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Understanding the Role of Phosphoinositide 3-Kinase and its Function as a Driving Force behind the ER Stress Response in Fibrostenotic Crohn's Disease-affected Ileal Smooth Muscle Cells

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Physiology and Biophysics at Virginia Commonwealth University

By,

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List of Abbreviations

1°	Primary
2°	Secondary
APS	Ammonium Persulfate
ATF4	Activating Transcription Factor-4
ATF6	Activating Transcription Factor-6
BCA	Bicinchoninic Acid
Bcl-2	B-cell Lymphoma 2
BiP	Binding Immunoglobulin Protein
BME	β-mercaptoethanol
CD	Crohn's Disease
CHOP	C/EBP-homologous Protein
DMEM	Dulbecco's Modified Eagle's Medium
EDEM	ER-degradation-enhancing-α-mannidose-like Protein
EGFR	Epidermal Growth Factor Receptor
eIF2α	α-subunit of Eukaryotic Initiation Factor 2
ER	Endoplasmic Reticulum
ERAD	ER-associated Degradation
FBS	Fetal Bovine Serum
GADD153	Growth arrest- and DNA Damage-inducible Gene 153
GRP78	Glucose-regulated Protein 78
IBD	Inflammatory Bowel Disease
IGF-I	Insulin-like Growth Factor-I

IRE1	Inositol-Requiring Protein-1
KO	Knockout
LY	LY294002
mTOR	Mammalian Target of Rapamycin
p-Akt	Phosphorylated Akt
PBS	Phosphate-buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PERK	Protein Kinase RNA (PKR)-like ER Kinase
PI3K	phosphatidylinositide 3-kinases
PVDF	Polyvinylidene Difluoride
RPM	Rounds Per Minute
S1P	Site 1 Protease
S2P	Site 2 Protease
SDS	Sodium Dodecyl Sulfate
SMC	Smooth Muscle Cells
TBST	Tris-buffered Saline with Tween 20
TEMED	Tetramethylethylenediamine
TM	Tunicamycin
TUNEL	Terminal Deoxynucleoidyl Transferase dUTP Nick-end Labeling
UPR	Unfolded Protein Response
XBP1	X-box Binding Protein 1

Abstract

UNDERSTANDING THE ROLE OF PHOSPHOINOSITIDE 3-KINASE AND ITS FUNCTION AS A DRIVING FORCE BEHIND THE ER STRESS RESPONSE IN FIBROSTENOTIC CROHN'S DISEASE-AFFECTED ILEAL SMOOTH MUSCLE CELLS

By Prashant Yadav, B.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Physiology and Biophysics at Virginia Commonwealth University

Virginia Commonwealth University, 2018

Director: John F. Kuemmerle, M.D. Professor, Department of Internal Medicine, Department of Physiology and Biophysics

Crohn's disease (CD) affects about 780,000 people in the United States alone, and it is estimated that 6-15 per 100,000 persons will receive a diagnosis of this disease each year. There currently is no cure for Crohn's disease, and available medical therapies simply serve to alleviate the inflammation. This does not help treat fibrostenosis that Crohn's disease patients may develop, which can only be treated surgically. Finding alternatives to treat CD requires an understanding of mechanisms at the biochemical level. In this thesis, we attempted to gain a better understanding of certain pathways found to be active in Crohn's disease-affected ileal smooth muscle cells. We found an upregulation of the ER stress pathway via expression of its surrogate, the GRP78 protein. We also showed evidence that the phosphoinositide 3-kinase (PI3K) pathway, a key proliferative pathway, is linked to ER stress in these cells, and is an upstream driving force of the ER stress response. Further research on the link between the PI3K and ER stress pathways needs to be conducted, and can potentially serve as a target for therapeutics to help reduce proliferation in fibrostenotic Crohn's disease-affected ileal smooth muscle cells.

Introduction

Crohn's Disease:

Inflammatory bowel diseases (IBD) are chronic inflammatory illnesses that affect the gastrointestinal tract. The two most common diseases that fall under this term are ulcerative colitis and Crohn's disease (CD). As the name suggests, ulcerative colitis describes a condition involving formations of ulcers and inflammation of the colonic regions. However, in the case of Crohn's disease, any area of the gastrointestinal tract can be affected (starting from the mouth to the anus). Pathologically, the inflammation seen in ulcerative colitis is limited to the mucosal layer. In Crohn's disease, the inflammation is transmural and can be seen in all layers of the gut (Qin, 2013)

Approximately 780,000 people in the United States are affected by CD (The Facts about Inflammatory Bowel Diseases, 2014). It is estimated that 6-15 per 100,000 persons receive a diagnosis of CD each year (Cosnes et. al., 2011). While CD can occur anywhere along the gastrointestinal tract, it is often found in the terminal ileum of the small intestine. One explanation for this could be due to alterations of certain genes expressed in this area (Caprilli, 2008). The polymorphism of these genes leads to favored colonization of adherent-invasive *E. coli*. The genetic defects contribute to an insufficient neutralizing of this bacteria, causing continuous immunological stimulation and inflammation (Caprilli, 2008). The exact cause of CD has not been determined, but a variety of environmental and genetic factors contribute to the onset of CD symptoms. CD is a chronic condition, and the constant damage from inflammation can lead to irreversible damage along the intestinal layers (Coskun, 2014). Patients suffering from CD will often present with diarrhea, abdominal pain, and weight loss (Ha & Khalil, 2015).

A patient's dysregulated immune system plays a significant role in the damage that is associated with CD (Anderson, 1979).

Different forms of Crohn's disease exist, and each individual's condition may vary. The most current form of categorization is the Montreal classification system, which separates CD type into three major 'behavioral' phenotypes - B1, B2, and B3. The B1 phenotype exhibit signs of inflammation, and does not present any evidence of stricturing or penetration (Satsangi et. al., 2006). The B2 phenotype indicates evidence of stricturing and stenosis, where there is narrowing of the lumen. B3 CD involves penetrance from the mucosal to the serosal layers, and leads to the formation of fistulae (Satsangi et. al., 2006).

There are treatments available to help manage symptoms, but there is no exact cure for CD. The medical therapy available follows an anti-inflammatory approach, but this may provide temporary relief. While this inflammation may underlie the onset of fibrostenosis, it is not required for fibrostenosis to continue (J. Kuemmerle, personal communication, April 2018). Fibrostenotic development is irreversible, and the only treatment available is surgical removal. With life-altering symptoms that come with a CD diagnosis, combined with the irreversible effects it can have on the body, research on this disease is crucial. It is important that efforts be made to better understand the mechanisms of CD in order to produce adequate treatment.

Fortunately, progress is being made in better understanding the underlying mechanisms of Crohn's disease at the biochemical level. Research is showing involvement of multiple signaling pathways and its relation to cellular behavior brought about by Crohn's disease (such as proliferation and fibrosis). One such pathway is the unfolded protein response initiated by endoplasmic reticulum stress.

