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Investigating TGF-β1 and Fibronectin Signaling in Low Oxygen Environments

Caitlin E. Williams
Virginia Commonwealth University

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A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science in Biomedical Engineering at Virginia Commonwealth University.

by

Caitlin E. Williams
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Director: Dr. Christopher Lemmon, Ph.D.
Professor and Associate Chair, Department of Biomedical Engineering

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List of Abbreviations

ECM	Extracellular matrix
EMT	Epithelial-Mesenchymal Transition
TGF-β1	Transforming Growth Factor- beta1
LDH	Lactate Dehydrogenase
FN	Fibronectin
HIF-1α	Hypoxia-Inducible Factor 1-alpha
FUD	The 49-residue functional upstream domain (FUD) of Streptococcus pyogenes F1 adhesion
PBS	Phosphate-buffered saline
BSA	Bovine serum albumin

Abstract

Breast cancer is the second leading cause of death in women, second only to lung cancer. It's one of the most well-known cancers, with almost 300,000 new cases estimated to be diagnosed in 2023 alone. Breast cancer often begins in ductal epithelial cells, and these cells will typically undergo a cellular transformation process known as epithelial-mesenchymal transition (EMT) in early stages. Breast cancer becomes deadly when it reaches stage IV, or a metastatic stage. As a part of the metastatic process, transformed cells will conduct remodeling of the extracellular matrix (ECM) to allow for migration to occur in an otherwise non-migratory cell type.

As dense tumors form, regions of low oxygen, or hypoxia, will begin to develop. This occurs in part due to cellular proliferation outpacing angiogenesis, resulting in reduced delivery of nutrients and oxygen to cells located more centrally in the tumor. In hypoxia, cells will undergo anaerobic respiration, creating lactic acid as a byproduct. Buildup of lactic acid in the environment leads to a decrease in extracellular pH. The lack of oxygen and decrease of extracellular pH facilitate cellular transformation, and further encourage cellular migration.

This work aims to better understand the role of changes in extracellular pH and low oxygen environments on EMT, and to further investigate the role of TGF-β1 signaling and fibronectin assembly on cellular transformation in low O_2. We cultured cells in acidic environments in normoxic conditions to investigate the role pH may play in hypoxia induced EMT. Cells were also cultured in hypoxia and treated with FUD or TGFB1 gene knockout, and we found that inhibition of TGF-β1 signaling or FN assembly can mitigate EMT in hypoxia. This work could provide insight into therapeutic targets against cellular transformation as a preventative treatment.

Chapter 1: Introduction

It is estimated that there will be over 297,000 new breast cancer diagnoses in women in 2023. Breast cancer has been one of the leading causes of death in women for multiple years, but there is still much to be learned about the complex disease.

Most breast cancers begin in ductal epithelial tissue, categorizing it as a carcinoma. Epithelial tissue covers all internal and external surfaces of the body, and one of their primary responsibilities is to maintain the structure of glands. Epithelial cells maintain a tight 'cobblestone' morphology, and have apicobasal polarity, E-cadherin containing adherens junctions, and cortical actin as some of their defining characteristics. However, these cells are able to undergo epithelial-mesenchymal transition (EMT), a necessary cell transformation process during wound healing and development. During EMT, cells will lose their epithelial phenotype, and transform into a more migratory mesenchymal phenotype. This increased migratory ability occurs through loss of apicobasal polarity, breakdown of cell-cell junctions, and development of F-actin stress fibers. Additionally, cells will upregulate expression of the ECM protein fibronectin and assemble fibronectin into fibrils in the ECM. While vital to wound healing and development, unregulated EMT is implicated in several disease states, including fibrotic diseases and cancer. Increased transforming growth factor beta-1 (TGF-β1) signaling is known to induce EMT, with addition of exogenous TGF-β1 causing transformation in breast epithelium [1].

As dense tumors form, cells will recruit new blood vessels to form around the tumor via angiogenesis to deliver more nutrients and oxygen. However, as tumor cell proliferation begins to outpace angiogenesis, hypoxic regions begin to develop within the tumor. Hypoxia in tissue occurs around 2-6 % oxygen, with 'normoxic' tissue receiving around 3-7% oxygen [2]. The exact hypoxic range in different tissues varies, causing hypoxia-responsive proteins to be

upregulated at different O_2 levels in different tissues [2]. Of these hypoxic-responsive proteins, hypoxia-inducible factor-1alpha (HIF-1α) is a dimeric transcription factor that is upregulated in low O_2 environments. The alpha subunit is degraded in oxygen-rich environments, but maintains its structure in low O_2, allowing it to catalyze low O_2 dependent signaling. As part of this signaling pathway, HIF-1α has been found to upregulate lactate dehydrogenase-5 expression in lung fibroblasts. Cytosolic enzyme lactate dehydrogenase-5 converts pyruvate, a common byproduct of anaerobic respiration, into lactate. As lactate binds to free H+ ions, lactic acid is formed, and the local cellular environment is acidified. This is a common process in anaerobic cellular respiration, but when prolonged can cause alterations in cellular metabolism, and cellular transformation. Cancerous cells are known to have altered metabolic processes, in which cells preferentially metabolize glucose, and over-consume resources from their environment.

Low O_2 environments have been shown to upregulate TGF-β1 signaling in lung fibroblasts [3], allowing for EMT to occur in epithelial tissue, as described previously. However, to our knowledge this pathway has not been investigated in mammary epithelium. This work aims to investigate the effect of low oxygen environments on EMT in mammary epithelium through TGFβ1 signaling and FN assembly. Additionally, we aim to understand the role of pH on fibronectin fibril assembly to better understand how the acidification of the local cellular environment can affect EMT pathways. Understanding of these mechanisms could provide potential therapeutics to pre-metastatic breast cancers.

Chapter 2: Background

2.1 – Epithelial-Mesenchymal Transition

It is estimated that around 30% of cancers diagnosed in women will be breast cancer in 2023 [4]. Breast cancer is the second most common cause of death among women, second to lung cancer [5]. While breast cancer treatment is advancing, there is still no cure, and there is still much to be learned about the mechanisms at play leading to cancer development and progression. Breast cancer occurs in ductal epithelial cells, categorizing it as a carcinoma. Carcinomas are the most common form of cancer, and occur within the breast, skin, liver, lung, and other epithelial tissues.

Epithelial-Mesenchymal Transition (EMT) is a necessary process during wound healing and embryonic development, playing a key role in tissue remodeling [6]. However, dysregulation of EMT is a part of several disease states, including fibrosis and cancer [7]. In early stages of carcinomas, epithelial cells are susceptible to undergo EMT, in which they lose many of their epithelial characteristics and take on a more migratory mesenchymal phenotype. Cells that have undergone EMT will present breakdown of E-cadherin-containing adherens junctions, formation of F-actin stress fibers, and increased fibronectin fibril assembly in early transformation [8]. Additionally, epithelial cells will demonstrate a loss of polarity, further allowing them to detach from the neighboring cells, and increase migratory abilities of the cell [9]. Targeting EMT has been a promising therapeutic for cancer treatment, as EMT plays a key role in allowing cancerous cells to metastasize to distant organs [10]. EMT is also suspected to contribute to cancer cells' chemotherapy resistance by altering the chemical pathways that the cell utilizes [11], [12].

A primary driver of EMT is Transforming growth factor beta-1 (TGF-β1). TGF-β1 is a versatile cytokine involved in development, wound healing, and cell proliferation [13]. In

healthy tissue, TGF-β1 works as a tumor suppressor by inducing cell cycle arrest and apoptotic events [14]. TGF-β1 has been found to induce EMT in epithelial cells, causing a loss of polarity, and development of a migratory phenotype [15]. When produced endogenously, TGF-β1 is secreted within a Latency Associated Peptide (LAP). LAP restricts TGF-β1 from binding to TGF- β receptors on the cell surface. LAP is stretched open mechanically, which releases active TGF-β1 into the extracellular space for autocrine or paracrine signaling. This mechanical stretching and opening can be facilitated by fibronectin (FN) fibrils, which anchor LAP in the extracellular matrix (ECM) so that integrins can stretch the peptide open [16]. LAP can also be opened via a decrease in pH, which is suspected to denature the peptide and release active TGF-β1 into the environment [16], [17].

Fibronectin is an extracellular matrix protein that plays a key role in wound healing and embryogenesis, and is expressed across a variety of cell types [18]. Fibronectin is secreted by cells as soluble, non-assembled proteins (often in plasma), and assembled into insoluble fibrils in the ECM by fibroblasts. Formation of fibronectin fibrils occurs through binding of fibronectin to $\alpha_5\beta_1$ integrins on the cell surface. Once bound to the integrin, fibronectin is stretched out of its compact state, exposing binding domains for FN-FN interactions [19]. This process repeats until a fibril is assembled. Fibronectin is able to bind to a variety of other extracellular matrix proteins as well, including heparin, collagen, and fibrin. Binding of these proteins facilitates the stiffening of the ECM in pathological conditions, and remodeling of the ECM can lead to metastatic cancer cells traveling through the matrix and to distal sites through vasculature [20]–[22].

