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Understanding vascular calcification through the lens of canonical WNT signaling

KarLee McNeel
Mississippi State University, mcneel77@gmail.com

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By

KarLee McNeel

Approved by:

C. LaShan Simpson (Major Professor)
Steven H. Elder
Matthew K. Ross
Reuben F. Burch V
Steven H. Elder (Graduate Coordinator)
Jason M. Keith (Dean, Bagley College of Engineering)

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Name: KarLee McNeel

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Every 37 seconds, someone in the United States dies from cardiovascular disease. Vascular calcification is one of the underlying causes of these fatal events. Medial calcification develops following arteriosclerosis, or hardening of the arteries. Medial calcification is characterized by the deposition of hydroxyapatite in the medial layer of the arteries after normal vascular smooth muscle cells undergo a phenotypic switch to resemble osteoblast-like cells. It is hypothesized that this switch is caused by the wingless related (WNT)-Signaling pathway. The WNT-Signaling pathway, upon activation, causes the upregulation of osteogenic markers for the development of osteoblast-like cells. Current treatments alleviate consequences of calcification but do not address the disease. Due to a lack of cures for calcification, a novel therapy for this disease is overdue. By studying human aortic smooth muscle cells and confirming the role of WNT-Signaling as it relates to calcification, a possible therapeutic target for calcification can be identified.

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INTRODUCTION

Background

Arteriosclerosis, characterized by hardening of the arteries, is a dangerous precursor to a degenerative condition called vascular calcification. Vascular calcification is a process by which a phenotypic transition of vascular smooth muscle cells causes generation of osteoblast mimicking cells within arteries[18]. This process is initiated by the wingless-related integrated (WNT) pathway mimicking osteogenesis as mechanical stress from arterial injury leads to this remodeling[25]. There are many causes of calcification, including diabetes, kidney failure, and obesity[35]. Based on data from the American Heart Association and the National Institutes of Health in 2017, someone in the United States dies of a cardiovascular related disease every 37 seconds[1]. This data further showed that globally, the number of cardiovascular disease related deaths increased 21.1% from 2007 to 2017 to a total of 18 million deaths annually[1]. The mechanisms that underlie common cardiovascular events like heart attack or stroke are not well understood. Vascular calcification can be an underlying cause of these often-fatal events and is becoming more heavily studied as it is underdiagnosed due to a lack of characteristic symptoms. Once calcification begins, buildup of hydroxyapatite in arteries can lead to hypertension and a decrease in arterial compliance and elasticity[1]. Often, calcification is not discovered until a heart attack or stroke occurs because it is not screened for and has no obvious symptoms. The only treatment protocols for calcification available, like angioplasty, are used to treat the disease and

not consequences associated with calcification. Angioplasty is a process using a stent inserted into the artery to open the area where calcification has occurred[33]. The stent is deployed where the artery has been affected by plaque buildup that blocks proper blood flow and increases blood pressure. The stent opens the artery back up for proper blood flow and decreases blood pressure as it expands and creates a channel for continuous blood flow[33]. This, however, does not address the fact that calcification associated with WNT Signaling initiated phenotypic switching of smooth muscle cells causes the reoccurrence of calcification along the length of the artery even though proper blood flow may have been returned at the initial site.

Thus, a cellular therapy focusing on the prevention and reversal of calcification is needed to reduce the risk of fatal cardiovascular events. Considering the lack of treatments to prevent and reverse calcification, a novel therapy for treatment of this disease is long overdue. By studying human aortic smooth muscle cells and confirming the role of WNT Signaling as it relates to calcification, a possible therapeutic target for calcification can be identified.

Vascular Calcification

Once thought to be a passive process, vascular calcification is now understood to be a cell-mediated process that is a major contributor to increased blood pressure, loss of arterial integrity, and ischemia of the body's major organs and extremities[2,3]. Manifesting itself in four distinguishable forms, vascular calcification generally presents itself as intimal or medial calcification occurring in the tunica intima and tunica media, respectively[3,6]. *Figure 1.1* presents the artery with its layers and the locations of the tunica intima and tunica media in relation to one another. The adventitia, while not given its own respective category of calcification, is less known to calcify. However, a study by Li, et al. shows that there is potential for calcification in

this layer as fibroblasts differentiate into myofibroblasts or smooth muscle cells before undergoing calcification[4].

