The effects of mechanical strain on vascular calcification and the canonical Wnt pathway

Hannah E. Douglas
Mississippi State University, hannahedouglas5@gmail.com

Follow this and additional works at: https://scholarsjunction.msstate.edu/td

Part of the Molecular, Cellular, and Tissue Engineering Commons

Recommended Citation
https://scholarsjunction.msstate.edu/td/5882

This Graduate Thesis - Open Access is brought to you for free and open access by the Theses and Dissertations at Scholars Junction. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of Scholars Junction. For more information, please contact scholcomm@msstate.libanswers.com.
The effects of mechanical strain on vascular calcification and the canonical Wnt pathway

By

Hannah E. Douglas

Approved by:

C. LaShan Hendrix (Major Professor)
Michael Jaffe
Filip Suminto To
Steven H. Elder (Committee Member/Graduate Coordinator)
Jason M. Keith (Dean, Bagley College of Engineering)

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Biomedical Engineering
in the Department of Agricultural and Biological Engineering

Mississippi State, Mississippi
August 2023
Cardiovascular disease is a significant health crisis, representing 32% of deaths worldwide in 2019. Vascular calcification (VC), a major contributor to cardiovascular disease, is a regulated biomineralization process whose exact mechanisms are unknown. Additionally, vascular smooth muscle cells (VSMCs) significantly contribute to VC by undergoing a phenotypic switch and differentiating into osteoblast-like cells. When factors like hypertension cause disturbed laminar flow in the body’s vasculature, the mechanical stress promotes the phenotypic switch and calcification of VSMCs via mechanotransduction. VC is also induced by the Wnt pathway, which is activated via mineral imbalance and mechanical stimulation. However, the exact mechanisms behind mechanotransduction in relation to VC, Wnt, and VSMC differentiation are unknown. If uncovered, knowledge of the mechanisms may be used to create effective treatments for VC.
DEDICATION

This work, and all the work I have endeavored to do throughout my academic career, is dedicated to my late Pappy, Charles Ray Burnham. It was your long struggle with cardiovascular disease that inspired me to find my lab at Mississippi State. You are the reason I chose to study not only cardiovascular disease, but also the field of biomedical engineering as a whole, so that I may contribute to finding a treatment for the disease that took you from us too soon. If it wasn’t for the inspiration and support you gave me, or your exemplary life, I would not be where I am today.
ACKNOWLEDGEMENTS

I would like to acknowledge Dr. Hendrix for mentoring and supporting me throughout my academic career. In addition, I would like to thank my fiancé and my family for always giving me the emotional support I need. A huge thanks to Dr. To for taking the time to aid me in troubleshooting our lab’s old Flexcell Tension System (may it rest in peace). Lastly, I’d like to acknowledge my entire committee, as each one of them has taught one or more of my classes that led to me being here today.
# TABLE OF CONTENTS

DEDICATION ........................................................................................................................................ ii

ACKNOWLEDGEMENTS ......................................................................................................................... iii

LIST OF TABLES ....................................................................................................................................... vi

LIST OF FIGURES .................................................................................................................................... vii

CHAPTER

I. INTRODUCTION ..................................................................................................................................... 1

  Background ........................................................................................................................................... 1
  Vascular Calcification ............................................................................................................................... 2
  Endothelial to Mesenchymal Transition ...................................................................................................... 4
  Hypertension and Mechanotransduction .................................................................................................... 6
  Vascular Smooth Muscle Cell Phenotypic Switch ....................................................................................... 8
  Hyperphosphatemia ................................................................................................................................ 9
  Hypercalcemia ....................................................................................................................................... 10
  Hypertension and Mechanotransduction .................................................................................................... 10
  Vascular Smooth Muscle Cell Stretch Biomarkers .................................................................................... 13
  The Wnt Pathway ................................................................................................................................... 15
  The Wnt Pathway and Vascular Calcification ............................................................................................ 16
  Hypertension and Mechanotransduction .................................................................................................... 18
  Blood Pressure-Induced Strain Simulation ................................................................................................. 18
  Conclusion ............................................................................................................................................ 21

II. AIM I: CREATE A SUITABLE CELL STRETCH REGIMEN AND PROTOCOL
    FOR VSMCS IN BOTH HYPERTENSIVE AND NORMAL BLOOD
    PRESSURE CONDITIONS ...................................................................................................................... 23

  Introduction .......................................................................................................................................... 23
  Aim I Specific Objectives ........................................................................................................................ 24
  Experimental Section ............................................................................................................................... 25
    Materials/Methods ................................................................................................................................. 25
      Model Regimen ................................................................................................................................. 25
      Regimen Trials ................................................................................................................................. 25
    Cell Culture and Stretching ................................................................................................................... 26
    Determination of Cell Viability and Appropriate Cell Seeding Density ................................................. 26

iv
III. AIM II: EXAMINE THE EFFECTS OF THE CELL STRETCH REGIMENS ON VSMC CALCIFICATION AND WNT PATHWAY ACTIVATION ...........................................34

Introduction .............................................................................................................34
Aim II Specific Objectives .........................................................................................34
Experimental Section ...............................................................................................35
  Materials/Methods .................................................................................................35
  Cell Culture and Stretching ....................................................................................35
  Biomarker Testing .................................................................................................36
  Statistical Analysis ...............................................................................................37
Results .......................................................................................................................37

IV. DISCUSSION .......................................................................................................46

V. FUTURE WORKS ..................................................................................................50

REFERENCES .........................................................................................................52
LIST OF TABLES

Table 1  Maximum Percent Elongations ................................................................. 30
LIST OF FIGURES

Figure 1  Arterial anatomy ........................................................................................................................................3
Figure 2  EndMT pathway schematic ..................................................................................................................5
Figure 3  VSMC phenotypic switch ......................................................................................................................9
Figure 4  Hypercalcemia in VC ..........................................................................................................................13
Figure 5  The Wnt pathway ...............................................................................................................................16
Figure 6  BioFlex® plates ....................................................................................................................................19
Figure 7  Waveform comparison ........................................................................................................................20
Figure 8  Flexcell Waveform Outputs ...............................................................................................................29
Figure 9  Standard blood pressure waveform parameters and comparison .......................................................31
Figure 10 Final waveform parameters ..............................................................................................................31
Figure 11 MTT Cell Viability Assay Results .......................................................................................................32
Figure 12 Total Protein Concentration per Cell Density ....................................................................................33
Figure 13 Quantification of α-SMA in HAVSMCs subjected to either the standard pressure regimen, the hypertension regimen, or no stretch (normalized to total protein content). ........................................................................38
Figure 14 Quantification of α-SMA in HAVSMCs subjected to either the standard pressure regimen, the hypertension regimen, or no stretch (not normalized). .................................................39
Figure 15 Quantification of calcium content in HAVSMCs subjected to either the standard pressure regimen, the hypertension regimen, or no stretch. .................................................................40
Figure 16 Quantification of TGF-β1 in HAVSMCs subjected to either the standard pressure regimen, the hypertension regimen, or no stretch (normalized to total protein content). ................................................................41
Figure 17  Quantification of TGF-β1 in HAVSMCs subjected to either the standard pressure regimen, the hypertension regimen, or no stretch (not normalized). ..................42

Figure 18  Quantification of β-catenin in HAVSMCs subjected to either the standard pressure regimen, the hypertension regimen, or no stretch (normalized to total protein content). .................................................................43

Figure 19  Quantification of β-catenin in HAVSMCs subjected to either the standard pressure regimen, the hypertension regimen, or no stretch (not normalized). .............44

Figure 20  Quantification of TGF-β1, β-catenin, and α-SMA in HAVSMCs subjected to either the standard pressure regimen, the hypertension regimen, or no stretch (not normalized). .................................................................45
CHAPTER I
INTRODUCTION

Background

The global leading cause of death is cardiovascular disease (CVD), representing 32% of deaths worldwide in 2019 [1]. Vascular calcification (VC), a major contributor to cardiovascular disease, is a regulated biomineralization process whose exact mechanisms are unknown [2]. Endothelial to mesenchymal transition (EndMT), the process through which vascular endothelial cells (ECs) lose their traits in exchange for mesenchymal cell phenotypes and markers, is a known cause of pathologies such as CVD and medial calcification [3], [4]. Additionally, vascular smooth muscle cells (VSMCs) significantly contribute to VC by undergoing a phenotypic switch and differentiating into osteoblast-like cells. When factors like high blood pressure cause disturbed laminar flow in the body’s vasculature, the mechanical stress promotes EndMT in ECs via mechanotransduction [5]. The same stress stimulates the phenotypic switch of VSMCs to osteoblast-like cells as well [5]. VC is also induced by the Wnt pathway, which is activated via mineral imbalance and mechanical stimulation. However, the exact mechanisms behind mechanotransduction in relation to VC, Wnt, and VSMC differentiation are unknown. This study aims to understand the role of VSMC osteogenic differentiation and the Wnt pathway in VC due to hypertension-induced mechanotransduction by performing mechanical testing. The FX-6000T™ Tension System, a computer-regulated bioreactor, executes the mechanical testing by
replicating the forces cells face during hypertension. If the knowledge gaps within these mechanisms can be filled, a suitable treatment for hypertension-related VC can be developed.