Fibronectin assembly is a critical part of wound healing, but uncontrolled fibronectin assembly can be indicative of disease states. Increased stiffness of the ECM from excess protein deposition causes alterations in cellular function, and can further facilitate disease progression. Functional Upstream Domain (FUD) is a 49-residue domain of the protein F1

Adhesin which is found in Streptococcus pyogenes; FUD has been found to inhibit fibronectin assembly [23]. Previous work from our lab has shown that fibronectin fibrils regulate TGF-β1 induced EMT, and blockage of FN fibril formation via FUD inhibits the TGF-β1 signaling pathway [24]. Utilization of FUD to block EMT is a promising therapeutic, as it does not seem to affect the epithelial characteristics of cells treated with it [24]. This makes utilization of FUD a potential avenue for prevention of metastasis in early stage cancers, and a potential method to block EMT in low oxygen environments.

In response to uncontrolled cell proliferation during tumor formation, a significant upregulation of vascular endothelial growth factor (VEGF) will occur to recruit new blood vessels to form around the developing tumor [25]. Within dense tumors, cellular proliferation often outpaces angiogenesis, resulting in low oxygen (hypoxic) regions. These hypoxic regions enable increased cellular transformation, and further alter the metabolism of the local cells. The dimeric transcription factor hypoxia inducible factor (HIF) is expressed in low oxygen environments. The beta subunit is continuously expressed, while alpha subunit expression is dependent on the partial pressure of oxygen in the cell [26], [27]. HIF-1α has been found to activate the TGF-β1/Smad signaling pathway, with inhibition of the HIF-1α pathway successfully inhibiting the activation of the TGF-β1 pathway [28]. This, coupled with the knowledge that low oxygen environments occur within the tumor microenvironment (TME), suggests that HIF behaves as a tumor activator; however, it has also been shown that deletion of HIF accelerates tumor growth, indicating the protein having a role in tumor suppression as well [26]. HIF-1α overexpression has also been shown to increase lactate dehydrogenase-5 (LDH5) expression. LDH5 drives pyruvate to lactate conversion, and increased expression of LDH5 facilitates fibroblast differentiation into myofibroblasts, in which cells will excrete more ECM components and cytokines in a manner similar to TGF-β1 induced transformation [29].

2.2 – Cellular Metabolism and the Effects of Low O_2 Environments on Metabolism

Cellular respiration is a complex process that includes glycolysis in the cytoplasm and the citric acid cycle in the mitochondria. Glycolysis breaks down glucose to produce ATP, NAD+, NADH, and pyruvate. Pyruvate is then oxidized into acetyl CoA, which enters the citric acid cycle, where it is used to ultimately generate ATP to power the cell.

Lactate dehydrogenase (LDH) is a versatile cytosolic enzyme responsible for the conversion of lactate into pyruvate, and vice versa. LDHA converts pyruvate to lactate during anaerobic respiration, while LDHB is responsible for converting lactate to pyruvate when oxygen is available [30]. Lactate dehydrogenase can further be described by the ratio of LDHA and LDHB subunits in a tetramer structure. LDH1 contains four LDHB subunits, while LDH5 contains four LDHA subunits, with LDH2-4 containing a combination of LDHA and LDHB. This combination of LDHA and LDHB subunits can make it difficult to pinpoint exactly which LDH tetramer is present, but comparison of one isomer to total LDH allows for the general activity of the cell to be monitored. Lactic acid formation is facilitated by LDHA. This reaction can be seen in Equation 1:

$$pyruvate \leftrightarrow lactate + H^+ \leftrightarrow lactic\ acid$$

LDH levels increase in low oxygen environments, likely regulated by hypoxia-inducible factor-1α(HIF-1α) [31]–[33]. The bidirectional conversion of lactate and pyruvate is important for cellular respiration; however, respiration has been found to be heavily affected by low oxygen and cancerous environments. Gossypol, a drug derived from cotton roots, inhibits LDHA activity and has promise as a potential therapeutic against fibrosis and cancer [34]. Inhibition of LDHA via gossypol inhibits myofibroblast differentiation by inhibiting collagens from assembling in the ECM and reducing active TGF-b through decreased lactic acid production [35].

When cells are exposed to a low oxygen environment for a prolonged period, they will preferentially produce lactate, even when re-introduced to a normoxic environment [36]. This was first observed by Warburg et al., when they observed an increased glucose consumption in cancerous cells compared to their non-cancerous counterparts [37]. Overall, the Warburg effect is still not fully understood, but hypotheses have been made that the acidification of the TME is beneficial for tumor growth and local invasion [38], and therefore part of the reason for the shift in metabolism. Additionally, it has been hypothesized that this shift occurs to out-compete tumor invading lymphocytes by over-consuming available glucose, and starving lymphocytes of their energy source [39]. Regardless of the reason for this behavior, the Warburg effect facilitates cancer cells to thrive in their environment and increase proliferation.

Cellular processes are tightly regulated, with deviations often causing disease states to occur. Low oxygen environments cause high rates of differentiation in lung myofibroblasts and transformation in lung epithelial cells, but to the best of our knowledge, the effects of these environments have not been investigated in breast epithelium. Since low O_2 increases TGF-β1 expression [40], it is expected that EMT will occur in breast epithelial cells in low O_2. However, since fibronectin assembly has also been seen to be a key regulator of EMT [24], we wanted to investigate the role of low O_2 environments on fibronectin fibril assembly, and the role that changes in pH may have on fibril assembly and EMT. We hypothesize that a decrease in extracellular pH will increase FN assembly through elongation of FN molecules to allow for greater assembly, and further facilitate EMT in low O_2 environments. We tested this by culturing human mammary epithelial cells in a low O_2 environment to measure EMT and compare it to TGFβ1-induced EMT in normoxic environments, and to attempt to block EMT via TGFB1 gene knockout or FUD treatment to block fibronectin assembly. Additionally, cells were cultured in low pH (pH >= 6) to determine if cells are able to assemble fibrils in these conditions.

Chapter 3: Methods and Materials

3.1 – Cell culture and Reagents

Human MCF10A mammary epithelial cells (Manassas, Va) were maintained under standard conditions of 37 °C at 5% CO_2 in DMEM/F-12 HEPES (Life Technologies, Carlsbad, Ca), supplemented with 5% horse serum, 0.05% hydrocortisone, 0.01% cholera toxin, 0.1% insulin, 0.02% EGF, and 1% antibiotics. Cells were lifted with 0.05% trypsin (Thermo Scientific) and passaged around 75% confluence. Prior to experimentation, cells were cultured in DMEM/F-12 media without HEPES supplemented with 0.05% hydrocortisone, 0.01% cholera toxin, 0.1% insulin, and 1% antibiotics for 2 hours. Normoxic conditions were cultured at atmospheric oxygen conditions (~22% O_2) and 5% CO2 at 37 °C, and hypoxic conditions were cultured in a 1.5% O_2 and 5% CO_2 tri-gas incubator at 37 °C.

Purified recombinant TGFβ1 was purchased from Sigma Aldrich (St. Louis, MO). TGF-β1 treatment was conducted at 4ng/mL, and FUD treatment was conducted at 125nM. Human MCF10A mammary epithelial cells with the TGFB1 gene knocked out were previously generated in our group (unpublished results). TGFB1 was knocked out via CRISPR-Cas9, and TGFB1 knockout was confirmed prior to this study.

3.2 – Immunofluorescence image acquisition and analysis

12mm glass coverslips were washed with ethanol, and coated with 50 µg/mL laminin (Sigma Aldrich, St. Louis MO) overnight at 4 °C. MCF10A cells were plated on the laminin-coated coverslips to achieve near confluent monolayers after overnight growth. Following treatment, cells were grown for 72 hours before fixing. Cells were rinsed with PBS, then permeabilized with 0.05% Triton in 4% paraformaldehyde for 2 minutes, then fixed in 4% paraformaldehyde for 20 minutes. Cells were rinsed with PBS, followed by blocking in 1%

BSA. Cells were incubated with primary antibodies Ms-anti-Hu E-cadherin (company), Rb-anti-Hu Fibronectin (company) for 30 minutes at 37 °C. F-actin was labeled with AlexaFluor555 Phalloidin (Life Technologies, Carlsbad, CA) during the primary antibody incubation step. Cells were blocked again with 0.1% BSA for 5 minutes, then incubated with secondary antibodies Gt-anti-Rb-Alexa Fluor 647 and Dk-anti-Ms-Alexa Fluor 488 (Thermo Scientific) for 30 minutes at 37 °C. Nuclei were counterstained using DAPI (NucBlue, Thermo Scientific) before mounting on glass coverslips. Images were acquired on a Zeiss AxioObserver Z1 fluorescence microscope using ZEN software.

3.3 – Image processing

After acquisition, images were analyzed using MATLAB code developed by Christopher Lemmon, and values from this analysis were used to quantify observations from images. Each image was filtered using a minimum and maximum object size, as well as an exclusion threshold to capture the desired components from the images.

3.4 – mRNA extraction, cDNA conversion, qPCR analysis

RNA was extracted using an RNeasy kit (Qiagen), and checked for concentration using the Nucleic Acid setting on a Nanodrop2000c (ThermoFisher). cDNA conversion was performed using reverse transcriptase from an iScript cDNA Synthesis kit (Bio-rad). cDNA was selectively amplified by predesigned primer sets to the gene of interest (IDT): TGFB1, FN1, LDHA, HIF1A, INTAV, PAI-1(Serpine-1). An 18s specific primer set (IDT) was used as a normalization control.