Endoplasmic Reticulum and Endoplasmic Reticulum Stress:

The endoplasmic reticulum (ER) is a large, membrane-bound organelle that is found in eukaryotic cells. It has a number of responsibilities, such as aiding in protein synthesis, lipid synthesis, storage of calcium, and transportation of synthesized materials to the Golgi apparatus for further processing. The rough endoplasmic reticulum is where protein synthesis tends to take place, while lipid synthesis occurs in the smooth endoplasmic reticulum. The ER is a very dynamic organelle, and alteration of the equilibrium required for its function can have detrimental effects. In fact, there are many illnesses that have shown links to a malfunctioning ER, such as Alzheimer's disease, Parkinson's disease, and type 2 diabetes (Ozcan & Tabas, 2012). A malfunctioning ER also has its connections to the enhancement of fibrosis (Heindryckx et. al., 2016).

Only correctly folded proteins pass through the ER for further processing in the Golgi apparatus. Nascent proteins are folded in the ER with the aid of ER chaperones. The two main chaperone systems are the classical chaperones and the carbohydrate-binding chaperones. While the carbohydrate-binding chaperones are more unique to the ER, classical chaperones can be found in various cellular units (Braakman & Hebert, 2013). Members of the classical chaperone classification include proteins of both the Hsp70 and Hsp90 families, while examples of carbohydrate-binding chaperones include calnexin and calreticulin (Schrag et. al., 2001).

ER homeostasis can be altered by pathological and physiological changes. These include synthesis of proteins that surpass chaperone capacity, oxidative stress, and alterations in calcium storage of the lumen (Tabas & Ron, 2011). The misfolding of proteins that results from these imbalances leads to a condition called 'ER stress'. ER stress is sensed by three major proteins,

which leads to activation of multiple corrective signaling pathways. These proteins are inositol-requiring enzyme-1 (IRE1), protein kinase RNA (PKR)-like ER kinase (PERK), and activating transcription factor-6 (ATF6). Collectively, these corrective signaling pathways are referred to as the 'unfolded protein response' (UPR), of which there are three main branches.

IRE1 is thought to be the evolutionarily oldest branch of the UPR (Tabas & Ron, 2011). There are two known mammalian isoforms: IRE1α (found in all cell types), and IRE1β (found in gastrointestinal and respiratory tract cells). When unfolded proteins are detected in the ER, IRE1 undergoes dimerization and autophosphorylation to become active. Activation of IRE1 leads to the splicing of X-box binding protein 1 (XBP1) mRNA. A spliced form of XBP1 contributes to the expression of various other genes involved in the UPR, such as ER-degradation-enhancing-α-mannosidase-like protein (EDEM), a protein involved with ER-associated protein degradation. Protein folding genes also become activated due to spliced XBP1, such as protein disulfide isomerase (Oslowski & Urano, 2011).

Activation of the kinase PERK is a second branch of the UPR. Similar to IRE1, PERK undergoes autophosphorylation and oligomerization to become active. Activated PERK phosphorylates the α-subunit of eukaryotic initiation factor 2 (eIF2α). A phosphorylated eIF2α hinders the formation of ribosomal initiation complexes, resulting in the attenuation of global mRNA translation (a known exception is the ATF4 gene) (Tabas & Ron, 2011). This may contribute to a decrease in protein synthesis, allowing for the ER to try and rectify the unfolded proteins.

ATF6 activation is the third branch of the UPR. Similarly to IRE1, there exists two isoforms of ATF6- ATF6α and ATF6β (Thuerauf et. al., 2004). It is a transmembrane ER

protein, and upon activation is dissociated from the membrane. The protein is then taken to the

Golgi apparatus to be cleaved by Site 1 and Site 2 proteases (S1P and S2P). The N-terminal

fragment of ATF6 is then moved into the nucleus, and promotes transcription of more UPR

genes that aid in protein processing, folding, and degradation (Oslowski & Urano, 2011; Bravo

et. al., 2013)

Activation of the UPR branches must only occur when there is ER stress. Therefore, a

mechanism is required to keep these branches inactive in a normal state. This mechanism is

provided by the immunoglobulin heavy-chain binding protein (BiP), also known as glucose-

regulated protein (GRP78). During normal conditions, GRP78 is able to keep the branches

inactive by binding to the luminal domains of the three UPR proteins. It is when there is ER

stress that GRP78 dissociates from the complex, resulting in activation of the UPR (Oslowski &
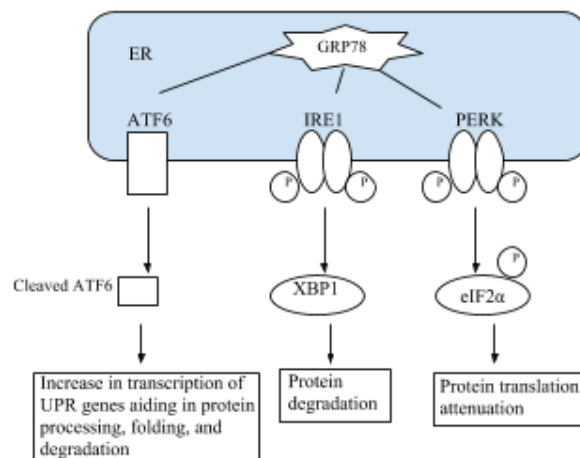
Urano, 2011).

Figure 1: GRP78 and its regulation of the UPR

GRP78 is a member of the Hsp70 family, and has multiple functions in addition to being

the master UPR regulator. It facilitates the folding and assembly of proteins, marks improperly

folded proteins for ER-associated degradation (ERAD), transfers synthesized polypeptides across

the ER, and aids in maintaining calcium homeostasis (Wang et. al., 2009). Because this protein is involved with UPR initiation, the presence of increased GRP78 is often used as a marker for ER stress.

Studies conducted on multiple cancer cell lines have shown elevated levels of GRP78 expression within the cells. With the connection of GRP78 and the three protein branches of the UPR established, along with its presence in cancer cells, GRP78 is often considered as a pro-survival protein. Overexpression of GRP78 has also shown enhanced proliferation in multiple cell types, such as chondrocyte development (Xiong et. al., 2015). The opposite occurred in experiments involving GRP78 knockout (KO); KO-GRP78 showed promotion of apoptotic activity in various cell lines, such as in pancreatic acinar cells (Liu et. al., 2014).

