THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF CHEMICAL ENGINEERING

THE EFFECT OF CALDESMON OVEREXPRESSION ON EPITHELIAL-MESENCHYMAL TRANSITION IN NORMAL MURINE MAMMARY GLAND EPITHELIAL CELLS

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A thesis
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ABSTRACT

The purpose of this thesis was to examine how the overexpression of caldesmon affected epithelial-mesenchymal transition (EMT) in normal murine mammary gland epithelial cells. EMT has been implicated in a number of biological processes including wound healing, tissue fibrosis, and cancer metastasis. EMT is characterized by a loss of cell polarity and cell-cell adhesion affinity and leads to the formation of cells possessing of mesenchymal phenotype that have increased motility and contractility. During this transition, actin filaments begin to bundle to form stress fibers and become linked to the extracellular matrix through focal adhesion assemblies. Caldesmon is an actin binding protein that has been found to stabilize stress fiber formation in epithelial cells. Specifically, the effect of caldesmon overexpression on different aspects of EMT was analyzed including stress fiber formation, α-smooth muscle actin expression, focal adhesion formation, and traction force generation.

Cells were transfected with a caldesmon wildtype plasmid to overexpress caldesmon and a green fluorescent protein plasmid as a control. The cells were then grown on polyacrylamide gels with a stiffness of 1800 Pa with either transforming growth factor (TGF)-β1 solution or control solution. The cells were then either analyzed using immunofluorescence microscopy or traction force microscopy.

The formation of stress fibers was found to be qualitatively higher in cells treated with TGFβ1 than the control treated cells. The expression of α-smooth muscle actin was significantly higher in cells treated with TGFβ1 where caldesmon was overexpressed than the other treatments. Focal adhesion numbers and sizes were slightly higher for cells where caldesmon was overexpressed for both TGFβ1 and control treatments. Finally, treatment with TGFβ1 increased the magnitude of traction forces for both caldesmon overexpression on normal caldesmon expression.

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Chapter 1

Introduction

The epithelium is a collection of cells that line the inner and outer surfaces of the body. The cells of the epithelium are called epithelial cells and possess specific characteristics such as columnar shape, apico-basal cell polarity, and a high cell-cell adhesion affinity. Through the process of epithelial-mesenchymal transition (EMT) cells can lose their epithelial characteristics and take on a mesenchymal phenotype. Figure 1 below details the major changes that occur in a cell during EMT. Specifically, cells that have undergone EMT exhibit decreased cell-cell adhesion affinity, lose their cell polarity, and gain increased migratory capabilities (1). A myogenic program can then be activated that leads to cellular differentiation of mesenchymal cells into myofibroblasts, specialized cells that can exert large contractile forces on their microenvironment and deposit extracellular matrix proteins. Accompanied with this whole transition are changes in protein expression and cytoskeletal rearrangements, most notably a decrease in expression of E-Cadherin, an increase in expression of α-Smooth Muscle Actin (α-SMA), and the formation of stress fibers from actin filaments (2).

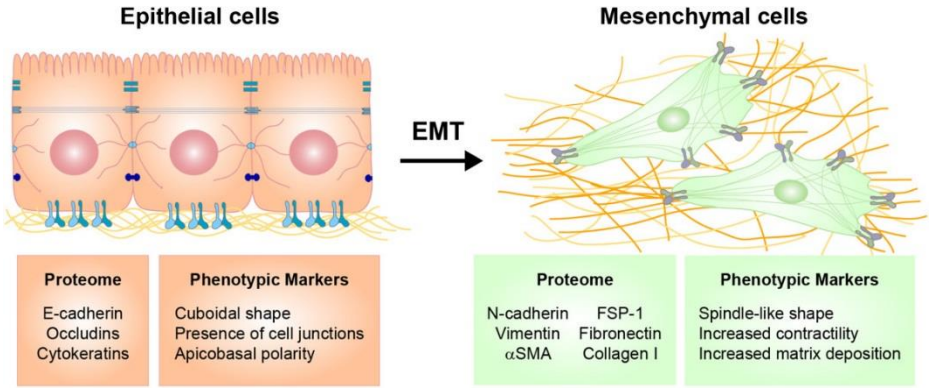

Figure 1. Representation of epithelial-mesenchymal transition detailing the changes in protein expression and phenotypic differences between epithelial and mesenchymal cells (3).

Myofibroblasts play an important in wound healing because of their ability to deposit extracellular matrix constituents to stabilize damaged tissues and exert large contractile forces to assist in wound closure. Chronic activation of myofibroblasts can lead to tissue fibrosis though tissue remodeling and stiffening. Aberrant activation of myofibroblasts has also been associated with tumor progression due to the increased migratory capabilities of these cells and their ability to secrete cytokines and other proteins at the invasive fronts of tumors (3).

The formation of myofibroblasts through EMT is a significant process that conveys many considerable changes to cells. EMT can be induced through a number of different pathways, but for the purposes of this thesis, only EMT induced through Transforming Growth Factor (TGF)-β1 will be studied. TGFβ1 is a cytokine signaling molecule that binds to serine/threonine kinase receptors that activate Smads, intracellular signaling molecules and transcription factors (4). Smads, along with other transcription factors, begin to induce changes in gene expression in the cell. An early marker for EMT is the decreased expression of E-Cadherin, a cell-cell adhesion protein (5). During EMT the cytoskeleton of the cell rearranges, specifically, actin filaments exhibit more anisotropy and bundle together to form stress fibers. These stress fibers lead to an increase in the stiffness of the cell (3). Another major marker of EMT is an increase in expression of α-SMA, an actin isoform that confers contractile ability to cells. During EMT, as α-SMA expression increases it is incorporated into stress fibers allowing the cell to exert large contractile forces (6). Furthermore, stress fibers are linked to the extracellular matrix through integrins, transmembrane proteins that link the cell to the extracellular matrix and transduce mechanical signals to the cell. During EMT, integrins cluster together to form focal adhesions, macromolecular complexes that act as signal transduction hubs between the cell and the extracellular matrix. Focal adhesions, through integrin binding to extracellular matrix proteins and actin stress fibers in the cell, enable cells to exert forces on the surroundings as well as sense mechanical properties (7). These forces generated by the cells are essential in cell spreading and migration (8).

Caldesmon is an actin binding protein that regulates the assembly and stability of actin microfilaments. Previous work has shown that depending on the cell type caldesmon can either stabilize or disrupt stress fibers and has been shown to be disruptive in lung fibroblasts and aorta smooth muscle cells (9,10). However, in mammary epithelial cells, caldesmon has been shown to be upregulated upon treatment with TGFβ1. Stress fiber formation also occurred in cells where caldesmon was upregulated indicating that it does not have a disruptive effect for this type of cell (11). The role caldesmon has in EMT is still largely unknown and the purpose of this thesis was to investigate how caldesmon affects different aspects of EMT and myofibroblast formation. The main areas of interest were to examine how overexpression of caldesmon affected the expression of α-SMA, as expression of this protein is necessary for the contractile ability of the cell, and then investigate how the forces generated by the cells changed as a result. Expression of α-SMA and stress fiber formation cannot exert forces on the extracellular matrix independently and must be linked to it through focal adhesions. Therefore, it is also important to examine how focal adhesions change as a result of overexpression of caldesmon. It was hypothesized that the overexpression of caldesmon would lead to higher α-SMA expression and that higher traction forces would be generated by the cells.