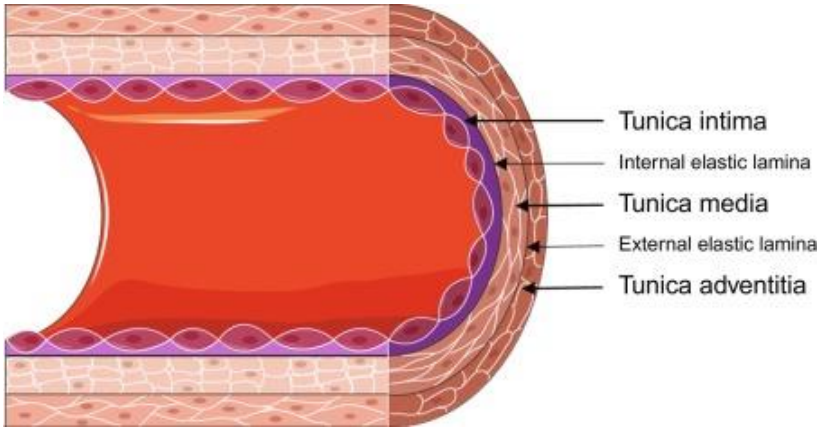

Figure 1.1 Different Parts of the Artery Showing Three Major Components: Tunica Intima, Tunica Media, and Tunica Adventitia[5]

Intimal vs Medial Calcification

When vascular calcification occurs in the two main layers of the artery: the tunic intima and tunica media, there are different names to denote the calcification of these layers. Calcification that occurs in the tunica intima is termed atherosclerosis while calcification in the tunica media is known as arteriosclerosis[34]. What often differentiates these two conditions is the mechanism that causes their onset[35]. Atherosclerosis is widely associated with inflammation. As seen in *Figure 1.1*, atherosclerosis occurs in the tunica intima which is in direct contact with blood flow through the arteries. This inflammation occurs primarily after injury to the endothelial cells that make up the tunica intima[8]. While injury is the most common precursor, other risk factors include a lipid-high diet, smoking, and obesity. If injury is the initial cause, this creates a pro-inflammatory, pro-thrombogenic environment in the artery that promotes the adhesion of lipids and foam cells in

different concentrations that depend on the type of lesion[8]. In addition, there is adhesion of monocytes, platelets, and lymphocytes that contribute to plaque formation along the inner layer of the arterial wall blocking the lumen[8,9]. Inflammatory cytokines mediate the process of atherosclerosis as they promote inflammatory pathways that recruit the aforementioned molecules. These molecules adhere to the site of injury by increasing vascular permeability and create this accumulation of plaque[9]. *Figure 1.2* denotes the difference in appearance of arteriosclerosis and atherosclerosis where the calcification of the artery in atherosclerosis is considered eccentric and is considered concentric in arteriosclerosis because it occurs within the layer of vascular smooth muscle cells[35].

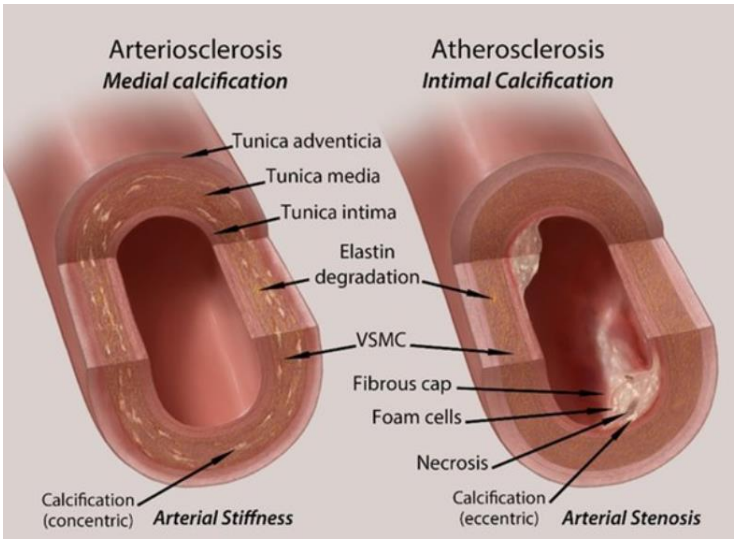

Figure 1.2 Image of Atherosclerosis vs Arteriosclerosis Calcification Differences[27]