**Vascular Calcification**

VC, which is the deposition of calcium and hydroxyapatite on arterial walls, is produced in a process resembling bone formation [6]. It is a common comorbidity of diseases like chronic kidney disease (CKD), hypertension, and diabetes [7]. In fact, VC is a common and dangerous complication for renal failure and hemodialysis patients [7]. No treatment is currently available that stops and/or reverses VC [8]. Balloon angioplasties or stent placements can be used as treatments, but they have poor patient outcomes and often cannot be deployed correctly due to calcified buildup in the target artery [9]. Additionally, stent placement causes tears and further mechanical strain on the artery, often worsening VC [5].

VC has long been considered a passive process. Now, it is known that it is a highly regulated process [6]. However, knowledge of its mechanisms is minimal compared to other, long-researched cardiovascular disease processes, such as atherosclerosis [6]. VC can occur in both the intimal and medial arterial wall layers. Figure 1 depicts these layers along with other vital arterial components, like the inner endothelium layer and the smooth muscle cell layer located in the tunica media.
Atherosclerosis is associated with calcification of the intimal layer. It is defined by the deposition of lipids in the intima that obstruct the lumen and thin the medial layer underneath [11]. Medial calcification, also known as Mönckeberg's sclerosis, is a type of arteriosclerosis (vessel stiffening) [12]. It is the focus of this study and involves the deposition of hydroxyapatite along with a decrease in vascular compliance and an increase in stiffness in the tunica media, leading to hypertension and cardiovascular mortality risk increase [5], [7]. It transpires along the elastic lamina, which is included in the tunica media along with VSMCs and extracellular matrix rich in elastin [7]. EndMT and a phenotypic switch of medial VSMCs to osteoblast-like cells play key roles in the formation of medial VC [13]. In medial calcification, mesenchymal cells differentiate osteogenically [11]. Their matrix mineralizes because vascular alterations upregulate osteogenic regulatory genes [11]. Unlike intimal calcification, medial calcification
does not require lipid deposition [11]. Left ventricular hypertrophy, arterial stiffness, and increased pulse pressure are due to medial calcification [11].

Healthy VSMCs in the tunica media regulate the hemodynamic balance and vascular tone because of their contractile phenotypes [14]. VSMC phenotypes can alter due to pathological or physiological changes like mechanical or oxidative stress [14]. Once altered in this process of phenotypic switching, the VSMCs are termed synthetic or dedifferentiated [14]. These synthetic VSMCs display upregulated migration and proliferation with a decrease in contractile protein expression (α-smooth muscle actin (α-SMA), myosin light chain, and more) [14]. Additionally, S100 calcium binding protein A4, a synthetic marker, was found to be increased in synthetic VSMC in vitro cultures when compared to contractile VSMCs [14]. Further differentiation of VSMCs into macrophages, chondrogenic, or osteogenic cells can follow the synthetic phenotype switch depending on the pathology [14]. In the context of VC, the phenotypic switch to osteoblast-like cells is of particular interest.

**Endothelial to Mesenchymal Transition**

EndMT, the process through which vascular ECs lining the blood vessel lose their traits in exchange for mesenchymal cell phenotypes and markers, is a known cause of pathologies such as cardiovascular disease and medial calcification [3], [4]. Its specific mechanisms are currently unknown. Mesenchymal cells are adult stem cells that can differentiate into various other cell types. Due to this characteristic, they are key in the repair and building of bone, cartilage, fat, and other tissue types. As shown in Figure 2, ECs that have begun the phenotypic transition to mesenchymal cells can contribute to calcification by transitioning into fibroblasts, which promote atherosclerosis [15]. Although they are distinct, atherosclerotic plaques and VC both express vital regulators in osteogenesis and bone structural proteins, implying that the presence
of one disease might eventually lead to the presence of the other [16]. In other words, a patient with end-stage atherosclerosis might also develop medial calcification and vice versa.

Figure 2  EndMT pathway schematic
Various inducers and markers involved in EndMT and its possible final products [17].

Similarly, EndMT instigates VC through the loss of the EC phenotype [5]. More specifically, anti-atherosclerotic substances are created and released by ECs [18]. The production of the most characterized of these substances, nitric oxide, can be stimulated under certain physiological conditions [19]. The nitric oxide then protects from cardiovascular disease by inhibiting the adhesion of leukocytes and platelets and relaxing VSMCs [19]. In diseased conditions such as EndMT, the protective effect of ECs is lost.

One study by Wu et al. demonstrates the significant contribution of EndMT to VC via the upregulation of serum parathyroid hormone levels followed by treatment with cinacalcet [20]. With the deterioration of renal function, serum parathyroid hormone concentrations increase [20]. The study shows that increased parathyroid hormone levels instigate VC by promoting the
EndMT pathway, and that the use of cinacalcet lowers parathyroid hormone levels and, subsequently, VC [20]. CD31, an EC marker, expression was even moderately restored in calcified aortic tissues treated with cinacalcet [20]. Overall, it can be concluded from this study that EndMT significantly contributes to VC.

EndMT is also instigated by the upregulation of bone morphogenetic protein 2 (BMP2), which is tightly linked to the maturation of osteoblasts [5]. The matrix Gla protein (MGP) regulates BMPs [5]. MGP acts as an inhibitor of VC and EndMT by inhibiting BMP activity, but there is often not enough expressed to equal the amount of BMPs [5]. Consequently, this unequal match fails to inhibit EndMT and VC [5]. Studies by Guihard et al. support this pathway by demonstrating that MGP deficiency instigates a substantial contribution of EndMT to VC [21]. The group revealed that ECs are pushed into a mesenchymal phenotype by the EndMT pathway, and that in aortas with low amounts of MGP, cells are directly provided to the process of VC by the pathway [21].

EndMT is also regulated by transforming growth factor-β (TGF-β) [22], [23]. This relationship is also depicted in Figure 2. In fact, when TGF-β was added to valvular ECs, they experienced EndMT [23]. Osteogenic markers such as osteopontin mRNA, RUNX2, and osteocalcin were subsequently increased, indicating that the ECs eventually gained osteoblast-like phenotypes capable of contributing to VC [23].

**Hypertension and Mechanotransduction**

Due to the location of ECs between vessel walls and circulating blood, they face exposure to mechanical forces like shear stress, stretch, and pressure, as well as molecules in the extracellular matrix (ECM) and blood [24]. These mechanical forces, generated by pulsatile blood flow and cyclic arterial wall distention, cause the alteration of vessel diameters [24]. They
also cause ECs to alter their morphology, gene expression, physiology, and more [24].

Connected to hypertension is vascular wall stress, cell proliferation, and vessel thickening due to increased collagen and fibronectin production [24]. Cell changes due to these stimuli cause an increase in vessel rigidity due to a process called vascular remodeling [24]. This process includes cell migration, apoptosis, and ECM protein turnover [24]. ECs have been found to align with the direction of flow when exposed to physiological shear stress [25]. Cellular responses resulting from mechanical forces are deemed products of mechanotransduction, a cell’s mechanism to sense physical stimuli and convert them to biological responses [24]. As a result of the combination of pathways, including those caused by TGF-β, Wnt (discussed below), disturbed flow, and mechanical stimuli, EndMT is promoted [26].

Furthermore, disease activates the normal, dormant phenotype of valvular interstitial cells [27]. Mechanical inflammation and stress can initiate EndMT in heart valves, and certain activated endothelial cells that undergo this process produce more valvular interstitial cells that may experience osteogenic transdifferentiation [27].