ER Stress and its Involvement with Apoptosis:

While GRP78 protein tries to keep a cell in a pro-survival/proliferative mode, prolonged ER stress will ultimately lead to the activation of apoptosis (programmed cell death) (Scull & Tabas, 2011). In fact, the major proteins involved in the previously discussed UPR play roles in the activation of multiple apoptotic pathways. One example is the phosphorylation of eIF2α induced by PERK phosphorylation. While a phosphorylated eIF2α attenuates the translation of a number of cellular proteins, it also promotes the translation of activating transcription factor 4 (ATF4) (Cullinan & Diehl, 2004). ATF4's key downstream target is the C/EBP-homologous

protein (CHOP), also known as growth arrest- and DNA damage-inducible gene 153
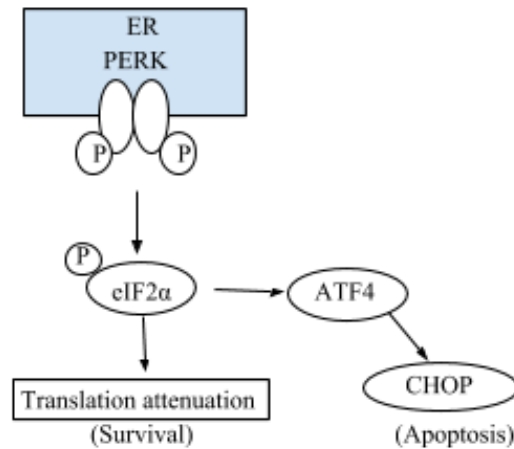(GADD153).

A largely accepted mechanism of CHOP is its role in the suppression of the B-cell lymphoma-2
(Bcl-2) protein, an anti-apoptotic protein. CHOP has also been shown to mediate the
upregulation of Bcl-2-like protein 11, commonly called "BIM", a pro-apoptotic protein
(Puthalakath et. al., 2007).

In addition to CHOP, caspase family proteins are used as markers when analyzing
apoptotic activity. These are cysteine proteases that maintain crucial links in both cell death and
inflammatory networks (McIlwain et. al., 2013). The apoptotic caspases can be divided into two
subgroups- initiator and executioner caspases. Caspases are generated as inactive zymogens, and
must undergo certain processes to become activated. All caspases share some common primary
structures, such as an amino-terminal prodomain and a carboxy-terminal protease domain. In the
case of initiator caspases, the prodomain tend to be longer as compared to that of executioner
caspases. Activation in initiator caspases occurs when adapter proteins interact with these longer

prodomains and encourages caspase dimerization. Executioner caspases, in turn, get cleaved by initiator caspases to become activated (Parrish et. al., 2013).

ER Stress and its Involvement with the PI3K/Akt Pathway and Proliferation:

Initiation of ER stress and the UPR has been shown to be a pro-survival and proliferative mechanism in multiple cell types. One pathway that has been shown to work in conjunction with the UPR is the phosphoinositide 3-kinases (PI3K) - Akt pathway (PI3K/Akt) (Kim et. al., 2014). Of the different classes of PI3K, it is the class IA that plays a role in regulating proliferation (Kim et. al., 2014). Initiation of the PI3K/Akt pathway begins once PI3K becomes activated. This involves stimulation from further upstream growth factors, such as insulin-like growth factor-I (IGF-I) (Povsic et. al., 2003). PI3K ultimately leads to the phosphorylation of Akt (p-Akt) at Ser473, Thr308, and Thr450, putting it in an active form (Hart & Vogt, 2011). p-Akt is involved with upregulation of pro-survival/proliferative pathways, and also contributes to the downregulation of proteins involved with apoptosis. A key target of Akt is the mammalian target of rapamycin (mTOR), a kinase involved with protein synthesis and promotion of other cell survival pathways. Akt also causes the activation of Bcl-2, a protein involved with the downregulation of apoptosis (Denduluri et. al., 2015).
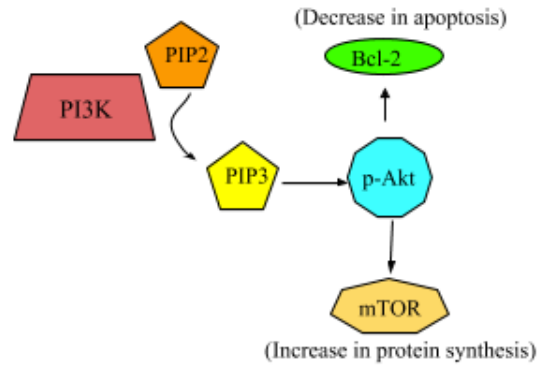
Figure 3: PI3K/Akt pathway

Experiments conducted on other mesenchymal cell types have shown the order of events in the PI3K/Akt and UPR pathways. A previous study showed a decrease in the ER stress response when lung fibroblasts were PI3K-inhibited, indicating that the PI3K/Akt pathway occurs upstream of the ER stress response (Hsu et. al., 2017).

Hypothesis:

The main hypothesis of this thesis is that the PI3K pathway plays a role with the initiation of the ER stress response in fibrostenotic CD ileal smooth muscle cells. Just as studies in other literature have shown the PI3K pathway being upstream of the UPR, we theorize to see similar results from experiments conducted in this thesis. While there may be many other pathways that facilitate the initiation of the UPR, we hypothesize that the PI3K pathway may be a significant driving force behind UPR activation in fibrostenotic CD ileal smooth muscle cells.

Methods and Materials

Cell Cultures:

Cells used for experiments were normal and fibrostenotic Crohn's disease-affected ileal smooth muscle cells. These cells were originally from tissue samples from VCU Health patients, undergoing intestinal surgical procedures. Cells were grown and maintained in 100mm petri dishes, and incubated at 37℃ in 10% CO_2. These cells were maintained in 10 ml of Dulbecco's Modified Eagle's Medium (DMEM), which was supplemented with 10% fetal bovine serum (FBS), 200 U/ml penicillin, 200 μg/ml streptomycin, 100 μg/ml gentamycin, and 2 μg/ml amphotericin B. The media was changed approximately every 2-3 days, and cells were rinsed with 5 ml/dish of phosphate-buffered saline (PBS) (Quality Biological™Catalog #114-056-101) before new media was given. Cells that needed to undergo serum starvation were given DMEM supplemented with 200 U/ml penicillin, 200 μg/ml streptomycin, 100 μg/ml gentamycin, and 2 μg/ml amphotericin B.

When splitting cells, old media was aspirated out of the dish and cells were rinsed with PBS. 2 ml of trypsin-EDTA (0.25%) was put into the petri dish and incubated for approximately 15 minutes at 37℃ in 10% CO_2. 8 ml of DMEM (with supplements listed above, including serum) was then given to neutralize the trypsin-EDTA. Cells were then spun down for 3 minutes, twice, at 23℃ and 1000 RPM, using an Eppendorf 5810 R centrifuge. Fresh media (with supplements listed above, including serum) was used to break up the pellet, and cells were then dispensed into the appropriate vessels.

Preparation of Cell Lysates:

The media in the wells of the 6-well plates were aspirated, and cells were rinsed with 1 ml/well of ice-cold PBS. PBS was then aspirated and cells were treated with lysis buffer. Lysis buffer was prepared using a T-PER™ Tissue Protein Extraction Reagent (ThermoFisher SCIENTIFIC Catalog #78510) and a protease cocktail inhibitor (QIAGEN Mat. No. 1065907). The inhibitor cocktail-T-PER™ solution was prepared in a 1:100 dilution. 100 µl of lysis buffer was aliquoted into each well, and the 6-well plates were put on ice for approximately 15 minutes.

