorescent bulb on the Discovery microscope burnt out and images for fluorescence were not able to be recorded after day 3. Figure 8 shows images captured from the Discovery microscope of the acini growth and the fluorescence of the mitochondria in cells pre-treated with *pMitoTimer*.

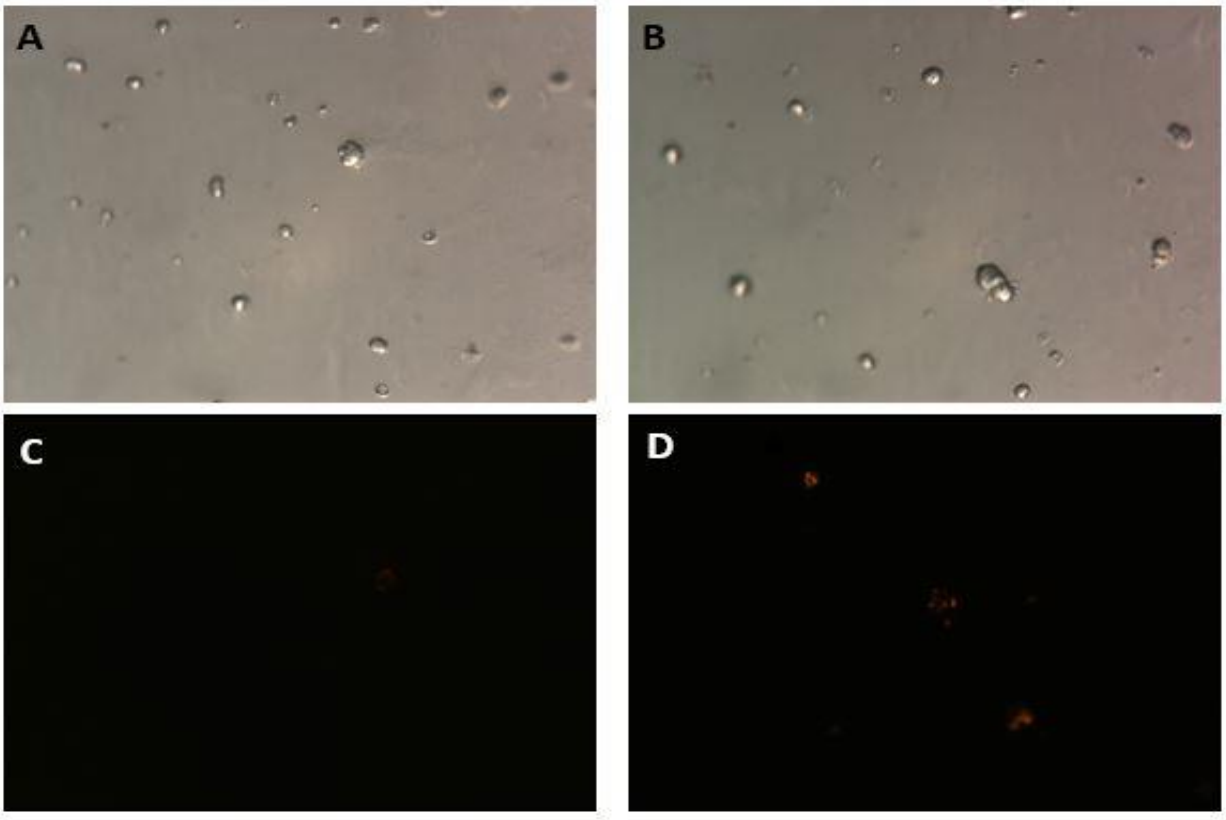

Figure 8. (A) Day 1 of EpH4 acini growth with *pMitoTimer*. (B) Day 3 of EpH4 acini growth with *pMitoTimer*. (C) Day 1 of EpH4 growth with red fluorescence. (D) Day 3 of EpH4 acini growth with red fluorescence.

**Tetracycline-Inducible Construct of *MitoTimer***

Since transient transfection is not a stable model, we turned to a tetracycline inducible model for the cells. EpH4 cells were retrovirally transfected with a tetracycline-inducible construct of *MitoTimer*, pTRE-tight-*MitoTimer*. Figure 9 shows the successful incorporation of the plasmid into EpH4 cells grown in a monoculture.

