2020

Epitranscriptomic Regulation Of Fibroblast Activation In Diseased Heart

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EPITRANSCRIPTOMIC REGULATION OF FIBROBLAST ACTIVATION IN DISEASED HEART

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A THESIS

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Master of Science in Biomedical Engineering

BIRMINGHAM, ALABAMA

2020

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ABSTRACT

Background: Transforming growth factor β (TGFβ) is a chemokine released during cardiac hypertrophy and remodeling. Evidence has shown that it plays a significant role in fibroblast differentiation/activation to myofibroblasts, which ultimately leads to adverse cardiac remodeling. It was previously suggested that m^6A methylation plays important role in cardiac pathology in both ischemic and hypertrophic heart failure [1, 2], however, its role in fibroblast activation/differentiation is largely unknown. Therefore, in this study we investigated the relationship between m^6A methylation and fibroblast activation using proinflammatory/profibrotic factor, TGFβ.

Methods: Fibroblasts were isolated from neonatal rat heart and activated with TGFβ for 48 hours. RNA and protein were harvested from fibroblasts after treatment to verify activation of fibroblasts via qPCR and Western blot analyses. METTL3 expression in fibroblasts was inhibited using METTL3 siRNA silencer system before treatment with TGFβ. RNA and protein were isolated from treated fibroblasts and the expression of profibrotic genes (Col1α, FN, αSMA and Periostin), and METTL3 expression were measured using RT-qPCR and western blot techniques. The m^6A RNA methylation was measured using Epigentek kit.

Results and Conclusion: TGFβ treatment significantly activated the expression of profibrotic genes (αSMA, Col1α, FN). It also significantly increased canonical TGFβ signaling as shown by Smad2/3 phosphorylation. M^6A methylation was significantly increased in TGFβ treated cells compared to the control. Inhibition of METTL3 in cells showed an inverse relationship to the fibroblast activation, i.e. fibroblasts with downregulated METTL3 showed less m^6A RNA methylation, and did not express as many fibrotic factors compared to control fibroblasts. Therefore, our data suggests that TGFβ-induced fibroblasts activation is partially dependent on RNA methylation.

Keywords: TGFβ, m^6A methylation, METTL3, fibroblast activation, cardiac fibrosis

ACKNOWLEDGEMENTS

I would like to thank God for making me all that I am and giving me the resilience to keep going on at every stage in my life. Without God, I would not be here.

I would like to express the deepest appreciation to my committee chair, Dr. Suresh K. Verma, who provided me with this opportunity. His patience and continual encouragement are what pushed me every day. Without his guidance and mentorship, this thesis would not have been possible.

I would like to thank my committee professors, Dr. Hind Lal and Dr. Prasanna Krishnamurthy, whose constructive criticism helped me improve my work ethic and delivery and always pushed my to do better.

In addition, thank you to my lab members, Dr. Rajesh Kumari, Ms. Jing Li, Dr. Prabhat Ranjan and Dr. Sumanta Kumar Goswami, for their assistance with my research and for coaching me on the right way to do many of the experiments. I would also like to thank the labs of Dr. Min Xie and Dr. Palaniappan Sethu for providing some of the materials and equipment that were used in this work.

And I am thankful to the Biomedical Engineering Graduate Program Director, Dr. Jack M. Rogers, and the Biomedical Engineering Program Administrator, Ms. Keri Dickens, for helping me meet all the requirements to complete my degree.

Finally, I would like to thank my family and friends for being my support system and my dad for always believing in me.

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INTRODUCTION

Cardiovascular diseases are among the leading causes of death in the US and the world. Heart is a multicellular organ and constitutes of different cells including cardiomyocytes, endothelial cells, fibroblasts and immune cells. Among these, a large percentage of the heart is made up of cardiac fibroblasts [3]. Cardiac fibroblasts are important for wound healing, inflammation response and remodeling [3, 4]. They help the heart adapt to physical and mechanical changes in its environment by providing structural support; this is due to their ability to secrete and breakdown extracellular matrix (ECM). A major component of the ECM secreted by cardiac fibroblast is collagen, an important protein that ensure proper alignment of the myocytes and places a limit on myocyte movement [5]. Usually, there is a balance between ECM secretion and ECM breakdown after injury, this leads to efficient remodeling of the injured tissue. However, in prolonged or persistent chronic injuries, the healing process has been impaired, there is excess deposition of collagen leading to formation of scar tissue of fibrosis. Cardiac fibrosis occurs in the heart in the late stages of diverse cardiac diseases and is a predictive factor for sudden cardiac death [6].

Various biological processes have been implicated in cardiovascular diseases; an important one being the epigenetic modifications in the RNA [7, 8]. Studies have shown that methylation in the N6 position of the adenosine base, known as m^6A methylation, is highly upregulated in many diseases, including cancer and cardiovascular diseases [9-11]. Although, this epitranscriptomic modification was first discovered in eukaryotic mRNA, m^6A methylation is the most prevalent RNA modification in different RNA classes, including tRNA, snRNA, rRNA, miRNA, lncRNA and mRNA [12]. Among these, m^6A mRNA methylation is most studied RNA modification. mRNA methylation is mediated by three classes of RNA modifiers: writers, erasers and readers. The writer for m^6A in mammals is a multi-faceted enzyme consisting of several components, including Wilm's tumor 1-associated protein (WTAP), methyltransferase-like 14 (METTL14), and methyltransferase-like 3 (METTL3) [1, 2]. These proteins are responsible for catalyzing the addition of methyl group at N6 position on adenosine (Figure 1). The downregulation or upregulation of METTL3 and METTL14 have been shown to decrease or increase m^6A methylation respectively [1]. Many components of RNA methylations machinery helps to