Additionally, a study by Balachandran et al. found that dual-mode EndMT was induced by and dependent on cyclic strain, with the TGF-β1-dependent EndMT being provoked under low strain (10%) [28]. EndMT even contributes to pulmonary hypertension, placing hypertension and EndMT in a deadly cycle [17]. However, ECs are not the only cells subjected to the stimulus of hypertension. Blood vessel walls majorly consist of VSMCs that are also subjected to the force of blood flow and respond to mechanical forces [24]. In standard pressure conditions, cyclic strain in cells, or the amount of stretch compared to original size, ranges from 5-10% [29]. Pathogenic strains of at least 20% arise with hypertension [29].
Vascular Smooth Muscle Cell Phenotypic Switch

VSMCs, as shown above in Figure 1, are in the artery’s tunica media. Their typical contractile phenotype is essential for arterial physiology, but vascular injury can drive them to switch to a proliferative synthetic phenotype [30]. These synthetic VSMCs contribute to VC through increased ECM and extracellular vesicle (EV) secretion, as well as further transdifferentiation [14]. VSMCs can further differentiate into an osteoblast-like phenotype during VC [5], [30]. This phenotypic switch contributes to calcification and results in the upregulation of BMP2 production [30]. Although the exact initial mechanism that drives the phenotypic switch is unknown, some pathways are being studied [5]. It is thought that this switch occurs through activation of the Wnt pathway, as well as other pathways and contributors discussed in this paper. Figure 3 shown below illustrates various pathways of VSMC phenotypic switch, along with several biomarkers discussed in this paper.
Figure 3  VSMC phenotypic switch

The phenotypic switch pathway of a contractile VSMC to a synthetic one and eventual transdifferentiation [31]. This study focuses on biomarkers and signals shown in the contractile phenotype, the switch to a synthetic phenotype, and the osteogenic VSMC.

Hyperphosphatemia

Through intricate signaling pathways, hyperphosphatemia produced from preexisting conditions like CKD and aging is one instigator of the phenotypic switch of VSMCs to osteoblast-like phenotypes [32]–[34]. The exact mechanisms in which this instigation occurs is unknown, but the mineralization of VSMCs has been found to be encouraged in a dose-dependent fashion by extracellular inorganic phosphate levels [34]. In dialysis patients, serum inorganic phosphate levels can be twice as high as normal levels [34]. It is known, however, that the main sodium-dependent phosphate cotransporter in VSMCs is one called PiT-1 [35]. In VC
caused by hyperphosphatemia, PiT-1 has been shown as a vital transporter and has even been determined as mandatory in vitro for the process of phosphate-induced calcification to occur [35]. Hyperphosphatemia is regularly treated with the use of calcium-based P binders, but these drugs interact with bone metabolism and worsen hypercalcemia [9]. Ca++-free phosphate binders are thus recommended but cannot reverse calcification [9].

**Hypercalcemia**

Hypercalcemia is another mineral imbalance that contributes greatly to VSMC calcification and phenotypic switch. A regulated amount of calcium is required for the body’s physiological processes, but a surplus can stiffen vessels, contribute to VC, and cause unexpected death and failure of the left ventricle [36]. A study by Furmanik et al. found that increased extracellular calcium levels led to synthetic VSMC calcification [14]. In dialysis patients, an increased serum calcium-phosphate product and the prescription of phosphate binders containing calcium have been linked to increased VC [36]. While the need for further research into the mechanisms behind this link remains, it is known that calcium levels can affect PiT-1 expression. Factors like hypercalcemia, platelet-derived growth factor, and BMP2 upregulate PiT-1 expression [34], [35]. The induction of Pit-1 expression in vitro has even been achieved with prolonged hypercalcemic treatment [34].

**Hypertension and Mechanotransduction**

As previously discussed, hypertension damages blood vessels and elicits responses on the cellular level. Specifically, it increases the rate and amplitude of arterial elastic distension, leading the vessel wall ECM to experience damage, degradation, and fatigue [37]. Additionally, elevated strain levels due to plaque cause arteries to narrow and blood velocity to increase,
boosting pressure [5]. VSMC proliferation, phenotyping, apoptosis, migration, phenotypic switching, and vascular remodeling are prompted by cyclic stretch [5]. However, some studies found that stretch led to increased VSMC apoptosis and decreased proliferation [38]. The studies that obtained these somewhat counteractive results cyclically strained cells using an FX-6000T™ Tension System waveform that mimics pulsatile pressure, while previous studies demonstrating increased proliferation used other waveforms [38]. This difference is further explored in a later section of this paper.

VSMCs are subject to various mechanical stimuli, including tension around the wall’s circumference and transmural pressure caused by pulsatile pressure [5]. VSMCs are also subject to triaxial loading due to blood pressure: hoop tensile strain, axial tensile strain, and radial compressive strain [5]. They obtain a perpendicular alignment to flow when exposed to physiological shear stress [25]. Additionally, they have been shown to align perpendicular to the axis of stretch in a frequency-dependent manner during both uniaxial and equibiaxial stretch [38]. Homeostatic responses are fueled by standard pressure conditions, but mechanical response could be excessive in hypertensive conditions [5]. Vascular remodeling responses could then be overactivated, and the mineralized ECM encouraged to expand [5].

When VSMCs are strained too much, they can switch to a synthetic or osteoblast-like phenotype and encourage fibrosis of the artery [5], [37]. In both the medial and intimal arterial layers, VSMCs with more synthetic phenotypes have been discovered [37]. When VSMCs undergo an osteogenic or synthetic phenotypic switch, they release EVs resembling those derived from true osteoblasts with osteoblast-like ECM creation and calcium-binding abilities [37]. However, VC is not induced by all EVs [37]. Although VSMC numbers dimmish with age
and contribute to vessel wall stiffening, calcification of the ECM also contributes to stiffening by promoting BMP2 and BMP4 fabrication in nearby VSMCs and ECs [37].

Furthermore, hypertension also affects calcium channels and uptake. Under normal conditions, calcium influx and blood pressure are closely regulated by calcium channels located in VSMCs [39]. During hypertension pathogenesis, however, intensified calcium uptake could lead to increased peripheral vascular resistance and atypical vascular tone [39]. Studies have shown that hypertension and an increased number of calcium channels in vascular beds are linked [39]. Pesic et al. investigated this link in vivo in one of their studies. The researchers banded rat aortas between their left and right renal arteries, exposing the proximal right renal arteries’ VSMCs to hypertension [40]. Within two days, the right renal arteries had more calcium channel subunits than the left renal arteries, which were exposed to lower blood pressure [40]. This upregulation of calcium channels was due to the depolarizing effect of hypertension [40]. Since hypertension increases VSMC calcium channel expression and it is known that hypercalcemia contributes to VSMC calcification, the possible increased calcium uptake during hypertension may contribute greatly to VC. Figure 4 below depicts the uptake of extracellular calcium as it leads to VC. It also shows the pathway of VSMCs from contractile to calcified with the role of EVs and other signals.

Various mechanosensors may play important roles in translating mechanical signals from stretch into biological activity. For instance, a type of trans-membrane receptor protein called an integrin has been shown to be non-specifically activated by stretch [41]. Additionally, PDGF receptor signaling is a signal that is related to the dedifferentiation of SMCs into a synthetic phenotype [41]. Specifically, cyclic strain can activate the receptor PDGF-Rβ independent of any
ligands, potentially leading to VSMC dedifferentiation [41]. Other trans-membrane mechanoreceptors in VSMCs include RTKs, LOX-1, GPCRs, RAGE, and ion channels [41].

Figure 4  Hypercalcemia in VC

A: EVs mediate extracellular calcium uptake, leading to VC [42]. B: The pathway of contractile VSMCs to synthetic and calcified VSMCs due to high calcium levels and leading to an increase in EVs and ECM production [42].

Vascular Smooth Muscle Cell Stretch Biomarkers

To confirm cell stretch in experiments and analyze cellular responses to stretch, reliable biomarkers are needed. In VSMCs, extremely promising potential stretch biomarkers have been identified. These biomarkers include TGF-β1 and reactive oxygen species (ROS) produced by Nox1.

VSMC differentiation, proliferation in response to stretch, and ECM component secretion are all associated with TGF-β1 [38]. One study by Joki et al. applied 60 cycles per minute of cyclic stretch with a maximum elongation of 20% and a maximum time of 24 hours to rat VSMCs [43]. The group found that mRNA expression of TGF-β1 was induced by the mechanical stretching and was dependent on both elongation and time [43]. It was concluded
that vascular remodeling due to hypertension is significantly influenced by stretch-induced TGF-
β1 expression, specifically that which is dependent on tyrosine-kinase [43].

