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The Effects of MRTF-A Phosphorylation State on TGFβ1-induced Epithelial-to-Mesenchymal Transition

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ABSTRACT

Epithelial-mesenchymal transition (EMT) is a biological process that results in the loss of cell-cell junctions and increased cell motility, invasiveness, and resistance to apoptosis[1-4]. Myocardin-related transcription factor (MRTF)-A is a G-actin-binding RPEL protein that works with serum response factor (SRF) to regulate cytoskeletal gene expression, contributing to transforming growth factor beta 1 (TGFβ1)-induced EMT. The polymerization of G-actin to F-actin frees the MRTF-A molecule from the G-actin and allows MRTF-A to enter the nucleus, where it can aid in promoting gene expression. MRTF-A subcellular localization can also be regulated by its phosphorylation state, which can be controlled by a variety of signaling molecules including extracellular signal-regulated kinase (Erk)[3,12,13]. To understand the effects of MRTF-A phosphorylation on TGFβ1-induced EMT, we examined cells transfected with MRTF-A and the MRTF-A mutants, S454A, which mimics the non-phosphorylated state, and S454E, which mimics the phosphorylated state. Using western blotting and immunofluorescence staining, we compared protein expression and localization and determined if cells underwent TGFβ1-induced EMT. We find that MRTF-A S454A promotes higher α-SMA expression than MRTF-A S454E. The results show that phosphorylation of MRTF-A decreases the expression of α-SMA, which is upregulated during EMT, while the de-phosphorylation of MRTF-A decreases the expression of E-cadherin, which is downregulated during EMT. Furthermore, treatment of cells with the inhibitor U0126, which blocks Erk activity, resulted in a decrease in the expression of the epithelial marker E-cadherin. These findings offer preliminary analysis toward future work to understand the mechanisms regulating MRTF-A phosphorylation and EMT.

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Chapter 1

Introduction

Epithelial-Mesenchymal Transition

Epithelial-mesenchymal transition (EMT) is a biological process that results in epithelial cells losing cell-cell junctions and gaining mesenchymal cell properties. These properties include increased motility and invasiveness, and resistance to apoptosis. In addition to these phenotypic changes, EMT results in a downregulation of epithelial markers, such as E-cadherin, and an upregulation of mesenchymal markers, such as alpha smooth muscle actin (α-SMA). Figure 1 depicts the process of EMT[1-4]. This process allows the cell to move away from the epithelial layer that lines the surfaces of body structures and compartments. This movement is significant in embryonic development, tissue repair, and malignant progression. These are classified as type 1, type 2, and type 3 EMT, respectively[1,5]. Regulation of EMT occurs via mechanical stimuli, such as contractile force and extracellular matrix stiffness[3,5]. Additionally, signaling pathways, such as epidermal growth factor (EGF), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), and transforming growth factor (TGF), have been shown to regulate EMT. In particular, TGFβ is a well-studied inducer of EMT[2,5].

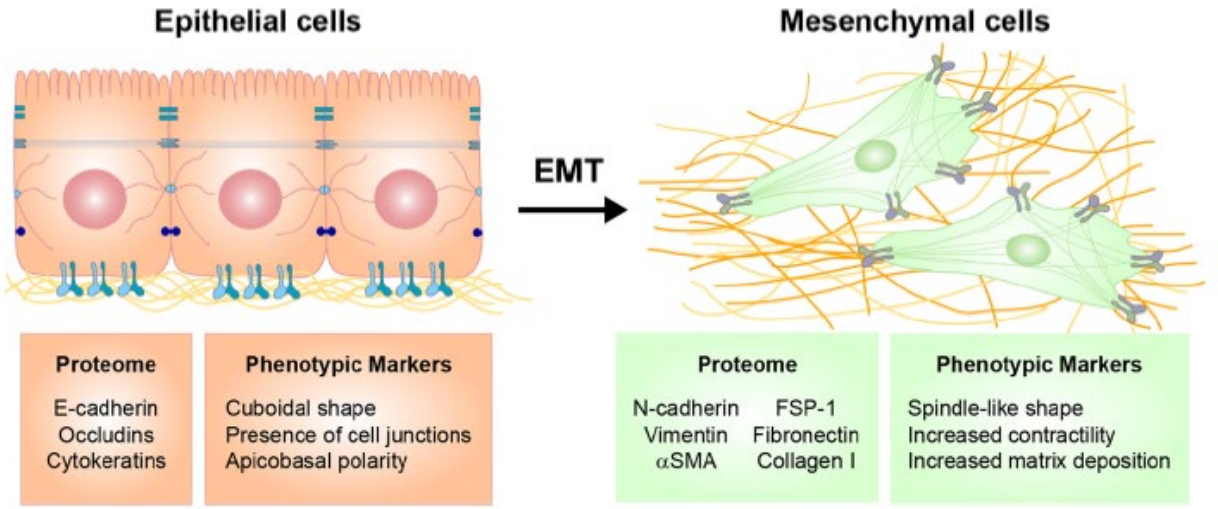

Figure 1: Schematic of epithelial-mesenchymal transition, a process in which epithelial cells lose polarity and cell-cell contact and gain mesenchymal cell characteristics such as increased motility and invasiveness. EMT results in the downregulation of epithelial markers, such as E-cadherin, and the upregulation of mesenchymal markers, such as α-SMA[3].