make this complex intact and functional such as WTAP. It interacts with other methylases (METTL3 and 14) and require for their localization into nuclear speckles enriched with pre-mRNA processing factors and for catalytic activity of the m^6A methyltransferase [13]. On the other hand, The Fat Mass and Obesity-associated protein (FTO) and ALKBH family proteins are responsible for catalyzing the removal of methyl group from RNA and called mRNA demethylase. Similar to the writers, the regulation of FTO has been shown to control the level of m^6A methylation present on mRNA, however in this case, the upregulation of FTO decreases the level of methylation present on mRNA, and vice versa. The third class of regulatory molecules, the readers, recognize and bind to specific sequences on the methylated RNA and mediate the effects of methylation. Examples of m^6A readers are the eukaryotic initiation factor 3 (elF3) and YTH proteins [5]. It is unclear how elF3 recognizes m^6A, however, the YTH proteins contain a (YT521-B homology) domain, which specifically recognizes the consensus sequence G[G/A]m^6ACU [5, 12, 14]. YTHDC1, a YTH domain-containing family, recognizes m^6A methylation in nuclear RNAs [12]. Another m^6A reader is the hnRNP family protein HNRNPA2B1 which recognizes and binds to the m^6A motif in primary miRNA transcripts [12]. Figure 1 below shows a schematic of these regulatory molecules.

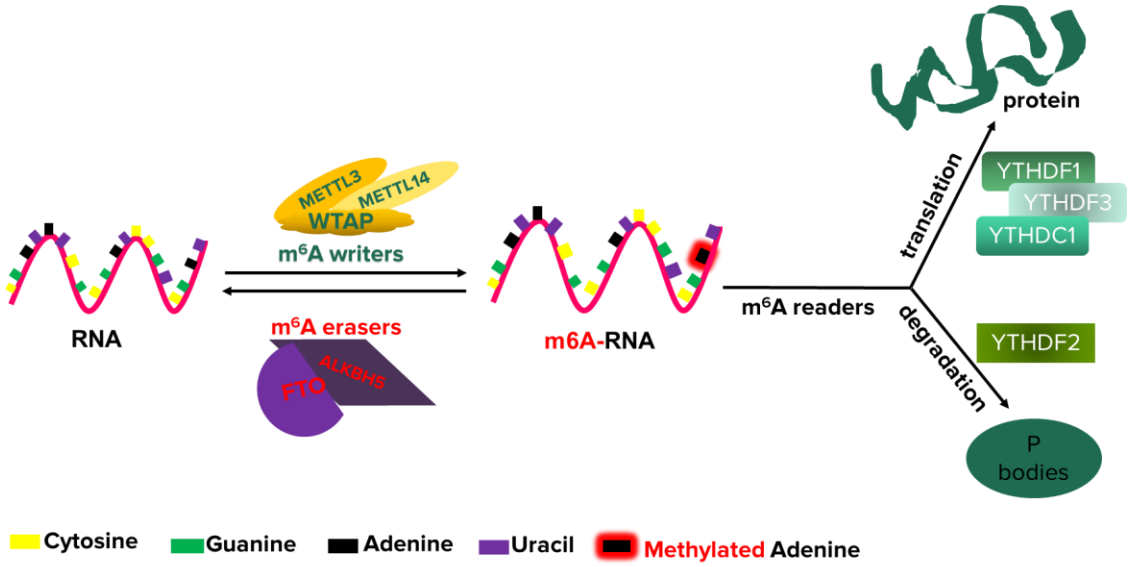


Figure 1: Methylation Machinery, showing writers and erasers on m^6A methylation on RNA.

The m^6A methylation plays an important role in various biological processes in multiple organisms, including plants, yeasts, flies, and mammals [1]. It is involved in synaptic

signaling, growth and metabolism, disease progression, stem cell differentiation, tissue homeostasis, and circadian clock regulation [1, 9]. In studies related to the heart, the focus of the m^6A methylation has been on cardiomyocytes. Kmietczyk et al showed that m^6A methylation in mRNA regulates the expression of cardiac genes and proteins, as well as the growth of cardiomyocytes [15]. In order to test the effect of methylation on cellular phenotype and gene expression in these studies, the writers and erasers, specifically METTL3, METTL14 and FTO, were manipulated. Methylation has also been connected to cardiovascular diseases and dysfunctions, such as, dilated cardiomyopathy, myocardial infarction, hypertrophy and ischemia [1, 15, 16]. Many studies have been conducted on DNA methylation in cardiac fibroblasts and suggests that it is important for fibroblast activation and fibrosis [17-19]. Pan et al showed that the regulation of DNA methylation in cardiac fibroblasts, controls the expression of collagen type I [17], suggesting that DNA methylation regulates cardiac fibrosis. However, as per our current knowledge, no data have been published regarding the effects of m^6A methylation on mRNA in cardiac fibroblasts. Thus, in this proposal we aim to investigate the role of m^6A methylation on cardiac fibroblast activation and trans-differentiation. It is important for this relationship to be looked into as fibrosis plays significant role in both hypertrophic and ischemic heart failure. Proposed experiments will be performed using neonatal rat ventricular fibroblasts (NRVF).

Fibrotic scars in the cardiac muscle can be caused by multiple diseases, including myocardial infarction, hypertensive heart disease, diabetic hypertrophic cardiomyopathy and idiopathic dilated cardiomyopathy [20]. As many heart diseases lead to pathological remodeling of the extracellular matrix of the heart, cardiac fibrosis is usually associated with these diseases [20, 21]. Since cardiac fibrosis is usually associated with other diseases, it can lead to multiple potentially fatal complications in the patient. Fibrotic diseases, mostly those of the heart and lungs lead to over 800,000 thousand deaths annually, worldwide and the treatment of cardiovascular related diseases and stroke costs $316.6 billion per year [20]. The study of cardiac fibrosis and the mechanisms that lead to it, will provide solutions to reducing the prevalence of this disease and cost of treatment.

