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Effect Of Endoplasmic Reticulum Stress On Vascular Smooth Muscle Cells And Its Regulation Of Sm22α

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EFFECT OF ENDOPLASMIC RETICULUM STRESS ON VASCULAR SMOOTH MUSCLE CELLS AND ITS REGULATION OF SM22*α*

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Approved By:

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Advisor Date

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DEDICATION

I would like to dedicate this dissertation to my family: my husband for encouraging and supporting me at every step of the way, my parents for their support and motivation and to my son Adhvith.

ACKNOWLEDGMENTS

I would like to thank my advisor Dr. Li Li for her guidance and support. I appreciate the freedom she gave me to explore various aspects of the project and for her patience during times when things did not work. I would like to thank all the present and previous lab members: Dr. Xiaohua Dai- for practically teaching me everything about managing a lab and for making my journey in the lab a pleasant one, Dr. Jingye Fang, Zhao Yang and all the summer students and volunteers.

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PREFACE

Vascular smooth muscle cells(VSMC) are the major cell type in the tunica media of the aorta. The many layers of the smooth muscle cells intertwined in the extracellular matrix provide structural integrity to the vessel wall. Smooth muscle cells maintain the caliber of the blood vessel by maintaining a contractile phenotype and secreting the extracellular matrix under normal physiological conditions. These cells are special, as they are not terminally differentiated. Under injury or cellular stress condition, they can switch from the contractile phenotype to a proliferative phenotype referred to as the synthetic phenotype. This switch, called the phenotypic modulation is accompanied by loss of contractile markers like smooth muscle α actin, myosin heavy chain and SM22α and increased deposition of the extracellular matrix components. This repair process often turns pathogenic and leads to apoptosis of the smooth muscle cells.

Though many different signaling pathways have been elucidated, there still exists a knowledge gaps in linking the various signaling processes to the phenotypic modulation, with endoplasmic reticulum stress being a comparatively new player in the field of cardiovascular diseases. Our preliminary data showed that *Sm22α* was one of the most downregulated genes in this stress response. SM22α is also known to be downregulated in various cardiovascular diseases like aneurysm and atherosclerosis, which supports the need to study its regulation in detail under the stress conditions. We studied ER stress and SM22 in aortic aneurysm background as it includes atherosclerosis as a pathological manifestation and downregulation of SM22α. We used *in vivo, ex vivo* and *in vitro* techniques to study SM22's protective role and the mechanism under ER stress.

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CHAPTER 1: INTRODUCTION

1. Aortic aneurysm

Aorta is the largest blood vessel in the body, carrying oxygenated blood. Aneurysms occurring in the aorta are called aortic aneurysms. Aortic aneurysm and dissections are a major cause for mortality, responsible for nearly 14,000 deaths in the US every year[1]. An aneurysm is a permanent focal dilatation of an artery to 1.5 times its normal diameter[2]. Depending on the region of the aorta that it occurs, they are identified as thoracic (TAA) or abdominal aneurysm (AAA). AAA represents most of the aneurysms that occur. TAA are rare and have a strong familial inheritance pattern. Syndromes associated with aneurysm include Marfan syndrome(MFS), familial thoracic aortic aneurysms and dissections (TAAD), Ehlers-Danlos syndrome (EDS), Loeys-Dietz syndrome (LDS), bicuspid aortic valve (BAV), and neurofibromatosis type 1 (NF1)[3]. The pathophysiology of aneurysms is defined by degradation of the vessel vasculature and loss of smooth muscle cells. The role of matrix metalloproteinases in the extracellular matrix degradation is well studied [4-6]. The weakened vessel is hence predisposed to dilation and rupture. The loss of smooth muscle cells in the aneurysmal tissue is attributed to apoptosis probably from elevated levels of ROS and DNA damage[7].The smooth muscle cells also repress the expression of the contractile genes and undergo a change in phenotype. This phenotypic switch might be the deciding factor for the cell survival. Mechanisms associated with this switch may hold the key to inhibit smooth muscle loss in the aneurysm tissue.

2. Vascular smooth muscle cells in cardiovascular diseases and the role of phenotypic modulation.

Vascular smooth muscle cells (VSMCs) are specialized cells that maintain the balance between vasoconstriction/vasodilation for the smooth functioning of the blood flow distribution. SMCs in the adult blood vessel possess a low rate of proliferation and express a unique repertory of contractile genes and signaling molecules that confers the ability to maintain the contractile phenotype[8]. VSMCs possess the ability to change their contractile phenotype to a synthetic phenotype. This switch is called phenotypic

modulation. The synthetic phenotype is characterized by an increase in proliferation, migration and increased synthesis of extracellular matrix[9]. The cells respond to a wide range of vasoactive stimuli by switching phenotype during pathology. Teleologically, this plasticity was probably a survival response to injury and stress. Under most pathological conditions though, this plasticity predisposes the cells to detrimental signaling and progression of vascular diseases. VSMC phenotypic modulation is a defining feature of many cardiovascular pathologies like aneurysms[10-12] and atherosclerosis[13-15]. One of the hallmark features of the synthetic or dedifferentiated phenotype is the loss of contractile markers like smooth muscle α actin, myosin heavy chain and SM22α [9]. Advances in the clinical and animal model studies have identified the synthetic phenotype in a wide range of pathologies like arteriopathy of diabetes and chronic kidney diseases[16-18]. The dedifferentiated cells depending on the environment may acquire additional phenotypic changes making them inflammatory[19, 20], adipogenic[16], osteogenic[16, 21] or osteochondrogenic[16, 21]. This pathogenic phenotype comes as a drawback for the high plasticity that these cells possess.

Research by previous lab members have helped establish the fact that the downregulation of the contractile gene expression plays an active role in the pushing the cells towards the pathogenic phenotype[22, 23]. My work aims at studying the regulation of one such contractile gene *Sm22*α during endoplasmic reticulum(ER) stress response**(Fig.1)**.

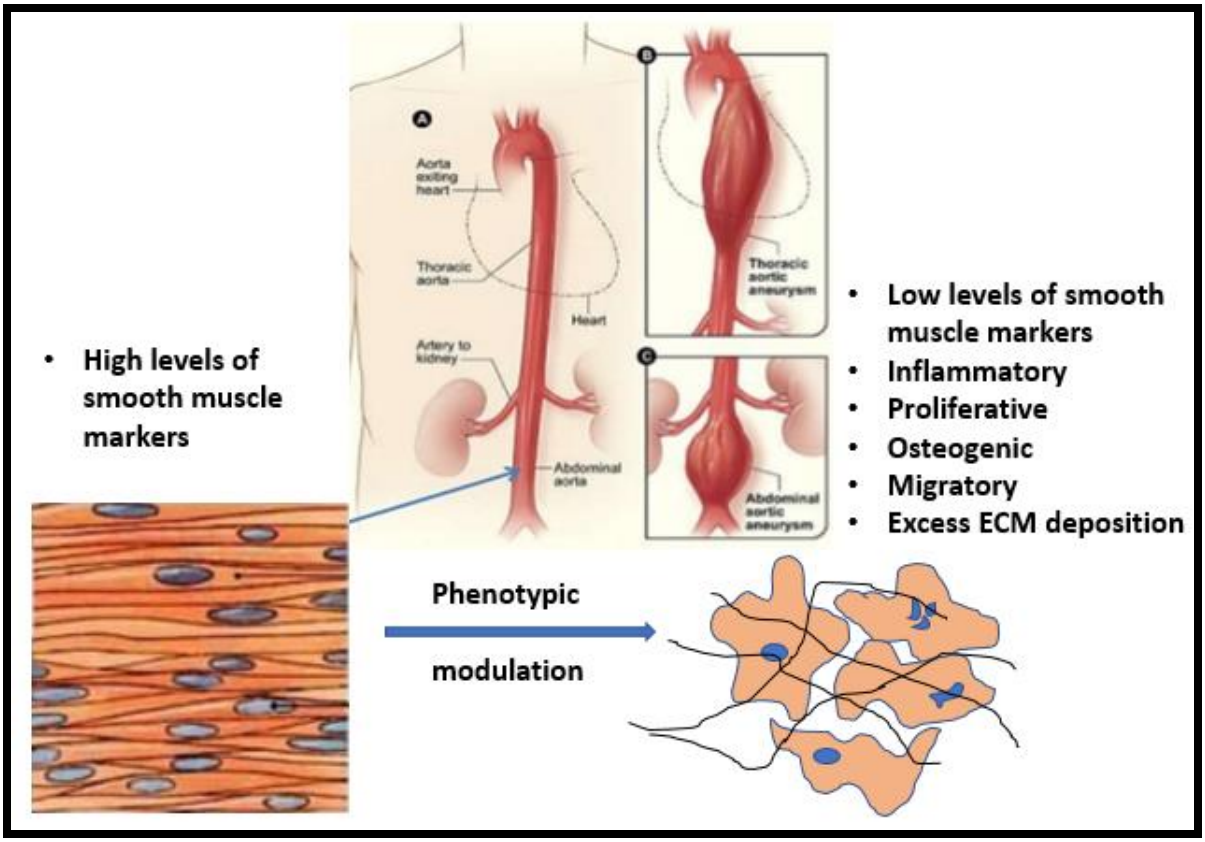


Figure 1 Vascular smooth muscle cells undergo phenotypic modulation in the cardiovascular diseases including aortic aneurysm.Vascular smooth muscle cells(VSMCs) are characterized by high expression of contractile marker genes.VSMCs are not terminally differentiated and undergo phenotypic modulation under many pathological conditions.The synthetic phenotype is marked by reduced expression of the smooth muscle markers and increased expression of inflammatory and osteogenic marker genes. The cells transform from spindle shaped to a cobblestone morphology.The cells also becomes more proliferative and migratory.(https://www.nhlbi.nih.gov/health/health-topics/topics/arm/types)

3. Cytoskeleton, Extracellular matrix and aortic aneurysm

In addition to their contractile capacity, VSMCs possess important secretory properties that ensure the synthesis and repair of various ECM components that regulate the structure of the vascular wall (collagen, elastin, fibrillin, fibronectin). Importantly, VSMCs can interact directly with the different ECM components through cell-surface integrin receptors, G-protein-coupled receptors and the discoidin domain receptor family. The fundamental unit of the cytoskeleton--cell receptor--ECM complex regulates

the function of the aortic wall. Indeed, VSMCs can transform mechanical stimuli into biological responses (also known as mechanotransduction), leading to intracellular responses (cytoskeletal rearrangement and stress fiber alignment) and extracellular changes (synthesis, alignment and repair of ECM components) that, in turn, can communicate directly with the cell.[24-27]. Mechanical stimuli are transmitted via integrins from the elastin to the intracellular compartment, which can lead to activation of biochemical signal activation and contraction of the contractile unit. The extracellular matrix has an important and complex function in maintenance of the integrity of the aortic wall. Elastin and its associated microfibrils interact with SMCs as described herein, but elastin also plays a critical role in regulation of the development of the aorta. The extracellular matrix also contains microfibril-associated proteins, such as fibulin or latent transforming growth factor (TGF)-β1 binding protein, which play a vital role in sequestration and regulation of the activity of cytokines, such as TGF- β [25, 28].Collagen fibers, primarily type I and III collagen, are also present in the aortic media and contribute to the stiffness of the aorta.

At the cellular and molecular level, the ability of the aortic wall to sense biomechanical forces via its unique cell-matrix interactions has an important bearing on its structural integrity and development. The aortic wall is constantly exposed to cyclic mechanical loads from pulsatile blood flow, with the ascending aorta exposed to forces delivered by the beating heart. Genetic mutations that affect mechanosensing via the elastin-contractile unit lead to aneurysm. Elastin, collagen fibers and the extracellular matrix (ECM) endure the bulk of stress that is exerted on the aortic wall, which is typically 100-200 kPa, such that only 3-5 kPa is exerted on the SMCs in the aortic wall. SMCs sense this stress via the elastin-contractile unit and regulate and remodel the ECM. In essence, the SMCs sense and monitor the mechanical stress of the aortic wall constantly via their contractile thin and thick filaments and integrin receptors and accordingly alter their cytoskeletal structure and the composition of the ECM via

various signaling cascades. Phenotypically modulated VSMCs are characterized by change in their secretory function. This change contributes to the pathology of the aneurysm tissue.

4. SM22α and its role in pathology of vascular diseases

SM22α or transgelin is a 22kDa protein was first identified in the chicken gizzard smooth muscle[29]. Its known to be expressed abundantly in the smooth muscle cells and at detectable levels in the fibroblasts. SM22 has two other homologs SM22β (transgelin 2) and NP22 (transgelin 3/Neuronal protein 22). All the homologs are cytoskeletal protein, but they express in different cell types[30, 31]. SM22β expresses in the smooth muscle and endothelial cells[30] whereas NP22 expresses in the neurons[31]. Structurally SM22 belongs to the calponin protein family. It has a N-terminal calponin homology domain(CH) and a C-terminal calponin like repeat. The protein structure has potential binding sites for calcium and phosphorylation sites[32]. The crystal structure was described by Li *et al*[33].SM22's role as an actin binding protein has been well characterized[34, 35].

Expression of SM22α has been reported to be downregulated in a variety of carcinomas like prostate cancer[36], breast cancer and colon cancer[37, 38]. SM22α's expression is also downregulated in cardiovascular diseases like aortic aneurysm[39] and atherosclerosis[40] as part of the phenotypic modulation of the smooth muscle cells. Sm22s role in modulating contractility in the SMCs is shown in *Sm22* knockout mouse model[41, 42]. *Sm22*$^{-/-}$ mice on the contrary have normal blood pressure, heart rate and vascular morphology[43]. SM22 is thus thought to be compensated under homeostasis but may play a role in a stress condition. My work focuses on regulation of SM22α under ER stress. ER stress is reported to be activated in cardiovascular diseases like atherosclerosis[44], aortic aneurysm[45] and vascular calcification[46, 47].

5. Endoplasmic reticulum stress and Cardiovascular diseases:

The ER stress is the cellular stress response associated with the functioning of the ER. The endoplasmic reticulum is an organelle required for many essential processes in the cell including protein synthesis, post translational modifications, synthesis of phospholipids and steroids, regulation of calcium homeostasis and many more[48]. The ER is a dynamic organelle and interacts with a wide range of other organelles including Golgi[49],Mitochondria [50],the Plasma membrane[51] and Nucleus. Disturbances in any of the major functions mainly the protein folding leads to a stressed ER. The stress caused due to unfolded proteins, triggers a series of signaling cascades termed the Unfolded Protein Response(UPR) [52].ER stress has been shown to be required for many physiological processes like insulin secretion from pancreatic β cells[53] and osteoblast maturation during bone formation[54, 55]. The UPR activation involves 3 distinct steps:1) Attenuation of protein translation to avoid buildup of misfolded proteins 2) Activation of chaperones to help fold the misfolded proteins 3) Activation of ER associated degradation to degrade the proteins whose structure cannot be rectified. The signaling is a 3-pronged cascade involving PERK (pancreatic ER kinase), IRE1 (inositol-requiring transmembrane kinase/endonuclease 1), and ATF6 (activating transcription factor 6). Prolonged interaction of the protein folding system with the chaperones activates the downstream apoptotic signaling**(Fig.2)**.

Presence of ER stress has been established in a wide range of clinical and animal model samples of vascular pathologies. Many compounds have been identified that could block atherosclerosis induced ER stress. Treatment with some of these compounds were shown to have therapeutic benefits[56, 57]. Deletion of myocardin – a transcriptional co-activator caused aortic aneurysm accompanied by ER stress[58]. Another study showed deletion of *Chop*, a signaling molecule of the ER stress process prevented aortic aneurysm[59].Attenuating ER stress have been shown to reduce vascular calcification [60-62].A recent review discusses ER stress in cardiovascular disease in detail[63]. However, the mechanism of the stress response and vascular remodeling is unclear and needs to be elucidated in detail.

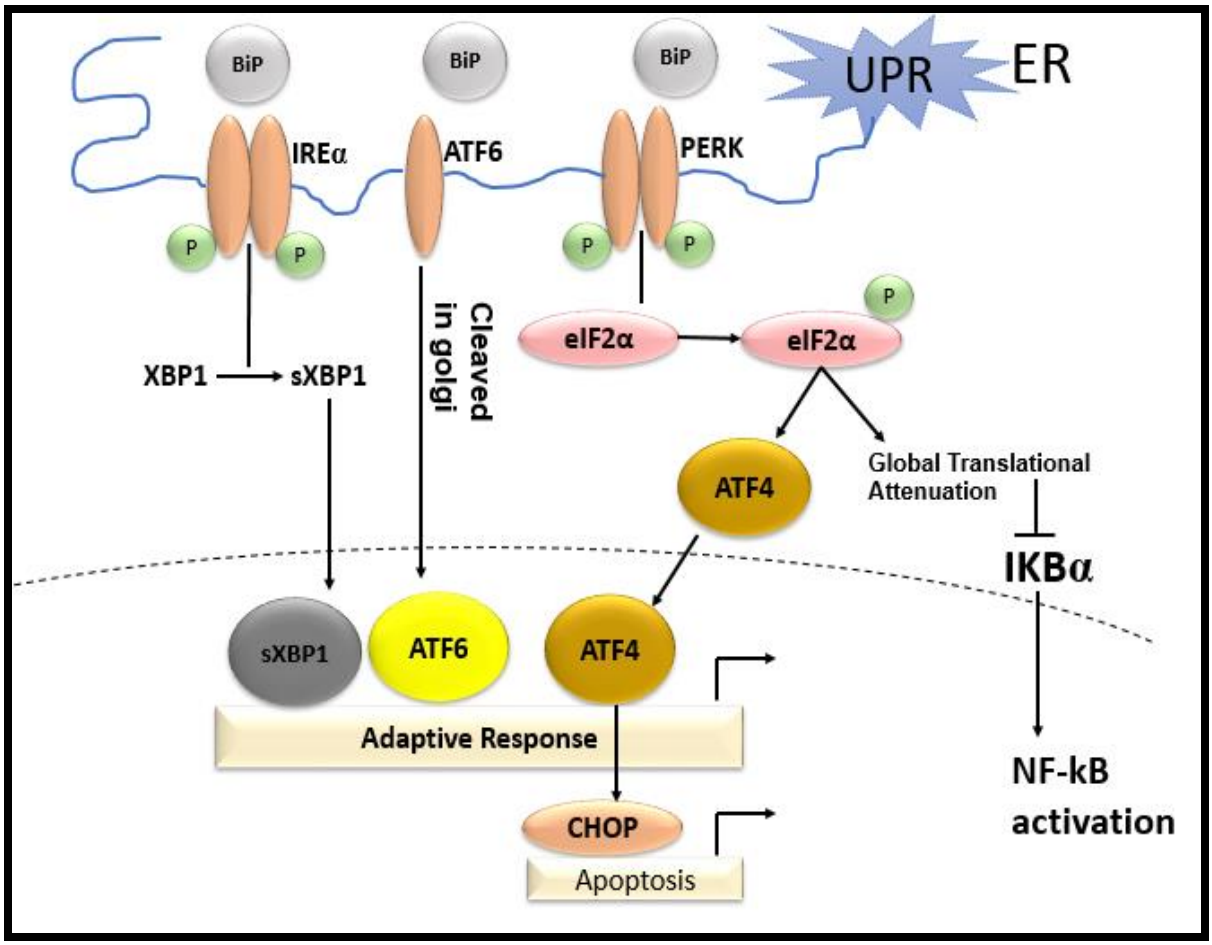


Figure 2 Unfolded protein response of the ER has two responses -the adaptive and the apoptotic UPR. An overload of unfolded proteins triggers the detachment of the ER chaperone Bip from the ER stress transducers and Bip binds to the unfolded proteins. The 3 transducers IREα, ATF6 and PERK activate downstream signaling. All the 3 arms of signaling cause nuclear translocation of factors that induce gene expression of adaptive response genes. Persistent stress then leads to activation of pro-apoptotic genes. Upon ER stress IREα, oligomerizes and undergoes autophosphorylation. It cleaves XBP1 and its spliced form enters the nucleus. ATF6 undergoes cleavage in Golgi and enter the nucleus. PERK also oligomerizes and auto phosphorylates like IREα. PERK then phosphorylates elF2α leading to increased expression of ATF4. ATF4 translocated to the nucleus and induces the expression of pro-apoptotic factors like CHOP. Phosphorylation of elF2α also leads to activation of NF-kB.

CHAPTER 2 - RESULTS

I. Establishing ER stress and apoptosis in the aortic aneurysms of *Sm22*$^{-/-}$ Marfan mouse model

1. Background

1.1 Marfan Syndrome (MFS)

Marfan syndrome is one the genetic disorders whose cardiovascular manifestations include aortic aneurysm. Marfan syndrome is an autosomal-dominant inherited disease affecting ~1 in 5000 births[64]. Mutation in Fibrillin-1(*FBN1*) gene is known to cause (MFS). Fibrillin-1 is one of the component of the cellular architectural matrix. Its two major roles in the context of aneurysm include – providing tensile strength to the cell and regulating the bioavailability of the cytokine, Transforming growth factor beta (TGFβ) via its interaction with latent TGFβ binding proteins (LTBPs)[65]. The fibrillin microfibrils can exist as individual structures or associate with elastin to form macro-aggregates. These elastic assemblies are important to carry out the physiological function like maintain the cardiovascular tone[66].Mutations in the Fibrillin gene leads to a compromised elastic assembly, predisposing the vasculature to structural incompetency. The defective fibrillin also causes increase in the TGFβ in the cell. TGFβ dependent increases in MMP2/9 leads to further breakdown of the extracellular matrix[67]. Role of TGFβ in the disease pathogenesis of MFS was established with help of the Marfan mouse model. The mouse had a heterozygous mutation for the *Fbn1* gene (*Fbn1*$^{C1039G/+}$)[68].

