UNIVERSITY OF CALIFORNIA SAN DIEGO

Understanding the Fibroblast-Extracellular Matrix Interaction regarding Tissue Remodeling in EoE

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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Ella Tour

2019

The Thesis of Lance Yan Hsieh is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California San Diego

2019

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I would like to thank Dr. Seema Aceves for not only serving as co-chair on my committee, but also for her continued guidance towards my academic, research, and professional career.

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VITA

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PUBLICATIONS

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FIELDS OF STUDY

Major Field: Molecular Biology

Research in Eosinophilic Esophagitis

ABSTRACT OF THE THESIS

Understanding the Fibroblast-Extracellular Matrix Interaction regarding Tissue Remodeling in EoE

by

Lance Yan Hsieh

Master of Science in Biology

University of California San Diego, 2019

Professor James Kadonaga, Chair
Professor Seema Aceves, Co-Chair

Eosinophilic Esophagitis (EoE) is an allergic/immune disease of the esophagus that is triggered by food or airborne allergens that invade and damage the esophagus and is distinguished by abnormal presence of eosinophils in the esophagus. This invasion can activate the epithelial layers of the esophagus to initiate a cascade of damaging immune responses and cause inflammation. Chronic inflammation and eosinophil persistence can lead to fibrosis and eventual remodeling of the esophagus that becomes stiff and responds poorly to current therapeutics. This process of remodeling is still not well understood, so we aimed to uncover how the esophageal extracellular

matrix environment may contribute to remodeling. It was hypothesized that the extracellular matrix (ECM) is able to alter esophageal fibroblast function. Primary healthy donor fibroblasts (n=5) and EoE active patient fibroblasts (n=5) were decellularized (20mM NH_4OH) to create an in culture ECM. Fibroblasts were then reseeded onto these decellularized ECMs. Proteomics analysis was performed on healthy and EoE decellularized ECMs. Once thrombospondin-1 (THBS1) was identified as a unique protein in EoE ECM in proteomics, recombinant human THBS1 was used to treat healthy and EoE fibroblasts to determine whether THBS1 was sufficient to induce fibrotic protein expression. Between 5 different experiments, EoE ECM induces collagen1a1 (2.5-fold, $p<0.005$) and a-smooth muscle actin (aSMA) (2-fold, $p=0.06$) protein expression in healthy fibroblasts compared to healthy (NL) ECM. Proteomics illustrates thrombospondin-1(THBS1) as a unique protein to EoE ECMs (fold change > 1.2, $p\leq0.05$) as well as increased expression of Protein disulfide isomerase A6 (PDIA6) in EoE ECM compared to healthy ECM. THBS1 expression is expressed more in ex vivo biopsy samples between biopsies from active state versus inactive state EoE patients ($p=0.05$). THBS1 also specifically induces collagen1a1 within healthy and EoE primary fibroblasts in a dose response, with significant induction at 10ng/mL ($p=0.04$). A disintegrin and metalloproteinase with thrombospondin motifs-1 (ADAMTS1) and tissue inhibitor of metalloproteinase 3 (TIMP3) proteins were identified in esophageal fibroblasts to determine any upstream targets of THBS1, but this data is currently inconclusive. Here, we demonstrate, in culture, that a diseased microenvironment can stimulate functional changes of healthy fibroblasts. Future research is still necessary to further dissect the interactions between the ECM and fibroblasts that will help us better understand how the esophagus maintains persistent remodeling.

Introduction

Eosinophilic Esophagitis (EoE) is an allergic/immune disease that is characterized by robust infiltration of eosinophils into the esophagus with at least 15 eosinophils per high power field (hpf) being the diagnostic minimal threshold. Basal zone hyperplasia is a classic histologic feature of EoE caused by active proliferation of basal stem cells (Aceves et. al, 2007 and Straumann et. al, 2012). In EoE, food or air borne antigens in the esophagus can activate the epithelial layer of the esophagus, which increases the production of thymic stromal lymphopoietin (TSLP) production. TSLP favors differentiation of Th2 cell type from naïve T cells in the sub epithelial regions. These Th2 cells produce IL-13 and IL-5 that stimulates inflammation of the epithelial esophagus and initiate remodeling throughout (Straumann et. al, 2012). Chronic inflammation of the esophagus can lead to pain, discomfort, and even food impactions due to esophageal thickening, narrower luminal esophagus, and linear furrows (Aceves et. al, 2009).

Activated EoE esophageal epithelium by IL-13 can distinctly induce eotaxin-3 (Blanchard et. al, 2006) to recruit eosinophils from the blood stream into the esophagus. Eosinophils, along with other cells in the sub epithelial region, produce transforming growth factor β-1 (TGFβ1), which stimulates subepithelial fibroblasts to deposit excess extracellular matrix (ECM) proteins, causing fibrosis (Straumann et. al, 2012). Fibrosis is the pathologic generation of excess ECM proteins and is caused by excess and dysregulated wound healing. Extracellular matrix proteins largely consist of collagen proteins, in addition to elastin, laminin and fibronectin proteins. Long standing fibrosis in of the subepithelial region of the esophagus can lead to eventual remodeling of the esophagus, in which strictures and esophageal rigidity can develop (Aceves et. al, 2007

and Straumann et. al, 2012). This can lead to dysphagia and tearing of the esophagus. Food impactions can also worsen, and together, overall quality of life can be greatly reduced.

EoE derived inflammation can be treated clinically with Oral Viscous Budesonide (OVB) (Aceves et. al, 2007). While OVB is effective at treating this inflammation, targeting the fibrotic complications in EoE has been more difficult, especially in steroid non-responsive patients. Patients who fail therapy, including OVB, tend to have the severe disease in terms of remodeling and fibrostenosis. While the progression from chronic inflammation to fibrosis has been well studied in EoE, the pathology between the EoE esophageal fibroblast and its ECM environment is still not well understood. We aimed to better understand the fibrotic aspect of EoE by studying the effects of the EoE ECM on esophageal fibroblasts. In a 2016 study by Amanda Muir and company, stiff and soft biomechanical microenvironments have been created to emulate the physical nature of the esophagus. Their studies show that stiff microenvironments are sufficient to promote the activation of fibroblasts to myofibroblasts as well as more robustly enhance TGFβ1 activation of fibroblasts compared to a soft culture microenvironment (Muir et. al, 2016). Preliminary studies in the Aceves Lab have also demonstrated rigid bioengineered matrices can activate fibroblasts and induce fibrotic gene expression. In idiopathic pulmonary fibrosis (IPF), studies have shown that normal lung fibroblasts, when in an IPF-derived ECM environment, can become activated to express more of the ECM proteins that are upregulated in IPF tissues (Parker et. al., 2014). To better understand how fibrosis is propagated in EoE and contributes to overall tissue remodeling, we hypothesized that the EoE ECM may be able to alter esophageal fibroblast function. We also aimed to find new proteins that are unique to EoE ECM

and/or fibroblasts that we could target for potential therapeutics for those with chronic or severe EoE.

Methods:

Matrix Swapping Experiments

Primary fibroblasts were isolated from healthy donor esophagi or EoE esophageal biopsies and cultured in 10%FBS/DMEM media (Gibco) in six well tissue culture plates, 9.5cm^2 growth area (Corning) to confluency. Cells were washed with PBS (Sigma-Aldrich) and matrix was decellularized with 20mM NH_4OH lysis buffer for 10 minutes at room temperature. This resulted in an in vitro extracellular matrix (ECM) environment from normal or EoE cells. Matrices were then briefly washed and 20mM NH_4OH lysis was repeated for 10 minutes. Last wash in PBS was performed until all cellular membrane/debris were removed. H&E and microscope verification showed no intact cells remaining.