Furthermore, increased expression of ROS has been observed in various hypertensive animal models, such as those injected with angiotensin II and spontaneously hypertensive rats [44]. In such conditions, nitric oxide levels are decreased while hydrogen peroxide and superoxide levels are increased [44]. Various hypertension-associated pathologies are regulated by overexpressed ROS: vasomotor tone managed by the endothelium, the mediation of blood pressure, and more [44]. Excess ROS production is caused by nicotinamide adenine dinucleotide phosphate oxidase (Nox) overactivation [45], [46]. In the cardiovascular system, Noxs 1-4 and 5 are present [45]. The mediation of blood flow, VSMC proliferation, ECM production, and migration are associated with Nox1 [45]. In disease models such as atherosclerosis, hypertension, and diabetes, the vasculature boasts upregulated Nox1 expression that can lead VSMCs to phenotypically switch [45], [47]. Specifically, mechanical shear stress, certain growth factors, and agents such as angiotensin II upregulate Nox1 in cultured ECs and VSMCs [45]. Angiotensin II is an inducer of Nox1 expression and may be calcium dependent [45]. Medial hypertrophy and hypertension due to angiotensin II is associated with the upregulation of Nox1 in VSMCs [48]. Furthermore, tumor necrosis factor alpha expression is stimulated by Nox1-derived ROS expression; tumor necrosis factor alpha leads to the phenotypic switch of VSMCs [48]. ROS produced by Nox1 can also be stimulated through the MEF2B-Nox1-ROS pathway, which has been shown to be activated under stretch [41]. More work needs to be done to characterize the role of Nox1 within cardiovascular disease in humans, but it is upregulated in human atherosclerotic vessels [45]. Nox4 and 5 have also been implicated in ROS production during hypertension, with some pathways even being calcium dependent [45], [49], [50].
In fact, the same studies that implicate Nox1, 4, and 5 demonstrate conflicting results regarding which Nox isoform is mainly responsible for ROS production due to hypertension and mechanical stretch in VSMCs. Nox5 may play a significant role in this pathway in humans but is difficult to study because it is absent in rodents [47], [51]. Figure 4 depicts the connection between extracellular calcium, NOX5, ROS production, and VC.

**The Wnt Pathway**

In addition to mineral imbalances, the Wnt signaling pathway also triggers VC via VSMC osteoblast-like differentiation. The Wnt cascade’s typical role, which is the regulation of multiple cellular functions, including organogenesis, the renewal of stem cells, and the determination of cell fate, takes place during embryonic development [52], [53]. Upon binding of an extracellular Wnt ligand to a Frizzled receptor, activation of the Wnt pathway occurs [52]. However, to understand the Wnt signal pathway’s effects, one must first understand how the involved molecules interact during non-Wnt-activated conditions. When Wnt is not activated, Axin acts as a scaffolding protein for a β-catenin destruction complex (Axin, the E3-ubiquitin ligase β-TrCP, adenomatous polyposis coli protein, casein kinase 1, and glycogen synthase kinase 3) [52]. Excess cytoplasmic β-catenin is phosphorylated and degraded by this complex [52].

When the Wnt pathway is activated, however, the regulation of β-catenin has a different fate. The cytoplasmic phosphoprotein Disheveled (Dsh) is sent to the plasma membrane upon Wnt activation, and the pathway deviates into at least three main branches: canonical, Planar Cell Polarity, and Wnt/Ca^{2+} [52], [53]. Though it is required that a Wnt signal binds to one of the ten different types of Frizzled receptors, co-receptors are also necessary for Wnt-signal mediation [53]. In the canonical Wnt pathway, which is the most studied and has suspected involvement
with VC, low-density-lipoprotein-related protein 5 and 6 act as the co-receptors [52]–[54]. Axin and a GSK3 complex are enlisted into the Wnt pathway, in part due to Dsh, and the previously described destruction complex is deconstructed [52]. As a result, β-catenin is upregulated and translocated to the nucleus, where it interacts with LEF-1/TCF DNA-binding transcription factors to form a transcriptional complex [52]. Lastly, Wnt target genes become upregulated due to the β-catenin complex associating with their promoters [52]. The pathway during both activation and inactivation is depicted in Figure 5.

![Figure 5](image)

**Figure 5**  
The Wnt pathway  
Schematic representation of the Wnt pathway during both activation and inactivation [53].

**The Wnt Pathway and Vascular Calcification**

The Wnt pathway has been shown to control growth in differentiation processes, like stem cell fate determination, and its ligands can be found in healthy VSMCs [55][52]. Canonical Wnt activation is a major contributor to VC [55]. Furthermore, mineral bone disease and CKD-associated VC have been related to the canonical pathway [56]. VC has even been shown to be
mitigated by Wnt pathway inhibition [55]. Both key players in VC, EndMT and VSMC transdifferentiation, are majorly affected by the Wnt pathway.

The integration of signaling pathways, such as the TGF-β and Wnt pathways, result in EndMT [26]. Additionally, hypercalcemia and hyperphosphatemia provoke Wnt [5]. As previously discussed, EndMT directly contributes to the VC process, linking VC to Wnt as well. This link also extends from Wnt to VSMC differentiation, and it is this connection that this study investigates.

The canonical Wnt pathway modulates the expression of RUNX2, in turn promoting VSMCs to transdifferentiate into osteoblast-like cells [55], [57]. The expression of RUNX2, one of the earliest osteoblast markers, was found by Ting et al. to be activated by the expression of β-catenin and its nuclear translocation in the canonical Wnt pathway [58]. Furthermore, a study by Zhou et al. succeeded in inhibiting VSMC osteogenic transdifferentation and calcium deposition in vitro and in vivo via Ginsenoside Rb1 treatment [55]. The Wnt pathway and β-catenin translocation to the nucleus of VSMCs were inhibited by the treatment, and an agonist of the pathway counteracted the treatment [55]. This phenomenon further demonstrates the relationship between the canonical Wnt pathway and VSMC phenotypic switch leading to VC.

Furthermore, it is thought that BMP2 expression is upregulated upon Wnt pathway activation. It has been shown that BMP2 expression is upregulated in osteogenic cells when WNT3a activates the Wnt pathway [5]. BMP2 induces bone formation by promoting the osteoblastic differentiation of mesenchymal cells, and mechanical stimulation during osteogenesis has led to the detection of elevated levels of it [5]. Rong et al. demonstrated in one study that the osteoblast-like transdifferentation of VSMCs is stimulated by BMP2 in a pathway
involving activation of canonical Wnt signaling [59]. They found that by knocking out β-catenin expression, VSMC transdifferentation by BMP2 was not observed [59].

**Hypertension and Mechanotransduction**

In the same way that hypertension induces mechanotransduction and cellular response in EndMT and VSMCs, it also affects the Wnt signaling pathway. The same study by Balachandran et al. also detected Wnt/β-catenin signaling activation in ECs when exposed to high cyclic strain (20%) [28]. If this pathway was activated in ECs, its other known effects, such as RUNX2 and BMP2 upregulation and integration with other pathways, likely also trigger VSMC osteogenic differentiation. In fact, osteogenic, Wnt-mediated vascular remodeling may be induced when VSMCs are subject to increased tension and compression strains [52]. A study by Csiszar et al. subjected rat arteries to high pressure [60]. They found that hydrogen peroxide and BMP2 expression were upregulated [60]. Through knockout of tumor necrosis factor alpha, the authors observed a direct effect of BMP2 upregulation due to hypertension [60]. Although this study was centered around ECs, this study was performed on intact rat arteries, which contain VSMCs.

Taken in combination with another study by Chen and Moe that demonstrates increased BMP2 and decreased α-SMA in calcified rabbit arteries, the effects of hypertension on VSMCs and calcification is apparent [61]. Because of the previous discussion on BMP2 activation of the canonical Wnt pathway and the effects of hypertension on BMP2 expression, hypertension-induced Wnt pathway activation is also made clear.

**Blood Pressure-Induced Strain Simulation**

To induce mechanical strain and investigate mechanotransduction, a bioreactor capable of applying cyclic equibiaxial strain via programming, i.e., the Flexcell® Dynamic Culture
System: FX-6000™ Tension system, will be used. This system is a computer-regulated bioreactor that utilizes positive air pressure and vacuum pressure to assert either static or cyclic strain on cells in unique culture plates with flexible bottoms [62]. Figure 6 demonstrates the strain placed on these BioFlex® plates. This system will be used to apply equibiaxial cyclic strain to a 2D monolayer of VSMCs, modeling both hypertension and normal blood pressure.