3.5 – ELISA analysis

ELISA analysis was conducted using conditioned media and cell lysates from MCF10A cells cultured for 72 hours. Sandwich (ELISA) against FN was performed based on manufacturer's instructions (R&D Systems). Plates were red using 450nm absorbance using a microplate spectrophotometer. A 4-parameter logistic regression was created as a standard curve using 7-points (200-3.125ng/mL), and absorbance values were compared to this curve to determine sample concentration. Conditioned media samples were diluted, and concentration values were then corrected based on the dilution factor to avoid oversaturation of the standard curve.

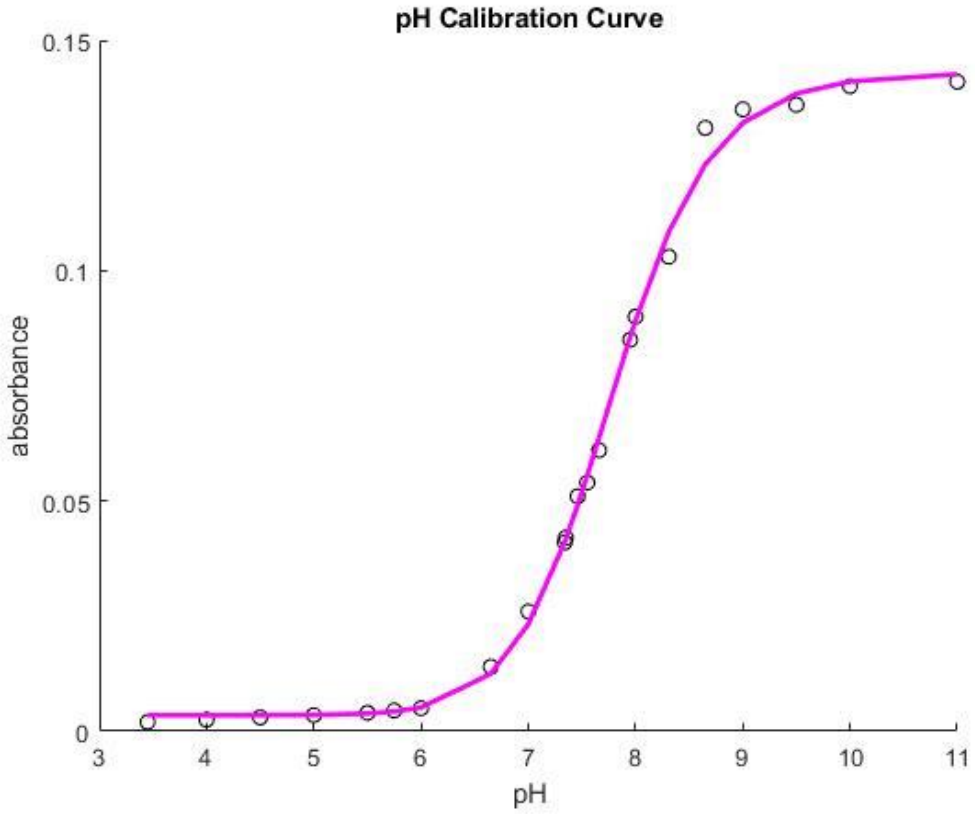
3.6 – Statistical Analysis

Statistical analysis was conducted using GraphPad PRISM software (San Diego, CA). One-way or two-way ANOVA tests were conducted as appropriate to determine significance.

Chapter 4: Results

4.1 – Role of pH on Fibronectin Expression and Assembly

A pH calibration curve (Figure 1) was generated by adding HCl and NaOH to MCF10A serum and HEPES free media, and measuring the pH and absorbance of the media using a pH meter and the NanoDrop2000c, respectively. These values were then put into a 4-parameter Hill function in MATLAB to generate the calibration curve. This curve was then used to estimate the pH of cell culture media by measuring the absorbance of small samples of media. However, it is important to note that the media buffered extremely quickly after removal from the 5% CO_2 incubator, so any pH values measured are estimates, not exact values. To determine the degree of buffering of the HCl occurring within the media after addition, a brief buffering period that lasted about 1 hour was observed in which the hue of Phenol red in culture media would noticeably change. Phenol red presents more yellow in acidic conditions, and more purple in basic conditions, so it is easily used for quick pH checks in cell culture. The buffering period was noticed primarily in the 10mM sample, and the 20mM sample underwent notably less buffering. Both samples maintained more acidic conditions than the control throughout the experiment.



$$pH = \left(\frac{-(k_1 * {k_3}^n) - ({k_3}^{n*y})}{k_1 + k_2 - y}\right)^{1/n}$$

Figure 1. pH calibration curve and equation used to estimate pH of MCF10A serum and HEPES free media using absorbance of the sample. Where k1 = 0.0034, k2 = 0.1397, k3 = 7.7870, and n = 16.8991

Immunofluorescence imaging was conducted on MCF10A cells using antibodies for E-cadherin (green), F-actin (red), and FN (white). Nuclei were tagged using DAPI (blue). Representative images for transformation occurring due to a low pH environment are shown in Figure 2. These images show an increase in FN composition with a decrease in pH of the extracellular environment. Additionally, extreme breakdown of E-cadherin junctions was observed in the 20mM condition, with some breakdown similar to that seen in TGF-β1-induced EMT occurring in the 10mM HCl condition. Interestingly, minimal actin stress fiber formation was observed in the 10mM HCl condition, and extreme actin breakdown was observed in the 20mM condition.

FN1, TGFB1, LDHA, HIF1α, INTAV, and SERPINE1 gene expression were measured via qPCR analysis. An extreme upregulation of FN was seen between the 20mM condition and control, however a non-significant increase in the gene occurred in the 10mM condition, indicating a potential pH threshold for increased expression. An increase in TGFB1 expression is expected to be observed in tandem with higher FN1 expression, however there was no change in expression of the TGFB1 gene across all conditions (Figure 3). This suggests that there may be another signaling mechanism at play to increase FN expression and production in acidic conditions. Further supporting this, there was not a significant increase in SERPINE1 activity in the acidic condition, which is a measure for TGFB1 receptor sensitivity (Figure 3).

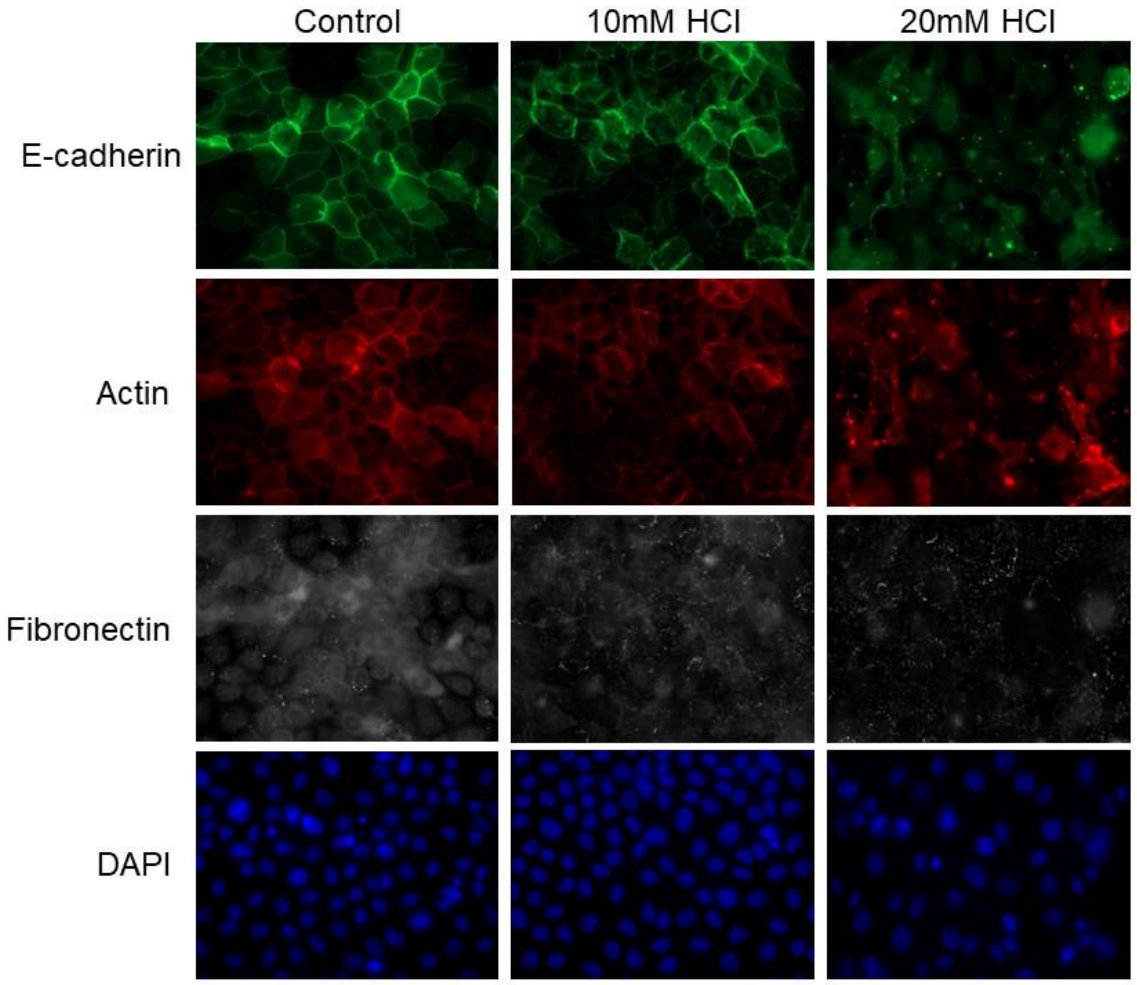


Figure 2. Representative images of HCl treated MCF10A cells after 72 hours of treatment. Samples stained for E-cadherin (green), F-actin (red), Fibronectin (white), and DAPI (Blue).