Corresponding fibroblasts (healthy or EoE) used for decellularization were then re-seeded onto their respective and opposite decellularized matrix and grown in 10%FBS/DMEM media for 7 – 12 days.

RNA collection and analysis of fibrotic genes by qPCR:

RNA was collected in RNA Stat solution (Tel-Test) and 500ng-1ug of RNA were synthesized into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen). Quantitative PCR was performed on a CFX96 thermocycler machine (Bio-Rad) using QuantiFast SYBR Green PCR Kit (Qiagen) for primers: Collagen1a1, aSMA, FN, PLN, PAI-1, COMP, and TGFβ1 (**Table 1**). RPL13a was used as a housekeeping gene for normalization purposes. See primers in table below. Relative levels of gene expression were compared using the ΔΔCT method and fold induction graphed as $2^{-\Delta\Delta CT}$.

Table 1: Primers for qPCR

Collagen1a1	FWD: CAGCCGCTTCACCTACAGC REV: TTTTGTATTCAATCACTGTCTTGCC
Alpha-Smooth Muscle Actin (aSMA)	FWD: CCGACCGAATGCAGAAGGA REV: ACAGAGTATTTGCGCTCCGAA
Fibronectin (FN)	FWD: TCAACAGTGGGAGCGGACCT REV: TGGCAGCGGTTTGCGATGGT
Phospholamban (PLN)	FWD: AAACTCCCCAGCTAAACACC REV: GAACTTCAGAGAAGCATCACGATGAT
Plasminogen Activator Inhibitor-1 (PAI-1)	FWD: TGGTTCTGCCCAAGTTCTCCCTG REV: TGCCACTCTCGTTCACCTCG
Cartilage Oligomeric Matrix Protein (COMP)	FWD: AACAGTGCCCAGGAGGAC REV: TTGTCTACCACCTTGTCTGC
Transforming Growth Factor β-1 (TGFβ1)	FWD: GGTGGAAACCCACAACGAAATC REV: AATTCCCCTCCACGGCTCAAC

Protein collection, immunoblot and analysis of fibrotic genes by densitometry:

Fibroblasts were lysed in RIPA buffer with protease inhibitor cocktail (PIC) (Sigma-Aldrich for protein collection. Lysed samples were centrifuged at 14,000xg for 15 minutes, and RIPA supernatant was collected.

Western Blot analysis was done on all matrix (ECM) derived fibroblast extracts and supernatant. Precision Plus Protein Dual Color Standards (Bio-Rad) was used as the protein ladder for all western blots. Samples were run in an 8% Bolt Bis-Tris Plus Gels (Thermo Fisher) in MES running buffer for 45 minutes at 150V in Blot Mini Gel Tanks (Novex). Gels were then placed in transfer cassettes (Novex) with 0.2mm PVDF membranes (Thermo Fisher) and transferred for 1 hour at 20V. Blots were then washed in TBST for 10 minutes and repeated 3 times then blocked with bovine serum albumin in TBST for 1 hour. Collagen1a1 (ab88147), alpha smooth muscle actin (ab7817), fibronectin (ab2413), thrombospondin-1 (THBS1) (ab85762), A disnintegrin and

metalloproteinase with thrombospondin type-1 motif 1 (ADAMTS1)(OABF00561), and β-Tubulin (CST9F3) were detected using primary antibodies at a concentration of 1:1000 or 1µg/mL. Appropriate rabbit or mouse secondary antibodies were used and then blots were imaged using Femto-peroxide (Prometheus) substrate mix and imaged in a G:BOX Chemi XRQ gel doc system via chemiluminescence (Syngene). Blots were analyzed using densitometry on ImageJ software by quantitating pixel intensity of protein bands of all proteins of interest and normalizing to pixel intensities of β-Tubulin loading control.

Tissue culture and proteomics analysis of extracellular matrix samples:

Three healthy and 3 EoE sets of fibroblasts were grown in 6 well plates to confluency (1.2 million cells), each in duplicate in 10%FBS/DMEM. Decellularization was performed on all 3 healthy and 3 EoE fibroblast plates using 20mM NH_4OH lysis buffer. Decellularized matrices of each healthy and diseased fibroblasts were scraped off in PBS and sent for proteomics analysis. Proteomics were performed by the Biomolecular and Proteomics Mass Spectrometry Facility (BPMSF) at UCSD. Data analysis was performed in collaboration with the Lewis Lab at UCSD. The differential expression criteria was set to a fold change cutoff of 1.2 and a p-value cutoff of 0.05.

EoE patient biopsies, protein collection, and western blot:

Esophagogastroduodenoscopy (EGD) collected biopsies obtained from active (average eosinophils per HPF ≥ 15) and inactive state (average eosinophils per HPF ≤ 15) EoE pediatric patients at Rady Children's Hospital, San Diego were flash frozen on dry ice, homogenized on ice, and lysed in RIPA with PIC for protein extraction. Western blot was performed on these protein extracts using THBS1 primary antibody (ab85762), and protein disulfide isomerase A6 (PDIA6) (ab226840). Blots were imaged and

analyzed in ImageJ for densitometry by quantitating pixel intensity of protein bands of all proteins of interest and normalizing to pixel intensities of the loading control.

Immunohistochemistry of esophageal tissue:

Healthy, inactive state EoE, and active state EoE esophagus tissue were formalin fixed and paraffin embedded on 75mm x 25mm Superfrost Plus Microscope slides (Fisher Scientific). At time of use, tissue sections were xylene histoclear citrisolve (National Diagnostics) and ethanol treated, then underwent heat mediated antigen retrieval in citric acid-based buffer (Vector). Slides were then washed and blocked with BSA in PBS for 30 minutes, then incubated with THBS1 primary antibody (ab85762) for 12 hours. Slides were then washed and incubated with appropriate biotinylated secondary antibody for 30 minutes, and then adivin-biotin complex solution for 30 minutes (Vector). AEC chromogen substrate (Enzo) was added for 15-20 minutes incubation then quickly 100% hematoxylin treated. Image sections were taken with a Zeiss Axiocam 105 Color microscope at 5, 10, 20 and 40 times magnifications.

rhThrombospondin-1 treatment of healthy and EoE fibroblasts and western blot analysis:

Primary fibroblasts from healthy donor esophagus and EoE esophagus were cultured in 6 well plates (Corning) to 75% confluency in 10%FBS/DMEM and serum starved for 24 hours. Fibroblasts were then treated with rhTHBS1 (3074TH050) at concentrations of 1, 5, 10, 50, 100ng/mL for 24 hours and collected in RIPA with PIC. Western blot was performed with collagen1a1 (ab88147), alpha smooth muscle actin (ab7817), fibronectin (ab2413), ADAMTS1 (OABF00561), tissue inhibitor of metalloproteinase inhibitor-3 (TIMP3) (ab39184), and β-Tubulin (CST9F3) primary antibodies. Blots were imaged and analyzed in ImageJ for densitometry by quantitating

pixel intensity of protein bands of all proteins of interest and normalizing to pixel intensities of the loading control.

Statistics:

Data was analyzed using a one-way ANOVA test (n≥3) or a parametric t-test (n=2). Alpha or significance level was set to 0.05, and comparisons with two-sided p-values less than 0.05 were considered significantly different means. Statistical tests were performed using GraphPad Prism 8 software (GraphPad).

