MODULATING THE FUNCTIONAL CONTRIBUTIONS OF C-MYC TO THE HUMAN ENDOTHELIAL CELL CYCLIC STRAIN RESPONSE

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MODULATING THE FUNCTIONAL CONTRIBUTIONS OF C-MYC TO THE HUMAN ENDOTHELIAL CELL CYCLIC STRAIN RESPONSE

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To my family, with love, for always encouraging me, believing in me, and for reminding me what really matters.

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LIST OF SYMBOLS AND ABBREVIATIONS

A	Adenine
AC	Adenylate Cyclase
ANOVA	Analysis of Variance
AS ONDs	Antisense Oligonucleotides
BMS	Bare Metal Stent
BrdU	Bromodeoxyuridine
C	Cytosine
CABG	Coronary Artery Bypass Graft
CDK4	Cyclin Dependent Kinase 4
CLB	Cell Lysis Buffer
CNBP	Cellular Nucleic Acid Binding Protein
CS	Cyclic Strain
CVD	Cardiovascular Disease
DES	Drug-Eluting Stent
DMSO	Dimethyl Sulfoxide
EC	Endothelial Cells
ECM	Extracellular Matrix
ELISA	Enzyme-Linked ImmunoSorbent Assay
FGF-2	Fibroblast Growth Factor
G	Guanine
hnRNP K	Heterogeneous Nuclear Ribonucleoprotein

HUVEC	Human Umbilical Vein Endothelial Cells
HSP60	Heat Shock Protein 60 kDa
kDa	Kilodalton
LDH	Lactate Dehydrogenase
MC	Motion Control
MMP-2	Matrix Metalloproteinase-2
MMP-9	Matrix Metalloproteinase-9
NHE III_1	Nuclease Hypersensitivity Element
OEI	Oxidative Endothelial Injury
PCNA	Proliferating Cell Nuclear Antigen
PDGF	Platelet Derived Growth Factor
PBS	Phosphate Buffered Solution
ROS	Reactive Oxygen Species
T	Thymine
UAZCC	University of Arizona Cancer Center
VEGF	Vascular Endothelial Growth Factor
VSMC	Vascular Smooth Muscle Cell
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
SAGE	Serial Analysis of Gene Expression
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
ss	Stainless Steel
SSRE	Shear Stress Responsive Elements

SUMMARY

With each heartbeat, major arteries experience circumferential expansion due to internal pressure changes. This pulsatile force is called “cyclic strain” and has been implicated in playing a pivotal role in the genetic regulation of vascular physiology and pathology. This dissertation investigates the hypothesis that in human umbilical vein endothelial cells (HUVEC), pathological levels of cyclic strain activate the c-Myc promoter, leading to c-Myc transcription and downstream gene induction. To determine expression and time-dependency of c-Myc in HUVEC, mRNA and protein expression of c-Myc under physiological (6-10% cyclic strain) and pathological conditions (20% cyclic strain) were studied. Both c-Myc mRNA and protein expression increased more than 3-fold in HUVEC (P4–P5) cyclically strained at 20%. This expression occurred in a time-dependent manner, peaking in the 1.5–2 hour range and falling to basal levels by 3 hours. Subsequently, the mechanism of c-Myc transcription was investigated by using a specific inhibitor to modulate c-Myc transcriptional activation. This compound, obtained from the University of Arizona Cancer Center, attenuates cyclic strain–induced c-Myc transcription by about 50% by binding to and stabilizing the silencer element in the c-Myc promoter. Having established this reduction in expression, it was investigated how these effects modulate downstream genes that are regulated by c-Myc. The results indicate that direct targeting of the c-Myc promoter may decrease stretch–induced gene expression of vascular endothelial growth factor (VEGF), proliferating cell nuclear antigen (PCNA), and heat shock protein 60 (HSP60). These findings may help in the development of a novel therapeutic opportunity in vascular diseases.

CHAPTER I: THESIS RATIONALE

1.1 Introduction

According to the Heart Disease and Stroke Statistics published by the American Heart Association, cardiovascular disease (CVD) remains the primary cause of mortality in both women and men in the United States. It is estimated that almost 80 million American adults (1 in 3) suffer from one or more types of CVD, which includes high blood pressure, coronary heart disease, heart failure, and stroke. Every day, nearly 2400 Americans die of CVD. In fact, CVD claims more lives each year than the next four leading causes of death (cancer, chronic lower respiratory diseases, accidents, and diabetes mellitus) combined. The estimated direct and indirect cost of CVD for 2007 exceeds $430 billion [1].

As a result of these staggering statistics, numerous studies have attempted to characterize the onset and progression of human CVD. With an enhanced biological understanding of the cell cycle events involved in this pathogenesis, it would be possible to better treat and/or prevent these diseases. Although numerous genes have identified roles in atherosclerosis, the mechanism of their induction is largely uncharacterized. Cyclic strain, which results as a consequence of pulsatile blood flow, is a principal factor in the localization of these diseases [2]. Under normal physiological conditions, arterial vascular endothelial cells (EC) are continuously subjected to cyclic strain (6–10%). When certain pathological conditions arise *in vivo*, such as hypertension, atherosclerotic plaque development, and intracoronary stenting, elevated cyclic strain (~20%) can induce vessel remodeling, leading to a pro-atherogenic endothelium [2, 3]. By implementing an

in vitro cyclic strain model, it is possible to mimic some of the hemodynamic conditions associated with atherosclerosis and thereby study it from a mechanistic point of view.

Recently, in an attempt to study the transcriptional changes that occur in vasculoproliferative vascular diseases, research has turned to finding parallel pathological mechanisms in cancer. These findings have identified the oncogene c-Myc, known to be involved in the modification of cell cycle-related events (proliferation, differentiation, and apoptosis), as a potential research target. This project will examine the functional contributions of c-Myc to the human EC cyclic strain response in an attempt to add insight into the pathogenesis of atherosclerosis/restenosis. This dissertation proposes that pathological levels of cyclic strain activate the c-Myc promoter, leading to c-Myc transcription and downstream gene induction, which, in sum, contribute to the development of cardiovascular disease. If true, these findings will help to develop a novel therapeutic opportunity in occlusive vascular diseases.

1.2 Hypothesis and Specific Aims

The principal goal of this project was to investigate the genetic expression of human umbilical vein endothelial cells (HUVEC) in response to applied mechanical stretch. Specifically, in order to model the circumferential expansion of arteries *in vivo*, HUVEC were subjected to uniaxial, cyclic strain *in vitro*. The overall hypothesis for this research, as outlined in the original thesis proposal, was:

Pathological levels of cyclic strain activate the c-Myc promoter, leading to c-Myc transcription and downstream gene induction, which, in sum, contribute to the induction of vascular obstructive diseases.

To evaluate this general hypothesis, the following three specific aims were formulated:

Specific Aim 1: Determine the expression and time-dependency of c-Myc in human umbilical vein endothelial cells subjected to cyclic strain

This aim will investigate the mRNA and protein expression of c-Myc under pathological conditions (20% cyclic strain) and compare these results to those under physiological (6–10% cyclic strain) and static conditions.

Specific Aim 2: Determine whether it is possible to use specific inhibitors to modulate c-Myc transcriptional activation and, thereby, determine the mechanism of c-Myc transcription by cyclic strain

By using compounds that directly target the silencer element in the c-Myc promoter, it will be determined if cyclic strain–induced c-Myc transcription can be attenuated.

Specific Aim 3: Determine both the mRNA and protein expression of downstream genes (especially those related to cardiovascular disease), which are regulated by c-Myc

Once establishing that c-Myc transcriptional activation can be attenuated (specific aim 2), it will be examined if this modulation can affect the transcription of c-Myc target genes. Cyclically-strained endothelial cells will be treated with and without compounds that prevent c-Myc transcription in order to examine potential downstream targets (vascular endothelial growth factor, proliferating cell nuclear antigen, and heat shock protein 60).

The completion of these studies helped to elucidate the genetic expression of HUVEC in response to cyclic strain. Hopefully, these results will contribute to the understanding of the link between hemodynamic forces and the physiology of arterial tissue, both in healthy and diseased states.

CHAPTER II: BACKGROUND & LITERATURE REVIEW

2.1 Arterial Physiology

Research on the structure and function of arteries is imperative to understanding the pathophysiology of cardiovascular disease, the primary cause of death in developed countries. Although there exists extensive knowledge in this field, arteries are complex and dynamic—arterial cells actively respond to and affect their surrounding mechanical and biochemical environments. This background and literature review offers a generalized understanding of arterial physiology, with an emphasis on one specific arterial cell type—the endothelial cell.

2.1.1 Structure

The human artery is composed of three concentric layers surrounding a lumen, through which the blood flows. The layers are the tunica intima, tunica media and the tunica adventitia, as idealized in Figure 2.1.

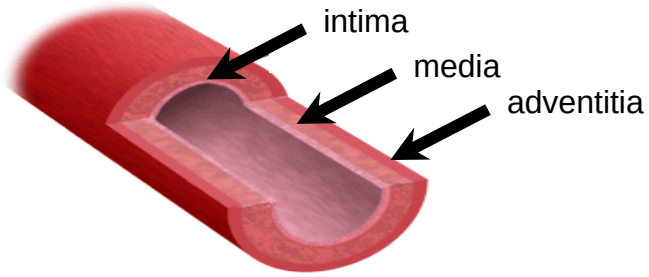


Figure 2.1: The artery [4]

The three layers are distinguished by their extracellular matrix and component cell type. They are bound together by connective tissue. The intima, the innermost layer, is in direct contact with the blood. It consists of a single layer of endothelial cells and rests on a connective tissue membrane that is rich in elastic and collagenous fibers. The media makes up the bulk of the arterial wall and is composed primarily of concentric layers of elastic laminae interposed with layers of extracellular matrix and contractile smooth muscle cells. The outermost, relatively thin layer, which attaches the artery to the surrounding tissues, is the adventitia. It consists chiefly of connective tissue with irregularly arranged elastic and collagenous fibers [5].

2.1.2 Function

The overall function of arteries is to deliver blood from the heart to the peripheral vessels, but these conduits also play important roles in vascular hemodynamics. Arteries regulate vascular mechanics primarily at the cellular level by controlling vascular tone, regulating the vessel diameter and by dampening pulsatile pressure waves [6].

2.1.3 Endothelial Cell Biology

Endothelial cells line the lumen of all blood vessels, serving as a barrier between blood-borne constituents and the underlying vascular tissue. Their function is complicated with multiple roles, including: cell growth, migration and differentiation, maintaining vascular tone, and responding to injury. EC accomplish these diverse tasks through cytokines, vasoactive peptides, and growth factors. Additionally, EC are directly exposed to the forces developed from blood flow and pressure. As a result of this direct contact, they are believed to be the primary mediators of response to shear stress and

cyclic strain [7, 8]. In fact, EC at sites of complex flow (i.e. vessel bifurcations and curvatures) exhibit increased permeability and a proinflammatory phenotype [9]. Disruptions in the hemodynamic forces imparted on vascular EC can cause their secretion of vasoactive factors including vasorelaxants (e.g. nitric oxide and prostacyclin) and vasoconstrictors (e.g. endothelin-1 and platelet-activating factor) and in the long-term, remodeling of the vessel wall [10].

2.2 Arterial Hemodynamics

The three hemodynamic forces imparted on vascular EC by blood flow are shear stress, hydrostatic compression and cyclic (circumferential) strain. Each of these forces has an associated phenotype and gene expression profile. Additionally, due to inherent differences in the varying types of blood vessels, the properties of the vessel wall vary along the vascular tree. Large and medium-sized arteries behave differently than capillary vessels, which in turn behave differently than low pressure veins. Within the arteries, typical physiological values of shear stress, cyclic strain, and transmural pressure range from 6 to 40 dyne/cm^2, 6% to 10% at 1 Hz, and 60 mmHg to 140 mmHg, respectively [11]. Although all three forces mediate vascular responses, some evidence indicates that shear stress and hydrostatic compression may not be the major factors influencing atherosclerosis or other vasculoproliferative cardiovascular diseases [2]. Rather, cyclic strain, which results from pulsatile blood flow, is the principal factor in the localization of these diseases [2]. Cyclic mechanical strain regulates the apoptosis, proliferation, and migration of vascular cells, and the degradation, synthesis, and reorganization of extracellular matrix [12]. Therefore, for the purpose of this dissertation, this review of arterial hemodynamics will emphasize cyclic strain in major,

typically elastic arteries, such as the coronary arteries. In these vessels, normal physiological cyclic stain is about 10% [6, 13].

The vascular wall exhibits complex material properties, including spatial heterogeneity, anisotropy, nonlinearity, and viscoelasticity [6]. Thus, the wall mechanics of the native artery are neither simple nor straightforward. However, by making a few assumptions, it is possible to derive a simplified relationship between transmural blood pressure and wall stretch.

Under normal conditions, the arteries are always "pressurized." Therefore, based upon the law of LaPlace, the wall stress in the circumferential direction for a thin-walled cylinder of wall thickness, h, and with transmural pressure, P, can be approximated as (inertial effects ignored):

$$\sigma_\theta (t) = \frac{P * r}{h}$$

where r is the deformed radius.

From Hooke's law for an elastic, thin-walled cylinder, circumferential strain (ε_θ) can be equated to circumferential (hoop) stress (σ_θ) through the definition of bulk Young's modulus, E, by the formula:

$$\varepsilon_\theta = (1/E)(\sigma_\theta)$$

Thus, if the artery is assumed to be a classic pressure, thin-walled, linear elastic cylinder, governed by LaPlace's and Hooke's laws, a very simple model can be developed to

provide a basis for *in vitro* work. By equating the stress and strain, the relationship between transmural blood pressure and wall stretch is derived:

$$\varepsilon_\theta = \sigma_\theta / E = \frac{(P*r)}{(h*E)}$$

Although this simplified equation is only applicable for infinitesimal strains of a thin-walled, linear elastic cylinder, it demonstrates that the relative circumferential expansion of the arterial wall is directly related to the wall composition and structure. Restated, arterial pressure, comprised of both blood pressure and pulsatile tensile stresses, causes a radial-directed stress in the wall, and the resulting strain directly depends on the constitutive properties of the arterial wall.

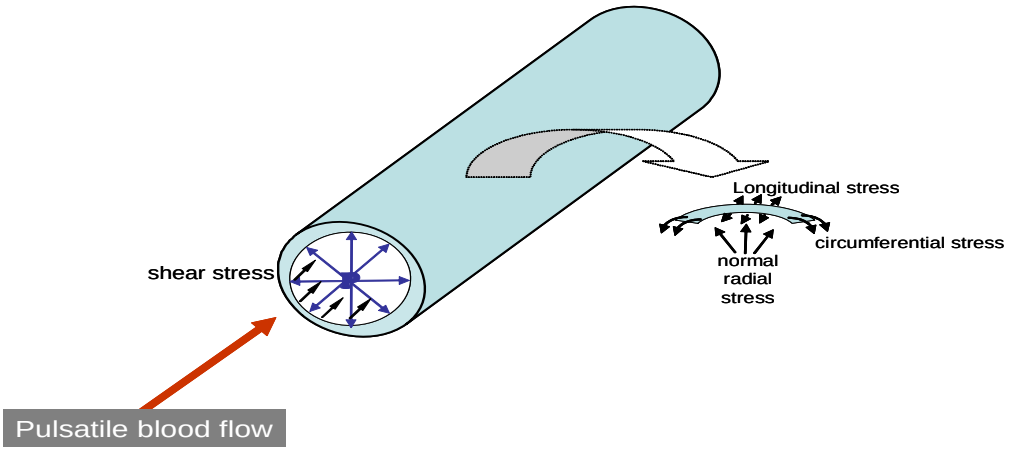


Figure 2.2: Mechanical forces acting on the artery
The figure at right shows the resultant stresses acting on an element of the vascular wall, where the endothelial cells reside.

2.3 Vascular Obstructive Diseases

Through autocrine and paracrine hormonal mechanisms, EC are able to react to local hemodynamic modifications. Almost immediately, vascular tone is used to compensate and restore any changes in mechanical forces. As reviewed in Section 2.1.3, one of the functions of EC is to respond to humoral agents and injury. If the endothelium is damaged, dysfunction is clinically characterized by a loss of nitric oxide production and therefore, reduced endothelium-dependent vasodilation. The shift in phenotype to a state of dysfunction is also characterized by increases in endothelial adhesiveness, permeability, proliferation, and thrombogenesis.

When the phenotype of vascular cells is altered or compromised, and thus not capable of restoring physiological levels through vascular tone alone, vascular remodeling occurs and can lead to vasculoproliferative diseases. These disorders can occur as a result of numerous pathological conditions, notably atherosclerosis, hypertension, and the consequence of mechanical interventions. The pathologies are similar in that they result in an abnormal narrowing of the blood vessel. Advanced lesion formation can result in mortality or serious tissue damage, including cerebral and myocardial ischemia and infarctions.

2.3.1 Atherosclerosis

Atherosclerosis is the pathological event leading to the development of plaque on the inner walls of arteries, decreasing lumen size. Specifically, vascular injury triggers a vasculoproliferative cascade that can be divided into three phases: (1) an early phase of platelet activation and thrombus formation, (2) an intermediate phase of vascular smooth muscle cell (VSMC) recruitment, and (3) a late proliferative phase [14]. It is known that

hemodynamics and the endothelium mediate these atherosclerotic events, but the balance amongst autocrine, paracrine, and endocrine cell signaling remains to be elucidated.

2.3.2 Hypertension

Hypertension is an increase in blood pressure due to a narrowing lumen and reduction in vessel elasticity. Instead of being focal in nature, like atherosclerosis, hypertension involves diffuse changes in hemodynamics and arterial structure. According to one hypothesis, high blood pressure causes chronic damage to the endothelium, promoting plaque formation [5]. Therefore, although not a "disease" by definition, chronic hypertension can lead to vessel stiffness via extracellular matrix (ECM) deposition, hypertrophy and hyperplasia of VSMC in the intima and media.

2.3.3 Surgical Intervention

In an effort to reduce CVD mortality, a variety of procedures, including balloon angioplasty, endarterectomy, and coronary artery bypass grafting and stenting, are performed. In 2004, an estimated 6,363,000 inpatient cardiovascular operations and procedures were performed in the United States, contributing to a decline in CVD mortality [1]. However, these treatments, like atherosclerosis, involve acute mechanical injury to the structure of the vessel wall, leading to increased susceptibility to atherosclerosis, aneurysm formation, and plaque disruption. Remodeling of the injured vasculature usually occurs within a few hours or days after injury [15] and often leads to neointimal hyperplasia and eventually, restenosis. It is estimated that 30 to 50% of patients undergoing percutaneous coronary interventions will experience restenosis [16, 17], and 20% of these will require additional operations, including coronary artery bypass

surgery, a procedure that in itself is limited by graft failure due to luminal obstruction [18]. Estimated costs as a result of restenosis complications exceed $3.5 billion per year in the United States alone [19].

2.4 Treatment of Vasculoproliferative Disease

2.4.1 Improved Stent Design

Stenting serves to unblock and hold open a narrowed vessel, ensuring perfusion to downstream tissue. Since it is a less caustic method than radiation or brachytherapy, stenting is a popular method to treat vasculoproliferative diseases. Recent reports state that more than 1.25 million stents are implanted in North America annually [20]. However, the consequences of stent placement are often deendothelialization, dissection of the media and occasionally the adventitia, and stretch of the entire artery [21]. In addition, a complex interaction occurs between the blood and the biomaterial surface. The complement system is activated, platelets and leukocytes become activated, and coagulation leads to thrombi formation [22]. These events initiate the arterial wall's healing response to mechanical injury, often leading to restenosis.

2.4.1.1 Drug-Eluting Stents (DES)

Since restenosis after stent placement is entirely the result of intimal hyperplasia, stent coatings with drugs or non-pharmaceutical agents may reduce in-stent restenosis. The metal stent is either coated with a matrix, allowing controlled drug release, or the pharmacological agent is incorporated into a polymer-metal composite stent [23]. Thus far, in comparison to bare metal stents (BMS), these "drug-eluting stents (DES)" have

been successful in reducing restenosis (from the 20–30% range to single digits [24]) and neointimal hyperplasia [25].

It is important to consider the local drug pharmacokinetics when designing a DES. Any pharmacological agent delivered locally is influenced by the local transport forces, which are related to the properties of the target tissue. Suboptimal transport could result in either toxic or nontherapeutic levels. The highly heterogeneous composition of the arterial wall and its asymmetric geometrical organization represent a challenge for most agents applied in DES technologies [21]. The ideal compound should contain hydrophobic elements to ensure high local concentrations as well as hydrophilic properties to allow homogeneous drug diffusion.