Here, we investigate the role of RNA methylation in fibroblast activation and fibrosis. Although RNA methylation has been connected to other cells, like cancers and cardiomyocytes, the overall hypothesis that RNA methylation regulates fibroblast activation in diseased hearts is novel, since no studies are currently available on this. If successful, the proposed study will provide relevant information on the mechanism of fibroblast activation, which could potentially lead to new ways of prevention and treatment of cardiac fibrosis. We will test our hypothesis in following two specific aims:

Specific Aim 1: First, we will test whether the activation of fibroblasts modulates m^6A methylation. Fibroblasts will be isolated from neonatal rat heart and activated with TGF-β for 48 hours. Cells will be harvested for RNA and protein analyses. Samples of RNA will be used for m^6A methylation measurements as well as for qPCR for myofibroblast marker genes (FN, Col1α, αSMA, Periostin). At protein level, Western Blot (WB) (for phosphorylation of, pSmad/tSmad) and immunostaining (for ECM proteins like αSMA, Col1α).

Specific Aim 2: Second, we will test the hypothesis that decrease or increase in m^6A expression in cardiac fibroblasts will influence gene expression related to fibroblast activation. This will be done by inhibiting METTL3, in order to decrease m^6A methylation in cardiac fibroblasts, and investigating the effects this change has on fibroblast activation.

MATERIALS AND METHODS

Cell Culture and Treatment

Neonatal Rat ventricular fibroblasts (NRVF) cells were isolated from neonatal rat pups and seeded in 150 mm dish for treatment. At 60-70% confluency, cells were treated with TGFβ for 48hrs. After different treatments, cells were harvested for biochemical and immune-cytochemical analyses.

Isolation of RNA

After 48hr treatment, 100mm dishes containing fibroblasts were washed two times with 5µL of 1 X Phosphate Bovine Serum (PBS). 350µL of RNA Lysis Buffer was added to

each 100mm dish to lyse cells. Using a cell scraper, cell lysate was harvested and collected in 1.5mL Eppendorf tubes and placed immediately in ice until the next step. Equal amount of 70% Ethanol as lysis buffer (350µL) was added to and mixed with lysate. Mixture was placed in RNeasy Mini Spin Columns and spun in centrifuge for 15s at 8000g. Samples were washed once with 700µL of RW1 wash buffer and spun in columns for 15s at 8000g. Then washed twice with 500µL of RPE wash buffer and spun in columns for 15s and 2min, respectively, at 8000g. Filtered liquid was discarded, and columns were placed in new 1.5mL Eppendorf tubes. 60µL of RNase free water was added and columns were spun for 1 min at 8000g. RNA samples were collected in the Eppendorf tubes and stored at -20^0C until further use, and columns were discarded.

Synthesis of cDNA

For each sample, 2µL of 10X RT Buffer was mixed with 0.8µL of 25X dNTP Mix, 2µL 10X RT Random Primer, 1µL MultiScribe Reverse Transcriptase and 4.2µL, making a total of 10µL Master Mix. 500ng of each sample was taken and mixed with appropriate volume of water to make a total of 10µL. The 10µL mixtures from step 1 and step 2 were added together to make a total mixture of 20µL. cDNA Synthesis was performed in standard PCR machine. cDNA was be stored at -20^0C. Before use for qPCR, cDNA was diluted by adding 80µL water to make a total of 100µL diluted cDNA.

RT-qPCR Procedure

For each sample, 10µL of TaqMan master mix was added to 6.5µL of water and 1µL of specific primer (Col1α, αSMA, and FN), making a total of 17.5µL. 2.5µL of each cDNA was added to the 17.5µL mixture, to make 20µL total mixture. The total reaction mixture was run in qPCR machine for gene expression. After the run was complete, the results were analyzed, and fold change of treatment samples were compared with the control.

Quantification of m^6A Methylation

In a 96-well plate, number of wells needed were predetermined, based on controls and number of samples available. 250ng of RNA and 80µL of binding solution (BS) were added to each well. 2µL of Negative Control and 80µL of binding solution were added to a separate well. 2µL of 0.2ng/µL Positive Control and 80µL of BS were added to a separate

well. Samples were incubated at 37^0C for 90minutes. Wells were washed 3 times with 150µL 1X Wash Buffer. Next, 50µL of diluted Capture Antibody (primary antibody) was added to each well and samples were incubated for 60min at RT. After incubation, wells were washed 3 times with 1X Wash Buffer. This was followed by the addition of 50µL of diluted Detection Antibody (secondary antibody) to each well. Samples were incubated at RT for 30min. After incubation, wells were washed 4 times with 1X Wash Buffer. 50µL of diluted Enhancer Solution was added to each well and samples were incubated for 30min at RT. Wells will be washed 5 times with 1X Wash Buffer. 100µL of Developer Solution was added to each well and samples were incubated for 10min (this step was done away from light). Blue coloration was observed in wells with methylated samples. 100µL of Stop Solution will be added to each well. Yellow coloration was observed in wells after the addition of Stop Solution. Wells were read at A420 within 2 to 15minutes of adding stop solution. Methylation was calculated using the following formula.

$$\%m^6A = \frac{\frac{Sample\ OD - NC\ OD}{S}}{\frac{PC\ OD - NC\ OD}{P}} * 100 \qquad \text{Eq. 1}$$

Where,

Sample OD=absorbance of the sample

NC OD=absorbance of negative control

PC OD=absorbance of positive control

S = concentration of sample

P = concentration of positive control

Immunocytochemistry

Cells were fixed on coverslip with 4% paraformaldehyde for 15 min, then washed two times with 1XPBS for 5 min each. Cells were incubated with permeabilization solution (0.1% triton-X in 0.1% sodium citrate) for 5 min, and then washed three times. Cells were then blocked for 1hr in blocking buffer (10% milk in 1% Bovine Serum Album in 1XPBS). Cells were washed three times and incubated in primary antibody (1:200 Col1α and 1:500 METTL3) at 4^0C overnight. Cells were washed three times and incubated in secondary antibody (1:1000 Goat anti Mouse IgG Alexa Flour 488 and 1:1000 Goat Anti-

Mouse) at RT for 1hr. Cells were washed three times, stained with Dapi and coverslip was attached to microscope slides. Using a fluorescence microscope, cells were viewed, and images were taken.