Results:

EoE fibroblast derived matrix alters healthy fibroblast protein gene expression:

Table 2: Label keys for matrix derived fibroblast conditions (matrix swapping)

NL-NL	Healthy fibroblasts on healthy ECM
NL-EoE	Healthy fibroblasts on EoE ECM
EoE-EoE	EoE fibroblasts on EoE ECM
EoE-NL	EoE fibroblasts on healthy ECM

Healthy and EoE fibroblasts were cultured on healthy and EoE fibroblast derived matrices (ECM) for 7-12 days to confluency, and then collected for RNA or protein (**Fig 1A**) quantification using 5 lines of healthy and EoE that were passage and sex matched. Transcriptionally, there was little induction or difference in relative gene expression of fibrotic genes when comparing NL-EoE to NL-NL (**Table 2**) condition with slight induction of phospholamban (PLN) and cartilage oligomeric matrix protein (COMP) gene expression. Similar results are shown when comparing EoE-NL to EoE-EoE (**Fig 1B**).

Protein was collected of the same matrix swapping samples using 5 lines of healthy and EoE that were also passage and sex matched. When comparing protein expression on immunoblots between matrix swapping conditions of all experiments, there was a 2.5-fold average increase of collagen1a1 (p=0.002) and a 1.5 average fold

increase of aSMA between NL-NL and NL-EoE conditions. Fibronectin levels did not show any average fold increase between NL-NL and NL-EoE (**Fig 1C**). Five experiments were conducted using 5 different healthy donor fibroblasts (n=5) and 5 different EoE patient fibroblasts (n=5), with three biological replicates in each set of experiments. Between all experiments, there is an induction of collagen1a1 and aSMA expression in NL-EoE as compared to NL-NL conditions. When comparing protein expression between EoE-EoE and EoE-NL, collagen1a1 and aSMA had an average of 1.5-fold decrease (not shown). Trends were seen however in 3 out of 5 total matrix swapping experiments, with expression of fold decrease being more variable. Fibronectin remained unvaried in expression between EoE-EoE and EoE-NL conditions.

Proteomics of ECM samples highlights proteins that are distinct in EoE ECMs.

Healthy and EoE derived ECM samples were analyzed using proteomics quantification (BPMSF, UCSD) to determine distinct protein profiles in the ECM from EoE versus normal FBL using an unbiased scientific approach. Twelve proteins had significantly lower expression in the EoE ECM compared to the healthy ECM (fold change ≤1.2, p≤0.05) shown in blue on the volcano plot, including fibronectin and periostin (**Fig. 2A**). Only one protein, protein disulfide isomerase A6 protein (PDIA6), had significantly higher expression in the EoE ECM compared to the healthy ECM, shown in red (**Fig. 2A**). PDIA6 is a protein important for the formation, reduction and isomerization of disulfide bonds.

Three proteins were detected to be unique to the EoE ECM (Z score log2(EoE:NL) >40) and not expressed in healthy ECM. One protein that was focused on was the matricellular glycoprotein thrombospondin-1 (THBS1) (**Fig 2B**), which is known to mediate cell-cell and cell-matrix interactions.

Thrombospondin-1 is elevated in EoE Cells:

Although THBS1 was shown to be unique to EoE ECM in the proteomics analysis, THBS1 was able to be detected by immunoblotting in all healthy and EoE matrix (ECM) derived fibroblast extracts and supernatants. THBS1 expression in the extracts, between all conditions in all experiments, is variable (p=0.1) and presents no clear trend (**Fig. 3A**). THBS1 is also expressed in all matrix derived fibroblasts supernatant, with more expression trending toward EoE supernatants. All supernatant samples also exhibit THBS1-specific doublet protein bands on the immunoblots, with EoE fibroblast derived supernatant showing increased THBS1 expression in the bottom band (**Fig. 3A**). Cleavage of THBS1 was hypothesized to be a potential mechanism for the increased fibrotic marker expression. ADAMTS1, known to cleave THBS1 (Iruela-Arispe 2008) was detected on immunoblots and shown to have increased expression in matrix derived EoE fibroblast extracts (**Fig 3D**).

Esophageal biopsies from pediatric, active and inactive state EoE patients were obtained and collected by esophagogastroduodenoscopy (EGD) and also immunoblotted for THBS1. When comparing biopsies between active and inactive patients, there was a 2-fold, significantly higher expression of THBS1 expression in the active disease biopsies compared to the inactive disease biopsies (p=0.05) (**Fig 3B**). Immunohistochemistry (IHC) of esophageal tissues sections also illustrates more numerous THBS1 specific cells in the epithelial and lamina propria areas of active EoE esophageal tissues compared to healthy and inactive esophageal tissues (**Fig. 3C**).

PDIA6 has unvaried expression in EoE patient biopsies:

Since PDIA6 was identified as an upregulated protein to EoE ECM, we decided to investigate its presence in protein extracted from EoE active and inactive state

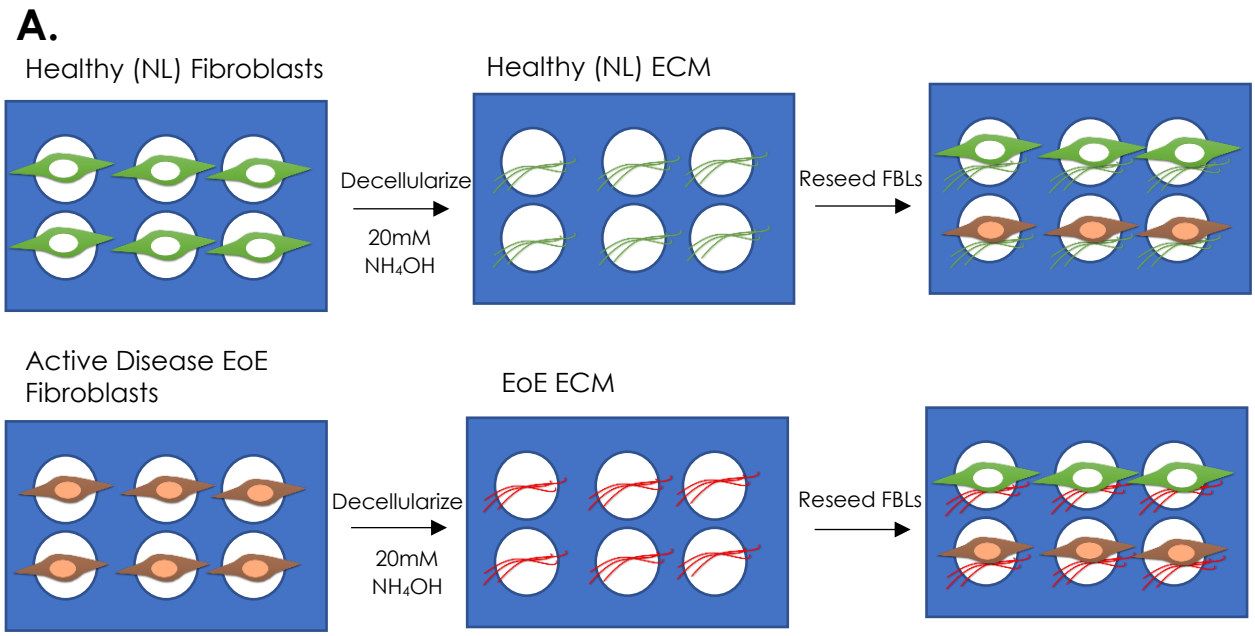
patient biopsies. When comparing PDIA6 amounts between active and inactive biopsies, there were on average relatively equal amounts of PDIA6, with inactive patient biopsies having slightly increased amounts of PDIA6 (**Fig 3E**). Healthy esophagus biopsies were unable to be obtained for further comparisons.

