

THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF CHEMICAL ENGINEERING

THE EFFECTS OF MICROENVIRONMENTAL STIFFNESS ON EXPRESSION OF
TROPOMYOSIN IN TGF β -INDUCED MAMMARY EPITHELIAL CELLS

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ABSTRACT

The effects of microenvironmental stiffness on mouse epithelial mammary gland cells undergoing epithelial-mesenchymal transition were observed in this experiment. The stiffnesses used represented the range of stiffness found in normal mammary gland epithelial tissue and cancerous breast tissue. Transforming growth factor (TGF)- β was used to induce the transition in the cells and the expression of tropomyosin was used as a read out of change in cell phenotype. The increased stiffness induced a higher degree of EMT as compared to the softer environments as observed by the increased expression of tropomyosin by immunofluorescence while EMT was not observed in TGF β treated cells on the soft environment as indicated by tropomyosin expression. There was no change in the cellular expression of tropomyosin in non EMT induced samples as stiffness varied. In TGF β -treated cells, the increased stiffness increased the tropomyosin expressed. The RhoA/SRF inhibitor, CCG-1423 caused the expression of tropomyosin to decrease, indicating the involvement of the RhoA pathway in the regulation of tropomyosin expression in EMT. This experiment showed EMT is controlled by both TGF β -signaling and the mechanical stiffness of the cellular microenvironment.

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Chapter 1

Introduction

One of the most frequently diagnosed cancers in women is breast cancer (1).

Breast cancer is uncontrollable growth of the breast tissue. The cancerous tissue is able to metastasize to other locations in the body, such as bone, lungs, and lymph nodes. In most cases, the primary cancer site is not the cancer that kills but rather the metastasis to other secondary sites. Because of the large impact breast cancer has, the field of breast cancer research is intensive. Cancers arise from the cellular level. Cells are not able to control their proliferation, and end up over growing with no check points, taking nutrients, supply and space from normal tissue.

A majority of breast cancers originate from epithelial cells. Epithelial cells are generally found as the lining of cavities and they are also found in many glands, which help with secretion. In mammary glands, the epithelial cells compose the ducts that secrete milk. Epithelial cells are marked by cell-cell junctions, cell-matrix junctions, and cellular polarity. The junctions between the cells and matrix are very important in the function of the cells (2). Tight junctions and cadherins comprise cell-cell junctions and function to keep cells connected to one another and polarize the epithelial cells. Integrins are proteins that bind the cell to the extracellular matrix. This protein structure allows for chemical and mechanical stimuli to pass between the cells and matrix.

Mesenchyme cells are a type of connective tissue cell that typically lack cell-to-cell connections. They do not have cell polarity and the cells have the ability to migrate

much more easily than epithelial cells. Myofibroblasts are specialized mesenchymal cells that are characterized by invasive motility and increased contractility.

Epithelial-mesenchymal transition (EMT) is a process where the cell morphology changes and epithelial cells lose cadherins, gap junctions, and cellular polarity, and gain increased motility. EMT is important in normal developmental processes such as the gastrulation stage of an embryo to form the 3 germ layers (3). EMT also plays an important role in initiating metastasis. EMT promotes breakdown of the cell-cell junctions in the primary tumor cells, allowing for the tumor to leave the primary tumor site and travel to other locations in the body, creating a secondary tumor. The cells can travel to nearby tissue, or they can penetrate the lining of blood vessels and travel via the circulatory system. This secondary tumor is often the cause of death.

EMT is observed by phenotype changes and the change in expression of certain proteins found in the epithelial cells. Epithelial cells are smaller, more rounded and tend to form groups with neighboring cells. Mesenchymal cells are spread out more, are not as clumped, and are elongated.