However, by concentrating on arteriosclerosis which is calcification of the tunica media between the tunica intima and the tunica adventitia through the lens of WNT Signaling, a significant number of affected patients can potentially be helped through this research. The tunica media is

made up of vascular smooth muscle cells. Arteriosclerosis is used to describe the hardening of this layer of cells[8,9]. Sometimes considered a broad term, arteriosclerosis is often used interchangeably with Monckeberg medial calcific sclerosis and arteriolosclerosis. However, it can be noted that Monckeberg medial calcific sclerosis and arteriolosclerosis are subsets of arteriosclerosis where Monckeberg's disease is termed as medial calcification caused by renal failure or diabetes mellitus[10]. Arteriosclerosis denotes medial calcification of smaller arteries throughout the body that are distinguished by major arterial stiffening, hypertension, and an increased risk of heart failure[10, 35-36]. For clarity, medial calcification can be used to denote this disease. In contrast with atherosclerosis, medial calcification is not a very well understood disease as there remains a clear amount of disagreement among research groups as to the origin of the disease. Some believe that medial calcification only involves the smooth muscle cells of the tunica media while others believe that migration of the myofibroblasts of the adventitia combined with initial injury to the tunica intima contribute to the future differentiation of smooth muscle cells [4,9]. Medial calcification manifests itself in the form of arterial stiffness, that promotes an increase in pulse wave speed and an increase in the cardiac load[14]. Medial calcification, termed the silent killer, can reach dangerous levels quickly and by the time it manifests itself in the form of a major event like a heart attack, it is often too late. Understanding the cells involved and the processes that cause increased vascular calcification is crucial to reducing the level of mortality by cardiovascular events.

Vascular Smooth Muscle Cells

Prior to 1967, it was thought that the tunica media was made up of fibroblasts and smooth muscle cells, but upon further study, it has been found that the tunica media is made up mainly of vascular smooth muscle cells[11,12]. Vascular smooth muscle cells are derived from mesenchymal

stem cells that come from bone marrow. These mesenchymal stem cells generally differentiate into smooth muscle cells or epithelial cells. These vascular smooth muscle cells develop a contractile phenotype within the tunica media of the arteries[35]. While it was once thought that the smooth muscle cells experienced only one phenotype, Wissler's discovery in 1967 promoted further study that discovered the different phenotypes that smooth muscle cells can experience when exposed to certain stimuli in the body. The contractile phenotype expressed by the stable vascular smooth muscle cells of the tunica media promotes slow proliferation, neurotransmitter responses, and expression of cell markers that discourage vascular calcification like α-smooth muscle actin and osteopontin[12]. This α-smooth muscle actin has normal levels in non-calcified arteries and is significantly decreased, as found by multiple studies to include that by Xin, et al., in calcified arteries[13].

When stressors target smooth muscle cells, the cells experience a phenotypic switch to an active, synthetic phenotype that is characterized by misplaced extracellular matrix production in the arterial wall and significantly reduced levels of α-smooth muscle actin and osteopontin making it possible for calcification to occur[12,13]. When injury occurs, the need for proliferation increases resulting in the need for the synthetic state that allows for vascular smooth muscle cells to proliferate and recruit cells to address the injury. In the synthetic state, vascular smooth muscle cells can differentiate into other cell types like adipocytes and osteoblasts[14]. *Figure 1.3* shows some of the possible cells that vascular smooth muscle cells can differentiate into when they transition to the synthetic phenotype. When vascular smooth muscle cells revert to this active phenotype in the tunica media, they resemble the immature, undifferentiated smooth muscle cells

of the tunica intima that can differentiate into multiple other types of cells like osteoblast-like cells.