1.2 Marfan - *Sm22*$^{-/-}$ mouse model

SM22 expression has been shown to be downregulated in the aneurysm tissue. Our lab made a *Sm22* deletion in the Marfan mouse to generate a *Fbn1*$^{C1039G/+}$: *Sm22*$^{-/-}$ mouse model. We observed increased severity of aneurysm formation in these mice **(Fig.3)** We used these mice to test for smooth muscle apoptosis and presence of increased ER stress signaling by histopathology.

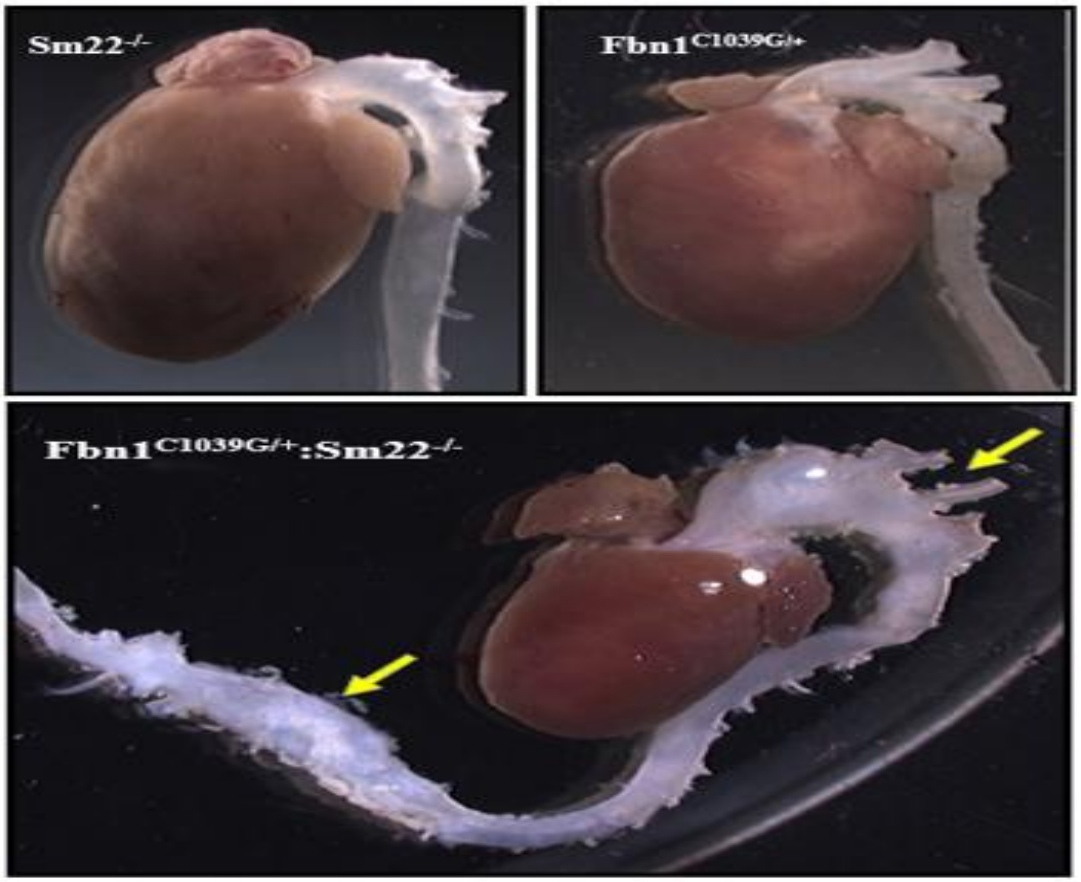


Figure 3: Aneurysm mouse model: SM22 deficiency exacerbated the aneurysm formation in Marfan mouse (*Fbn1*$^{C1039G/+}$). Upper panel: Left -Right: normal aorta from *Sm22*$^{-/-}$ mice, normal aorta from Marfan mouse. Lower panel: Colored arrows indicate the Thoracic aneurysm closer to the heart and Abdominal aneurysm in the lower part of the aorta from *Fbn1*$^{C1039G/+}$*: Sm22*$^{-/-}$ mouse. SM22 deficiency leads to a higher incidence and severity of aneurysm in the MFS mice. Picture credits: Dr. Xiaohua Dai, Li Li lab

1.3 Significance of *Ex vivo* culture system

Ex vivo culture system refers to isolation of organs from the organism and culturing them in an external environment with minimal alterations of natural conditions. The advantage of this system lies in the fact that it allows the cells to be in their natural setting. In aorta, it allows the cells to maintain the structural scaffolding with the ECM. It helps reduce the non-aortic signaling crosstalk.

1.4 Pharmaceutical ER stress inducers

There are several chemicals used regularly to induce ER stress. These include tunicamycin, brefeldin A and thapsigargin. All of them induce unfolded protein response (UPR) in the ER by creating an unfolded protein overload through various mechanisms. Tunicamycin blocks the initial step of glycoprotein biosynthesis by inhibiting the enzyme UDP-N-acetylglucosamine-dolichol phosphate N-acetylglucosamine-1-phosphate transferase (GPT). Brefeldin A causes accumulation of unfolded protein by inducing retrograde protein transport from the Golgi to the ER and blocking transport of proteins from the ER to Golgi. Thapsigargin works by decreasing ER store calcium and increasing the cytosolic calcium levels in the cell. It does this by inhibiting the sarcoplasmic/endoplasmic reticulum Ca2+-ATPase(SERCA) which sequesters calcium from the cytosol, thereby raising intracellular calcium. Store depletion also secondarily activates plasma membrane channels and causes more influx of calcium into the cytosol. As the calcium levels in the ER decrease, the calcium dependent chaperones of the ER lose their activity and hence can no longer fold the protein. This leads to overload of unfolded proteins[69, 70].Thapsigargin has been shown to induce apoptosis[71, 72]. We selected thapsigargin as it induces apoptosis, which was the hypothesized endpoint of the cells in our aneurysm model.

2. Results

2.1 Aneurysm tissue from the *Fbn1*$^{C1039G/+}$: *Sm22*$^{-/-}$ mice stained positive for apoptosis

The aneurysm tissue from the *Fbn1*$^{C1039G/+}$: *Sm22*$^{-/-}$ mouse was used to perform the TUNEL assay to establish apoptosis. TUNEL assay stands for Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling. TdT is a specialized DNA polymerase and is capable of labelling blunt ends of double stranded breaks. The assay uses this ability to label apoptotic cells that undergo severe DNA degradation as part of the apoptosis mechanism. The aneurysm tissue was found positive for higher number of apoptotic cells compared to the aortic tissue from the WT mice **(Fig.4)**. Extensive degradation of the elastin layers can be observed in the aneurysm sample.

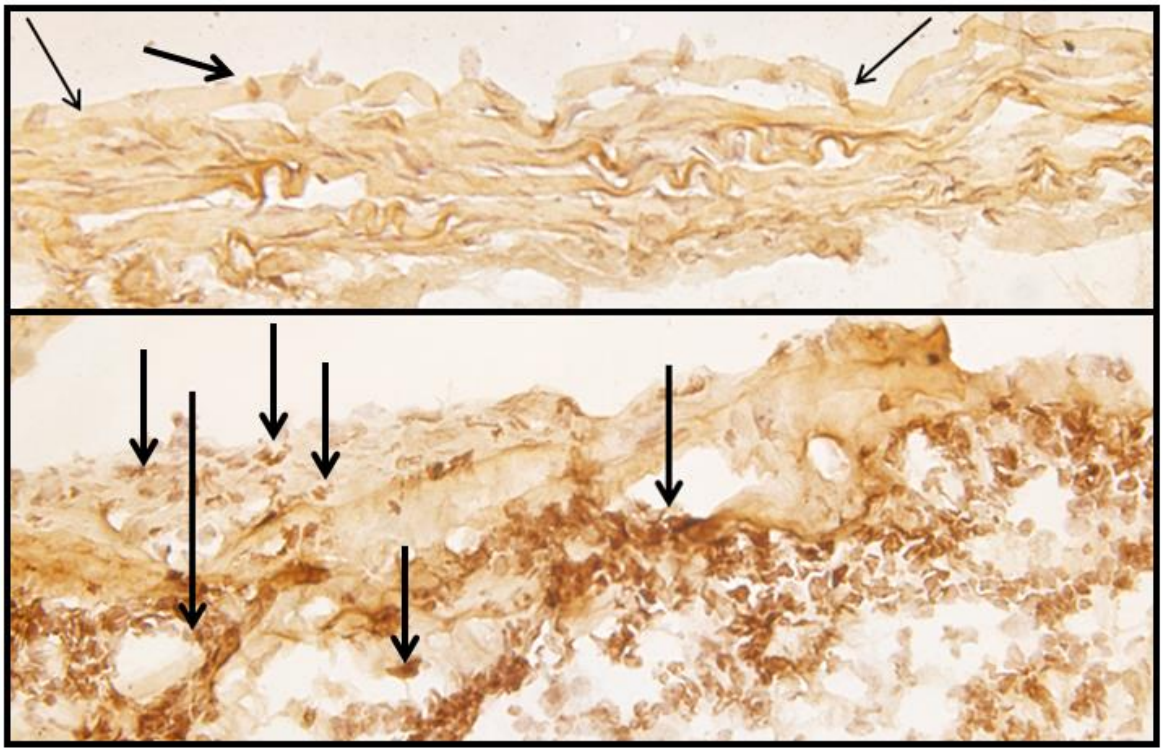


Figure 4: VSMCs undergo apoptosis in aortic aneurysm tissue from *Fbn1*$^{C1039G/+}$: *Sm22*$^{-/-}$ mouse. The apoptotic cells are detected by TUNEL assay. Terminal deoxynucleotidyl transferase (TdT) is a specialized DNA polymerase and is capable of labelling blunt ends of double stranded breaks. Multiple clinical studies have shown presence of apoptotic VSMCs in the aneurysm samples. Upper panel: Aorta tissue from WT mouse. The structural integrity of the elastin fibers can be seen by distinct layers. Lower panel: Aorta from *Fbn1*$^{C1039G/+}$*: Sm22*$^{-/-}$ mouse. The aorta tissue from these mice undergo extensive degradation. This leads to loss of distinct elastin membrane layers. The black arrows point to apoptotic cells.

2.2 Aneurysm tissue from the *Fbn1*$^{C1039G/+}$: *Sm22*$^{-/-}$ mice stained positive for ER stress markers.

We stained the tissue for Activating transcription factor 4 (ATF4). It is downstream in the PERK arm of the ER UPR signaling pathway. We found that the tissue stained positive for higher ATF4 showing activated ER stress in the aneurysm tissue **(Fig.5)**. We then stained for CHOP, a pro-apoptotic factor downstream to the ATF4. CHOP expression was also upregulated in the aneurysm tissue **(Fig.6)**. This showed that ER stress induced apoptosis was being induced in the aneurysm tissue.

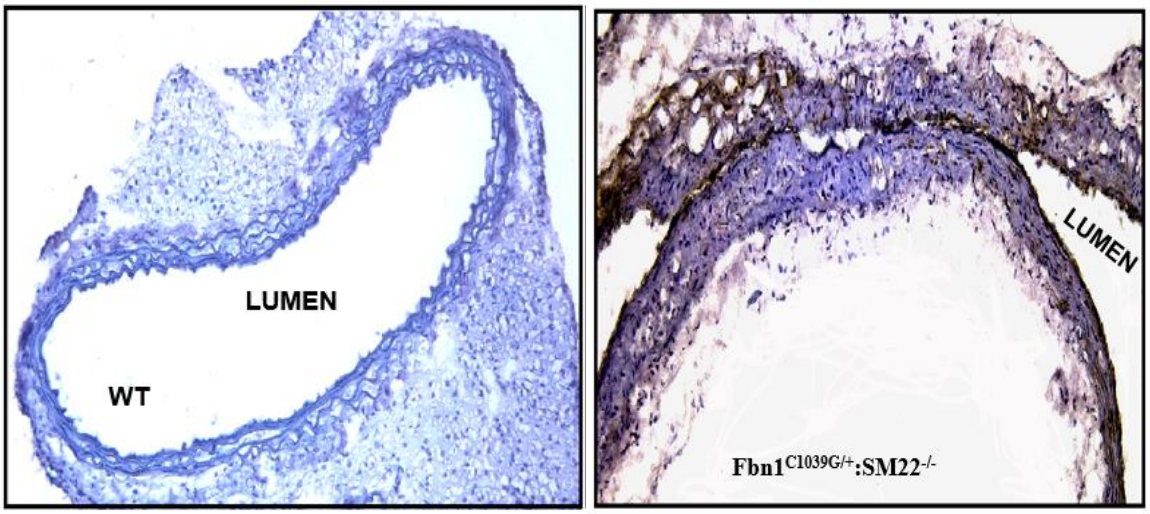


Figure 5: Upregulated ATF4 expression was observed in aneurysm tissue from *Fbn1*$^{C1039G/+}$: *Sm22*$^{-/-}$ mouse by IHC: (L)Aortic tissue from WT mouse. (R) Aortic tissue from *Fbn1*$^{C1039G/+}$: *Sm22*$^{-/-}$ mouse. N=3Activating transcription factor 4 (ATF4) is upregulated by phosphorylated elF2α. It translocates to the nucleus to induce transcription of adaptive genes. In chronic ER stress Atf4 induce the pro-apoptotic factor Chop. N=3

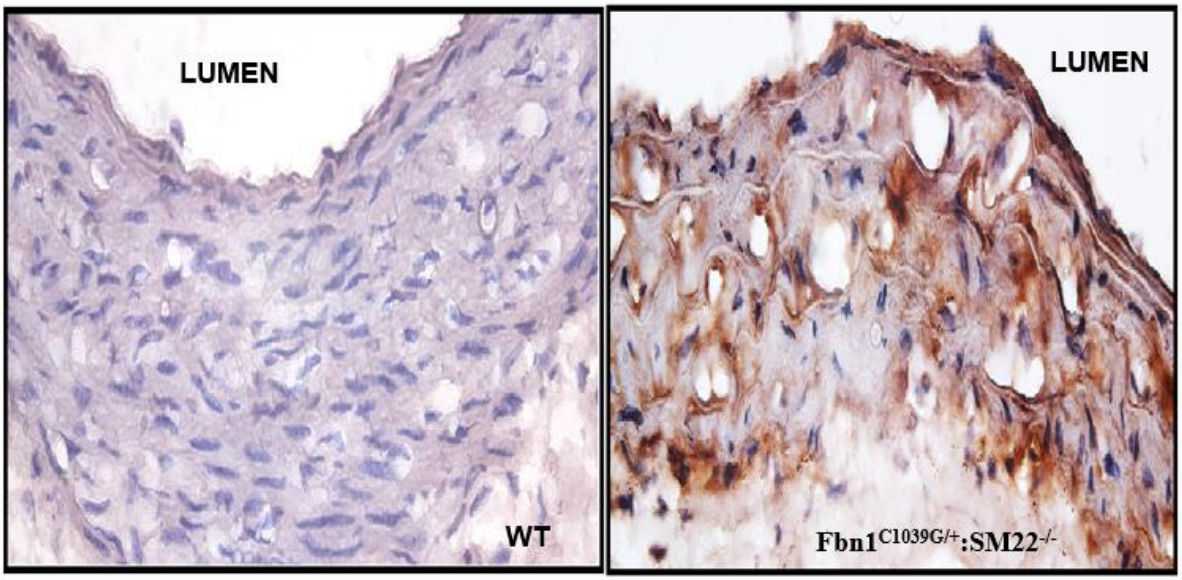


Figure 6: Upregulated CHOP expression was observed in aneurysm tissue from *Fbn1*$^{C1039G/+}$: *Sm22*$^{-/-}$ mouse by IHC: Left panel: Aorta from WT mouse. Right panel: Aorta from Fbn1$^{C1039G/+}$: Sm22$^{-/-}$.CHOP (GADD153) is ubiquitously expressed at very low levels in the cells. Under non-stressed conditions, it is present in the cytosol. Robust stress conditions induce elevated levels of CHOP and its accumulation in the nucleus. N=3

2.3 *Sm22α* deficiency results in a higher ER stress response in the *ex vivo* model of aorta culture

After the activation of ER stress markers were confirmed by immunohistochemistry in the aneurysm tissue, we carried out an *ex vivo* aorta culture. We wanted to test the significance of *Sm22* deficiency in the ER stress process. To test this, we used aorta from WT, *Sm22*+/- and *Sm22*-/- mice. The aortas were isolated and incubated in culture medium containing thapsigargin. After 24 hrs. of incubation RNA was isolated to perform qPCR analysis for the expression of *Atf4* and *Chop*. We found that *Sm22α* deficiency caused a higher expression of *Atf4* and *Chop* **(Fig.7)**.

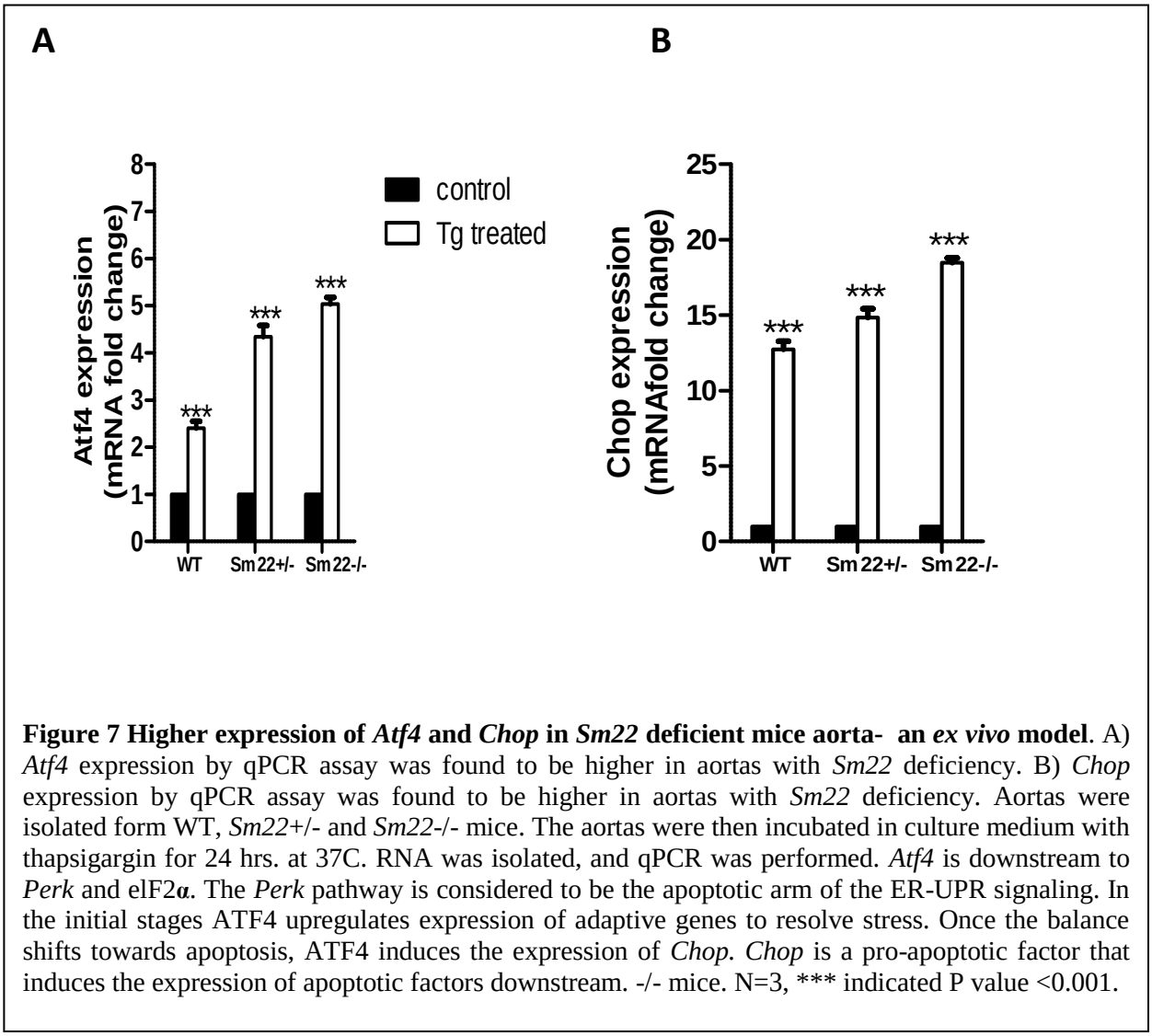


Figure 7 Higher expression of *Atf4* and *Chop* in *Sm22* deficient mice aorta- an *ex vivo* model. A) *Atf4* expression by qPCR assay was found to be higher in aortas with *Sm22* deficiency. B) *Chop* expression by qPCR assay was found to be higher in aortas with *Sm22* deficiency. Aortas were isolated form WT, *Sm22*+/- and *Sm22*-/- mice. The aortas were then incubated in culture medium with thapsigargin for 24 hrs. at 37C. RNA was isolated, and qPCR was performed. *Atf4* is downstream to *Perk* and elF2**α**. The *Perk* pathway is considered to be the apoptotic arm of the ER-UPR signaling. In the initial stages ATF4 upregulates expression of adaptive genes to resolve stress. Once the balance shifts towards apoptosis, ATF4 induces the expression of *Chop. Chop* is a pro-apoptotic factor that induces the expression of apoptotic factors downstream. -/- mice. N=3, *** indicated P value <0.001.