EMT is induced by both chemical and mechanical stimulations from the cell and the extracellular environment (4). Extracellular signals can activate various pathways which in turn activate and deactivate different transcription factors. Pathways such as Ras and Src can affect the transcription factors Snail and Slug (5). Slug and Snail repress the expression of E-cadherin, a cell-cell junction protein in epithelial cells. Additionally, the RhoA pathway is activated. The RhoA pathway helps with restructuring of cellular microfilament and stress fibers and plays a role in controlling the mechanical state of the

cell (6). The molecule CCG-1423 acts as an inhibitor to the RhoA pathway, which has an effect on the mechanical state of the cell.

Transforming growth factor (TGF)- β is used in this experiment to induce EMT. TGF β controls cell proliferation, differentiation, apoptosis, and the cell cycle (7). TGF β is normally released to regulate epithelial division. In tumor progression, TGF β activates pathways that cause the cell to lose their epithelial characteristics. TGF β binds to a transmembrane receptor, leading to signaling within the SMAD pathway (8). Cells exhibit a decrease in amount of cadherins and increase in motility.

Studies have demonstrated that stiffness of breast tissue increases during the development of breast cancer, with the stiffness of normal mammary tissue being approximately 200 Pa and that that of an average breast tumor being approximately 5000 Pa (9). Recent studies have shown that the microenvironmental stiffness regulates aspects of EMT. Here, we seek to determine whether the expression of tropomyosin, an actin-binding protein, is also controlled by TGF β and matrix stiffness (10). In this experiment tropomyosin is the marker used to observe the change in phenotype of the NMuMG cells. Tropomyosin is a protein that regulates the organization of the actin cytoskeleton (11).

The experiment will utilize normal mouse mammary gland normal epithelial cells (NMuMG cells). NMuMG cells are widely studied as a model system to investigate EMT. Cell adhesion and transcription factor effects are able to be studied easily with NMuMG cells.

To better understand how EMT is induced and to suggest ways to block its induction during cancer, it is essential to understand signaling pathways involved in the

transition (12). Understanding the signaling pathways can provide targets for treatment and prevention of tumor progression and metastases.

The purpose of the experiments outlined in this thesis is to observe the effects that microenvironmental stiffness has on NMuMG cells treated with TGF β . Furthermore, the effects of the RhoA pathway inhibitor, CCG-1423, are explored.

Immunofluorescence is used to quantify the changes in expression of tropomyosin. It is hypothesized that expression of tropomyosin will increase as stiffness increases, and will increase in TGF β treated cells. This is due to the induced contractility of cells as a function of increased substrate stiffness. In addition, CCG-1423 is hypothesized to lower the expression of tropomyosin, due to it inhibiting the RhoA pathway which is responsible for the reconstruction of actin in stiffer environments.

Chapter 2

Experimental Methods

Solution Preparation:

Two buffer solutions were used within the experiment. 1× phosphate buffered saline (PBS) was prepared from 10× PBS by dilution. Buffer solution 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was prepared at 50 mM and the pH was adjusted to 8.5 by addition of sodium hydroxide.

Cell Culture:

NMuMG mouse mammary gland epithelial cells were cultured in DMEM, 10% FBS, 10 µg/ml insulin and 50 µg/ml gentamicin.

Polyacrylamide Gel Preparation:

Square glass slides (22 mm x 22 mm) were placed in a 0.1 M NaOH solution for 15 minutes. The slides were then rinsed thoroughly with deionized water (dH₂O). The slides were then incubated in a solution of 2% (v/v) 3-aminopropyltrimethoxysilane (APTMS) in acetone and rinsed with acetone. The slides were dried completely and were then placed in 0.5% (v/v) glutaraldehyde in 1× PBS for 30 min and rinsed with dH₂O. Circular slides (22-mm diameter) were placed in 2% dichlorodimethylsilane in toluene for 30 min and were rinsed with methanol.