After the 15-minute time period, cells were scraped off each well using a cold cell scraper. Contents of each well were then placed into microcentrifuge tubes, which were also stored on ice to keep cold. The microcentrifuge tubes were centrifuged for 10 minutes at 12,000 RPM using an Eppendorf 5417R centrifuge. Supernatants of the samples were then transferred into new microcentrifuge tubes, also kept on ice. These samples were to be stored in a -20℃ freezer.

Preparing Lysates to Load into Gel:

A β-mercaptoethanol (BME) and 2x Laemmli solution (BIO-RAD catalog #161-0737) was prepared, using a 1:20 dilution (ex. 950 µl of 2x Laemmli, and 50 µl of β-mercaptoethanol). Each sample's original lysates was treated with this prepared BME/2x Laemmli solution in a 1:1 dilution (ex. 50 µl of lysate and 50 µl of BME/2x Laemmli solution). These newly prepared samples were then heated at 100℃ for 10 minutes. After heating, samples were spun down at 4℃, 12,000 RPM, for 15 minutes, using an Eppendorf 5417R centrifuge. Samples were then stored at -20℃.

Bicinchoninic Acid (BCA) Assay for Protein Quantification:

To measure the protein concentrations of newly obtained cell lysates, BCA assay was conducted. An Abcam quantification kit (Abcam ab102536) was used. The protocol provided by Abcam was used when conducting this assay, and each standard/sample was prepared in duplicates in a 96-well plate. The stock solution used for preparing the 8 standards was diluted in T-PER™ reagent. Original cell lysates were also diluted in T-PER™ reagent, at a 1:10 dilution per sample. When the Abcam-instructed Working Solution was aliquoted into each well containing a standard/sample, the 96-well plate was incubated at 37℃ in 10% CO_2 for approximately 80 minutes. At the end of the incubation period, a spectrophotometer (VICTOR 3V™ 1420 Multilabel Counter) was used to measure absorbances following Abcam protocol.

Determining Protein Concentrations and Volume to Load into Gel:

The absorbance readings of each standard/sample were transferred into Microsoft Excel for further analysis. Averages of each reading were taken, and then the mean value of the 0 μg/μl standard absorbance reading was subtracted from all other collected mean value absorbance readings. Newly calculated absorbance readings of the standards were then used to generate a scatter plot, with the absorbances on the x-axis, and the concentrations of the 8 standards on the y-axis. A trendline line was then added to the scatter plot, displaying the regression line equation.

To determine the protein concentration of each sample, the mean absorbance value of each sample (with the 0 μg/μl mean value subtracted) was used as the x-value of the generated regression line. The newly obtained y-value was the protein concentration of that sample, in μg/μl. Depending on the protein amount to load into each well of a gel, this amount was divided

by the newly calculated protein concentration. This value was then multiplied by 2 (to take into account the 2x Laemmli solution used when preparing samples to be run in the gel). The final value is the volume, in μl, to load into the gel.

SDS-PAGE:

10% gels were either prepared in the lab or 10% precast gels were used, when running SDS-PAGE experiments. When preparing a gel, resolving gel was prepared first, followed by the stacking gel. Resolving gel was prepared using 3.042 ml double-distilled water, 2.5 ml of pH 8.8 Tris-HCl (1.5M stock concentration), 1.0 ml of 1% sodium dodecyl sulfate (SDS), 3.35 ml of 30% acrylamide solution, 100 μl of 10% ammonium persulfate (APS), and 8 μl of tetramethylethylenediamine (TEMED). Solution was poured into cast to solidify, before adding stacking gel.

The stacking gel required 1.4 ml distilled and double-distilled water, 125 μl of pH 6.8 Tris-HCl (1.0M stock concentration), 200 μl of 1% SDS, 335 μl of 30% acrylamide solution, 20 μl of 10% APS solution, and 4 μl of TEMED. This stacking solution was added into the cast after resolving gel was solidified. Immediately after pouring stacking gel into the cast, a 15-well comb (26 μl volume/well) was added. After stacking gel solidified, newly prepared gels were stored at 4℃, wrapped in a wet paper towel. These gels were used within a week of being prepared.

Precasted 10% gels used for experiments were made by BIO-RAD (catalog #456-8033).

Running buffers consisted of 3 g tris base, 1 g SDS, 14.4 g glycine, and 1000 ml double-distilled water. Transfer buffers consisted of 3.03 g tris base, 14.4 g glycine, 200 ml methanol, and 800 ml double-distilled water. Transfer buffers were stored in ice-cold conditions at all times.

SDS-PAGE gel was loaded into the chamber and filled with running buffer. Once samples were loaded, gels were run at 180 volts for 50-60 minutes, at room temperature.

All gels had their protein contents transferred to polyvinylidene difluoride (PVDF) membranes upon completion of running. PVDF membranes were immersed in methanol prior to be loaded into the transfer apparatus. Once the gel and PVDF membrane were loaded into the transfer cassette, cassette was placed into the transfer apparatus. Ice-cold transfer buffer was then poured into the apparatus. The apparatus was kept in ice-cold conditions throughout the transfer process. Once the transfer apparatus was completely set up, the transfer was run at 350 milliamperes for 75 minutes.

Membranes were treated with blocking buffer for one hour with gentle agitation, before antibody solutions were given. This buffer consisted of tris-buffered saline with tween 20 (TBST) (1%) with 5% composition of nonfat dry milk (BIO-RAD #1706404). Primary (1°) and secondary (2°) antibody solutions were prepared as instructed in the antibody datasheets, adjusting the dilutions as needed. PVDF membranes treated with 1° antibodies were kept at 4℃ overnight, prior to being treated with 2° antibodies. Before a PVDF membrane was treated with the required 2° antibody solution, the membrane was rinsed three times (10 minutes each) with TBST. Membranes were then incubated with 2° antibody solution at room temperature, with gentle agitation, for one hour. Upon completion of incubation with 2° antibody solution, the membrane was rinsed three times (10 minutes each) with TBST. Membrane was then treated with Pierce™

ECL Plus Western Blotting Substrate (ThermoFisher SCIENTIFIC Catalog #32132) for one minute.

Membrane would then be exposed to a blank x-ray film (30 minutes to 3 hours), followed by development of film using an appropriate film developer.