Figure 6  BioFlex® plates
Equibiaxial strain applied via FX-6000™ Tension System on flexible culture plates [63].

As far as the author is aware, no current work utilizing the Flexcell® Tension system with VSMCs, a realistic waveform, and a focus on VC and the Wnt pathway exists. While this study includes hypertensive and standard pressure regimens, along with a static control, studies found upon literature review only included two groups: a control and one strained group. Furthermore, most studies described below utilize a sinusoidal waveform for cyclic strain application; others do not mention waveform shape. While this simple and common waveform allows for higher,
and therefore more physiologically accurate, frequencies, it is not the same shape as the pulsatile pressure waveform produced by the body. The Flexcell® Tension system possesses a preset heart (P) waveform that imitates the beating heart’s generated pressure [38], [64]. The specific anatomical location of the strain simulated by this waveform is not stated by the company. Figure 7 gives a visual representation of the two different waveforms.

![Waveform comparison](image)

**Figure 7**  Waveform comparison

Depiction of Flexcell® Tension system program with comparison of sine and heart (P) waveform shapes [64].

The specific type of waveform used may seem trivial, but study results between groups who used the heart (P) waveform and other waveforms differ [38]. One study by Arnold et al., for example, studies mechanical strain via the Flexcell® Tension system on human umbilical artery smooth muscle cells but does not mention VC, the Wnt pathway, or which waveform was used [65]. In addition, the group applied a maximum stretch of 13% to the cells, significantly
less than the stretch of at least 20% found in pathological hypertension [29], [65]. Additionally, some studies focus on straining different types of myocytes, but again do not mention VC, Wnt, or waveform type, or use a sinusoidal waveform [66]–[69]. The aforementioned study by Balachandran et al. stretched cells up to 20% with a 1 Hz frequency and included a 10% strain study but did not mention waveform or focus mostly on VC [28].

Other studies that do use the heart (P) waveform do not use a high enough strain level and/or don’t have the same focus as this study. One study by Groenendyk et al. used this waveform at 1 Hz to apply a maximum of 10% strain to cardiac fibroblasts and used similar parameters but with 12% maximum stretch applied to cell/hydrogel constructs [70], [71].

Some studies use smooth muscle cells or VSMCs but lack a focus on the Wnt pathway and VC [72]–[74]. One study was found that concentrates on VSMC strain with a little emphasis on the Wnt pathway, but it is not in the context of VC and did not mention a waveform used for strain application [75]. A few studies tested VSMCs with the heart (P) waveform but lack a VC focus and have a maximum strain less than the pathological 20% [76], [77].

**Conclusion**

Overall, this project will significantly advance current knowledge in the key mechanisms that cause and affect VC through hypertension induced Wnt activation and VSMC strain.

More importantly, the results of this endeavor will unlock doors into subsequent investigative research projects that will further define the relevant mechanisms and impacts. If a certain signaling molecule or pathway is discovered to play a key role in the onset of VC due to mechanotransduction in this project, then perhaps a treatment involving the inhibition of that molecule could be developed. In the end, this research could play a vital role in reducing the
number of CVD-related deaths each year while improving patients’ prognosis. The following aims guide this project:

**Specific Aim 1:** Create a suitable cell stretch regimen and protocol for VSMCs in both hypertensive and normal blood pressure conditions

**Specific Aim 2:** Examine the effects of the cell stretch regimen and protocol on VSMC calcification and Wnt pathway activation

It is hypothesized that VSMCs will display upregulated Wnt, cell stretch, and calcification markers alongside downregulated VSMC markers post-stretch. Therefore, it is thought that VSMC stretch in hypertensive conditions will greatly contribute to VC through activation of the canonical Wnt pathway.
CHAPTER II

AIM I: CREATE A SUITABLE CELL STRETCH REGIMEN AND PROTOCOL FOR VSMCS IN BOTH HYPERTENSIVE AND NORMAL BLOOD PRESSURE CONDITIONS

Introduction

Because a cell-stretch study such as this one had not previously been performed in this laboratory, an established stretch regimen suitable for the purposes of this study did not exist. Thus, the need to establish stretch regimens in the Flexcell® Tension system with appropriate parameters was established. For the purposes of this study, regimen suitability is defined by one with the most physiologically accurate waveform and highest possible frequency that is able to stretch cells to the maximum % elongation that occurs during hypertension.

When creating a new regimen in the Flexcell® Tension system, parameters that can be altered include waveform shape, minimum and maximum percent elongation, frequency, duty cycle percentage (DC%), cycles, duration, and the number of regimen steps. The waveform shape chosen (custom, 1/2 sine wave, sine wave, triangle wave, square wave, heart (E) wave, heart (P) wave, and static wave) determines the pattern of strain applied to the cells contained in flexible-bottomed culture BioFlex® plates. The minimum and maximum percent elongation inputs possess an overall range of 0-30%, while frequency ranges from 0.01-5 Hz [64]. Furthermore, DC% represents the opportunity to define a duty cycle. The time percentage that the waveform continues at the waveform’s rising or high portion is determined by the duty cycle.
input [64]. The cycle number determines how many times the regimen repeats and ranges from 0-65,535 [64]. The duration parameter automatically updates based on the cycle number and other parameters, and the cycle number automatically updates based on the duration entry and other parameters [64].

Additionally, the appropriate number of cells to seed into each BioFlex® plate well warranted investigation. In their BioFlex® plate product information sheet, Flexcell recommends plating 1.2x10^5 cells/well [78]. They also, however, suggest experimenting with various cell seeding densities to find one suitable for the exact cell type and application used. However, plating too many cells may lead to detachment during stretching due to being overly confluent and not fully attaching to the flexible-bottomed plate [79]. Upon literature review, it was discovered that other researchers using the Flexcell® Tension system with BioFlex® plates seeded cells at various densities. Three different studies, for instance, plated either rat VSMCs or human aortic SMCs at densities of 2x10^5, 4x10^5, and 6x10^5 cells/well [75], [77], [80].

**Aim I Specific Objectives**

This aim consists of three main objectives: create regimens to simulate cyclic strain placed on cells due to the excess pressure in hypertension, create a regimen to simulate cyclic strain placed on cells during normal physiological conditions, and determine the appropriate cell seeding density. The density with the highest amount of viable cells and total protein concentration (a statistically significant difference) may be the most viable. To create the regimens, the appropriate maximum and minimum elongations for both physiological conditions need to be determined. The best waveform shape and frequency also require establishment.
Experimental Section

Materials/Methods

*Model Regimen*

Because the hypertensive regimen would be the more straining of the two regimens due to its higher amount of stretch, it was modeled first. The model regimen parameters were determined by review of literature and the Flexcell® Tension system’s user manual. These parameters include waveform shape, maximum and minimum elongation, duration, and frequency. First, the preset heart (P) waveform in the Flexcell® software was selected due to its physiological accuracy in simulating the pressure wave produced a beating heart. The appropriate maximum % elongation was discovered in literature, with cellular strain in standard blood pressure conditions ranging from 5-10% and at least 20% in hypertensive conditions [29]. A maximum strain of 10% was chosen for the standard pressure regimen because it falls within the physiological range and would allow the author to study the direct effects of doubling the maximum % elongation on cells. Duty cycle, number of regimen steps, and cycle were not relevant to the required regimen. The duty cycle is only relevant for square and triangle waveforms, the regimen created will need to consist of only one continuous step, and the cycle number is automatically updated by the Flexcell software based on the duration entered.

*Regimen Trials*

To visualize the model regimen, a simulation of it was performed within the Flexcell® software. Any live trials were performed by placing 3-4 mLs of phosphate-buffered saline (PBS) in each well of a BioFlex® plate and running the desired regimen in the software as directed. The % elongation waveforms of the flexible-bottomed plates were saved for analysis.
**Cell Culture and Stretching**

The cells, specifically primary human aortic vascular smooth muscle cells (HAVSMCs) were cultured until passage 7 using a growth medium that consists of Dulbecco’s Modified Eagles Medium (DMEM), 10% Fetal Bovine Serum (FBS), and 1% Penicillin. Throughout the study, cell morphology was viewed via light microscopy. Cell density was obtained via the Countess II Automated Cell Counter (Invitrogen) and cells were seeded with 3 mLs of complete DMEM into collagen type 1-coated 6-well BioFlex® Culture Plates. 1x10^5, 2x10^5, and 4x10^5 cells were seeded into each well (n=3, sample size was limited due to a maximum stretching capacity of 4 BioFlex® plates at a time). Then, the cells were allowed to attach to the plates overnight. The following morning, each well was washed with 3 mLs of PBS and supplied with 5 mLs of complete DMEM before being stretched on the FX-6000T™ Tension System with the 24-hour hypertension regimen.