DES also have potentially dangerous complications. Recent studies report increased risk of stent thrombosis, myocardial infarction, and death associated with the use of this type of stent [26-28]. Since the currently employed drugs fail to specifically target the factors involved in the vascular injury caused by surgical implantation, there are complications known as "late stent thrombosis." The antiproliferative agents prevent the growth of endothelial cells onto the stent, thereby increasing the tendency for blood clotting inside the stent. This complication is generally avoided in BMS because they promote the growth of a smooth, thin layer of endothelial cells, incorporating the device into the artery [29]. A possible solution could be designing a drug-eluting stent which employs a novel drug designed with a molecular-target approach, specifically targeting the factors involved in vascular injury.

2.4.1.2 Alternative Stent Improvements

The alternative approach to improving stent design acts to accelerate, not inhibit, the healing process. Healing-enhancing stents aim to reduce VSMC migration and proliferation, promote local angiogenesis, and improve re-endothelization. These stents are coated with growth factors, hormones or antibodies [30] to prevent endothelial denudation and subsequent thrombosis. For example, researchers have designed a stent coated with anti-human CD34 antibodies in order to capture circulating endothelial progenitor cells [31]. The estrogen-releasing stent [32], the nitric-oxide-releasing stent [33], and the cell migration inhibitor (batimastat)-releasing stent [34] use elution to control inflammation and accelerate endothelial regeneration. Animal studies from these novel stents ascertain that this new technology may be an effective means to treat restenosis. However clinical trials are still underway and future investigations are necessary to overcome methodological limitations, such as dosing and vehicle delivery.

2.4.2 Intraluminal Radiotherapy

Intraluminal radiotherapy of arteries has been shown to inhibit restenosis by suppressing neointimal growth. The irradiation can target cells in all three layers of the blood vessel, i.e. from the intima to the adventitia. This technology attempts to inhibit new cell growth without causing long-term vascular necrosis. Several irradiation techniques are currently being investigated, including temporary intravascular insertion of gamma or beta-emitting wires, inflating balloon catheters with radioactive liquid, and using radioactive impregnated stents. However, uncertainties in dose-volume effects and the biological time course of restenosis make intraluminal radiotherapy as yet difficult to both plan and execute [35].

2.4.3 Gene Therapy

Since in-stent restenosis remains an important clinical problem, gene therapy offers an alternative approach for treating vessel remodeling. The genes delivered to vascular wall cells can encode for proteins that are either directly or indirectly cytotoxic or cell cycle-inhibitory. There is great interest in developing a strategy for safe, efficient, targeted gene delivery into the vessel wall. However, as a result of the nature of atherosclerotic lesions and lipid-rich atheroma, there is a potential for harmful biodistribution of vector.

Under evaluation are several approaches using gene therapy to prevent or reduce intimal hyperplasia. Viral vectors have the advantage of high gene transfer efficiency [36], but their success has limitations. Adenoviruses efficiently transfect both proliferating and quiescent cells, but they often have a short duration of expression and transfect untargeted organs and cells [37]. Retroviruses, lentiviruses, and baculoviruses are limited by host reactions and safety concerns. An attractive alternative approach is plasmid DNA with or without carrier molecules, because it is easy to produce and has a high level of safety. However, it is limited by low efficiency of gene transfer in vascular applications [37].

In spite of recent promise in the field of gene therapy, including success in preclinical animal models [38], there are not yet any therapeutic effects in clinical trials [39, 40]. Additionally, gene therapy is best suited for treating genetic disorders with single gene defects [36] and therefore, is unlikely to be successful in treating most vascular obstructive diseases, which involve multiple genes. Thus, this CVD treatment

option faces the same problem as the DES and radiotherapy fields—a need to develop an optimally-targeted pharmacological solution.

2.5 Role of c-Myc in Vascular Disorders

Cardiovascular disease and cancer are the predominant causes of mortality in industrialized countries [41]. Biological evidence suggests that these human pathologies may share pathological mechanisms. The three stages of carcinogenesis—induction, growth and invasion of tissue, and neoangiogenesis—parallel the course of atherosclerosis—initiation, progression, and complication. As a result of these commonalities, genes that have classically been studied in regards to carcinogenesis (c-Myc, Ras, and the Insulin-like growth factor-1 cascade) are now being linked to endothelial dysfunction and atherogenesis [42].

Specifically, a growing body of evidence implicates that the c-Myc proto-oncogene can play a pivotal role in cardiovascular disease. c-Myc, whose overexpression is associated with a significant number of human cancers [43, 44], controls numerous functions, including cell cycle progression, differentiation, and apoptosis [45-47]. In fact, c-Myc expression occurs within 30 minutes of mitogenic stimulation of quiescent cells, and peaks around 2 hours [48]. Disruption of c-Myc gene expression lengthens both the G_1 phase of the cell cycle (prior to DNA synthesis) and the G_o to G_1 transition [49, 50]. Moreover, c-Myc plays a key role in p27 sequestration through modulation of the level of regulatory cyclin D and E proteins [51].

Specific to the development of obstructive vascular disease, c-Myc is quickly induced in VSMC after arterial injury [50] and activated by proliferative signals, including a number of mediators of vascular EC biology, such as LDL [52], thrombin

[53], endothelin [54], and angiotensin II [55]. Inhibition of c-Myc has been shown to inhibit smooth muscle cell proliferation *in vitro* and in several animal models [56]. Several studies suggest that c-Myc may be involved in the regulation of angiogenesis [57]. Research with c-Myc knockout mice indicates that a deficiency in this gene causes serious defects in the development of blood vessels, angiogenesis and erythropoiesis [58, 59]. c-Myc also regulates the downstream genes causing cell migration and adhesion, collagen formation, secretion of extracellular matrix, and cell proliferation [60]. When compared with healthy conditions, there is increased c-Myc expression in atherosclerotic plaques, after carotid injury, and in hypertensive rats [61].

2.5.1 Targeting of c-Myc to Treat Vascular Diseases

To combat atherosclerosis and other cardiovascular diseases effectively, drugs are needed to downregulate the overexpressed genes comprising the vasculoproliferative response. Transcriptional factors such as c-Myc, which are downstream of the signaling cascades, have advantages because, unlike targeting a signaling molecule, there is less likely to be redundancy due to upregulation of parallel pathways, resulting in drug resistance.

Additionally, since c-Myc is an early-activated oncogene, a drug regulating its expression would only need to be released in the first 48 hours following intervention. In fact, *in vivo* studies have shown that single-dose suppression of c-Myc can prevent neointimal formation months later because the proliferation is critically dependent upon mitogenic events that occur very shortly after injury [62]. Not only does this treatment generate an easily controllable local pharmacokinetic profile, but it also has enormous potential for drug-eluting stents [63]. After the relatively brief delivery of the anti-

restenosis drug, the stent would become a bare metal stent—which may eliminate the concern of late stent thrombosis present in currently-designed drug-eluting stents. With bare metal stents, endothelial cells are able to grow, incorporating the device into the artery. A major complication of drug-eluting stents in use today is that the drugs that coat them have a long-term potency that prevents regrowth of endothelium, increasing the risk of thrombosis months later.

For all of these reasons, inhibition of c-Myc-dependent signaling has become a novel therapeutic opportunity in vascular obstructive diseases. Strategies have been undertaken to downregulate c-Myc, such as through intervention with antioxidants, RNA synthesis inhibitors, and antisense oligonucleotides against c-Myc [64]. However, an effective means to modulate c-Myc gene expression in humans has yet to be developed.

2.5.1.1 Antisense Oligonucleotides

The mechanism by which antisense oligonucleotides (AS OND), the most-researched therapy, inhibit expression of c-Myc involves interference with translation of the target c-Myc mRNA [65]. Thus far, these strategies have shown prevention of restenosis in animal models [66]. In rat studies, c-Myc AS OND reduce negative remodeling induced by arteriotomy, significantly reducing cell proliferation, inflammatory cell infiltration, and medial oedema [50]. However, when c-Myc AS OND were studied in humans, there was no reduction in angiographic or clinical restenosis after bare metal stenting [39] or in randomized human clinical trials [56]. It is generally believed that the ineffectiveness of these therapies in humans is due to an inherent nonspecificity, a slow uptake across the cell membrane, and rapid intracellular degradation of the oligonucleotide [67]. AS OND have unfavorable pharmacokinetics

because a significant fraction of antisense may be retained by membrane lipids [63] and large amounts of drug are required to produce an effect.

Instead, what is needed to effectively treat these vascular obstructive diseases is a *de novo* drug design. A novel and potentially more effective drug would prevent c-Myc transcription (and therefore downstream gene expression) by directly targeting the c-Myc silencer element.

2.5.1.2 Drug Targeting: Arizona Cancer Center Collaboration

Since there is an established relationship between vasculoproliferative cardiovascular diseases and cancer, a logical next step would be the investigational application of cancer drug therapies in the cardiovascular field. c-Myc has been a particularly attractive target for cancer drug development because it is overexpressed in the majority of tumor types and its inactivation can induce regression of malignant cancers. The University of Arizona Cancer Center (UAZCC) in Tucson, Arizona actively researches and develops cell-selective molecular targets for antitumor application, specifically identifying compounds targeted to the c-Myc promoter. Their drug design involves the traditional approach of using a small molecule as the starting point for drug optimization.

c-Myc transcription takes place when transcription factors heterogeneous nuclear ribonucleoprotein (hnRNP K) and cellular nucleic acid binding protein (CNBP) bind the c-Myc promoter. The compounds identified by UAZCC are able to silence the c-Myc promoter and thus, repress gene expression by preventing this binding of hnRNP K and CNBP to the c-Myc promoter. As proof of principle, the Cancer Center has performed

chromatin immunoprecipitation (ChIP) analysis. In unpublished data, their results verify that these compounds almost completely inhibit hnRNP K and CNBP binding.

As justification of the modulation capability of these compounds, UAZCC has performed serum-starvation experiments. It is well established that serum induces c-Myc expression in numerous cell types. In order to monitor basal levels of c-Myc, cells are often serum-starved, thus inducing a quiescent growth state. In a serum-starvation study with cancer (HeLa) cells, UAZCC researchers showed that compounds prevent the recovery of c-Myc levels after starvation. HeLa cells were grown to confluence, then rinsed and placed in media without serum for 12 hours. After serum-starvation, cells were then given media containing serum with and without drug. After starvation, c-Myc mRNA expression levels are about 0.4. When serum is added back to the cells and no drug is given, c-Myc levels increase to 0.75. On the contrary, when cells are given serum AND drug, there are no noticeable differences from serum-starved cells—the levels remain around 0.4. Therefore, these findings indicate that University of Arizona has identified drugs that are able to modulate the transcriptional activation of c-Myc.

Thus far, these UAZCC compounds have only been studied in oncology, but there is also a clear application in cardiology. Additionally, these compounds have both hydrophobic and hydrophilic properties—desirable traits for allowing homogeneous drug diffusion on a DES.

Therefore, a Material Transfer Agreement has been established between University of Arizona and Georgia Institute of Technology. This project utilized University of Arizona's lead compounds to investigate the modulation of c-Myc

transcriptional activation in my own experimental setup, which mimics the hemodynamic conditions associated with vessel remodeling and atherosclerosis.

2.5.2 Structure of c-Myc Promoter Region

The c-Myc gene is located on human chromosome 8q24 and consists of three exons. Translation at the AUG start site in the second exon produces a major 439 amino acid, 64 kDa c-Myc protein [68]. The transcriptional regulation of c-Myc expression is complex and involves multiple promoters and transcriptional start sites. The nuclease hypersensitivity element (NHE III_1) in the c-Myc promoter controls up to 90% of the transcriptional activity of this gene that occurs at the P1 and P2 promoters (the predominant c-Myc promoters) [69] (Figure 2.3).

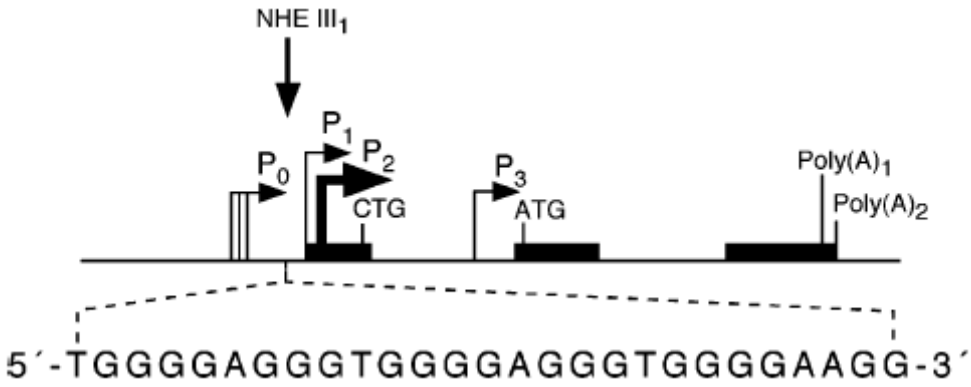


Figure 2.3: c-Myc sequence [70]

The NHE III_1 sequence includes an inordinate number of guanine residues on one strand and the corresponding cytosine residues on the opposing strand. The unique G-rich and C-rich sequences impart a special property to the DNA in this region—the ability of this duplex NHE III_1 to convert to a structure in which the purine-rich strand

adopts a G-quadruplex structure [71-73] (Figure 2.4). When NHE III_1 takes on this form, either side of these structures is single-stranded for at least one turn on each side. By stabilizing this silenced form, the binding proteins hnRNP K and CNBP, which directly mediate transcriptional activation, can be prevented from binding. In other words, by transforming the purine-rich sequence of the NHE III_1 to a parallel G-quadruplex structure, hnRNP K and CNBP cannot bind. Thus, gene expression is silenced [74]. Potential drugs could succeed in preventing transcription of c-Myc by stabilizing this region of NHE III_1.

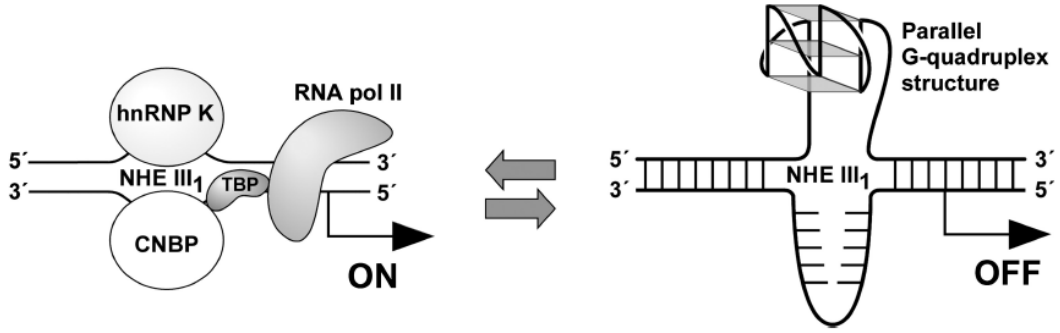


Figure 2.4: Proposed structure of c-Myc promoter [70]

2.6 Genetic Markers of Vasculoproliferative Diseases

Direct targets of c-Myc are defined as genes whose expression is directly regulated by binding of the c-Myc protein to the promoter region. These target genes have been identified by several techniques including DNA microarray analysis and serial analysis of gene expression (SAGE). It is important to point out that there may be differences in the c-Myc targets induced in normal cell homeostasis and those induced in pathophysiological conditions when c-Myc is overexpressed. The following are potential

downstream targets of c-Myc that have also been implicated in promoting vasculoproliferative diseases.

2.6.1 Proliferating Cell Nuclear Antigen (PCNA)

Proliferating cell nuclear antigen (PCNA) has been identified by microarray as a transcriptional target of c-Myc (3.2–fold upregulation) in rat endothelial cells [75]. This protein was originally identified as an antigen expressed in cell nuclei during the DNA synthesis phase of the cell cycle. PCNA encircles double-stranded DNA as a trimer, forming a sliding clamp that tethers proteins such as polymerases to DNA [76]. Based upon its essential role in DNA synthesis and therefore, increased concentrations during the cell cycle, this protein is considered to be a valid indicator of cell replication. Cells traversing the cell cycle are often identified by PCNA immunostaining.

PCNA is implicated as a downstream gene of c-Myc [77] and in relation to CVD, PCNA has been identified as playing an important role in vascular remodeling (proliferation, migration, and phenotypic change). Its expression is frequently used to determine the proliferation activity of endothelial cells in restenotic tissue [78] and matrix accumulation in neointimal vasculature [79] obtained from porcine coronary angioplasty. Smooth muscle cell proliferation, an important stage in the pathogenesis of vascular obstructive diseases, can be inhibited *in vitro* and in several animal models by inhibiting PCNA expression [56]. Because of its role in vascular disease, in addition to targeting the c-Myc gene for AS OND, research is also directed towards developing AS OND for PCNA [80].

2.6.2 Heat Shock Protein 60 (HSP60)

HSP60, a protein which is expressed on the endothelial surface, has been identified as a transcriptional target of c-Myc [81] through SAGE and confirmed with microarray (5-fold induction). It is a suspected contributor to the initiation and aggravation of vascular pathologies like atherosclerosis and restenosis [82]. The first published discovery of human HSP60 detection in diseased vascular tissue was in the early 1990s [83], with the identification of HSP60 expression in atherosclerotic lesions. Subsequent research has validated this discovery, showing localization of HSP60 in neointimal, but not healthy, vasculature [84] and soluble HSP60 levels associated with intima–media thickness [85].

With increasing studies, HSP60 is now being recognized as a marker for early cardiovascular disease. Elevated HSP60 levels have been identified in patients with borderline hypertension and there is an association between early atherosclerosis and HSP60 levels [86]. Further, HSP60 both activates and is induced in endothelial cells, smooth muscle cells, and monocyte-derived macrophages in atherosclerotic lesions [85]. Endogenous as well as exogenous HSP60 have been implicated in stimulating vascular smooth muscle cell proliferation in a concentration-dependent fashion [87, 88].

The association of HSP60 with the induction/progression of both chronic hypertension and atherosclerosis can be further substantiated from a vascular mechanics point of view. Disturbed flow patterns have been found to induce HSP60 and have been suspected to provoke autoimmune reactions which result in atherogenesis [89]. Altered hemodynamic conditions, like those found in venous bypass grafts and atherosclerosis-prone sites of the vascular tree, are also possible causes of HSP60 induction. In

particular, increased levels of fluid shear stress (30 dynes/cm^2) increased HSP60 expression in endothelial cells *in vitro* [81, 90].

2.6.3 Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor (VEGF) is well characterized as a transcriptional target of c-Myc at both the mRNA and protein levels [91]. In fact, upregulation of c-Myc has been shown to cause almost a 10-fold induction of VEGF expression [92]. Within the vasculature, VEGF is a potent mitogen, identified as a mediator of c-Myc–induced angiogenesis.

VEGF plays a key role in both physiological and pathological conditions. This growth factor is involved in processes ranging from wound healing and neovascularization to carcinogenesis and cardiovascular disease [36]. It acts on endothelial cells, inducing various effects, including increased vascular permeability, angiogenesis, vasculogenesis, and endothelial cell proliferation and migration [37].

Although VEGF is necessary for maintaining hemostasis, studies also indicate that increased expression and activity of endogenous VEGF are essential to the development of restenosis. Injurious conditions due to pathologies or surgery alter the mechanical environment, leading to a thickening and subsequent increased stretch of the vessel wall. In VSMC, the increasing stretch induces a significant increase in VEGF expression both at the mRNA and protein levels [93] and in a time- and amplitude-dependent manner [94]. Since VEGF enhances neointimal formation and atherogenesis *in vitro* and *in vivo* [21], these findings suggest that VEGF plays a role in arterial disease. In fact, higher levels of VEGF are found in atherosclerotic and restenotic legions as compared with normal, healthy tissue [95, 96].

2.7 Cell Culture Models of Mechanical Forces

The fluid mechanical forces imparted by blood flow have been implicated in playing a pivotal role in the initiation and progression of CVD. Stenotic lesions preferentially develop in regions of disturbed blood flow, implicating that local hemodynamic forces directly influence pathobiological processes. Endothelial cells grown statically on tissue culture fail as an appropriate *in vivo* model of EC behavior because this oversimplified system lacks the critical feature of mechanical stress. *In vivo*, EC experience stress from the flow of blood, pressure fluctuations, and pulsatile stretch (see Section 2.1.3). Relevant to this dissertation, c-Myc has been reported to respond not only to shear stress, but also to other types of mechanical forces, such as pressure overload and stretch in cardiac myocytes [97].