Antibodies Used:

GRP78 (A-10) mouse monoclonal IgM (Santa Cruz Biotechnology, sc-376768), 1:200; Monoclonal **anti-β-actin** antibody produced in mouse (Sigma-Aldrich, A5441), 1:2,000; **Cleaved Caspase-3** (Asp175) Antibody (Cell Signaling Technology, 9661S), 1:500; **GADD 153/CHOP** (Santa Cruz Biotechnology, sc-575), 1:250; **PCNA** (PC10) Mouse monoclonal antibody (Cell Signaling Technology, 2586S), 1:1,500; **Phospho-Akt** (Ser473) rabbit monoclonal antibody (Cell Signaling Technology, 4060S), 1:1,500-300; **Cyclin D1** rabbit monoclonal antibody (Cell Signaling Technology, 2978S), 1:500; **Cell Cycle and Apoptosis WB Cocktail** (pCdk/pHH3/Actin/PARP) (Abcam, ab139417), 1:250 for primary antibody solution and 1:2,500 for secondary antibody solution; **Goat anti-Mouse IgG antibody** (ThermoFisher, 32430), 1:2,000; **Anti-rabbit IgG antibody** (Cell Signaling Technology, 7074S), 1:2,000

Confirming Tunicamycin's Efficacy in Inducing ER Stress via GRP78 Analysis:
CD-affected ileal SMC were grown in 6-well plates, until they reached approximately 90% confluency. Cells underwent serum starvation once the desired confluency was reached. Cells were then treated with 5 μg/ml of tunicamycin (diluted with serum-free media) for 0, 6, 12, and

24 hours. Tunicamycin inhibits enzymes involved with N-linked glycosylation in protein biosynthesis, thus causing an accumulation of improperly folded proteins. Lysates of these samples were then prepared for analysis of GRP78 expression via Western blotting. HeLa cell lysates were loaded into the gel to serve as a positive control. After the PVDF membranes were blotted for GRP78, membranes were then blotted for β-actin as a loading control.

Analysis of CD-affected and Normal Ileal SMC for Apoptosis:

CD-affected ileal SMC were grown in 6-well plates until ~90% confluency was reached. Cells were treated with 5 μg/ml of tunicamycin (diluted with serum-free media) for 0, 24, 48, and 72 hours. Each well underwent overnight serum starvation before tunicamycin was administered. Lysates of these samples were prepared at the end of the treatment course, and analyzed for apoptotic proteins via Western blotting. The newly prepared PVDF membranes were blotted using the Abcam apoptosis cocktail, CHOP, and cleaved caspase-3. Membranes were then blotted for β-actin as a loading control. Bortezomib-treated H929 cell lysates were loaded into the gel as a positive control.

Normal ileal SMC were grown in 6-well plates until ~90% confluency was reached. Two of the wells served as controls and did not undergo serum starvation or treatment. The next two wells underwent serum starvation for 24 hours, with one of the wells also receiving a 5 μg/ml dosage of tunicamycin. The same was done for 48 and 72 hours. After the 72-hour period, cells were prepared into lysates and analyzed via Western blotting, using the Abcam apoptosis cocktail. Bortezomib-treated H929 cell lysates were loaded into the gel as a positive control. Membrane was then blotted for β-actin as a loading control.

Analysis of CD-affected Ileal SMC for Proliferation:

CD-affected ileal SMC were split into 6-well plates and grown to reach ~90-100% confluency. Once desired confluency was reached, cells underwent overnight serum starvation before a 5 µg/ml dosage of tunicamycin was administered. Time course for this assay was 0 (control), 24, 48, and 72 hours. Samples were then prepared into lysates upon completion of the time course. These samples were analyzed for expression of proliferating cell nuclear antigen (PCNA), via Western blotting.

Other CD-affected ileal SMC samples underwent similar treatment, except for a time course of 0, 6, 12, and 24 hours. These samples were then analyzed for cyclin-D1, via Western blotting.

Hela lysates were loaded into the gel to serve as a positive control for both PCNA and cyclin-D1 analyses, and blotting for β-actin was conducted as a loading control.

Measuring Effects of PI3K Inhibition on GRP78 Expression:

CD-affected ileal SMC were split into 6-well plates and grown to reach ~90-100% confluency. Cells underwent overnight serum starvation before any treatment was given. The time course of treatment for this assay was 0, 6, 12, and 24 hours. LY294002 was the PI3K inhibitor used for this experiment. LY294002 works by blocking the ATP binding site on the PI3K protein. Cells were pre-incubated with 10 µM of LY294002 for 30 minutes, followed by a 5 µg/ml dosage of tunicamycin. On the other plate, cells were only treated with tunicamycin at the same time points. Samples were then prepared into lysates and analyzed for GRP78 expression, via Western

blotting. HeLa lysates were loaded into the gel as a positive control, and blotting for β-actin was conducted as a loading control.

Densitometry:

ImageJ software was used to perform densitometric analyses on Western blots conducted. All images were converted to 32-bit in the ImageJ software before values were obtained. Blots were normalized to β-actin.

Results

Evidence of ER stress response in Crohn's disease-affected cells:

GRP78 protein is often used as a marker of the ER stress response. To illustrate that Crohn's disease-affected intestinal smooth muscle cells were under ER stress, Western blotting was conducted on multiple CD-affected cell culture samples to look for GRP78 expression. These samples were grown in 6-well dishes, and certain wells were dedicated to cells that were not to undergo any treatment (i.e. tunicamycin exposure). These cells served as the control for assays, and protein expression in these cells are at the basal level. Prior to conducting the assay, we had hypothesized that intestinal smooth muscle cells affected by Crohn's disease are in a state of ER stress. Figure 4 shows the results from Western blots conducted on multiple samples, illustrating the presence of GRP78 protein at the basal level. These findings confirmed that the ER stress response is active in CD-affected ileal SMC, validating the hypothesis.
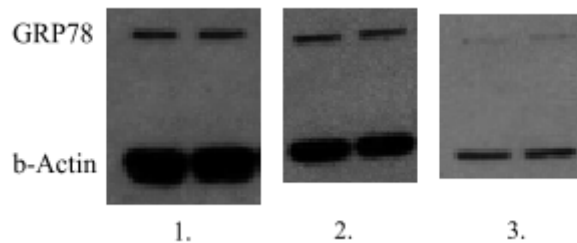

Figure 4: GRP78 expression from three different samples of CD-affected intestinal SMC without any treatment

Tunicamycin is an ER stress inducer in CD-affected intestinal SMC:

With activation of the ER stress response evident in CD-affected intestinal SMC, the next step involved determining an adequate chemical and dosage to enhance the ER stress response even further. Tunicamycin (TM) was used as the ER stress inducer for experiments conducted in this thesis, at a final concentration of 5 µg/mL. Cells were exposed to TM at different time points