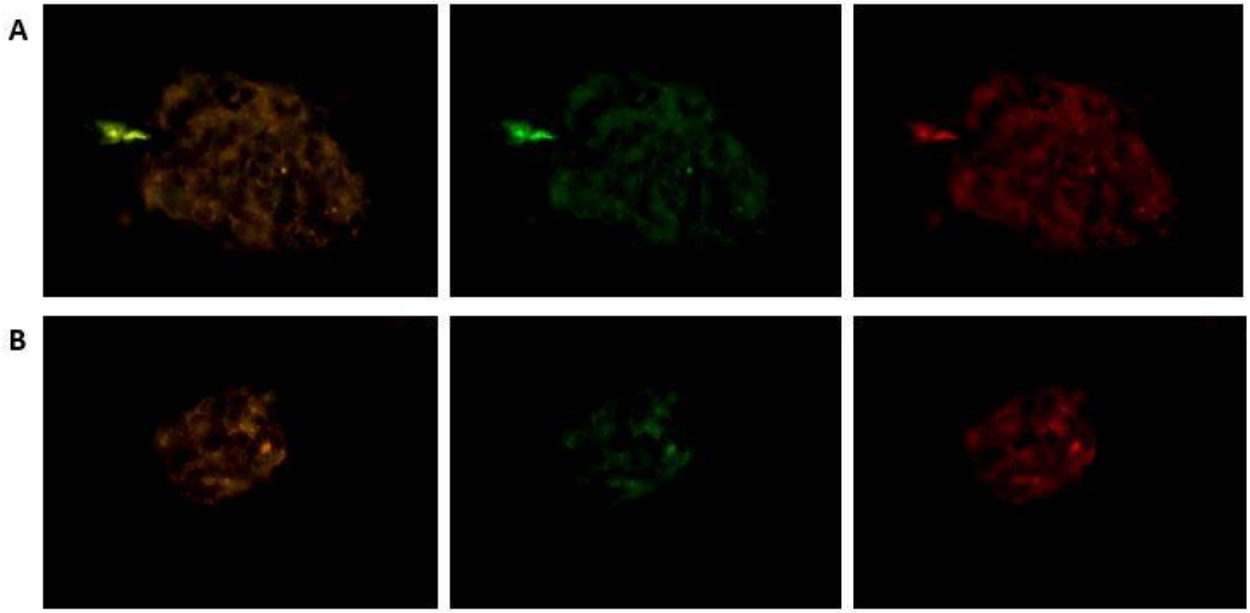


Figure 9. (A) Fluorescence 3 hours after doxycycline treatment. (B) Fluorescence 24 hours after doxycycline treatment.

***siOpa1* with Acini Formation**

The *siOpa1* was transiently transfected in the EpH4 cells prior to plating in Matrigel. The cells were transiently transfected with three different silencing RNAs in order to target three regions of *Opa1*. These silencing RNAs are denoted as *siNEG* for our control, *siOpa1 A*, *siOpa1*

*B*, and *siOpa1 C*. RNA was harvested from the cells when there was a noticeably different phenotype shown in the growing acini as seen in Figure 10.