Protein Analyses

A. Protein Isolation

After washing fibroblasts in 100mm dishes with 1XPBS, 2µL 0.25% trypsin was added to cells and incubated for 2 min. Cells were collected in falcon tube and spun in centrifuge at 4^0C and 1400 rpm for 5 min. Excess media was discarded and pellets were transferred into 1.5mL Eppendorf tubes. 1000µL of 1XPBS was added and tubes were spun in centrifuge at 4^0C and 14000g for 5 min. PBS was discarded and 50µL of working protein lysis buffer with 1XPIC (Protease Inhibitor Cocktail) was added. Samples were vortexed for 15s three times and stored overnight at -20^0C. The next day, samples were vortexed for 15s three times and spun in centrifuge at 4^0C and 14000g for 20 min. Supernatant was collected as isolated protein samples and pellet was discarded. Protein was stored in -20^0C.

B. Protein Estimation

Eight Eppendorf tubes were labeled Standard 1-7. 25µL of lysis buffer was added to each tube. 25µL of 2mg/mL Standard BSA was serially diluted in each Eppendorf from the previous step (total dilution: 7). This makes eight BSA concentrations including the original: 2, 1, 0.5, 0.25, 0.125, 0.063, 0.031, and 0.016 mg/mL. 3µL of protein was diluted in 27µL working lysis buffer. Working Reagent (WR) was prepared with a ratio 50 to 1 of Solution A to Solution B. For example, 6.25mL of Solution A was mixed with 125µL of Solution B. In a 96-well plate, number of wells based on protein samples and serially diluted BSA were predetermined. 200µL of WR was dispensed in each designated well of 96-well plate. 10µL of working lysis buffer was dispensed in wells designated as blank. 10µL of each BSA concentration were dispensed in wells designated as standard. 10µL of protein samples were dispensed in wells designated as samples (the remaining protein was stored at -20^0C until needed for further analyses). The 96-well plate was incubated at RT for 30 min. OD was measured at 562nm in EPOCH2. Sample ODs and concentrations were

used to plot curve. Linear equation slope $y = Ax \pm B$ and R^2 value were displayed on plot. Concentrations of protein samples were calculated using the following equation.

$$\frac{OD\ of\ sample - (\pm B)}{A}$$

Where, A and B are values from the linear equation.

C. Protein Preparation for Gel Loading

Equal concentration of each protein sample was put in 1.5mL Eppendorf tubes. Samples were mixed with 4XLaemmli dye (reducing) and vortexed briefly. Tubes were heated at 95^0C for 7min and then allowed to cool down at RT.

D. Western Blotting

Gel loading instrument was set up and filled with 1X SDS running buffer. Protein samples prepared in the previous step were placed in electrophoresis gel, along with 3µL of protein ladder and the electrophoresis instrument was run for 2 hours. Nitrocellulose membrane was charged in methanol for 2 min. After completing gel electrophoresis, gel was placed in transfer instrument with charged membrane. Instrument was filled up with transfer buffer (containing methanol), placed in ice, and run for 1 hr to transfer protein to membrane. Blocking buffer containing 2 g milk in 30mL TBST was prepared. After transfer, membrane was placed in blocking buffer on put on shaker for 1hour at 52rpm. Membrane wash washed 3 times with TBST on shaker at 85rpm for 7 min each time. Primary antibody was prepared in TBST+3% BSA in 1:1000 dilution. Membrane was in primary antibody and put on shaker at 52rpm overnight in 4^0C. The next day, membrane was washed wash 3 times with TBST on shaker at 85rpm for 7 min each time. Secondary antibody was prepared in TBST+3% BSA in 1:1000 dilution. Membrane was placed in secondary antibody and put on shaker at 52rpm for 1 hour. Membrane was washed 3 times with TBST on shaker at 85rpm for 7 min each time. Membrane was developed and picture was taken.

Statistical Analyses

Statistical analyses were carried out using Prism software (Graph Pad). Unpaired, 2-tailed Student's t test was used for determining significance between 2 groups, while ANOVA followed by Turkey post hoc test was applied to calculate significance when more than 2 groups were involved. P values of <0.05 were considered significant. Data are presented as mean ± s.e.m. of minimum 3 biological replicates.

RESULTS AND DISCUSSION

A. TGFβ treatment activates cardiac fibroblast trans-differentiation to myofibroblast: After treatment with TGFβ for 48hrs, expression of the profibrotic markers, fibronectin, Col1α and αSMA, were compared between control and treated samples. As shown In Figure 2, TGFβ treatment significantly increased fibronectin (Figure 2A), Col1α (Figure 2B), and αSMA (Figure 2C) genes expression as compared to control. The increase in expression of these markers verifies that treatment with TGFβ was successful in activating fibroblasts. SMAD 2/3 is a protein in the canonical signaling pathway of TGFβ; a widely recognized pathway that induces cardiac hypertrophy and fibrosis in the failing heart [22, 23]. In its active form, SMAD 2/3 is phosphorylated into phospho-SMAD 2/3, which plays a role in the activation of the fibronectin, Col1α and αSMA genes, which lead to cell growth and differentiation. A high ratio of phospho-SMAD 2/3 to SMAD 2/3 is an indicator of TGFβ-induced fibroblast activation. Phospho-SMAD2/3 forms a complex with SMAD 4 in the pathway. This complex is translocated into the nucleus and activates these genes, which lead to the synthesis of important extra-cellular matrix protein fibronectin, and secretion of cytokines, procollagen and αSMA, which in turn induce fibroblast differentiation into myofibroblasts [23, 24]. In Figure 2D, the expression of phospho-SMAD 2/3 and SMAD 2/3 are shown in control and treated samples. GAPDH is used as a control to normalize protein loading and expression. Figure 2E is a densitometry plot of these protein expressions, showing a statistically significant increase in phospho-SMAD2/3 to SMAD2/3 ratio following TGFβ treatment, compared to control. The higher ratio of phospho-SMAD2/3 to SMAD 2/3 in the treated samples

verifies the activation of fibroblasts via TGFβ signaling. This supports the changes in gene expression increase shown in the preceding qPCR plots.