(0, 6, 12, and 24 hours) and Western blotting was done to measure GRP78 expression over the time course. At least three different samples of CD-affected ileal smooth muscle cells underwent this treatment, and all results successfully showed an increase in GRP78 expression as TM time exposure increased. This confirmed that TM at 5 µg/mL can increase the ER stress response over time. Figure 5 shows the results taken from one sample.
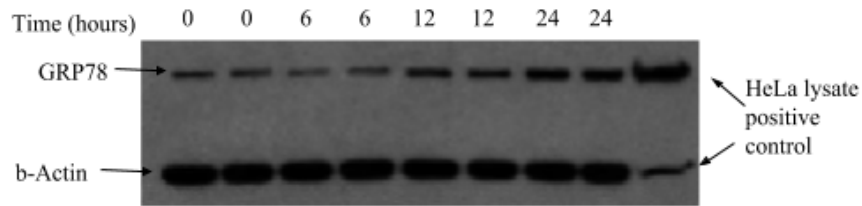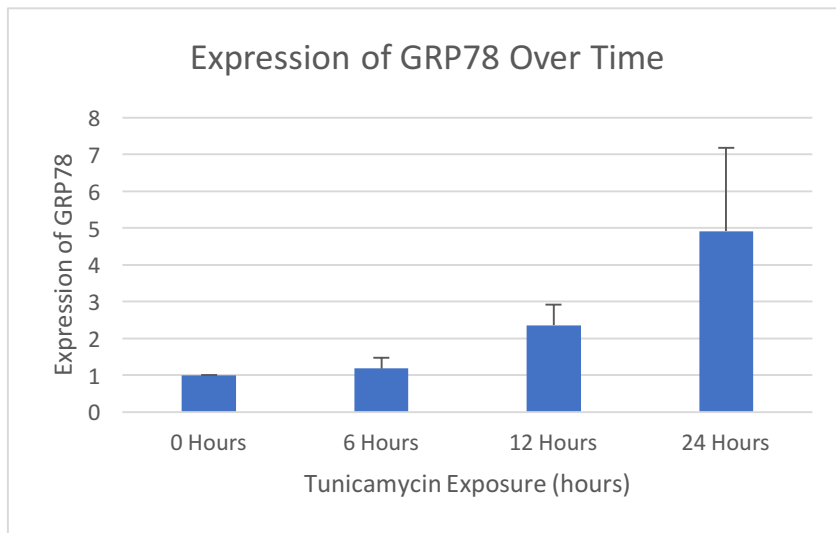

Figure 5: Expression of GRP78 protein over 24-hour time period in the presence of 5 µg/mL tunicamycin

Table 1: Densitometry results of GRP78 expression in CD-affected cells treated with 5 µg/mL tunicamycin. n=3

Apoptotic activity:

For cells that are in a state of chronic ER stress and are unable to rectify unfolded proteins, activation of apoptotic pathways may result. In order to see if these CD-affected cells had high apoptotic activity, Western blotting was conducted to detect certain apoptotic protein markers. Initially, Western blotting was done to look for expression of cleaved caspase-3 and CHOP, on CD-affected cells that were treated with 5 μg/mL of tunicamycin for 0, 24, 48, and 72 hours. These proteins were not detected, which may suggest that endogenous levels apoptotic activity were low.

CD-affected intestinal smooth muscle cells treated with tunicamycin for 0, 24, 48, and 72 hours were then analyzed via Western blotting, using an Abcam apoptosis cocktail (ab139417). Major proteins that this cocktail targets includes cleaved poly [ADP-ribose] polymerase 1 (PARP) and phosphorylated histone H3. Both proteins increase in expression when cells undergo apoptosis. The results of this Western blot did not show any expression of these proteins, further pointing to the possibility that these cells are in a more proliferative than apoptotic state.
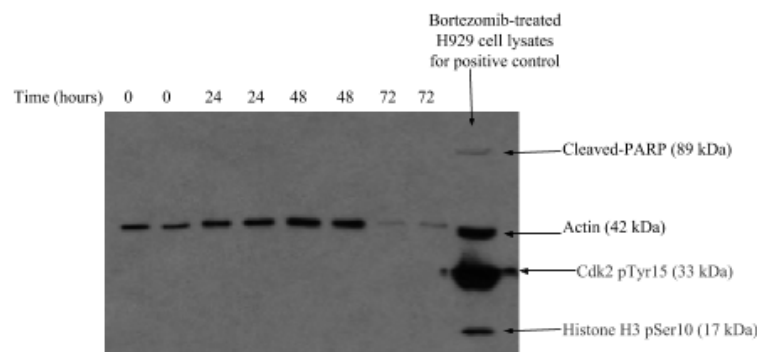

Figure 6: Western blot of samples treated with tunicamycin (5 μg/mL for 0, 24, 48, and 72 hours) using apoptosis cocktail

Normal margin ileal smooth muscle cells were then grown in 6-well dishes and underwent similar treatment. However, during this assay we also attempted to see if serum

starvation, alone, would be enough to upregulate apoptotic pathways. These normal margin cells were either serum starved for 0, 24, 48, and 72 hours, or serum-starved and received a dosage of 5 μg/mL tunicamycin at the same time points. We had hypothesized to see an increase in apoptotic proteins in the cells that underwent serum starvation and tunicamycin exposure, as compared to cells that only underwent serum starvation. However, except for actin (loading control), no apoptotic proteins were detected using the Abcam apoptotic cocktail.

Proliferative activity:

With previous data not showing any pronounced apoptotic activity, we explored the possibility that the CD-affected SMC are in a pro-survival/proliferative state. To test for proliferative activity, Western blotting was conducted on CD-affected SMC treated with 5 μg/mL tunicamycin for 0, 24, 48, and 72 hours. Initially, samples were blotted for PCNA. Because we were not able to detect any PCNA expression from our samples, we decided to measure proliferation via cyclin-D1 expression. Time points for this assay were 0, 6, 12, and 24 hours. Cyclin-D1 plays an important role in cell cycle progression, promoting the progression through the G_1-S in various cell types. There is often overexpression of cyclin-D1 in a number of cancers, such as breast and colon cancer (Fu et. al., 2004). Because the cells showed an increase in ER stress as tunicamycin exposure time increased, we had hypothesized to see an increase in proliferative activity (increased expression of cyclin-D1). After conducting the assay on three different samples our hypothesis showed to be incorrect. Graphical analysis of values obtained

via densitometry showed a significant decrease ($p < 0.05$) in cyclin-D1 expression at the 24-hour mark. This may indicate a shift away from a pro-survival/proliferative state.
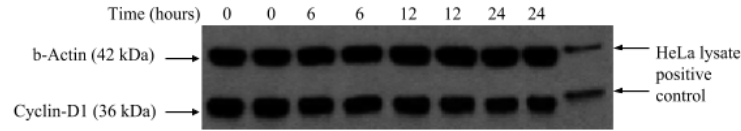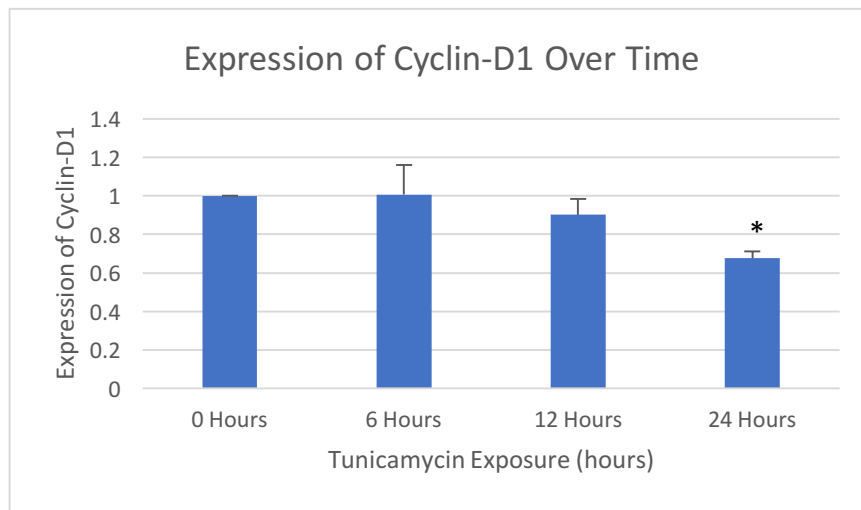

Figure 7: Western blot illustrating cyclin-D1 expression from a CD-affected SMC sample, treated with 5 µg/mL tunicamycin for 0, 6, 12, and 24 hours.