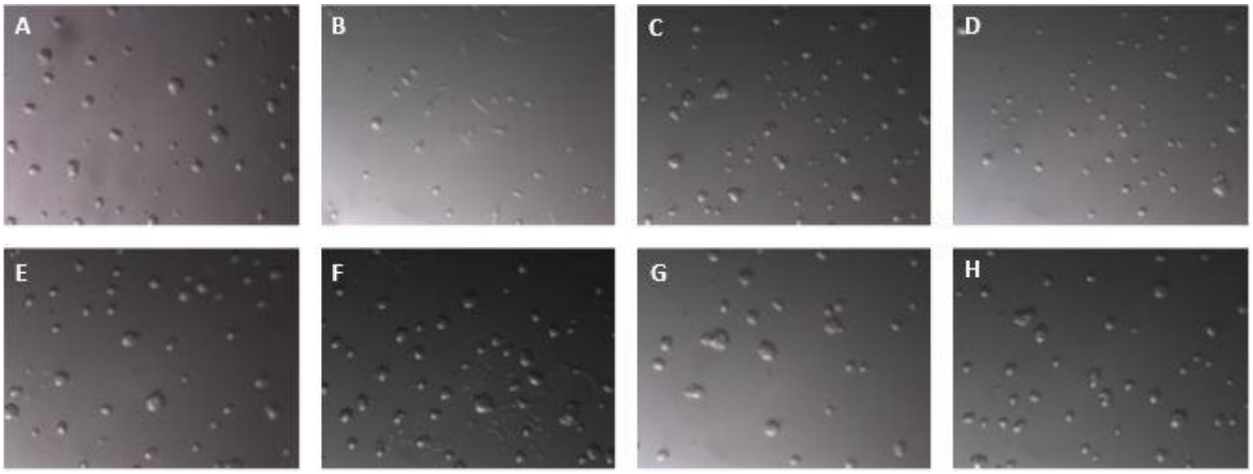


Figure 10. (A) *siNEG* Control in EpH4 cells on day 1. (B) *siOpa1 A* in EpH4 cells on day 1. (C) *siOpa1 B* in EpH4 cells on day 1. (D) *siOpa1 C* in EpH4 cells on day 1. (E) *siNEG* Control in EpH4 cells on day 4. (F) *siOpa1 A* in EpH4 cells on day 4. (G) *siOpa1 B* in EpH4 cells on day 4. (H) *siOpa1 C* in EpH4 cells on day 4.

By quantitative PCR analysis, the relative expression of *Opa1* and *Csn2* was analyzed as shown in Figure 11.

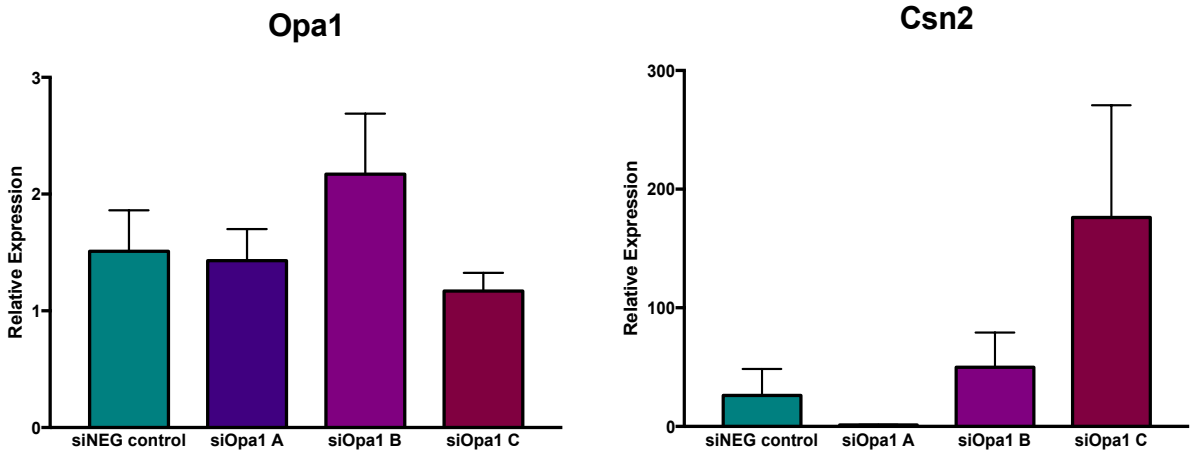


Figure 11. *Opa1* and *Csn2* expression in EpH4 cells.

**Bafilomycin A1 with Acini Formation**

EpH4 cells were treated with Bafilomycin A1 at different time points in the acini protocol. One group of cells was treated prior to plating the cells in Matrigel (pre-treated) with 1nM of Bafilomycin A1. One group was treated with Bafilomycin A1 after plating in Matrigel. There was a control group that was treated with DMSO as a vehicle control. Figure 12 shows the differences in acini growth of the EpH4 cells.