In this study, Normal Murine Mammary Gland (NMuMG) epithelial cells were cultured for experiments. To investigate the effects of caldesmon, the cells were transfected with a caldesmon wild type plasmid (CADWT) and for a control the cells were transfected with a green fluorescent protein plasmid (pEGFP). By transfecting the cells with the CADWT plasmid, caldesmon was overexpressed in the cells and its effects on EMT could be analyzed. The pEGFP plasmid was used as a control that simulated the stress of transfection of the cells but did not change the expression level of caldesmon from normal levels. After transfection, the cells were grown on polyacrylamide gels with a Young's Modulus of 1800 Pascal to mimic stiffness of a typical tumor environment (12). The cells were then treated with either a TGFβ1 or a control solution and incubated for 48 hours. Following incubation, the cells were either fixed and stained for a specific protein or analyzed for force generation using traction force

microscopy. Western blotting was also performed to probe for the expression levels of caldesmon, E-cadherin, and α-SMA. By applying these different techniques, the effect of overexpression of caldesmon on α-SMA expression, E-cadherin expression, focal adhesion formation, stress fiber formation, and cellular traction force generation was analyzed.

Chapter 2

Procedure

Glass Slide Preparation

Glass slides (22x22mm) were incubated in a 0.1N solution of sodium hydroxide for fifteen minutes and then thoroughly rinsed with MQ water. The slides were completely dried and then incubated in a solution of 2% (v/v) (3-aminopropyl) trimethoxysilane in acetone for fifteen minutes. The slides were washed with acetone and completely dried. Lastly, the slides were incubated in 0.5% glutaraldehyde diluted in 1× Phosphate Buffered Saline (PBS) for thirty minutes and then rinsed with MQ water.

Polyacrylamide Gel Fabrication

Polyacrylamide solutions were prepared from stock solutions of 40% acrylamide, 2% bis-acrylamide, and MQ water to make a gel of 1800 Pa stiffness after polymerization according to the Young's Modulus. The prepared solution was degassed for thirty minutes. The polymerization reaction was initiated by the addition of TEMED and a 10% (w/v) Ammonium Persulfate solution. For single layer gels, 20 µL of the solution was pipetted on top of a treated slide and a 22 mm circular slide treated with Rain-X was placed on top to facilitate spreading of the solution. For double layer gels, 8 µL of the solution was pipetted on top and a 15 mm circular slide treated with Rain-X was placed on top. The second layer of the gels were made in similar fashion where 12 µL of solution with fluorescent beads was pipetted on top of the previous gel layer and a 22 mm circular slide treated with Rain-X was placed on top.

Cell Culture

NMuMG, Normal Murine Mammary Gland epithelial cells (American Type Culture Collection), were cultured in DMEM supplemented with 10% Fetal Bovine Serum, 50 μg/ml Gentamicin, and 10 μg/ml Insulin.

Transfection

Cells were plated in a six well plate at 300,000 cells/well 24 hours prior to the transfection with Lipofectamine LTX Plus Reagent (Life Technologies). 5 μg of DNA and either the CADWT or the pEGFP plasmid were added to 500 μL of OPTIMEM media along with 5 μL of Plus Reagent and incubated for ten minutes. 20 μL of LTX Reagent and 500 μL of OPTIMEM were added to each solution and incubated for thirty minutes. After the incubation 1 mL of each solution was added to separate wells containing cells.

Gel Activation

Polyacrylamide gels were activating using 0.5 mM Sulfa-SANPAH in 50 mM HEPES (pH 8.5) by pipetting 200 μL onto the surface of each gel. The Sulfa-SANPAH was then activated and crosslinked to the gels by applying UV light for ten minutes in a UV cross linker. The gels were washed with a 50 mM HEPES solution and the process was repeated. After the second crosslinking the gels were washed three times with sterile 50 mM HEPES. After the washes, 10μg/mL fibronectin in HEPES was applied to the gels and they were incubated over night at 4°C. The gels were then incubated for thirty minutes in

70% ethanol solution to sterilize them. Following sterilization, the gels were washed three times with sterile 1× PBS and stored in DMEM at 37°C.

Plating Cells

Transfected cells were then plated at 150,000 cells/gel for immunofluorescence staining and at 20,000 cells/gel for traction force microscopy experiments. For Western Blot experiments cells were plated at 300,000 cells in 60 mm cell culture plates filled with 5 mL of DMEM. The day after being plated, cells were treated with either control solution (4 mM HCl, 1 mg/mL BSA, in autoclaved water) or with of 2 μg/mL TGFβ1 diluted in the control solution.

Fixing Cells

Cells for focal adhesion and actin filament structural analyses were fixed with 3.7% Paraformaldehyde in 1× PBS solution for fifteen minutes. The cells were then washed three times with 1× PBS and stored at 4°C in 1× PBS for later analysis. Cells for α-SMA analysis were fixed with a 50:50 solution of acetone and methanol at -20°C for ten minutes. The cells were then washed three times with 1× PBS and stored at 4°C in 1× PBS.

Immunofluorescence Staining

Focal Adhesions

Cells previously fixed with 3.7% Paraformaldehyde were permeabilized in a 0.1% solution of Triton X-100 in 1x PBS for five minutes. The cells were then washed twice with a 0.05% Tween-20 in 1× PBS wash buffer. The cells were then incubated in a blocking solution of 1% BSA in 1× PBS for

thirty minutes. The slides were then incubated for one hour with a mouse anti-Vinculin antibody diluted 1:200 in the blocking solution. Following the incubation the cells were washed three times with the wash buffer and then incubated for one hour with a goat anti mouse Alexa Fluor 594 conjugated secondary antibody in 1× PBS at a 1:150 dilution. The cells were then rinsed with 1×PBS to remove unbound secondary antibody.

α-Smooth Muscle Actin

Cells previously fixed with a 50:50 methanol: acetone solution was incubated in a blocking buffer (0.3% Tween-20, 10% goat serum, 1x PBS) for one hour. An mouse anti-α-smooth muscle actin antibody was diluted at 1:200 in blocking buffer and incubated with cells for one hour. Following incubation, the cells were washed three times with 1× PBS. The cells were then incubated for one hour with a goat anti mouse Alexa Fluor 594 conjugated secondary antibody in 1× PBS at a 1:150 dilution. Cells were then rinsed with 1×PBS to remove unbound secondary antibody.

Actin Filament Staining

Cells previously fixed with 3.7% Paraformaldehyde were permeabilized for five minutes with a solution of 0.1% Triton X-100 in 1× PBS and then washed two times with 1× PBS. The cells were then incubated in a blocking buffer (1% BSA in 1× PBS) for thirty minutes. The cells were then incubated for twenty minutes in a solution of phalloidin in 1× PBS at a dilution of 2.5:100. Cells were then rinsed with 1×PBS to remove phalloidin.