**Determination of Cell Viability and Appropriate Cell Seeding Density**

Total protein content was quantified with the BCA Protein Kit (Pierce). To determine cell viability, the CyQUANT™ MTT Cell Proliferation Assay Kit (Invitrogen) was adapted for 6-well plates. Using the same ratios of kit components as the original protocol, the sterile assay was performed directly in the 6-well plates. One well containing only cell culture media was used as the blank control. First, each well was washed with 3 mLs of PBS before 3 mLs of PBS were added back into each well. 300 μLs of MTT stock solution was added into each well and plates were incubated for 2 hours. Next, all but 0.75 mLs of solution was removed from each well and 1.5 mLs of DMSO per well was added. After 10 minutes of formazan solubilization, the solution from each well was transferred to a 96-well microplate, and results were obtained according to the established protocol.
**Statistical Analysis**

Single Factor Analysis of Variance (ANOVA) and a post-hoc Tukey test were conducted in SPSS with an $\alpha$ value of 0.05 to determine statistical significance.

**Results**

The model hypertensive regimen was determined to include the heart (P) waveform, a minimum and maximum elongation of 0.8% and 20% (respectively), a duration of 24 hours, and a frequency of 1 Hz. The 0.8% minimum was listed in the Flexcell user manual as the minimum strain reached for the specific loading posts used in this study. The duty cycle field was not altered because, according to the manual, only the triangle and square waveforms are affected by it. Lastly, because the regimen only warranted one step, the number of regimen steps and cycle fields were not altered. Figure 8 depicts the simulated model hypertensive regimen generated by the Flexcell software. Live trials revealed that the parameters with the selected waveform were unrealistic. General troubleshooting (shortening the connecting Flexcell tubing, ruling out baseplate issues) and waveform comparison confirmed that the differences were due to limitations specific to the heart (P) waveform. Parameters were modified for additional trials to determine their optimal values.

Shortening the FLEX IN and FLEX OUT tubing to the recommended length and readjusting the baseplate’s components did not resolve the issue of the ideal hypertensive regimen not obtaining the programmed 20% maximum cell elongation. The static and sine waveform trials were successful, indicating no fault in the Tension System. Any discrepancy between the true maximum % elongation obtained by the sine wave at 0.5 Hz and the programmed maximum of 20% was deemed normal at such a frequency by Flexcell Technical Services. Thus, the heart (P) waveform must have maximum elongation limitations when set to a
high frequency (1 Hz). The frequency was lowered by more than 50% to determine the largest value that allows a maximum strain closest to 20%. 0.2 Hz was found to be the ideal frequency because it reached a maximum of 19.28% stretch. Although the previously mentioned literature shows a maximum strain of at least 20% in hypertension, the Flexcell system setup possessed by this lab only allows for an absolute maximum of 21.8% stretch, as stated by the user manual. It is due to this limitation that a hypertensive maximum stretch of 20% was desired for this study. Furthermore, the minimum % elongation was set to 0 and the maximum % elongation set to 23 (the highest value allowed for the field) to encourage further strain. Figure 8 displays each set of parameters that was tested. The sine waveform within Figure 8 does not resemble a typical sine waveform until around second 17, likely due to a required alteration of the positive air pressure output. After second 17, one can see the sine wave becomes that of a typical sinusoidal waveform. Table 1 displays the true maximum % elongations obtained by each waveform described above. Figure 9 displays PBS trial runs of the ideal standard blood pressure regimen and the determined regimen with the frequency to match the hypertensive regimen. Lastly, Figure 10 depicts the final waveform regimens chosen to simulate hypertension and standard blood pressure.
Figure 8  Flexcell Waveform Outputs

The graphs obtained when running the simulation of the first ideal hypertension regimen and when running it as a live trial with PBS, along with the resulting changes when parameters such as frequency were altered.
Table 1  Maximum Percent Elongations

<table>
<thead>
<tr>
<th>Model Regimen Trial (1 Hz, 0.8% Min, 20% Max)</th>
<th>True Obtained Maximum % Elongation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Static (0% Min, 20% Max, 0.5 Hz)</td>
<td>20.11%</td>
</tr>
<tr>
<td>Sine (0% Min, 20% Max, 0.5 Hz)</td>
<td>18.26%</td>
</tr>
<tr>
<td>Heart (P), 0.5 Hz, 0% Min, 20% Max</td>
<td>13.06%</td>
</tr>
<tr>
<td>Heart (P), 0.4 Hz, 0% Min, 23% Max</td>
<td>15.14%</td>
</tr>
<tr>
<td>Heart (P), 0.3 Hz, 0% Min, 23% Max</td>
<td>17.2%</td>
</tr>
<tr>
<td><strong>Heart (P), 0.2 Hz, 0% Min, 23% Max</strong></td>
<td>19.28%</td>
</tr>
<tr>
<td>Heart (P), 1 Hz, 0.8% Min, 10% Max</td>
<td>7.56%</td>
</tr>
<tr>
<td><strong>Heart (P), 0.2 Hz, 0% Min, 10% Max</strong></td>
<td>9.83%</td>
</tr>
</tbody>
</table>

The true maximum % elongations reached for each set of parameters when run in a live trial with PBS. Parameters in bold are the ones selected to be used as regimens in later experiments for both hypertensive and standard blood pressure regimens.
Figure 9  Standard blood pressure waveform parameters and comparison

Graphical comparison of the ideal regimen for standard blood pressure (left) and the realistic one (right) determined as previously described by testing of the hypertensive regimen parameters.

Figure 10  Final waveform parameters

Final Waveform parameters for standard blood pressure (left) and hypertension (right).

The MTT viability assay revealed no statistically significant differences between the three groups. Although the $4 \times 10^5$ cells/well group visually presents higher in viability and total protein concentration, neither of the differences were significant due to the large standard error.
deviation between those samples. Figure 11 displays the MTT results, while Figure 12 depicts BCA total protein results. The large standard deviation in the $4 \times 10^5$ cells/well group is evident by the error bars in Figure 12. Furthermore, there proved to be no statistically significant differences in total protein concentration between each of the three $4 \times 10^5$. This finding further validates the high variability depicted by the large standard error bar. Because the manufacturer recommended density is $1.2 \times 10^5$ cells/well and there were no statistically significant differences between the various densities tested, it was decided that a seeding density of $1 \times 10^5$ cells/well was suitable for the purposes of this project.

![MTT Cell Viability Assay Results](image)

**Figure 11  MTT Cell Viability Assay Results**

No statistically significant differences were determined using Single Factor ANOVA and a post-hoc Tukey test with an $\alpha$ value of 0.05.
Figure 12  Total Protein Concentration per Cell Density

No statistically significant differences were determined using Single Factor ANOVA and a post-hoc Tukey test with an $\alpha$ value of 0.05.

In summary, the objectives of this aim were investigated using MTT cell viability and BCA total protein content analysis. No statistically significant differences between cells plated at densities of $1\times10^5$, $2\times10^5$, and $4\times10^5$ cells/well were noted. Although, the data from the $4\times10^5$ cells/well group appears higher than the other groups, it possesses high variability amongst its samples. It is also possible that the extraction buffer used for BCA analysis was inefficient, but the overall results are at least comparable. Because the $1\times10^5$ cells/well group had lower standard deviations and the manufacturer recommends a plating density of $1.2\times10^5$ cells/well, this group was chosen as the plating density for all future work.
CHAPTER III
AIM II: EXAMINE THE EFFECTS OF THE CELL STRETCH REGIMENS ON VSMC CALCIFICATION AND WNT PATHWAY ACTIVATION

Introduction

As aforementioned, the author is currently unaware of a study that uses the same combination of stretch regimen parameters to examine the effects of mechanotransduction on VC and Wnt pathway activation in VSMCs as this one. Many other studies either do not include a standard pressure control group, focus on a different cell type, or use a less physiologically accurate waveform. For this study, the two regimens determined in Aim I will be used to apply cyclic stretch to cells.