3. Discussion

Apoptosis of smooth muscle cells is thought to the primary reason for smooth muscle cell loss in aneurysm[73, 74].Among the many stress signals activating apoptosis, ER stress is the lesser known pathway in aneurysm. A few studies have shown activation of ER stress in Ang II or Cacl2 induced aneurysm[45, 75, 76].Consistent with these studies we show presence of ER stress induced apoptosis in our mouse model. This also points to the association of ER stress with aortic aneurysm irrespective of the etiology. The results also highlight the role of *Sm22α* under stress. It suggests that under quiescent conditions *Sm22α* might be compensated but it may play an active role in stress conditions

II. ER stress triggers a signaling cascade inducing phenotypic modulation leading to apoptosis in smooth muscle cells

1. Background

The cells need protein homeostasis or proteostasis to maintain optimal functioning of the cells in response to various stimuli. Hence the cells have a very sophisticated mechanism of sensing cell changes and responding to it. There exists an intricate network of cytosolic proteostasis and organelle proteostasis that work together to reduce proteotoxic stress. The cytosolic proteostasis include the chaperones like the heat shock proteins to fold the proteins and degradation system like the proteasome pathway to unfavorable proteins. The organelle proteostasis include the ER. The ER keeps its protein folding in check using the unfolded protein response (UPR). The homeostasis is maintained by the adaptive response of the UPR. This function of the UPR tries to resolve the protein overload by increasing the expression of the protein folding chaperones. The predominant function of the UPR is to maintain the cell function and structure in response to stress. Once the adaptive response proves insufficient to maintain normal functioning, the apoptotic UPR signaling is triggered. The main function of this signaling is to get rid of the cells whose normal functioning cannot be restored. Cells subjected to severe or chronic stress are cleared through this pathway[77]. The trigger, signaling the switch from adaptive to apoptotic remains

unclear. Although in most cases, once the balance shifts to apoptotic pathway, several pathogenic signaling are also induced.

The role of long term ER stress has been suggested in the inflammatory and osteogenic signaling in many pathologies[63]. There is some research though that suggest that ER stress has an anti-inflammatory role[78]. Our work aims at highlighting the regulation of cytoskeletal markers in the context of ER stress and its associated signaling. SM22α and SMAα are the characteristic cytoskeletal proteins of the smooth muscle cell. The most well elucidated gene regulatory mechanism of the smooth muscle genes is through SRF/myocardin ternary complex. SRF along with the its transcriptional co-activator myocardin binds to the CArG boxes in the promoter of the cytoskeletal gene to induce its expression. There are many ternary complex factors that compete with the SRF/myocardin complex to repress the transcription.

We treated our cells with 2 pharmaceutical ER stress inducers- 1) tunicamycin (Tm) 2) thapsigargin (Tg) and found that they produced similar preliminary results. We chose to continue our work with thapsigargin as it causes ER stress through changes in intracellular calcium. Perturbed calcium signaling has been implicated in aneurysm pathology[79].Also changes in calcium levels lead to vascular calcification through matrix vesicle release by synthetic VSMCs[80].

2. Results

2.1 Chronic ER stress causes a transient upregulation of SM markers

The smooth muscle cell line was treated with thapsigargin for up to 24hrs. We observed the expression pattern of *Sm22α* and *Smaα* over the course of 24 hrs. We found that the gene expression was transiently upregulated before downregulating **(Fig.8)**. Calponin1(*Cnn1*), *Sm22α, Smaα* were among the most downregulated cytoskeletal genes. The other smooth muscle genes that were downregulated at the 24hr time point were vimentin (*Vim*), Filamentin A(*Flna*), Myocardin (*Myocd*), Myosin heavy chain (*Myh11*), Tubulin beta (*Tubb*) and Transgelin 2 (*Tagln 2*) **(Fig.10)**. The activation of ER stress was

confirmed by the upregulation of of ER stress markers *Atf4*, *Chop* **(Fig.9 and Fig.11)**. It was accompanied by upregulation of *Klf4* and *Pdgf-bb* expression.ER stress chaperones *Calr* and *Grp78*(Bip) were also found to be upregulated. Tunicamycin and thapsigargin resulted in downregulation of *Sm22α* to a similar extent. Thapsigargin treatment also caused downregulation of ECM markers – Collagen (*Col3a*), Fibrillin(*Fbn1*) and Fibronectin(*Fn1*) **(Fig.10)**.

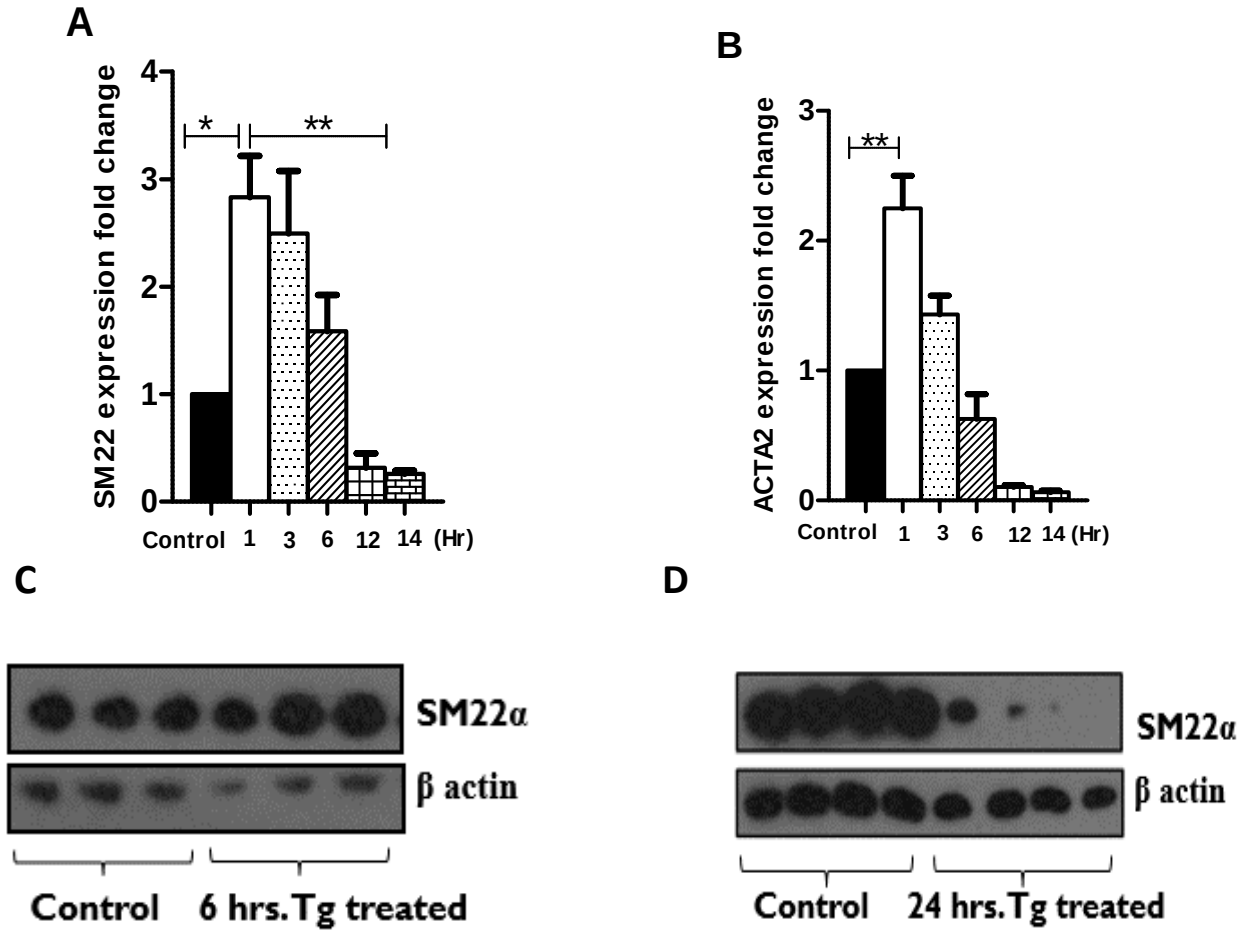


Figure 8: Thapsigargin treatment caused transient upregulation of smooth muscle markers.: A) Time dependent gene expression pattern of *Sm22* by qPCR assay with thapsigargin treatment. B) Time dependent gene expression pattern of Smooth muscle actin by qPCR assay with thapsigargin treatment C) Western blot showing upregulation of SM22 at 6 hrs. with thapsigargin treatment. Upregulation of protein was only detected at 6hrs and not at 1-3hrs. Translation follows transcription and delay between the processes is a normal phenomenon D) Western blot showing downregulation of SM22 after 24 hrs. of 1uM thapsigargin treatment. Increase in the expression of smooth muscle markers might be indicative of the adaptive response of the ER-UPR. Loss of smooth muscle markers induce phenotypic changes in the smooth muscle cell that could induce pathogenic signaling. The downregulation of the proteins probably marks the transition of the adaptive response to the apoptotic branch of the signaling.SM22 and SMA are the two characteristic markers of smooth muscle phenotype. N=3-6, P value *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ respectively vs the control(cntrl)

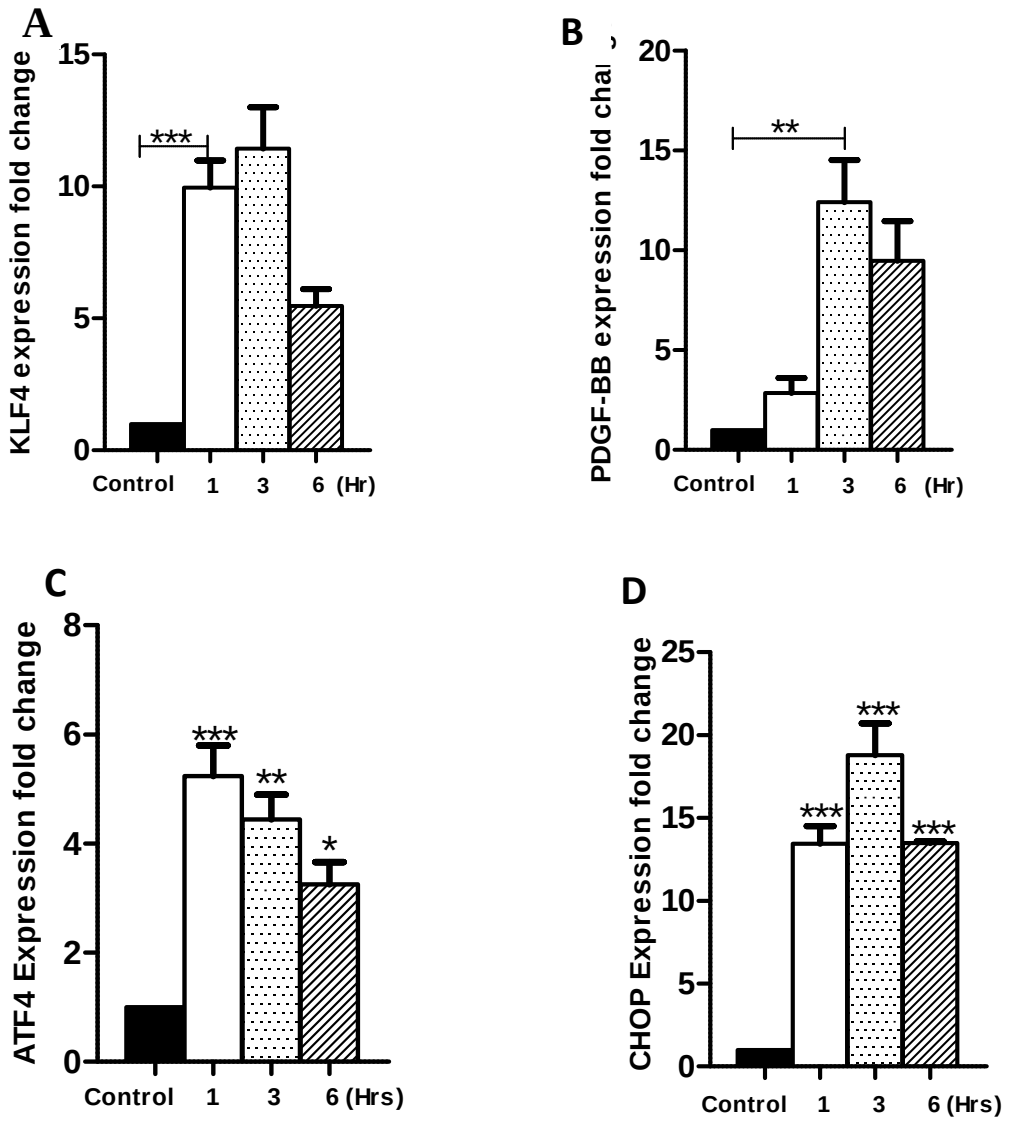


Figure 9: Transient upregulation of smooth muscle markers is accompanied by an increase in *Klf4, Pdgf-bb* and ER stress markers expression. PAC1 cells were treated with thapsigargin for 1-6 hrs. and RNA isolated for qPCR assays. A) Bar graphs shows an increase in *Klf4* expression. B) Bar graphs shows an increase in *Pdgf-bb* expression. C-D) Bar graphs showing increase in expression of *Atf4* and *Chop* expression. *Atf4* and *Chop* are downstream to PERK in the ER-UPR pathway and part of the apoptotic signaling. N=3-6, P value *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ respectively vs the control(cntrl).

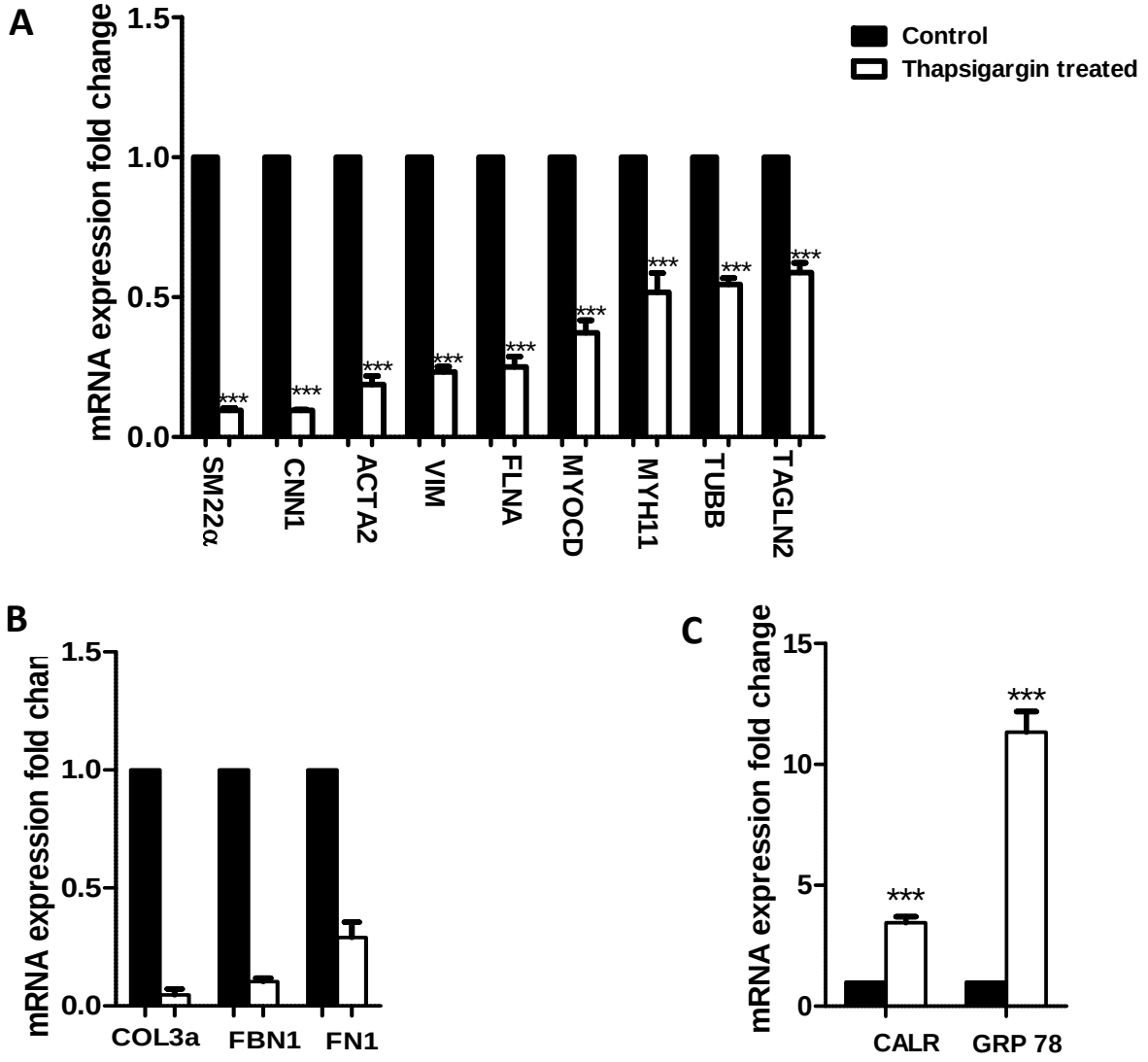


Figure 10: Thapsigargin treatment (24 hrs.) caused downregulation of smooth muscle markers, ECM genes and upregulation of ER chaperones: RNA was isolated from PAC1 cells and qPCR assay performed. A) Gene expression pattern of smooth muscle genes. All the smooth muscle genes that we tested were downregulated with thapsigargin treatment. *Sm22* was among the most downregulated genes. B) Downregulation of ECM genes with thapsigargin treatment - *Col3a, Fbn1* and *Fn1*. C) Upregulation of ER chaperones *Grp78*(*Bip*) and *Calr* (Calreticulin). N=3-6, P value *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ respectively vs the control(cntrl).

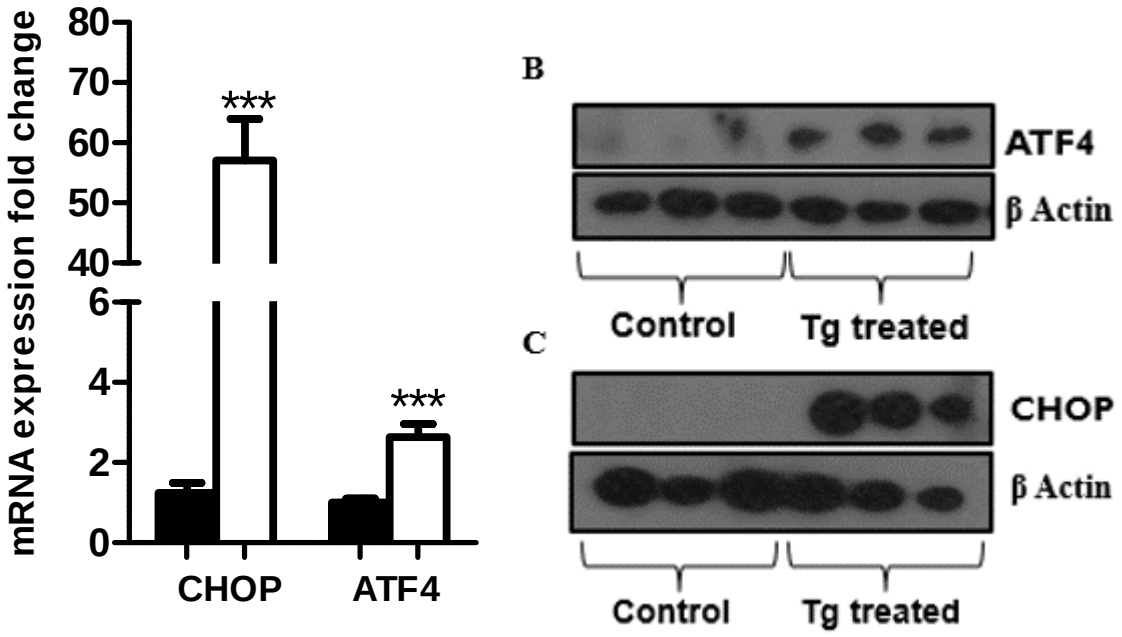


Figure 11: Upregulation of ER stress markers *Atf4* and *Chop* at gene and protein levels after 24 hrs. of thapsigargin treatment. A) Upregulation of gene expression of *Atf4* and *Chop* after 24 hrs. treatment with thapsigargin in PAC1 cells by qPCR assay. B-C) Immunoblots showing upregulation of ATF4 and CHOP protein levels after 24 hrs. treatment with thapsigargin in PAC1 cells. ATF4 is a transcription factor downstream of Perk and elF2α. Atf4 translocates to the nucleus and induces expression of adaptive response genes initially. *Atf4* induces the pro-apoptotic gene *Chop*. CHOP induces other apoptotic genes. N=3-6, *** indicate $p<0.001$ respectively vs the control(cntrl).