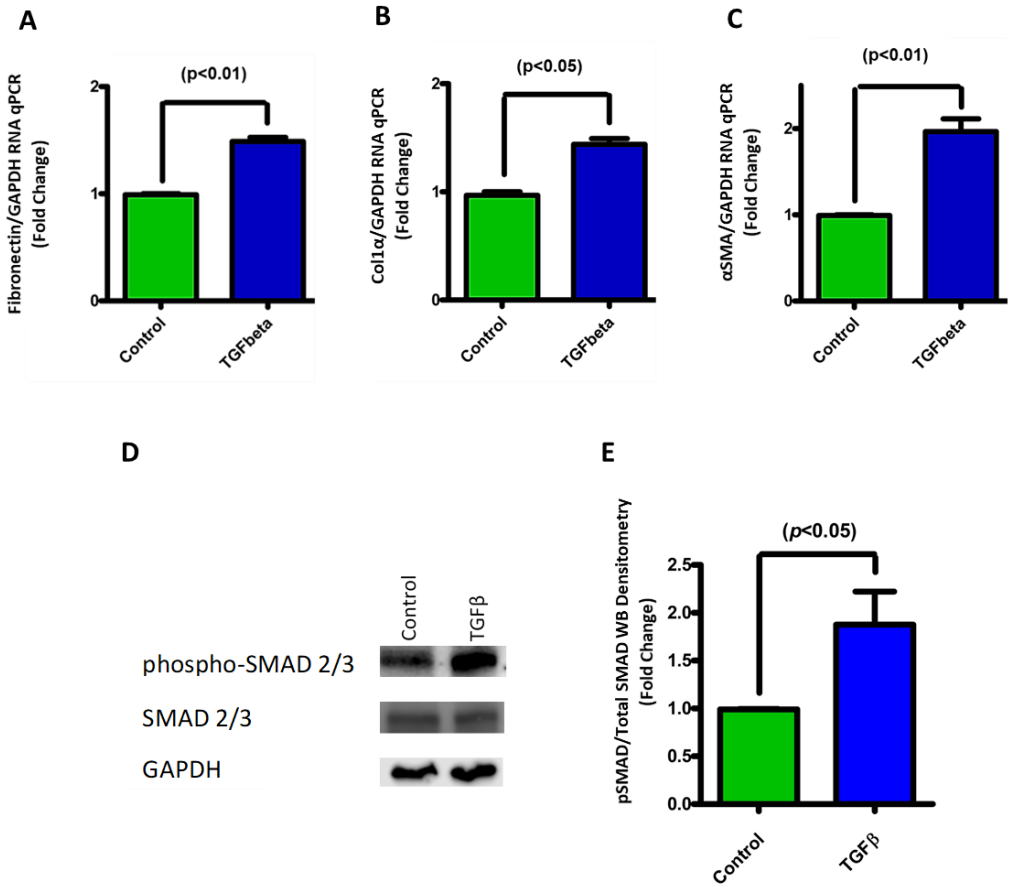


Figure 2: Results after qPCR and m^6A quantification showing fibroblast activation into myofibroblasts and fold increase in methylation, respectively. **A)** Increase in αSMA expression in cells treated with TGFβ for 48 hours. **B)** Increase in Fibronectin expression in cells treated with TGFβ for 48 hours. **C)** Increase in COL1α expression in cells treated with TGFβ for 48 hours. **D)** Results after western blot, showing increase in phospho-SMAD/Total SMAD ratio in TGFβ treated samples, compared to control. **E)** Densitometry plot of western blot comparing control and TGFβ treated samples.

B. TGFβ treatment increased RNA methylation in cardiac fibroblast: After confirming the activation of fibroblasts, the next step was to investigate the effects of this activation on m^6A methylation. A methylation quantification assay was done, using an anti-m^6A specific, antibody-based methylation kit, in order to explore this potential correlation. Methylation was measured in both control and TGFβ treated samples and compared; and as shown in Figure 3, there was an increase in the expression m^6A RNA methylation in the treated samples, supporting the hypothesis of specific aim 1 that the activation of

fibroblasts modulates m^6A methylation. This data suggests that RNA methylation may play an important role in fibroblast activation after TGFβ treatment.

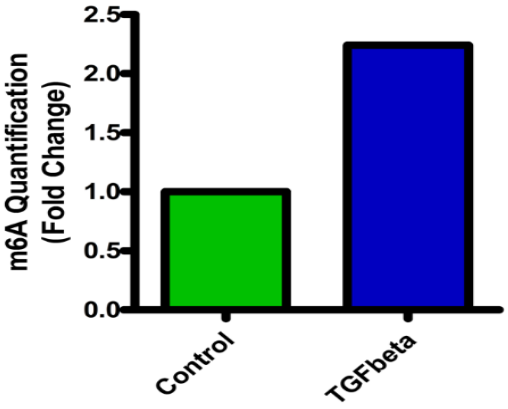


Figure 3: Results after m^6A methylation quantification showing fold increase in methylation after treatment with TGFβ for 24 hours. TGFβ treatment significantly increased RNA methylation.