Polyacrylamide (PA) gels were prepared with the following stiffnesses: 330 Pa, 2030 Pa, and 6230 Pa. Table 2-1 shows the composition of the gels

Table 2-1: Composition of various stiffness PA gels

Stiffness (Pa)	Ratio (A/bisA)	Acrylamide monomer (μL)	bis-acrylamide (μL)	dH₂O (μL)
330	5-0.015	125	7.5	826
2030	5-0.06	125	30	839.5
6320	7.5-0.2	187.5	100	707

5 μL of 1% ammonium persulfate and 0.5 μL of TEMED were added to the solutions to initiate polymerization. The solution was thoroughly mixed and 20 μL of the solution was placed on the center of an activated square slide. A circular slide was placed on top of the solution as a cover. The samples polymerized within 30-45 minutes. After polymerization was complete, the slides and gels were placed in 1 \times PBS until ready for activation.

Polyacrylamide Gel Activation:

200 μL of 0.5 mM sulfo SANPAH was added on top of each of the gels and placed in the CL-1000 Ultraviolet Crosslinker for 10 minutes. SANPAH was aspirated off the gel, rinsed with HEPES and 200 μL of the sulfo SANPAH solution was added on top of the gels again and treated with UV light for 10 more min.

The gels were taken to the cell culture lab, and rinsed with HEPES buffer solution. 100 μL of fibronectin (10 $\mu\text{g}/\text{mL}$) was added on top of the gels, and the gels were incubated in the 4 $^{\circ}\text{C}$ fridge overnight.

Cell Plating and Treatment:

Gels were rinsed with 1 \times PBS to remove excess fibronectin. Cells were plated at 150,000 cells/well. The cells were treated with either 10 $\mu\text{g}/\text{ml}$ (2 $\mu\text{g}/\text{mL}$ stock solution) TGF β or 1% BSA, 4 mM HCl and with either 10 mM CCG-1423 or ethanol for 48 hours.

Table 2-2: Treatment for each sample

	Cell Plate 1	Cell Plate 2	Cell Plate 3	Cell Plate 4
Treatment	10 μ L TGF β 0.5 μ L EtOH	10 μ L TGF β 0.5 μ L CCG	10 μ L Control 0.5 μ L EtOH	10 μ L Control 0.5 μ L CCG

Tropomyosin Staining:

Cells were fixed with 4% formaldehyde and rinsed with PBS. Cells were blocked in 10% goat serum and 0.1% Triton-X-100 and incubated with primary antibodies that bind to tropomyosin. The samples were rinsed with PBS and stained with secondary antibodies. The nuclei were stained with Hoechst 33342.

Sample Imaging and Analysis:

Samples were imaged with a 20 \times objective using a Nikon Eclipse Ti-E fluorescence microscope. Integrated intensities were calculated using ImageJ software. Cell area was calculated in ImageJ by outlining individual cells. The area was converted from pixels² to μ m² for analysis.

Chapter 3

Results and Discussion

Tropomyosin expression levels were observed to increase in NMuMG cells treated with TGF β compared to the control cells on a glass slide substrate