Table 2: Graphical representation of cyclin-D1 expression in a 24-hour period of CD-affected intestinal SMC. Samples were treated with 5 µg/mL tunicamycin (n=3). * $p < 0.05$, comparing to 0-hours samples

Activation of the PI3K/Akt pathway:

The PI3K/Akt pathway plays an important role in cell survival and proliferative activities. Downstream results of PI3K activation include increased protein synthesis and glucose metabolism, and decrease in apoptotic activity. With the expression of cyclin-D1 evident in the CD-affected intestinal SMC, we wanted to see if the PI3K/Akt pathway was also active. This was conducted by Western blotting for phosphorylated Akt (p-Akt Ser473). Analysis was done on CD-affected intestinal SMC. The time points of cells that received 5 µg/mL of tunicamycin

were 10, 20, 40, 60, and 120 minutes. We had hypothesized that there would be an increase in expression of p-Akt (Ser473) as the time course increased. Results showed presence of p-Akt (Ser473) from all time points, including the 0-minute control. However, a steady increase in p-Akt (Ser473) expression was not observed, inconsistent with our hypothesis. It is important to note that attempts were made to repeat this assay two more times. However, we were not able to successfully obtain results. We attempted stripping the membranes and reblotting for total-Akt, and then again for b-actin. But we were not able to obtain any signal.
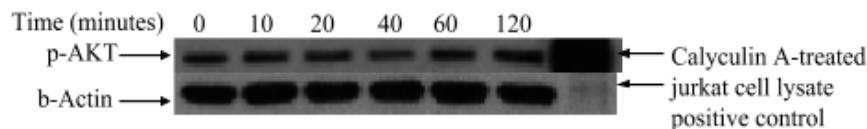

Figure 8: Western blot displaying results of p-Akt (Ser473) expression in CD-affected intestinal SMC's. Sample was treated with 5 µg/mL tunicamycin for 0, 10, 20, 40, 60, and 120 minutes.

Inhibition of PI3K and its effect on the ER stress response:

With activation of Akt evident in CD-affected intestinal SMC's, we wanted to explore the relationship between the PI3K/Akt pathway and the ER stress response. Multiple literature have shown evidence that the PI3K/Akt pathway occurs upstream of GRP78 expression, and we attempted to see if this was the case in CD-affected intestinal SMC. A sample of CD-affected intestinal SMC was grown in 6-well plates, and treatments involved the usage of tunicamycin (5 µg/mL) and LY294002 (10 µM), a PI3K inhibitor. Cells were treated with either just tunicamycin or tunicamycin and LY294002, for 0, 6, 12, and 24 hours. Western blotting was performed to see the expression of GRP78 in these cells. With past literature showing the PI3K/Akt pathway occurring upstream of GRP78 expression, we had hypothesized the same in this sample. We expected to see a decrease in GRP78 expression for cells treated with

tunicamycin and LY294002, as compared to cells treated with tunicamycin alone, at any given
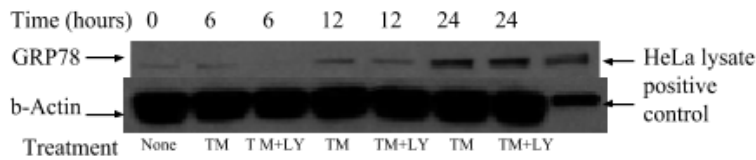
time point.

Figure 9: Western blot results of a CD-affected intestinal SMC sample treated with TM and TM+LY294002. Cells requiring tunicamycin were given a dose of 5 µg/mL and cells requiring LY294002 were given a dose of 10 µM. HeLa lysate was loaded into the gel as a positive control for GRP78 expression.

Our results showed an overall increase in GRP78 expression over the 24-hour period.

However, there was a decrease shown in the cells treated with tunicamycin + LY294002, as

compared to cells treated with tunicamycin alone, at a given time point. There was also a very

noticeable decrease in GRP78 expression in cells treated with tunicamycin + LY294002 at the 6-

hour time point the first time this assay was conducted. This apparent difference could indicate

blockage of TM induced up-regulation of GRP78.

Table 3: Graphical representation of Western blot results as shown in Figure 9
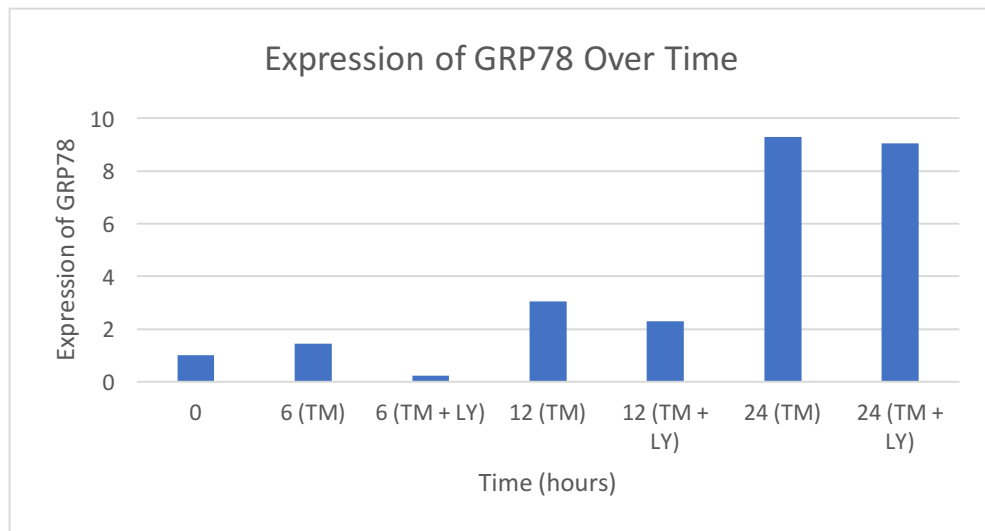
It is important to note that this assay was repeated two more times on two different samples. In

the second sample, bands for GRP78 were obtained. However, a consistent b-actin blot was not

achieved. Densitometry was conducted on the GRP78 bands, but could not be normalized to the

b-actin. Analysis showed a decrease in GRP78 in cells treated with TM+LY. There was an

exception at 12-hour time point, where densitometric analysis showed a slight increase in GRP78

expression. In the third sample, some GRP78 signals were detected. However, it was difficult

locating all the time points' bands. b-Actin signal was not successfully obtained.
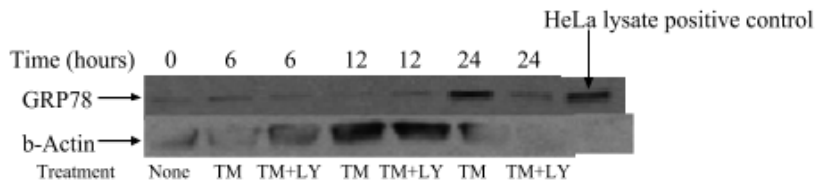
Figure 10: Western blot results from the second attempt of TM and TM+LY assay. Cells requiring tunicamycin were given a dose of 5 µg/mL and cells requiring LY294002 were given a dose of 10 µM. HeLa lysate was loaded into the gel as a positive control for GRP78 expression.