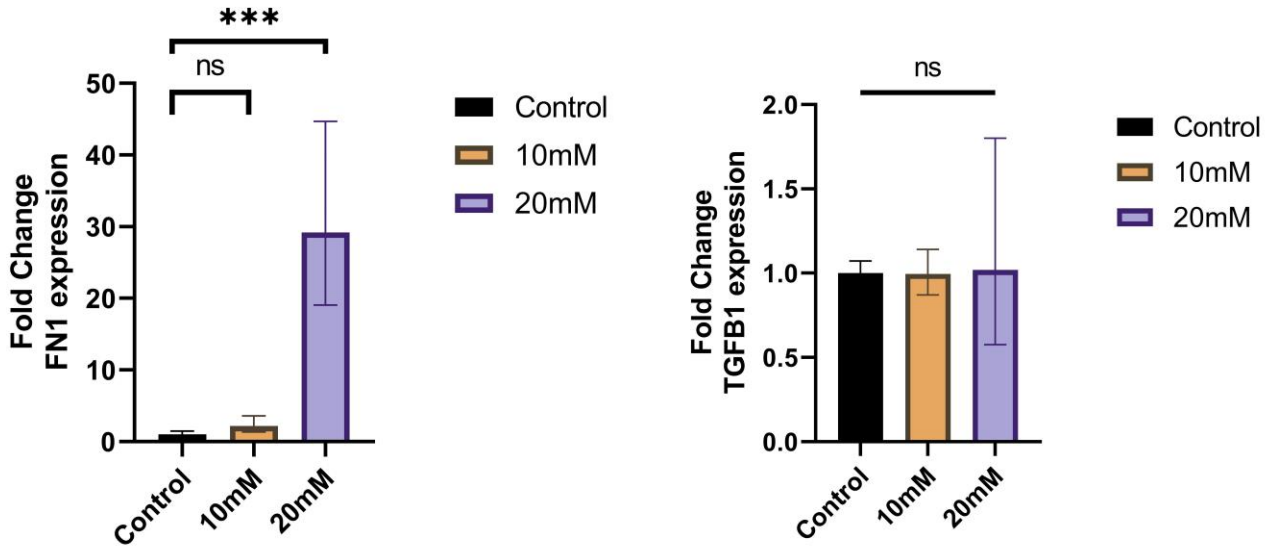


Figure 3. Gene expression of FN1, TGFB1, and in cells cultured in 0, 10, or 20mM HCl for 48 hours. Changes in gene expression were averaged between 5 trials and normalized to the control (0mM HCl) condition. Grouped statistical analysis using a one-way ANOVA was conducted. (*** $p < 0.0005$)

Serp1 expression was measured to better understand the role TGF-β1 signaling is playing in any cellular transformation occurring. There was no significant change in Serp1 expression across the conditions, but there was a slight increase in the 20mM sample. Integrin-α_V expression was measured due to its role in RGD binding, and a decrease in expression was observed in the 20mM sample (Figure 4).

HIF1α and LDHA gene expression were measured to consider the role changes in pH may play in hypoxic conditions to enhance or inhibit activity caused from TGF-β1 and low O_2 signaling. A decrease in HIF1α expression was observed, which was of interest due to the increase of lactic acid that occurs in low O_2 in lung fibroblasts[3], [29]. No change in LDHA expression occurred between conditions, which was expected due to the function of LDHA being oxygen dependent (Figure 5).

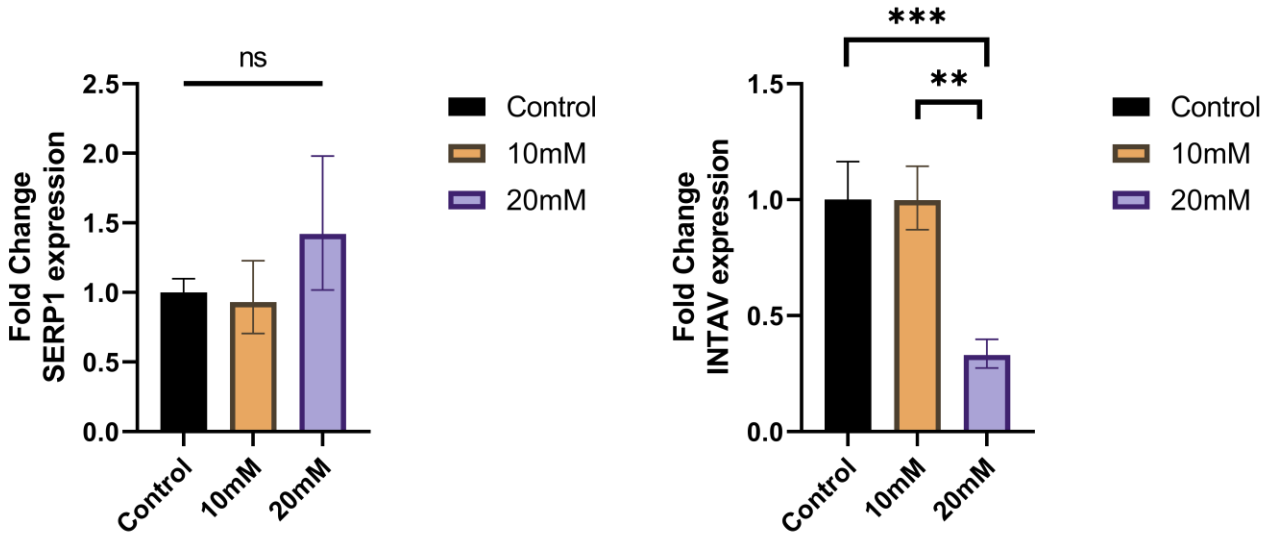


Figure 4. Gene expression of SERP1 and INTAV in cells cultured in 0, 10, or 20mM HCl for 48 hours. Changes in gene expression were averaged between 5 trials and normalized to the control (0mM HCl) condition. Grouped statistical analysis using a one-way ANOVA was conducted. (** $p < 0.005$, *** $p < 0.0005$)

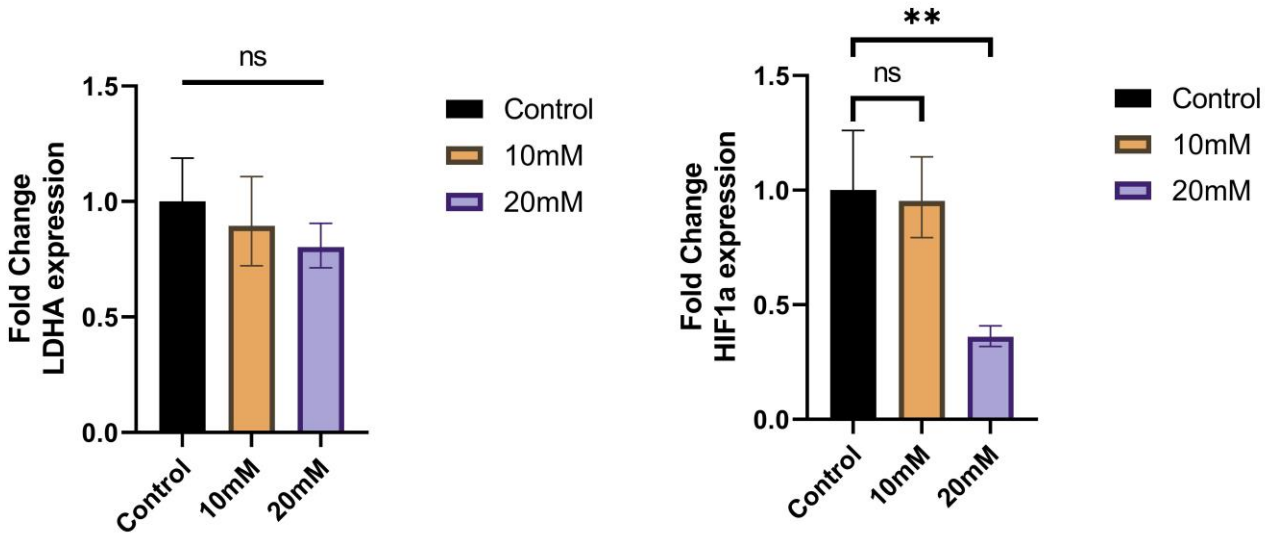


Figure 5. Gene expression of LDHA and HIF1α in cells cultured in 0, 10, or 20mM HCl for 48 hours. Changes in gene expression were averaged between 5 trials and normalized to the control (0mM HCl) condition. Grouped statistical analysis using a one-way ANOVA was conducted. (** $p < 0.005$)

4.2 – TGFβ1 and Fibronectin Signaling in Low O_2

Previous work from our lab has shown that a 49-amino acid fragment of the bacterial protein Adhesin F1 (FUD) is an effective means of blocking EMT via inhibition of fibronectin fibril assembly [24]. Figure 6 shows immunofluorescence images demonstrating utilization of 125nM FUD in low O_2 environments, and a decrease in overall FN assembly observed in these conditions.