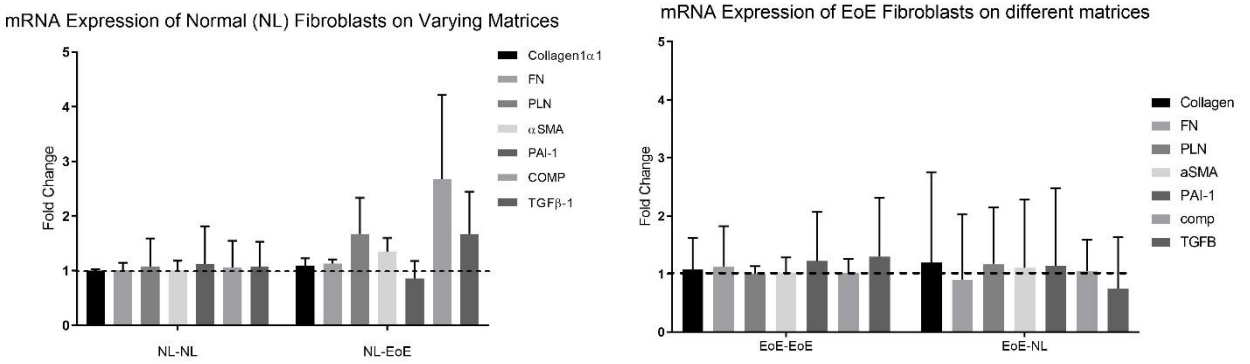
rhTHBS1 treatment of healthy and EoE primary fibroblasts stimulates collagen1a1 production:

Because THBS1 is known to activate TGFβ1 (Zenaida Lopez-Dee et. al, 2011) and TGFβ1 is a key pro fibrotic cytokine in EoE, we hypothesized that treating cultured healthy and EoE primary fibroblasts with recombinant human THBS1 (rhTHBS1) may similarly induce fibrotic expression such as collagen1a1. We verified that THBS1 was inducible by TGFβ1 both on mRNA and protein levels (**Fig. 4A-B**).

Healthy and EoE primary fibroblasts were treated with recombinant human THBS1 protein in a dose response of 1ng/mL, 5ng/mL, 10ng/mL, 50ng/mL, and 100ng/mL. Three biological replicates of healthy and EoE fibroblasts were used to test this dose response, with 1 unique healthy primary fibroblast and 2 unique EoE active primary fibroblasts. After a 24-hour treatment, healthy and EoE fibroblasts demonstrated gradual increased collagen1a1 production with the dose response. Between all experiments, peak induction of collagen1a1 yielded an average of 2.5-fold increased in healthy fibroblasts ($p=0.04$) and an average of 2-fold increase in EoE fibroblasts. Levels of FN, ADAMTS1, and TIMP3 show no average change in response to rhTHBS1 dose treatment in both healthy and EoE fibroblasts (**Fig 4C-D**). aSMA detection in EoE fibroblasts show an average trend of increased expression with high variability (high standard error of the mean), and no average induction in healthy fibroblasts (**Fig 4C**).

Figure 1: EoE fibroblast derived ECM alters function of normal fibroblasts. (A) Experimental set up of matrix decellularization and reseeding of healthy donor and EoE patient fibroblasts. **(B)** qPCR analysis of relative gene expression of fibrotic genes in all matrix swapping conditions. No significant changes observed. Five experiments using 5 unique healthy and EoE fibroblasts (n=5). **(C)** Western blot analysis of healthy donor and active EoE patient fibroblast protein shows induction of collagen1a1 ($p<0.005$) and aSMA ($p=0.06$) from NL-NL to NL-EoE. Collagen1a1 and FN graphs represent immunoblot densitometry quantification of all 5 experiments and their biological replicates. aSMA graph represents 1 representative experiment and its biological replicates. Five experiments using 5 unique healthy and EoE fibroblasts (n=5), with biological triplicates per experiment.


A.
Healthy (NL) Fibroblasts
Decellularize
20mM
NH4OH
Healthy (NL) ECM
Reseed FBLs
Active Disease EoE
Fibroblasts
Decellularize
20mM
NH4OH
EoE ECM
Reseed FBLs


B.
mRNA Expression of Normal (NL) Fibroblasts on Varying Matrices
Fold Change
Collagen1α1
FN
PLN
αSMA
PAI-1
COMP
TGFβ-1
NL-NL
NL-EoE
mRNA Expression of EoE Fibroblasts on different matrices
Fold Change
Collagen
FN
PLN
aSMA
PAI-1
comp
TGFB
EoE-EoE
EoE-NL