2.2 ER stress induces an upregulation of transcription factor *Klf4* and *Mmp9*

ER stress induced the expression of *Klf4*, Pdgf-bb and *Mmp9***(Fig.12)**. We found that *Klf4* and *Pdgf-bb* expression is upregulated as soon as 1hr of thapsigargin treatment **(Fig.9)**. The phenotypic marker *Pdgf-bb* is known to induce expression of *Klf4* through Sp1 binding to the *Klf4* promoter. Traditionally *Klf4* is known to repress the transcription of smooth muscle markers. Recent studies however show that the post translational modification of *Klf4* determines its function as enhancer or

repressor. The non SUMOylated *Klf4* is known to induce the expression of smooth muscle genes whereas the SUMOylated form represses the SM gene expression. This is probably why we see an increase in the smooth muscle genes accompanied by increase in *Klf4* expression. KLF4 is known to repress the expression of smooth muscle markers through several mechanisms. KLF4 binds the CArG box in the *Sm22* promoter replacing the *Sm22* inducing SRF-MYOCD complex. It also recruits HDAC2 to block the SRF complex from binding the promoter. KLF4 also represses the expression of smooth muscle master regulator MYOCD. MMP upregulation is characteristic of the aneurysm and atherosclerosis pathology. It is responsible for the degradation of the vessel wall. Apart from repressing the gene expression of the ECM genes, ER stress also degrades the vessel wall by inducing expression of *Mmp9*

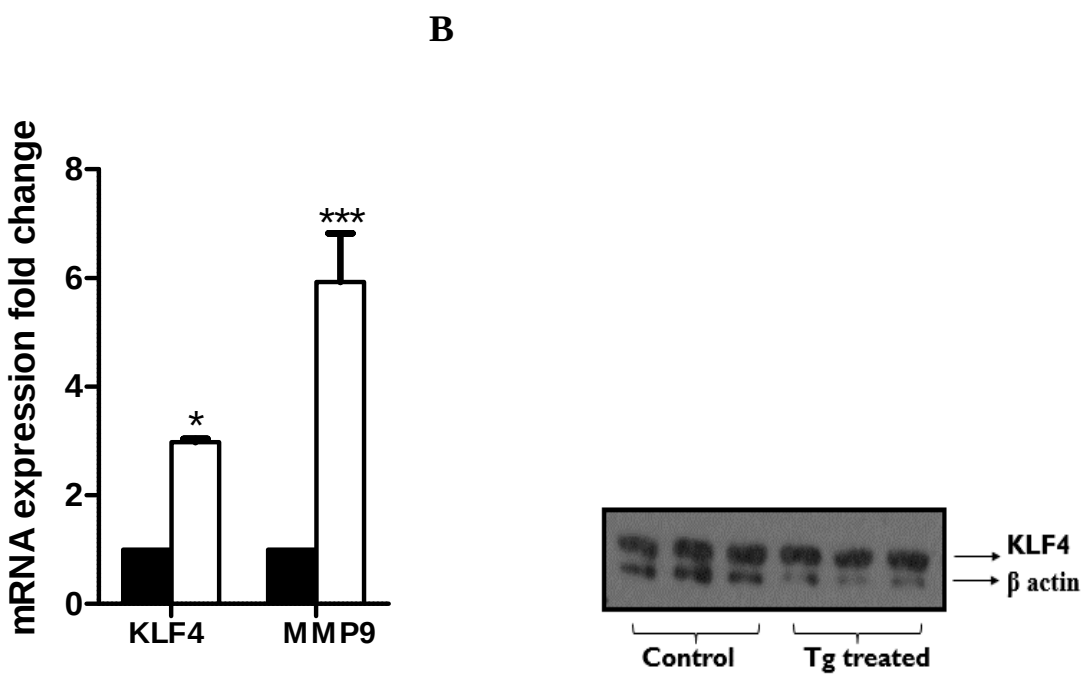


Figure 12: Thapsigargin treatment (24 hrs.) caused upregulation of *Klf4* and *Mmp9*. In PAC1 cells treated with thapsigargin for 24 hrs. A) qPCR showed upregulation of *Klf4* and *Mmp9*. B) Western blot assays showed the upregulation of KLF4. N=3-6, P value * and *** indicate $p<0.05$ and $p<0.001$ respectively vs the control(cntrl).

2.3 ER stress induces inflammation and osteogenic markers

Thapsigargin treatment induced the inflammation markers *Vcam-1* and *Ccl2* in the smooth muscle cells. Along with markers of inflammation, osteogenic markers were also upregulated. The osteogenic genes whose expression was increased were *Pit-1, Runx2, Msx2* and *Ocn* **(Fig.13)**. Some these markers are known to bind to *Sm22α* promoter directly or displace the SRF/MYCOD to repress *Sm22α* expression. *Runx2* is a transcription factor whose targets include *Ocn*. *Msx2* is also a transcription factor contributing to the vascular calcification. *Runx2* and *Msx2* regulate expression of many ECM genes. *Pit-1* is a sodium dependent phosphate transporter whose expression along with *Runx2* and *Ocn* is mediated by the ER stress induced transcription factor *Atf4*. Spliced XBP1 is also known to mediate *Runx2* expression. Thapsigargin also induced apoptosis at the same time point **(Fig.14)**

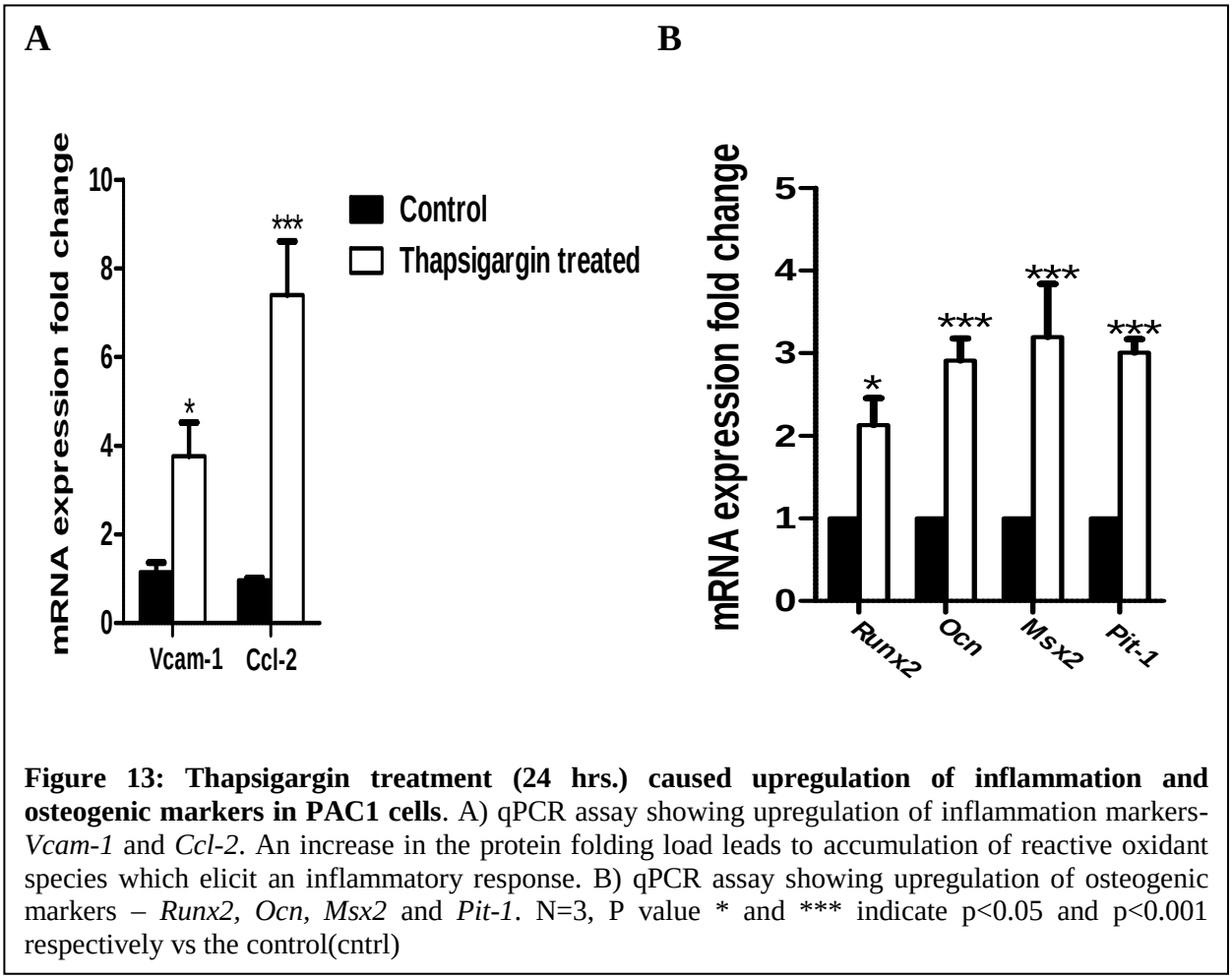
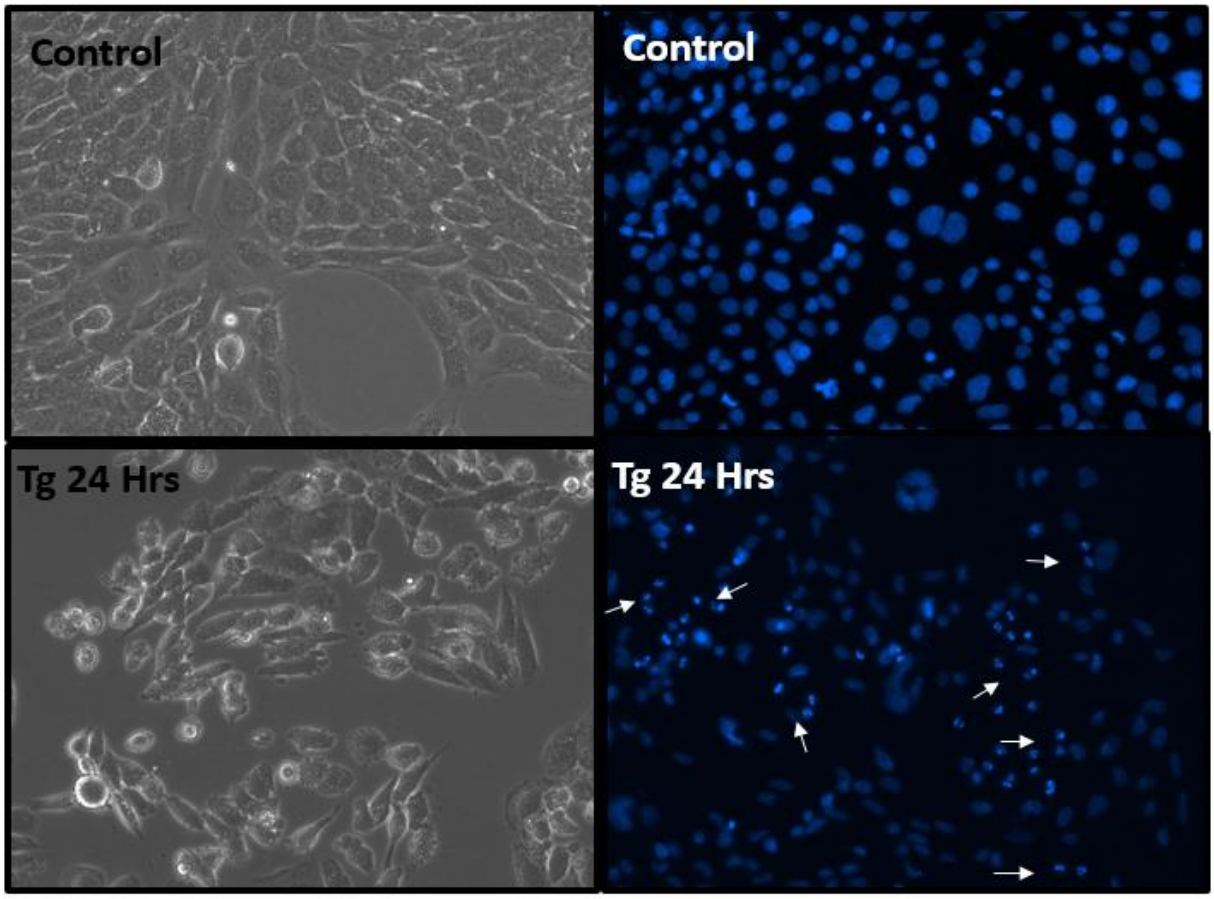


Figure 13: Thapsigargin treatment (24 hrs.) caused upregulation of inflammation and osteogenic markers in PAC1 cells. A) qPCR assay showing upregulation of inflammation markers- *Vcam-1* and *Ccl-2*. An increase in the protein folding load leads to accumulation of reactive oxidant species which elicit an inflammatory response. B) qPCR assay showing upregulation of osteogenic markers – *Runx2, Ocn, Msx2* and *Pit-1*. N=3, P value * and *** indicate $p<0.05$ and $p<0.001$ respectively vs the control(cntrl)

A



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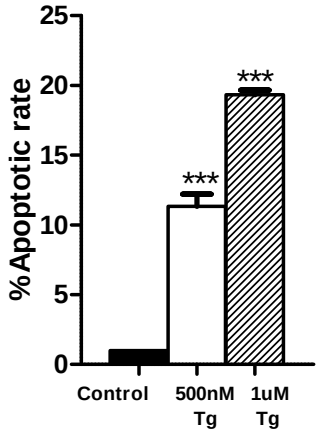

Figure 14: Thapsigargin treatment (24 hrs.) causes apoptosis in PAC1 cells in a dose dependent manner A) Top- Left panel – Bright field, Control PAC1 cells, Right panel- Stained for nuclei with Hoechst stain. Bottom-Left panel- Bright field, Thapsigargin treated for 24 hrs. at 1uM conc., Right panel- Stained with Hoechst stain. The white arrows pointing to some of the apoptotic nuclei. B) Quantitative analysis of apoptosis induced by Thapsigargin. N=3-6, *** indicate $p<0.001$ vs the control(cntrl)

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2.4 Effect of physiological ER stress inducers on VSMCs

Free fatty acids (FFA) is one among the many risk factors for the cardiovascular diseases and elevated FFAs have been shown to be associated with increased aortic stiffness, seen in atherosclerosis[81, 82].Palmitate, a saturated fatty acid accounts for 30-40% of the serum FFAs. Palmitate has been shown to alter the extracellular matrix in cultured smooth muscle cells, causing lipoprotein deposition and favoring atherogenesis [83]. Palmitate is also known to induce various cellular stress responses like endoplasmic reticulum (ER) stress, phenotypic modulation and apoptosis[84, 85]. We found that palmitate like thapsigargin induced downregulation of smooth muscle markers along with upregulation of *Mmp9* and *Chop* **(Fig.15)**. It is possible that the phenotypic modulation caused by palmitate is due to its induction of ER stress in the VSMCs. Further analysis is needed to elucidate the mechanism.

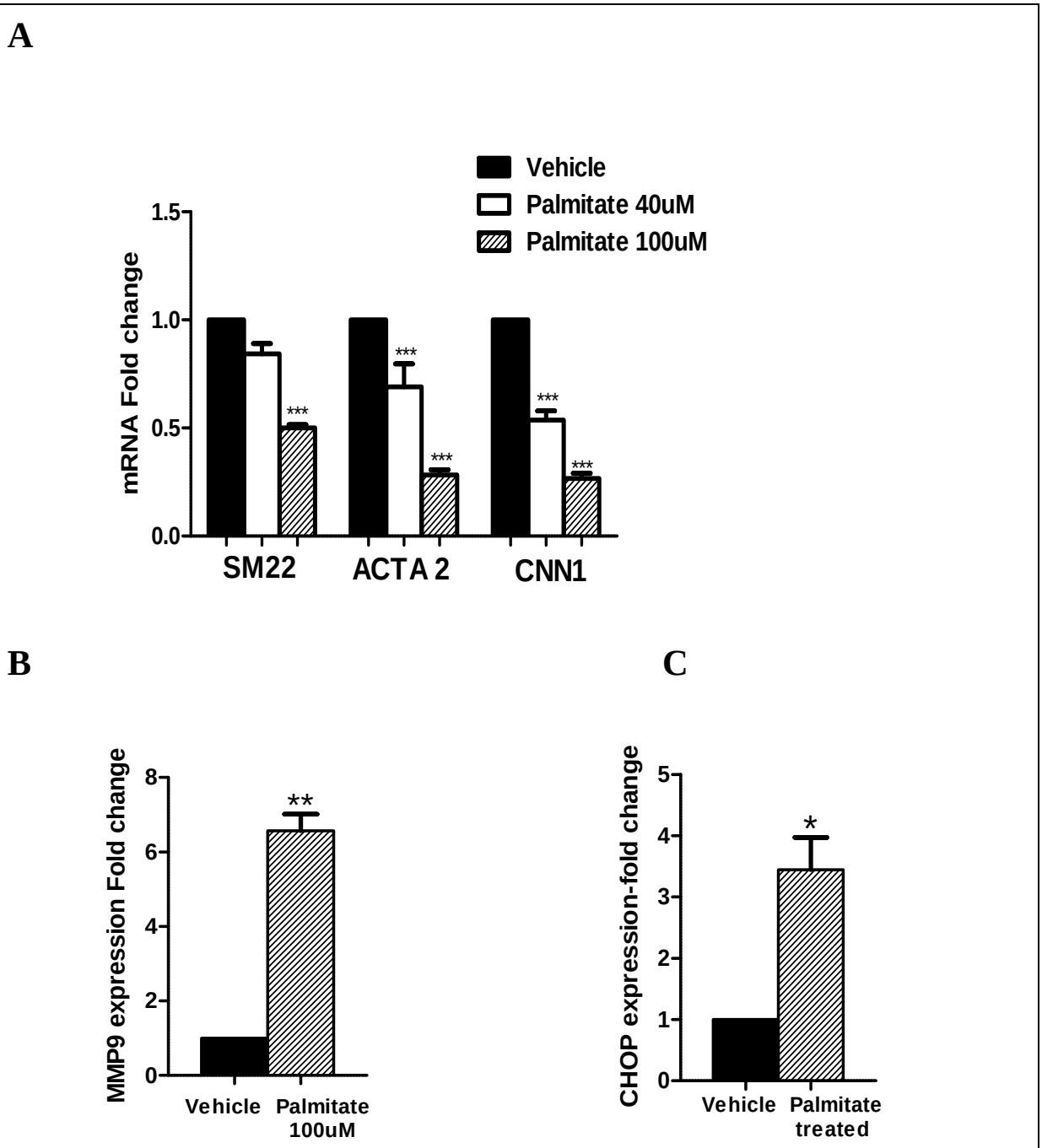


Figure 15: Physiological ER stress inducer palmitate causes phenotypic modulation in PAC1 cells. Palmitate is a saturated fatty acid that accounts for 30-40% of serum free fatty acids(FFA). FFAs have been associated with cardiovascular events. PAC1 cells were treated with BSA conjugated palmitate at 40uM and 100uM conc. for 18 hrs. and qPCR assay was performed. A) Smooth muscle markers *Sm22, Acta2* and *Cnn1* gene expression was downregulated in a dose dependent manner. B) *Mmp*9 gene expression was upregulated indicating changes related to phenotypic modulation. C) *Chop* gene expression was upregulated showing the induction of ER stress with palmitate. N= 3-6. P value *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ respectively vs the control(cntrl).

3 Discussion

Our results consolidate the data that ER stress induces a cascade of pathogenic signaling pathways that lead to downregulation of smooth muscle markers leading to phenotypic modulation of VSMC. These smooth muscle cells in the synthetic state then progress to apoptosis. The results show that a range of factors induced as part of the ER stress response further induce factors that trigger inflammation and osteogenesis. The numerous factors involved in these processes have been shown to downregulate the smooth muscle marker SM22α in several ways[86-90]. *Sm22α* was found to be one of the most downregulated genes in the stress response. SM22α is also known to be downregulated in various cardiovascular diseases like aneurysms[39, 91] and atherosclerosis[40, 92, 93] , making it an ideal candidate for further analysis. Our results show upregulation of both phenotypic markers *Pdgf-bb* and *Klf4. Pdgf-bb* is known to be a potent repressor of SMC genes. Part of its repressor function is shown to be mediated through upregulation of *Klf4* expression. The phenotypic marker Kruppel like factor 4 (KLF4) has been shown to downregulate the *Sm22* expression through a few different mechanisms. *Klf4* can bind to the two distinct regions of the *Sm22* promoter and recruit HDAC2 to interfere with Srf/Myocd dependent transcription. KLF4 also represses the expression of *Myocd,* hence limiting its availability for *Sm22* expression[86-88]. The transcription factor ATF4 (PERK pathway) has been shown to upregulate KLF4 by increasing its transcription and blocking its protein degradation.

The Perk pathway is also involved in triggering NF-kb mediated inflammation which represses *Sm22α* transcription. The nuclear factor NF-kB induces a repertoire of pro-inflammatory genes which include cytokines, chemokines and adhesion molecules. Under normal conditions NF-kB is bound by the inhibitory molecule IkBα. PERK induced phosphorylation of eIF2α which induces a translational repression to combat ER overload. This repression leads to reduced IkBα leading to less sequestration of NF-kB. The free NF-kB then translocates to the nucleus to induce expression of inflammatory markers like *Vcam-1, Ccl2* etc. NF-kB has also been shown to bind to CArG box of *Sm22α* promoter and repress

its transcription. A parallel signaling involves degradation of IkBα through IKKα enzyme. IRE1 dependent regulation of the IKKs basal activity through TRAF2 plays a vital role in IkBα reduction under ER stress[94, 95].

ER stress induced transcription factors ATF4 and CHOP have also been implicated in vascular calcification[47, 96].Vascular calcification is the most extreme form of phenotypic modulation in SMCs.RUNX2 an osteogenesis specific transcription factor upregulated in two different arms of the ER stress response ATF4(PERK pathway) and XBP1(IREα pathway).RUNX2 interacts with SRF thus blocking the SRF/MYOCD complex binding on CArG and repressing SM22α transcription[90]. Another osteogenic factor involved in SM22α downregulation is PiT-1[97].ATF4 and CHOP (PERK pathway) have been shown to regulate type III sodium-dependent phosphate cotransporter, PiT-1 expression through interaction with C/EBPβ[47, 89]. Pit-1 has been shown to repress SM22α by methylating its promoter[98]. Pit-1 is also known to regulate RUNX2 too, thus amplifying the osteogenic effect[99].Palmitate has been implicated in cardiovascular diseases. We show that palmitate has the same effect on cytoskeletal markers as thapsigargin. Hence it might be that the phenotypic modulation and inflammation caused by palmitate is partly through the ER stress process.

III Mutational analysis of *Sm22α* promoter under ER stress

1. Background

*Sm22*α is a 5.4 kb gene located on 11q23.2. It has 5 exons, 1 large and 3 small introns. Cis regulatory elements in the *Sm22* promoter include two CArG boxes (near and far), Smad binding element (SBE) and TGFβ control element (TCE) **(Fig.16)**. Our lab made a series of promoter mutations based on these different cis regulatory elements[100]. Many factors induced during inflammation and calcification

have been shown to regulate *Sm22α* expression. To check which binding sites of the promoter was involved in its downregulation under ER stress, luciferase assay was carried out to check for SM22α expression in cells transfected with different mutants and treated with thapsigargin. Myocardin is the master regulator of SM22α expression [101], so we overexpressed Myocardin to see if it could rescue the downregulation of SM22α.