Aim II Specific Objectives

For this experiment, the objectives include examining the direct effects of mechanical stretch on HAVSMCs. Specifically, any change in the following markers between the 3 cell stretch groups will be assessed: α-SMA, a marker of typical HAVSMCs; β-catenin, an indicator of Wnt pathway activation; TGF-β1, a stretch biomarker; and intracellular free calcium, a marker of calcification. HAVSMCs will undergo either the hypertension regimen or the normal blood pressure regimen; another group will face no cyclic mechanical strain. Each group of HAVSMCs will then be examined for the rate of calcification and Wnt signaling activation through the examination of markers such as α-SMA, β-catenin, TGF-β1, and intracellular free calcium. It is hypothesized that the mechanically strained HAVSMCs in the hypertension regimen will exhibit
a decrease in VSMC markers and an increase in VC, osteogenic, and Wnt pathway markers when compared to the controls.

**Experimental Section**

**Materials/Methods**

**Cell Culture and Stretching**

HAVSMCs were cultured in complete GIBCO 231 media (base of VSMC basal medium, 1% penicillin, Smooth Muscle Growth Supplement (SMGS)) until passage 6. Cell culture, counting, seeding, overnight attachment, washing, and pre-stretch feeding were performed as previously described. For this study, however, cells were switched to complete DMEM for the remainder of the study when seeded into BioFlex® plates. Per the result of aim I, $1 \times 10^5$ cells were plated into each well. Finally, cells were either stretched for 24 hours with the hypertension or standard blood pressure regimen or not stretched at all. Because none of the testing kits could be run with the exact same sample due to having different extraction buffers and protocols, enough plates were plated so that each kit would end up with a sample size of 6 ($n=6$) for both the stretched groups and the respective static control groups. Each stretched group of cells was given its own static control group because the different stretch regimens were tested at different times. The Flexcell® Tension system setup only allows for one regimen to be run at a time, and this setup allows for the stretching of four plates at a time. Because of these two limiting factors, each of the two stretch regimens had to be performed at different timepoints. They were each given their own static control groups to account for this difference.
Biomarker Testing

Each specific protein marker was quantified using an enzyme-linked immunosorbent assay (ELISA). The following ELISA kits were used to detect their respective proteins: Human alpha SMA SimpleStep ELISA® Kit (Abcam), the Human beta-Catenin SimpleStep ELISA® Kit (Abcam), and the TGF beta 1 Human ELISA Kit (Abcam). Intracellular free calcium was quantified via the Calcium Colorimetric Assay Kit (Sigma-Aldrich). After experimental conditions (stretching or control) were completed, cell culture supernatant was collected and immediately used for the TGF-β1 ELISA kit according to the manufacturer’s protocol. For the standard pressure trial, TGF-β1 samples were diluted by a factor of 3 after the activation of latent target protein. Samples from the hypertensive trial were diluted by a factor of 4. Samples for the β-catenin and α-SMA ELISA kits were extracted with their respective extraction buffers and manufacturer protocol was followed. To collect calcium kit samples after cell stretching, the designated plates were rinsed with nonsterile PBS and allowed to rest in 5 mL of 0.6 M HCL for 24 hours before collection. Samples were either used immediately or stored at -80° C and thawed for later testing.

Total protein content was quantified with the BCA Protein Kit (Pierce). Calcium content was normalized to the respective well’s total protein content. The results of each ELISA sample were normalized to the total protein concentration of either that same sample or its respective well. For each assay performed, the standard solution containing no protein or calcium was used as the blank control.

RIPA buffer (Pierce) was used for protein extraction and normalization for the TGF-β1 standard pressure study and for both calcium content studies. For the hypertensive condition study with TGF-β1, β-catenin samples were taken from the same wells whose supernatant was
tested for TGF-β1, so the β-catenin samples were used to run a BCA assay for normalization. Other than the buffer difference used for total protein analysis between the two TGF-β1 studies, each biomarker was normalized to total protein content measured from the exact same samples.

**Statistical Analysis**

Single Factor Analysis of Variance (ANOVA) and a post-hoc Tukey test were conducted in SPSS with an α value of 0.05 to determine statistical significance.

**Results**

As depicted in Figure 13 and 14, subjecting the cells to the hypertensive stretch regimen was successful in initiating a phenotypic switch through the downregulation of α-SMA. However, this same effect was not observed within the standard pressure regimen category. Statistical analysis revealed a statistically significant difference between the cells stretched with the hypertension regimen and control (unstretched) cells of the same group, with the control cells exhibiting a significantly higher amount of α-SMA than the stretched cells. There was no significant difference between the stretched and control cells within the standard pressure regimen group or between the two different groups of stretched cells. There was a statistically significant difference between the hypertension control group and every other group within the normalized data (Figure 13). However, it is important to note that ELISA results are typically analyzed as unnormalized (shown in Figure 14); thus, the unnormalized results for each ELISA in this study are the focus of analysis.
Figure 13  Quantification of α-SMA in HAVSMCs subjected to either the standard pressure regimen, the hypertension regimen, or no stretch (normalized to total protein content).

Single-factor ANOVA and a post-hoc Tukey test with a significance level of p<0.05 were performed to determine statistical significance (n=6). Each matching symbol located above the respective error bars is indicative of a pair of groups with a statistically significant difference.
Quantification of $\alpha$-SMA in HAVSMCs subjected to either the standard pressure regimen, the hypertension regimen, or no stretch (not normalized).

Single-factor ANOVA and a post-hoc Tukey test with a significance level of $p<0.05$ were performed to determine statistical significance ($n=6$). Each matching symbol located above the respective error bars is indicative of a pair of groups with a statistically significant difference.

In addition to $\alpha$-SMA indicating a phenotypic switch in the HAVSMCs, the calcium content results found the stretched cells in the hypertensive regimen to have been successfully calcified when compared to their respective control group. A statistically significant difference, with the stretched cells containing a greater amount of mineralization, was found between the hypertensive control (unstretched) cells and the hypertensive stretched cells. No statistically significant difference was found between the standard pressure stretched cells and the standard pressure control cells. These results are depicted in Figure 15.
Figure 15  Quantification of calcium content in HAVSMCs subjected to either the standard pressure regimen, the hypertension regimen, or no stretch.

Single-factor ANOVA and a post-hoc Tukey test with a significance level of p<0.05 were performed to determine statistical significance (n=6). Each matching symbol located above the respective error bars is indicative of a pair of groups with a statistically significant difference.

TGF-β1, a well-studied biomarker of stretch, showed no overall statistically significant differences. No statistically significant difference was found between the standard pressure stretched cells and the standard pressure control cells or between the hypertension stretched and control cells. In Figure 16, there was a significant difference between the standard stretched cells and their respective control group. There was also a significant difference between the standard control cells and every other group. Figure 17, which depicts the unnormalized data and is the focus of analysis, does not show that same statistical significance between groups. The unnormalized data also displays a higher concentration of TGF-β1 in each stretched group when compared to its respective control, though the difference is not statistically significant.
Figure 16  Quantification of TGF-β1 in HAVSMCs subjected to either the standard pressure regimen, the hypertension regimen, or no stretch (normalized to total protein content).

Single-factor ANOVA and a post-hoc Tukey test with a significance level of $p<0.05$ were performed to determine statistical significance ($n=6$). Each matching symbol located above the respective error bars is indicative of a pair of groups with a statistically significant difference.
Figure 17  Quantification of TGF-β1 in HAVSMCs subjected to either the standard pressure regimen, the hypertension regimen, or no stretch (not normalized).

Single-factor ANOVA and a post-hoc Tukey test with a significance level of p<0.05 were performed to determine statistical significance (n=6).

The canonical Wnt pathway marker, β-catenin, showed no statistically significant differences between groups as seen in Figures 18 and 19. However, the unnormalized results in Figure 19 show a trend of each stretched group of cells containing more β-catenin than its respective control group. This difference is not statistically significant.
Figure 18  Quantification of β-catenin in HAVSMCs subjected to either the standard pressure regimen, the hypertension regimen, or no stretch (normalized to total protein content).

Single-factor ANOVA and a post-hoc Tukey test with a significance level of p<0.05 were performed to determine statistical significance (n=6).
Quantification of β-catenin in HAVSMCs subjected to either the standard pressure regimen, the hypertension regimen, or no stretch (not normalized).

Single-factor ANOVA and a post-hoc Tukey test with a significance level of $p<0.05$ were performed to determine statistical significance ($n=6$).

Lastly, Figure 20 depicts the results of each ELISA assay combined into one bar chart.
Figure 20  Quantification of TGF-β1, β-catenin, and α-SMA in HAVSMCs subjected to either the standard pressure regimen, the hypertension regimen, or no stretch (not normalized).