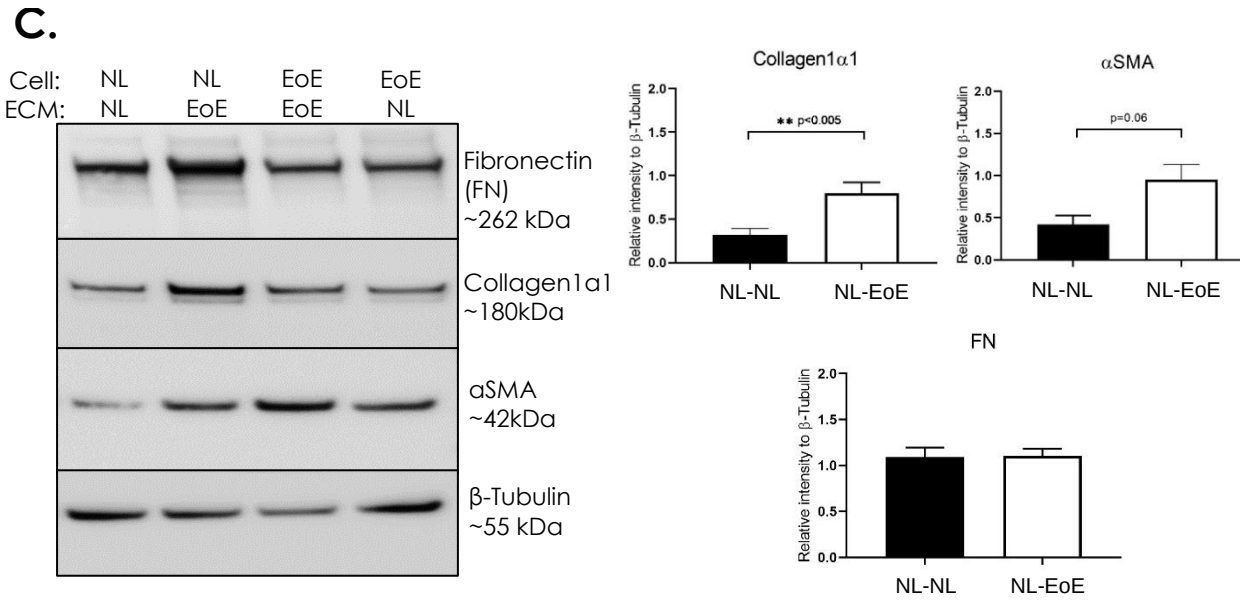
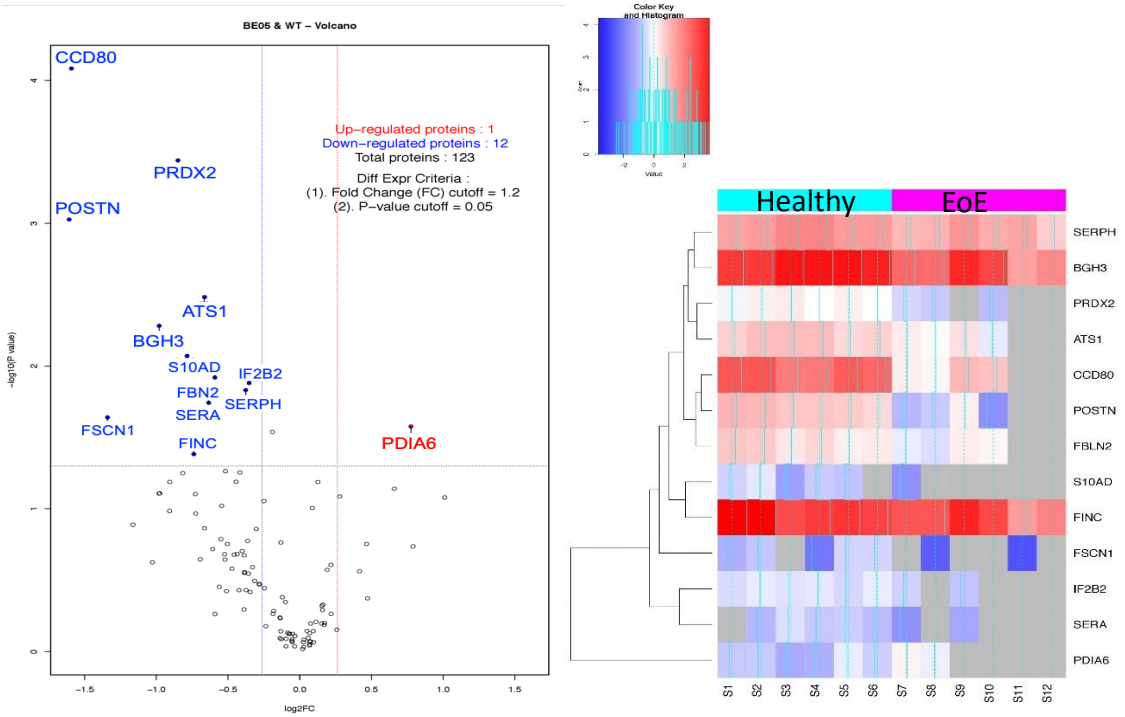

C.
Cell: NL NL EoE EoE
ECM: NL EoE EoE NL
Fibronectin
(FN)
~262 kDa
Collagen1a1
~180kDa
αSMA
~42kDa
β-Tubulin
~55 kDa
Collagen1α1
** p<0.005
Relative intensity to β-Tubulin
NL-NL
NL-EoE
αSMA
p=0.06
Relative intensity to β-Tubulin
NL-NL
NL-EoE
FN
Relative intensity to β-Tubulin
NL-NL
NL-EoE

Figure 2: Proteomic analysis of EoE vs. healthy extracellular matrix (ECM) samples. (A) Volcano plot of differentially expressed proteins between EoE ECM and healthy ECM. Log2(fold change) is graphed against log10 of the significance (p-value), with significant fold change cutoff at 1.2 and p-value cutoff of 0.05. Blue proteins represent proteins that are significantly expressed less in EoE ECM compared to healthy ECM. Red proteins represent proteins that are significantly expressed higher in EoE ECM compared to healthy ECM. **(B)** Differential expression of all significant proteins based on each protein's EoE:Normal ratio. Red proteins indicate proteins that are expressed in EoE ECMs but not healthy ECMs.

A. Volcano Plot comparing healthy (NL) to EoE ECM



B.

Differential Expression based on EoE:NL Ratio

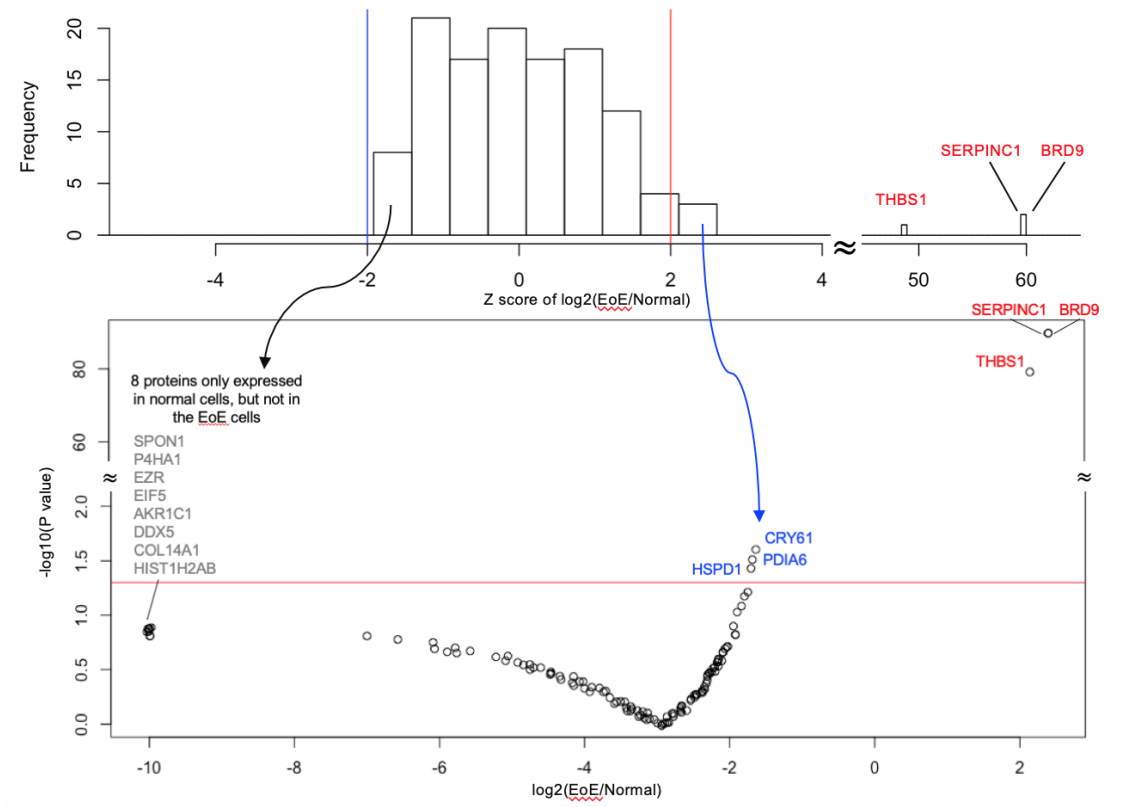
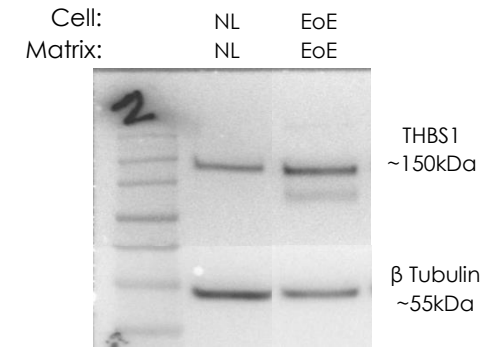
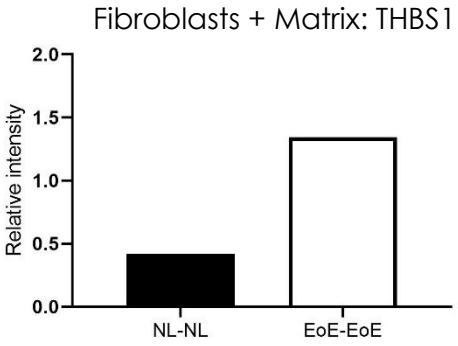