Transforming Growth Factor Beta-mediated EMT

Transforming growth factor beta 1 (TGFβ1)-mediated EMT works via the binding of TGFβ1 to type I and type II receptor serine/threonine kinases. This binding results in the association and phosphorylation of Smad2 and Smad3 transcription factors. A complex consisting of phosphorylated Smad2 and Smad3 and Smad4 translocates into the nucleus and regulates gene transcription. Other pathways activated by TGFβ1 include focal adhesion kinase (FAK), p38 mitogen-activated protein kinase (p38MAPK), and, of relevance to this thesis, extracellular signal-regulated kinase (Erk)[3].

Erk Signaling in EMT

Erk is a type of serine/threonine protein kinase that transmits mitogen signals. Upon activation, Erk travels from the cytoplasm into the nucleus to regulate transcription factor activity and gene expression. As a member of the MAPK family, Erk plays a role in signaling cascades and transmits extracellular signals to intracellular targets which regulates processes, such as cell proliferation, differentiation, and stress responses[6].

Activation of the Erk signaling pathway was found to be required for TGFβ1-mediated EMT in normal murine mammary gland (NMuMG) and mouse proximal tubule epithelial (MCT) cells. TGFβ1 treatment results in increased levels of phosphorylated Erk activity. However, treatment with U0126, a MEK inhibitor, blocks the Erk signaling pathway, and as a result, blocks TGFβ1-mediated EMT[7]. However, the role of the Erk signaling pathway on EMT appears to be different depending on the cell type. Other cell lines in which blocking the Erk signaling pathway has mediated TGFβ-induced EMT are renal tubular cells[8] and MDA-MB-231[9], a triple negative breast cancer cell line. On the other hand, TGFβ-induced EMT has been shown to not activate the Erk signaling pathway in mouse hepatocytes[10].

Myocardin-Related Transcription Factor A

Motile functions of cells, such as adhesion, cell-cell contact, and cell-extracellular matrix interactions, require dynamic changes in the actin microfilaments of the cell. This reversible polymerization of globular actin (G-actin) into filamentous actin (F-actin) is facilitated by actin

binding proteins. Furthermore, the actin cytoskeleton undergoes dramatic remodeling during TGFβ1-induced EMT. Epithelial cells lose cortical actin and gain F-actin stress fibers[11].

Myocardin-related transcription factor (MRTF)-A is a G-actin-binding RPEL protein that works with serum response factor (SRF) to regulate cytoskeletal gene expression[3,12,13]. The polymerization of G-actin to F-actin frees the MRTF-A molecule from the G-actin. The free MRTF-A molecule can then enter the nucleus, where it can aid in promoting gene expression. This process is depicted in Figure 2. Of relevance, MRTF-A has been shown to contribute to TGFβ1-induced EMT[3,14].

MRTF-A is regulated by G-actin through three methods: nuclear import, nuclear export, and activation or deactivation of MRTF-dependent transcription. MRTF-A bound to nuclear G-actin is unable to work with SRF to promote gene expression. In studying the phosphorylation state of MRTF-A, two important findings were that N-terminal phosphorylations affected nuclear import, and G-actin binding negatively regulated phosphorylation[12,13]. Additionally, Erk-mediated S98 phosphorylation promotes nuclear import by inhibiting assembly of G-actin complexes on the MRTF-A regulatory RPEL domain. In contrast, S33 phosphorylation promotes nuclear export[13]. The S454 site resembles a MAPK phosphorylation site, and its modification can be blocked by U0126, implying Erk is the kinase that phosphorylates MRTF-A at this site[15].