At present, however, it is not possible to monitor and control the hemodynamic factors in the vicinity of the vessel wall. Since an *in vivo* model does not exist, the mechanism by which mechanical forces modulate the morphology, metabolism and genetic expression of EC must be studied with appropriate *in vitro* models. Various types of experimental apparatuses have been developed to impose different forms of mechanical forces on culture cells. Thus far, limited studies have been performed using direct hydrostatic pressure separate from circumferential wall tension.

2.7.1 Shear Stress Models

In order to mimic the effect of arterial wall shear stress due to blood flow, *in vitro* models study fluid flows on cultures. These designs usually involve a parallel plate, cone and plate, or flow loop apparatus. Published research studying the gene expression

effects of shear stress indicate that flow induces c-Myc expression, peaking in the 1.5–2 hour time range. Specifically, Li *et al.* found that both steady and 1 Hz pulsatile flow (average shear stress of 16 dynes/cm^2) caused slight increases in c-Myc mRNA levels which peaked at 2 hours and returned to basal levels by 4 hours [98]. Hsieh *et al.* investigated c-Myc protein levels in HUVEC subjected to steady shear stress at very low levels (4 and 10 dynes/cm^2). Their results show a peak in expression at 1.5 hours, which falls to basal levels by 2.5 hours [99]. Both of these studies substantiate the role of c-Myc expression in the pathogenesis of vascular disease.

2.7.2 Cyclic Strain Models

Although several types of devices have been used to induce mechanical stretch *in vitro*, in general, researchers have employed two different stretch regiments to mimic pulsatile circumferential wall stretch: uniaxial and biaxial. Different types of mechanical stretch elicit different molecular and cellular responses. Uniaxial systems mimic *in vivo* pulsatile (one-dimensional) expansion of the artery wall. Biaxial systems are advantageous because multiple systems can be run in parallel, but they incur a non-uniform strain field and are therefore inconsistent with *in vivo* mechanics.

In the biaxial stretch apparatus, cells are generally cultured on a circular membrane, constrained at the periphery. Cyclic stretching is imposed either by vacuum suction of the air or fluid below the membrane or by moving a central piston. The strain field produced is non-uniform throughout the membrane—maximal strain occurs near the outer edges, where the strain is primary radial as a result of clamping. Approaching the membrane center, strain levels decrease and the strain field is isotropic and biaxial. At the center, strain is close to 0%. A commercially available system called “Flexercell”

utilizes a vacuum-operated biaxial system. Extensive studies reveal that the biaxial strain induced in this system are insufficient to model vascular cyclic strain and in fact, are more appropriate for studying heart tissue and bone mechanics, where biaxial strain is apparent *in vivo* [100-102]. Additionally, during stretching, microscopic observation is impaired as a result of vertical movement in the center of the membrane.

Another model for cyclic strain implements an equibiaxial system. Indenter devices apply a uniform and equibiaxial strain to the membrane, but they have a limited range of cyclic strain (0.04–0.4%). These low percentages are suitable for bone cells, but not for vascular applications [103]. In one equibiaxial strain model [104], research found that endothelial cells were exposed to the same stress field and showed no morphological changes after 20% cyclic strain at 1 Hz. Since *in vivo* morphology indicates that cells orient themselves perpendicular to circumferential stretch, this lack of cytoskeletal reorganization makes results suspicious for *in vivo* application.

In uniaxial stretching devices, such as employed in this dissertation, the cells are cultured on a rectangular membrane, with one end anchored and the other end pulled at a certain frequency. Since the membrane in these devices moves only in one direction, microscopic observation of the cultured cells is relatively simple. Additionally, consistent with *in vivo* observations, morphological studies show that endothelial cells subjected to uniaxial cyclic strain orient themselves so that their long axis is perpendicular to the direction of stretch. This reorganization may play a fundamental role in regulating vascular tone and blood fluidity. Theories on cell alignment conjecture that the perpendicular alignment may be an attempt to reduce the strain energy exerted on the cell

[105]. Cells may be trying to minimize stretching of microtubules or other cytoskeletal networks during pulsation.

Published studies measuring c-Myc expression levels in cyclic strain models are limited. Thus far, no one has reported on the effect of c-Myc gene expression in uniaxial stretched HUVEC. However, preliminary research has investigated the effect of 20% uniaxial stretching of cardiac myocytes [106]. This group reported a transient stretch–induced upregulation of c-Myc mRNA that peaked around one hour.

CHAPTER III: MATERIALS AND METHODS

3.1 Experimental Procedures

3.1.1 Cyclic Strain Model

The system was designed to mimic the "normal" *in vivo* conditions that arteries experience during circumferential expansion. Since the radius of curvature of large arteries is much greater than the dimensions of the cell, the *in vitro* design was simplified by "cutting" a vessel along its axis and laying it flat, as shown in Figure 3.1. In this way, fluid shear stress and wall strain act together to cause alignment of the EC parallel to the flow axis of the vessel. In short, cyclic strain is imposed by culturing EC on a flexible membrane that is inserted into a chamber with one side of the chamber oscillating. To mimic the heart beating, a simple sinusoidal function (uniaxial cyclic strain) was imposed at 1 Hz.

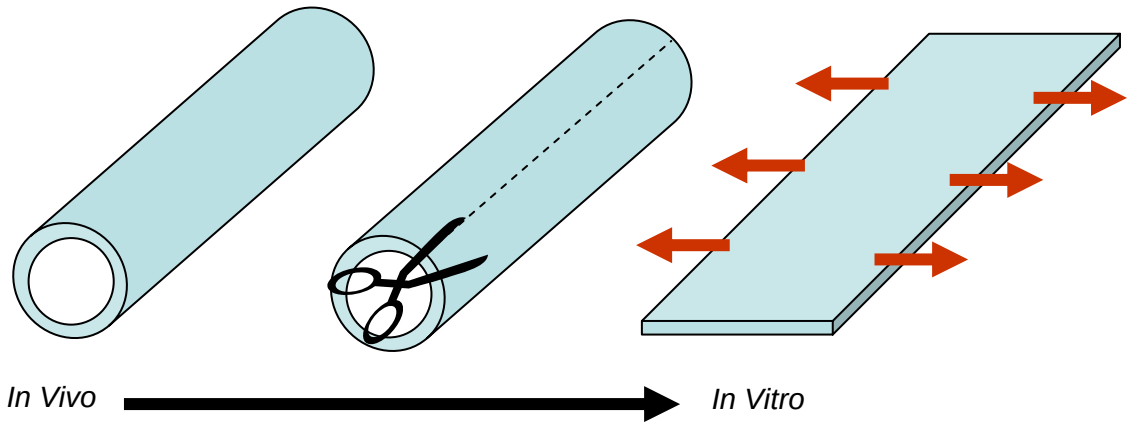


Figure 3.2: Rationale for cyclic strain model

3.1.1.1 Elastic Substrate

Silicone sheeting (Specialty Manufacturing Inc) was chosen as the substrate material because it is elastic, relatively inexpensive, readily available, non-toxic, and chemically inert. It is also optically clear, allowing for microscopic visualization of the cells. Another advantage of the silicone is that it does not lose its elasticity with autoclaving sterilization and can be stored at this stage for several weeks. The elastic substrate was cut to 8 centimeters long x 4 centimeters wide, a uniform geometry that provides uniform strain with axial loading. Since the membrane is very thin (0.005 inches), relative to its width and length, edge effects on the strain distribution are minimized (St. Venant's principle) [107].

After cutting membranes, they were placed in sealed autoclaving bags and sterilized for 10 minutes at 135°C. At the time of cell seeding, autoclaved membranes were moved to a laminar hood and assembled into the cyclic strain chamber. The detailed procedure is given in Section 3.1.1.3

3.1.1.2 Surface Modifications of Silicone Membrane

Although the silicone is medical implant grade and therefore biocompatible, its main disadvantage is its hydrophobic nature which prevents cell attachment. In order to improve this property, the silicone surface was modified through gelatin and gluteraldehyde cross-linking. Biological candidates for extracellular matrix include structural proteins (i.e. collagen and elastin), adhesive proteins (i.e. fibronectin and laminin), and polysaccharide glycosaminoglycans (i.e. heparin and hyaluronic acid). Cells will secrete their own ECM after attachment, but it is important to select an artificial matrix that will encourage this process.

For the purposes of these cyclic strain experiments, gelatin (Sigma) was chosen as an appropriate substrate for growing a confluent monolayer and retaining attachment, even during stretching. Once the stretch chambers were assembled, 1% gelatin dissolved in 1x phosphate buffered solution (PBS) was added to the membranes and the entire chamber was incubated at 37°C for at least one hour.

In order to crosslink the gelatin to the membrane, 0.5% (final concentration) gluteraldehyde [108] was added to the gelatin and the membranes were incubated at room temperature for 25 minutes. Immediately following, since residual gluteraldehyde is very toxic to cells, the membranes were thoroughly rinsed with 1 x PBS. The PBS wash was repeated a minimum of 4 times.

3.1.1.3 Cyclic Strain Apparatus

The apparatus for stretching consists of a polycarbonate chamber divided into two identical compartments. Polycarbonate is non-toxic, sterilizable and optically clear, allowing for visualization of the culture before and after stretching. All polycarbonate and stainless steel (ss) parts were rinsed thoroughly with deionized water, dried, and autoclaved unassembled. Chambers were assembled under sterile conditions in a laminar flow hood. The silicone membrane is mounted with ss clamps at either end of the chamber so that it is positioned close (within 1mm) and parallel to the floor of the chamber. This dynamic model takes into account the effect of not just cyclic strain, but also fluid agitation by including a "motion control."

Specifically, ss plates were fastened to the rear of the unit and were connected to ss bars at the front of the unit. The bars were set into the arm of the camshaft whose displacement was regulated through an adjustable pin mounted atop a rotating cylinder.

Displacement frequency is regulated by a DC motor. A detailed description of the strain apparatus is given in Figure 3.2.

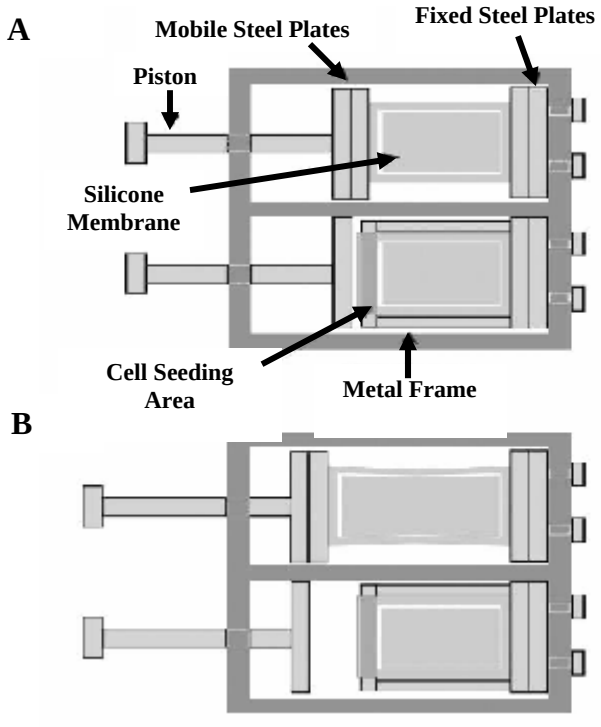


Figure 3.3: Cyclic strain model
Cyclic strain apparatus *A*: relaxed position. *B*: stretched position. Upper chamber in both A and B contains the cyclically-strained (CS) membrane, whereas bottom chamber contains the motion control (MC) membrane [109].

3.1.2 Human Endothelial Cell Culture

Pooled HUVEC obtained from Cambrex were grown to confluence on pre-gelatin-coated tissue culture flasks in media [composed of Media-199 supplemented with 20% fetal bovine serum (Cellgro), 25 mg/mL purified endothelial mitogen (Biomedical Technologies), 2 mM L-glutamine (Cellgro), 2.5 U/mL heparin sodium (American Pharmaceutical Partners), 50 U/mL penicillin (Cellgro), and 50 mg/mL streptomycin (Cellgro)] and maintained at 37°C in the presence of humidified 5% CO_2/95% air. Pre-warmed (to 37°C) media was replaced every 2–3 days, thus removing metabolic wastes and supplying fresh nutrients. HUVEC growth was monitored by assessing culture confluence and the cells were generally passaged every 4–6 days, just prior to reaching over-confluence. Cells were passaged by washing with 1xPBS and then incubating with 0.25% trypsin-EDTA solution (Gibco), centrifuged, and resuspended in complete HUVEC media. Cells were only used up to passage 5 since stretch-induced gene expression has been found to change in late passage EC [97].

The same HUVEC lot was used for each independent experiment to minimize lot-to-lot variability. Time-matched static controls were maintained for each stretching condition. The cell suspensions (approximately 1 x 10^6 cells in 1 ml of media) were carefully placed onto the pre-treated membranes and allowed at least 4 hours in the incubator for attachment. Cells were uniformly seeded and confluent. Although many cells remained unattached, the entire wetted surface was completely covered, and the cell appearance was the typical HUVEC cobblestone morphology characteristic of tissue culture. After cell attachment, 13 ml of complete media were added to each compartment and the chamber was placed in the incubator.

In order to synchronize the cells, they were incubated in quiescent media for 12 hours prior to stretching. This step also removed the unattached HUVEC. Quiescent media is identical to normal media, except it has a low serum content (5% versus 20%) and does not contain endothelial growth factor or heparin. Cyclic strain experiments were performed in quiescent media.

3.1.3 Exposure to Cyclic Strain

During an experiment, the polycarbonate unit was transferred to the motor-gear assembly inside a 37°C incubator and in the presence of humidified 5% CO_2/95% air. If necessary, the central shaft was coated with antibiotic ointment to provide lubrication and minimize contaminate entry via the drive shaft surface. The motor assembly was plugged into an extension cord which was specifically routed into the culture incubator and turned on. The motor speed and cam eccentricity were adjusted to subject the experimental membrane to cyclic deformation of 5–20% of its unstretched length at 1 Hz. The movement of the clamp in the motion control (MC) compartment was set to give the same media movement as in the experimental cyclic strain (CS) compartment. Thus, although the membrane was not stretched in the MC chamber, fluid motion due to clamp motion was nearly identical in both chambers. In the CS chamber, the movement of the clamp provided a cyclic stretching and relaxation of the membrane together with viscous drag-induced movement of the media bathing the membrane. An additional control utilized cells cultured on membranes maintained under static incubation without any fluid motion.

3.2 Experimental Assays

3.2.1 Quantitative RT-PCR

3.2.1.1 Isolation of mRNA

Immediately after stretch and, if applicable, drug exposure, membranes were removed from the cyclic strain chambers under a laminar hood and placed in a sterile Petri dish. The stainless steel clamps were cut off at the point of attachment with a sterile scalpel blade. The HUVEC were washed with ice-cold 1x PBS. Cells were removed from the membrane with a sterile cell scraper and collected in fresh 1x PBS. The resulting cell suspension was centrifuged for 10 minutes at 1500 rpm, 4°C. After aspirating the PBS from the resultant pellet, cells were lysed in Buffer RLT from the RNeasy RNA Mini Extraction kit (Qiagen) and homogenized using a QIAShredder (Qiagen). To prevent DNA contamination, on-column DNase digestion was performed using RNase-free DNase and Buffer RDD (Qiagen). The DNase was efficiently washed away in subsequent steps.

The integrity and quantity of RNA was verified through spectroscopic analysis, except with very small yields of RNA, which cannot be accurately quantified photometrically. In these cases, RNA was instead quantified by quantitative real time polymerase chain reaction (qRT-PCR). Readings were taken at ultraviolet wavelengths of 260, 280, and 320. The A260/A280 ratio, an indication of RNA purity, was at least 1.8 for all isolations. The A320 reading was used to provide a background reading. The A260 reading provided a measure of the concentration of RNA, such that 1 absorbency

unit equaled 40µg of RNA/ml of solution [110]. Purified RNA was stored at -80°C in RNase-free water, but used within one year to ensure stability.

3.2.1.2 cDNA Synthesis

For qRT-PCR, 0.1 µg of total RNA was reverse transcribed into cDNA with SuperScript II (Invitrogen) according the manufacturer's instruction. The resulting template cDNA was purified through Micro Bio-Spin P-30 Chromatography Columns (BioRad) to reduce background fluorescence.

3.2.1.3 RNA Primers

Quantitative RT-PCR primers were designed with Primer3 software [111] with the following criteria:

- primer size should be between 20 and 22 (optimally 22)
- T_m should be matching and within 55°C to 65°C (optimally 65°C because this matches the melting temperature for the c-Myc primer set)
- product T_m optimally 80°C
- the percentage of G to C binding should be between 50–60% (optimally 55%)
- the last 5 bases at the 3 prime end should have no more than two G's or C's
- annealing temperatures of the primers as close as possible
- primer pairs with minimal number of potential primer dimers and primer hairpins as possible

From these criteria, four possible binding pairs were selected for each gene (Sigma Genosys). Primer efficiency was determined for each primer set at 8

temperatures ranging from 55–65°C. The optimal primer set for each gene was used at its respective melting temperature.

Table 1: Primer Sets for qRT-PCR on MyiQ Cycler

Gene	Primer Sequence	T_m (°C)
c-MYC	5'- GCTGCTTAGACGCTGGATTP -3' 5'- TCCTCCTCGTCGCAGTAGA -3'	65
ACTIN	5'- CTGGAACGGTGAAGGTGACA -3' 5'- AAGGGACTTCCTGTAACAACGCA -3'	65
HSP60	5'- AGTCAAGGCTCCAGGGTTTGG -3' 5'- TGGCATCGTCTTTGGTCACA -3'	65
PCNA	5'- CCTGACAAATGCTTGCTGACC -3' 5'- CTTGAGGATGGAGCCCTGGA-3'	63.1
VEGF	5'- TTTGCTTGCCATTCCCCACT -3' 5'- GGTCACTCACTTTGCCCCTGT -3'	61.2

Genes were analyzed on a MyiQ Cycler (Bio-Rad). Quantitative RT-PCR was performed according to the following protocol: each 16µl reaction contained 1x iQ Sybr Green Supermix (Bio-Rad), 0.3 µM of forward and reverse primers, and 1:2 dilution of cDNA. Reactions were carried out in a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad). Amplification conditions were as follows: T_m (50 sec), 95°C (60 sec); 40 repetitions. The appropriate number of cycles was determined in preliminary experiments to ensure that PCR was taking place in the linear range, in order to guarantee a proportional relationship between input RNA and densitometry readout. Absolute concentrations were quantified from C_T values with a linear standard curve and normalized to β-actin expression. Each PCR was repeated at least 4 times.

3.2.2 Western Blotting

HUVEC exposed to cyclical stretch (0–20% elongation) were harvested by scraping and then centrifuged (1500 rpm) for 10 min at 4°C. The pellet was resuspended and homogenized by syringe needle in 100–150 µl Cell Lysis Buffer (CLB) [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 2 mM EDTA], and the supernatants were collected and stored frozen at -80°C. Protein content was determined by Bradford assay (Bio-Rad) using CLB as the standard. Equal protein was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% Tris-HCl precast polyacrylamide gels (Bio-Rad). Proteins were electroblotted onto nitrocellulose. Blots were incubated for 1 hour at room temperature with 3% non-fat dried milk to block non-specific binding. Proteins were revealed with primary antibodies [rabbit anti-(c-Myc) monoclonal (Cell Signaling); mouse anti-(VEGF) polyclonal (Santa Cruz Biotechnology); goat anti-(HSP60) polyclonal (Assay Designs); rabbit anti-(PCNA) polyclonal (Santa Cruz Biotechnology)] (1:1000) overnight at 4°C. Next, blots were incubated with secondary antibodies [horseradish peroxidase-conjugated polyclonal anti-(rabbit IgG), anti-(mouse IgG), or anti-(goat IgG)] (1:10000) for 1 hour at room temperature. Protein was detected with an enhanced chemiluminescence detection system (Amersham Biosciences). Equal protein loading was verified by mouse-anti-(ß-actin) monoclonal antibody (Sigma-Aldrich). Western blots were quantified using densitometry (Kodak EDAS 1D Imaging System).