We also investigated the gene expression of FN1, TGFB1, LDHA, and HIF1α in cells cultured in hypoxic conditions treated with FUD to determine if inhibition of FN fibril assembly could have an effect on LDHA or HIF1α expression in hypoxia, as well as EMT. From our treatment with TGF-β1 or hypoxia, there was not a notable increase in FN1 or TGFB1 expression across conditions. However, previous work has shown increased expression of these genes after TGF-β1 treatment [24]. This difference is likely due to timepoints of mRNA extraction (24 vs. 48 hours) or cell density upon seeding. We did see an increase in TGFB1 expression in the hypoxia +FUD condition, which was expected from the hypoxic culture conditions (Figure 7).

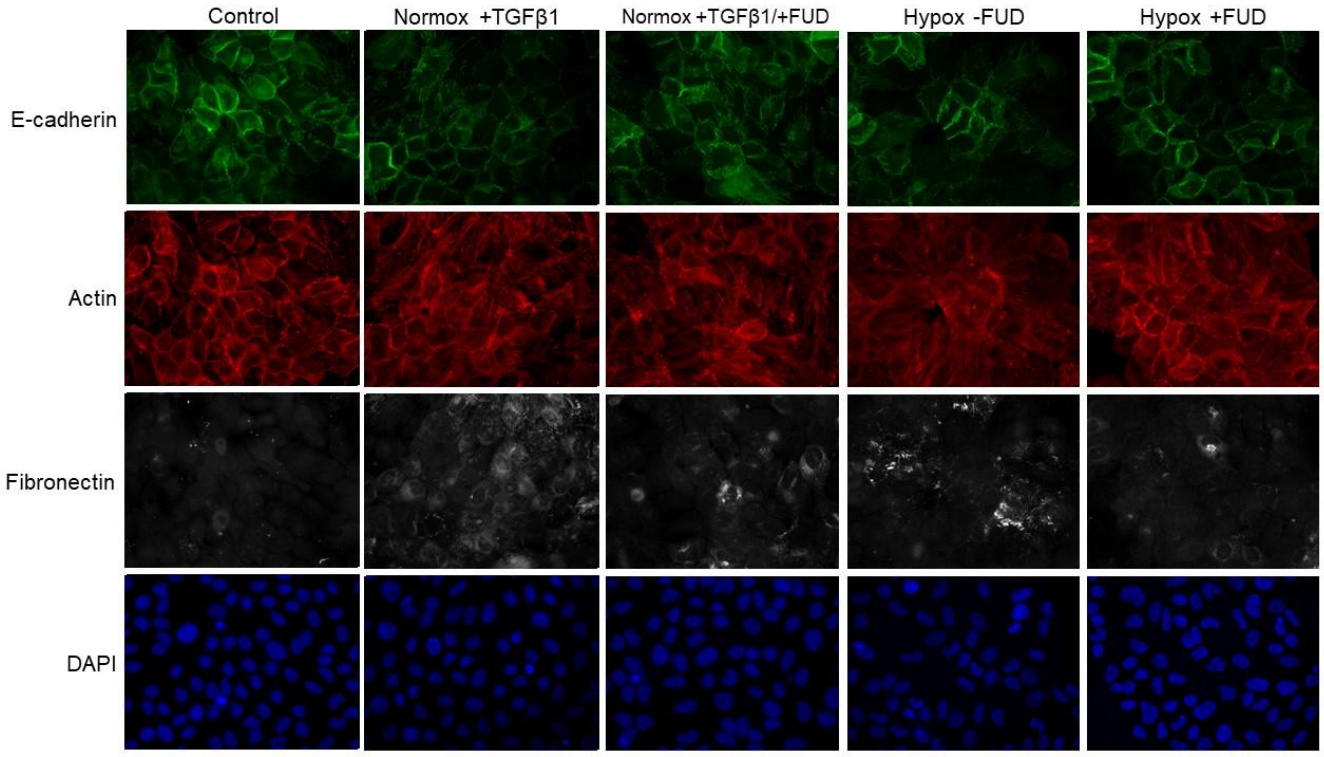


Figure 6. Representative images of MCF10A cells after 72 hours of treatment in normoxia or hypoxia, with or without FUD. Samples stained for E-cadherin (green), F-actin (red), Fibronectin (white), and DAPI (Blue).

A Normoxia +TGF-β1 condition to was included induce fibrosis in normoxia, and used to compare fibrosis in normoxic conditions to hypoxia-induced fibrosis. LDHA expression was expected to increase in hypoxic environments due to altered cellular respiration, but the change was not as great as expected, and only significant in the hypoxia +FUD condition. Similarly, HIF-1α expression was expected to increase in hypoxic environments, but there was no increase across conditions (Figure 8). However, this is not an indicator of transcription factor activity, and it is possible that different time points could yield different expression levels.

To quantify the amount of FN and TGF-β1 protein being expressed, an ELISA was conducted to measure FN and LAP (Latency Associated Peptide of TGF-β1) concentrations in both media and lysates from each condition. Minimal LAP was found to be in the media, and there was not a significant difference in LAP concentrations between conditions in lysates (Figure 9). There was not a significant change in FN in media or lysate with addition of FUD (Figure 10).

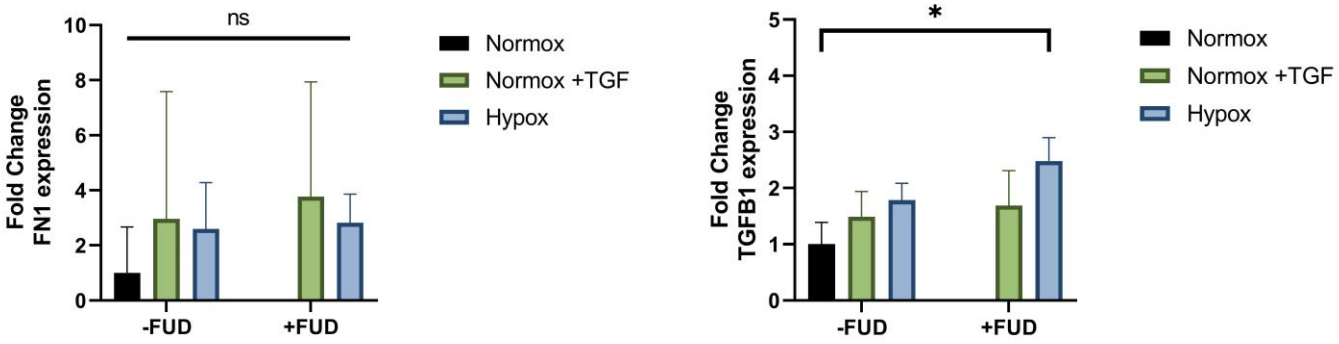


Figure 7. Gene expression of FN1 and TGFB1 in MCF10A cells treated for 48 hours in normoxia or hypoxia, with or without FUD. Grouped statistical analysis using two-way ANOVA without correcting for multiple comparisons. (* $p < 0.05$)

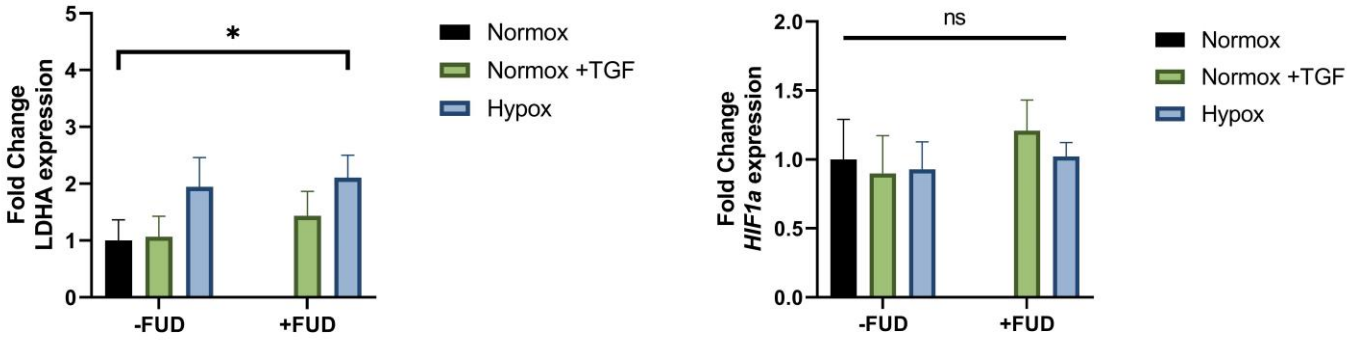


Figure 8. Gene expression of LDHA and HIF1α in MCF10A cells cultured for 48 hours in normoxia or hypoxia, with or without FUD. Grouped statistical analysis using two-way ANOVA without correcting for multiple comparisons. (* $p < 0.05$)

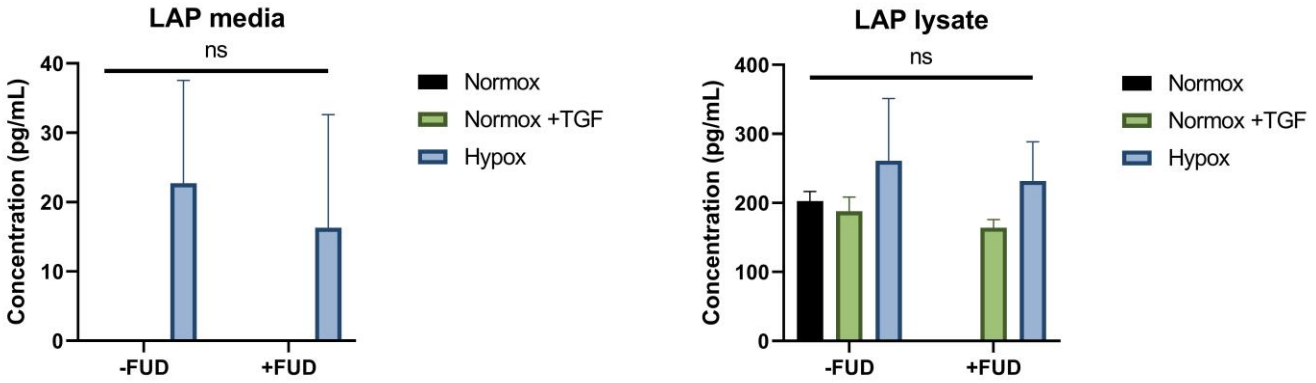


Figure 9. LAP ELISA analysis of conditioned media and cell lysate after 72 hours of treatment in normoxia or hypoxia, with or without FUD treatment. Grouped statistical analysis using two-way ANOVA without correcting for multiple comparisons.