Nuclei Counterstaining and Mounting

Following secondary antibody or phalloidin staining the cells were then incubated in a solution of Hoechst 33342 diluted 1:10,000 in 1× PBS for ten minutes. The cells were washed three times with 1× PBS and then mounted to cover slides using Fluoromount-G mounting solution.

Immunofluorescence Microscopy and Image Analysis

Samples were imaged using either a 20× or 40× air objective on a Nikon Ti-E inverted microscope fluorescence microscope equipped with a Photometrics HQ2 CCD camera. For analysis of α-SMA expression the 20× objective was used and 7 to 10 images were aquired to image approximately 200 cells for each treatment per trial. The expression of α-SMA was evaluated by dividing the number of cells that expressed α-SMA by the total number of cells. For analysis of focal adhesions, a 40× objective was used and 5 to 7 images were acquired for each treatment per trial. ImageJ was used to isolate single cells from the acquired image and then stack the images. The image stack was then analyzed for focal adhesions by using a focal adhesion analysis web server hosted by Shawn Gomez's research group at Chapel Hill (13). The images for F-actin were obtained at 20× and 5 images were obtained per treatment. Finally, the cells from the α-SMA images were analyzed for cell area and aspect ratio using ImageJ.

Traction Force Microscopy

Cells were plated onto double layer polyacrylamide gels with beads at 25,000 cells per gel and grown for 48 hours after treatment with either TGFβ1 or control solution. The samples were imaged immediately at the end of the incubation. To conduct the imaging a sample was placed into a live cell chamber that controlled the temperature, relative humidity, and carbon dioxide levels. The temperature

was kept at 37°C, the relative humidity was kept at 70%, and the carbon dioxide level was kept at 5%. Brightfield and TxRed images were taken of the cells and the beads respectively and the position of each image was recorded using the microscope coordinates. The cells were the treated with a 1% sodium dodecyl sulfate solution for ten minutes to detach the cells. Once the cells were detached the positions were reimaged to obtain a relaxed image of the beads. The data was analyzed using LIBTRC v2.0 software (generously provided by Dr. Micah Dembo at Boston University).

Protein Extraction

Transfected cells were plated on 60 mm diameter plates at 150,000 cells per plate. The cells were transfected with either a CADWT or pEGFP plasmid and incubated with either the TGFβ1 or control solution and incubated for 48 hours as previously described. After the incubation the cells were rinsed twice with cold 1× PBS. Following the washes, 200 µL of RIPA Buffer with 1% of both Phosphatase and Protease Inhibitor and EDTA Solution was added to each plate. The plates were kept on ice for ten minutes and swirled once per minute to ensure even coating of RIPA Buffer. The entire contents of the plates were scraped and transferred to a centrifuge tube and spun for fifteen minutes at 13,200 RPM at 4°C. The protein was collected and stored at -20°C until quantification.

Protein Quantification

Protein quantification was done using a Pierce BCA Protein Assay Kit a microplate reader and Magellan Software. In Eppendorf tubes, bovine serum albumin (BSA) was serially diluted in RIPA buffer from a starting concentration of 2,000 µg/mL to 25 µg/mL. In total, nine different solutions were prepared ranging in concentration of BSA. In a 96 well plate 10 µL of each standard or protein solution from extraction was added in duplicate along with 200 µL of the Working Reagent (50:1 Reagent A:B) to

the wells. The plate was incubated for thirty minutes at 37°C and then immediately analyzed using the aforementioned plate reader at a wavelength of 562 nm. The protein concentration was calculated by comparing the absorbance values against the standard calibration curve for BSA.

Gel Electrophoresis

A solution of 90 µL of protein, 90 µL of Tris Glycine SDS, and 20 µL of NuPage was heated for five minutes and 95°C and then put on ice for five minutes. The samples were run in a NuPage 4-12% Bis-Tris Gel at the amount specified from the quantification step. The proteins were run against a Page Ruler Plus Prestained Protein Ladder. The proteins were run for fifty minutes at 200V in a 1× MOPS SDS Running Buffer.

Western Blotting

Following gel electrophoresis, the proteins were transferred to a PVFD membrane by Western Blotting. The PVFD membrane was placed in methanol for one minute and was then incubated in a transfer solution (25 mL of 20× NuPage Transfer Buffer, 50 mL of methanol, and 425 mL of MQ water) with two pieces of blot paper for ten minutes. The gel was also incubated in transfer solution for ten minutes. The BioRad Transblot SD was slightly wet with the transfer solution and then the presoaked blot paper was added followed by the membrane, then the gel, and finally the other piece of blot paper. The Transblot was run for one and a half hours at 12V. When the run was completed the membrane was put in Ponceau S solution for five minutes to verify protein transfer and then rinsed with MQ water. The membranes were then incubated in a TBST solution (50 mL Tris Buffered Saline, 500 µL Tween 20, and 450 mL MQ water) for ten minutes and then they were incubated in 5% milk in TBST blocking solution for one hour while mixing. The primary antibodies, E-Cadherin (1:1000), α-SMA (1:2500), α-Tubulin

(1:2000), and Caldesmon (1:10,000) were added at the specified dilution rate and incubated overnight at 4°C. Following incubation, the membranes were washed three times with TBST for ten minutes on a shaker. The membranes were then incubated with a horseradish peroxidase (HRP)-linked secondary antibody (1:1000) in TBST for one hour on the shaker at room temperature. The membranes were washed as previously described and then immediately imaged.

Gel Doc Imaging

The blots were incubated in SuperSignal West Dura Extended Duration Substrate for three and a half minutes and then imaged on a FluorChem FC2 system.

Chapter 3

Results and Discussion

Cells were transfected with a caldesmon wild type plasmid (CADWT) to overexpress caldesmon or with a green fluorescent protein plasmid (pEGFP) to serve as a control. The cells were additionally treated with TGFβ1 or control solution to yield four experimental treatments groups.

Actin Cytoskeleton

The transfected cells were analyzed for stress fiber formation by staining for filamentous actin. Figure 2 below shows representative images for the formation of stress fibers in the cells of the different treatment groups. Independent of caldesmon expression levels, cells not treated with TGFβ1 showed very little stress fiber formation, cortical actin staining around the cell peripheries, and a slightly disorganized array of actin filaments throughout. Cells that were treated with TGFβ1 showed much more ordered actin filaments and the formation of stress fibers throughout the cell. The inability of stress fibers to form without treatment with TGFβ1 indicates that caldesmon alone cannot induce stress fiber formation. It is worth noting that these results were purely qualitative and no quantitative analysis on the stress fibers was done. The formation of stress fibers indicates that the cells treated with TGFβ1 have gained mesenchymal cell characteristics whereas the control treated cells have not.