3.2.3 Quantitative Protein ELISA

TransAM (Active Motif) is a sensitive, quantitative enzyme-linked immunosorbent assay (ELISA) for transcription factor activation. This kit uses a plate to which the consensus-binding site oligonucleotide for c-Myc has been immobilized. Nuclear extract from cyclic strain samples is added to each well and c-Myc binds specifically to this bound oligonucleotide. A primary antibody specific for an epitope on the bound and active form of c-Myc is then added, followed by secondary antibody and developing solution. Absorbance was measured using a Wallac Victor microplate reader (Perkin-Elmer) at 450 nm.

3.2.4 Cell Viability Assays

3.2.4.1 CellTiter Cell Proliferation Assay

In vitro cytotoxicity assays were performed using the CellTiter 96 nonradioactive cell proliferation assay (Promega). Cells were plated in 0.1 mL of media on day 0 in 96-well microtiter plates. On day 1, 10 μL of serial dilutions of the investigational compound were added in replicates of four to the plates. After incubation for 4 days at 37°C in a humidified incubator, 20 μL of a 20:1 mixture of 2 mg/mL MTS [3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] and an electron coupling reagent, phenazine methosulfate (0.92 mg/mL in DPBS), were added to each well followed by incubation for 4 hours at 37°C. Absorbance was measured using a Wallac Victor microplate reader (Perkin-Elmer) at 490 nm. Data were expressed as the percentage of survival of the control calculated from the absorbance corrected for background absorbance. The surviving fraction of cells was determined by

dividing the mean absorbance values of the test agents by the mean absorbance values of the untreated control.

3.2.4.2 BrdU Cell Proliferation Assay

Chemicon's BrdU Cell Proliferation Assay Kit was used to evaluate cell cycle progression of HUVEC subjected to quiescent media. This kit uses bromodeoxyuridine (BrdU) to immunochemically detect the population of cells which are synthesizing DNA. BrdU is incorporated into newly synthesized DNA strands of actively proliferating cells and absorbance was measured using a Wallac Victor microplate reader (Perkin-Elmer) at wavelength 450nm. Data are expressed as the BrdU absorbance corrected for background absorbance.

3.2.4.3 Cell Cytotoxicity Assay

CytoTox-ONE Homogeneous Membrane Integrity Assay (Promega) is a cytotoxicity assay used specifically to measure necrosis in a system. Since lactate dehydrogenase (LDH) is produced in HUVEC with damaged membranes, by quantifying the amount of LDH released into cell culture media, the number of lysed cells can be determined. The enzymatic assay functions by measuring the fluorescence created in the reaction of LDH with resazurin. Immediately after stretch and, if applicable, drug exposure, cyclic strain chambers were relocated to a laminar hood and a 100 μL sample of cell culture media was removed for analysis. The kit was used according to the manufacturer's protocol. Absorbance was measured using a Wallac Victor multilabel counter (Perkin-Elmer) at 490 nm. Data were expressed as relative necrosis, which is the absorbance of the stretched fraction divided by the absorbance of the static fraction.

3.2.4.4 Cell Apoptosis Assay

Apo-ONE Homogeneous Caspase-3/7 Assay (Promega) is used to detect apoptosis in a system. This kit fluorescently detects the presence of caspase-3 and -7 (apoptosis markers) in cells by utilizing Promega's proprietary lysis/activity buffer, in conjunction with the (Z-DEVD)2-Rhodamine 110 substrate. Absorbance was measured using a Wallac Victor multilabel counter (Perkin-Elmer) at 490 nm. Data were expressed as relative apoptosis, which is the absorbance of the stretched fraction divided by the absorbance of the static fraction.

3.2.5 Preparation of Drug Compound

Compounds were synthesized at University of Arizona Cancer Center and authenticated through nuclear magnetic resonance and liquid chromatography-mass spectrometry. Lyophilized drug compound was shipped overnight from the University of Arizona Cancer Center to Georgia Tech. Upon arrival, the compound was resuspended in 100% 0.02 μm filtered-sterilized DMSO at 10mM and frozen at -20°C until use. Repeated freeze/thaws of the compound were avoided. Upon use, the compound was thawed at room temperature and diluted to 100 μM in 0.02 μm filtered deionized water. The compound was then diluted to its final concentration of 5 μM in the quiescent media. Specifically, the quiescent media was removed, drug added, and then added back to the cyclic strain chamber.

3.3 Data Analysis

Significance in protein expression was assessed by Student's t-test. Data are expressed as mean ± SE. Student's t-test was used for the comparisons between the different groups. Differences were considered significant ($P<0.01$ or $P<0.05$) as indicated on the graphs.

CHAPTER IV: RESULTS

4.1 Morphological Analysis

To test the accuracy of the cyclic strain system, it was imperative to examine whether stretching the silicone substrate resulted in cell stretch and to determine if HUVEC responded appropriately to the application of this cyclic strain. Previously published studies [105] using a precursor of the stretching apparatus employed for this dissertation examined the mechanical effects of cyclic strain on the morphology of HUVEC. The results found that EC most frequently aligned 90° away from the direction of stretch. Additionally, in visualizing the cytoplasmic microtubule complexes, there was a greater density of microtubules running parallel to the major axis of the cell after stretch–induced alignment. Upon replication of these previous morphological analyses, it was determined that in fact, the cells do align perpendicular to the direction of stretch, not only at pathological levels of cyclic strain, but also at percentages as low as 5%. This altered morphology occurs as soon as 15 minutes after the initiation of stretch.

Comparable to previous studies on cyclic strain, cells in less confluent sections of the membrane aligned more rapidly. Static membranes failed to align at a specific angle and had an essentially random pattern. Figure 4.1 shows the morphology of stretched and static HUVEC.

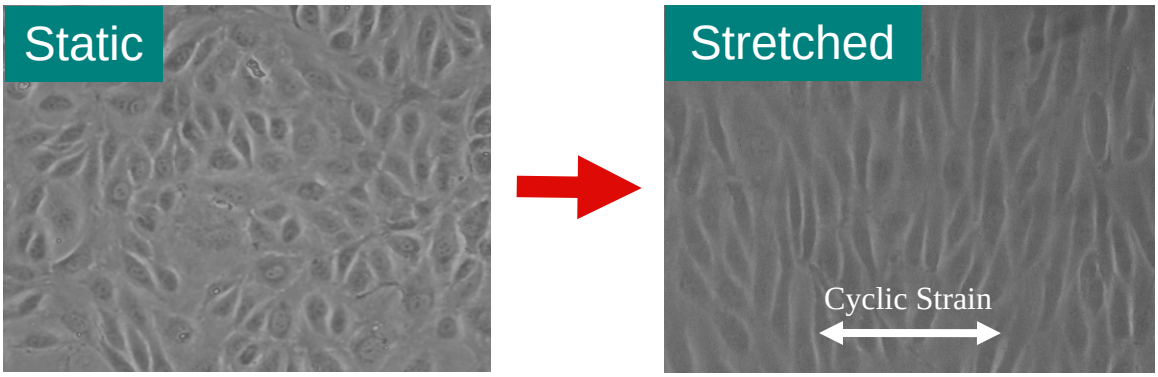


Figure 4.1 Morphology of static and stretched HUVEC

In order to discount the possibility of apoptotic and necrotic induction by the experimental system, it was necessary to assay cell necrosis and apoptosis simultaneously. The CytoTox-ONE Homogeneous Membrane Integrity Assay (Promega) and the Apo-ONE Homogeneous Caspase-3/7 Assay (Promega) were multiplexed. Although necrosis and apoptosis both ultimately conclude with cell death, necrosis is traumatic cell death resulting from acute cellular injury, while apoptosis is carried out in an orderly process [112].

The CytoTox-ONE Homogeneous Membrane Integrity Assay measures the release of lactate dehydrogenase (LDH) into the cell culture media. LDH levels were measured when the cells were cyclically strained at the maximum percentage, 20%, because higher strains implicate a higher probability for cell injury. As shown in Figure 4.2, there was no increase in release of LDH by 20% stretch for the 0–3 hour time range. Thus, 20% cyclic strain of HUVEC had comparable results to static conditions, implicating that cyclic straining of cells causes cell deformation without significant cell injury.

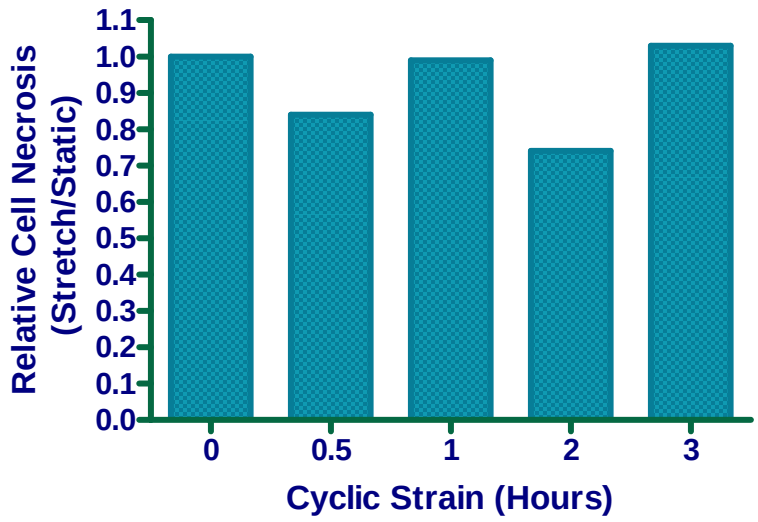


Figure 4.2: Cyclic strain of 20% does not increase cell necrosis in HUVEC, n=1.

Next, the effect of cyclic strain on EC apoptosis was detected using the Apo-ONE Homogeneous Caspase-3/7 Assay, which employs a luminescent substrate to detect caspase-3/7 activity. The caspases, a family of cysteine proteases, mediate apoptosis at different stages. With activation, they disable cellular housekeeping and repair programs and cleave important structural components in the cells [112].

Numerous studies on VSMC and EC using various techniques for apoptosis detection have shown that physiological levels of cyclic strain (5% to 10%) do not induce apoptosis [3, 113, 114]. However, higher potentially pathological levels of strain (15% to 20%) stimulate apoptosis on both vascular cell types [3, 12, 114-116]. Cyclic strain has been shown to activate p53 and Bcl-2 and cause the production of reactive oxygen species [116]. These events result in direct oxidative DNA damage. Therefore, a slight increase in apoptosis was expected with 20% cyclic strain. Figure 4.3 demonstrates that 20% cyclic strain causes a slight increase in caspase activity.

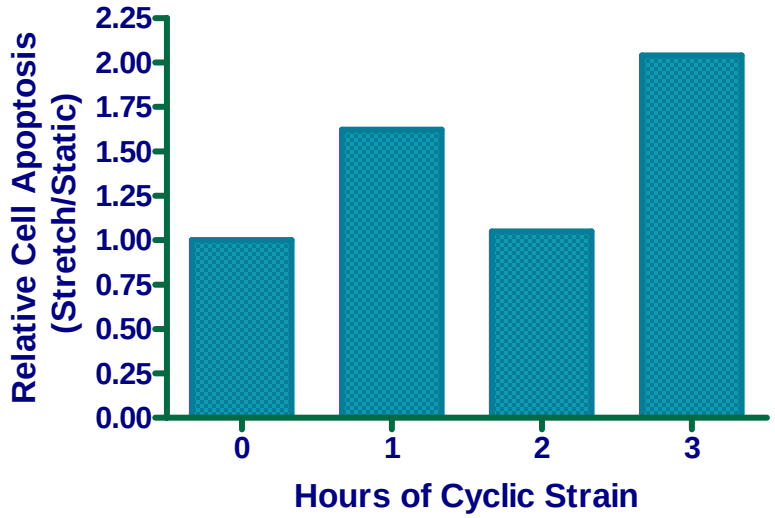


Figure 4.3: Cyclic strain of 20% slightly induces apoptosis in HUVEC, n=1.

4.2 Quiescent HUVEC

It is well-known that serum exerts strong and rapid control over c-Myc expression in numerous cell types [117]. Therefore, in order to monitor basal levels of c-Myc, cells are often serum-starved in "quiescent media," inducing a quiescent growth state. Quiescent media is identical to normal media, except it has a low serum content (5% versus 20%) and does not contain endothelial growth factor or heparin. It has been established that c-Myc is not expressed in quiescent cells [118], but the length of time necessary for specific cell types to become quiescent is imprecisely known. For that reason, before any c-Myc analysis could be undertaken, it was imperative to determine a sufficient time period for pre-incubation of HUVEC with quiescent media.

Bromodeoxyuridine (BrdU) is commonly used to determine if cells are proliferating, and thus, is an indicator of whether cells are in a quiescent (G_0) state. During the S-phase of the cell cycle, BrdU is incorporated into the newly synthesized

DNA of replicating cells, substituting for thymidine. Although this method gives an indication of cell quiescence, unfortunately, it has limitations. Cells in serum-starved experiments have previously been determined to not only be quiescent, but also maintain positive BrdU staining [119]. This apparent contradiction is explained by the process of apoptosis. Serum and growth factor deprivation induce cells, such as HUVEC, to undergo apoptosis. Researchers report that during this programmed cell death, cells depart from their quiescent state and experience some part of the G_1 phase of the cell cycle, thus explaining their positive BrdU staining. It is hypothesized that the reason may be due in part to the process of apoptosis requiring the presence of newly synthesized proteins [119].

Thus, bearing these limitations in mind, Chemicon's BrdU Cell Proliferation Assay Kit was employed as an efficacious means to evaluate alterations in cell cycle progression of HUVEC subjected to quiescent media. HUVEC change morphology and die when deprived of serum and growth factors for more than 16 hours (based upon morphological analysis). For this reason, and taking into account that HUVEC will spend up to 4 hours in quiescent media during cyclic strain experiments, 12 hours was determined to be the maximum incubation period. Therefore, cells were incubated up to 12 hours in quiescent media and assayed at 2–hour intervals. Results are shown in Figure 4.4.

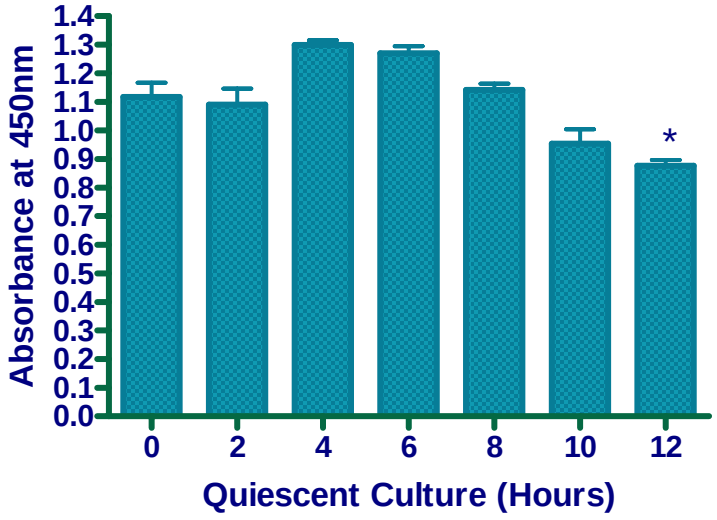


Figure 4.4: BrdU staining of HUVEC cultured in quiescent media for 0–12 hours, n=4.
* Significance vs. cells not subjected to quiescent media

Based upon these results, it was determined that a 12–hour incubation with quiescent media was necessary in order to attain a significant change in BrdU incorporation. Although there was not a major decrease in positive BrdU staining, the deficit was sufficient to assume cell quiescence without incurring apoptosis. Consequently, EC were incubated in quiescent media for 12 hours prior to stretch experiments. Subsequently, all cyclic strain experiments were performed in the same quiescent media, ensuring no addition of serum or growth factors, which could alter c-Myc expression.

4.3 c-Myc Expression in EC subjected to cyclic strain

To examine the impact of cyclic strain on c-Myc expression, an *in vitro* system mimicking the hemodynamic conditions associated with cyclic strain was employed. With any *in vitro* cyclic strain model, cells are concomitantly exposed to low fluid

motion–induced shear stress and convective transport. Since fluid agitation may alter gene expression, the utilized cyclic strain system accurately takes into account the fluid agitation inherent in the strain apparatus. This is accomplished by including a motion control (MC) condition. The devices used in many strain studies do not allow for a motion control; therefore, many of the reported cellular responses to cyclic strain (CS) have not been confirmed as actual stretch responses (for a detailed description of the strain unit, please see Section 3.1.1.3).

It has been well documented that normal physiological cyclic strain can be modeled as about 10% [6, 13], and pathological strain as about 20% [3, 12]. Since it has also been established that higher c-Myc levels correspond with pathological states [50, 61, 66], a direct correlation can be made between c-Myc expression and cyclic strain percentage. Preliminary studies using the imposed cyclic strain model examined if fluctuations in cyclic strain percentage were associated with changes in c-Myc expression. HUVEC were subjected to 10% or 20% cyclic strain for 1.75 hours. Results are shown in Figure 4.5.

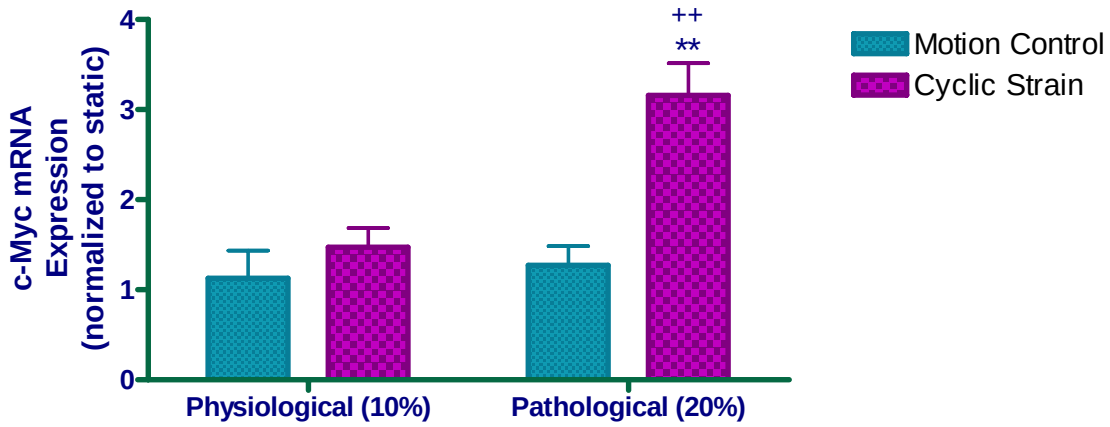


Figure 4.5: Physiological and pathological c-Myc mRNA expression in HUVEC
HUVEC were subjected to physiological and pathological levels of cyclic strain for 1.75 hours, n=4–9.
** Significance (P<0.01) vs. static control
++ Significance (*P*<0.01) vs. motion control

In comparing 20% CS to static and 10% CS samples, it is apparent that c-Myc mRNA expression at 20% CS is almost 3 times that of the other conditions. Since expression of c-Myc in the normal cell is tightly regulated, this expression change has enormous implications for downstream targets. In fact, the normal cell expresses little c-Myc unless prompted by external signals, such as growth factors and extracellular matrix contacts. Even then, changes are on the order of only a 1- to 1.5-fold increase.

Additionally, 20% CS is significant over the motion control, meaning that the increased c-Myc expression is due to the actual stretching of the cells and not just to the motion of fluid across them. Based upon these results, it can be summarized that 20% cyclic strain does indeed infer a more “pathological” state of cyclic strain.

To further investigate the correlation between *in vitro* cyclic strain and *in vivo* conditions, the cyclic strain regimen from 0–20% was examined. Figure 4.6 shows

c-Myc mRNA expression in HUVEC subjected to 1.75 hours of cyclic strain at 5%, 10%, 15%, and 20% CS.