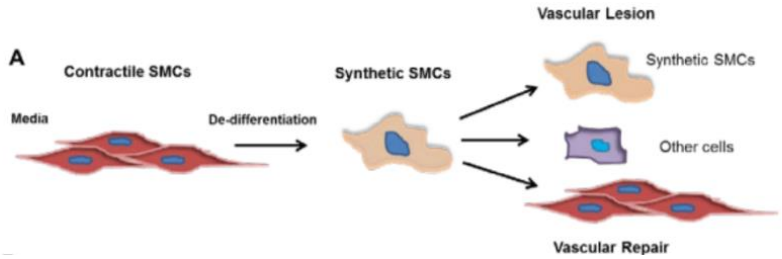

Figure 1.3 Possible Differentiation Methods of Vascular Smooth Muscle Cells After Phenotypic Switch from Contractile to Synthetic[28]

Conditions that promote the initiation of medial calcification include hyperphosphatemia, hypercalcemia, and hypercholesterolemia that cause the downstream process of vascular calcification that resembles the process of osteogenesis[34-36]. When comparing the process of osteogenesis (bone formation) with vascular calcification, it becomes apparent that their parallels can be used to gain a better understanding of the initiation and downstream positive feedback loop that causes the severe calcification. After the onset of calcification, those who experience cardiovascular events have significantly increased mortality. Vascular smooth muscle cells are unable to terminally differentiate which makes them susceptible to phenotypic change. Although not completely understood, the process of arterial remodeling following the phenotypic switch of vascular smooth cells involves osteoblast-like cells that develop from these phenotypically switched vascular smooth muscle cells[12,14]. The change to osteoblast-like cells is key to the beginning of calcification of the tunica media as the process begins to mimic osteogenesis with the deposition of hydroxyapatite in the arterial wall.

Another stressor that can cause vascular smooth muscle cells to undergo further phenotypic switching is mechanical stress. Mechanical stress comes in the form of hypertension which occurs due to an accumulation of plaque in the tunica media as previously switched cells deposit hydroxyapatite in the walls of the artery. As this hydroxyapatite is deposited, strain within the artery is elevated due to the plaque causing the arteries to narrow and velocity of the blood to increase, boosting blood pressure[34]. Under this increased pressure, vascular smooth muscle cells may experience triaxial loading, which is the combination of hoop tensile strain, axial tensile strain, and radial compressive strain[34]. This, in combination with shear stress, causes vascular smooth muscle cells to align themselves perpendicular to the flow of blood and the axis of stretch during uniaxial and equibiaxial stretch[61-62]. In the face of hypertension, these vascular smooth muscle cells can become overstrained, and this overstraining can stimulate a phenotypic switch from their natural contractile phenotype to a synthetic phenotype with the ability to become osteoblast-like cells[34,63]. Vascular smooth muscle cells normally possess the ability to regulate serum calcium levels and blood pressure, but hypertension can cause increased calcium uptake leading to vascular resistance and abnormal vascular tone[64]. This increased calcium uptake leads to hypercalcemia and contributes to further vascular calcification suggesting that mechanical stressors like hypertension have a great impact on the progression of calcification.

Osteogenesis

Osteogenesis is defined simply as bone formation. Under normal circumstances during development, osteogenesis is the complex process that adds bone via osteoblastic activity. Though not completely understood, it is hypothesized that the process of osteogenesis is mimicked in vascular calcification as hydroxyapatite crystals are deposited along the artery walls. After the phenotypic switch of the vascular smooth muscle cells, synthetic vascular

smooth muscle cells may develop into osteoblast-like cells. As medial calcification occurs, there is also an associated decreased bone density that occurs in what is known as the calcification paradox[17]. This association is not understood as there seems to be no determinable reason why this correlation would occur.

Mimicking Osteogenesis

In almost all forms of vascular calcification, including medial calcification, elastin degradation is one of the early events that happens alongside phenotypic change of the vascular smooth muscle cells to begin the process of calcification[18]. In the tunica media of the artery, elastin and vascular smooth muscle cells are the main components. Unlike in intimal calcification, inflammatory cells and other responding cytokines are not necessary to the progression of calcification. Likely as a response to vascular injury or systematic apoptosis, medial calcification can begin when apoptotic bodies of vascular smooth muscle cells are not able to be cleared by other vascular smooth muscle cells nearby[18]. This is often due to the elastic laminae that line the areas between elastin and the vascular smooth muscle cells serving as a barricade for other vascular smooth muscle cells to not be able to phagocytose the debris[18]. When this lack of phagocytosis occurs, it can lead to an increase in phosphate concentrations beginning the process of calcification. In response to injury and increased serum phosphate levels, there is a phenotypic switch of the vascular smooth muscle cells that causes them to differentiate into osteochondrogenic cells that synthesize matrix vesicles[16,19]. These matrix vesicles then interact with the extracellular matrix via matrix metalloproteinases which lead to elastin degradation and release of elastin peptides[19,20]. These elastin peptides initiate a positive feedback loop as they interact with the elastic laminae and release more matrix metalloproteinases[20].