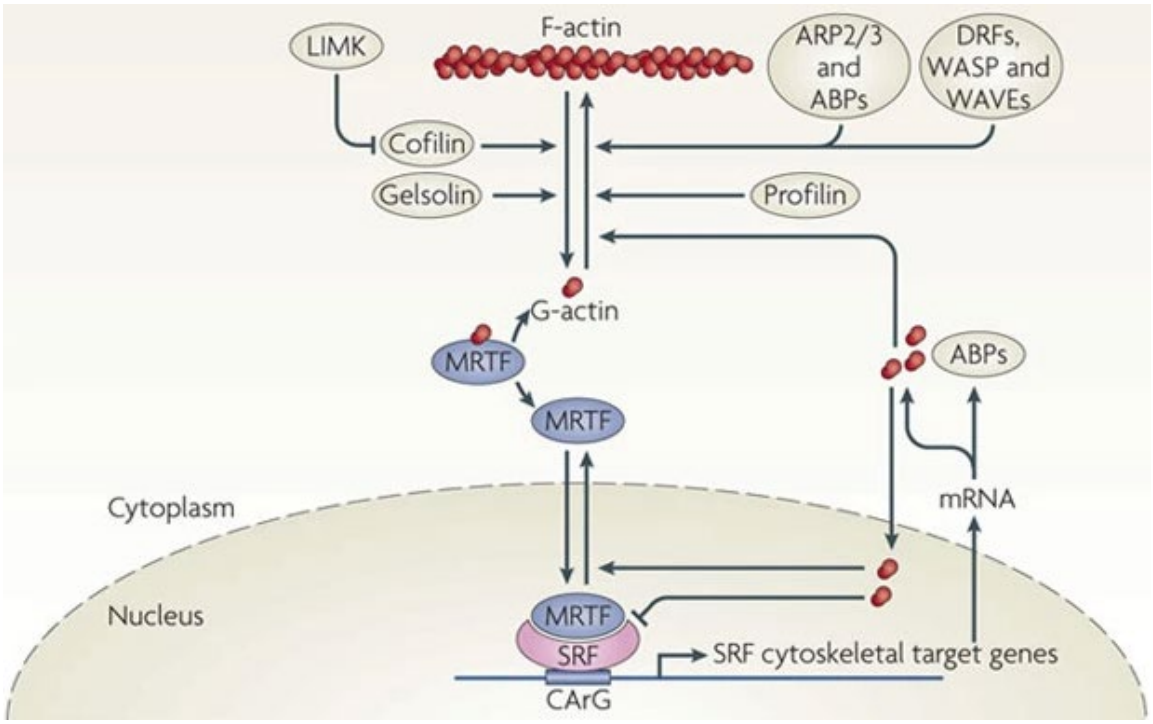

Figure 2: Schematic of the nuclear localization of MRTF-A. The dissociation of G-actin from the MRTF-A molecule to polymerize into F-actin allows MRTF-A to enter the nucleus. Inside the nucleus, MRTF-A works with SRF to regulate cytoskeletal gene expression[12]. Reprinted by permission from Springer Nature: Nature, Nature reviews molecular cell biology, Linking actin dynamics and gene transcription to drive cellular motile functions, Olsen E. N. & Nordheim, A., 2010.

Effects of TGFβ1-induced EMT on E-cadherin

Cadherins are transmembrane glycoproteins involved in tissue morphogenesis and coordinated cell movement by mediating calcium-dependent cell-cell adhesion. The cadherin family can be split into three major families: cadherins, protocadherins, and cadherin-related proteins. Classical cadherins span the membrane once and have five extracellular cadherin repeat domains. These domains bind with catenins, forming a cadherin-catenin complex, to mediate the actin cytoskeleton. Classical cadherins can be further divided into type-I and type-II based on the

molecular features of their interaction. Of relevance, E- and N-cadherin fall into the type-I group[16,17].

Epithelial cells express E-cadherin, which is important in regulating cell-cell adhesion and cell migration through various signaling pathways. On the other hand, mesenchymal cells express other cadherins, such as N-cadherin. Transcription factors, such as Snail, Slug, ZEB1, and ZEB2, have been shown to directly downregulate the expression of E-cadherin to induce EMT. Furthermore, MRTF-A has been shown to regulate Slug[16-19].

Effects of TGFβ1-induced EMT on α-SMA

The mammalian actin gene family consists of six isoforms including cytoplasmic actins, such as β non-muscle actins, and tissue-specific actins, such as α-SMA. α-SMA is a cytoskeletal protein that contributes to cell-generated mechanical tension. While expression is normally restricted to cells of vascular smooth muscle, non-muscle cells, such as myofibroblasts, can express α-SMA. Myofibroblasts are important cells for healing wounds, scars, and fibro contractive lesions[20].

Cells expressing α-SMA can arise from EMT, and its expression can be controlled by growth factors and specialized extracellular membrane proteins. Ras homolog family member A (RhoA) is a part of the Rho family proteins and can mediate SRF activity by inducing actin polymerization. This regulation can in turn affect the transcriptional activity of genes regulated by SRF, such as α-SMA[20,21]. In addition, expression of α-SMA has been found to be regulated by cell shape partially through controlling the subcellular localization of MRTF-A. MRTF-A and

SRF work together to regulate the transcription of many genes associated with actin dynamics and cell contractile function, including α-SMA[3].

Purpose and Objective

The purpose of this study is to understand the effects of MRTF-A phosphorylation on TGFβ1-induced EMT. Previous studies have shown that nuclear localization of MRTF-A during EMT regulates the expression of some mesenchymal genes. We hypothesize that the phosphorylation state of MRTF-A will regulate the nuclear import of the MRTF-A molecule during TGFβ1-induced EMT and affect gene expression. This hypothesis will be tested by examining cells transfected with MRTF-A and the MRTF-A mutants, S454A, which mimics the non-phosphorylated state, and S454E, which mimics the phosphorylated state. Western blotting and immunofluorescence staining are performed to compare protein expression and localization and to determine if cells have undergone TGFβ1-induced EMT. Specifically, we compare the expression levels of the epithelial marker E-cadherin, and the mesenchymal marker α-SMA. A better understanding of the mechanisms behind EMT and regulation of this process can assist toward the development of new therapies for diseases such as cancer and fibrosis.