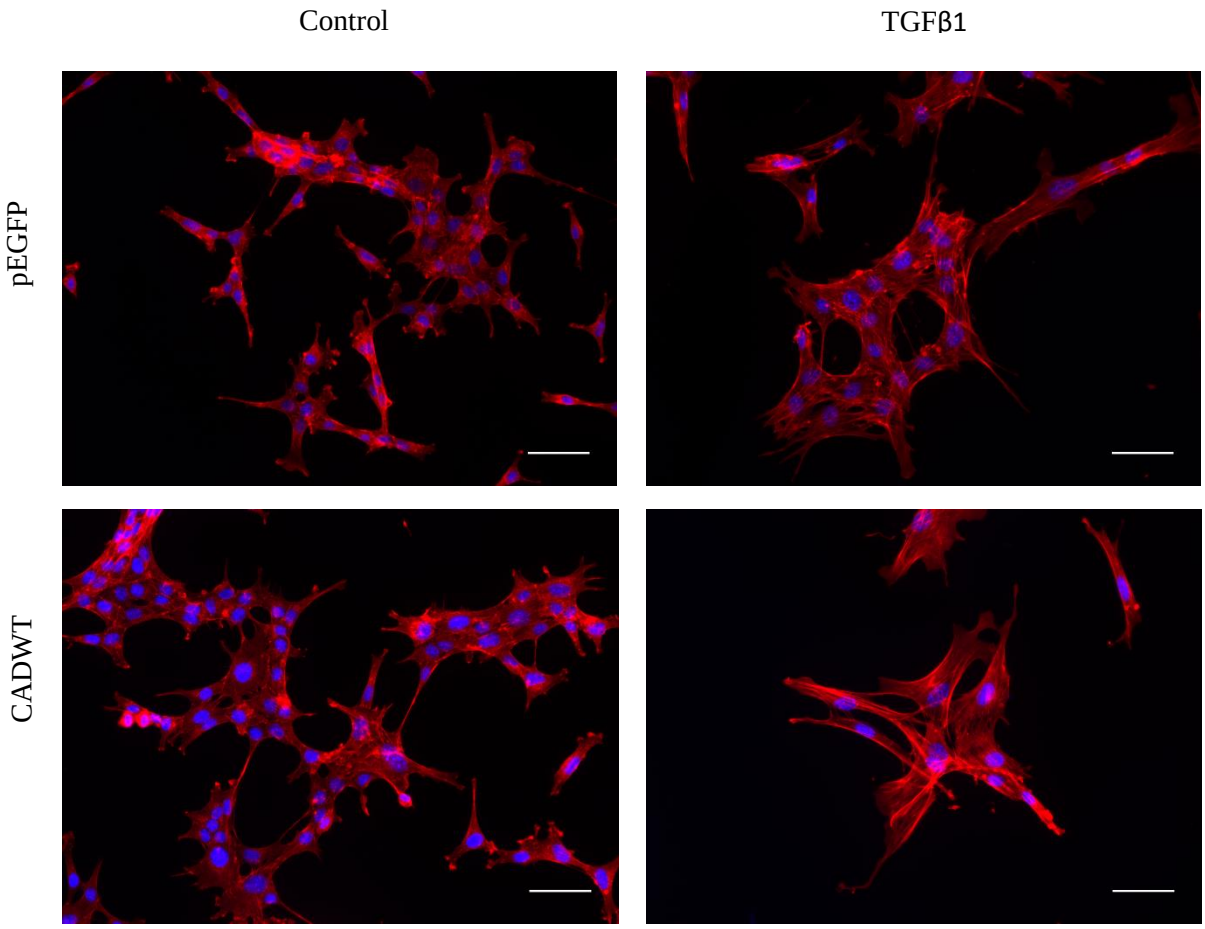

Figure 2. Fluorescence microscopy images for actin filaments for control transfected cells and cells overexpressing caldesmon with and without TGFβ1 treatment. Actin filaments are visualized in red and nuclei are blue. (20× objective and scale bar equivalent to 25 um)

α-Smooth Muscle Actin Expression

Cells corresponding to the different experimental treatment groups described above were stained for α-smooth muscle actin, a mesenchymal cell marker. Representative pictures are shown in Figure 3 below. A quantitative analysis was performed to determine the percentage of cells expressing α-smooth muscle actin and the results have been summarized in the graph in Figure 4.

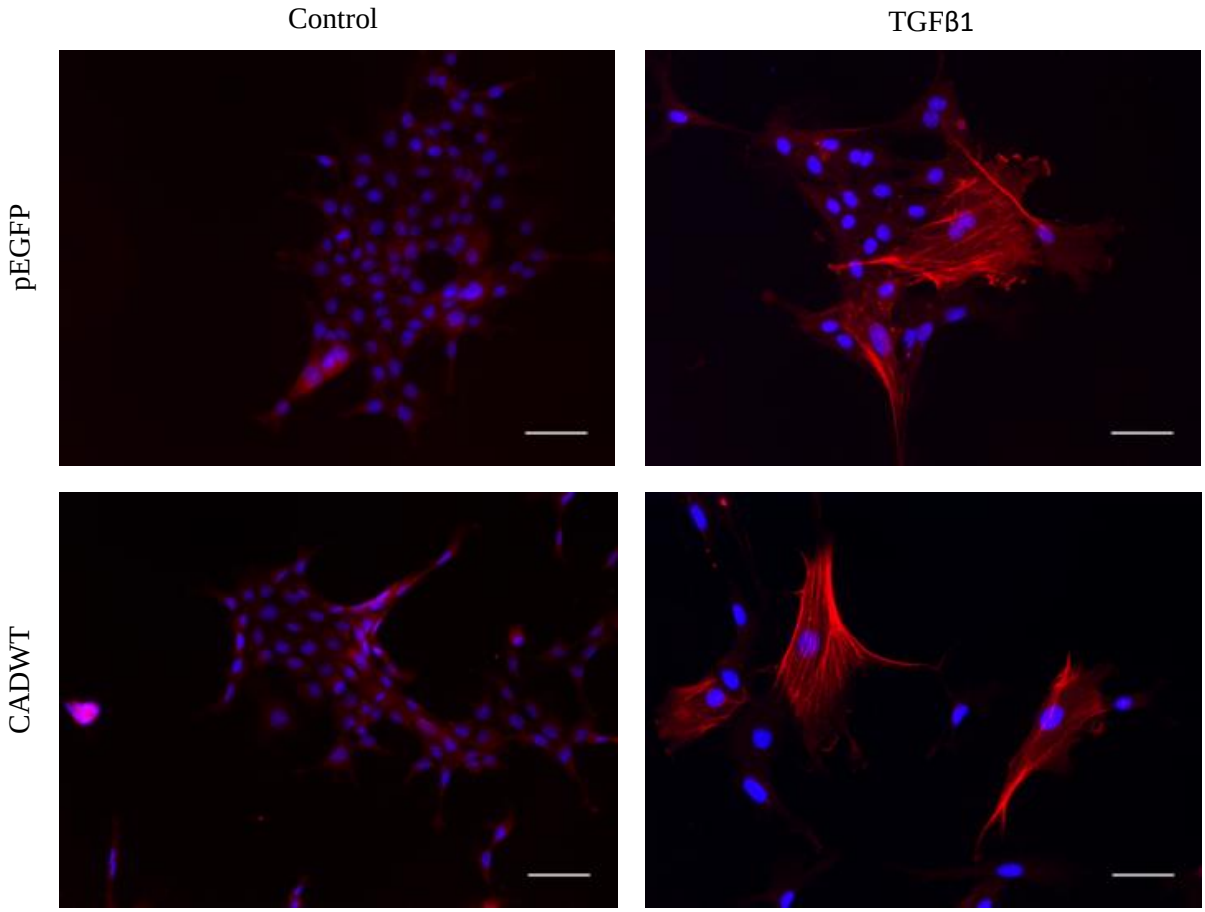

Figure 3. Immunofluorescence microscopy images for α-SMA filaments for control transfected cells and cells overexpressing caldesmon with and without TGFβ1 treatment. α-SMA has been stained red and the nuclei appear blue. (20× objective and scale bar equivalent to 25 µm)