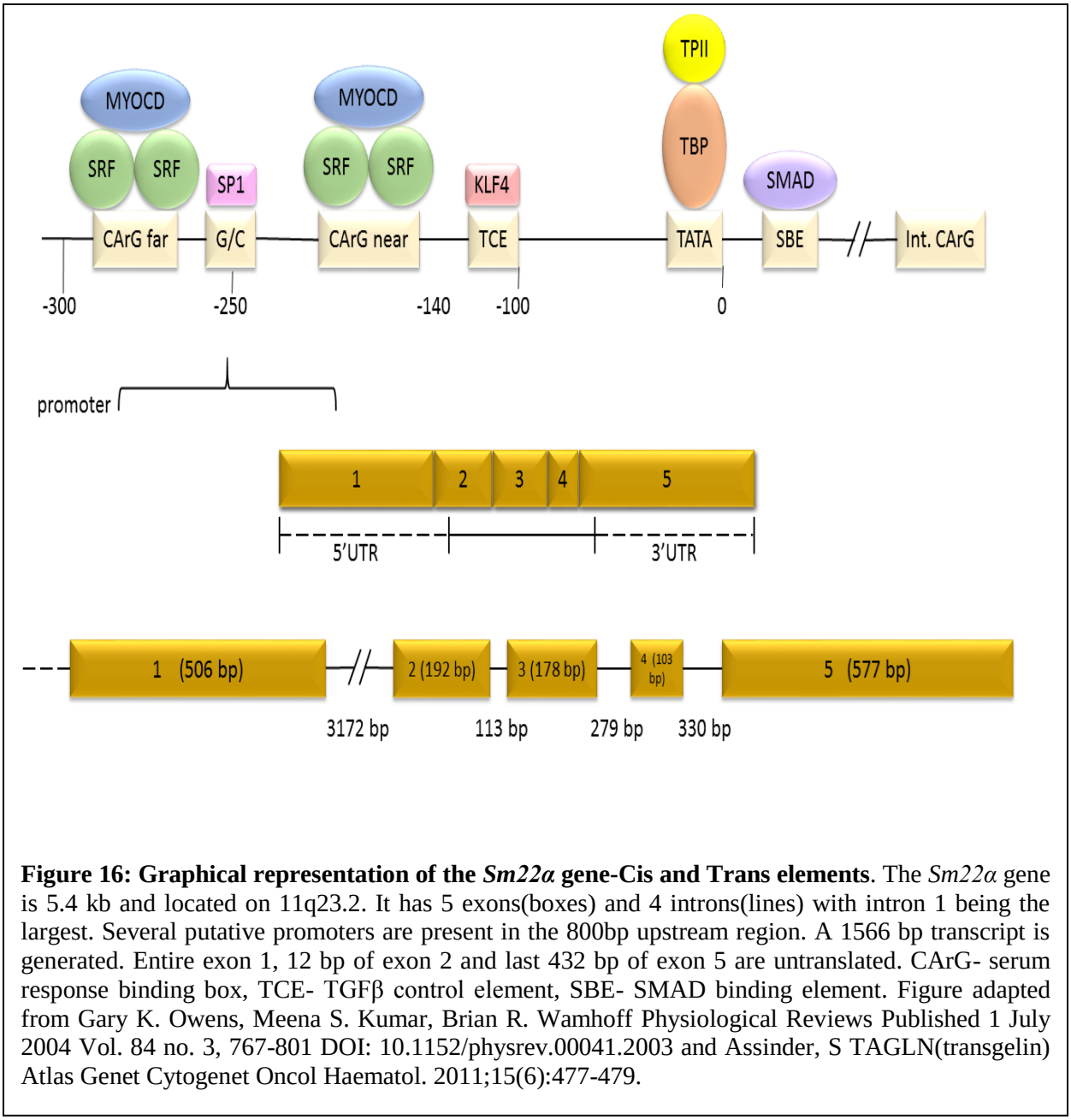
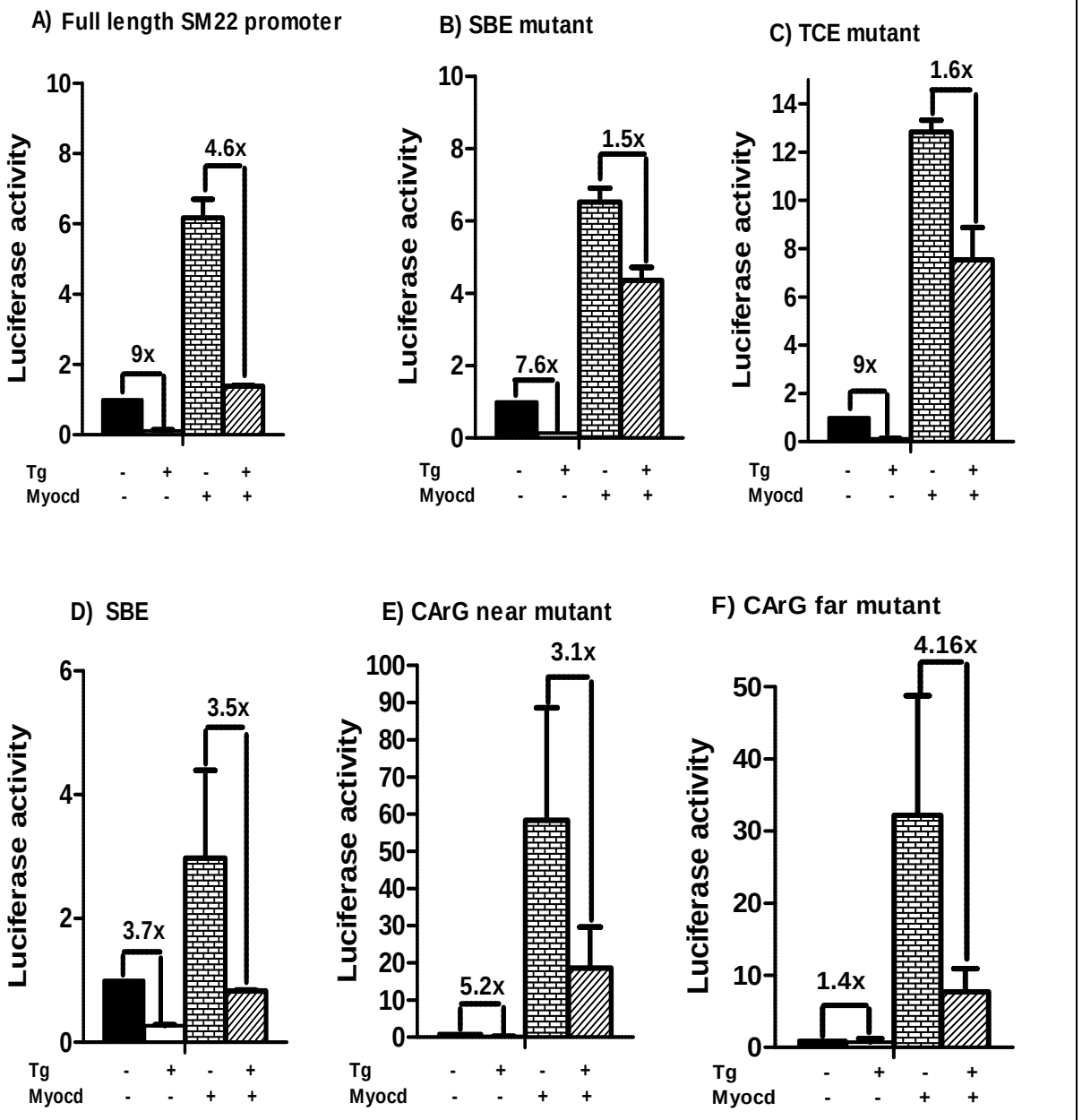


Figure 16: Graphical representation of the *Sm22α* gene-Cis and Trans elements. The *Sm22α* gene is 5.4 kb and located on 11q23.2. It has 5 exons(boxes) and 4 introns(lines) with intron 1 being the largest. Several putative promoters are present in the 800bp upstream region. A 1566 bp transcript is generated. Entire exon 1, 12 bp of exon 2 and last 432 bp of exon 5 are untranslated. CArG- serum response binding box, TCE- TGFβ control element, SBE- SMAD binding element. Figure adapted from Gary K. Owens, Meena S. Kumar, Brian R. Wamhoff Physiological Reviews Published 1 July 2004 Vol. 84 no. 3, 767-801 DOI: 10.1152/physrev.00041.2003 and Assinder, S TAGLN(transgelin) Atlas Genet Cytogenet Oncol Haematol. 2011;15(6):477-479.

2. Results

The different plasmid constructs that were used include a full length *Sm22* promoter, SBE mutant, TCE mutant, CArG near mutant, CArG far mutant, CArG null-TCE mutant and SBE alone. The cells were transfected with these plasmids and treated with thapsigargin**(Fig.17)**. We found that SM22 downregulated in all the cases showing that all the different sites were involved in its repression. SBE site was found to be sufficient to repress the expression of SM22 with thapsigargin. Many transcription factors induced by ER stress have been shown to bind *Sm22* promoter and cause its repression.

As myocardin can stimulate *Sm22* expression through different promoter mutants, we wanted to test if it could stop SM22 downregulation in an analogous manner using the luciferase assay We tested to see if myocardin overexpression could rescue SM22 downregulation under thapsigargin treatment. We found that myocardin overexpression could reduce the downregulation of SM22 in all the cases except when CArG far was used. This can be explained by the fact that myocardin induces SM22 at very low levels through this mutation. Hence a significant change in regulation could not be observed.


A) Full length SM22 promoter
B) SBE mutant
C) TCE mutant
D) SBE
E) CArG near mutant
F) CArG far mutant
Luciferase activity
9x
4.6x
7.6x
1.5x
1.6x
3.7x
3.5x
5.2x
3.1x
1.4x
4.16x
Tg - + - +
Myocd - - + +

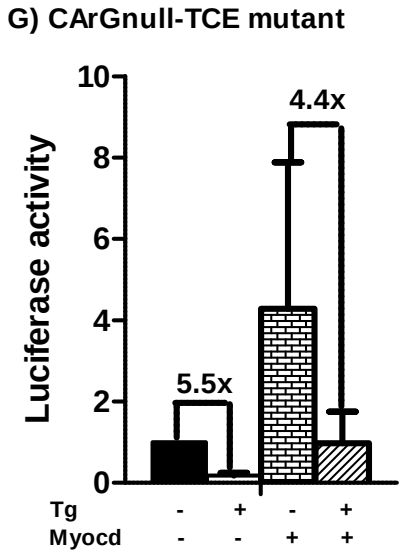


Figure 17: Promoter analysis by luciferase assay shows more than one cis element of *Sm22* is involved in its downregulation under ER stress. Luciferase assay was carried out using the different promoter mutants. No single or double mutant could prevent SM22 downregulation with thapsigargin treatment. It can be inferred that more than one cis element is involved. The data is backed by literature showing various trans elements induced by ER stress capable of binding different regions of the *Sm22* promoter and repressing its expression. Various promoter mutation plasmids with luciferase were transfected into PAC1 cells A) A full length *Sm22* promoter was used. B) Promoter with mutation in the smad binding element (SBE) is used. C) Promoter with mutation in the TGF control element (TCE) is used. D) Plasmid construct with only the SBE site was used. E) Promoter with mutation in the CArG near region was used. F) Promoter with mutation in the CArG far region was used. G) Promoter with double mutation in the CArG and TCE region was used. Myocardin is known to induce SM22 expression through all these promoter mutants. The same effect was observed even with thapsigargin treatment. Overexpression of myocardin can block SM22α downregulation to some extent under the ER stress condition. N=3

3. Discussion

A consensus sequence motif of nucleotides that form a signal is called a box. The sequence with the nucleotides [$CC(A/T)_6GG$] is referred to as the CArG box. Almost all the smooth muscle gene promoters have been shown have at least two of these. The CArG boxes play a pivotal role in their transcription. SRF has been shown to bind the CArG box selectively in SMCs and myocardin increased SRF binding. The binding of the SRF/myocardin complex to the promoter requires some epigenetic

modifications. Myocardin/SRF complexes physically interact with H3K4dMe and is sensitive to expression levels of myocardin. As expression of myocardin is reduced under ER stress, the SRF binding to the chromatin is reduced thus repressing *Sm22* expression. Parallelly, *Klf4* induced under ER stress can bind the TCE site on the promoter and recruit histone H4 deacetylase to compact the chromatin and render the CArG box inaccessible to SRF[102]. Myocardin binds to SMAD3 and coactivates the *Sm22* expression via the SBE site. Hence the repression of SM22 expression through this site is caused by reduced myocardin availability[103]. NF-kB is also known to bind myocardin preventing the SRF/myocardin complex from binding to the CArG box[104]. RUNX2, induced during ER stress also binds SRF and inhibits SRF dependent transcription[90].

Hence inhibition of SRF/myocardin complex plays a key role in *Sm22* repression under ER stress. We therefore see an increase in SM22 expression when myocardin is overexpressed. It is possible that the ER stress induced repressors are more potent than myocardin and hence myocardin overexpression is not sufficient to block the *Sm22* repression completely.

IV. SM22α overexpression can reduce expression of inflammatory and ER stress markers

1. Background

SM22α deficiency has been shown to induce inflammation and chondrogenesis in an arterial injury model[22, 23].As SM22α expression downregulates under ER stress, we overexpressed SM22α to see if it could reduce the expression of inflammation, osteogenic or ER stress markers. We also overexpressed myocardin to see if it could reduce the inflammation and osteogenesis.

2. Results

We only saw reduction in the expression of ER stress (*Atf4*, *Chop*) and inflammation markers(*Ccl2*) but no change in the expression of calcification markers **(Fig.18A-B)**. Myocardin induced SM22 expression could only reduce *Vcam-1* expression **(Fig.18C)**. It was not enough to reduce *Ccl2, Atf4* or *Chop* expression.

3. Discussion:

NF-kB has also been shown to repress *Sm22* expression in a CArG box dependent manner[104]. Reduced SM22α works in a loop mechanism to induce inflammation. SM22α is required to form a complex with IKBα to sequester NF-kB. Reduced binding of SM22 to IKBα leads to more nuclear translocation of NF-kB[105].By overexpressing SM22α, we might be reducing the nuclear translocation of NF-kB to some extent and hence we see reduce *Ccl2* expression. Reduction in inflammation probably also lead to less ER stress shown by reduced expression of *Atf4* and *Chop*. Myocardin could only reduce *Vcam-1* expression under ER stress.

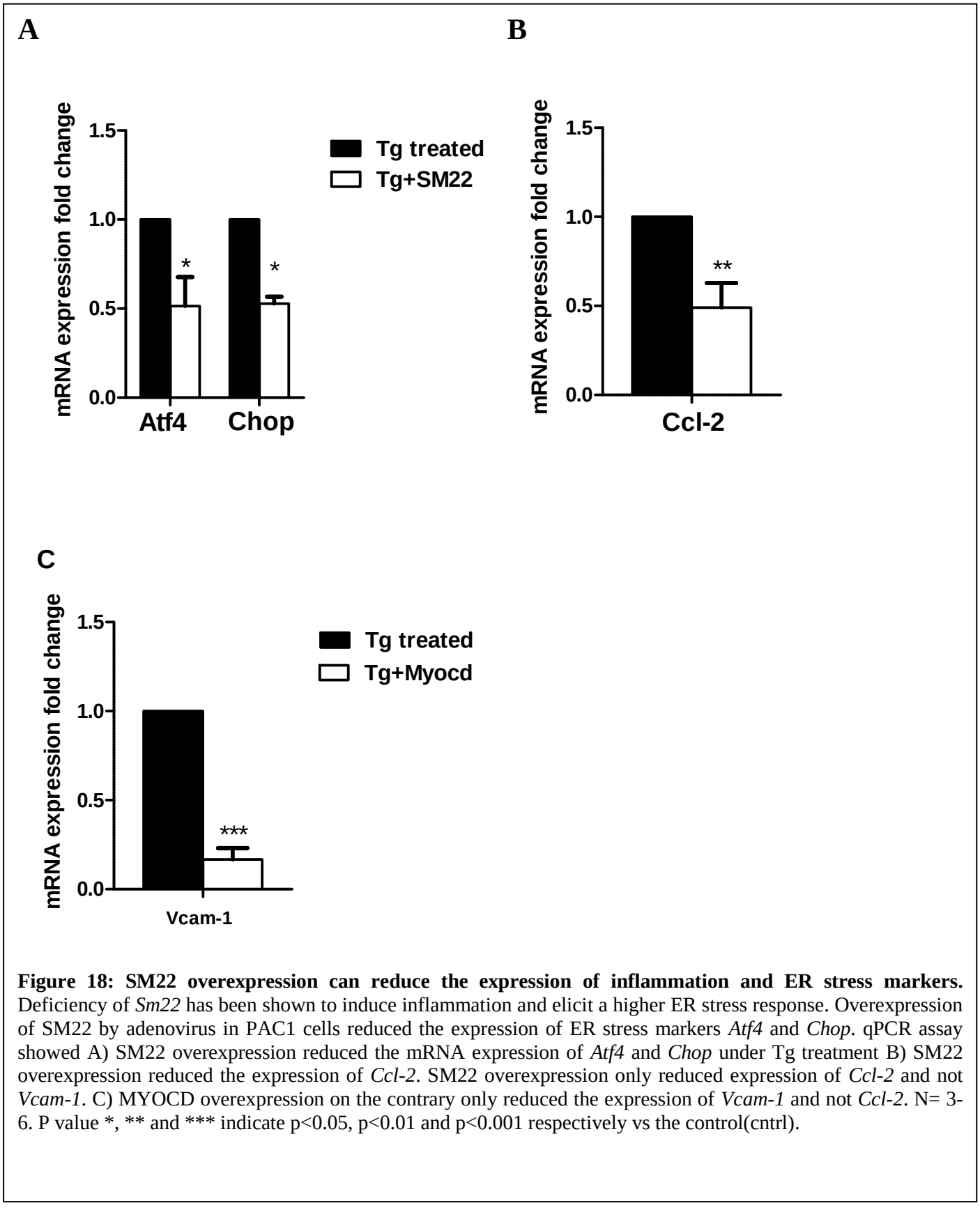


Figure 18: SM22 overexpression can reduce the expression of inflammation and ER stress markers. Deficiency of *Sm22* has been shown to induce inflammation and elicit a higher ER stress response. Overexpression of SM22 by adenovirus in PAC1 cells reduced the expression of ER stress markers *Atf4* and *Chop*. qPCR assay showed A) SM22 overexpression reduced the mRNA expression of *Atf4* and *Chop* under Tg treatment B) SM22 overexpression reduced the expression of *Ccl-2*. SM22 overexpression only reduced expression of *Ccl-2* and not *Vcam-1*. C) MYOCD overexpression on the contrary only reduced the expression of *Vcam-1* and not *Ccl-2*. N= 3-6. P value *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ respectively vs the control(cntrl).

V. Cytoskeletal dynamics regulate the ER stress response

1. Background

Disruption of actin cytoskeleton has been shown to induce NF-kB nuclear translocation and dependent inflammation induction[106, 107].Also *Sm22* deficiency has been shown to collapse actin cytoskeleton[23].Studies show that inflammation could induce ER stress[108].Hence we treated the cells with actin depolymerizer cytochalasin D to see if it induced ER stress. We also observed changes in ECM expression during actin depolymerization with latrunculin B. We treated the cells with CytoD and thapsigargin to see if ER stress influences this interplay between ECM components and the cytoskeletal dynamics.

2. Results

Cytochalasin D was sufficient to induce ER stress and when used in combination with thapsigargin, caused a more severe stress response shown by the expression levels of *Atf4* and *Chop* **(Fig.19 A)**. Depolymerizing actin with CytoD caused upregulation of expression of ECM markers like *Col3a*, *Fbn1* and *Fn1*.This upregulation of ECM proteins is blocked when treated with thapsigargin **(Fig.19 B)**.

3. Discussion:

SM22α has long been studied as an actin binding protein. The earliest known function was to bind actin and help in the formation of cytoskeletal structures like stress fibers[34].Follow up studies showed that SM22 deficiency lead to actin cytoskeleton collapse[23]. This cytoskeleton collapse probably induced inflammation and inflammation dependent ER stress.