Chapter 2

Materials and Methods

Cell Culture

Normal murine mammary gland (NMuMG) epithelial cells (American Type Culture Collection) were cultured in media comprised of Dulbecco's Modified Eagle Medium (DMEM), 0.005 mg mL^{-1} insulin, 0.05 mg mL^{-1} gentamicin, and 10% fetal bovine serum (FBS). The cells were incubated at 37°C and at 5% CO_2. Cells were plated 10,000 cells cm^{-2} and treated with 10 ng mL^{-1} of recombinant TGFβ1 for 72 hours prior to downstream analysis. For inhibitor studies, cells were treated with 10 μM of U0126 from UMD Millipore.

Transfection

Lipofectamine LTX Reagent was diluted in Opti-MEM Medium. MRTF-A, S454A, S454E, and yellow fluorescent protein (YFP) were each diluted in Opti-MEM Medium and mixed with PLUS Reagent. In a 1:1 ratio, the diluted DNA solutions were each mixed with the diluted Lipofectamine LTX Reagent and incubated at room temperature for 5 minutes. Plated cells were treated with the DNA-lipid complexes.

Immunofluorescence Staining

For the staining of E-cadherin, cells were fixed with 4% paraformaldehyde for 15 minutes. Cells were washed twice with 1× phosphate buffered saline (PBS) and stored at 4°C. Cells were then permeabilized by incubating twice with 0.5% v/v IGEPAL solution in 1× PBS for 10 minutes and twice with 0.1% v/v Triton X-100 solution in 1× PBS for 10 minutes. Blocking buffer was prepared using 5% v/v goat serum and 0.3% v/v Triton X-100 in 1× PBS. Cells were incubated at room temperature with the blocking buffer for 90 minutes. Primary antibody recognizing E-cadherin (Cell Antibody 24E10 #3195) diluted 1:200 in the blocking buffer was pipetted onto parafilm. Samples were placed cell side down on the parafilm and incubated overnight at 4°C. Cells were washed with 1× PBS three times and incubated in a secondary antibody (Invitrogen A-11037) diluted 1:500 in 1× PBS for 1 hour at room temperature. Cells were washed with 1× PBS three times again. To stain the cell nuclei, the sample was incubated for 15 minutes at room temperature in a 1:10000 Hoechst 33342 to 1× PBS solution. The cells were then washed with PBS twice.

Imaging

A Nikon Eclipse Ti-E inverted fluorescence microscope with a 20× objective and a Photometrics CoolSNAP HQ2 CCD camera was used to image the stained samples. Image J was used to process the images taken to compare the protein expression levels of control and TGFβ1-treated samples.

Protein Extraction and BCA Assay

Cold RIPA buffer with Halt Protease and Phosphatase Inhibitor Cocktail was added to treated cells for 5 minutes on ice, occasionally swirling for uniform spreading. Lysate was collected via cell scraping and centrifuged at 4 ºC for 15 minutes at 14,000 rpm. The supernatant was stored at -80 ºC.

The Pierce BCA Protein Assay Kit was used to determine protein concentration. Standards with bovine serum albumin (BSA) concentrations ranging from 0 to 2 mg mL^{-1} in 0.9% sodium chloride solution were prepared to calibrate the assay. Standards and samples were pipetted into microplate wells. BCA reagent was added to each well and incubated at 37 ºC for 30 minutes. Using a Tecan Infinite M200 Pro microplate reader, the relative absorbance of each sample was measured. A standard curve was generated and used to determine the protein concentration of each sample.

Western Blotting

Samples were prepared using NuPAGE LDS sample buffer and NuPAGE sample reducing agent at 70 ºC for 10 minutes. 20× 4-morpholinepropanesulfonic acid (MOPS) solution was diluted with deionized water to a 1× MOPS solution. Samples loaded on a NuPAGE 4-12% bis-tris gel were separated using gel electrophoresis at 120 V with NuPAGE MOPS SDS running buffer in an XCell SureLock Cell powered by a Bio-Rad Power Pac HC. The gel was transferred onto a nitrocellulose membrane and placed in an XCell II Blot Module at 30V for membrane transfer. 10× tris buffered saline was diluted with deionized water and 0.1% tween concentration

to prepare a 1× tris buffered saline with tween (TBST) buffer solution. 5% non-fat dry milk in 1× TBST was used to block the membrane, which was incubated with primary antibodies against α-SMA (Sigma 1A4) diluted 1:2500 overnight at 4 ºC. Following three rinses with 1× TBST, the membrane was incubated with IRDye secondary antibodies for 1 hour at room temperature. Three additional rinses were performed after the incubation. A Licor Odyssey imaging system was used to capture and Image J was used to analyze images of the western blots.