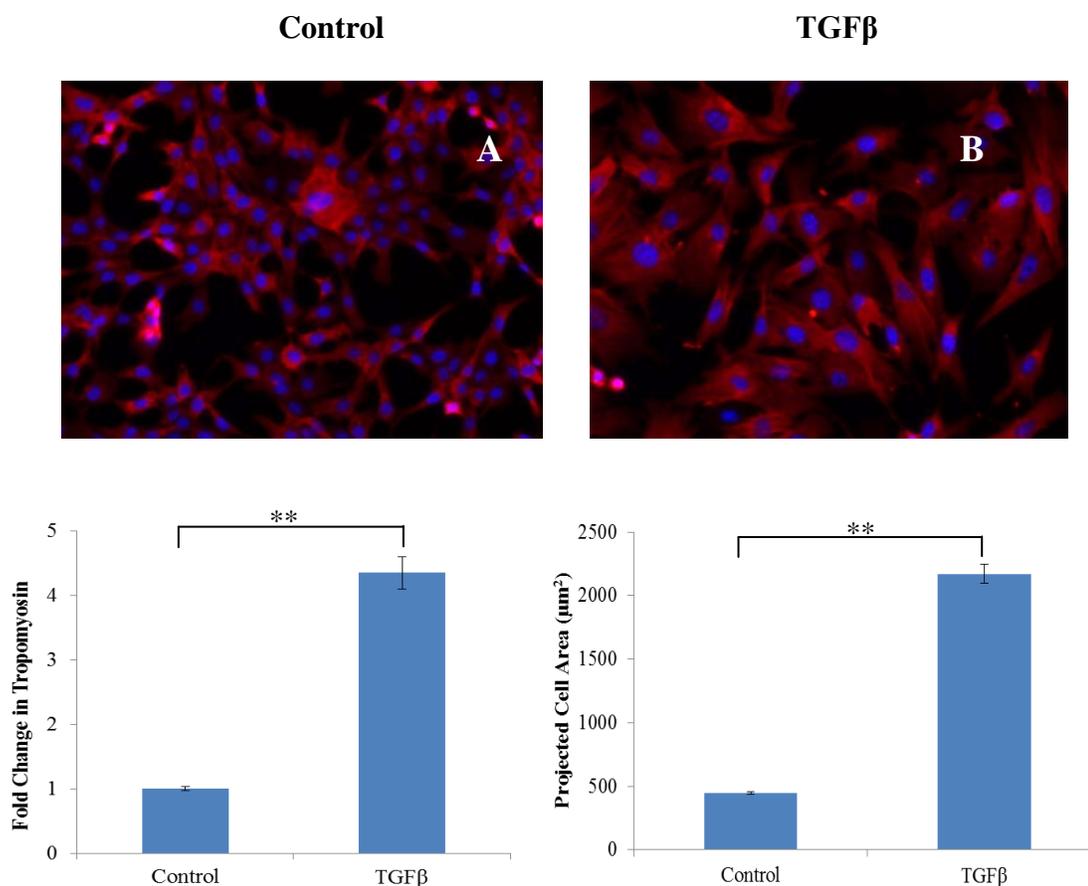


Figure 3-1. Tropomyosin expression of NMuMG plated on a glass substrate on the control (A) and TGF β (B).
**p<0.0001

As seen from Fig. 3-1, the change in tropomyosin was significant with TGF β treatment. These results indicate that tropomyosin expression and projected cell area increases as cells are treated with TGF β on glass which is a popular cell culture substrate.

Because glass is much stiffer than the normal environment in which these cells grow in the body, we hypothesized the expression of tropomyosin will also varies with TGF β treatment for cells cultured in environments with physiological stiffnesses.

Effects of Stiffness

The cell shape was affected by the stiffness of the substrate. Fig. 3-2 shows the tropomyosin stain in red and the nucleus stain in blue. There is a drastic change in the shape of the cell. The softer environment cells are much more rounded and clumped together, while cells on the stiffer environment are spread out more. Because of the stiff environment, the cells are able to adhere to pull on the substrate more, causing them to spread out more. In the control cells, the expression of tropomyosin stayed relatively the same as a function of matrix stiffness because EMT was not induced. EMT is not induced with a stiff substrate alone. A chemical stimulus is needed to induce the transition.