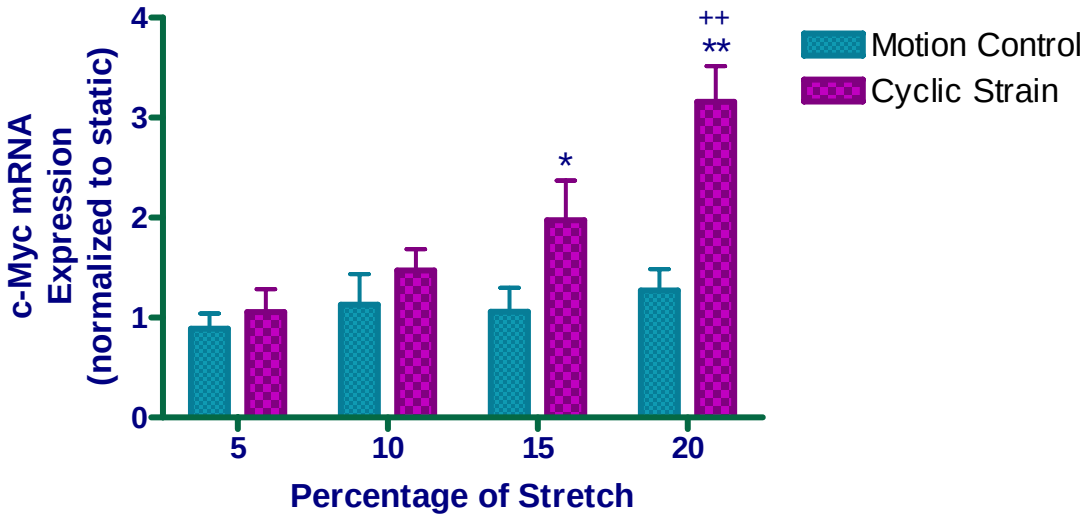


Figure 4.6: c-Myc mRNA expression in HUVEC cyclically strained 0–20%.
HUVEC were subjected to cyclic strain at percentages of 0-20% for 1.75 hours, n=4–9.
** Significance (P<0.01) vs. static control
* Significance (P<0.05) vs. static control
++ Significance (P<0.01) vs. motion control

From the results in Figure 4.6, we can again see that c-Myc expression is significant in the 15–20% range—the range previously implicated as modeling a pathological state in the vasculature. Thus, having established and confirmed the *in vitro* conditions necessary to model *in vivo* conditions, experiments could now be confidently and competently preformed to examine the time-dependency of c-Myc expression.

c-Myc mRNA expression, as measured by qRT-PCR, was examined at the following time-points: 0, 0.5, 1, 2, 3, 4, 5, 6, and 12 hours. As shown in Figure 4.7,

expression increased almost 3-fold in HUVEC cyclically-strained at 20% for 1.5 to 2 hours over control conditions.

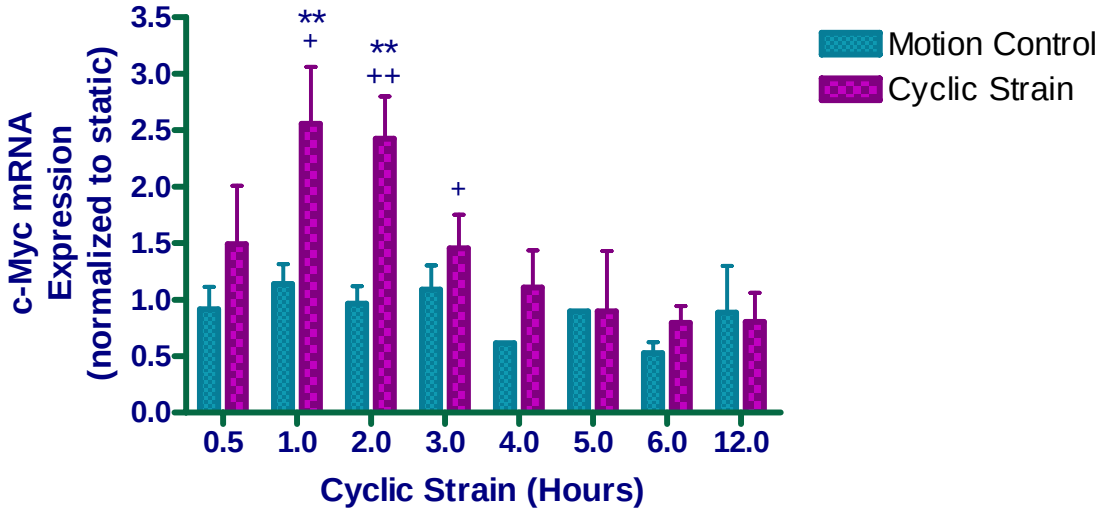


Figure 4.7: c-Myc is induced by cyclic strain and is time-dependent
HUVEC were subjected to 20% cyclic strain for 0–12 hours and c-Myc mRNA expression was examined in comparison to motion control and static samples, n=4–13.
** Significance (P<0.01) vs. static control
++ Significance (P<0.01) vs. motion control
+ Significance (P<0.05) vs. motion control

This upregulation is time-dependent, returning to basal levels by 3 hours. Also, the motion controls did report a very slight, but not significant, increase in expression. This finding implicates that the slight movement of media is not a factor in determining the cyclic strain–induced gene expression profile. Therefore, for the purposes of simplification, the motion control data will only be reported in Appendix A.

Western analysis also indicates that 20% cyclic strain induces a time-dependent increase in c-Myc protein expression, which peaks around 2 hours (Figure 4.8).

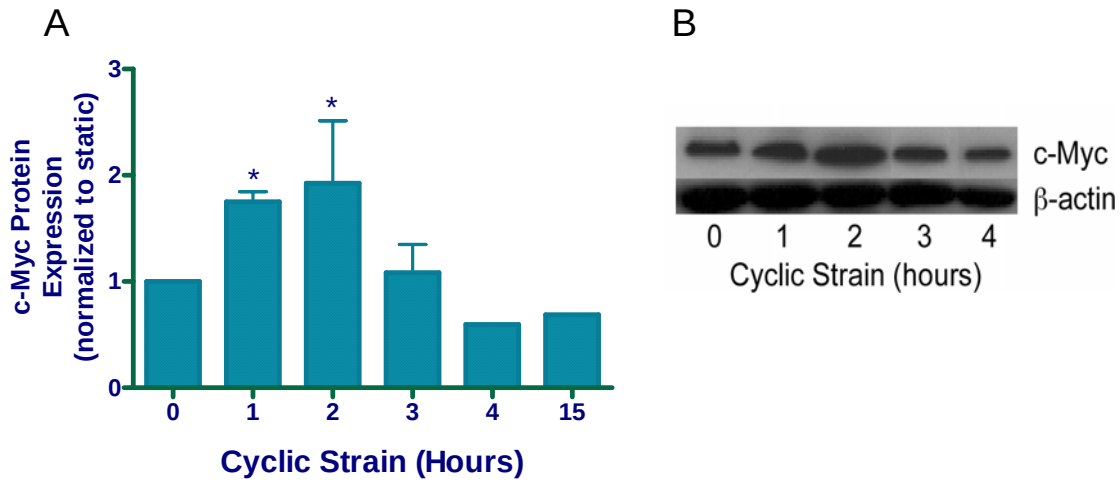


Figure 4.8: Western blot analysis of c-Myc protein
A. Band intensities from densitometry of 6 independent experiments were normalized to ß-actin.
* Significance (P<0.05) vs. static control
B. Representative immunoblot of c-Myc and ß-Actin.

These results are further confirmed through quantitative ELISA (Figure 4.9).

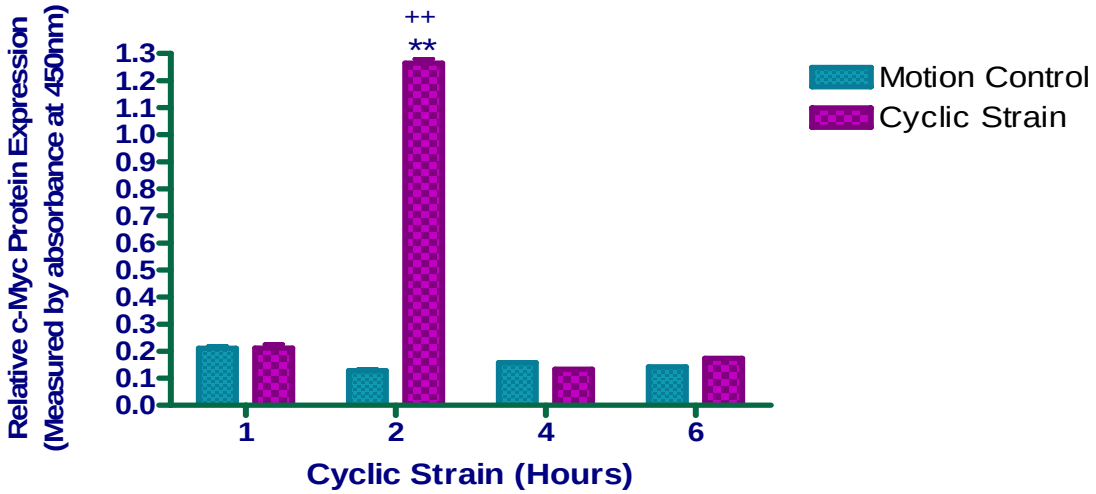


Figure 4.9: c-Myc protein expression as measured by TransAM ELISA, n=2–3.
** Significance (P<0.01) vs. static control
++ Significance (P<0.01) vs. motion control

Through the use of transcriptional profiling, these results demonstrate significant changes in endothelial cell c-Myc gene expression with imposition of pathological levels of cyclic strain. Specifically, both mRNA and protein expression of c-Myc is induced in a time-dependent manner by 20% cyclic strain.

4.4 Compound screening to modulate c-Myc transcription

Having established that c-Myc expression is induced by pathological levels of cyclic strain, the question arises whether it is possible to modulate this activation. As detailed in Section 2.5.1.2, University of Arizona Cancer Center (UAZCC) in Tucson has identified compounds targeted to the c-Myc promoter, which are able to prevent c-Myc transcription in cancer cells. In order to study whether cyclic strain–induced c-Myc transcription can also be attenuated, three lead UAZCC compounds were investigated—compounds 0005, 0010, and 0012.

The toxicity of each drug was determined by using a cell proliferation assay (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay, Promega). The LD_{50}, the concentration of drug at which 50% of cells die, was calculated to be the following (Table 2, Appendix B).

Table 2: LD_{50} of UAZCC Compounds

	COMPOUND 0005	**COMPOUND 0010**	**COMPOUND 0012**
LD_{50}	56.5 μM	5.4 μM	> 100 μM

These results indicate a high level of toxicity for Compound 0010. Compounds 0005 and 0012 are more viable options for HUVEC treatment.

In discussing toxicity, it should also be noted that the lyophilized compounds have been dissolved in dimethyl sulfoxide (DMSO). DMSO is often chosen as an ideal solvent due to its ease in dissolving solutes. However, with *in vivo* use, this same property raises safety issues. These concerns are easily avoided in this experimental setup, however, because the compounds are in cell culture for a very short incubation time and the final concentration of DMSO is extremely low (0.05%). In fact, the FDA has approved the clinical use of DMSO at 100-fold higher concentrations. Patients inflicted with interstitial cystitis and intractable cerebral edema are routinely treated with 50% and 10% DMSO, respectively [120].

As further confirmation that DMSO is not hindering the cells or inducing secondary responses, HUVEC were treated with 0.05% DMSO. The minimal addition of DMSO did not induce any noticeable cell differentiation, such as death, change in morphology, or alteration in cell doubling time. DMSO-treated samples yielded responses comparable to untreated samples (Figure 4.10).

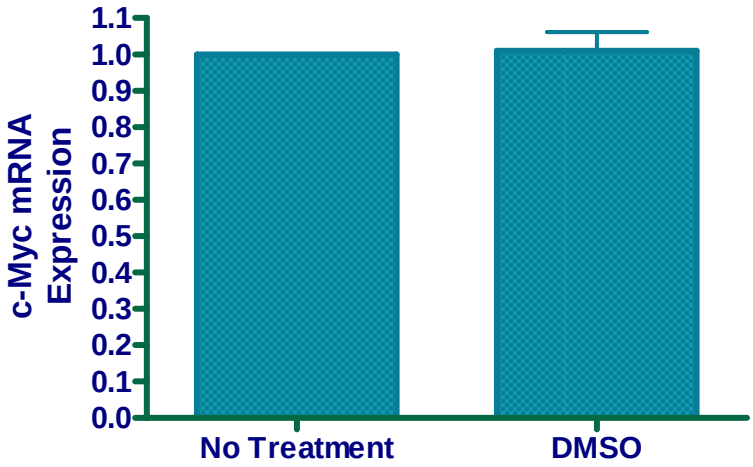


Figure 4.10: Effect of DMSO on HUVEC culture, n=2.

Next, to evaluate the efficacy of each of these compounds in the cyclic strain experimental setup, a preliminary time course experiment was performed. Since cyclic strain's effect on c-Myc levels parallels that of treating cells with serum, results were expected to mirror those of University of Arizona's serum-starvation experiment in cancer cells (see Section 2.5.1.2). For this preliminary experiment, all drugs were used at 1 µM for at least 24 hours. Cells were seeded in normal HUVEC media. For 12 hours, HUVEC were placed in normal media containing 1 µM of drug, washed, and then treated for an additional 12 hours in quiescent media containing drug. cDNA from 20% cyclic strain and motion control samples was then collected from the following time-points: 0, 0.5, 1, 1.5, 2, 2.5, and 3 hours. The results from each drug were as follows (Figures 4.11, 4.12, and 4.13).

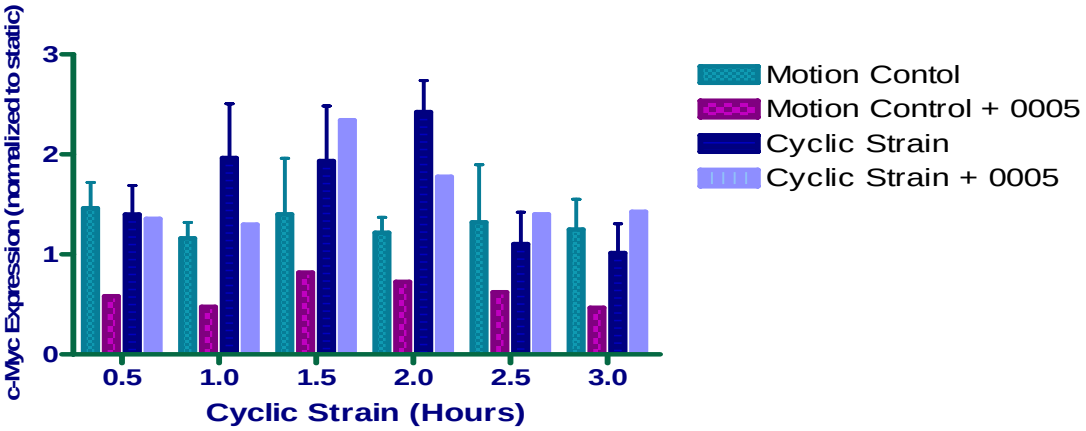


Figure 4.11: Compound 0005, n=1.

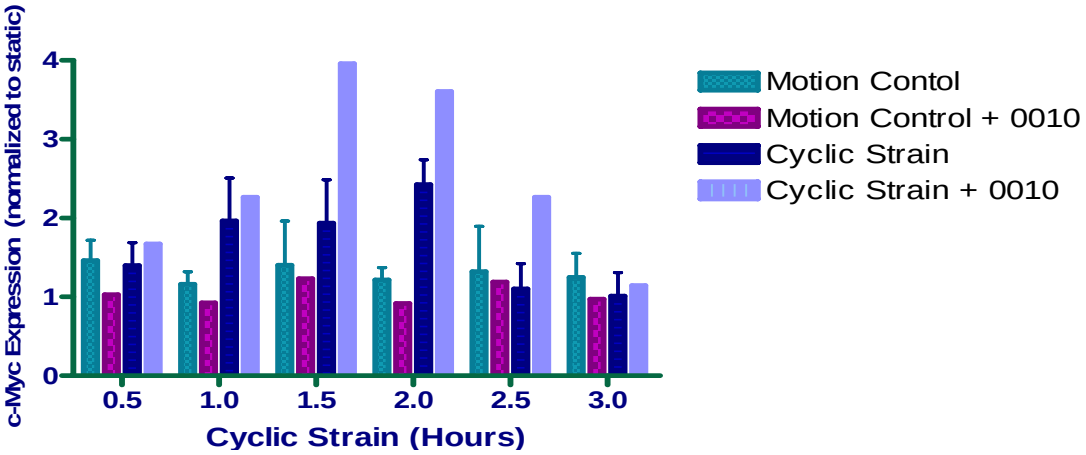


Figure 4.12: Compound 0010, n=1.

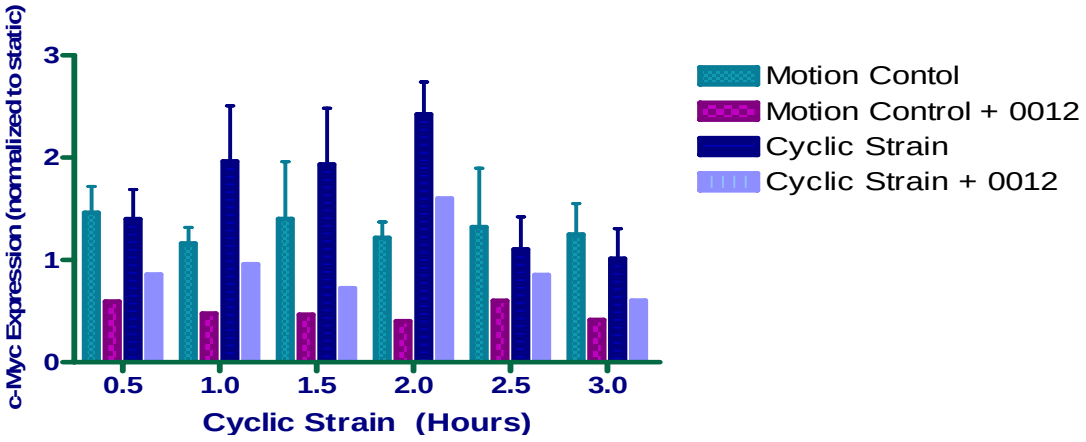


Figure 4.13: Compound 0012, n=1.

From these preliminary evaluations, it was concluded that compound 0012 is non-toxic and the most effective at attenuating c-Myc levels, while 0010 and 0005 are essentially inconsequential. From a drug development point of view, the failure of a compound is due to a number of limitations, which are disease and cell-type specific. A compound with suboptimal chemical properties may be toxic or unable to penetrate the cell nucleus due to its stereochemistry and/or hydrophobicity. The efficacy of compound 0012 reveals that it is indeed possible to attenuate c-Myc expression by targeting the silencer region of the c-Myc promoter.

4.5 Compound Optimization

As the target compound for further analysis, the usage of compound 0012 was further optimized to maximize its ability to attenuate c-Myc expression.

4.5.1 Compound 0012 Dosage

In order to determine the optimal concentration of 0012 to use, cells were subjected to 1.75 hours of cyclic strain and treated with the following concentrations of 0012: 0μM, 0.5μM, 1μM, 2.5μM, 5μM, 10μM, 15μM, 25μM, and 50μM. Results are graphed in Figure 4.14.