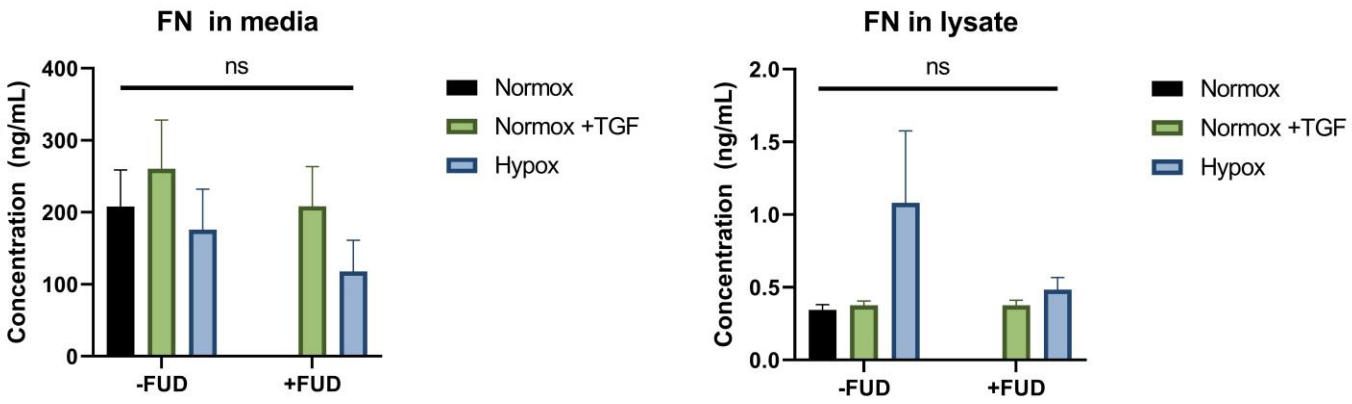


Figure 10. FN ELISA analysis of conditioned media and cell lysate after 72 hours of treatment in normoxia or hypoxia, with or without FUD treatment. Grouped statistical analysis using two-way ANOVA without correcting for multiple comparisons.

Our lab has established a KO cell line in which the TGFB1 gene was disrupted using CRISPR-Cas9 technology. Studies from others in the lab have demonstrated that TGF-β1 expression is nearly completely eradicated in these cells. Figure 11 shows immunofluorescence images of MCF10A wild type (WT) and MCF10A cells with the TGFB1 gene knocked out (K.O.). These cells were cultured in normoxic or hypoxic conditions to better understand the role TGF-β1 signaling plays in EMT in low oxygen environments. Knocking out the TGFB1 gene facilitated a decrease in FN in the cells, as well as maintenance of E-cadherin junctions and cortical actin.

FN1, TGFB1, LDHA, and HIF1α gene expression were measured to better understand their role in the TGF-β1 signaling pathway. FN1 expression was seen to decrease significantly ($p < 0.05$) when the TGFB1 gene is knocked out, and only slightly increase in response to hypoxia (Figure 12). TGFB1 expression also increased in hypoxia, and there was an increase in expression even in the knockout cells in hypoxia compared to normoxia. This is potentially supported by an increase in LDHA expression (Figure 13). Note that while TGFB1 mRNA increased in the KO cell line, this did not result in expressed protein (Figure 16, discussed later). The disruption of the TGFB1 gene can result in some transcribed mRNA, suggesting that cells are attempting to upregulate TGF-β1 in hypoxia. Similar to the FUD experiments, there was no increase in HIF1α expression, even in hypoxic conditions (Figure 14).

Consistent with the increased gene expression of FN1, an increase in FN content in media was observed in hypoxic conditions. However, there wasn’t an increase in the lysate (Figure 15). There was also more LAP in the WT than the knockout condition, showing that even though the TGFB1 gene is being expressed, the protein expression is negligible in the K.O. cells (Figure 16).

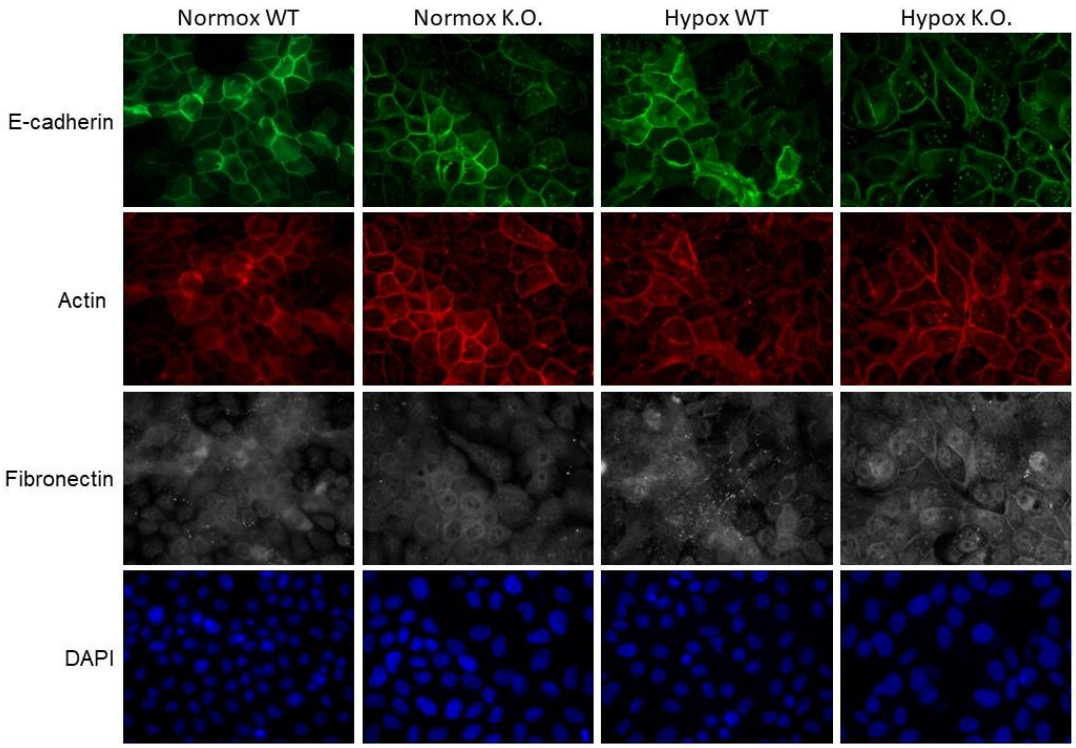


Figure 11. Representative images of MCF10A cells after 72 hours of treatment in normoxia or hypoxia, with or without the TGFB1 gene knocked out. Samples stained for E-cadherin (green), F-actin (red), Fibronectin (white), and DAPI (Blue).

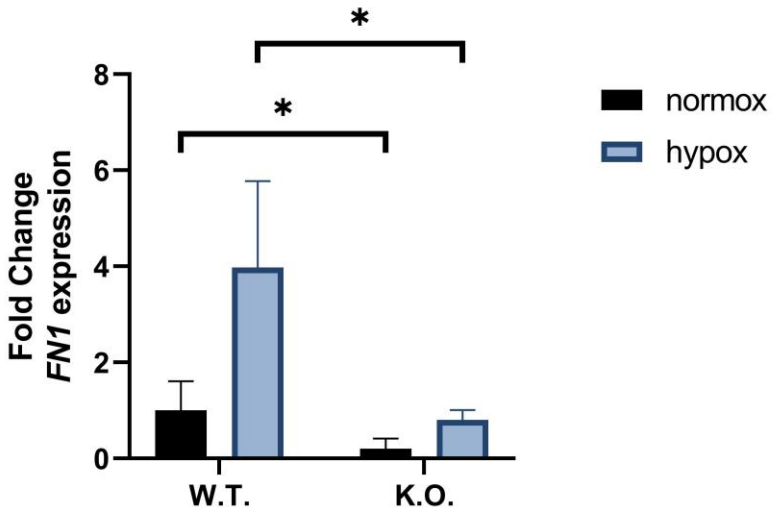


Figure 12. FN1 gene expression in WT and TGFB1 K.O. MCF10A cells cultured for 72 hours in normoxic or hypoxic environments. Grouped statistical analysis using two-way ANOVA without correcting for multiple comparisons. (n = 5) (*$p < 0.05$)