Single-factor ANOVA and a post-hoc Tukey test with a significance level of p<0.05 were performed to determine statistical significance within each ELISA assay (n=6). Each matching symbol located above the respective error bars is indicative of a pair of groups with a statistically significant difference.

In summary, the objectives of this aim were investigated using ELISA and calcium content analysis. α-SMA was significantly lowered between the hypertension stretched group and its respective control, and calcium content was significantly heightened between the same two groups. In the unnormalized results for TGF-β1 and β-catenin, which are the results typically used for analysis in this laboratory, no statistically significant differences were observed. However, the data’s overall trend corresponds with each protein’s well-known reaction to mechanical stretch.
CHAPTER IV
DISCUSSION

These experiments’ original goals were to create regimens in the Flexcell® Tension system to simulate both standard blood pressure and hypertensive conditions, determine the appropriate cell seeding density, and examine the effects of those regimens on vascular calcification and Wnt activation through mechanotransduction. First, the appropriate parameters for each regimen needed to be established. Literature review provided the physiological values of percent stretch that cells undergo (5-10% for standard blood pressure and at least 20% in hypertension) [29]. Thorough perusal of the Flexcell® Tension system’s user manual, along with testing various waveforms and frequencies, led to the finalization of the two regimens to be used thereafter: the standard pressure regimen (Heart (P) waveform, 0% minimum elongation, 10% maximum elongation, 0.2 Hz frequency, 24 hour duration) and the hypertensive regimen (Heart (P) waveform, 0% minimum elongation, 23% maximum elongation, 0.2 Hz frequency, 24 hour duration). The low frequency of the regimens may make results less accurate to physiological conditions with a normal heartbeat but was necessary to use the physiologically accurate Heart (P) waveform at the maximum elongation values.

The number of cells plated per well in the BioFlex® plates also needed to be tested and established. After testing various seeding densities, relative cell viability and overall protein content revealed no statistically significant differences between groups. A density of $1 \times 10^5$ cells/well was chosen to be used for all subsequent studies because it more closely aligned with
the manufacturer’s recommendations and displayed less overall standard deviation than other groups.

ELISA and calcium colorimetric kits were used to measure the impact of both the hypertensive and standard pressure regimen on HAVSMCs. It was hypothesized that the cells would demonstrate upregulation in Wnt, cell stretch, and calcification markers. It was also hypothesized that healthy VSMC markers would exhibit downregulation. α-SMA, a healthy contractile VSMC marker, showed no statistically significant differences between the standard pressure regimen cell group and its control. However, cells stretched with the hypertensive regimen possessed a statistically significant smaller amount of α-SMA when compared to its respective control group. This result answers the study’s main objectives and supports the hypothesis by indicating that HAVSMCs under hypertensive conditions can undergo dedifferentiation into a synthetic phenotype through mechanotransduction alone, pointing towards calcification and additional disease.

Furthermore, calcium content was also statistically insignificant between the standard pressure regimen group and its control group. On the other hand, cells stretched with the hypertension regimen possessed a statistically significant greater amount of calcium compared to their respective control group. These findings signify that the mechanical strain from hypertension alone induces calcification in HAVSMCs and supports the hypothesis.

The findings from TGF-β1 quantification did not support the hypothesis. In fact, there were no overall significant differences between groups except for within the normalized data, which is not typically used for analysis when utilizing ELISAs. In the unnormalized data (Figure 17), there is no statistically significant difference between any groups of cells. There is also a trend in which each stretched group contains more TGF-β1 per mL than its respective control
group, contrary to the normalized data trends in Figure 16. It is thought that this difference in trends between normalized and unnormalized data may be due to the use of different extraction buffers used for the BCA assay between this particular standard stretch and hypertensive stretch study. It may be possible that the two buffers have different levels of effectiveness, and so normalizing the data to the BCA assay altered the overall trend shown in Figure 17 and produced less comparable results.

β-catenin content was analyzed as a marker for Wnt pathway activation. Although there were not any significant differences between any groups in the study, the unnormalized data depicts an overall trend of each stretched group containing a higher concentration of β-catenin than its respective control group. This observation concludes that although the canonical Wnt pathway was not significantly activated, further studies with altered parameters may yield more apparent differences between groups that correspond with the overall trend found in this study. The same may be true for TGF-β1, as it is a well-studied biomarker of stretch.

It was found in this study that the unnormalized data trends for TGF-β1 and β-catenin, though statistically insignificant, match what is well-known from other previously discussed literature. Joki et al. stretched rat VSMCs with an older Flexcell® Tension system model. Their study found that when stretched for up to 24 hours at a frequency of 1 Hz and a maximum elongation of 20%, expression of TGF-β1 was time dependent [43]. Additionally, they saw that this protein expression was dependent on percent elongation and reached its highest point at 15% maximum stretch [43]. It is important to note that their cells were stretched “using alternating cycles of 0.5 s stretch and 0.5 s relaxation” instead of the heart (P) waveform [43]. It is possible that by altering parameters in this present study, the differences in TGF-β1 and β-catenin concentration between groups will become more significant. The heart (P) waveform was chosen

48
in this present study to be as physiologically accurate as possible with the pressure wave simulation, as this may impact results. To obtain the maximum elongation of 20%, the frequency was limited to 0.2 Hz. The stretch duration was set at 24 hours. Altering this study by including longer timepoints for stretch duration with the same waveform or studying the effects of a sinusoidal waveform at a higher frequency may increase the significance of protein expression between stretched and control groups. Increasing stretch duration without changing the waveform would keep the most physiologically accurate waveform. However, stretching cells with a sinusoidal waveform, which can reach high maximum elongations at higher frequencies, would allow a more physiologically accurate frequency and its effects on cells to be studied. In conclusion, this study succeeded in creating a regimen baseline and cell seeding density/protocol for all future studies. It also demonstrated significantly increased calcification in VSMCs as a direct result of mechanotransduction within hypertensive conditions.
CHAPTER V
FUTURE WORKS

This study succeeded in creating regimens with the Flexcell® Tension system to simulate hypertension and standard blood pressure mechanical strain on VSMCs. Furthermore, appropriate cell seeding density was also confirmed. These findings were subsequently applied to HAVSMCs. Thus, the cyclic mechanical strain placed onto VSMCs due to hypertension was confirmed to lead to a significant increase in VC, as indicated by a loss of α-SMA and an increase in calcium content. Although there were no significant differences in β-catenin and TGF-β1 concentrations between groups, the overall trend of the data is promising and matches results obtained in other literature. While this study focuses on VSMCs in the context of Wnt activation and VC, further work is needed to investigate ECs and EndMT in the same context, as well as how the two cell types interact in a hypertensive environment. To further investigate the mechanisms involved in the canonical Wnt pathway and its role in VC upregulation in hypertension, the following objectives should be completed:

1. Expand the duration of stretch to multiple timepoints (2 and 3 days, for example) while holding all other parameters constant to identify the effects of time on VC, Wnt pathway, and stretch biomarker activation.

2. Examine cell alignment post-stretch.

3. Repeat study with this lab’s established calcification media/protocol to examine the effects of a calcific and hypertensive environment on each biomarker.
4. Perform study with ECs instead of VSMCs to examine the effect of mechanotransduction on EndMT and VC with the specific regimen parameters.

5. Perform study with VSMC and EC coculture to determine their interactions within the setting of hypertension, mechanotransduction, VC, and canonical Wnt pathway activation.

6. Create an altered stretch regimen (sinusoidal waveform, same maximum elongation as present study, highest frequency possible giving other parameters) and compare its effects on each biomarker to the present study.

Although the present study produced promising results regarding the effect of hypertension on the Wnt pathway and VC, these additional objectives will serve to further confirm both the significant and insignificant data trends described here. This study brings technology one step closer to understanding the hidden mechanisms and interactions of hypertension with the canonical Wnt pathway and calcification. As the suggestions above are implemented and more information is uncovered, a therapeutic target for VC may become apparent, leading to an attainable, effective treatment for VC.
REFERENCES


54


S. Rong *et al.*, “Vascular Calcification in Chronic Kidney Disease is Induced by Bone Morphogenetic Protein-2 via a Mechanism Involving the Wnt/β-Catenin Pathway,” *Cellular Physiology and Biochemistry*, vol. 34, no. 6, pp. 2049–2060, Jan. 2014, doi: 10.1159/000366400.