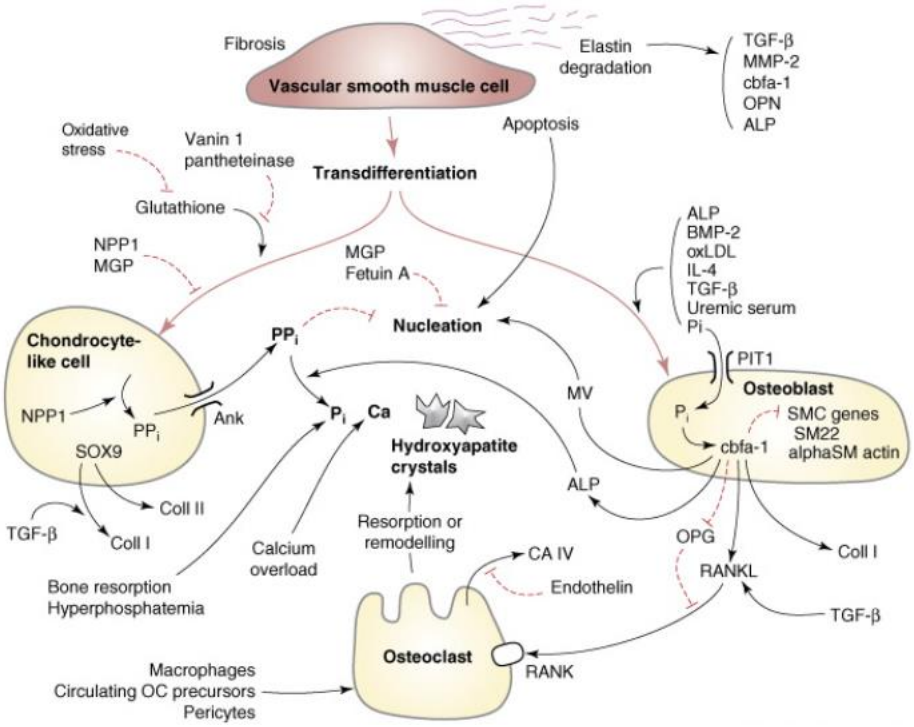

Figure 1.4 Pro-calcifiying Conditions Cause Smooth Muscle Cells to Phenotypically Switch into Osteoblast-like Cells via the Expression of Bone-Associated Proteins[17]

As shown in Figure 1.4, under these pro-calcifying conditions, phenotypically switched smooth muscle cells begin to express bone-associated proteins to include core binding factor α-1 (Cbfa-1), osteopontin, bone morphogenetic protein-2a (BMP-2a), osteocalcin, matrix Gla protein, and alkaline phosphatase[20]. BMP-2a is one of the many proteins thought to induce expression of Cbfa-1 causing the phenotypically switched smooth muscle cells to become osteoblast-like cells[15]. In normal osteogenesis, the activation of expression of Cbfa-1 causes mesenchymal stem cells to differentiate into full osteoblasts[15]. Cbfa-1 is also known as runt-related transcription factor 2 (RUNX2). RUNX2 is a product of canonical Wingless (WNT) Signaling which is initiated by WNT proteins combining with the Frizzled receptor and low-density lipoprotein receptor-related protein (LRP) 5/6 in the plasma membrane of the vascular smooth muscle cells.

An important regulator of this mineralization of the tunica media is the phosphate to pyrophosphate ratio in serum, determined by pyrophosphate, secreted extracellularly[17]. Another source of pyrophosphate is that which is cleaved by alkaline phosphatase[17]. Alkaline phosphatase, in the presence of enough phosphate, can cleave its inhibitor, pyrophosphate, to begin forming hydroxyapatite[17]. Like the normal process of bone mineralization, in medial calcification there is an increased expression of alkaline phosphatase within the differentiated vascular smooth muscle cells that begins the process of hydroxyapatite formation and deposition as shown in studies by Shanahan, et al.[21]. The way to understand how these differentiated synthetic vascular cells acquire RUNX2 to become osteoblast-like cells is through the lens of WNT Signaling.