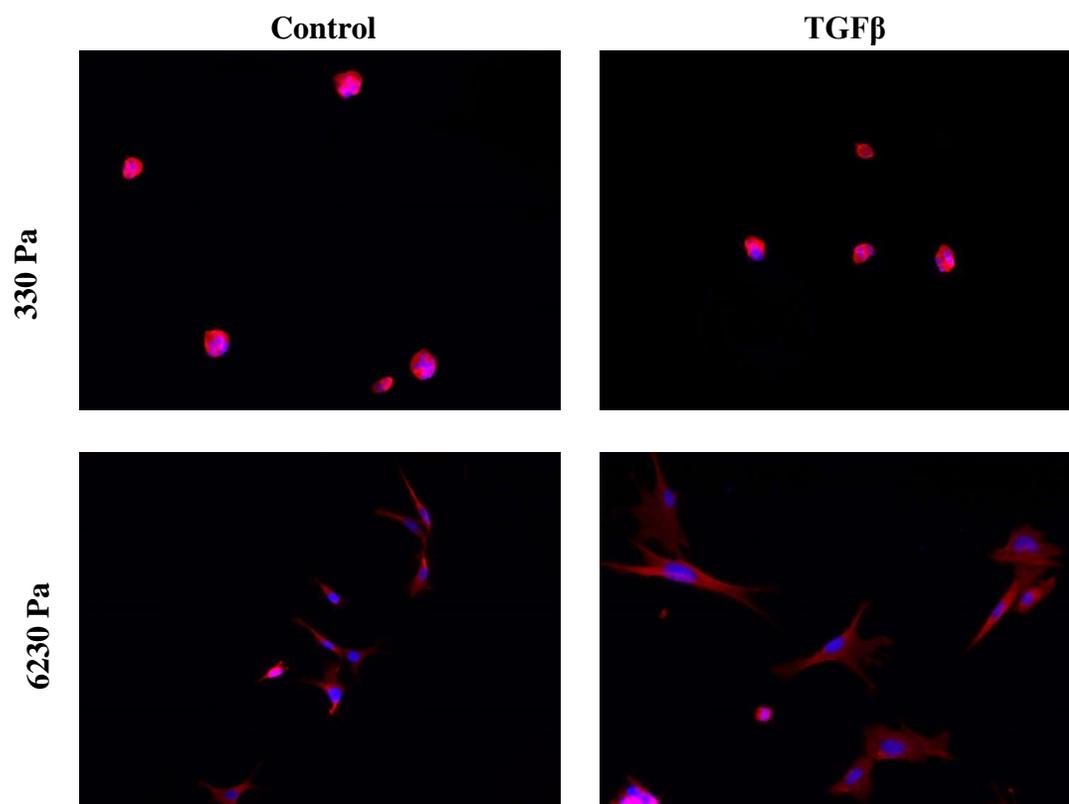


Figure 3-2: Fluorescence images of tropomyosin stained cells of soft, normal environment (330 Pa) and stiff, tumorigenic/cancer substrates (6230 Pa)

Area of Cells

The area of the cells varied significantly with each sample. Similar to tropomyosin expression, area of the cell was dependent on TGF β and stiffness. Projected cell area was calculated by outlining cells, as shown below.

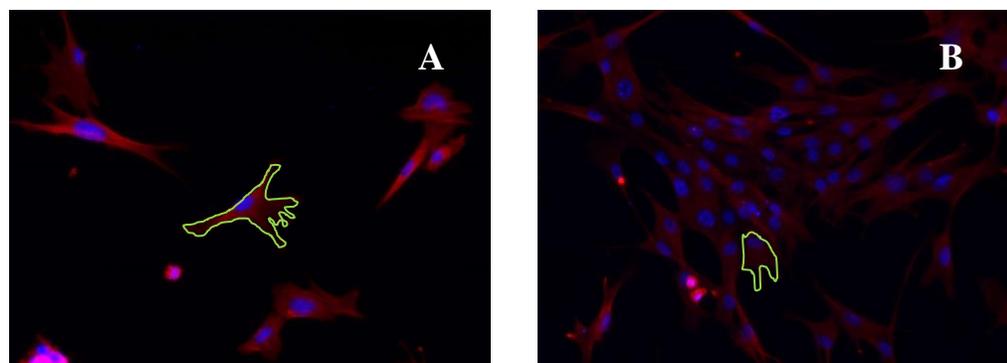


Figure 3-3: Comparison of cell size between EMT induced samples with treatment with EtOH (A) and the RhoA inhibitor, CCG-1423(B)

EMT was induced with treatment with TGF β and the role of RhoA signaling was investigated by treatment with the pharmacological inhibitor CCG-1423.