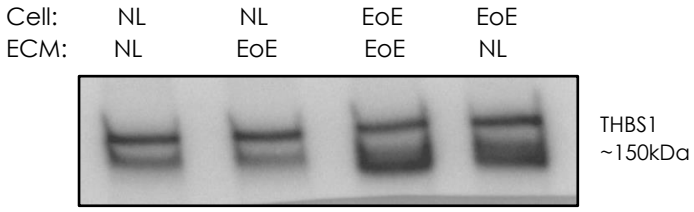
Figure 3: Verification of significant proteomic markers in ECM derived fibroblast extracts and supernatants, and EoE esophageal biopsies. (A) THBS1 expression in ECM derived fibroblast extracts shows trend of elevated expression in EoE-EoE compared to NL-NL. THBS1 expression in ECM derived fibroblast supernatant shows similar expression trends. N=3 for healthy and EoE fibroblasts used for both immunoblots. **(B)**. THBS1 expression in active and inactive state EoE biopsies shows 2-fold elevated expression in the active biopsies (p=0.05). 3 active and 5 inactive biopsies were extracted for protein and immunoblotted. **(C)** Immunohistochemistry (IHC) illustrates increased THBS1 specific cells in active EoE esophageal tissues compared to healthy and inactive esophageal tissues. N=3 for tissues from healthy, inactive and active esophagi used for IHC. **(D)** Immunoblot for ADAMTS1 shows a trend of increased expression in EoE fibroblasts compared to healthy fibroblasts, n=1. **(E)** PDIA6 expression in biopsies from active and inactive state EoE patients shows minimal difference in expression between active and inactive biopsies. Densitometry quantification performed on all immunoblots.

A.

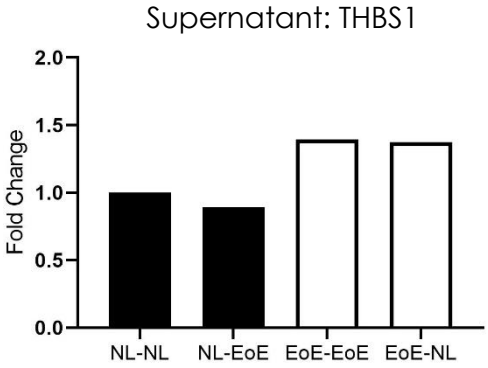


Fibroblasts + Matrix





Supernatant



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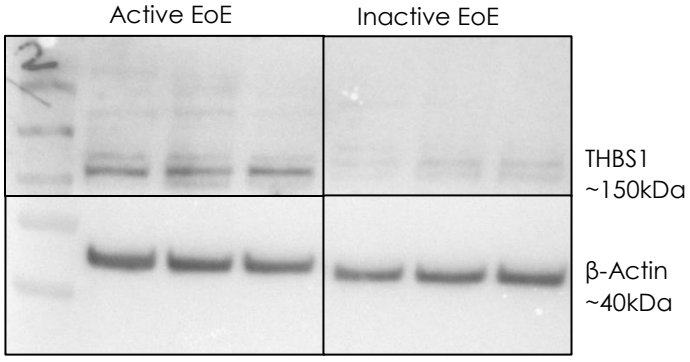


Esophageal Biopsies

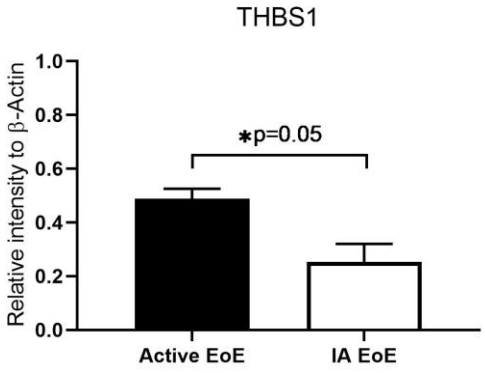
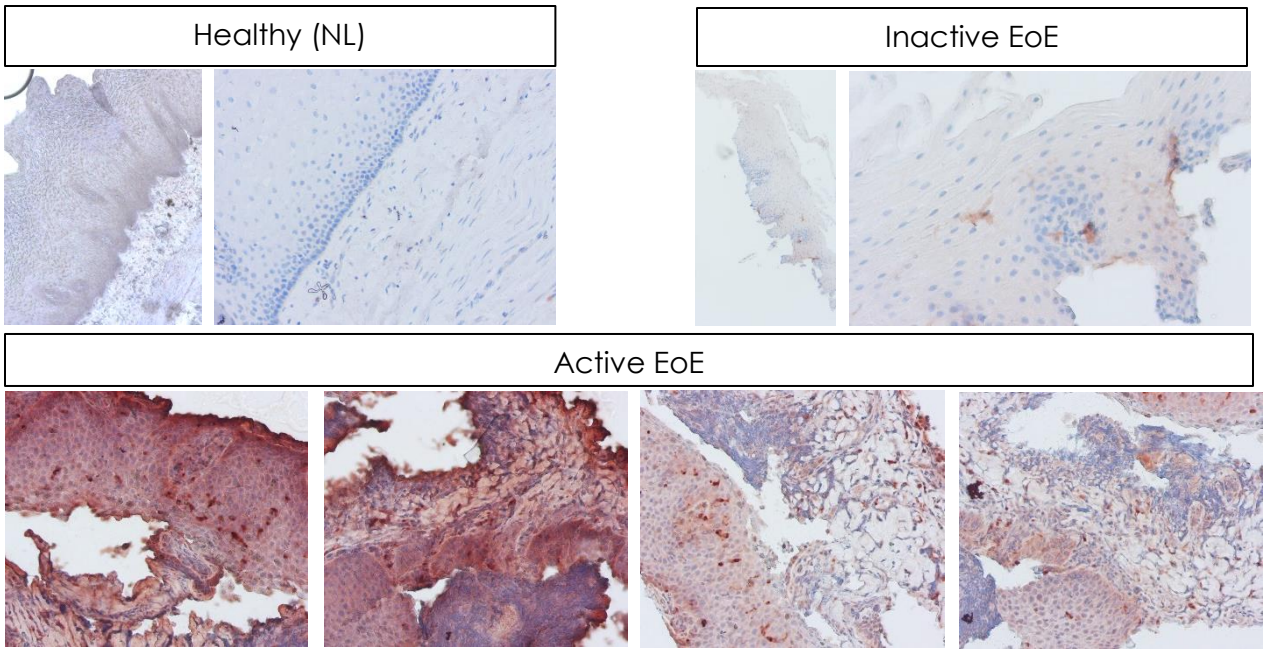
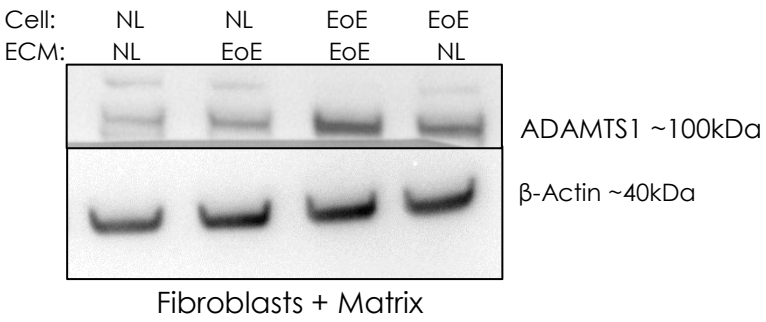


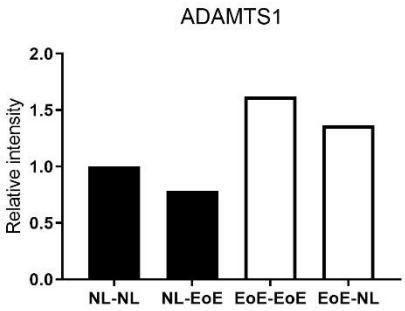
Figure 3 continued.