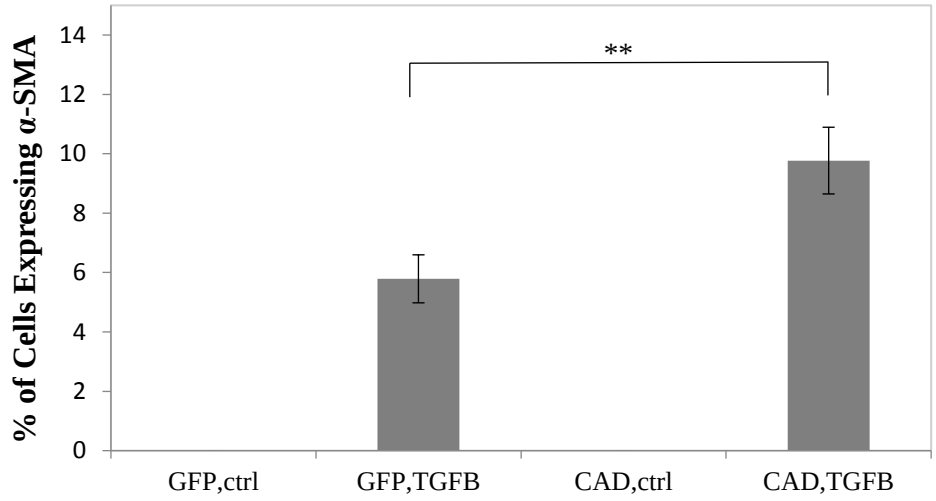

Figure 4. Percentage of cells expressing α-Smooth Muscle Actin for control transfected cells and cells overexpressing caldesmon with and without TGFβ1 treatment. (p<0.05)**

Figure 3 shows representative images from the α-SMA staining experiments and shows only expression of α-SMA in cells that have been treated with TGFβ1. Figure 4 shows the average percent of cells expressing α-SMA for each treatment condition. There was essentially no expression of α-SMA in cells treated with only the control solution regardless of the expression level of caldesmon. Cells that were treated with TGFβ1 showed increased expression of α-SMA from the control cells. It was also found that overexpression of caldesmon in cells treated with TGFβ1 showed a significant increase in the percentage of cells expressing α-SMA as compared to cells with normal expression levels of caldesmon. These results suggest that caldesmon on its own is not enough to induce α-SMA expression, but leads to a significant increase in expression in combination with TGFβ1.

Western Blotting

Western blots were also performed to verify the presence and quantify the proteins of interest. For the western blot experiments the cells the cells were transfect with the same plasmid and treated with either TGFβ1 or control solution, but the cells were grown on petri dishes instead of polyacrylamide gels.

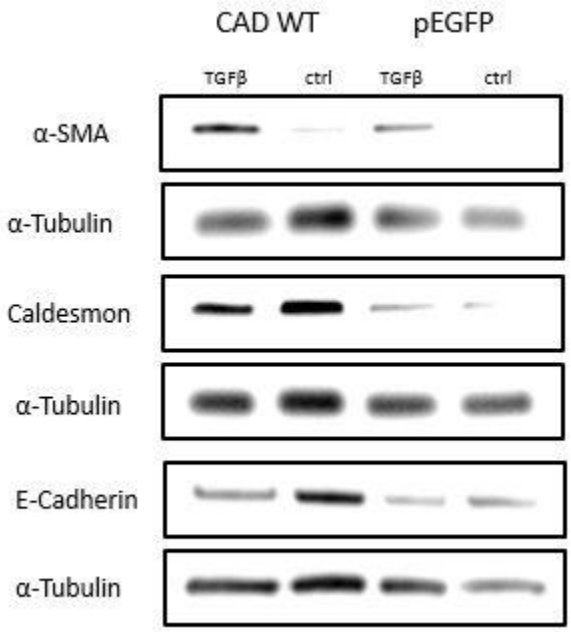

Figure 5. Western control transfected cells and cells overexpressing caldesmon with and without TGFβ1 treatment. A-Tubulin was used as a loading control. Proteins of interest were α-SMA, caldesmon, and E-cadherin

In the above western blots α-Tubulin served as the loading control to ensure that the same amount of protein was loaded into each well. The proteins of interest were α-SMA, caldesmon, and E-cadherin. Figure 5 shows a decrease in expression of E-Cadherin, and epithelial marker and cell-cell adhesion protein, upon treatment with TGFβ1. Both cell types transfected with the CADWT plasmid show increased expression of caldesmon over the pEGFP transfected cells. This proves that the transfection for

caldesmon was successful. Finally, the results for α-SMA actin expression are in agreement with the results obtained from immunofluorescence experiments where there is either none or only very slight expression for cells treated with the control solution and cells treated with TGFβ1 show increased expression. Additionally, for the cells treated with TGFβ1 the expression of α-SMA appears greater in cells where caldesmon is overexpressed as compared to the normal caldesmon expression level, which is also consistent with the immunofluorescence experiments for α-SMA.