Densitometric Analysis

Image J was used to quantify the relative expression levels of control and TGFβ1-treated samples. The mean gray value for the protein bands and the background were quantified. To get expression levels, the background value was subtracted from the protein band values. The normalized intensity was obtained by dividing the intensity of the protein band by the intensity of the loading control of each sample. Relative expression levels were determined by dividing the normalized intensity of the samples by the control of that set of samples.

Chapter 3

Results and Discussion

E-cadherin Immunofluorescence Staining of DMSO and U0126-Treated Samples

To investigate the effects of Erk phosphorylation on EMT, NMuMG epithelial cells were treated with U0126, a MEK inhibitor, and treated with TGFβ1. Immunofluorescence staining was performed to compare the E-cadherin expression levels. Figure 3 shows the E-cadherin immunofluorescence staining results of cells treated with U0126 with (right) and without (left) TGFβ1. For both DMSO and U0126 samples, expression of E-cadherin is higher in control samples than their respective TGFβ1-treated samples. However, staining is less pronounced for U0126 samples compared to DMSO-treated samples. These results suggest blocking Erk phosphorylation via U0126 treatment regulates the expression of E-cadherin.

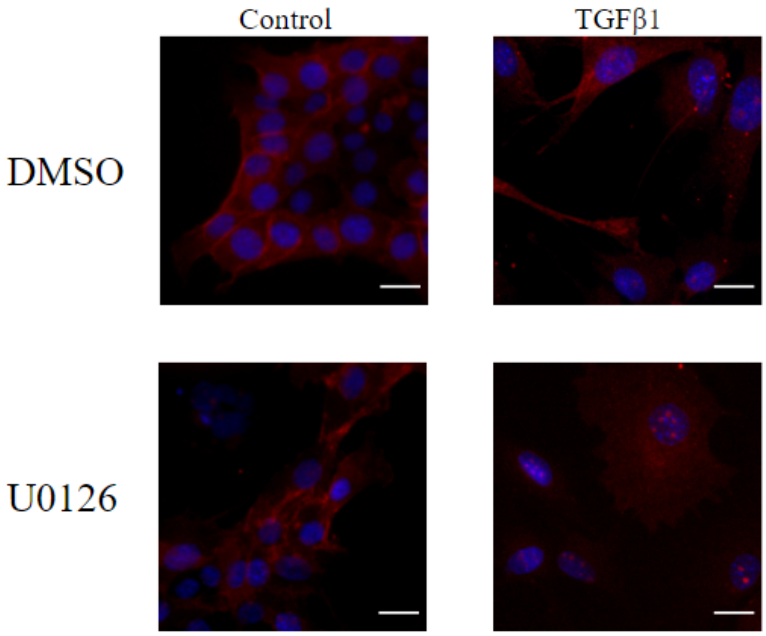

Figure 3: Nuclear (blue) and E-cadherin (red) immunofluorescence staining of cells treated with DMSO or U0126 and with (right) and without (left) TGFβ1. Scale is 20 µm.

Protein Expression of Transfected Samples

Previous studies show that Erk can phosphorylate MRTF-A and can impact its subcellular localization and the expression of MRTF-A target genes[3]. To investigate the impact of MRTF-A phosphorylation, NMuMG cells were transfected with MRTF-A and the MRTF-A mutants, S454A, which mimics the non-phosphorylated state, and S454E, which mimics the phosphorylated state. Cells transfected with a YFP plasmid were used as a control. The concentration of protein extracted from transfected cells was determined using a BCA assay. Figure 4 shows the normalized curve for the bovine serum albumin standards with known concentrations that was generated as a function of absorbance. This curve was used to determine the bulk protein concentration of each sample. The fit equation along with the average absorbances for each sample were used to calculate the protein concentration. These

concentrations are tabulated in Table 1. The concentrations for each sample found were used to determine the volume to load into the gel for western blotting. This is done to ensure that equal amounts of total protein are loaded to each well so that protein levels can be compared across samples.