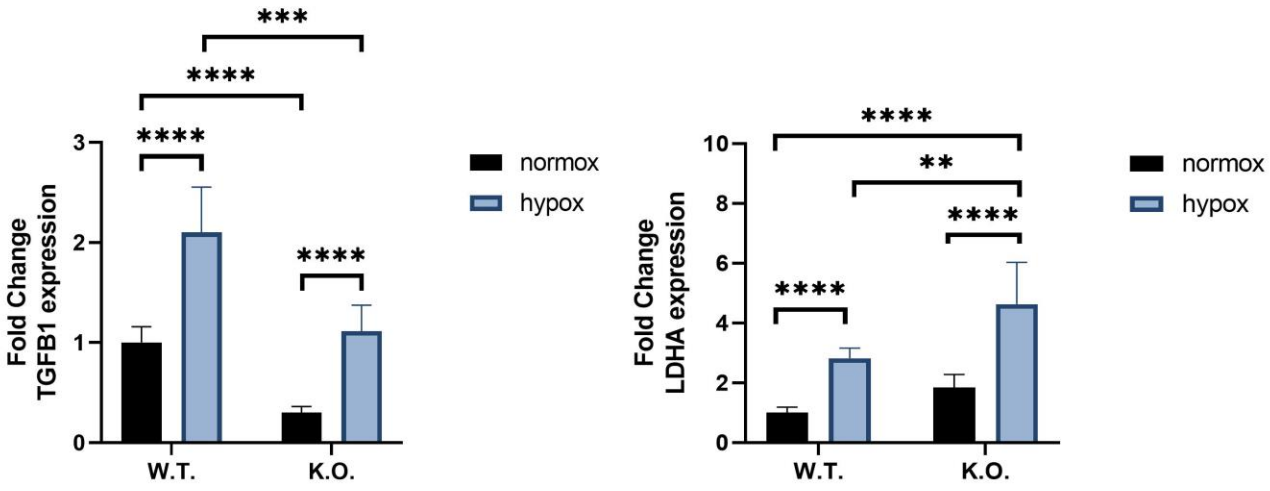


Figure 13. TGFB1 and LDHA gene expression in WT and TGFB1 K.O. MCF10A cells cultured for 72 hours in normoxic or hypoxic environments. Grouped statistical analysis using two-way ANOVA without correcting for multiple comparisons. (n = 5)(**p < 0.005, ***p < 0.0005, ****p < 0.00005)

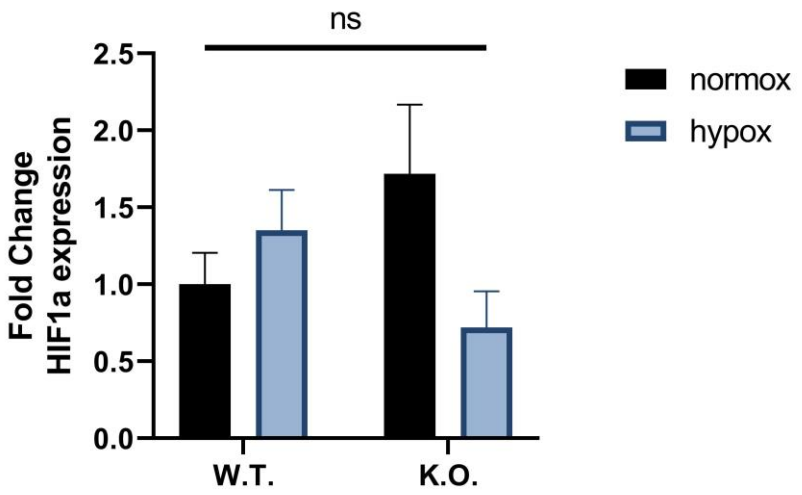


Figure 14. HIF1α gene expression in WT and TGFB1 K.O. MCF10A cells cultured for 72 hours in normoxic or hypoxic environments. Grouped statistical analysis using two-way ANOVA without correcting for multiple comparisons. (n = 5)(* $p < 0.05$)

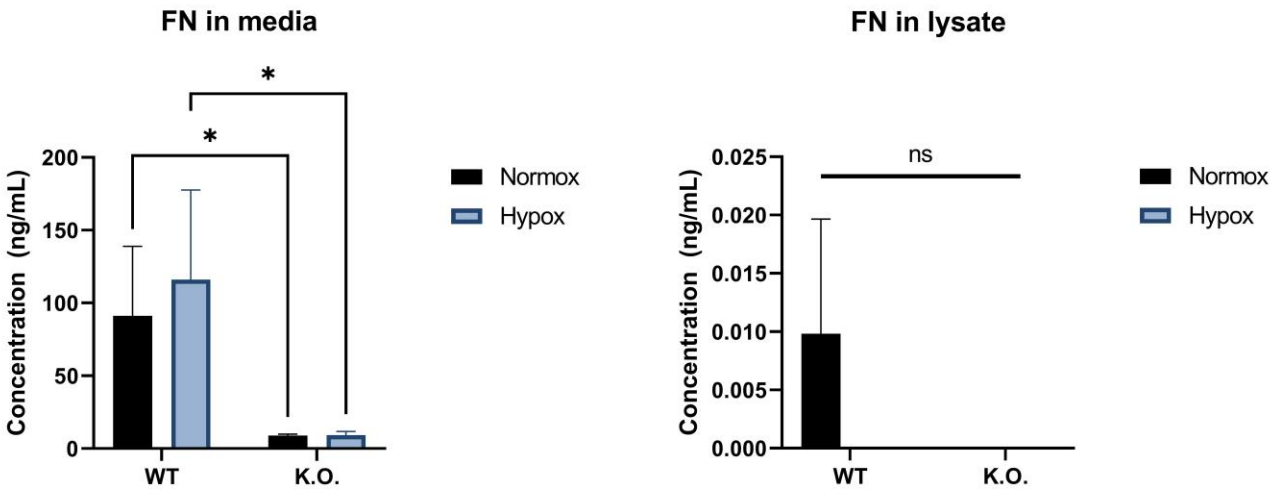


Figure 15. FN ELISA analysis of conditioned media and cell lysate after 72 hours of treatment in normoxia or hypoxia, with or without the TGFB1 gene knocked out. Grouped statistical analysis using two-way ANOVA without correcting for multiple comparisons. (n = 3) (* $p > 0.05$)

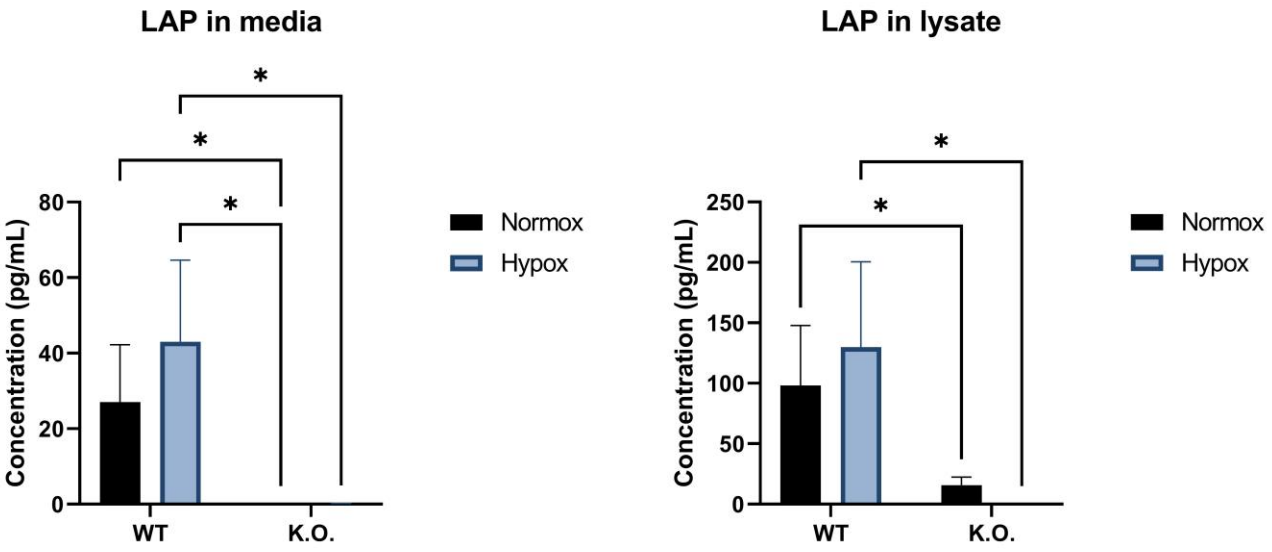


Figure 16. LAP ELISA analysis of conditioned media and cell lysate after 72 hours of treatment in normoxia or hypoxia, with or without the TGFB1 gene knocked out. Grouped statistical analysis using two-way ANOVA without correcting for multiple comparisons (n = 3) (* $p > 0.05$)

Chapter 5: Discussion

Cells were treated with 0, 10, or 20mM HCl to determine the role that changes in pH may play in cellular transformation. With the 10mM HCl addition, little to no changes were noticed in gene expression, but 20mM HCl was able to elicit a drastic increase in fibronectin expression. With a 20mM HCl treatment, an extreme breakdown of E-cadherin junctions was observed, as well as increased fibronectin deposition into the extracellular matrix. Breakdown of cell-cell junctions and increased ECM deposition suggest that cells in this environment are experiencing increased migratory abilities. Additionally, it was expected that an increase in fibronectin deposition would occur in tandem with an increase in TGF-β1 expression. However, there was no change in TGFB1 gene expression after 48 hours of culture. This could be the result of either alternative pathways of fibronectin expression, or an initial increase in TGF-β1 expression at an earlier time point, and a return to baseline after a short time. A non-significant change in SERP1 expression also suggests that an alternate pathway is activating FN1 expression, since Serpine is a measure of TGFb receptor sensitivity. It was also of interest that there was a decrease in INTAV expression in the 20mM treatment group, suggesting that fibronectin fibrils may not be assembled as readily in the acidic environment. The lack of change in LDHA expression was expected since the cells were cultured in a normoxic environment, however the decrease in HIF-1α expression in response to low pH was of interest. This could suggest a potential regulation point, since low oxygen environments often yield lower pH environments from changes in cellular respiration, but more experiments would be required to confirm this.