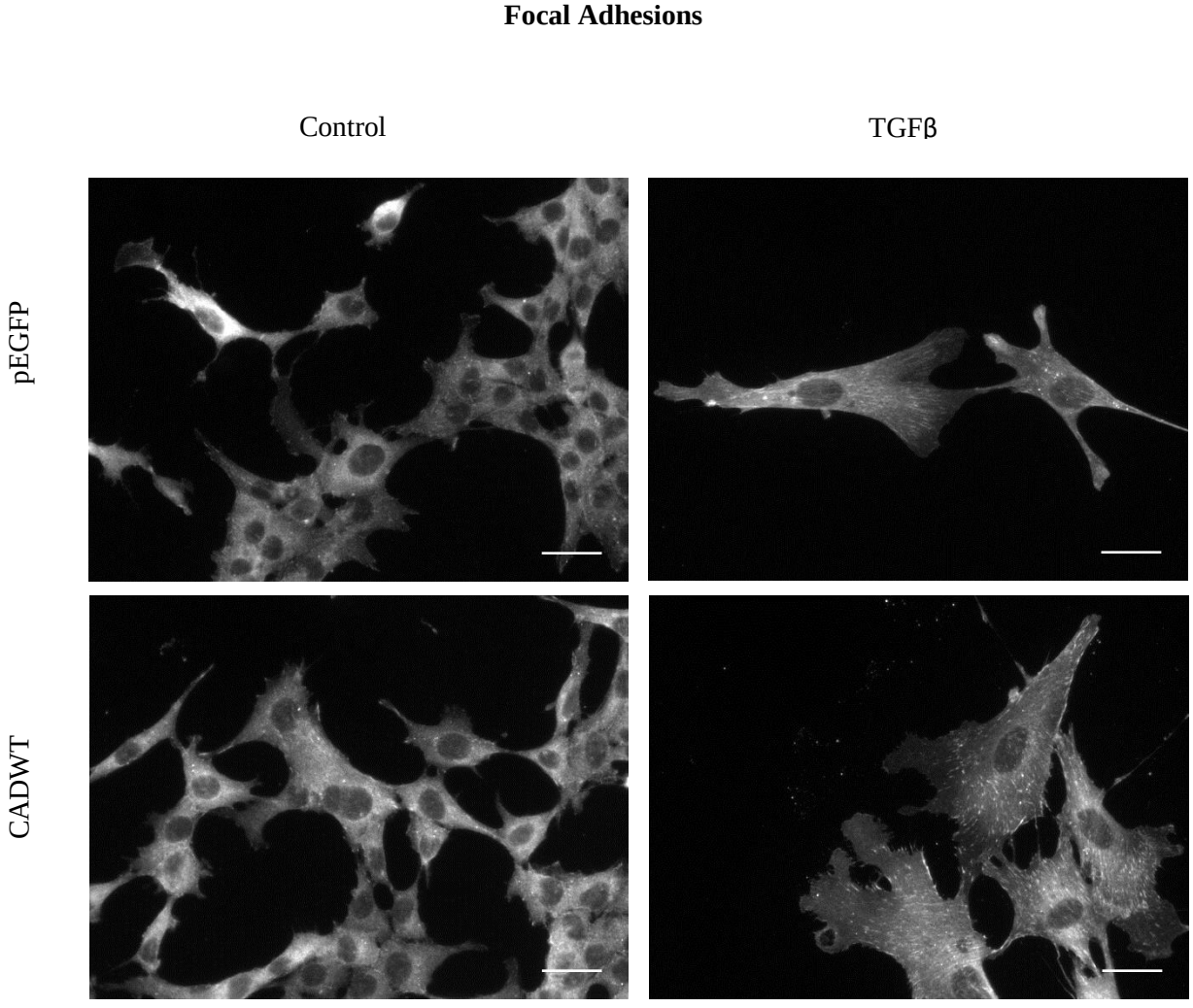

Figure 6. Immunofluorescence microscopy images of focal adhesions for control transfected cells and cells overexpressing caldesmon with and without TGFβ1 treatment. Images shown in greyscale to highlight focal adhesions. (40× objective and scale bar equal to 25 μm)

As discussed earlier, integrins begin to cluster during EMT to produce focal adhesions, which can transduce mechanical regulatory signals between the cell and the ECM and link stress fibers to the ECM. The number and size of focal adhesions were analyzed for the different transfections for cells treated with either TGFβ1 or control solution. As shown qualitatively in Figure 6, focal adhesions for cells treated with TGFβ1 appear more frequently and are larger. A quantitative analysis was performed and the results are shown in the Figures 7 and 8 below. Figure 7 shows the average number of focal adhesions where cells treated with TGFβ1 have a greater number than cells treated with control solution. There is also a slight increase in the number for cells where caldesmon is overexpressed as compared to cells that have normal caldesmon expression levels.

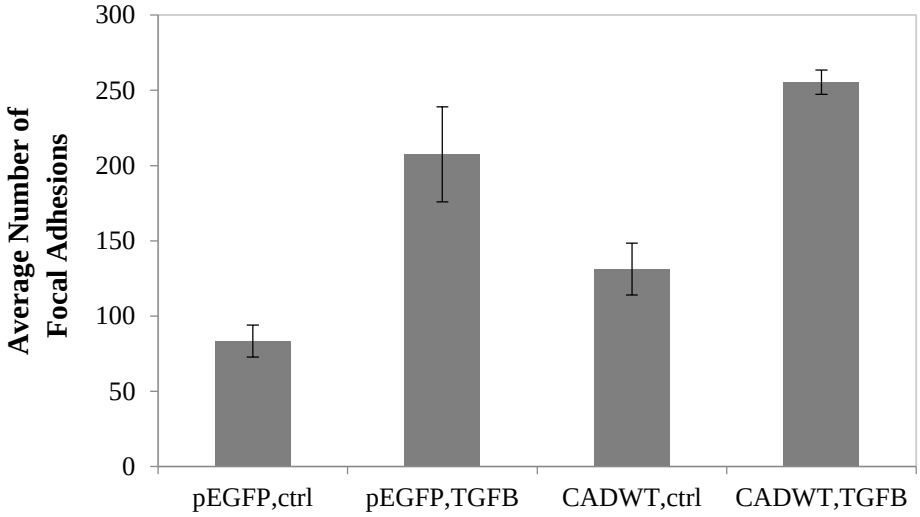

Figure 7. Average number of focal adhesions per cell for control transfected cells and cells overexpressing caldesmon with and without TGFβ1 treatment

Figure 8 shows the average area of focal adhesions per cell and this follows a similar trend as the previous graph where cells treated with TGFβ1 have larger focal adhesions than cells treated with control solution. There again is a slight increase in size of focal adhesions for cells where caldesmon is overexpressed for both treatment types as compared to cells with normal expression levels of caldesmon.

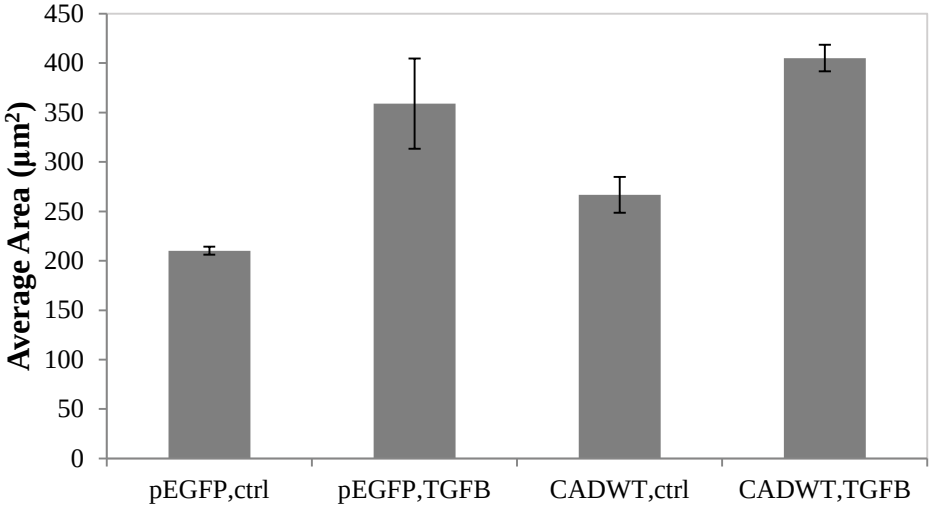

Figure 8. Average area of focal adhesions per cell for control transfected cells and cells overexpressing caldesmon with and without TGFβ1 treatment

Cell Area and Aspect Ratio

Epithelial cells tend to have a cuboidal morphology while mesenchymal cells are elongated. To determine whether overexpression of caldesmon impacts cell morphology, the cell area and aspect ratio were quantified. The results from the two analyses are shown in Figures 9 and 10 below.