Dephosphorylation of eukaryotic translation initiation factor 2a (eIF2a) restores protein synthesis at the end of the adaptive UPR. It requires a PP1 catalytic subunit and a regulatory subunit, PPP1R15A(GADD34)/ G actin[109].Sm22α deficiency is also known to increase the Gactin/Factin ratio[23]. Increased availability of G actin allows the complex formation of GADD34, PP1 and actin

which dephosphorylates elF2alpha. This helps resume the protein synthesis following its inhibition. It is suggested that this might be a pro survival response during transient ER stress but may prove fatal during chronic ER stress[110].We show that cytochalasin D treatment alone is sufficient to induce ER stress. This can be explained by the fact that cytochalasin D depolymerizes actin by competitive binding to the barbed ends. This creates a pool of the G actin and association with the GADD34 [111]. eIF2α is dephosphorylated leading to unresolved protein accumulation and probably a switch to apoptotic UPR

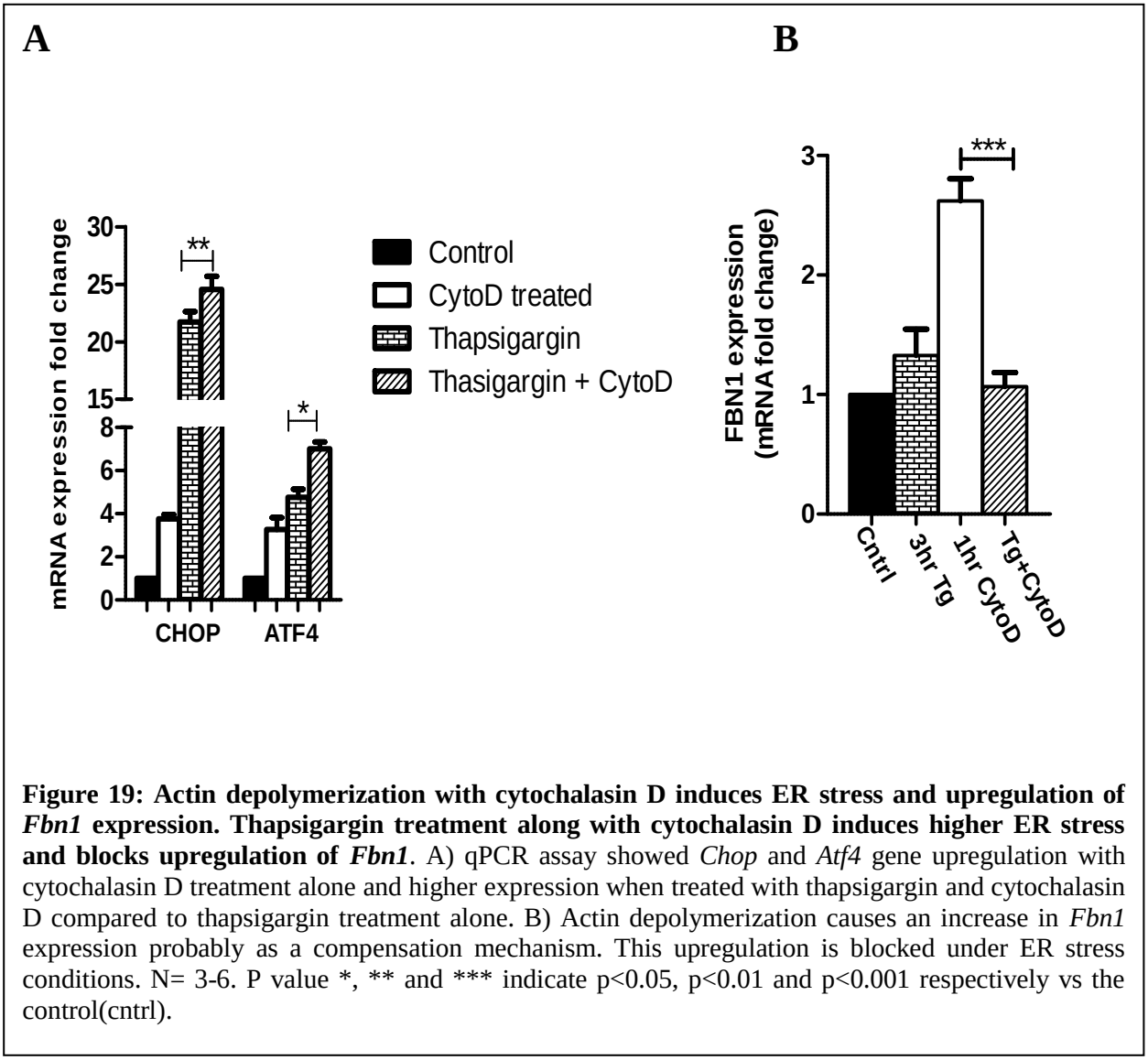


Figure 19: Actin depolymerization with cytochalasin D induces ER stress and upregulation of *Fbn1* expression. Thapsigargin treatment along with cytochalasin D induces higher ER stress and blocks upregulation of *Fbn1*. A) qPCR assay showed *Chop* and *Atf4* gene upregulation with cytochalasin D treatment alone and higher expression when treated with thapsigargin and cytochalasin D compared to thapsigargin treatment alone. B) Actin depolymerization causes an increase in *Fbn1* expression probably as a compensation mechanism. This upregulation is blocked under ER stress conditions. N= 3-6. P value *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ respectively vs the control(cntrl).

VI. Drug screen for natural compounds that can positively regulate *Sm22* expression

1. Background:

As SM22 overexpression showed some reduction in ER stress response and ER stress induced inflammation, hence we screened for natural compounds that could increase SM22 expression. The potential of SM22 as a therapeutic target is also strenghtened by the fact that SM22 expression is downregulated in a number of cancers too.The screen for the compounds was carried out at our collaborator- Andrew Fribley's robotic screen core facility. A repoter cell line using CHO cells transfected with *Sm22* promoter-luciferase was constructed.CHO cells do not express any SM22 endogenously.The candidate compounds from the screen were then validated in our mouse fibroblast cell line 10T1/2, a progenitor for SMCs.

2. Results:

We found a group of compounds called isoflavenoids which were able to induce *Sm22* expression **(Fig.20)**. We then verified those compounds in our fibroblast cell line. The fibroblasts were used for the reporter as they endogenously express low levels of SM22 unlike the SMCs which express very high levels of SM22.This helps detect any small increase in SM22 expression during the drug screen. The cells in the preliminary screen wre treaed with the drug for 16-48 hrs. but the same treatment time in the fibroblast cells did not yield the same result. Subsequent efforts at earlier time points revealed significant and robust increases of *Sm22* between 1–6 hours. The proteins levels were found to be upregulated at the same treatment time as the screen **(Fig.21)**

3. Discussion:

Isoflavones belong to a group of phytoestrogens.Isoflavones are commonly found in soy and red clover. Phyestrogens have been shown to have a cardioprotective effect. Two phytoestrogens biochanin A and formononetin have been shown to inhibit pdgf induced migration in aortic smooth muscle cells.

These among the top hits in our drug screen. We chose to do further analsysis with biochanin A as it was shown to be more potent than other phytoestrogens from our list in some instances[112].

Biochanin A(BCA) is an O-methylated isoflavone. Its found in red clover, soy, alfalfa sprouts,peanuts and in legumes.Biochanin A has been shown to attenuate LPS induced NF-kB activation and dependent expression of VCAM-1 and ICAM-1by activating PPAR-γ[113, 114].

We are proposing that its anti-inflammatory effects could be through the increased expression of SM22. The isoflavones have been shown to inhibit the cytochrome P450[115, 116]. The cytochrome P450 are a superfamily of hemoproteins. They are either located in the inner membrane of the mitochondria or the ER. The enzyme is responsible for metabolising a wide range of endogenous or exogenous chemicals and drugs.There are 18 families of CYP genes. Biochanin A has been shown to reduce the activity of Cyp2e1 in Ccl4 treatment[117]. This is of significance as , *Cyp2e1* gene was found to be upregulated in *Sm22*α-/- mice[118].Hence the isoflavone's therapeutic properties might be SM22 dependent and further analysis could lend more significance to SM22's role in cardiovascular pathologies.

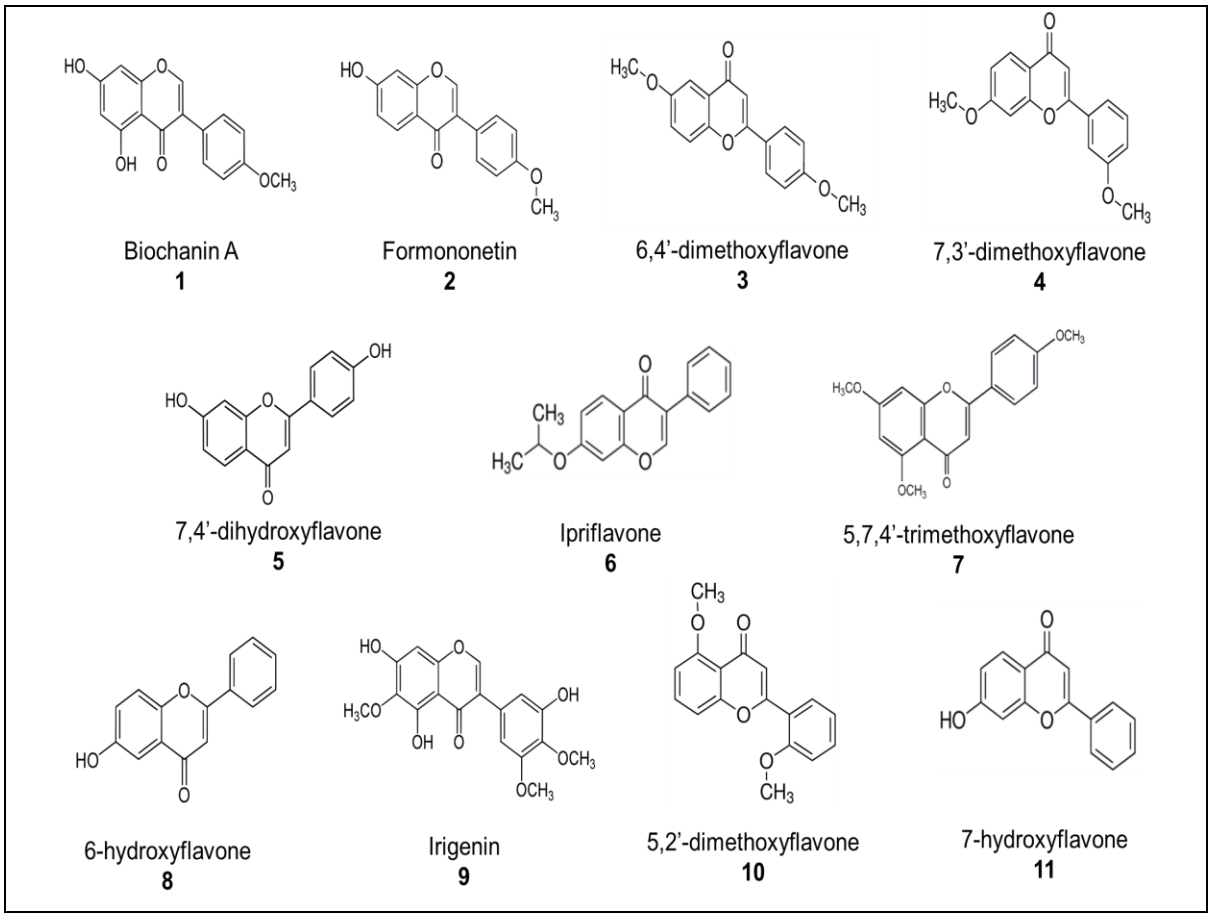


Figure 20: Flavones and isoflavones were the top hits in the drug screen for *Sm22* expression inducing agents. 75 hits were parsed to identify compounds amenable to use in cell culture and preclinical small animal models, with a preference for known utility for use in humans. Eleven flavone compounds were chosen for confirmatory secondary assays based on structural similarity and broad reported use in pre-clinical and clinical human studies. Flavones and isoflavones belong to the class of phytoestrogen and have effects on CYP450 activity. CYP450 are the enzymes that metabolize most of the drugs in the body. Phytoestrogens have been shown to have cardioprotective effect and commonly found in soy and red clover. Biochanin A and formononetin have been shown to have anti-inflammatory effects.

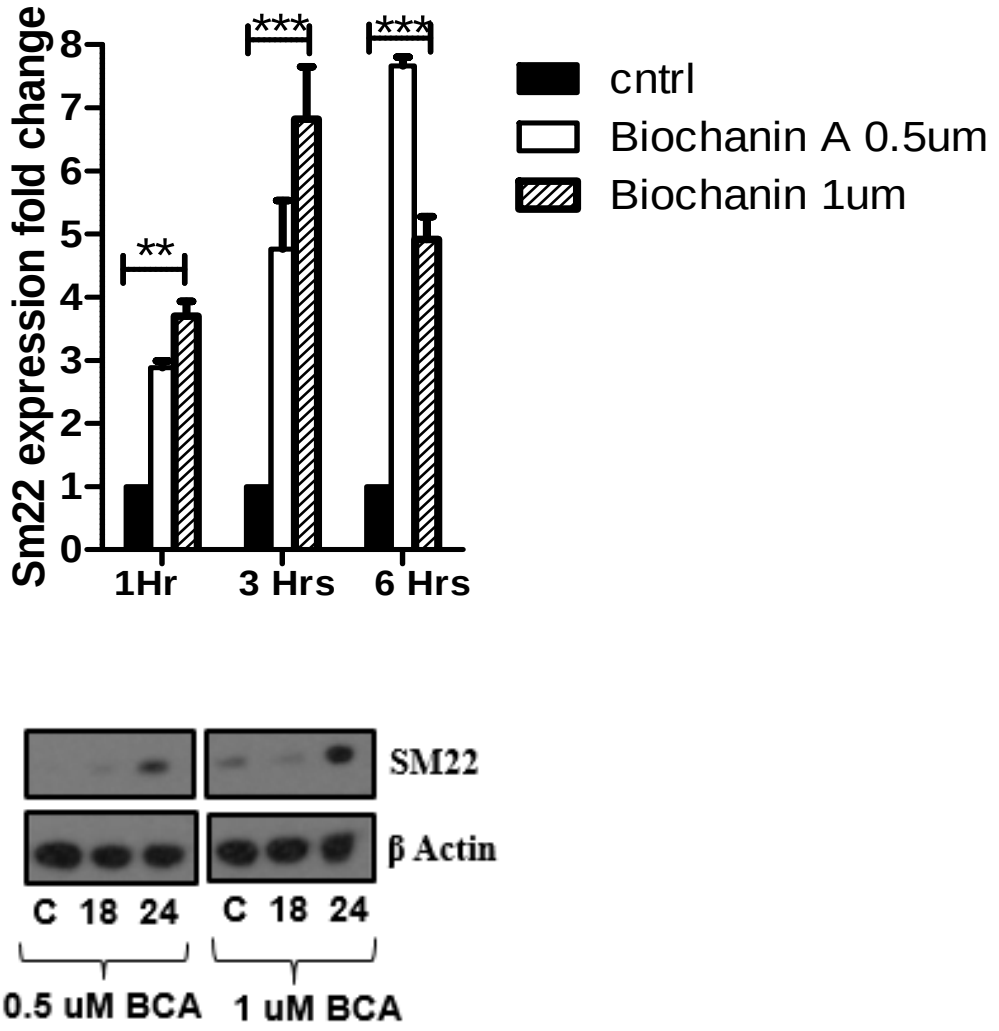


Figure 21: Biochanin A upregulated *Sm22* expression in the fibroblast cell line 10T1/2. The phytoestrogen biochanin A (BCA) was chosen for further scrutiny with experiments focused to validate the ability of the primary CHO-SM22-luc HTS assay to identify hits able to modulate SM22 gene and protein expression. (A)10T1/2 cells were treated with 0.5 or 1.0 uM BCA for 16 – 48 hours (same as the HTS) without significant increase in *Sm22* gene expression. Subsequent efforts at earlier time points revealed significant and robust increases of *Sm22* between 1–6 hours. (B)Western immunoblot analysis during the same time frame demonstrated that BCA could induce SM22 in a time- and dose-dependent fashion. N= 3-6. P value ** and *** indicate $p<0.01$ and $p<0.001$ respectively vs the control(cntrl).

CHAPTER 3 -MATERIALS AND METHODS

3.1 Tissue culture

Rat pulmonary smooth muscle cell line PAC1 was used for all experiments except for the drug screen. For the drug screen validation mouse fibroblast cell line 10T1/2 (ATCC C3H/10T1/2,clone 8,CCL-226) was used.The cells were grown and maintained in DMEM (Invitrogen,11995073) supplemented with 10% Fetal bovine serum (FBS,Invitrogen, 26140079) , antibiotics pencillin and streptomycin (P/S,Invitrogen 15070063) and fungicide (Invitrogen, 15290026) were used at a final concentration of 1%.The cells in general were grown for 24-48 hrs before any drug treament.PBS (#10010049) and Trysin (#25200072) were ordered from Invitrogen.For transfections ,serum free medium,opti-MEM (Invitrogen # 31985062) was used.

Cell passaging and freezing – Cells in T75 flask were washed with PBS. 1ml trypsin is added and put in the 37C cell culture incubutor for 3mins. The trypsin is inactivated by adding medium. The cells are then split into new flasks.For freezing,the cells are centrifuged and the supernatant discared. Cells from a T75 flask are resuspended in 2-3 ml of cell recovery freezing medium(Fisher # 12-648-010)and aliquoted into labelled freezing tubes and placed in the cell freezing box at -80C. To thaw, the cells are placed in a 37C water bath for 1 min and then warmed medium is added to the vial. The cells are then put in the cell culture flask and allowed to attach overnight.

3.2 Genotyping and Aorta isolation

C57BL/6 mice were used for the *Sm22* knockout colony.The *Sm22* gene is knocked out using LACZ insertion.Mouse toe tissue was used to genotype the mice. The tissue was digested overnight in 100ul of digestion buffer(Viagen,#102-T) with 4ul (20mg/ml) of proteinase k (501-PK) at 37C. The proteinase K is inactivated by heating the sample on a heating block at 95C for 15 mins.The DNA sample is stored at -20C. For genotyping, a reaction mix for a single sample is made using 10ul of red master mix (ASI # C225), 1ul DMSO, 0.4ul of forward primer, 0.4ul of reverse primer and 8.2 ul of water.To

this 2.5 ul of digested DNA is added.The primer sequences for genotyping are mentioned in the table 1.The PCR conditions sre as follows: for *Sm22*: 94C – 3:00 mins, 94C – 0:30 min, 52C- 0:30, 72C- 0:40, 39 cycles and then 72C for 10:00 mins. *LacZ*: 94C – 3:00 mins, 94C – 0:30 min, 58C- 0:30, 72C- 1:00, 39 cycles and then 72C for 10:00 mins.

Table 1 : Primer sequences used for genotyping

SM22 forward	CCCAGCCCAGACACCGAAGCTAC
SM22 reverse	TCCCTTGGCCTCATTTGTCACCTC
LacZ forward	TACCACAGCGGATGGTTCGG
LacZ reverse	GTGGTGGTTATGCCGATCGC

3.3 Protein isolation

The protein from cells was isolated using the reagent M-PER from (Thermofisher#78501).The cells are washed twice with PBS and then M-Per reagent was added. Protease (Thermofisher#78429) and phosphatase inhibitors(Fisher, #PI78443) were added to the M-Per reagent at 1:100 dilution.The cells are incubated on ice on a rotor for 5 mins. The cells were then scraped and the lysate collected.The lysate was then incubated in ice while shaking for 20-40 mins.The lysate was then centrifuges at ~14000g for 10mins.The supernatant was transferred to a new tube and stored at -20C.

3.4 Protein quantification

The protein quantification was done using the Quant-iT™ protein quantification kit (Fisher#Q33212) and qubit benchtop fluorometer from Fisher# Q32857.The pre-diluted BSA standards provided in the kit were used. A working solution is prepared by diluting (1:200) the Quant-iT reagent in Quant-iT buffer provided in the kit.1 ul of protein sample is added to 199ul of working solution , vortexed and incubated for 15 mins. 10ul of standards us added to 190ul of working solution.Readings are taken in the fluorometer.

3.5Western blot

Equal amount of whole cell lysates from PAC1 cell or 10T1/2 samples were loaded on a 4-12% Bis-Tris NuPAGE Mini-gel (Invitrogen# NP0321) for electrophoresis, followed by transfer onto an Immobilon-P membrane (Millipore # IPVH00010). The running (# NP0001) and transfer buffer (#NP 00061) from Invitrogen was used. The buffers were used at a dilution of 1x. The membrane was blocked with 5% milk (Fisher# NC9121673) in TBST for 1 Hr., followed by primary antibody (diluted in milk) incubation overnight at 4 °C. The membrane is washed 3 times in TBST for 5 mins and then incubated with biotinylated secondary antibody for 2 Hrs. The membrane is washed in TBST again 3 times for 5 mins. The membrane was subject to enhanced chemiluminescence detection using Super Signal West Pico Chemiluminescent Substrate (Thermo # 34080). The primary antibodies used are listed in the Table 2.

Table 2. Antibodies used for Western

Gene	Company	Catalog No.	Dilution
Sm22	Abcam	ab14106, ab10135	1:1500
Sma	abcam	7817	1:2000
Klf4	Sigma	HPA002926	1:250
Atf4	Santa Cruz	Sc-200	1:200
Chop	Santa Cruz	Sc-7351(WB), sc-793(IHC)	1:200
B actin	Cell signaling technology	4967S	1:1500
Secondary ab			
Anti-mouse	Southern biotech Santa cruz	1070-05(for sc 7351) Sc 2962	1:2000

Anti-goat	Santa cruz	Sc 2961	1:2000
Anti-rabbit	Santa cruz, sigma	Sc 2955, SAB3700941	1:2000

3.6 RNA isolation

RNA isolation for the cells was carried out using the Direct-zol RNA Miniprep kit from Zymo research(#R2073). The cells were treated with trizol and buffers from the kit are used to carry out the subsequent steps. For cells grown in a 6 well plate 800ul of trizol was added and incubated for 5 mins. The trizol was collected and added to DNase/RNase free tubes with 800ul of 100% ethanol and mixed well. The solution was then added to the columns provided in the kit. The columns were centrifuged at 16000g for 30sec (for all steps except where mentioned). The columns were then washed with 400ul of RNA wash buffer and centrifuged. The column was then treated with DNase I (75ul of digestion buffer + 5ul of DNase I) and incubated at room temperature for 15 mins.400ul of directzol buffer was then added to the column and centrifuged and this step was repeated. 700ul of RNA wash buffer was then added to the column and centrifuged for 2 mins.50 ul of DNase/RNase free water was added directly to the column to elute the RNA. The RNA concentration was determined using the nanodrop ND-1000 spectrophotometer and stored at -80C. A sample was considered pure enough for experiments if the absorbances at 260/280 and 260/230 ratio were at least 1.8 and 1, respectively.