C.

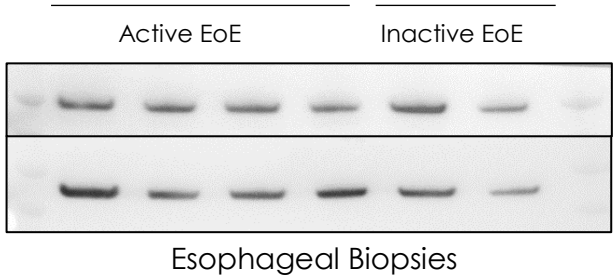


D.





E.



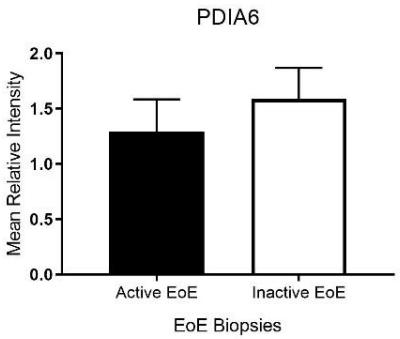
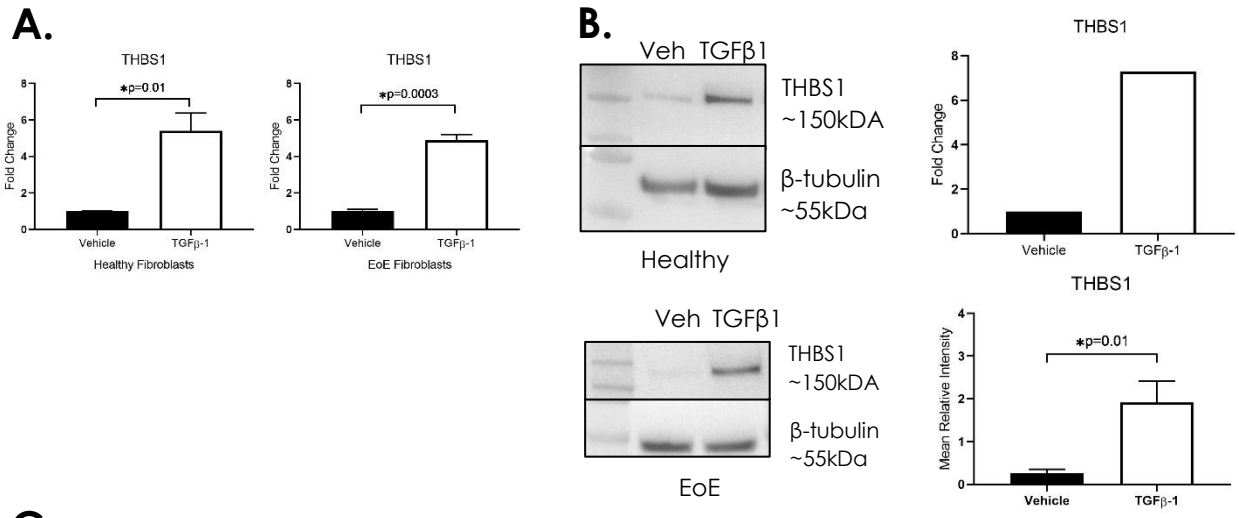
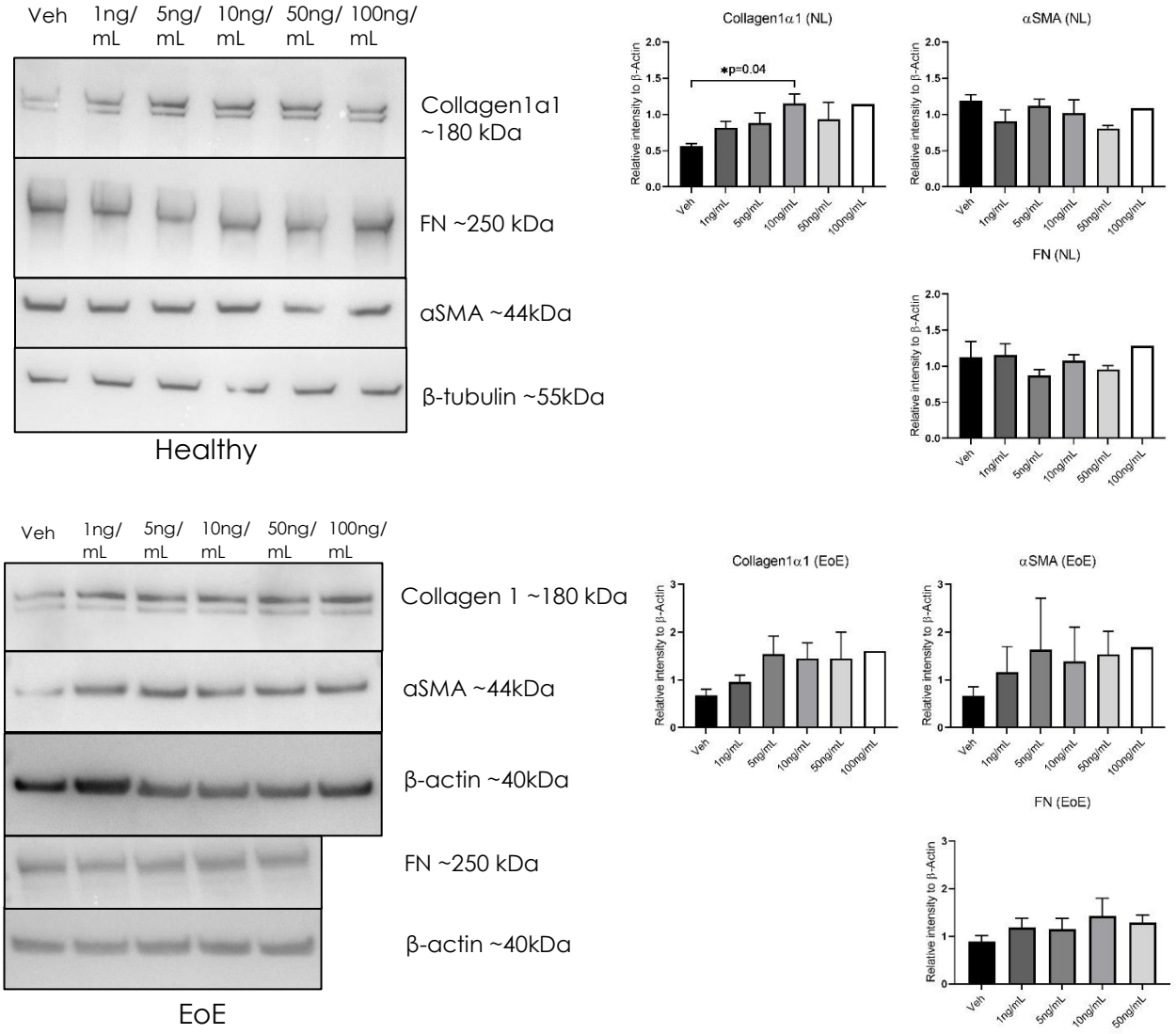