C. METTL3 inhibition using METTL3 siRNA: The results of specific aim 1 show a positive correlation between fibroblast activation and m^6A expression; however, it is unclear whether TGFβ-induced RNA methylation leads to fibroblast activation. That is, whether a change in RNA methylation have a similar effect on fibroblast activation? To answer this question, METTL3 expression (RNA methylation) in fibroblasts was inhibited before TGFβ treatment using a siRNA against METTL3. In Figure 4 below, the inhibited METTL3 fibroblasts (labeled siMETTL3) are compared with a control group (labeled siControl). In the control group, lipofection was done using lipofectamine to produce pores in the cells, however no siRNA was introduced. In the siMETTL3 group, siRNA specific to METTL3 was introduced in the cells along with lipofection. In Figure 4A, METTL3 protein expression is shown in both groups, with a decrease in the expression for the siMETTL3 group, compared to siControl. Figure 4B quantifies the fold change in METTL3 band intensity, showing a significant decrease in METTL3 protein expression, thereby confirming METTL3 inhibition.

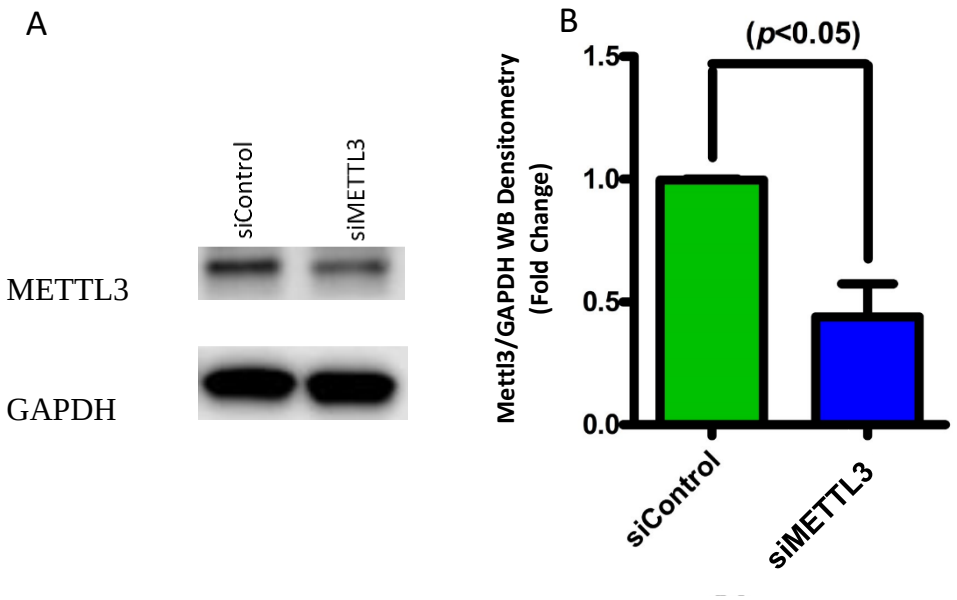


Figure 4: Western Blot (WB) analysis was done to verify the inhibition of METTL3. **A)** Western blot images showing a decrease in methylation following siRNA inhibition of METTL3 50nM. **B)** Densitometry plot of METTL3WB bands. METTL3 was normalized with GAPDH, to compare siControl and siRNA inhibition of METTL3.

D. Effect of METTL3 inhibition on TGFβ-induced fibroblasts activation: Fibroblasts were divided into six groups, Control (no treatment or alteration), TGFβ treated, lipofection without siRNA METTL3 inhibition, TGFβ treated lipofection without inhibition, siRNA inhibited, TGFβ treated siRNA inhibited. These groups are designated "Control", "TGFβ", "siControl", "siControl+TGFβ", "siMETTL3", and "siMETTL3+TGFβ", respectively, in Figure 5. Transfection and TGFβ treatment were done in the appropriate groups. After 48hrs, protein and RNA were harvested for biochemical analyses. As shown in Figure 5A, there is an increase in TGFβ treatment significantly increased METTL3 protein expression as compared to their respective non-treated counterparts. This supports the positive correlation between TGFβ-induced activation and methylation expression in the forward reaction from specific aim 1. METTL3 inhibition using METTL3 siRNA significantly reduced METTL3 expression as compared to control, which also supports the information in Figure 4. Interestingly, METTL3 inhibition noticeably reduced TGFβ-induced METTL3 expression in siMETTL3+TGFβ group compared to TGFβ alone.

To further confirm the role of METTL3 in TGFβ-induced fibroblasts activation, we performed fibrotic markers (Periostin and Fibronectin) genes expression in above treated cells. Notably, there is a significant decrease in the expression of both markers in the siMETTL3+TGFβ groups compared to the TGFβ groups. Therefore, inhibition of METTL3 expression had a directly proportional effect to the treatment of TGFβ. In other words, fibroblasts with less METTL3 expression did not activated as successfully as fibroblasts with normal METTL3 expression, when treated with TGFβ. Therefore, in response to the question posed earlier, the positive correlation seen between TGFβ-induced fibroblast activation and m^6A methylation expression can be seen in both forward and backward directions, regardless of whether activation or change in methylation expression occurs first. This conclusion is further supported by Figure 6 below, showing immunocytochemistry images of the six groups. The nucleus is stained blue with Dapi; Colα expression is shown in green in the cytoplasm; siMETTL3 inhibition shows less Col1α expression compared to Control, and siControl. The expression of Col1α increased in the siMETTL3+TGFβ group, compared to siMETTL3.