Wingless (WNT) Signaling

The WNT-Signaling pathway is initiated by WNT proteins. WNT proteins are growth signaling factors that come in nineteen variable forms in mammals. The discovery of the first WNT protein is credited to Nüsslein-Volhard and Wieschaus during a study of *Drosophila* conducted in 1980[52]. Table 1 shows the different WNT proteins and the WNT-Signaling pathway they correspond with as noted by varying researchers. There is some varying disagreement among researchers as the which WNT proteins belong under each branch of WNT signaling (as denoted by those listed twice). However, some researchers also believe that the branch WNT signaling that is activated is context dependent based on the expression of WNT antagonists and co-receptors[37, 39]. Further still, other researchers believe that some of the non-canonical WNT proteins like WNT5a, WNT9b, WNT10a, and WNT10b play a role in blocking canonical WNT signaling to promote liver development and regeneration[41]. This action was first categorized well

in 2003 by Moon et al. who showed that non-canonical WNTs could serve an important role in antagonizing the canonical WNT pathway[56].

Table 1.1 WNT Proteins and the WNT-Signaling Pathway They Correspond to Normally

WNT-Signaling Proteins	
Canonical	Non-Canonical
WNT1[37]	WNT2[38]
WNT3[37, 38]	WNT4[37, 41-42]
WNT6[40]	WNT5a[37, 38, 41-42]
WNT8a[38]	WNT5b[38, 42]
WNT8b[38]	WNT6[40]
WNT10a[42]	WNT7a[42]
WNT10b[37, 42]	WNT7b[42]
N/A	WNT9b[41]
N/A	WNT10a[41]
N/A	WNT10b[41]
N/A	WNT11[37-38, 42]

Incorporated into exosomes, little is known about the process by which WNT proteins are called to the surface of the vascular smooth muscle cells and the WNT proteins are released to initiate the process of WNT Signaling[22,23]. Under normal circumstances in other parts of the body, proteins that are incorporated into the surface of exosomes are “called” by factors on target cells that cause the proteins to be secreted to initiate a particular process. In WNT Signaling, this target could be Frizzled and its coreceptor LRP 5/6. The Frizzled receptor was first categorized in 1993 by Bhanot et al. who used a study of *Drosophila* cells to show that cells expressing a

gene family called Dfz2 responded to WNT proteins and WNT proteins were able to bind to those cells expressing Dfz2 indicating that Frizzled proteins are receptors for WNT molecules[53]. Once called to the surface of the target cells, WNT proteins are released to bind to Frizzled and initiate the process of canonical or non-canonical WNT Signaling. After in-depth analysis of the WNT pathways and their contributions to the development of medial calcification as well as many types of cancer, there have been many that have sought out a method to completely turn off WNT Signaling. This would be an error, as shutting off the WNT Signaling pathway would ensure a lack of development. In 1990, a study by McMahon et al. showed in a mouse model that the loss of the WNT1 alone could lead to severe underdevelopment of the central nervous system[51]. In the early stages of life, the WNT-Signaling pathway is vital to proper embryonic skeletal development and growth in later years[2]. However, with age also comes an increased risk of medial calcification.

WNT Signaling comes in two major forms: canonical and non-canonical. Until 1993, it was thought that the canonical WNT Signaling pathway was the only WNT pathway. It was the work of Moon et al. in 1993 studying WNT5a, a specific WNT protein, that showed that WNT5a initiated cellular processes that were different from those initiated during canonical WNT signaling[54]. Later, in 1997, Varmus et al. discovered that in addition there was an entirely different receptor, not within the Frizzled family, that captured WNT5a to initiate intracellular signaling[55]. This work generated the term non-canonical which simplistically referred to the fact that the cellular process was not like the canonical pathway. Non-canonical WNT Signaling also has two major subsets, including the planar polarity pathway and the Ca^{2+} dependent pathway. However, as shown in *Figure 1.5*, there is one component that links all three independent

pathways: Frizzled[60]. Frizzled is a receptor for the specific WNT proteins for each WNT Signaling pathway coupled with a co-receptor that is specific to each respective pathway.