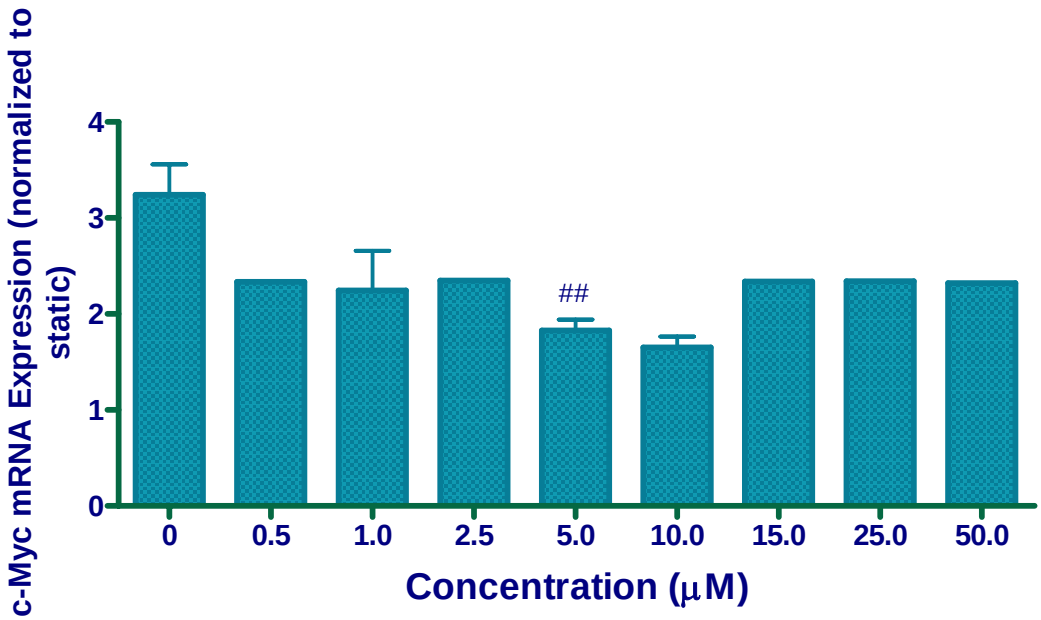


Figure 4.14: Compound 0012 dose response
c-Myc mRNA expression was examined in HUVEC that were cyclically strained for 1.75 hours and treated with varying concentrations of Compound 0012, n=1–5.
Significance (*P*<0.01) vs. untreated HUVEC

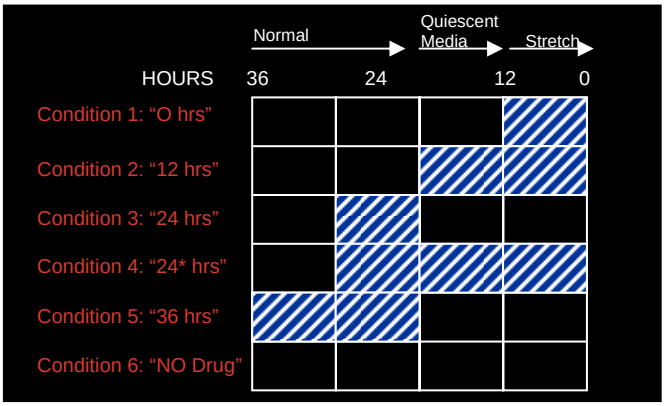
By comparing the treated cyclic strain samples to 0 μM, it is apparent that compound 0012 is most effective at concentrations of 5 μM and 10 μM. Since 5 μM is a slightly lower concentration of DMSO (0.05% as compared to 0.1%), it was chosen as the optimal treatment concentration.

4.5.2 Compound 0012 Time Treatment

The next step in optimizing compound 0012 efficacy was to determine the optimal dosing schedule. A treatment time course was investigated as follows (Table 3). Shading is indicative of 5 μM of compound 0012 in the media. For all samples, at 12 hours prior to stretch, normal media was removed and replaced with quiescent media. For example, "36 hours" refers to cells that were given drug in regular media 36 hours prior to stretch, then replaced with quiescent media without drug. Since normal cells are

in a G_0 state and only express the gene when they actively divide, the “0 hrs” time point mimics physiological conditions.

Table 3: Time Treatment Course



The results of the time treatment experiment are shown in Figure 4.15.

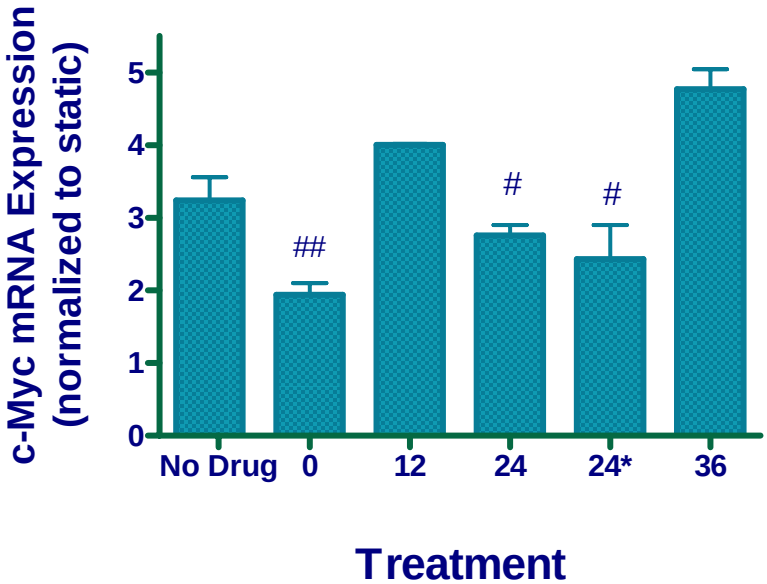


Figure 4.15: Time treatment experiment, n=2–5.
Significance (p=0.01) vs. untreated HUVEC
Significance (*P*<0.05) vs. untreated HUVEC

Interestingly, the optimal results were seen in the "0 hrs" treatment in which the drug was added as stretch began. The "0 hrs" treatment resulted in an attenuation of c-Myc expression by 40%. Comparing this data to UAZCC's serum-starvation experiment with cancer cells, "0 hrs" treatment parallels adding serum back in the cancer cell experiment.

The other time points also yielded interesting results. At "12hrs", there was no effect. This result could indicate that HUVEC must be in a nondividing G_0 state in order for the drug to be effective. The results for the "24 hrs", "24* hrs", and "36 hrs" indicate that perhaps the drug is being metabolized. This could explain why "24 hrs" and "24* hrs" attenuated cyclic strain effects by 15% and 25%, respectively, and "36 hrs" had no effect at all.

4.6 Modulation of c-Myc Transcriptional Activation

After determining the best possible conditions for using compound 0012, its success in modulating cyclic strain induced c-Myc expression was evaluated. HUVEC were seeded onto silicone membranes in normal media. Twelve hours prior to the initiation of cyclic strain, quiescent media replaced the normal media. At the time of stretch, the quiescent media was removed and mixed with compound 0012 to give a final concentration of 5 μM. The compound-containing quiescent media was then returned to the stretching apparatus and cyclic strain experiments commenced. c-Myc mRNA expression with and without compound 0012 was examined at the following time-points: 0, 0.5, 1, 2, 3, 4, 5, 6, and 12 hours. As shown in figure 4.16, at peak c-Myc expression, compound 0012 succeeds in attenuating mRNA transcriptional activation about 33% and 28% at 1 and 2 hours, respectively.

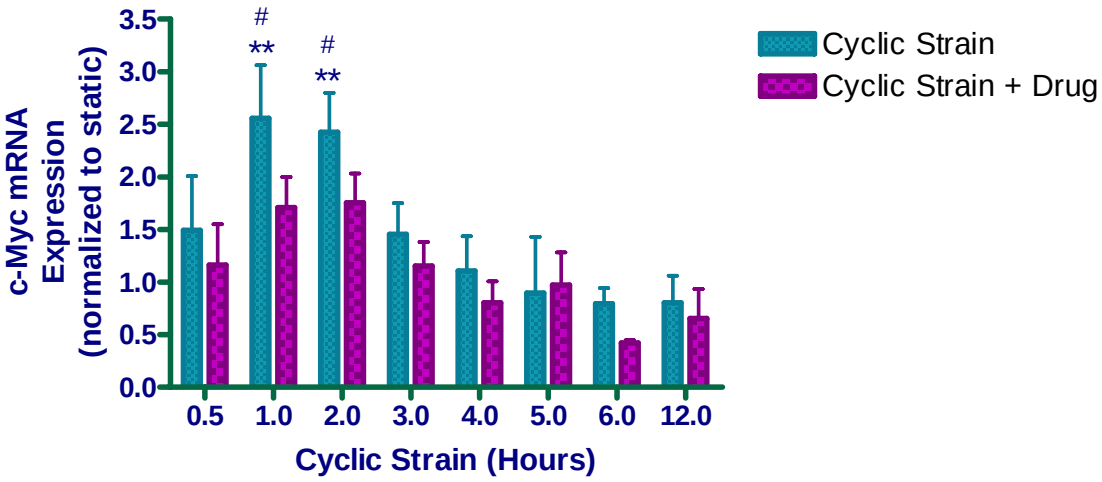


Figure 4.16: Compound 0012 attenuates c-Myc mRNA expression, n=4–13.
** Significance (P<0.01) vs. static conditions
# Significance (P<0.05) vs. untreated HUVEC

Western analysis also indicates that cyclic strain–induced c-Myc expression can be modulated at the protein level. As shown in Figures 4.17, c-Myc protein expression is attenuated 55% and 64% at 1 and 2 hours, respectively, by the addition of compound 0012.

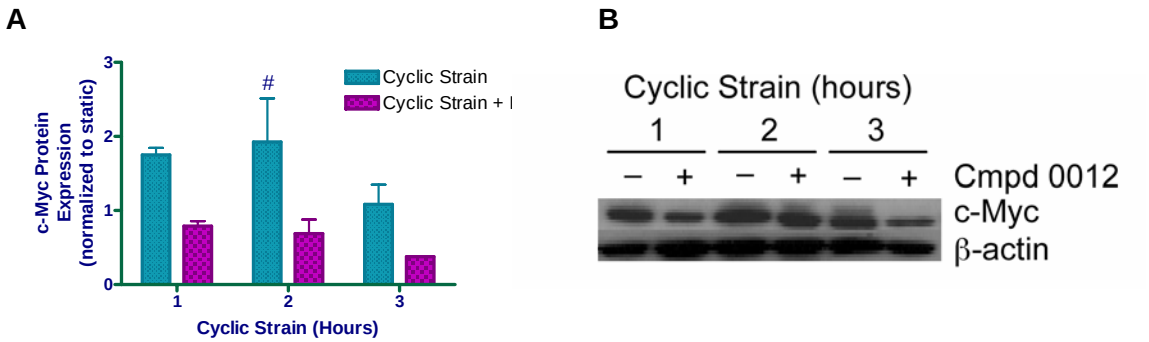


Figure 4.17: Compound 0012 attenuates c-Myc protein expression
A. Compound 0012 attenuates c-Myc protein upregulation by cyclic strain. Band intensities from densitometry of 5 independent experiments were normalized to ß-actin. # Significance (P<0.05) vs. untreated HUVEC
B. Representative immunoblot of c-Myc and ß-Actin.

4.7 Necrosis and Apoptosis

Although results indicate that c-Myc transcription can be attenuated through the use of compound 0012, it is imperative to ensure that this outcome is due to c-Myc promoter targeting, and not as a secondary result of necrosis and/or apoptosis. As detailed in Section 4.1, the CytoTox-ONE Homogenous Membrane Integrity Assay and the Apo-ONE Homogenous Caspase-3/7 Assay were used to determine levels of necrosis and apoptosis. These two assays were performed as described before—HUVEC were cyclically strained at 20%, but now, 5 μM of compound 0012 was added to the quiescent media at the time of stretch. Results for necrosis and apoptosis are shown in Figures 4.18 and 4.19, respectively.

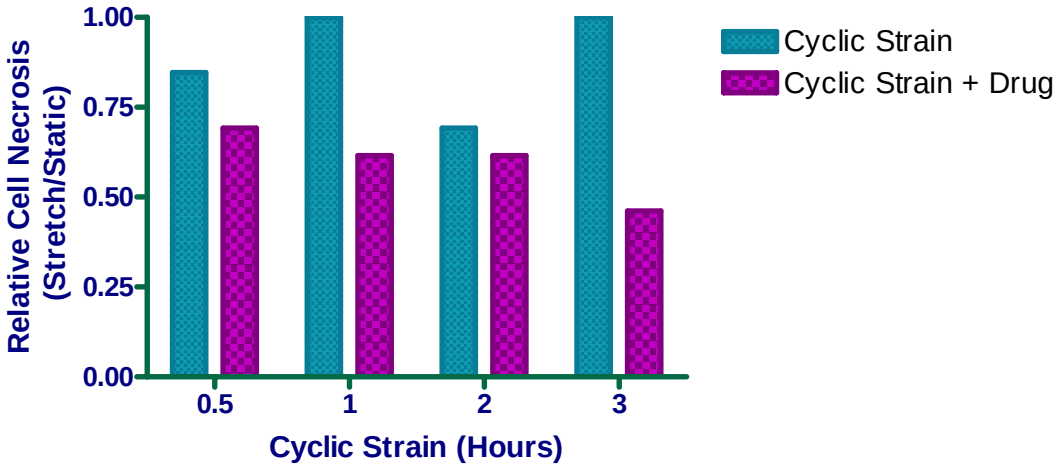


Figure 4.18: Necrosis caused by compound 0012, n=1.

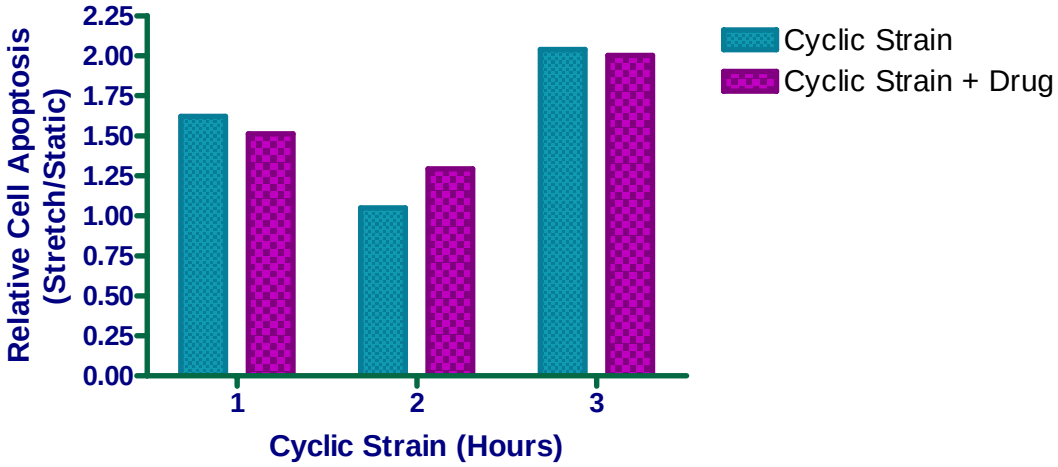


Figure 4.19: Apoptosis caused by compound 0012, n=1.

From these figures, it is apparent that the addition of compound 0012 does not induce HUVEC necrosis or apoptosis. Therefore, it is unlikely that the attenuation of c-Myc gene expression is due to these cellular death processes.

4.8 c-Myc Downstream Target Genes

The subsequent step in studying the modulation of c-Myc focuses investigations on the genetic profile downstream targets of c-Myc. These are genes whose expression is directly regulated by the c-Myc protein. It is well known that c-Myc induction leads to activation of numerous genes with a wide range of functions, including regulating cell proliferation, apoptosis, differentiation, morphology, migration, and secretory function. Of interest to this dissertation are genes related to cardiovascular disease. Three genes in particular have been identified as both regulated by c-Myc and as playing a significant role in the progression of vasculoproliferative cardiovascular diseases. These c-Myc downstream target genes are proliferating cell nuclear antigen, heat shock protein 60, and vascular endothelial growth factor (see Section 2.5).

4.8.1 PCNA

Proliferating cell nuclear antigen (PCNA) mRNA expression of HUVEC subjected to cyclic strain is shown in Figure 4.20. As expected from the review of relevant literature, this graph shows that cyclic strain does indeed induce PCNA expression and that the addition of compound 0012 succeeds in attenuating it.

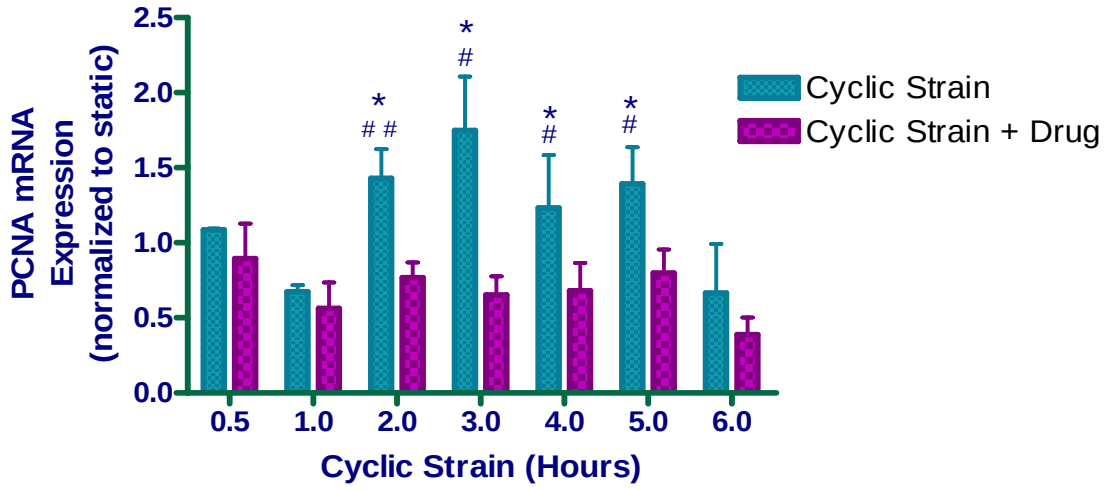


Figure 4.20: PCNA mRNA expression, n=3–10.
* Significance (P<0.05) vs. static control
# Significance (P<0.05) vs. untreated HUVEC
Significance (P<0.01) vs. untreated HUVEC

Western analysis (Figure 4.21) also indicates that 20% cyclic strain induces PCNA protein expression, which can also be attenuated by treatment with compound 0012. As expected, the protein expression peaks later than the mRNA expression.

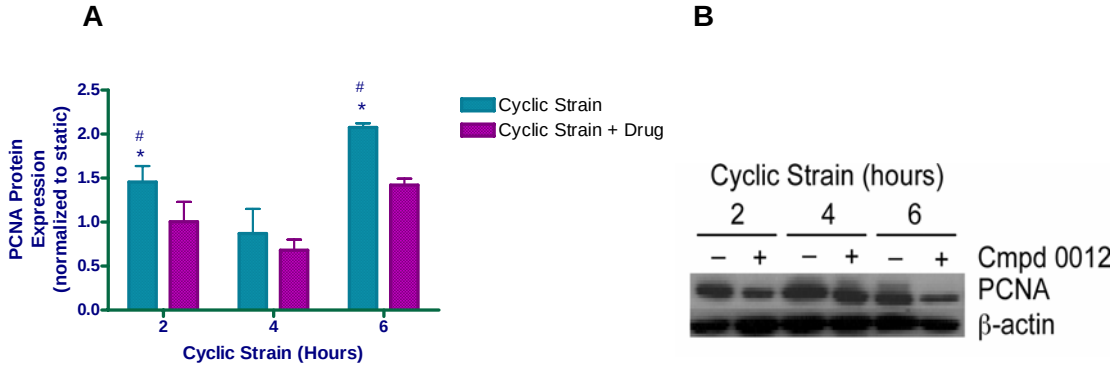


Figure 4.21: PCNA protein expression
A. Compound 0012 attenuates PCNA protein upregulation by cyclic strain. Band intensities from densitometry of 5 independent experiments were normalized to ß-actin.
* Significance (P<0.05) vs. static control
# Significance (P<0.05) vs. untreated HUVEC
B. Representative immunoblot of PCNA and ß-Actin.

4.8.2 VEGF

Vascular endothelial growth factor (VEGF) mRNA expression of HUVEC subjected to cyclic strain is shown in Figure 4.22. As expected from the review of relevant literature, this graph shows that cyclic strain does indeed induce VEGF expression and that the addition of compound 0012 does succeed in attenuating it.

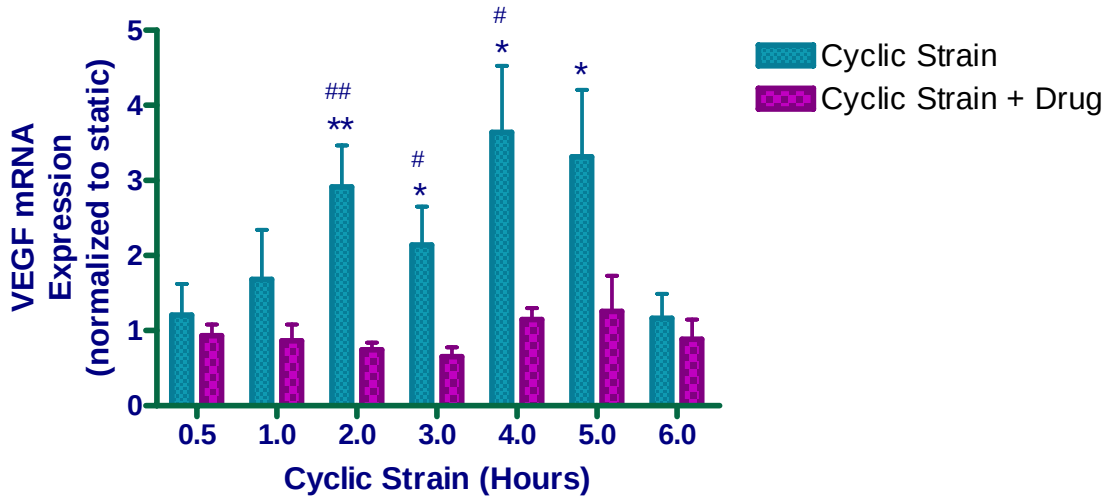


Figure 4.22: VEGF mRNA expression, n=3–12.
** Significance (P<0.01) vs. static control
* Significance (P<0.05) vs. static control
Significance (P<0.01) vs. untreated HUVEC
Significance (P<0.05) vs. untreated HUVEC

Western analysis also indicates that 20% cyclic strain induces VEGF protein expression, which can also be attenuated by treatment with compound 0012 (Figure 4.23).