The relative size can be seen by the green highlighted cell as an example. The shape of the cells in the TGF β /ethanol-treated sample is much more spread out and elongated. This can indicate EMT and an increased motility. The TGF β /CCG-1423 treated cells but do not have the elongated appearance like the ethanol sample.

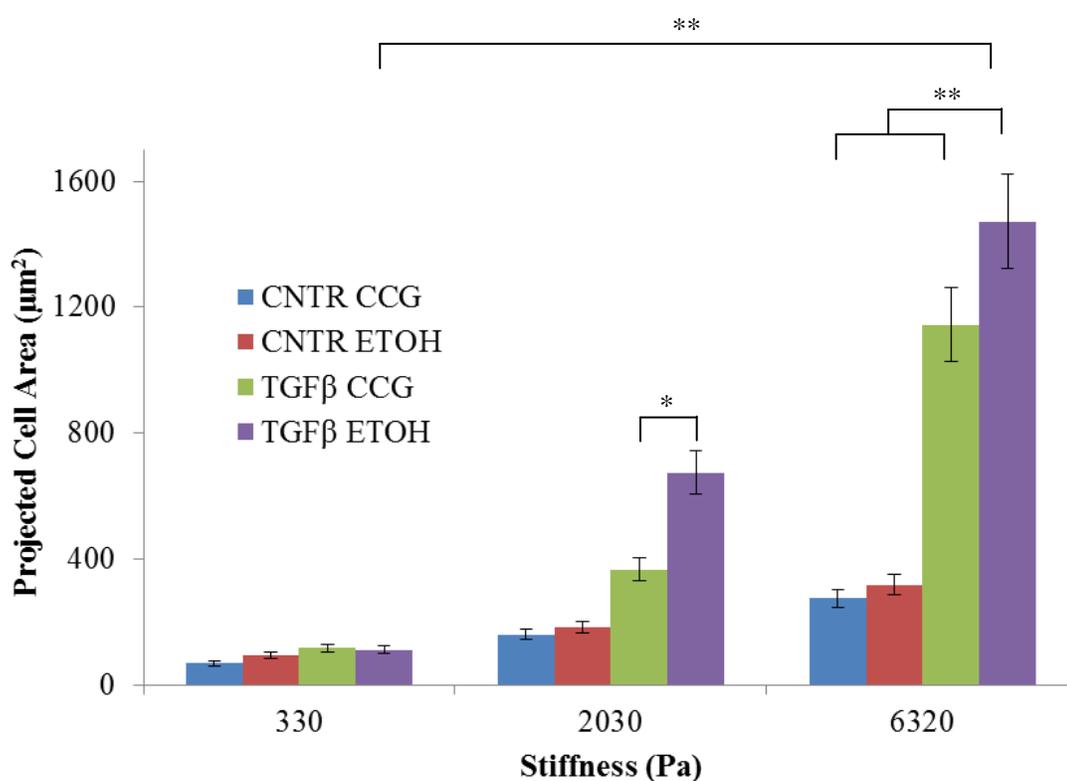


Figure 3-4: Projected cell area varies as a function of TGF β treatment and matrix stiffness, * $p < 0.001$, ** $p < 0.0001$

The area of the cells increased as stiffness increased, in both control and TGF β treated cells. Fig. 3-4 shows the difference in TGF β and control is significantly larger at higher stiffness. TGF β with EtOH in the stiffer environments was significantly larger than TGF β and CCG-1423. Thus, signaling through the RhoA pathway may contribute to the increased cell area induced by TGF β .