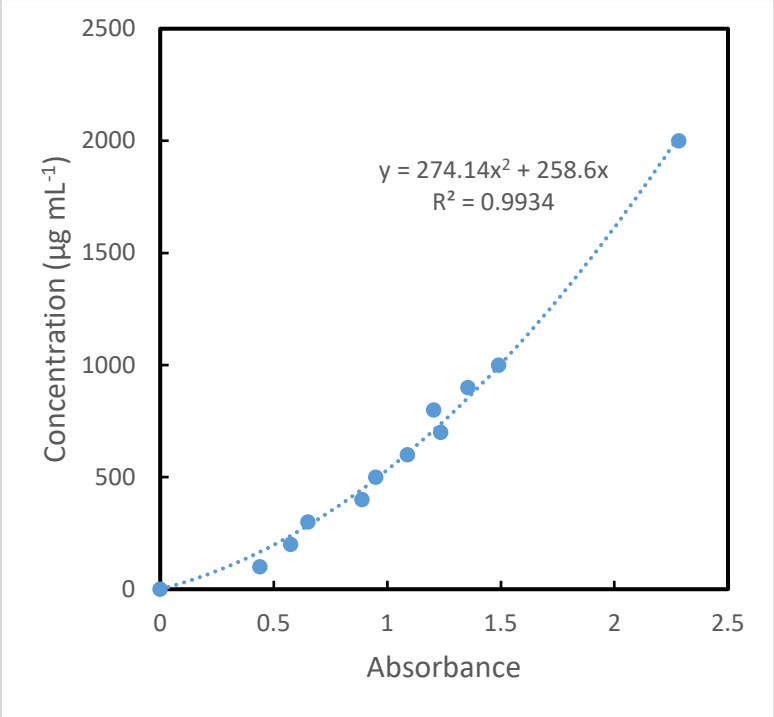

Figure 4: Standard curve for the BCA Assay.

Table 1: Concentrations for the transfected control and TGFβ1-treated samples calculated using the standards curve.

Sample	Concentration (µg mL^{-1})
YFP Control	339.0
YFP TGFβ	326.1
MRTF-A Control	309.3
MRTF-A TGFβ	243.8
S454A Control	347.4
S454A TGFβ	349.3
S454E Control	259.8
S454E TGFβ	352.8

Western blotting was then performed to compare the levels of EMT-associated proteins in transfected cells. The results of the western blot shown in Figure 5 show that the expression of α -SMA is higher in samples treated with TGFβ1 than those not treated. TGFβ1 has been shown to induce EMT, and as such, a higher expression of α-SMA, a mesenchymal marker, is expected for treated samples. The expression of α-SMA for the MRTF-A sample is more pronounced than that of S454A, S454E, and YFP samples. This is likely due to overexpression of the MRTF-A protein. Specifically, YFP samples have the lowest expression of α-SMA, followed by S454E samples, and then S454A samples. Comparing the expression levels of the S454A and S454E samples, which mimic the non-phosphorylated and phosphorylated states respectively, suggests the phosphorylation state of MRTF-A impacts the expression of α-SMA in TGFβ1-induced EMT. Additionally, for the S454E sample, α-SMA expression was expected to be greatly diminished with TGFβ1 treatment due to phosphorylation enhancing nuclear export. This data suggests there is a regulator that contributes to α-SMA expression in addition to MRTF-A.

A densitometric analysis was performed on the western blot results to quantify the relative expression levels of α-SMA. The results of the densitometric analysis shown in Figure 6 support the earlier conclusion comparing S454A and S454E samples. S454E samples shows slightly less α-SMA expression compared to S454A samples. TGFβ1-treated samples likely maintain some levels of α-SMA expression due to the regulation of endogenous MRTF-A expressed by the cells. Additionally, MRTF-A samples had significantly higher α-SMA expression than YFP samples, These results agree with previous studies from the Gomez group showing nuclear localization of MRTF-A during TGFβ1-induced EMT regulating the expression of some mesenchymal genes.

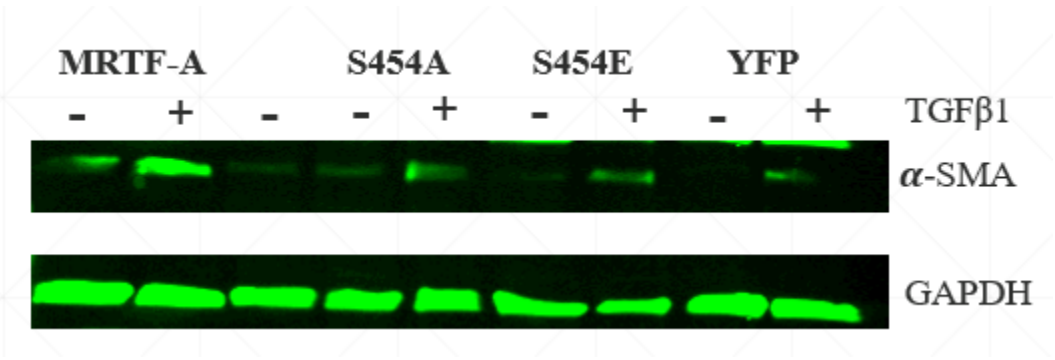

Figure 5: Western blot of transfected (MRTF-A, S454A, S454E, and YFP) control and TGFβ1-treated samples for GAPDH, a loading control, and α-SMA, a mesenchymal marker. Samples treated with TGFβ1 show greater expression of α-SMA than the control.