For ex vivo organ culture, the tissue was homogenized using a homogenizer. The tissue was either used fresh or stored in RNA later solution (Ambion# AM7021) and RNA isolation was carried out at a later time point.

3.7 cDNA preparation

500ng -1000ng of RNA was used to make the cDNA. The cDNA was synthesized using the Superscript II reverse transcriptase (18064014), Random primers (48190011) and buffers from Invitrogen.2ul(3ug/ul) of random primer and 2ul of dNTP mix (10mM each, Invitrogen #18427013) was

added to 500-1000ng of RNA and volume made up to 23ul with distilled water. The mixture was heated to 65C for 5 mins and quick chilled in ice. The contents were briefly centrifuged. 8 ul of 5x buffer and 4ul of 0.1M DTT was added. The contents were mixed gently and incubated at room temperature for 2 mins. 200U of superscript was added. It was incubated at 25C for 10 mins. The mixture was then heated at 42C for 50mins. The reaction was inactivated by heating at 70C for 15mins.

3.8 Quantitative PCR

Real-time PCR was performed using SYBR Green (Invitrogen # 4385616) on a StepOne Plus system (Applied Biosystems). Gapdh and snRNA U6 were used as internal controls in ΔΔCt method. The primers were ordered from IDT. The sequences for the primers for mouse and rat are listed in Table 2.A total of 10 ul reaction mixture is added to each well.5ul of SYBR and 4ul of primer(2.5uM). To this 1ul of cDNA made from 500-1000ng of RNA is used. The reaction run had 3 stages- Holding stage- 95C for 20sec, cycling stage- 95C for 3 sec, 60C for 30 sec repeated 40 times and Melt curve stage – 95C for 15sec and 60C for 1 min.

Table 3 Primer sequences for RT-PCR

Gene	Forward (5'-3')	Reverse (5'-3')
RAT		
Sm22	TCCTTCCAGCCCACAAACGACCAA	CTTGGACTGCACTTCACGGCTCAT
Acta 2	GAGAAGCCCAGCCAGTCG	ATCTTTTCCATGTCGTCCCAGTTG
Cnn1	GCGTCACCTCTATGATCCCAAACT	GTTGAGCGTGTCACAGTGTTCCAT
Myocd	CAGTGAAGCAGCAAATGACTCGG	GTCGTTGGCGTAGTGATCGAAGG
Flna A	ACTGTTTCTAGCCTTCAGGAG	GCACAGCATACTTATCTTGGTC
Vim	CGGAAAGTGGAATCCTTGCA	CACATCGATCTGGACATGCTGT
Tubb	GCAGTGCGGCAACCAGAT	AGTGGGATCAATGCCATGCT

Tagln 2	ATTGAGAAGCAGTACGACCCAGAT	AGAGGCCTGGATCTTCTTTACTGG
Myh11	AACGCCCTCAAGAGCAAACTCAGA	TCCCGAGCGTCCATTTCTTCTTCA
Atf4	GGGTTCTGTCTTCCACTCCA	AAGCAGCAGAGTCAGGCTTTC
Chop	CCTAGCTTGGCTGACAGAGG	CTGCTCCTTCTCCTTCATGC
Klf4	AGGCACACCTGCGAACTCA	ACAGCCGTCCCAGTCACAGT
Pdgf-bb	CGCACAGAGGTGTTCCAGATC	CCAGGAAGTTGGCGTTGGT
Mmp9	TCTTCGACTCCAGTAGACAATCCT	ACTTCCAATACCGACCGTCCTTGA
Grp78(Bip)	TCCTGCGTCGGTGTATTC	CGTGAGTTGGTTCTTGGC
Calr	GAATACAAGGGCGAGTGGAA	GGGGGAGTATTCAGGGTTGT
Runx2	CAGGTTCAACGATCTGAGATTTGT	TGAAGACCGTTATGGTCAAAGTGA
Msx2	GCCATTTTCAGCTTTTCCAG	CCCTGAGGAAACACAAGACC
Ocn	AAGCCCAGCGACTCTGAGTCT	GCTCCAAGTCCATTGTTGAGGTA
Pit-1	CCGTCAGCAACCAGATCAACTC	CCCATGCAGTCTCCCACCTTG
Vcam-1	TGTGAAGGGATTAACGAGGCTGGA	GCACACTTCCACAAGTACAGGAGA
Ccl2	CAGTTAATGCCCCACTCAC	GTTTCTGATCTCACTTGGTTCT
Mouse		
Sm22	TCCTTCCAGTCCACAAACGACCAA	TTTGGACTGCACTTCTCGGCTCAT
Same for mouse and rat		
Gapdh	TGAATACGGCTACAGCAACAGGGT	TTGTGAGGGAGATGCTCAGTGTTG
U6	CTCGCTTCGGCAGCACATATACTA	CGCTTCACGAATTTGCGTGTCATC

3.9 Immunoflorecence

PAC1 cells attached to chamber slides were rinsed twice with PBS and liquid removed by gentle aspiration. The control and treatment groups are on the same slide. The cells were then fixed in -20C methanol for 5 mins. The cells were washed with PBS 3 times for 5 mins. The cells were then blocked and permeabilized using the blocking solution (1ml chicken serum, 20ul of Triton X-100(Fisher #BP151-500) and 9ml PBS, vortexed until dissolved) for 1 hr. at 37C.The primary antibody was diluted in 5% serum at a dilution of 1: 100.The cells were then incubated 1-2 hrs. at 37C in a moist chamber to prevent drying. The cells were then washed 3 times in PBS for 5 mins, then 2 times with 10% serum. The cells were then incubated with secondary ab diluted in 5%serum at a dilution of 1:200 for 1hr at 37C in the moist chamber. The cells are then washed 3 times with PBS for 5 mins. Cover slips were mounted with Vectashield with DAPI (Vector labs# H-1200). The images were captured using the Nikon Eclipse Ti - inverted microscope.

3.10 Immunohistochemistry

The slides with OCT (VWR, #102094-106) sections was first air dried for 5 mins. The sections were fixed in pre-chilled methanol containing 0.3%H_2O_2 for 10 minutes at -20 °C. Its then washed in PBS for 5 mins. The sections are incubated for 20 minutes with diluted normal blocking serum which was prepared from the species in which the secondary antibody is made. Blot the excess serum from the section. sections are incubated for 30 minutes with primary antiserum diluted in PBS. The slides are then washed in PBS for 5 mins. The sections are then incubated with biotinylated secondary antibody for 30 mins. The slide is then washed in PBS for 5 mins. The slide is the incubated for 30 minutes with VECTASTAIN® ABC Reagent (Vector labs, PK-6100). The reagent is made and let to stand for 30 mins before use. The slides are then washed in PBS for 5 mins. The sections are then incubated in peroxidase substrate for until desired intensity is achieved (The substrate solution should be made just before use). slides are then immersed in tap water for 2 minutes to get rid of PBS residue, otherwise you may get high

pink background after hematoxylin counterstaining. Its counterstained with Gill's formula hematoxylin for 10 to 20 seconds. slides are then immersed in bluing solution for 1 minute (Bluing solution: 1.5 ml NH4OH (30% stock) + 98.5 ml of 70% ethanol), then rinsed in tap water. Immerse into 100% ethanol for 5 minutes and airdry. Then in xylene for 6 mins and air dry. The coverslip is mounted using permount mounting medium. Both positive and negative controls are one single slide.

3.11 TUNEL assay

The TUNEL assay was performed using the DeadEnd colorimetric TUNEL system from Promega (# G7130). Both the control and test sample sections are located on the same slide. The slide is immersed in 0.85% NaCl for 5 mins at room temperature. The slide is then washed in PBS for 5 mins at RT. The sections are then fixed in 4% paraformaldehyde for 15 mins at RT. The slides are immersed in PBS for 5 mins and repeated.100ul of 20ug/ml proteinase K is added to the tissue sections and incubated for 30 mins at RT. The slide is washed in PBS for 5 mins at RT. The slide is re-fixed and immersed in PBS for 5 mins. The sections are then incubated in the equilibration buffer for 10 mins at RT. The biotinylated nucleotide mix is thawed on ice and the rTdT reaction mixture is prepared. Each section was covered in 100ul of reaction mixture (98ul equilibration buffer+ 1ul nucleotide mix+1ul rTdT). For negative controls, the rTdT is replaced by deionized water. The slides are incubated in a humidified chamber at 37C for 1hr.The reaction is terminated by immersing it in 2x SSC for 15 mins at RT. The slide is washed in PBS for 5 mins,3 times to remove unincorporated biotinylated nucleotides. Endogenous peroxidases are blocked by immersing the slide in 0.3% hydrogen peroxidase solution. It is washed in PBS for 5mins 3 times. The streptavidin solution is diluted in PBS 1:500 and 100ul is added to each section. The slide is incubated for 30mins.the slide is then washed in PBS for 5 mins ,3 times each. The DAB solution should be prepared just before use and kept away from light.50ul of DAB buffer is added to 950ul of ionized water.50ul of chromogen and 50ul of hydrogen peroxidase is then added to the buffer solution.100ul of

this solution is added to each section. The sections are incubated until the desired intensity is achieved. The slide is rinsed in water several times and cover slip is mounted.

3.12 Adenovirus Transfection

Adenovirus was grown in HEK 293 T cells and harvested. The cells were grown in 10cm dishes and let to reach a confluency of 80%. Once the cells had reached the desired confluency, they were infected with the virus. The virus positive cells were positive for GFP. The cells were monitored and expanded when enough GFP signal was detected(~48 hrs).The virus was expanded in a similar manner to 5 – 10cm dishes.For harvesting, the cells were collected in a 50ml conical tube.They were pelleted at 1000rpm for 10mins.The supernantant was discarded.The cells were rinsed in PBS and pelleted at 1000rpm for 10mins. The cells were resuspended in 10mM Tris, pH 8.1(1ml for each 10cm dish).The cells were then lysed in ice/water bath at 37C. This step was repeated a total of 3 times.It was then centrifuges at 12000rpm for 10 mins.The supernatant is discarded. The virus is stored at -70C.The virus was then used to infect PAC1 cells. Once the cells gave desired GFP signal, the cells were treated with Thapsigargin and RNA isolated.

3.13 Plasmid transfection for MYOCD overexpression

PAC1 cells were grown in 6 wells plates. Cells were plated at a conc. of $10x10^4$ cells/well. They were transfected once they reached 80% confluency. Lipofectamine and Plus reagents (Invitrogen) were used.1 ug of plasmid DNA was used to transfect cells in each well of the 6 well plate. Plasmid DNA was diluted in 210 ul of Opti-mem and 6.3 ul of plus reagent. It is necessary to dilute DNA in medium before the reagent to avoid DNA precipitation. In a second tube 4.2ul of lipofectamine is added to 210 ul of media. The tubes are incubated for 15 mins at room temperature. The contents of the two tubes are mixed and incubated for an additional 30mins at room temperature. During the incubation, the cells medium is removed and washed with serum free medium and 1ml of Opti-mem is added to the cells. The transfection mix is then added to the wells and put at 37C in the incubator. After 3 hrs. the medium is

changed to normal medium (10% FBS). RNA was harvested 72hrs after transfection. Thapsigargin (1uM) was added to the treatment wells 24 hrs. before RNA isolation.

3.14 Plasmid transfection for Luciferase assay

PAC1 cells were grown in 96 well plates. The cells were plated at a conc. of $7.5x10^3$/well. They were transfected once they reached 80% confluency. Lipofectamine and Plus reagents (Invitrogen) were used. For the renilla luciferase pRL-CMV vector from Promega (Cat# 2261) was used as internal control. The different promoter mutations are detailed in the publication from our previous lab member[100].In tube one, to 10ul of media 1ul of plasmid(50ng/ul), 1ul of renilla plasmid(1ng/ul) and 1ul of plus reagent is added. In tube two, 10ul of media and 0.5ul of lipofectamine is added. After an initial incubation of 15 mins at RT, the contents of the two tubes are mixed and incubated for an additional 15 mins. The reaction mixture of total 24.5ul is added to each well. After 3 hrs. of incubation another 125.5ul of normal 10% FBS medium is added to the wells. Thapsigargin (1uM) was added to the treatment wells 24 Hrs. before the assay.

3.15 Luciferase assay

The dual luciferase reporter assay system from Promega (Cat# E 1960) was used. All reagents are thawed at room temperature for at least 1 hr. 5x passive lysis buffer was diluted to 1x using distilled water. Once the cells in 96 well plates are ready after transfection, the cells are washed with PBS and 22ul of passive lysis buffer was added to each well. The plate was put on a shaker for 15 mins. 20ul of the lysate is transferred to a white 96 well plate to be read in the luminometer. In this assay the activities of firefly (*Photinus pyralis*) and *Renilla* (*Renilla reniformis* or sea pansy) luciferases are measured sequentially from a single sample. The firefly luciferase reporter is measured first by adding Luciferase Assay Reagent II (LAR II) to generate a luminescent signal. After quantifying the firefly luminescence, this reaction is quenched, and the *Renilla* luciferase reaction is initiated simultaneously by adding Stop & Glo® Reagent to the same sample. The firefly luciferase value was normalized using the renilla luciferase

value. The lyophilized luciferase assay substrate was resuspended in 10ml of luciferase assay buffer II. The 50x stop and glo substrate was diluted to 1x using the stop and glo buffer. Both the final reagents were used to prime the tubing of the luminometer before the sample was read. Veritas 96 well luminometer was used to read the plate.

3.16 Chemicals

Thapsigargin (Sigma # T9033) was diluted in DMSO and 10uM stock solution was prepared. Thapsigargin was used at a conc. of 1uM. Cytochalasin D (Sigma # C8273-1mg) was dissolved in DMSO and used at a final conc. of 1uM. Palmitate solution was made by conjugating it with BSA. Palmitic acid (#P0500-10g) and BSA(#A8806-1g) was ordered from Sigma. BSA was dissolved in DMEM to give 10%FFA BSA. Palmitic acid was dissolved in ethanol to make 200mM stock solution. 40ul of palmitate and 1960ul of 10%FFA-BSA was mixed to make a 4mM stock and incubated overnight at 37C to conjugate.

3.17 High Throughput Screening (Performed by Dr. Andrew Fribley's lab)

Cell lines and reporters- CHO-K1 cells (ATCC, Manassas, VA) were grown in F-12 medium with glutamax (# 31765-035, Life Technologies, Carlsbad, CA), supplemented with penicillin and streptomycin in a standard tissue culture incubator at 37 °C with 5% CO2. A pool of cells was transfected with pSM445GL4.21luc2P-puro (SM22-luc) and selected with 1.0mg/ml of puromycin for ten days. For screening, a single high-expressing clone that provided Z' values = 0.7 was identified from the pool. Screening - 5000 CHO-SM22-luc reporter cells were plated onto white 96-well tissue culture plates and incubated for 16 hours (overnight) at 37 °C, 5% CO2, prior to compound addition. Compounds from the MicroSource Spectrum Collection, containing ~2400 biologically well-characterized small molecules and natural products including many FDA-approved drugs dissolved at 10mM in DMSO, were added using a high-density replication (HDR) tool on a Biomek FX liquid handler (Beckman Coulter, Brea, CA) in a 0.05ul volume (DMSO concentration = 0.1% in all wells). All compounds were tested as singletons at

final concentration of 10mM. 0.3 uM panobinostat (Pano) or an equal volume of DMSO was each added to one row of wells on each plate for assay controls. Sixteen hours later the medium was aspirated to 25 ul and 25 ul of Bright-Glo (E2620, PromegaCorp, Madison, WI) was added to each well and allowed to come to equilibrium at room temperature for 10 minutes. Luminescence was measured on a Flexstation 3 multi-mode plate reader (Molecular Devices, Sunnyvale, CA). A hit was defined as a compound able to induce luciferase to a level ≥120% of the panobinostat control.

3.18 Statistical analysis

Data analysis was carried out in the software GraphPad Prism. Graphs show mean with SEM. Where appropriate t-tests or one-way ANOVA with Tukey's or Dunnett's post hoc tests were performed. Results were considered statistically significant when $p<0.05$. Statistical significance is indicated with asterisks: * denotes p between 0.05 and 0.01, ** denotes p between 0.01 and 0.001, *** denotes $p<0.001$.

DISCUSSION

Most of the stress signaling mechanisms in the cell are meant to restore homeostasis by diverting resources to the injured or stressed area. In many cases though, the signaling due to several reasons might become deleterious and the cells need to switch to a damage control mode. The smooth muscle cells inhabiting the vessel wall undergo wear and tear due to mechanical stress and various environmental stimuli. As a result, various signaling pathways are activated such as inflammation, oxidative stress and cyclic strain signaling. When the cells become overwhelmed and need to stop further damage, they signal for apoptosis. Apoptosis when unchecked can lead to loss of tissue viability and irreversible functional damage. Hence it is necessary to understand the triggers and factors leading to apoptosis under various stress mechanisms. Among the various stress responses in the VSMCs, ER stress seems to be one of the lesser studied mechanisms. Its activation in a wide range of cardiovascular diseases, warrants the need for further analysis.

Presence of ER stress (ATF 4 and CHOP) and apoptosis was established in our mouse model of genetic stress induced aortic aneurysm. *Sm22* deletion was made in the Marfan (MFS) mouse to generate a *Fbn1*$^{C1039G/+}$: *Sm22*$^{-/-}$ mouse model. SM22 deficiency has been shown to cause decreased vascular contractility but otherwise have a normal development. SM22 deficiency by itself does not have any significant impact on aneurysm formation. Excessive TGFβ signaling has been shown to contribute to aneurysm formation in MFS mice. Deletion of *Sm22* in the Marfan mice exacerbated the aneurysm formation. Apoptosis of smooth muscle cells is thought to be the primary reason for smooth muscle cell loss in aneurysm[73, 74].Consistent with these studies we show presence of ER stress induced apoptosis in our mouse model. This also points to the association of ER stress with aortic aneurysm irrespective of the etiology. The results also highlight a potential protective role of *Sm22α* under stress. It suggests that under quiescent conditions *Sm22α* might be compensated but it may play an active role in stress conditions.

The PERK arm of the ER stress signaling is thought to be the apoptotic arm of the stress process. PERK-dependent phosphorylation of eIF2α enhances ATF4 protein levels during ER stress. ATF4 is known to induce the pro-apoptotic factor CHOP. Microarray studies have shown that CHOP is the highest inducible gene during ER stress. CHOP protein undergoes phosphorylation at Ser78 and Ser81 by p38 MAP kinase family. Phosphorylation enhances transcriptional activation and elicits a maximal apoptotic effect of CHOP[119]. Deletion mutant analysis of CHOP revealed that bZIP domain is important for CHOP-induced apoptosis[120, 121].

The effect of ER stress induced signaling in smooth muscle cells was studied. The smooth muscle cell line PAC1 was treated with a pharmaceutical ER stress inducer thapsigargin. Thapsigargin has been established to induce ER stress dependent apoptosis. Thapsigargin induces ER stress by inhibiting SERCA and causing ER store depletion of calcium. SM22α is an actin binding cytoskeletal protein.SM22α and SMA are characteristic markers for smooth muscle phenotype. The cytoskeletal proteins work in tandem with the extracellular proteins to maintain the mechanical and functional integrity of the vessel wall. The expression of several cytoskeletal, smooth muscle and ECM genes with thapsigargin treatment was observed. At 24 hrs. treatment point significant apoptosis was observed. Hence 24hrs was chosen as the maximum treatment time. All the smooth muscle, cytoskeletal and ECM genes observed were found to be downregulated at the 24 hrs. treatment time. A time course analysis of *Sm22* and *Sma* showed a transient upregulation at 1-6 hrs. treatment time before downregulation. This increase in the expression could be a compensatory mechanism of the adaptive phase of the ER stress. Studies have shown that the ER depends on the actin cytoskeleton for their morphology and functions like ER-Golgi trafficking[122]. These aspects of the ER play a vital role in the ER-UPR.

Thapsigargin treatment also results in upregulation of ER stress chaperones calreticulin and *Grp78*(Bip). Calreticulin (CRT) is shown to mediate ECM production by TGFβ in fibrotic conditions[123]. As mentioned before excessive TGFβ signaling is present in aneurysm of Marfan mouse.

ER stress induced CRT might contribute to excessive amounts of ECM deposition, a characteristic of synthetic phenotype of VSMCs.