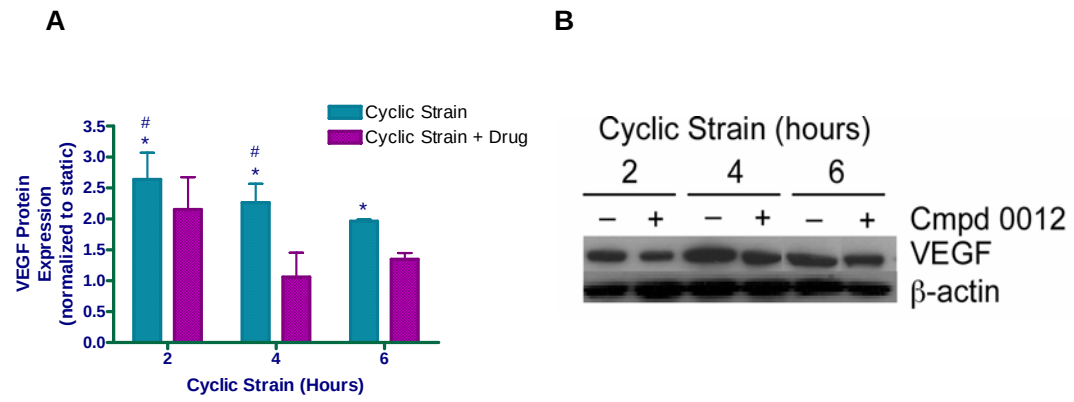


Figure 4.23: VEGF protein expression

A. Compound 0012 attenuates VEGF protein upregulation by cyclic strain. Band intensities from densitometry of 5 independent experiments were normalized to ß-actin.
* Significance (P<0.05) vs static control
# Significance (P<0.05) vs. untreated HUVEC
B. Representative immunoblot of VEGF and ß-Actin.

These results are consistent with other researchers who have found VEGF expression can be induced for several hours [94, 121].

4.8.3 HSP60

Heat shock protein 60 (HSP60) mRNA expression of HUVEC subjected to cyclic strain is shown in Figure 4.24. As expected from the review of relevant literature, this graph shows that cyclic strain does indeed induce HSP60 expression and that the addition of compound 0012 does succeed in attenuating it.

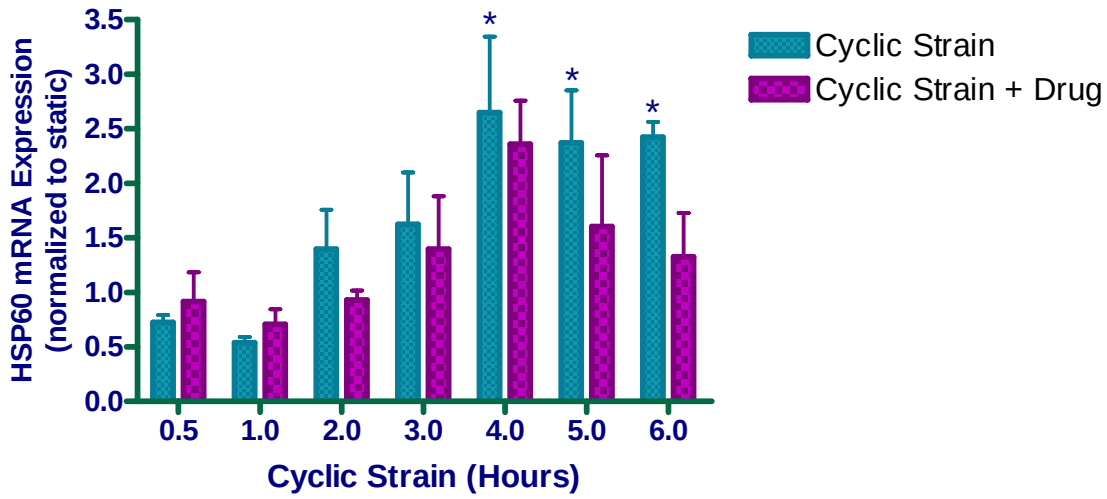


Figure 4.24: HSP60 mRNA expression, n=3–9.
* Significance (P<0.05) vs. static control

Although the effect of compound 0012 is not significant for mRNA expression, there is a slight decrease in expression. For all of these downstream genes, the genetic profile is essential at the mRNA level, but more important at the protein level, where cellular functions are directly affected. In the case of HSP60, the effect of the compound effect is significant at the protein level (Figure 4.25).

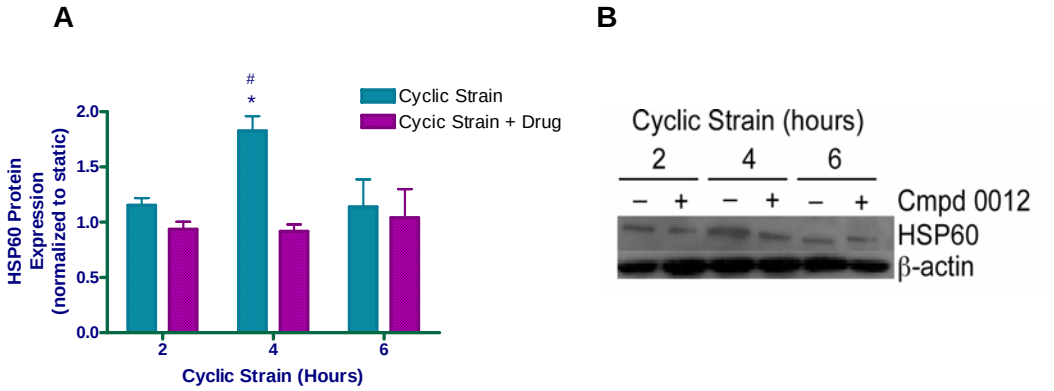


Figure 4.25: HSP60 protein expression

A. Compound 0012 attenuates HSP60 protein upregulation by cyclic strain. Band intensities from densitometry of 4 independent experiments were normalized to ß-actin.
* Significance (P<0.05) vs. static control
# Significance (P<0.05) vs. untreated HUVEC
B. Representative immunoblot of HSP60 and ß-Actin.

4.8.4 Overlay of c-Myc Downstream Target Genes

By plotting the mRNA expression of all three target genes onto one graph, we can easily see the time-dependent expression of c-Myc downstream target genes (Figure 4.26).

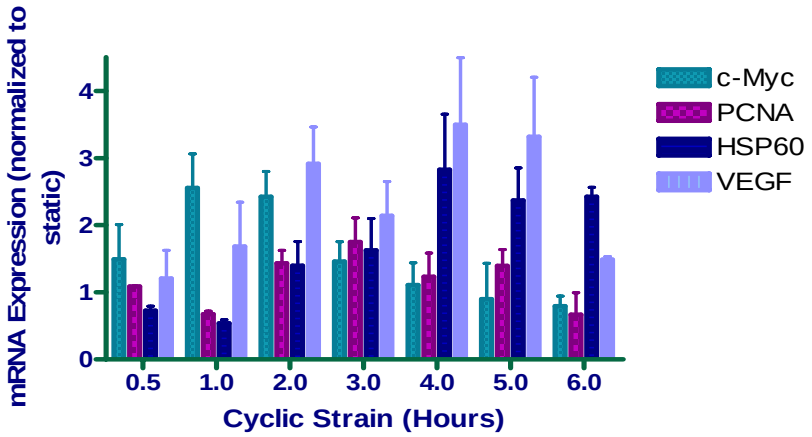


Figure 4.26: Combined mRNA expression of c-Myc downstream genes

The mRNA expression of these genes after treatment with compound 0012 is compiled in Figure 4.27. By directly comparing Figures 4.26 and 4.27, it can be inferred that compound 0012 succeeds in attenuating the expression of PCNA, VEGF and HSP60. Hence, by inhibiting cyclic strain–induced c-Myc transcription, compound 0012 modulates c-Myc downstream gene expression.

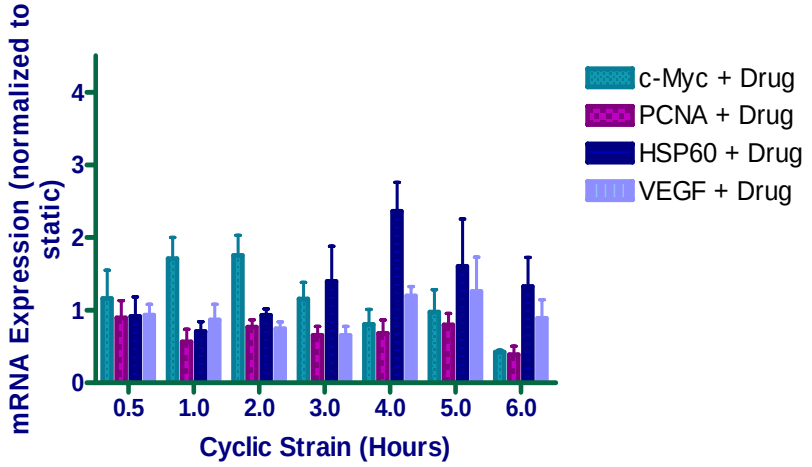


Figure 4.27: Compound 0012 attenuates c-Myc downstream gene expression
Compound 0012 attenuates c-Myc, PCNA, HSP60 and VEGF mRNA expression in HUVEC subjected to cyclic strain.

CHAPTER V: DISCUSSION

5.1 Mechanotransduction

The primary focus of this research is on the nuclear events that occur in response to pathological levels of cyclic strain, specifically, transcription factor c-Myc and its downstream gene targets. However, in studying the effects of mechanical forces on EC, it is also important to discuss the mechanisms by which vascular cells sense the extracellular mechanical stimuli and convert them into chemical signals. The aim in postulating this mechanotransduction pathway is to aid in the understanding of physiological and pathological processes involved in vascular remodeling and cardiovascular disease.

Specific mechanotransductive signaling pathways have not yet been elucidated; however, some possible mechanisms can be hypothesized. Stretch-mediated responses can be simplified in five generalized steps. First, applied mechanical forces are transmitted thorough the extracellular matrix to the cytoskeleton. Second, the cytoskeleton responds by activating membrane receptors, including integrins, ion channels, platelet-derived growth factor receptors, and G proteins [122]. Third, these mechanosensors convert the mechanical stimuli into chemical signals, activating second messenger systems. Next, these molecules transduce the signals from the mechanoreceptors to the nucleus. Finally, the nuclear events result in changes in cellular function [123]. It is known that there are different levels of sensing and control at each step, but current research is still being undertaken to determine the specific mechanisms.

Research shows that transduction of cyclic strain to the nuclear level seems to involve the same second messenger pathways and genes as those involved in other mechanical forces [124]. These second messengers include mitogen-activated protein kinases, protein kinase C, and Akt. Important to this discussion is second messenger adenylate cyclase/cAMP, which has not only been identified as activated by cyclic strain, but also has an implicated role in c-Myc expression. Studies with bovine aortic endothelial cells indicate that the activity of adenylate cyclase/cAMP increases within 15 minutes of 10% cyclic strain. This induction of adenylate cyclase/cAMP, which does not occur at 6% stretch, more than doubles the basal expression levels [115]. These experiments provide evidence that when cyclic strain reaches a threshold, the adenylate cyclase (AC) signal transduction pathway is activated, stimulating the expression of genes containing cAMP-responsive promoter elements.

Important to this dissertation is the discovery that c-Myc is a gene with this promoter element, meaning cAMP is able to activate transcription factor c-Myc [125]. Therefore, a possible pathway for cyclic strain induced c-Myc is via the activation of adenylate cyclase/cAMP. The diagram shown in Figure 5.1 outlines the possible pathway of cyclic strain-mediated EC responses.

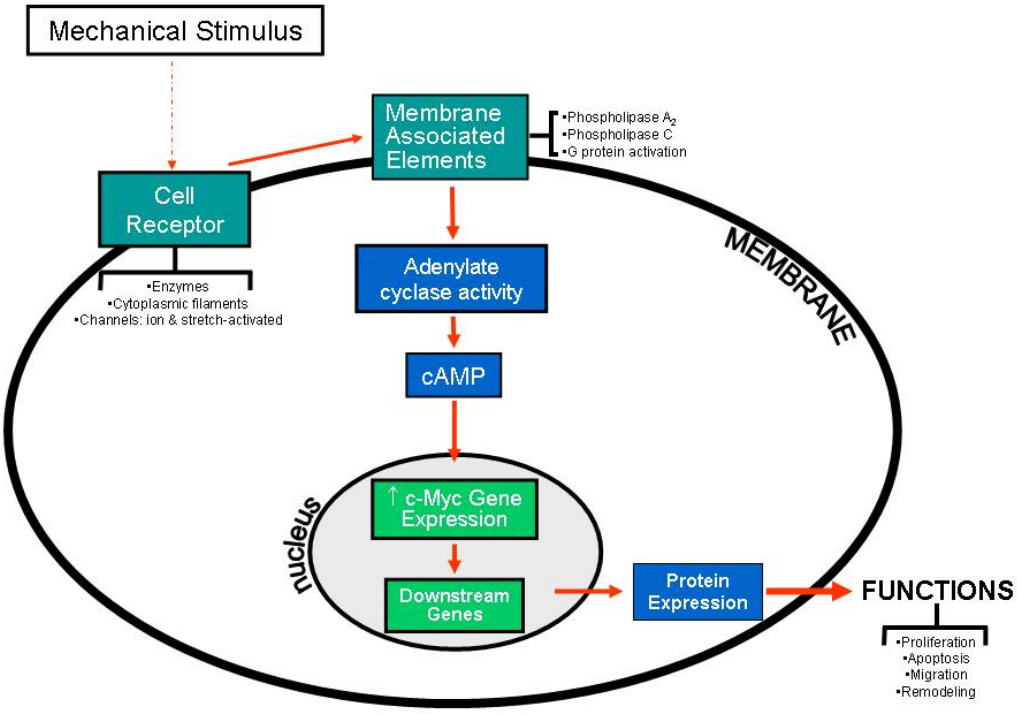


Figure 5.1: Proposed mechanotransduction pathway for c-Myc
Pathological levels of stretch induce adenylate cyclase/cAMP which leads to increased c-Myc expression, and subsequent gene expression.

5.2 c-Myc Control of Gene Expression

Although the mechanical forces initiating vascular disease have been studied extensively, their implicated differential gene expression profiles remain relatively unknown. As a starting point for investigation, immediate-early proto-oncogenes, such as c-Myc, have been identified as the earliest genetic responses to stimuli. c-Myc is a particularly promising choice because it is shear-inducible [126] and due to its vast network of downstream target genes, is referred to as a "global regulator of transcription" [127]. In all, c-Myc controls the expression of about 15% (3000-4000 genes) of the genome [127]. These target genes are involved in proliferation, apoptosis, metabolism, migration, and remodeling.

Although vast, high-throughput genomic and functional analyses, such as SAGE and microarray, have been performed on c-Myc, very few target genes are identified as being the most relevant to the progression of vascular diseases. This lack of data is not due to insufficient accomplishment in the field. Rather, despite numerous studies documenting the proliferation markers in human atherosclerotic plaques and restenotic lesions, universal acceptance of the "pertinent genes" remains controversial. Research does reveal that the starting events of the stenotic process include cell proliferation, migration, apoptosis, deposition of extracellular matrix, and inflammatory reactions [14].

Accordingly, pertinent c-Myc target genes were chosen based on their roles in these initiation events. Together, the selected genes, VEGF, HSP60 and PCNA, are three markers often simultaneously monitored for initiation and progression of vascular pathologies [128]. Although each gene can be isolated to determine its individual role in proliferative cardiovascular diseases, it is their concerted effects which result in the culmination of abnormal blood vessel narrowing. Together, these genes comprise the steps implicated in the vasculoproliferative cascade, beginning with regulation of the cell cycle and the inflammatory response, and culminating with vascular remodeling.

Examination of the mRNA and protein expression profile of each gene (Section 4.8.1–4.8.4) presents the opportunity to hypothesize a potential timeline governing this proliferative process. It is reasonable to postulate that upregulation of c-Myc quickly triggers the expression of PCNA and HSP60, while VEGF is induced slightly later and sustained longer. These time points can be further substantiated by extrapolating to the known progression of events *in vivo*. Animal studies suggest that vascular cell proliferation is the prevailing event in the initiation of vascular diseases [51]. Not only

are all three genes intimately linked to regulating the cell cycle, each gene mediates its effects at a different phase. Thus, their concerted expression allows direct cell cycle regulation at the G_1, S, and G_2 phases. VEGF can directly stimulate cell cycle progression from the G1 to S phase [129] and can mediate indirect effects through cell cycle regulators [130]. PCNA is essential for the S phase of the cell cycle. Lastly, heat shock proteins protect cells from apoptosis and reverse cell-arrest in the G_2 phase [131].

After early cell proliferation, likely mediated by the expression of HSP60 and PCNA, arterial remodeling begins at the site of intimal injury. This later process, alike to the reported expression of VEGF, is sustained. In addition, the implicated roles of VEGF implicated roles, such as wound healing, neovascularization, and EC migration [37], are pivotal to vascular remodeling. Hence, these parallels corroborate the argument that remodeling is at least partly accomplished through the activation of VEGF.

In conclusion, the upregulation of c-Myc by cyclic strain leads to the expression of c-Myc downstream targets. These genes can be implicated in comprising the steps of the vasculoproliferative cascade, beginning with regulation of the cell cycle and the inflammatory response, and culminating with vascular remodeling.

5.3 Drug-Eluting Stents

A promising contribution of this research is the identification of a possible novel treatment for vascular disease. Restenosis after surgical intervention is a major clinical problem, occurring in 30-50% of patients [16, 17]. Anti-coagulants, anti-inflammatories, and anti-proliferative drugs have been unsuccessful at preventing restenosis [132]. Ideally, the most effective pharmacological solution to preventing restenosis is the controlled-release of an anti-proliferative drug directly targeted to the factors involved in vascular injury.

Based upon these ideal drug characteristics and the data reported in this dissertation, compound 0012 is an excellent candidate for the treatment of in-stent restenosis. Results demonstrate not only the effectiveness of compound 0012 in reducing c-Myc protein expression (by more than 50%), but also in attenuating (to different extents) the expression of downstream genes (VEGF, HSP60, and PCNA) that contribute to vascular remodeling pathologies. It can be postulated based upon the roles of these genes that compound 0012 inhibits key targets in the cell cycle, while also potentially preventing inflammation and vascular remodeling.

Additionally, the chemistry of compound 0012 makes it an ideal anti-restenotic drug because it contains hydrophobic elements, which ensure high local concentrations, and hydrophilic elements, which allow homogeneous drug diffusion. Most importantly, since compound 0012 has been identified as specifically targeting the factors involved in vascular injury, its use can be short-lived and thus, will not inhibit re-endothelialization of the intima. Therefore, the fear that drug-eluting stents (DES) cause late stent thrombosis is abrogated.

Despite these encouraging *in vitro* results with compound 0012, several questions remain to be answered. Recalling that c-Myc controls up to 15% of the genome, the use of compound 0012 must be tethered by the concern for attenuating essential downstream genes. Additionally, compound 0012 must be proven to be anti-thrombotic, anti-inflammatory, anti-proliferative, and non-toxic *in vivo*. Once confirmed to be safe and efficacious, it needs to be determined if compound 0012 readily diffuses across vascular tissue and achieves high local tissue concentration. The standard pharmacology for *in vivo* animal models and, later, human clinical trials will also need to be determined. Specific to the use of compound 0012 in DES, research will have to be completed to identify a biocompatible polymer able to deliver the drug at a sustained, controlled, and predictable rate. However, the successful completion of these studies could culminate in the development of a novel therapeutic opportunity to treat vasculoproliferative diseases.

5.4 Limitations of Experimental Setup

The extrapolation of these results to *in vivo* conditions must be tempered by the understanding that all *in vitro* experimental models have limitations. Specific to this setup are cell culture and hemodynamic restrictions.