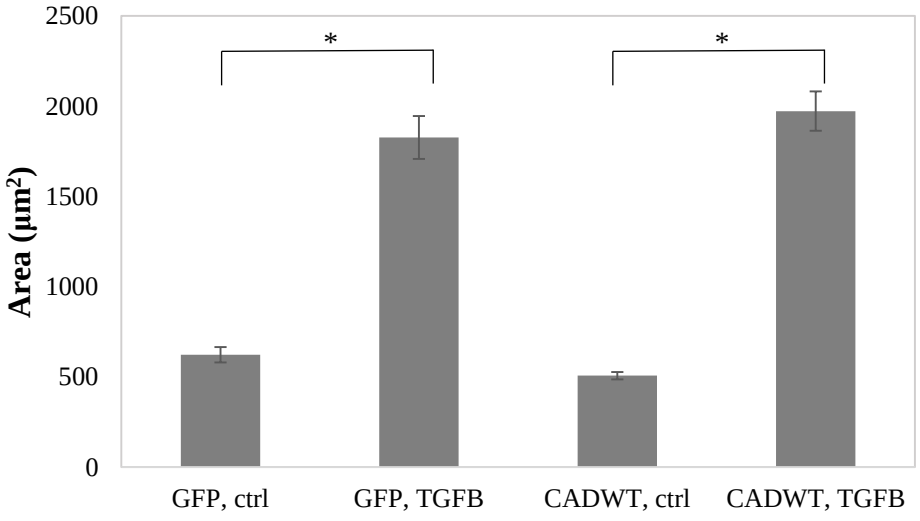

Figure 9. Average cell area for control transfected cells and cells overexpressing caldesmon with and without TGFβ1 treatment

Figure 9 shows that the average area is larger for cells treated with TGFβ1 in comparison to the control cells. This is consistent with cells that have undergone EMT, as cells tend to spread out and become larger as a result of the transition.

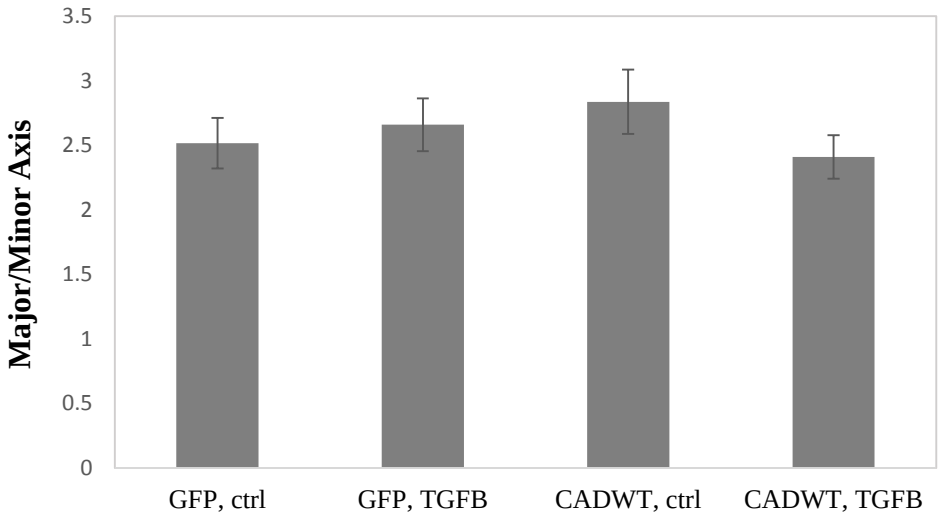

Figure 10. Average Aspect Ratio for control transfected cells and cells overexpressing caldesmon with and without TGFβ1 treatment

The aspect ratio is the ratio of the length of the cell's major axis to its minor axis. Figure 10 shows that cells all have about equal aspect ratios, as there is no significant difference between any of the treatment groups. This is atypical for mesenchymal cells to have similar aspect ratios to epithelial cells as mesenchymal cells tend to be more spread out.

Traction Force Microscopy

Traction force microscopy was a technique that was utilized to analyze the forces that the cells were exerting on their surroundings for each different treatment. The magnitudes of the traction forces are shown in the maps in Figure 11 below.

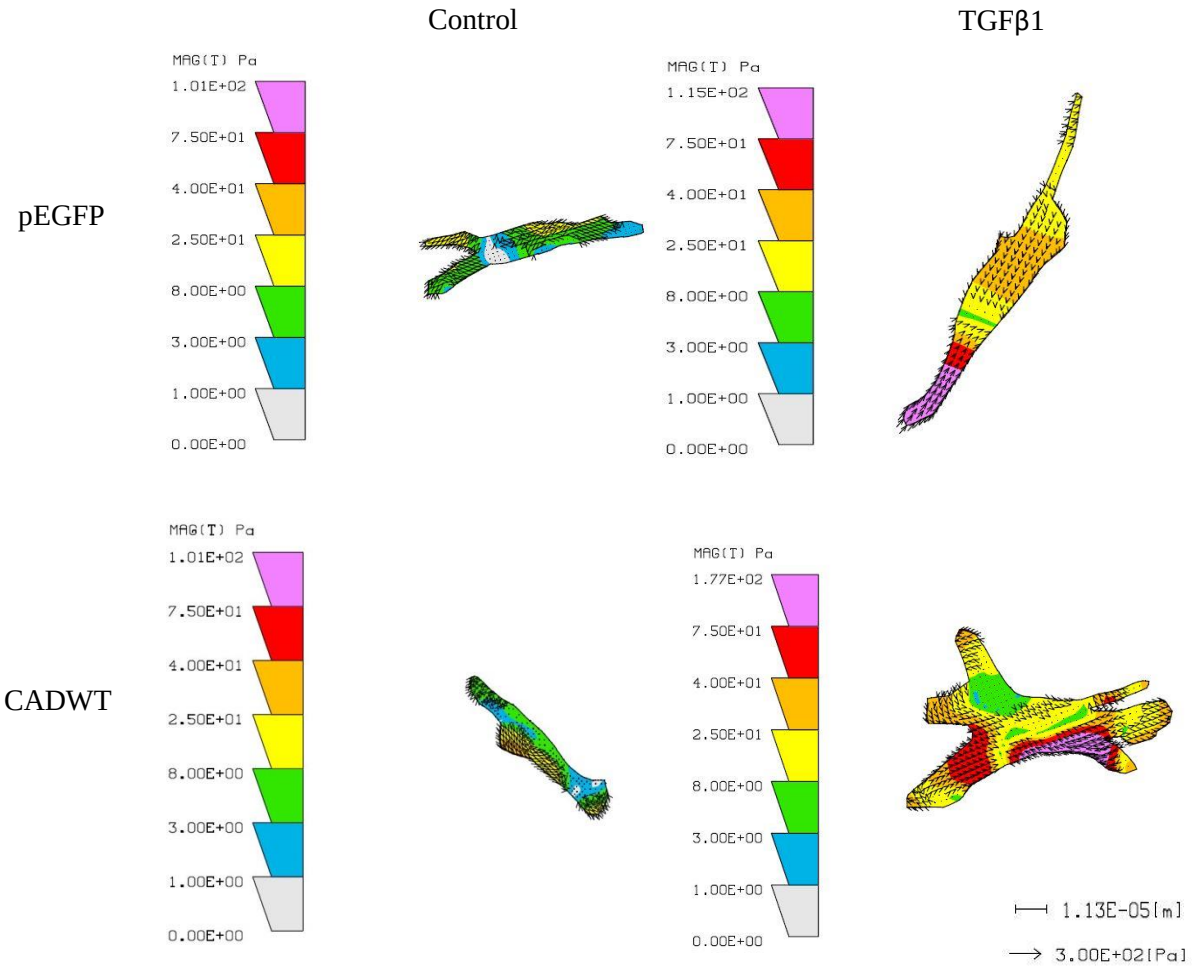

Figure 11. Representative traction stress maps for control transfected cells and cells overexpressing caldesmon with and without TGFβ1 treatment.