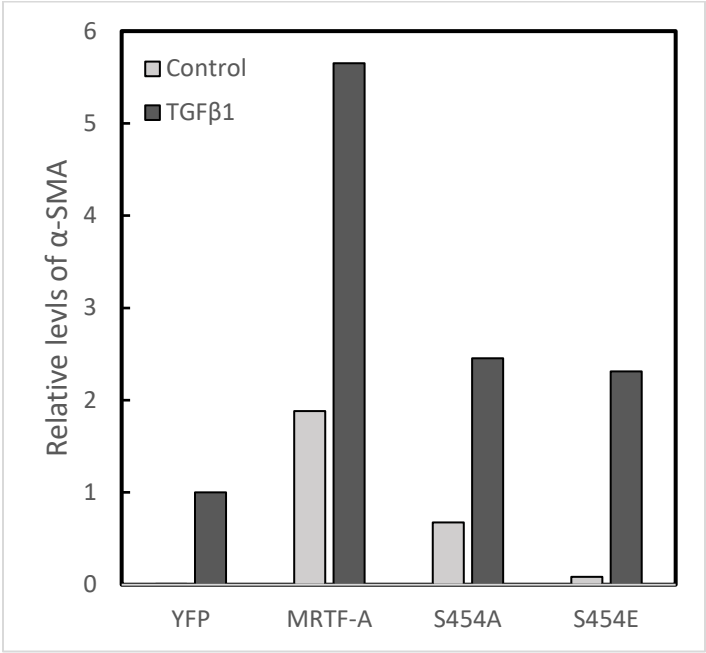

Figure 6: Densitometric analysis of western blot results. The S454E mutant shows lower expression of α-SMA than the S454A mutant.

E-cadherin Immunofluorescence Staining of Transfected Samples

In addition to the western blot results, immunofluorescence staining was performed to determine the expression of E-cadherin, an epithelial marker, in cells that were transfected with MRTF-A mutants. Figure 7 shows the E-cadherin immunofluorescence staining of transfected

(YFP, MRTF-A, S454A, S454E) cells with (right) and without (left) TGFβ1. YFP serves as a control to compare to the other transfected samples. Across all transfected samples, the control groups show greater levels of E-cadherin staining than TGFβ1-treated samples. While the expression of E-cadherin is higher for the S454A control samples than the corresponding TGFβ1-treated samples, staining of the control is minimal. E-cadherin staining in S454E control samples is more pronounced than the staining in S454A control samples. This data suggests that the nuclear export of phosphorylated MRTF-A helps regulate the expression of E-cadherin during EMT.