Table 4: Graphical representation of Western blot results shown in Figure 10. Normalization to b-actin was not possible due to inconsistent b-actin signaling from the blot.
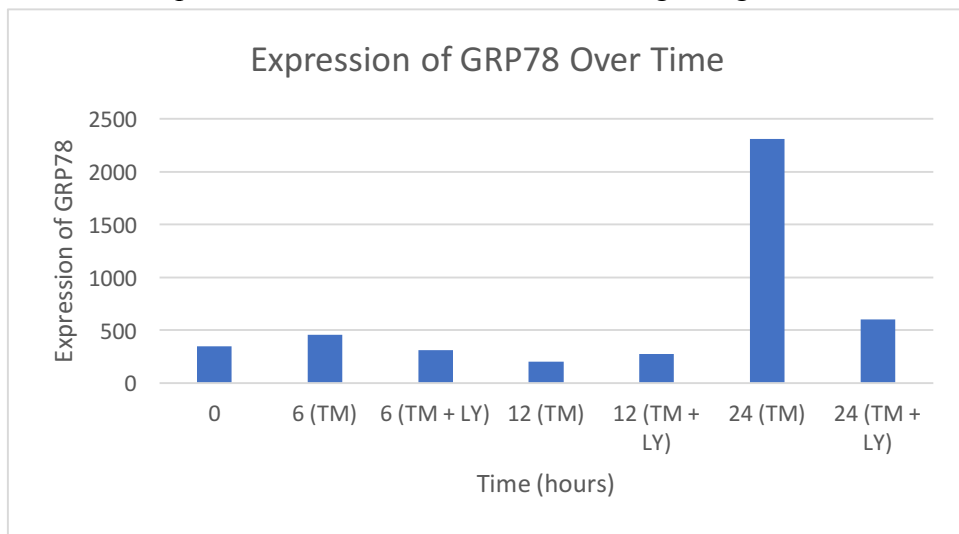
Discussion

Apoptotic Assays:

Before analyzing CD-affected ileal SMC for apoptotic markers, we had hypothesized that we would not see elevated levels of apoptotic proteins. When cells are initially in a state of ER stress, signaling pathways involved with cell survival are upregulated. This is partly mediated by the dissociation of GRP78 from the UPR proteins, allowing for further activation of survival proteins. The results from apoptotic assays conducted on fibrostenotic ileal cells came as expected, since apoptotic markers were not detected.

When conducting the apoptotic assay on the normal margin intestinal SMC, we had hypothesized to see elevated levels of apoptotic markers, as the serum-starvation and tunicamycin exposure time increased. After analyzing our Western blot data using the Abcam apoptosis cocktail, we were not able to see any presence of apoptotic proteins. While this may discredit our hypothesis, it cannot be said with 100% certainty that apoptotic pathways were not active. There are a number of pathways and proteins related to apoptosis activation, aside from the proteins we attempted to detect. Organelles such as the mitochondria also play a role in the onset of apoptosis. Therefore, we do believe that further studies need to be done to detect for signs of cell-death activity. One example of an assay that can be conducted in the future includes the annexin V assay, which helps detect apoptotic activity in its early stage. Another apoptotic assay is the terminal deoxynucleoidyl transferase dUTP nick-end labeling (TUNEL) assay. This assay is useful in detecting late-stage apoptotic activity.

Proliferative Assays:

It was hypothesized that there would be signals of proliferation activity in the CD-affected ileal SMC. It was also hypothesized that as the ER stress response increased (by increase in tunicamycin exposure time) we would see an increase in proliferative protein. However, our hypothesis was partially disproven based on the results of cyclin-D1 expression. We were able to see cyclin-D1 expression at the basal levels of CD-affected ileal SMC, and the expression was fairly consistent throughout the time points in the 24-hour period. However, there was a significant decrease ($p<0.05$) at the 24-hour time point. This significant decrease could potentially indicate a point where the cells started to downregulate their proliferative activity.

PI3K Activation:

With evidence of proliferative activity present in the CD-affected ileal SMC's (by cyclin-D1 expression), it was important to search for a pathway linked to proliferation. The PI3K pathway is an important pathway in cell proliferation, and downstream results of PI3K activation include mTOR activation, increase in protein synthesis, and downregulation of proteins involved with apoptosis. Our assay successfully showed that the PI3K pathway was active in the CD-affected ileal SMC. This was due to the expression of p-Akt (Ser473), which was even present at the basal level.

Effect of PI3K Inhibition on the ER Stress Response:

Using LY294002 as the PI3K inhibitor, we attempted to show a correlation between the PI3K pathway and the ER stress response. We had hypothesized to see a decrease in GRP78 expression in cells treated with both LY294002 and tunicamycin, as compared to cells treated with only tunicamycin. Overall the results did show a decrease in GRP78 expression from cells

treated with LY294002 and tunicamycin (with one exception in the 12-hour time point for the second sample). For a period of time, the PI3K pathway could be the major driving force behind the ER stress response. But as time increases, other pathways may come into play in helping facilitate the ER stress response. This may explain why there was still an overall increase in GRP78 expression as the time point increased.

Conclusion:

The overall results from this thesis could indicate that the PI3K pathway occurs upstream of the ER stress response in CD-affected ileal SMC, and that it may have a significant driving force behind the ER stress response for a certain period of time. This is consistent with the thesis's hypothesis. However, there is still a lot more experimentation that needs to be done that can improve the quality of results given in this thesis. One example is conducting more tests to detect apoptosis in normal margin cells. This is because we would expect to see normal margin cells to exhibit apoptotic behavior more readily when compared to CD-affected cells. Just because apoptotic markers were not detected from the assays conducted, does not mean that the cells are in an anti-apoptotic state.

If further experimentation continues to show a strong link between the PI3K pathway and ER stress, then the mechanisms of these pathways may serve as a target for therapeutic development for the treatment of fibrostenotic Crohn's disease.

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Vita

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      Prashant then completed the Premedical Graduate Certificate Program through the Virginia Commonwealth University's School of Medicine, Richmond, Virginia in May of 2017. He then continued his education to obtain his Master of Science in Physiology and Biophysics through VCU's School of Medicine. He will begin medical school at the West Virginia School of Osteopathic Medicine in Summer of 2018.