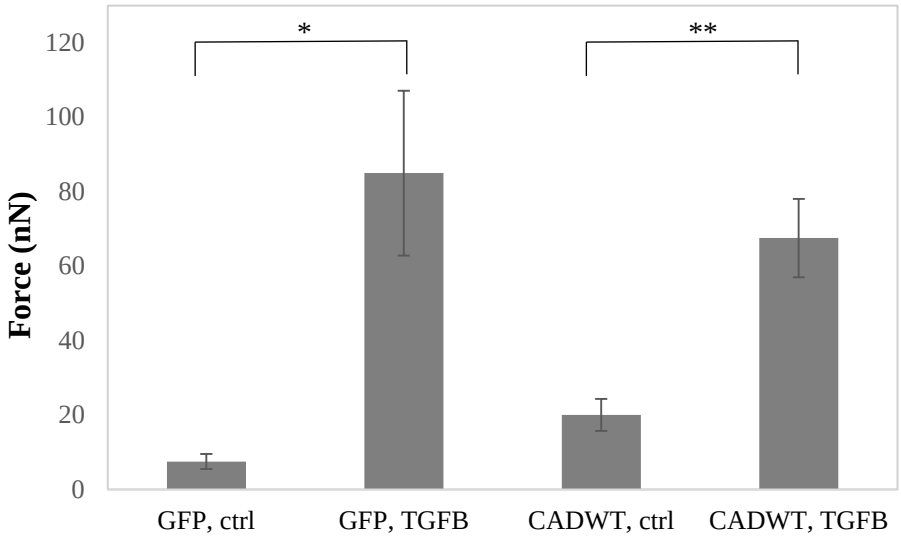

Figure 12. Total force for control transfected cells and cells overexpressing caldesmon with and without TGFβ1 treatment. (* p<0.005 and ** p<0.001)

Figure 12 shows the total force calculated by traction force microscopy for the different treatment groups of cells. The difference between the forces for cells treated with TGFβ1 as compared to control cells was significantly different regardless of treatment type. However, there was no significant difference between cells treated with TGFβ1 for cells with normal expression and overexpression of caldesmon.

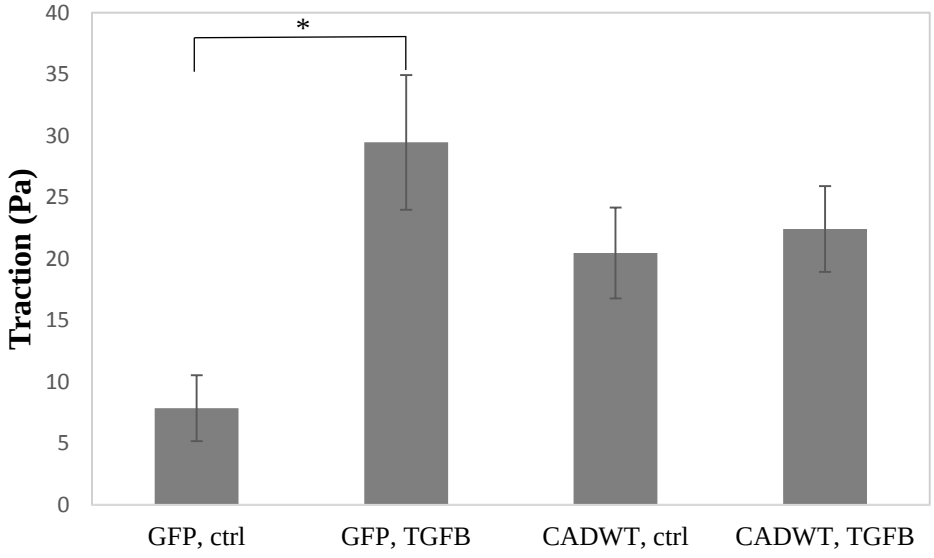

Figure 13. Force per unit area for control transfected cells and cells overexpressing caldesmon with and without TGFβ1 treatment. (* p<0.005)

Figure 13 above shows the force per unit area for the different treatment cell groups. For cells with normal expression levels of caldesmon the force per unit area was significantly higher for cells treated with TGFβ1 as compared to those treated with the control solution. However, for cells where caldesmon was overexpressed there was no significant difference between the TGFβ1 and control treatments. There was also no significant difference between cells treated with TGFβ1 for normal caldesmon expression or overexpression.

Chapter 4

Conclusions and Future Work

It was found through immunofluorescence staining and western blotting that overexpression of caldesmon led to an increase in α-SMA expression, a myofibroblasts marker. It was also shown that upon treatment with TGFβ1 for both normal expression and overexpression of caldesmon, stress fiber formation occurred. Interestingly, this is opposite to what has been observed for overexpression of caldesmon in some other cell types where it has been shown to lead to a disruption in stress fiber formation (9,10). Focal adhesions were analyzed and it was found that overexpression of caldesmon led to a slight increase in the number and area of focal adhesions in comparison to cells where caldesmon expression was at a normal level for treatment with either TGFβ1 or the control solution. Traction force microscopy was used to analyze the traction force generated by the cells and it was found the total forces were significantly higher for cells treated with TGFβ1 as compared to cells treated with control solution regardless of caldesmon expression levels. The force per area was significantly higher for cells with normal and overexpressed caldesmon levels when compared to control treated cells with normal caldesmon expression levels. However, cells where caldesmon was overexpressed and treated with control solution the force per area was not significantly lower than cells treated with TGFβ1.

The overexpression of caldesmon only gives half the information on the effects of caldesmon on EMT and myofibroblast formation. Another student in the group conducted experiments that knocked down the expression of caldesmon to analyze what happens during EMT when the cell is deficient in caldesmon. It was found that for cells where caldesmon was knocked down there was a decrease in α-SMA expression in comparison to the control cells that were treated with TGFβ1. Knockdown of caldesmon also led to decreased stress fiber formation, reduced focal adhesion formation, and cells that generated smaller traction forces.

In terms of continued work on the overexpression of caldesmon western blots can be done for cells grown on gels with a stiffness of 1800 Pa to observe how protein expression levels change. Another trial to analyze the aspect ratio of the cells could also be performed since the results are atypical, as epithelial and mesenchymal cells do not tend to have similar aspect ratios. Finally, more traction force experiments can be performed to confirm the force per area being generated by cells overexpressing caldesmon that where treated with control solution and look further into what causes its increase.

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