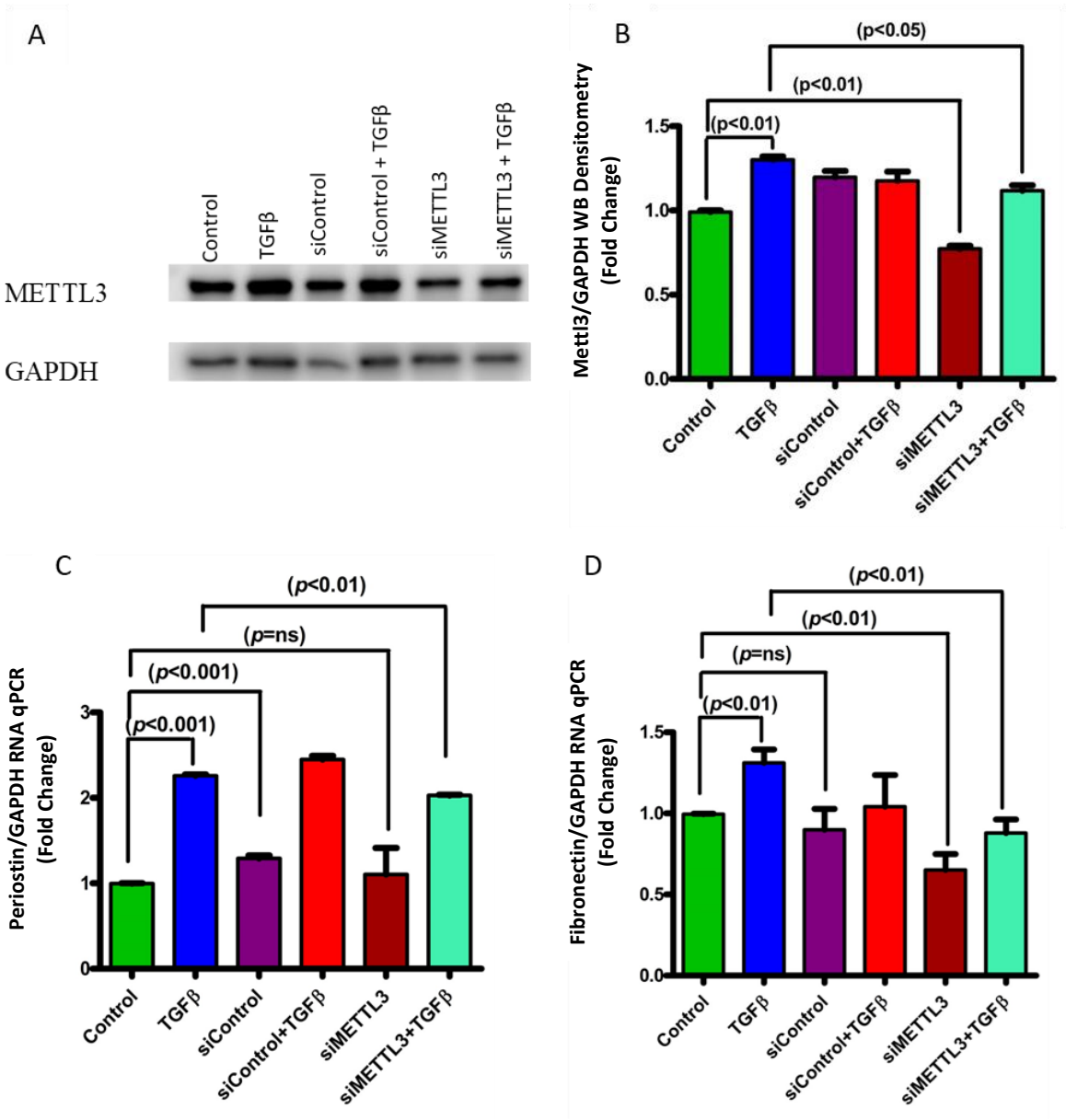
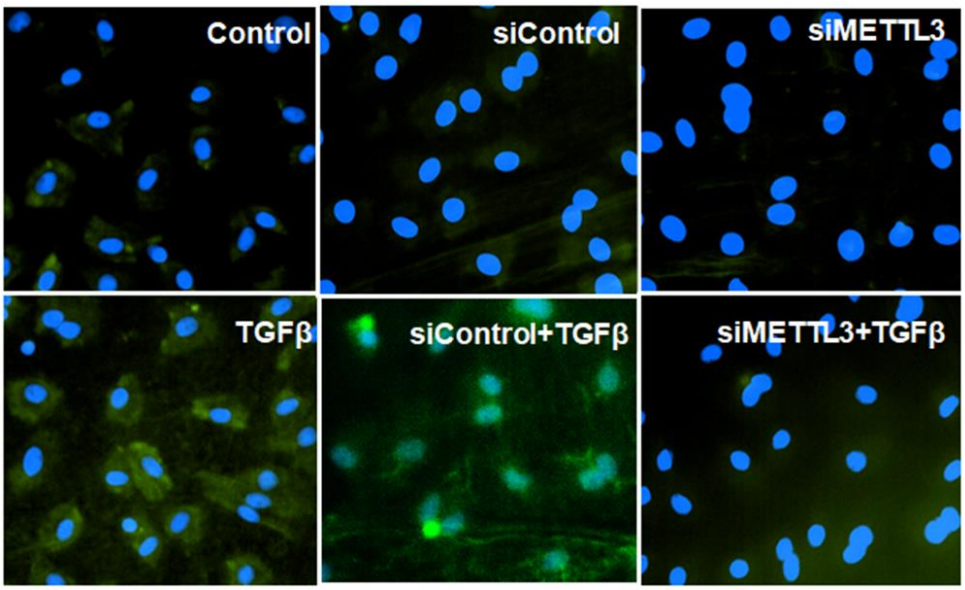


Figure 5: Results after siMETTL3 inhibition, qPCR and Western blot (WB). Fibroblasts were divided into six groups, Control(not treated), TGFβ, siControl (lipofectamine added to control), siControl+TGFβ, siMETTL3 (METTL3 inhibition using siRNA silencer), siMETTL3+TGFβ (fibroblasts with METTL3 inhibition were treated with TGFβ) and treated for 48H. Treated samples were compared with untreated samples (n=3). **A)** Western blot protein expression of six sample groups. **B)** Densitometry plot of Western data. **C)** qPCR plot shows periostin expression between control and treated samples before and after inhibition of METTL3. **D)** qPCR plot shows fibronectin expression between control and treated samples before and after inhibition of METTL3.