Next, fibronectin assembly was inhibited in hypoxia to see if inhibition of this would be enough to inhibit EMT in hypoxia. Qualitatively, it appears as though there is less fibronectin assembly in both normoxic conditions treated with TGF-β1 and FUD, and hypoxic conditions

treated with FUD only. There was not a significant difference in Fibronectin gene expression between conditions, but previous work has shown that treatment with TGF-β1 should induce higher FN1 gene expression, so it is expected that this is due to the large error bars, or measuring gene expression after 48 hours instead of 24 hours. Interestingly, there was a significant increase in the hypoxic condition treated with FUD, suggesting a potential over-expression of the gene in response to inhibited FN assembly, and that the cell is attempting to force transformation. An increase in LDHA expression was also observed in the hypoxia +FUD group, but not in the hypoxia -FUD group. This was of interest because LDHA expression was expected to increase in response to low oxygen. A slight, but non-significant ($p = 0.0828$) increase in LDHA was also seen in the normoxic +TGF/+FUD condition. Of particular interest was the lack of change in HIF1α expression across all conditions. An increase in HIF1α expression was expected in the hypoxic conditions, but it is possible that expression peaks at an earlier time point, and then returns to baseline when the transcription factor is not degraded by local oxygen. An ELISA was conducted to measure the protein content of Latency Associated Peptide (LAP) and fibronectin to compare the gene expression to protein transcription occurring in the cells. There was not a significant difference in LAP across conditions, but there was a 10-fold difference in concentration overall in the lysate compared to the media, showing that LAP is anchored in the ECM instead of free-floating. Fibronectin showed the opposite trend, with much more fibronectin present in the media compared to the lysate, but still no significant difference in composition across conditions.

Knocking out the TGFB1 gene also appeared to successfully mitigate EMT characteristics in cells cultured in low oxygen environments. qPCR analysis showed significantly ($p < 0.05$) less fibronectin expression in the knockout cells compared to wild type cells both in normoxia and hypoxia. Additionally, there was less TGFB1 expression overall in

the knockout cells compared to wild types, but there was still some TGFB1 expression in the knockout cells, especially when cultured in hypoxia. This expression of TGFB1 even though the gene is knocked out could be due to the qPCR primer sequence being slightly upstream to the knockout site, allowing for some TGF-β1 to be transcribed, but the protein will be unable to be assembled properly. There was also increased LDHA expression in the cells cultured in hypoxia. Interestingly, there was increased LDHA expression in the TGFB1 K.O. cells cultured in hypoxia compared to their WT counterparts. This shows there may be a connection between TGFB1 and LDHA, but the exact importance of this connection is unclear. It was also observed that the TGFB1 K.O. cells would create slightly more acidic environments than their wild type counterparts, both in normoxic and hypoxic environments. Given the increase in LDHA expression, this is likely due to an alteration in cellular metabolism caused by knocking out the TGFB1 gene. Consistent with the Hypoxia +/-FUD experiments, there was not a change in HIF1α expression across conditions. ELISA for LAP and FN were used to further determine the protein transcription occurring. The knockout cells produced less fibronectin in media, and an undetectable amount in media. Knockout cells also did not produce LAP in media nor lysate, but their wild type counterparts showed some in each, again with more LAP present in lysate than media.

This work shows promising potential therapeutic options for pre-metastatic cancers. MCF10A cells were used, which are a pre-cancerous cell line that are susceptible to transformation. Mitigation of EMT through blocking fibronectin assembly provides a potential therapeutic target, especially through inhibition of some ECM remodeling that occurs with EMT. Knocking out the TGFB1 gene also mitigated EMT, providing another therapeutic target for pre-cancerous treatment. However, the increased acidification of the local environment in the knockout cells is important to consider due to the downstream effects that may occur.

This work is limited since it was conducted in 2D cell culture on tissue culture plastic. 2D cell culture is known to be not physiologically relevant, and the stiffness of the tissue culture plastic is not applicable to breast tissue in vivo. Additionally, since a pre-cancerous cell line was utilized, this work does not apply to cancerous cell types. Full blocking of EMT cannot be claimed without further investigation of EMT specific gene markers.

Chapter 6: Conclusions and Future Directions

We studied the effects of acidic environments on fibronectin fibril assembly by treating cells with HCl. We saw an extreme increase in fibronectin expression, but not via TGF-β1 signaling. We investigated this because of the decrease in pH that occurs due to hypoxia. We were also successfully able to mitigate the effects of EMT on cells cultured in hypoxia by blocking fibronectin fibril assembly with FUD. Additionally, we saw that knocking out the TGFB1 gene was able to mitigate EMT in hypoxia.

Future work on this project can further elucidate the tumor microenvironment, and how cells work together to allow for metastatic processes to occur. MCF10A cells can be grown in hypoxic conditions, and then returned to normoxia to recover function, and determine how cell processes are altered by time spent in hypoxia. Additionally, treatment with FUD or other treatments during this process could reveal potential therapeutic options for dense tumors that have not yet metastasized.

Conducting a co-culture of MCF10A and fibroblasts would also allow for more of the FN assembly dynamics to be understood in hypoxia, or in recovered cells. MCF10As are able to assemble fibronectin fibrils, but they are not known for assembling fibrils to the same extent as fibroblasts. Introducing fibroblasts to the low oxygen environment may facilitate additional fibril assembly. Additionally, conducing similar experiments with a cancerous cell line such as MDA-MB-231 cells, to observe how a cancerous cell line differs from a pre-cancerous cell line.

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Curriculum Vitae

Caitlin Williams

(571)577-7430 ● cewilliams0105@gmail.com

Education

Virginia Commonwealth University, Richmond, VA
Bachelor of Science in Biomedical Engineering August 2019 – May 2022
Master of Science in Biomedical Engineering May 2022 – August 2023
GPA: 3.5 Dean's List (Fall 2020, Fall 2021, Spring 2022)

Skills

Lab Skills: Cell Culture | Immunofluorescence Staining & Microscopy | RT-qPCR analysis | ELISA analysis
Computer Skills: MATLAB | SolidWorks | Microsoft Office | Adobe Illustrator | Figma

Relevant Coursework

Mechanobiology | Cell Engineering | Biomaterials | Regenerative Medicine | Biomechanics | Cell Biology

Relevant Experience

Research Assistant, Cell-Matrix Mechanobiology Lab, VCU Spring 2020 - Present

- Researching the role of low oxygen environments on cellular transformation, and ECM composition
- Create work plans for each semester to maintain flow of research, and ensure goals are being reached in a timely manner
- Mentor undergraduate students to help them build the skills they need to thrive in lab. Aid in conducting experiments, collecting and analyzing data, and promoting independent work

Publications

Kim, K.P.; Williams, C.E.; Lemmon, C.A. Cell–Matrix Interactions in Renal Fibrosis. Kidney Dial. 2022, 2, 607-624. https://doi.org/10.3390/kidneydial2040055

Other Work Experience

Hourly Grader – VCU College of Engineering September 2022 – May 2023

- Provide meaningful feedback to students in a timely manner after assignments have been submitted
- Work closely with course instructor to understand rubric expectations, and host review sessions
- Aid in proctoring exams, and guest lecture when needed

Logistics Coordinator--Office of New Student & Family Programs, VCU, August 2021–January 2022

- Helped to ensure that logistic components of the Orientation program ran smoothly through answering emails and phone calls from students and families
- Assisted in Orientation Leader recruitment, training, and recognition to develop a strong team

Operations Assistant – Office of New Student and Family Programs January 2021-August 2021

- Help to ensure that logistic components of the Orientation program run smoothly through answering emails and phone calls from students and families
- Attend monthly team meetings to build relationships within the team

Orientation Leader – Office of New Student and Family Programs March 2020 – January 2021

- Worked as an Orientation Leader, where students were led in weekly meetings in preparation for the transition from high school to college
- Sent weekly reminder emails to students regarding what would be discussed during the meeting and mandatory tasks to be completed before enrollment at VCU

Volunteer, INOVA Loudoun Hospital, Leesburg, Va, June 2019 – August 2019

- Aided Patient Relations representative in creating grievance binders for Joint Commission review, and input complaints into SafetyAlways for easy access
- Used Microsoft Word to produce a new template for documenting complaints received via phone
- Aided Patient Relations representative by helping clean out their filing cabinet and run errands in hospital

Leadership

Theta Tau – Professional Engineering Fraternity – Virginia Commonwealth University
January 2020

- Served as Vice Regent in the Spring 2021 semester, in charge of organizing committees, and keeping chapter on track to complete necessary events.

Achievements

Finalist – VCU Three Minute Thesis Competition Fall 2022

- Presented a condensed and easily understandable form of Master's project for a general audience
- Competed against 20 individuals in the semi-final round, and against ten finalists