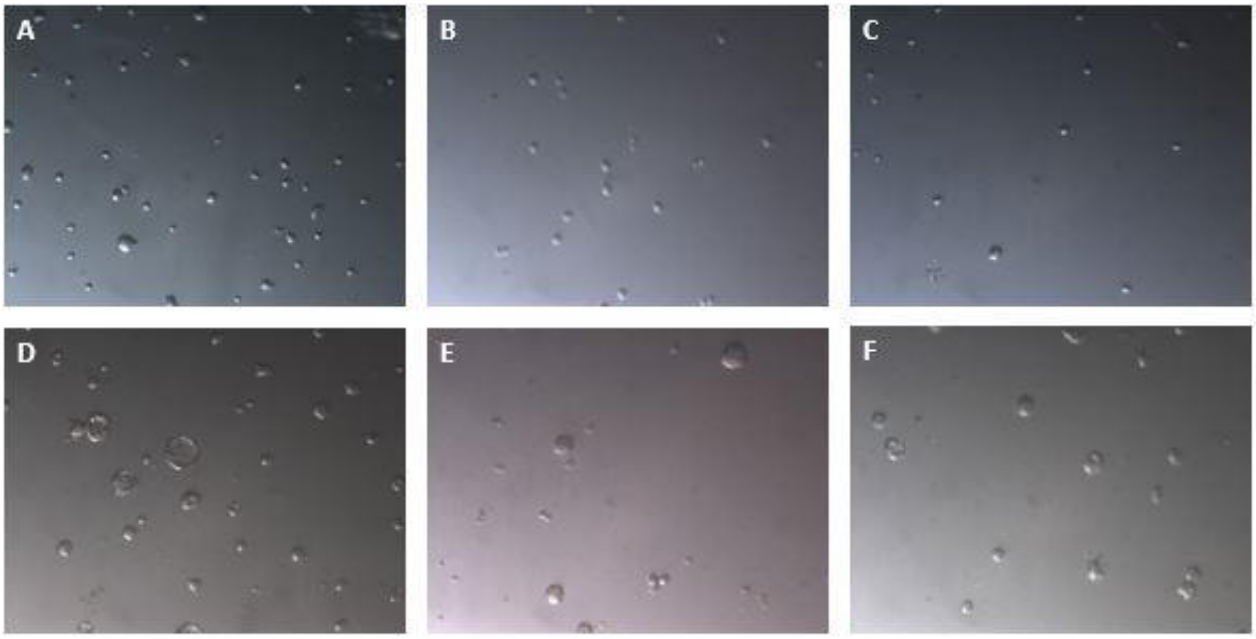


Figure 12. (A) Bafilomycin A1 pre-treated EpH4 cells on day 1. (B) Bafilomycin A1 EpH4 cells on day 1. (C) DMSO control EpH4 cells on day 1. (D) Bafilomycin A1 Pre-treated EpH4 cells on day 6. (E) Bafilomycin A1 EpH4 cells on day 6. (F) DMSO control EpH4 cells on day 6.

# CHAPTER IV

# DISCUSSION

We know from previously published work from our lab that *Sim2s* plays a role in mammary epithelial development (1). We also know from ongoing research in the lab that mitochondrial turnover, fusion, and autophagy are essential mechanisms for mammary epithelial cell differentiation. The three-dimensional, acini model allowed for us to observe these known phenomena at a new angle, which had not previously been done before in our lab. The acini model better emulates normal cell biology and furthered our understanding of the cellular mechanisms behind mammary epithelial cell differentiation.

To initially test the three-dimensional acini model, we examined the effect of *Sim2s* on EpH4 cell differentiation. In Figure 7A and B, we knocked down the expression of *Sim2s* and observed the resulting acini growth. The control cells developed large, hollow acini structures, as expected. The *Sim2s* knock-down cells were considerably smaller and lacked the formation of a lumen. This suggests that *Sim2s* is essential for mammary gland formation, because the lack of *Sim2s* failed to produce the alveolar structure typical of a mouse mammary gland. Furthermore, in Figure 7C and D, EpH4 cells overexpressing *Sim2s* were subjected to acini formation. The control cells showed well-developed, large, hollow acini structures. The *Sim2s* overexpressing EpH4 cells formed hollow acini structures that were larger overall than the control. This suggests that *Sim2s* contributes to and enhances the development of mammary alveoli. To address our question, these data support past research of the developmental role of *Sim2s* in mammary ductal development.