Effects of TGF β

Cells treated with TGF β compared to the control exhibited an increase in tropomyosin expression only in the stiff environment as shown in Fig 3-5. At 330 Pa, there was a 1.3 fold increase in tropomyosin expression between control/ethanol and TGF β /ethanol treated samples. At 2030 Pa, there was a 3.1 fold increase in tropomyosin between non-TGF β and TGF β treated cells. The stiffest matrix had the largest fold increase. Tropomyosin expression levels increased by a factor of 4.9. At 330 Pa, the cell shape did not change substantially. Cells in both samples were small, rounded and balled together. In the 6320 Pa samples, the control cells were larger than cells grown in the softer environments, but the TGF β treated cells were much more spread out. There was a large difference in phenotype, which is consistent with EMT induction in the stiffer environment.

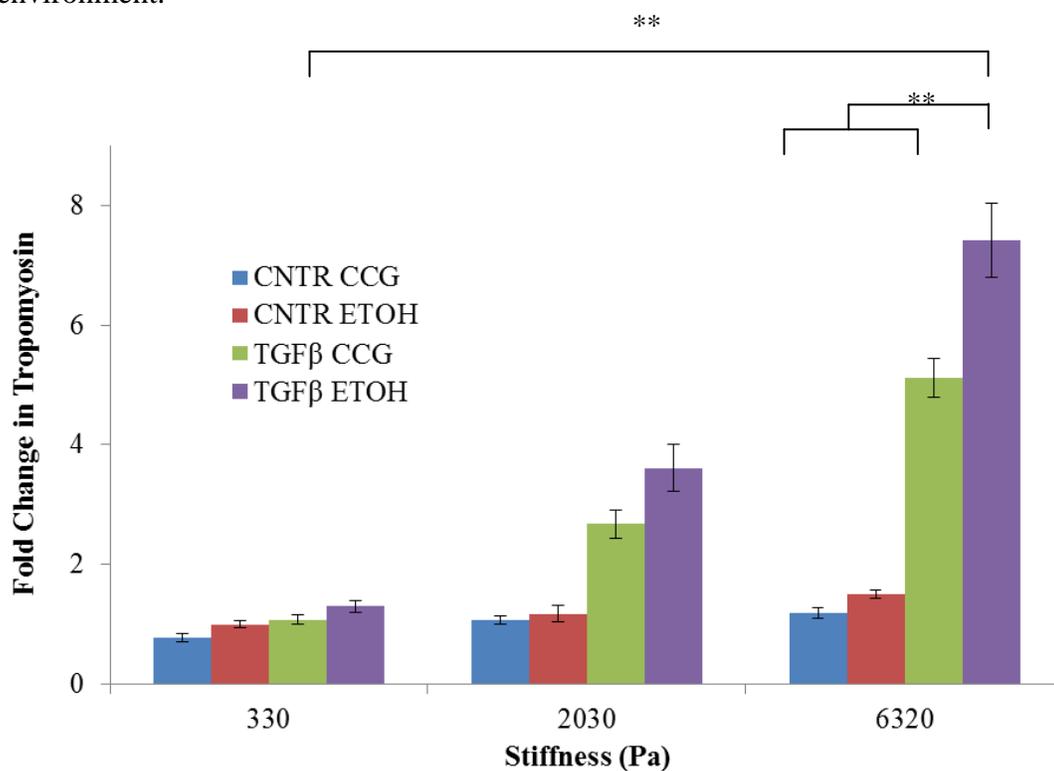


Figure 3-5: Tropomyosin expression levels vary as a function of matrix stiffness, * $p < 0.001$, ** $p < 0.0001$

The tropomyosin expression for the stiffer environment treated with TGF β and EtOH was significantly larger than the same treatment at softer environments and non TGF β treated samples.

Effects of CCG-1423

Treatment with CCG-1423, a RhoA pathway inhibitor, caused a decrease in tropomyosin expression levels in all TGF β -treated samples compared to the respective ethanol sample. There was a significant decrease in tropomyosin expression in the stiff environment treated with TGF β . This indicates that the RhoA pathway is important for the regulation of tropomyosin expression by TGF β and matrix stiffness.