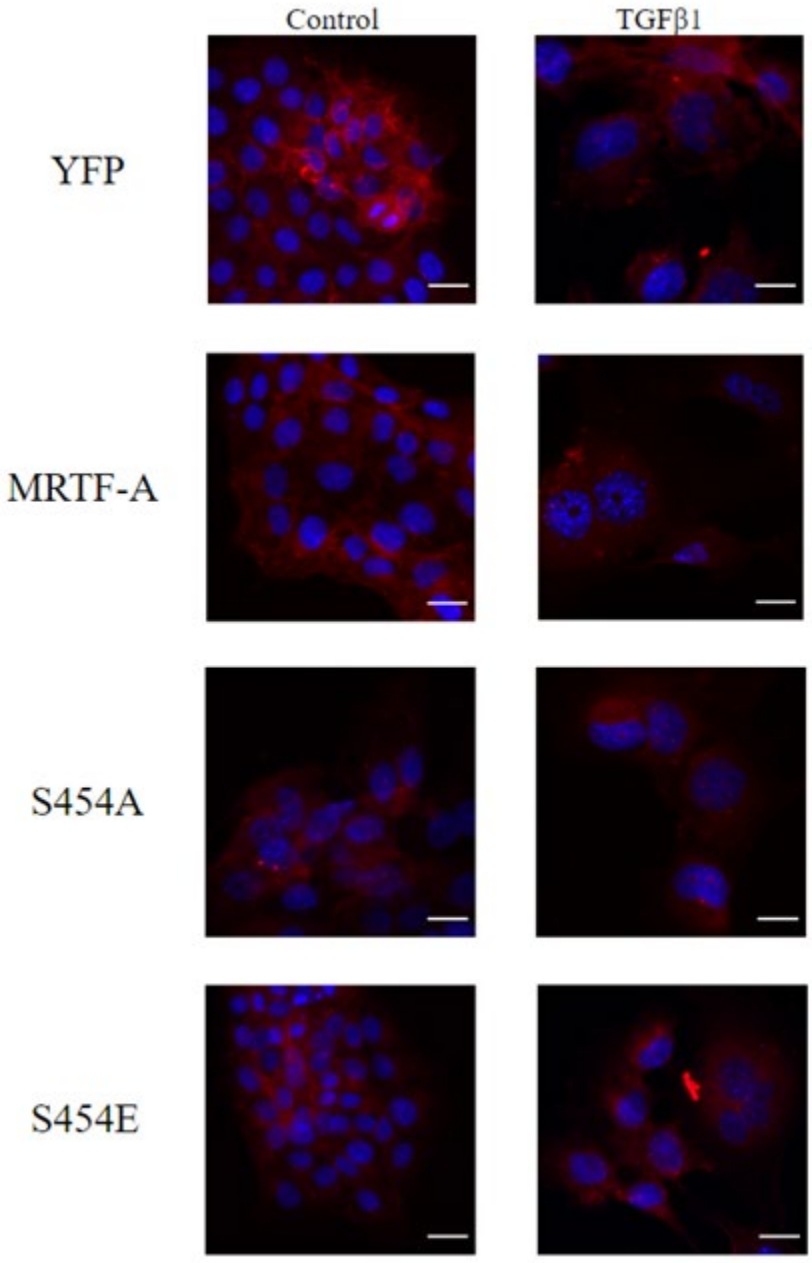

Figure 7: Nuclear (blue) and E-cadherin (red) immunofluorescence staining of transfected (YFP, MRTF-A, S454A, S454E) control and TGFβ1-treated cells. Scale is 20 µm.

Chapter 4

Conclusions and Future Work

These results suggest that there is a relationship between the phosphorylation state of MRTF-A and TGFβ1-induced EMT. Previous studies from the Gomez group have shown that nuclear localization of MRTF-A during TGFβ1-induced EMT regulates the expression of some mesenchymal genes. From the western blot analysis of the transfected samples, cells treated with TGFβ1 showed higher levels of α-SMA. Additionally, the S454A mutant, which mimics the de-phosphorylated state of MRTF-A, showed greater expression of α-SMA than the S454E mutant, which mimics the phosphorylated state of MRTF-A. Immunofluorescence staining of E-cadherin was also performed to support these results. In the transfected samples not treated with TGFβ1, cells expressing the S454E mutant had more pronounced E-cadherin staining than cells transfected with the S454A mutant. This data suggests that phosphorylation of MRTF-A decreases the expression of α-SMA, while the de-phosphorylation of MRTF-A decreases the expression of E-cadherin. It is important to note this experiment was performed once. Replicate experiments should be performed to confirm reproducibility of results and to determine if observed differences are statistically significant.

For NMuMG cells, expression of E-cadherin for U0126 treatment was found to be decreased relative to the DMSO control when blocking the Erk signaling pathway using immunofluorescence staining. There was no difference in E-cadherin expression for both samples when treated with TGFβ1 to induce EMT. Further quantification, such as western blotting, should be performed to confirm the immunofluorescence staining results. Additionally,

experiments can be performed to look at the expression of other epithelial and mesenchymal markers when blocking the Erk signaling pathway.

For cells expressing the S454E mutant and treated with TGFβ1, α-SMA expression, while diminished, was not completely gone, suggesting an additional regulator contributing to α-SMA expression. Exploration of other proteins or pathways that could contribute to α-SMA expression in addition to MRTF-A could help provide more details on EMT and α-SMA regulation. Understanding the mechanisms regulating MRTF-A phosphorylation and EMT can help understand diseases, such as cancer, and develop therapies to those diseases.

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ACADEMIC VITA

STEVEN H. PHAN
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EDUCATION

Bachelor of Science in Chemical Engineering (August 2017 - May 2021)
The Pennsylvania State University, University Park, PA
Schreyer Honors College

Thesis: The Effects of MRTF-A Phosphorylation State on TGFβ1-induced Epithelial-to-Mesenchymal Transition
Thesis Supervisor: Dr. Esther Gomez

EXPERIENCE

Undergraduate Research Assistant (September 2019 - Present)
Dr. Esther Gomez Laboratory

- Performed the passaging of mammary epithelial cells following recommended ATCC procedures
- Reviewed literature and reported results and key points to the graduate student
- Performed protein assays, Western Blotting, and immunofluorescence staining and imaging

Intro to Chemical Engineering Thermodynamics Instructional Aide (January 2021 - May 2021)
The Pennsylvania State University

- Organized personal office hours to assist students with problem sets and answer questions
- Directed recitation sessions to review course material and solve problem sets
- Communicated and implemented ideas for improving the course and recitation sessions with the instructor

Chemical Engineering Phase and Chemical Equilibria Grader (August 2020 - December 2020)
The Pennsylvania State University

- Analyzed and provided feedback on the work of 90 students
- Responded to grading mistakes and re-grading requests from students
- Communicated with the other grader to review individual responsibilities for the week

LEADERSHIP

Logistics Chair (May 2020 - Present)
Penn State American Institute of Chemical Engineers

- Participated in weekly meetings with 18 other executive board members to assess events held the week prior and to discuss plans for the upcoming week
- Collaborated with co-chair to schedule events and ensure they ran smoothly

Equipment Captain (August 2019 - February 2021)
Apollo Benefiting THON

- Participated in fundraising events to support pediatric cancer research and families affected by cancer
- Designed and created signs for members to hold during the 46-hour dance marathon
- Organized team meetings to plan and discuss new sign ideas

Orientation Leader (May 2018 - August 2019)
The Pennsylvania State University

- Instructed a group of 20 first year students about the university systems
- Coordinated with other team members to guide and assist approximately 650 first year students
- Communicated and planned unexpected changes in schedule due to weather and time with team members