An idealized *in vivo* experiment is limited at even the cellular level. The use of HUVEC is often criticized as an inaccurate representation of EC behavior because they are vein-, not arterial-, derived endothelial cells [109]. Further, once in culture, researchers argue whether passaged HUVEC accurately represent *in vivo* genetic profiles. However, since HUVEC are the most economically feasible EC, low passages are generally accepted as an accurate representation of arterial EC. A final concern in cell

culture is the fear that by not including VSMC and macrophages, HUVEC are permitted to respond differently than they would *in vivo*.

From a mechanistic point of view, it is important to point out that, although cyclic strain is a major contributor to vascular disease, the arterial blood vessel is also subjected to two other forces: hydrostatic compression and shear stress. An ideal model would allow the study of the concerted effects of all three forces. Additionally, this dissertation's setup incorporated the use of a motion control to account for the small movement of media, but these controls are not a perfect reproduction. Fluid motion is possibly slightly greater when the entire substrate is agitated rather than a single edge moving.

The only plausible solution to combat these limitations is the development of a novel setup integrating a whole vessel model with all of the hemodynamic forces implicated in vascular mechanics. Since this experimental setup does not yet exist, this project employed an optimal uniaxial system to experimentally investigate cyclic strain. Very recent data validate this type of bioreactor as the most suited at replicating the physiological environment [133]. Therefore, the minimal limitations imposed on this experimental setup deem it a reliable method for monitoring the interrelationship between hemodynamics, vascular biology, and disease progression.

CHAPTER VI: CONCLUSIONS

6.1 Summary of Results

Each heartbeat creates a pressure difference between systole (peak pressure, corresponding to the opening of the heart valve) and diastole (closing of the valve). This fluctuating difference in pressures causes a pulsatile circumferential expansion of the artery called "cyclic strain," which is normally about 10%. When certain pathological conditions arise *in vivo*, such as hypertension, atherosclerosis, or after surgical intervention, elevated cyclic strain (~20%) can induce vessel remodeling, leading to a pro-atherogenic endothelium [3]. Research thus far has been successful in demonstrating that different cellular functions are modulated by changes in cyclic strain, but elucidation of the mechanisms by which EC alter these functions remains to be detailed.

This dissertation's chief goal is to offer a possible mechanism and promising clinical treatment for vascular diseases initiated by increased cyclic strain. The unifying theory is that pathological conditions increase cyclic strain in the endothelium, leading to plaque formation and intimal thickening, occurring directly due to the induction of c-Myc. In order to substantiate this hypothesis, this study verified that elevated levels of cyclic strain (20%) induce c-Myc mRNA and protein expression. In fact, by justifying these pathological levels of cyclic strain, this dissertation presents novel evidence that HUVEC c-Myc mRNA and protein expression are induced by 20% cyclic strain in a time-dependent manner.

This study provides evidence that these pathogenic levels of cyclic strain specifically activate the c-Myc promoter, leading to c-Myc transcription and downstream

gene induction. Further, by specifically targeting the c-Myc promoter, it is possible to not only modulate c-Myc, but also the downstream gene targets of c-Myc, which are prevalent in the induction of vascular disease. The compounds targeting c-Myc could provide a potential therapeutic opportunity in treating vasculoproliferative diseases.

6.2 Recommendations for Future Work

6.2.1 Cyclic Strain Model Enhancements

Based upon experiences with the current cyclic strain apparatus, it is possible to offer several possible modifications. The current methodology poses serious problems with HUVEC seeding and adherence, including spillage, patchy seeding, and cell detachment. Furthermore, there are several technical problems, including difficulty in sterilizing the multi-component stretch chamber and limitations of the substrate size, which often required the pooling of multiple chambers to provide enough cells for experimental assay.

6.2.2 Additional Areas for Research

This *in vitro* work elucidates some of the possible mechanisms of HUVEC gene regulation by mechanical stimuli. However, there are several areas for continued research.

- Since this experimental setup only utilized endothelial cells, it would be valuable to investigate smooth muscle cell behavior individually and in co-culture with EC.

- An interesting study would be to precondition the cells to “normal” physiological conditions (10% CS) and then introduce pathological stretching conditions (20%).

- This dissertation focused on three c-Myc downstream gene targets, however, other possible genes exist for future investigation.
    - Preliminary research was attempted without success on two c-Myc target genes implicated in vascular remodeling: matrix metalloproteinase-9 (MMP-9) and cyclin dependent kinase 4 (CDK4). However the limited achievement could be due to experimental methodology and further studies are warranted. Both genes have been identified as transcriptional targets of c-Myc [90, 134]. CDK4 is an especially interesting target because it directly correlates to the ability of c-Myc to promote vasculoproliferative responses and cell-cycle progression. MMP-9 is only induced by vascular cells during pathogenesis and is neither produced under basal conditions nor detectable in healthy human arteries.

    - Three genes, matrix metalloproteinase-2 (MMP-2), fibroblast growth factor (FGF-2), and platelet derived growth factor (PDGF) have been shown to have increased expression with cyclic strain and are implicated in the progression of vascular obstructive diseases [135-137]. However, current publications have not yet identified these genes as downstream c-Myc targets. The establishment of these genes as c-Myc targets could lead to the elucidation of more genetic targets in restenosis that can be modulated by attenuating c-Myc transcription.

APPENDIX A: MOTION CONTROL DATA

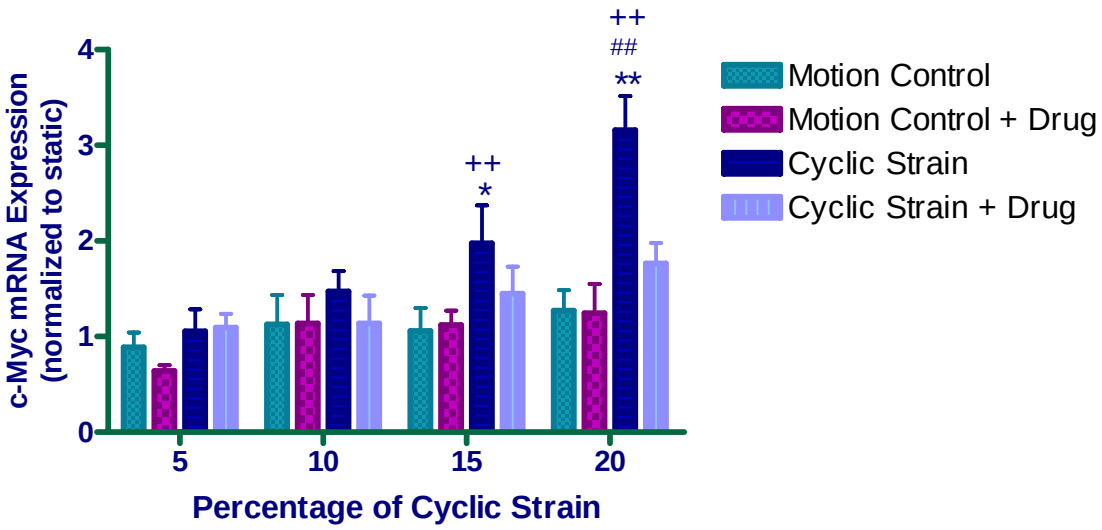


Figure A.2: c-Myc mRNA expression in HUVEC cyclically strained 0–20%.
HUVEC were subjected to cyclic strain at percentages of 0–20% for 1.75 hours, n=4–13.
** Significance (P<0.01) vs. static control
* Significance (P<0.05) vs. static control
++ Significance (P<0.01) vs. motion control
Significance (P<0.01) vs. untreated HUVEC

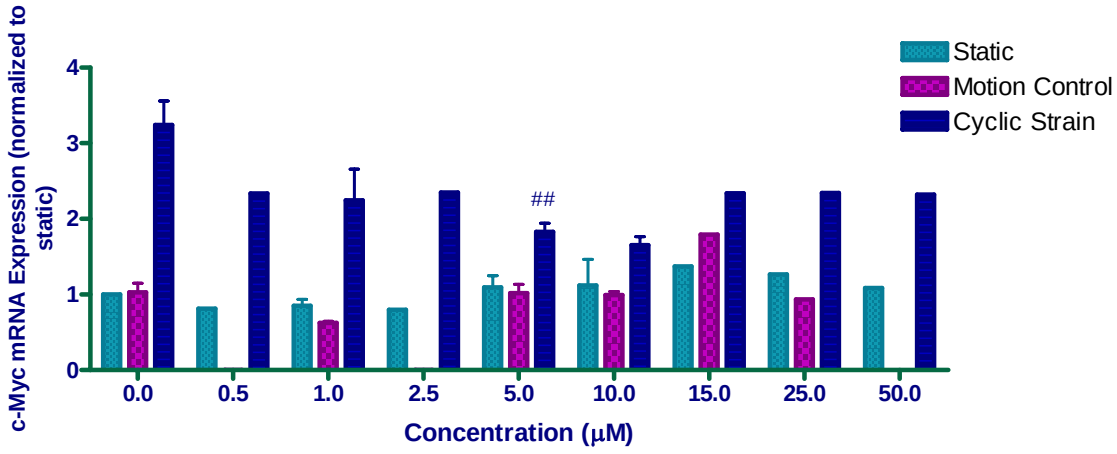


Figure A.3: Compound 0012 dose response
c-Myc mRNA expression was examined in HUVEC that were cyclically strained for 1.75 hours and treated with varying concentrations of Compound 0012, n=1–5.
Significance (P<0.01) vs. untreated HUVEC

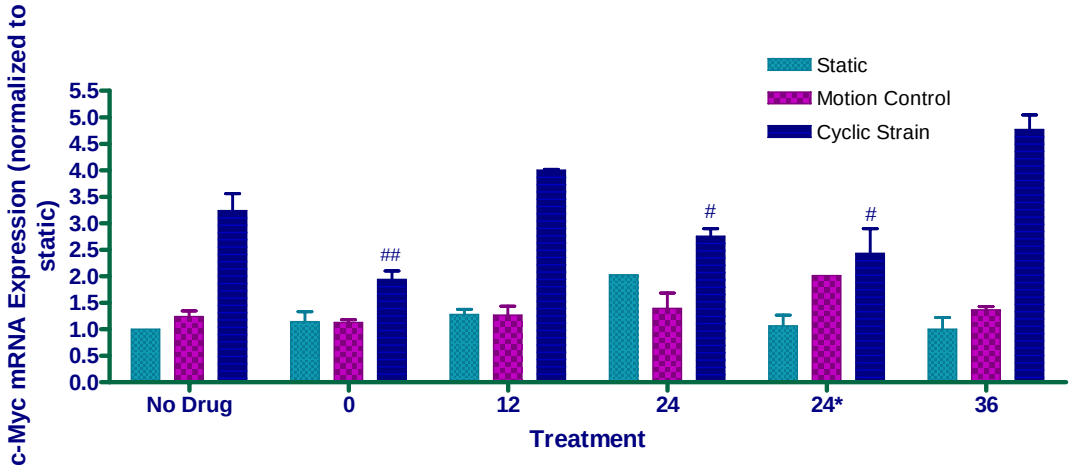


Figure A.4 Time treatment experiment, n=2–5.
Significance (p=0.01) vs. untreated HUVEC
Significance (*P*<0.05) vs. untreated HUVEC

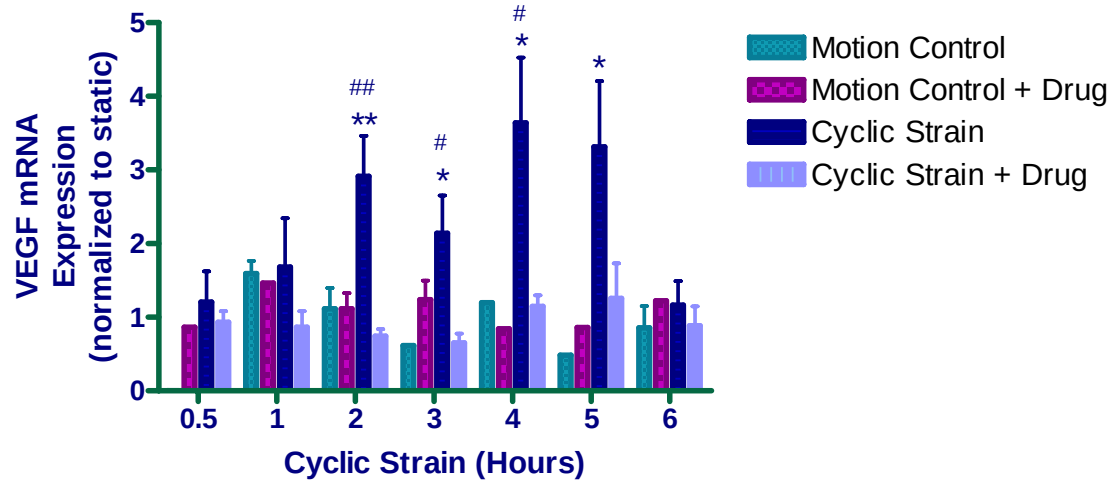


Figure A.5: VEGF mRNA expression, n=3–12.
** Significance (*P*<0.01) vs. static conditions
* Significance (*P*<0.05) vs. static conditions
Significance (*P*<0.01) vs. untreated HUVEC
Significance (*P*<0.05) vs. untreated HUVEC

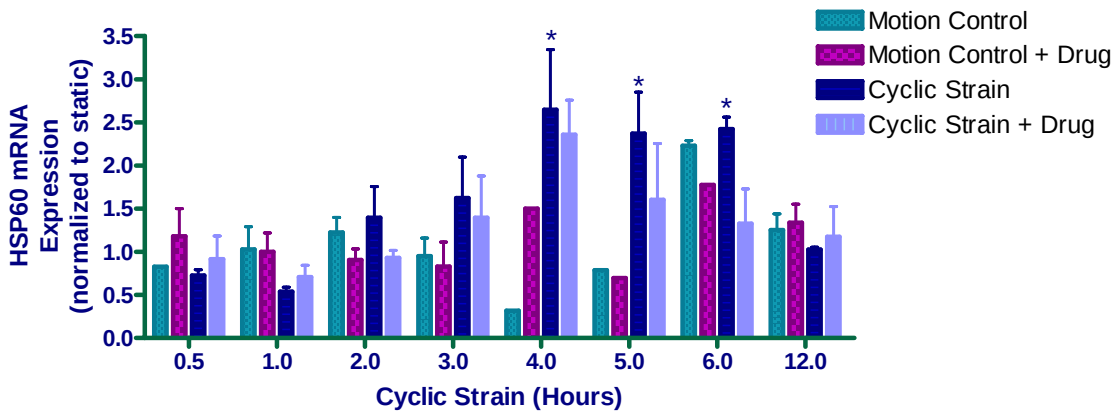


Figure A.6: HSP60 mRNA expression, n=3–9.
* Significance (P<0.05) vs. static control

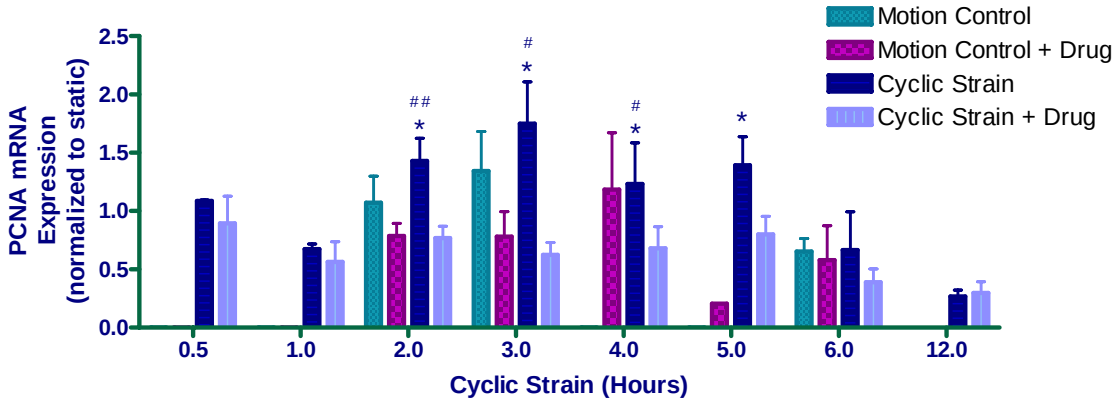


Figure A.7: PCNA mRNA expression, n=3–10.
* Significance (P<0.05) vs. static control
# Significance (P<0.05) vs. untreated HUVEC
Significance (P<0.01) vs. untreated HUVEC

APPENDIX B: CELLTITER PROLIFERATION ASSAY

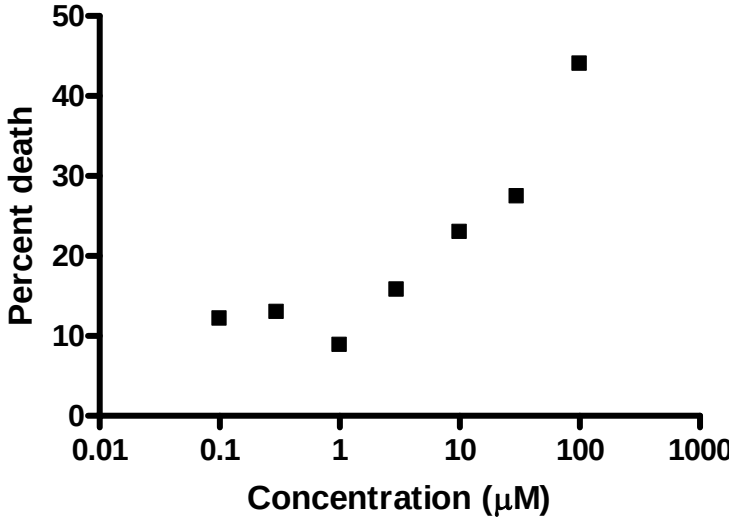


Figure B.8: Percent death in HUVEC treated with compound 0012

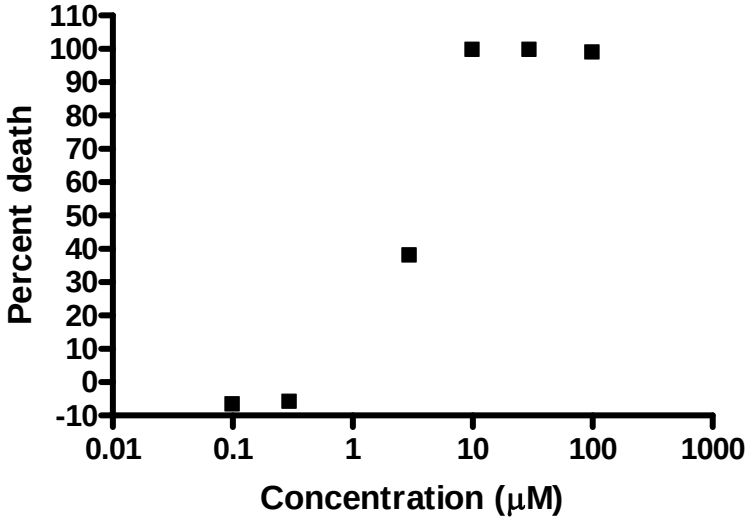


Figure B.2: Percent death in HUVEC treated with compound 0010

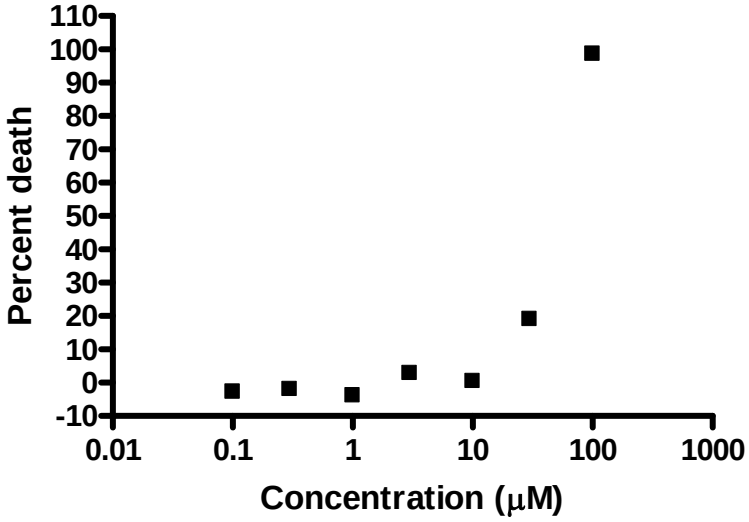


Figure B.3: Percent death in HUVEC treated with compound 0005

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