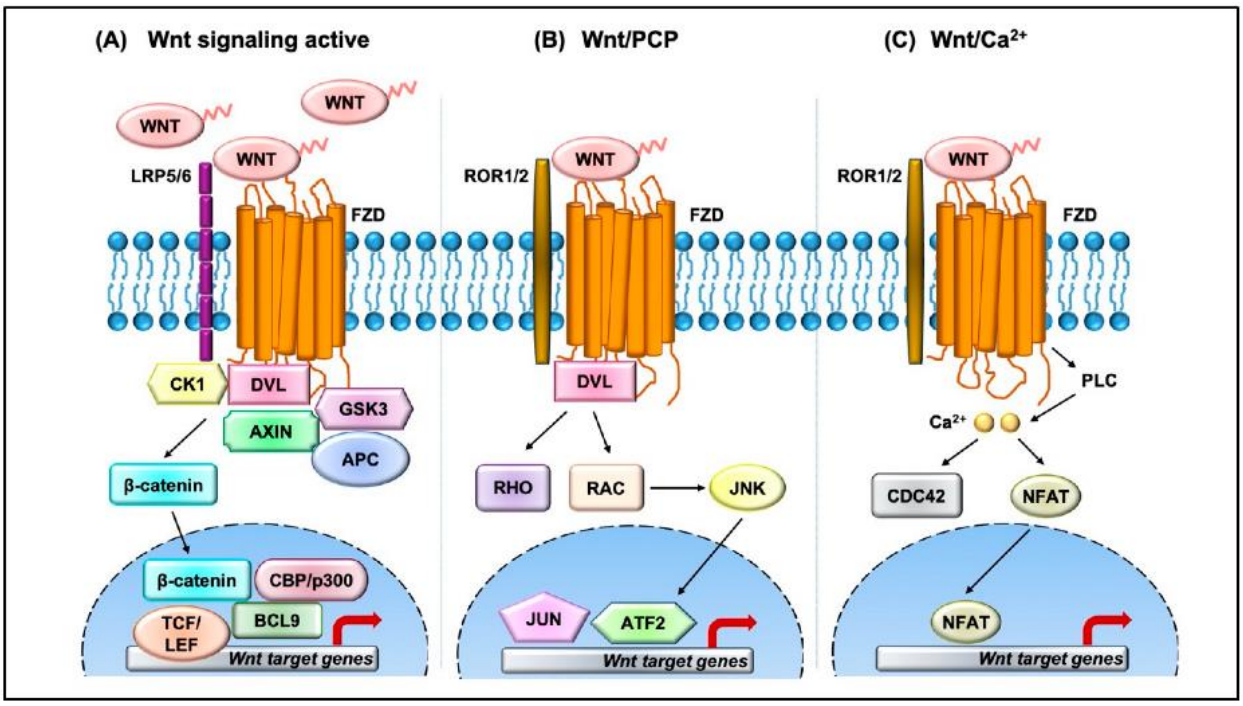

Figure 1.5 The Three WNT Signaling Pathways: Canonical WNT Signaling, Planar Polarity Non-Canonical Pathway, and Ca^{2+} Non-Canonical Pathway[60]

Medial calcification, as expressed earlier, is mediated by RUNX2 and β-Catenin levels that are part of the canonical WNT-Signaling pathway[2,24,25]. Embedded in the plasma membrane of the vascular smooth muscle cells are the receptor and coreceptor Frizzled and LRP 5/6, respectively. Frizzled is a G-protein coupled receptor and LRP 5/6 is a lipoprotein receptor-related protein, both of which are necessary to the progression of the WNT-Signaling pathway[24,26]. It is agreed upon that these two components are necessary for the progression of WNT Signaling as the signal is passed from the extracellular components to the intracellular components.

Intracellularly, within the cytosol of the vascular smooth muscle cells, there is a destruction complex. The components of the destruction complex are widely debated upon with some of the more simplistic models including Axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3 (GSK3), Disheveled, and β-Catenin[2]. More complex models also include components such as casein kinase-1 (CK-1) and β-transducin repeat containing protein (β-TrCP)[24]. Other models include CK-1, but do not suggest that there is involvement by β-TrCP[25,26]. In non-calcifying conditions, β-Catenin is phosphorylated and ubiquitinated before undergoing proteosomal degradation. *Figure 1.6* shows the more simplistic method of addressing WNT Signaling[2]. A common player in the destruction complexes proposed, the method by which β-Catenin is phosphorylated is by GSK-3 leading to the release of β-Catenin from the destruction complex[2,24-26]. Upon release from the destruction complex, β-Catenin is targeted for ubiquitination before undergoing proteasomal degradation that allows for β-Catenin levels to remain at normal/low levels. When β-Catenin levels remain low, RUNX2 levels remain low and keep calcification at bay.