The ER stress also induced transcription factors like *Klf4*, *Pdgfbb* and metalloproteinase like *Mmp9*. These markers contribute to the switch from contractile VSMCs to the synthetic phenotype. The VSMCs in the synthetic state secrete increased amounts of extracellular matrix components to repair the vessel wall integrity. Another hallmark of the synthetic state is loss of contractile markers. The phenotypic modulation though in most cases proceeds to apoptosis. The apoptosis of the smooth muscle cells leads to loss of cells in the vessel tissue which leaves it more prone to damage. The phenotypic marker *Pdgf-bb* is known to induce expression of *Klf4* through Sp1 binding to the *Klf4* promoter[124]. Traditionally, *Klf4* is known to repress the transcription of smooth muscle markers. KLF4 is known to repress the expression of smooth muscle markers through several mechanisms. KLF4 binds the CArG box in the *Sm22* promoter replacing the *Sm22* inducing SRF-MYOCD complex. It also recruits HDAC2 to block the SRF complex from binding the promoter. KLF4 also represses the expression of smooth muscle master regulator MYOCD. Recent studies however show that the post translational modification of *Klf4* determines its function as enhancer or repressor. The non SUMOylated *Klf4* is known to induce the expression of smooth muscle genes whereas the SUMOylated form represses the SM gene expression[125]. This is probably why we see an increase in the smooth muscle genes accompanied by increase in *Klf4* expression

We checked for parallel signaling mechanism that were induced due to ER stress and ones that could also induce phenotypic modulation. We found that the inflammation markers *Vcam-1* and *Ccl2* were upregulated. *Vcam-1* is a cell adhesion molecule. Cell adhesion molecules are transmembrane glycoproteins that mediate cell-cell and cell-extracellular matrix interactions. *Ccl-2* is a chemokine. Chemokines are a superfamily of secreted proteins involved in inflammation and immunoregulation. In cardiac cell *Ccl-2* manifests a protective effect by induction of ER stress chaperones. Prolonged exposure

to CCL-2 breaks down the protection and results in heart failure[126, 127]. Hence a pre-existing inflammation may trigger ER stress as a repair mechanism but may turn pathogenic. The inflammation - ER stress loop results in extreme response mechanism and ultimately apoptosis. Along with inflammatory markers, osteogenic markers were also found to be upregulated. The various osteogenic markers were *Runx2, Msx 2, Ocn* and *Pit-1*. *Runx2* is a transcription factor whose targets include *Ocn*. *Msx2* is also a transcription factor contributing to the vascular calcification. *Runx2* and *Msx2* regulate expression of many ECM genes. *Pit-1* is a sodium dependent phosphate transporter whose expression along with *Runx2* and *Ocn* is mediated by the ER stress induced transcription factor *Atf4*. Spliced XBP1 is also known to mediate *Runx2* expression[128]

As discussed earlier all these inflammatory and osteogenic markers contribute directly or indirectly to the repression of the contractile markers. Mutational analysis of the *Sm22* promoter confirmed that many of the cis acting elements in its promoter are involved in its downregulation under ER stress. We hypothesized that the downregulation of SM22 contributed to more severe inflammation and ER stress as part of the loop mechanism. This was based on published data from our previous lab members[22] and confirmed by independent studies[105]. These studies showed SM22 deficiency caused inflammation and oxidative stress. We overexpressed SM22 by adenovirus under the ER stress condition and found that it could reduce the expression ER stress and inflammation markers.

Myocardin is known to be the master regulator of smooth muscle genes and myocardin itself is downregulated under ER stress. We overexpressed myocardin to see if it would rescue the SM22 downregulation and dependent pathogenic signaling. Myocardin reduced the downregulation of SM22 in all the promoter mutants. Myocardin overexpression reduced *Vcam-1* expression but not *Ccl-2* or the ER stress markers.

Another aspect of the regulation of contractile markers is the cytoskeletal dynamics. Apart from the gene expression, the state of the contractile protein also plays a key role in the signaling process.

Sm22 deficiency can collapse the actin cytoskeleton and induce oxidative stress. We used an actin depolymerizer cytochalasin D to treat the cells. We found that depolymerizing the actin cytoskeleton was sufficient to induce ER stress. The oxidative stress induced due to the actin cytoskeleton collapse could be the trigger for inducing ER stress. Adding the depolymerizers along with thapsigargin leads to a higher stress response. Another contributing factor is the availability of G-actin. G actin can bind to factors like PP1/GADD34 complex to dephosphorylate elF2α and resume global protein translation prematurely. This leads to termination of the adaptive phase of the response before the problem has been resolved causing higher stress.

We show that SM22 plays a protective role in the ER stress process and upregulating its expression might help downregulate other pathogenic signaling like inflammation and osteogenesis. A drug screen was therefore carried out to identify compounds that could increase *Sm22* expression. The compounds that scored the highest in the drug screen belonged to the group of phytoestrogens called isoflavonoids and its metabolites. This class of compounds is known to repress the expression of cytochrome P450.*Sm22* deficiency is also known to increase cytochrome P450 expression. Hence it could be a potential mechanism through which flavonoids increase *Sm22* mechanism. Biochanin A, a phytoestrogen was chosen to validate the result in the fibroblast cell line, a SMC precursor. Biochanin A upregulated *Sm22* expression at gene and protein level. The screen is strengthened by studies that show the anti-inflammatory role of biochanin A against LPS induced inflammation[113]. Hence *Sm22* might be a potential drug target to attenuate the pathogenic effects of ER stress.

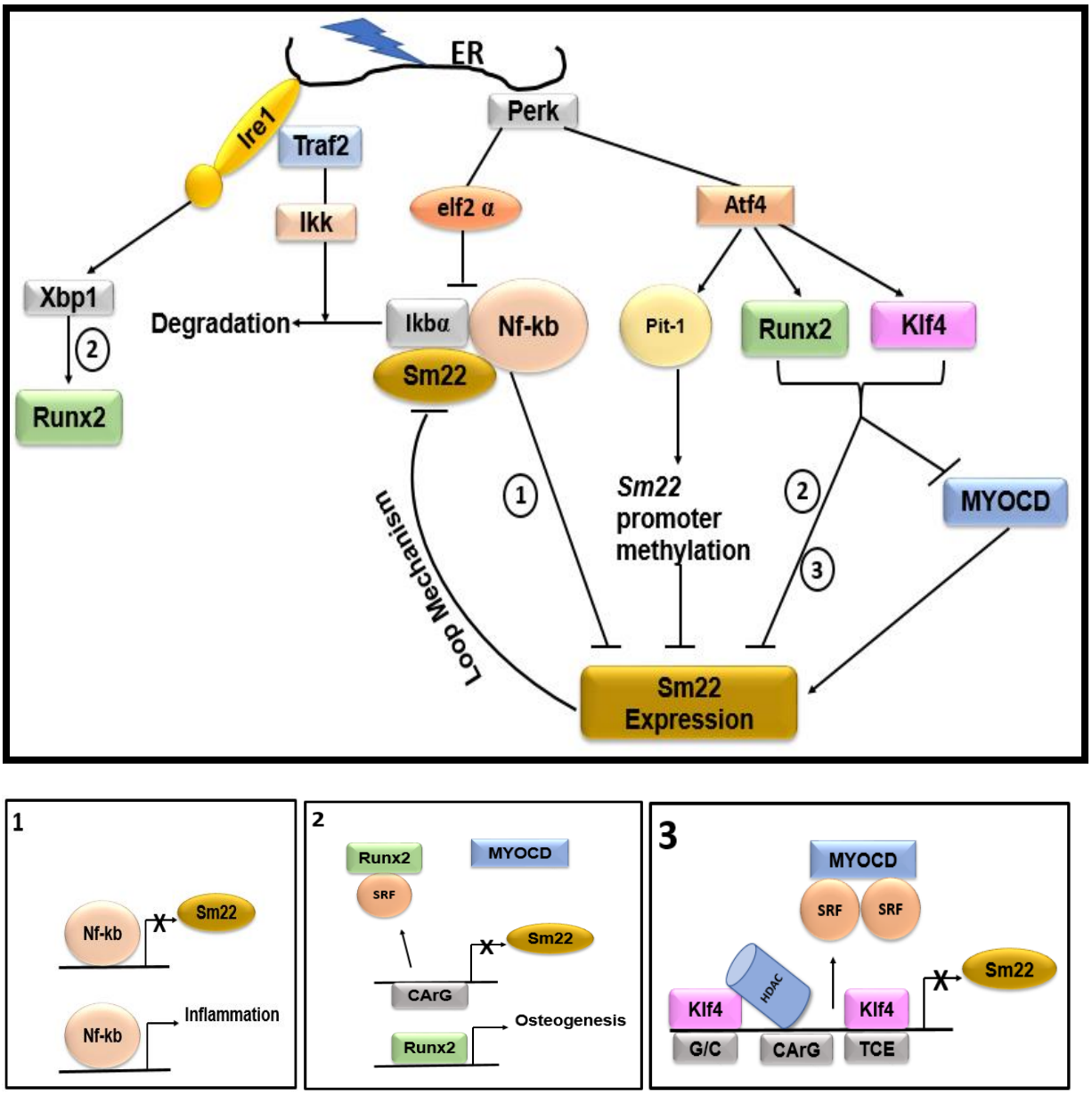


Figure 22: Working model of *Sm22* regulation under the ER stress and role of inflammation and osteogenic pathways activated under ER stress. The ER stress transducers IREα and PERK activate signaling that leads to repression of *Sm22* expression. 1)PERK phosphorylates elF2α which activates a translational attenuation. This attenuation causes reduced IkBα and SM22 that sequester NF-kB in a complex. The free NF-kB translocates to the nucleus to repress *Sm22* expression and induce expression of inflammatory markers. The *Sm22* repression by NFkB then works in a loop mechanism to increase translocation of NFkB. 2-3) Downstream to PERK and elF2α is ATF4. ATF4 induces the expression of RUNX2 and KLF4. RUNX2 represses MYOCD, the master regulator (positive) of *Sm22* expression. RUNX2 also displaces the SRF-MYOCD complex form the CArG box leading to reduced *Sm22* expression. RUNX2 also induces the expression of osteogenic markers. XBP1 downstream to IREα induces RUNX2. KLF4 works in comparable manner by repressing MYOCD and displacing the SRF-MYOCD complex. ATF4 also induces PIT-1 which represses *Sm22* by methylating its promoter.

CONCLUSION

Cardiovascular diseases (CVDs) are a major health and economic burden globally.It is estimated that >1 in 3 American adults have CVD. Often a person will have more than 1 type of CVD due to interdependent complications.Data from 2013 shows that ~2200 people die of CVD every day, averaging to one every 40 secs [129].It is estimated that if all the forms of CVD are eliminated the life expectancy could rise by 7 years[130]. It is therefore of utmost importance to find therapies to treat these pathologies.

The CVDs are complicated by a number of secondary signaling processes like inflammation and vascular calcification. These symptoms are called atherogenic, indicating the development of atherosclerosis. Aortic aneurysms are often accompanied by inflammation and atherosclerosis. This subtype is often referred to as the atherosclerotic aneurysm[131].The pathology also differs between TAA and AAA due their embryonic origin. The SMCs of the AA retain the genetic programming of the embryonic state whereas the SMCs in the thoracic area are populated by cells that can adapt better to the rigour and stress of the blood pressure. Hence the cells from these two regions also differ in their response to different stimuli[132]. Therefore understanding the pathology at the molecular level is vital.

The smooth muscle cells are the predominant cell type involved in these pathologies and hence the focus is on the unique repertoire of contractile genes that these cells express.The dynamic nature of the cells in regulating the expression pattern of these genes is a double edged sword.The phenotypic modulation of the cells under stress results in reduced expression of the contractile markers and increased expression of inflammatory and osteogenic markers that lead the cells to apoptosis. The ER stress is a multitier signaling pathway. The two phase ER stress response may be useful to find a time point to intervene and stop the adaptive UPR from becoming apoptotic. The downregulation of SM22α under ER stress may not be just a passive outcome. Overexpression of SM22 reduced inflammation and ER stress markers. It is also regulated by a number of pathways, so there might be more than one path to regulate SM22 expression, which gives it an advantage as a therapeutic target. Our drug screen identified

phytoestrogens as positive regulators for *Sm22* expression.Given that these candidates are natural compounds and present in small quantities in the common foods, the chances of toxicity are greatly reduced. This might be a benfit for the therapeutic development.

FUTURE DIRECTIONS

Our work shows that the ER stress signaling and the gene regulation of Sm22 is a multitier complex signaling cascade. We mainly focussed on the gene expression patterns and gene regulation but the ER stress is regulated at many different levels. Role of miRNAs in the stress response is an emerging field. miRNAs are small non-coding RNAs that silence RNA and function as post-translational gene regulation. Several miRNAs have been identified that work as the stress sensors or regulate other trancriptional factors of ER stress.MiR-211 was shown to be directly induced by PERK[133].MiR-702 was found to regulate ATF6[134]. So studying the regulatory role of miRNAs under the stress response would open up the doors to deeper signaling pathways.

Another aspect of the project that needs attention is the epigenetic regulation.As dicussed before KLF4 recruits HDAC2 to block the SRF binding to CArG box. Pit-1 also downregulates Sm22 expression by methylating the Sm22 promoter. Also the histone acetyltransferase enzyme p300 is recruited to the Grp78 promoter under ER stress. It then acetylates histone H4 to activate its expression[135].These are just a few examples.The contribution of epigenetics and chromatin remodelling play a very significant role in the stress response.

Another layer of regulation that needs to be taken into account is the post translational modification of different proteins involved in the signaling process.SM22α phosphorylation has been shown to be the link between actin cytoskeletal remodelling and oxidative stress[136].Though the major trasnscritption factors like elF2α have been studied , the downstream proteins still need to be explored in detail.

The newly emerging field though is the exosome biology. Studies have shown that phenotypically modulated VSMCs show increased secretion of exosomes.These contain miRNA for inflammatory and osteogenic signals.Exosome biogenesis is not clearly understood in this aspect and might be an important therapeutic target[137].

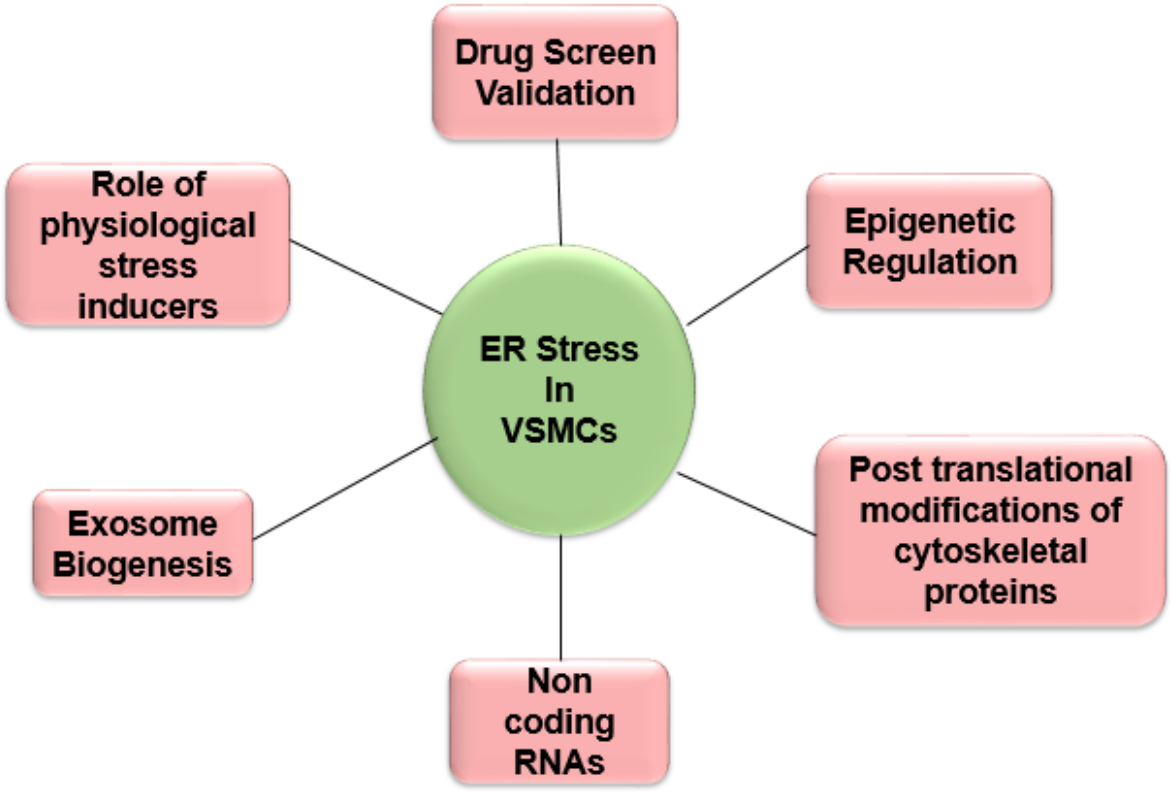


Figure 23: Diagrammatic representation of future directions for the project- ER stress in VSMCs.1) Drug screen validation- Only one of the drug screen candidate Biochanin A was validated in the 10T1/2 fibroblast cell line. Other isoflavones and flavones need to be tested for activation of *Sm22* expression in the fibroblast cells or stresses VSMCs.2) Epigenetic regulation- KLF4 is known to recruit HDAC2 to the CArG site to displace the SRF-MYOCD complex, repressing the expression of *Sm22*.PIT-1 also represses *Sm22* expression by methylating its promoter. Hence epigenetic regulation might be an important regulation pathway.3) Post translational modification of cytoskeletal proteins- AngII has been shown to induce ROS by phosphorylating SM22. How ER stress modifies SM22 post translationally might be crucial in the stress response.4) Several miRNAs have been implicated in the regulation of the factors involved in the stress process.5) Recent studies have shown that VSMC exosomes might play a significant role in calcification of the tissue. Therefore, this novel aspect of signaling might be a good direction for the project to move forward.6) Effect of physiological ER stress inducers on smooth muscle phenotypic modulation. Physiological stress inducers might be clinically relevant to the pathology and hence might be an important aspect of the ER stress project.

ABBREVIATIONS

10T1/2, mouse fibroblast cell line

ACTA2, smooth muscle α actin;

ATF4, Activating transcription factor 4

ATF6, Activating transcription factor 6;

BCA, Biochanin A;

bp, base pairs;

CArG, $CC(A/T)_6GG$;

CCL2, monocyte chemotactic protein 1;

cDNA, complimentary DNA;

CHO cells, Chinese hamster ovary cells;

CHOP, C/EBP homologous protein;

CNN1, calponin 1;

Cyp2e1, cytochrome P450 2e1

CVD, cardiovascular disease;

CytoD, cytochalasin D;

DAPI, 4',6-diamidino-2-phenylindole;

DMEM, Dulbecco's Modified Eagle's medium;

ECM, extracellular matrix;

elF2α, Eukaryotic Initiation Factor 2 α;

ER, endoplasmic reticulum;

FBS, fetal bovine serum;

G/F – actin, globular actin /filamentous actin;

GADD34, Growth Arrest And DNA-Damage-Inducible34

GAPDH, glyceraldehyde 3-phosphate dehydrogenase;

IF, immunofluorescence;

IHC, immunohistochemistry

IKB, I-kappa-B;

IKK, IκB kinase;

KLF4, kruppel like factor 4;

LPS, lipopolysaccharides;

MMP9, matrix metalloproteinase 9

MSX2, muscle segment homeobox 2;

MYH11, smooth muscle myosin heavy chain;

MYOCD, myocardin;

NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells;

OCN, osteocalcin;

OCT, optimal cutting temperature compound;

PAC1, a pulmonary arterial SMC cell line;

PBS, phosphate-buffered saline;

PERK, PRKR-Like Endoplasmic Reticulum Kinase;

Pit-1, The *sodium-dependent* phosphate cotransporter;

PP1, Protein phosphatase 1;

PPARγ, Peroxisome proliferator-activated receptor gamma;

PPP1R15A, Protein Phosphatase 1 Regulatory Subunit 15A

rtRT-PCR, real-time RT-PCR;

RUNX2, Runt-related transcription factor 2

ROS, reactive oxygen species;

SBE, smad binding element;

siRNA, small interfering RNA;

Sm22-/-, *Sm22* knockout;

Sm22+/+, *Sm22* wild type;

TAGLN 2, transgelin 2

TCE, TGFβ control element

Tg, Thapsigargin;

TUBB, tubulin beta

TUNEL, Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling;

UPR, unfolded protein response

UTR, untranslated region;

SMA, smooth muscle α actin;

SMC, smooth muscle cell;

snRNA, small nuclear RNA;

SRF, serum response factor;

VCAM1, vascular cell adhesion molecule 1;

VIM, vimentin;

VSMC, vascular smooth muscle cell;

WB, western blotting.

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ABSTRACT

EFFECT OF ENDOPLASMIC RETICULUM STRESS ON VASCULAR SMOOTH MUSCLE CELLS AND ITS REGULATION OF SM22α

by

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Background: The vascular smooth muscle cells(VSMC) possess the ability to differentiate into a synthetic phenotype in response to stress. This phenotypic modulation may be accompanied by inflammatory or osteogenic response in chronic stress. The synthetic state is characterized by low levels of contractile markers unlike the differentiated state.

Hypothesis: Endoplasmic reticulum (ER) stress causes phenotypic modulation in VSMCs leading to apoptosis. Many transcription factors induced by ER stress contribute to the downregulation of *Sm22α.* Perturbation in cytoskeletal dynamics exacerbates the ER stress response.

Methods: *Ex-vivo* culture was used to establish importance of *Sm22* in ER stress. *In vitro* analysis was carried out in PAC1 cells to elucidate the signaling induces by ER stress in smooth muscle cells and its contribution to the downregulation of *Sm22α.*

Results: ER stress caused a transient upregulation of smooth muscle markers during 1-6 hrs. of treatment with the ER stress inducer thapsigargin. The downregulation of contractile markers at 24hour of

thapsigargin treatment was accompanied by upregulation of phenotypic regulators- *Klf4, Mmp9* and *Pdgf-bb*, inflammatory markers *Vacm-1* and *Ccl-2* and osteogenic markers-*Runx2*, *Msx2*, *Ocn* and *Pit-1*. Overexpression of SM22 reduced the expression of ER stress markers (*Atf4* and *Chop*) and inflammation marker (*Ccl-2)*. Myocardin overexpression could reduce the downregulation of SM22α. Promoter analysis of *Sm22α* showed that all the cis acting elements were involved in its downregulation under ER stress. As our results indicated a protected role of SM22α under ER stress conditions, a drug screen was carried out to identify potential regulators of *Sm22α*.The screen identified isoflavonoids as positive regulators for *Sm22α* expression. Biochanin A, a candidate from the drug screen was subsequently validated in the fibroblast cell line

Conclusion: SM22α plays a protective role in the ER stress condition. Many of the ER stress induced factors associate with different cis acting elements in the *Sm22α* promoter to repress it. *Sm22α* could be a potential drug target to reduce the pathogenic effects of ER stress in cardiovascular diseases. Preliminary drug screen suggests that the phytoestrogens – flavonoids and isoflavonoids could be potential candidates with therapeutic benefits.

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