Mitochondrial turnover in acini formation was observed when *pMitoTimer* was transiently transfected into the EpH4 cells. The transfection of *pMitoTimer* and growth of acini was performed twice to select a time point for transfection that would be successful and to determine if successful transfection could be repeated. For the pre-treated cells, it was determined that *pMitoTimer* was consistently observed in the cells on days 1 through 4. For the cells that underwent the transfection on days 1 and 4 there was no evidence of successful transfection. The day 1 and day 4 cells did not fluoresce under the microscope, and it was concluded that the cells would not uptake the plasmid as the cells began to differentiate. For replication of this experiment, we can conclude that transient transfection prior to plating is best for the successful uptake of *pMitoTimer* into the cells. Additionally, the cells did not fluoresce past day 5. This was not surprising because as the mitochondria turnover, this tag gets cycled out of the cell. In a second run of the experiment with only *pMitoTimer* pre-treated cells, the experiment was not completed. This protocol stills shows that transfecting prior to plating in Matrigel was a successful technique and allowed for the mitochondrial turnover to be observed for at least three days. Due to equipment failure, the fluorescence was not able to be observed past day 3 of growth. To address our question, this information shows that mitochondria do in fact turn over during the course of differentiation in a three-dimensional model.

As *pMitoTimer* is cycled out of the mitochondria as they turnover, we turned to a more permanent model. The transient transfection, though successful, limits our ability to observe turnover throughout the full differentiation of EpH4 cells. By utilizing a tetracycline-inducible version of *MitoTimer*, we were able to successfully create a stable EpH4 cell line. These cells are denoted as pTRE-tight-*MitoTimer* EpH4 cells. We found that treating cells with 1μM of doxycycline could initiate the expression of *MitoTimer*. We treated the cells with doxycyclin and

observed their fluorescence after 3 and 24 hours. Although the cells shown in Figure 9 are in two-dimensional culture, future steps would be to utilize these cells in the three-dimensional, acini model. By having this permanent construct in the cells, we will be able to observe mitochondrial turnover over a longer period during cell differentiation.

Mitochondrial fusion is an essential process in two-dimensional cellular differentiation. In Figure 10, we saw significant acini growth. It was expected that silencing *Opa1* would affect the development of the acini structures. Using quantitative PCR analysis, we found that the transfection was not successful due to remaining expression of *Opa1* in all three variants of *siOpa1*. The growth was expected to be impaired between day 1 and day 6 of differentiation. One anomaly that we saw was there were cells that did not form acini structures and grew as if they were in a two-dimensional culture. This can be seen in Figure 10B where the EpH4 cells both formed acini and a flat growth pattern. We harvested these cells for RNA when we saw cells that did not form acini structures, which we hypothesized was indicative of the silencing of *Opa1*. However, as we saw from the quantitative PCR analysis, that was not the case. The cells that failed to form acini structures was possibly due to lack of Matrigel where they were plated. This prevents the cells from establishing a base for an acini structure to develop. Though the data here is not conclusive for what we sought to answer, this data does point us in a new direction for this phenomena. As with *pMitoTimer*, it might be better to try this with a tetracycline-inducible model. This would establish a more permanent model that would allow for the observation of the effects of silencing *Opa1* over the course of differentiation. This would also allow us to confirm the role *Opa1* plays in normal mammary epithelial cell differentiation.

Autophagy is another key cellular mechanism in the differentiation of mammary epithelium. Our images in Figure 12, show the results of treating EpH4 cells with Bafilomycin

A1 and allowing them to grow in Matrigel. The difference in day 1 of pre-treated cells (Figure 12A) versus day 6 (Figure 12D) is interesting, because the hollow acini structures were not expected to be seen. Figure 12B and E show the EpH4 cells that were treated with Bafilomycin A1 after plating. These cells formed acini structures, and on day 6 the cells from each group were harvested for RNA. The RNA quantity was too low to continue to quantitative PCR and was therefore inconclusive. This was suspected to be due to the low cell count plated in Matrigel. Based on these results, a greater number of cells will be used in future repetitions of this experiment. Another way of answering this question would be to employ a silencing RNA in a fashion similar to what we tried with *Opa1*. Autophagy related 5 (*Atg5*) is known mediator of autophagy, and by silencing its expression in EpH4 cells, we could observe how autophagy plays a role in differentiation (5).

This was the first time our lab looked at EpH4 cell differentiation in a three dimensional model. Utilizing the acini growth protocol helped us to visualize the process of differentiation in a model that is similar to normal cell biology of a mouse mammary gland. This information is important because it confirms the known cellular mechanisms that we have seen previously in the published literature and in current ongoing research in the lab. The data found in this project also provides future directions for studying cellular mechanisms in mammary epithelial cell differentiation. From this project, we were able to confirm that mitochondria due in fact adapt in a three-dimensional model.

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