Col1α: GREEN, Nucleus: BLUE

Figure 6: Results after immunocytochemistry showing Col1α (green) expression in the cytoplasm of cells after treatment with TGFβ for 48hrs. Nucleus is stained with Dapi (blue)..

In summary, the relationship between fibroblast activation and m^6A methylation was investigated. In specific aim 1, fibroblasts activated via TGFβ canonical signaling pathway, expressed an increase in m^6A methylation. Alternatively, the reverse reaction was done in specific aim 2-methylation writer, METTL3 expression was inhibited (to decrease methylation expression) and the change in fibroblast activation was assessed. Fibroblasts with inhibited METTL3, did not activated as well as fibroblasts with normal METTL3 expression, implicating the m^6A methylation levels in the regulation of fibroblasts activation/transdifferentiation to myofibroblasts. These findings support the two hypotheses that m^6A and fibroblast activation regulate each other. In the future, it will be important to explore the mechanisms by which these regulations occur. The m^6A RNA methylation is controlled by a complex machinery of writers, readers, and erasers, each having a potential role in regulating fibroblast activation.

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ABSTRACT

IACUC Approval

UNIVERSITY OF ALABAMA AT BIRMINGHAM
GRADUATE SCHOOL

APPLICATION FOR ADMISSION TO CANDIDACY FOR MASTER'S DEGREE

Name: ZAINAB G SULEIMAN Email: zainabgs@uab

Blazer ID: zainabgs Student Number: B01259087 Date: 05/19/2020

Graduate major: BIOMEDICAL ENGINEERING

Total hours completed: 30

Total hours yet to be completed:
(including courses in progress) 6

I hereby petition the Graduate Dean to be admitted to candidacy for the degree of MS in BIOMEDICAL ENGINEERING **under Plan 1.**

Thesis title: EPITRANSCRIPTOMIC REGULATION OF FIBROBLAST ACTIVATION IN DISEASED HEART

☐ Yes ☑ No Project involves human subjects. *(If yes, please attach approval or exemption)*

☑ Yes ☐ No Project involves animal subjects. *(If yes, please attach approval or exemption)*

Signature of candidate

SUPERVISORY COMMITTEE: We, the undersigned, approve the thesis title. We recommend the applicant to the Graduate Dean for admission to candidacy for the degree.

Suresh Verma (PI)

Prasanna Krishnamurthy

Ramaswamy Kannappar

HIND LAL

06/02/2020

Program Director/Department Chair date

Graduate School Use Only

August 3, 2020

Approval of Graduate School Dean Date

RCR verified by: Juanitra Moore

Updated 03/2019

Admission to Candidacy
Research Compliance Verification Form

Instructions:
Complete this form, including all applicable approvals and the signatures of the student, the student's advisor, and the Graduate Program Director.
For research approval forms, contact the Institutional Review Board (IRB) (http://www.uab.edu/irb or 934-3789) or the Institutional Animal Care and Use Committee (IACUC) (http://www.uab.edu/iacuc or 934-7692).

Human Subjects
The University of Alabama at Birmingham defines a human subject as not only a living human being, but also human tissue, blood samples, pathology or diagnostic specimens, study of medical records, observation of public behavior, and all questionnaires or surveys.

Does the research proposed by the student involve human subjects? ☐ Yes (continue below) ☐ No
The research is:
Approved _____ Date: _____

IRB Protocol No. _____
Attach a copy of your IRB approval. Your name and the protocol number **must** appear on the original approval or on an attached amendment.

Animal Subjects
The University of Alabama at Birmingham defines a laboratory animal as any vertebrate animal (e.g., traditional laboratory animals, farm animals, wildlife, and aquatic animals) and certain higher invertebrate animals used in research, teaching, or testing at UAB or sponsored through UAB but conducted off-site (i.e., field research or at collaborating institutions, etc.).

Does the research proposed by the student involve animal subjects? ☒ Yes (continue below) ☐ No
The research is
Approved Yes Date: 2/14/2020

IACUC Protocol No. 21369
Attach a copy of your IACUC Notice of Approval, showing your research subject and the animal project number. If your name does not appear on the Notice of Approval, take this form to the IACUC office for verification of approval.

The IACUC office verifies that Zainab Suleiman (name of candidate) is covered under the attached approval.

Signature of IACUC representative Amanda J Weller Date: 5/26/2020

NOTE: The student's advisor, the student, and the Graduate Program Director agree that no research will be initiated until an application is submitted for review and approved by the appropriate review boards (IRB and/or IACUC) if the proposed thesis or dissertation project requires approval. If approval already exists, this student's name must be added to the existing protocol before candidacy will be approved by the Graduate School. It is the responsibility of the student's advisor and the student to comply with federal and UAB regulations associated with this research. Documentation of continuous, appropriate approval will be required before degree conferral; all required IAB and/or IACUC approval must be current at the time final versions of theses or dissertations are submitted to ProQuest.

Student's Signature — Dept. BIOMEDICAL ENGINEERING — Date 05/17/2020

Signature of Student's Advisor — Dept. Medicine - CVD — Date 5/26/2020

Signature of Student's Graduate Program Director — Dept. Biomedical Engineering — Date 06/02/2020

Updated 05/2019

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

MEMORANDUM

DATE: 13-Jan-2020

TO: Karthikeyan, Mythreye

FROM: Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 13-Jan-2020.

Protocol PI:	Karthikeyan, Mythreye
Title:	TGFb superfamily in ovarian cancer
Sponsor:	UAB DEPARTMENT
Animal Project Number (APN):	IACUC-21887

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

This protocol is due for full review by 12-Jan-2023.

Institutional Animal Care and Use Committee (IACUC) | Mailing Address:
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