Chapter 4

Conclusion

Environmental stiffness played a significant role in the induction of tropomyosin expression in NMuMG cells. In this process, both TGF β and a stiffer substrate were required. Tropomyosin immunofluorescence indicated a 7.1 fold increase in the protein between the softest and stiffest environment. In TGF β treated cells, expression of tropomyosin did not change for cells cultured on soft environments that mimic the stiffness of the normal mammary gland, indicating that TGF β alone does not induce EMT. The projected cell area increased for cells grown on the stiffer substrate, as predicted, due to the ability of cells to pull more on their environment. However, this increase in matrix rigidity was not sufficient to increase tropomyosin expression levels in control cells. A combination of TGF β and a stiff environment was necessary for increased expression of tropomyosin. CCG-1423 was shown to decrease the expression of tropomyosin in TGF β -treated cells. These data suggest a role for RhoA/SRF signaling in the induction of EMT and control of tropomyosin expression in mammary epithelial cells. By understanding the environmental effects of EMT, further understanding of the mechanisms of breast cancer metastasis can be achieved. In the long run, the hopes are to find ways to limit and prevent the formation of secondary tumors from breast cancer.

Chapter 5

Future Work

There are many areas to study in cell signaling and TGF β signaling cascade in NMuMG cells. In this experiment, only tropomyosin expression levels were analyzed. Expression of other mesenchymal or epithelial markers can be studied. Utilizing western blotting techniques to quantify the concentration of tropomyosin in cells can also be done.

REFERENCES

- 1) Buchholz S, Schally A, Englel J, Hohla F, Heinrich E, Koester F, Varga J, Halmos G. 2006. Potentiation of mammary cancer inhibition by combination of antagonists of growth hormone-releasing hormone with docetaxel. National Academy of Sciences
- 2) Radisky D. 2005. Epithelial-mesenchymal transition. *J Cell Sci.* 118: 4325-4326
- 3) Nakaya Y, Sheng G. 2008. Epithelial to mesenchymal transition during gastrulation: An embryological view. *Develop. Growth Differ.* 50:866-766
- 4) Voulgari A, Pintzas A. 2009. Epithelial-mesenchymal transition in cancer metastasis: Mechanisms, markers and strategies to overcome drug resistance in the clinic. *Biochimica et Biophysica Acta.* 1796: 75-90
- 5) Thiery J. 2002. Epithelial-mesenchymal transitions in tumor progression. *Nature.* 2: 442-454
- 6) Kalluri R, Neilson E. 2003. Epithelial-mesenchymal transition and its implications for fibrosis. *J Clin Invest.* 112:1776-1784
- 7) Massague J. 2008. TGF β in Cancer. *Cell.* 134: 215-230
- 8) Fafet P, Vignais M. 2000. TGF β -dependent epithelial-mesenchymal transition. Madame Curie Bioscience Database.
- 9) Lopez J, Kang I, You W, McDonald D, Weaver V. 2011. In situ force mapping of mammary gland transformation. *Integr. Biol.* 3:910-921

- 10) Eisenber J, Safi A, Wei X, Espinosa H, Budinger S, Takawira D, Hopkinson S, Jones J. 2011. Substrate stiffness regulates extracellular matrix deposition by alveolar epithelial cells. *Research and Reports in Biology*. 2:1-12
- 11) Kubo E, Hasanova N, Fatma N, Sasaki H, Singh DP. 2013. Elevated tropomyosin expression is associated with epithelial-mesenchymal transition of lens epithelial cells. *J Cell Mol Med*. 17:212-221
- 12) Gomez W, Chen Q, Gjorevski N, Nelson C. 2011. Tissue geometry patterns epithelial-mesenchymal transition via intercellular mechanotransduction. *J Cell Biochem*. 110: 45-51

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