Figure 4: THBS1 affects esophageal fibroblast protein production. **(A)** TGFβ1 induces THBS1 mRNA expression of healthy ($p<0.01$) and EoE fibroblasts ($p<0.001$). N=2 total healthy fibroblasts and n=4 total EoE fibroblasts used. Graph represent one representative healthy and EoE fibroblast. **(B)** TGFβ1 induces THBS1 protein expression in healthy and EoE fibroblasts ($p=0.01$). N=1 total healthy fibroblast and n=5 total EoE fibroblasts used, as represented by the graphs. **(C)** Healthy and EoE fibroblasts treated with recombinant thrombospondin-1 (rhTHBS1) at concentrations of 0 (Vehicle), 1, 5, 10, 50, 100ng/mL for 24 hours show dose-response induced collagen1a1 expression, but not aSMA and FN expression. **(D)** ADAMTS1 and TIMP3 expression show no dose-response induction due to rhTHBS1 treatment Densitometry quantification analysis performed on all immunoblots.


A.
THBS1
*p=0.01
Fold Change
Vehicle
TGFβ-1
Healthy Fibroblasts
THBS1
*p=0.0003
Fold Change
Vehicle
TGFβ-1
EoE Fibroblasts
B.
Veh TGFβ1
THBS1
~150kDA
β-tubulin
~55kDa
Healthy
THBS1
Fold Change
Vehicle
TGFβ-1
Veh TGFβ1
THBS1
~150kDA
β-tubulin
~55kDa
EoE
THBS1
*p=0.01
Mean Relative Intensity
Vehicle
TGFβ-1


C.
Veh
1ng/mL
5ng/mL
10ng/mL
50ng/mL
100ng/mL
Collagen1a1
~180 kDa
FN ~250 kDa
αSMA ~44kDa
β-tubulin ~55kDa
Healthy
Collagen1α1 (NL)
*p=0.04
Relative intensity to β-Actin
αSMA (NL)
FN (NL)
Veh
Collagen 1 ~180 kDa
αSMA ~44kDa
β-actin ~40kDa
FN ~250 kDa
β-actin ~40kDa
EoE
Collagen1α1 (EoE)
αSMA (EoE)
FN (EoE)

Discussion:

Here, it is demonstrated that ECM derived from EoE fibroblasts, in culture, can alter and activate the function of healthy fibroblasts. Specifically, components of the EoE ECM can increase the production of collagen1a1 and a-smooth muscle actin (aSMA), enhancing healthy fibroblasts profibrotic activity as well as activating its myofibroblast transition. We suggest that EoE ECM induced collagen1a1 production in fibroblasts may cause increased collagen1a1 deposition back into the ECM, leading to a more fibrotic ECM that can further activate fibroblast fibrotic expression to create a potential positive feedback loop between the ECM and fibroblasts. Increasing aSMA production also can lead to increased contractility and stiffness of fibroblasts. We show that, in addition to a stiff microenvironment (Muir et. al, 2016), the presence of an EoE ECM environment also can propel dysregulation of healthy fibroblasts. Overall, the interplay between the ECM and fibroblasts in a diseased state may attribute to the overall persistence and robustness of fibrosis in EoE and may be one attributing factor to the difficulty of treating long-standing EoE, even if the esophageal inflammation can be subdued.

Proteomic analysis of the decellularized ECM environment (**Fig. 2A-B**) distinguished the expression profiles between healthy and EoE ECM and highlights intrinsic differences between the two. Surprisingly, fibronectin and periostin were both shown to have significantly lower expression in EoE ECM compared to healthy ECM. This may be due to an insoluble fraction of the ECM collection sample that was unable to be analyzed for proteomics collection. The cultured ECMs were collected in PBS, as opposed to RIPA collection solutions, for optimization of the proteomics procedure and preservation of the purity of protein in the ECM.

Thrombospondin-1 (THBS1) was one uniquely expressed EoE ECM protein that was analyzed to better understand the mechanisms between the ECM and fibroblasts, due to its role in cell-cell adhesion, cell-matrix adhesion (Sage and Bornstein 1991, Bornstein and Sage 2002). On immunoblots, THBS1 was verified to have increased expression in EoE fibroblasts and supernatant. To provide more biological relevance to EoE, inactive and active disease state esophageal biopsies were obtained for additional immunoblotting and found to have elevated expression of THBS1 in active state biopsies compared to inactive state biopsies (**Fig 3A-D**), further alluding to THBS1 importance in EoE.

THBS1 is also known to activate TGFβ1 cytokine (Stacey Schultz-Cherry et. al. 1994 and Zenaida Lopez-Dee et. al, 2011). While this activation of TGFβ1 has shown to have protective mechanisms such as inhibition of tumor growth in murine melanomas and lung carcinomas (Miao et. al., 2001), and prevention of angiogenesis in pancreatic cancer (Zhang et. al. 2005), THBS1 activation of TGFβ1 may inadvertently lead to other consequences of activating TGFβ1, such as esophageal fibrosis. TGFβ1 is widely known as a key mediator of esophageal tissue remodeling and fibrosis in EoE, and classically shown to induce fibrotic markers in esophageal fibroblasts such as collagen1a1, aSMA, and fibronectin (Aceves et. al, 2009). Additionally, we confirmed that TGFβ1 also significantly induces THBS1 mRNA and protein levels. We identify THBS1 as a component of the EoE ECM that increases collagen1a1 expression in healthy and EoE fibroblasts to a dose response treatment of recombinant human THBS1. This data suggests that EoE ECM is sufficient to induce specific fibrotic expression of esophageal fibroblasts. While potential upstream targets of THBS1 were identified as well on immunoblots of dose response treatment of rhTHBS1, further experiments will need to be explored to better

uncover how THBS1 signaling and activity attributes to the pathogenesis of tissue remodeling in EoE. ADAMTS1 is a protein known to cleave THBS1 with TIMP3 known to inhibit ADAMTS1 activity (Rodriguez-Manzaneque et. al, 2002 and Iruela-Arispe 2008), however identification of both potential upstream proteins gave any clear trends to suggest a mechanism.

In summary, we show that EoE ECM derived from diseased fibroblasts is sufficient to alter the functions of healthy fibroblasts. Further studies are needed to investigate the mechanisms of how ECMs interact with fibroblasts to change their function and how thrombospondin-1 particularly might be regulating esophageal cell-matrix interactions or cell function. Proteomic analysis of fibroblasts grown in decellularized ECM may give us some insight on how proteins are being regulated by the matrix, and what cellular functions, if any, are altered in an EoE ECM environment. By better understanding the interaction between the ECM and fibroblasts in EoE, we may be better able to determine how fibrosis progresses within the esophagus and find better targets for treatment of severe and/or long standing EoE. Furthermore, we will want to continue to distinguish what cellular functions within the fibroblasts are being altered by the ECM and how these altered functions may attribute to overall cell and tissue remodeling

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