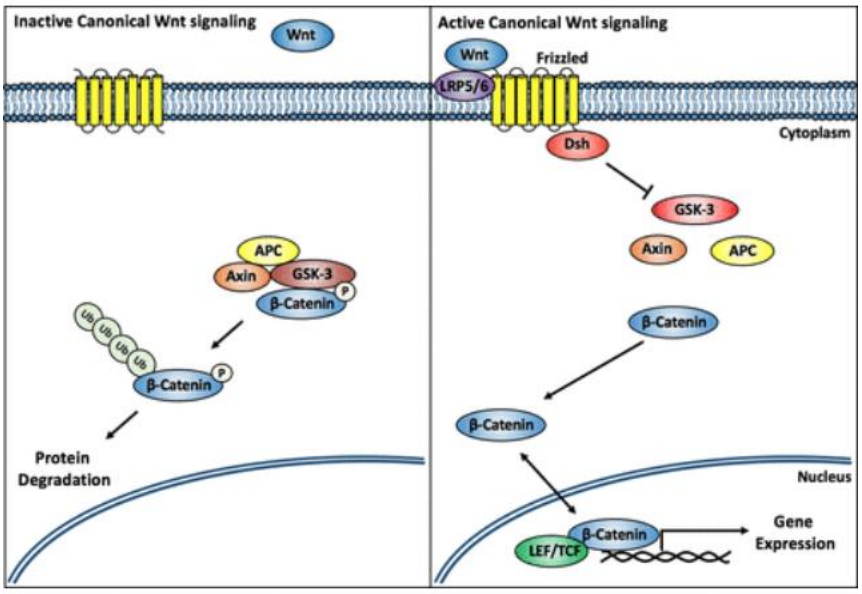

Figure 1.6 Canonical WNT-Signaling Pathway Both Active (right) and Inactive (left)[2]

When a WNT protein is released from an exosome due to high serum phosphate levels, it binds to the Frizzled receptor and LRP 5/6 coreceptor prompting the recruitment of Disheveled on the destruction complex as shown in *Figure 1.6*[2,24-26]. Once Disheveled is recruited, the destruction complex dissociates and β-Catenin levels in the cytosol rise as GSK-3 is no longer able to phosphorylate β-Catenin for targeting for proteasomal degradation. Once β-Catenin reaches high levels in the cytosol, it undergoes transport to the nucleus of vascular smooth muscle cells where it interacts with the lymphocyte enhancer factor (LEF)/T-cell factor (TCF) complex that regulates gene transcription within the vascular smooth muscle cells[2,24-26]. As levels of β-Catenin rise and translocate to the nucleus to interact with this complex, the LEF/TCF complex upregulates its transcription of RUNX2. This upregulation of RUNX2 leads to the permanent differentiation of vascular smooth muscle cells into osteoblast-like cells that can create hydroxyapatite and deposit it within the arterial wall of the tunica media. This deposition in

combination with the apoptotic debris within the tunica media leads to the arterial stiffness that creates a higher cardiac load and results in major cardiovascular events like heart attack or stroke.

It is often asked why turning off the expression of RUNX2/Cbfa-1 would not serve as a potential cure or inhibitor of vascular calcification. In a study by Speer, et al., it was found that RUNX2/Cfba-1 knockout mice died quickly after birth because they were unable to undergo skeletal development and died, most commonly, of respiratory failure[16]. Therefore, the answer lies not with turning off an entire process, but rather with inhibiting a small factor like the binding of β-Catenin to the LEF/TCF complex of the canonical WNT-Signaling pathway.
The current treatments for medial calcification, or vascular calcification in general, include methods like cutting away the diseased part of the artery or inserting a stent to alleviate pressure from arterial stiffening[33]. Neither of these treatment options is especially conducive to a full recovery as patients often have a relapse of the disease that often leads to death during the second reoccurrence. That is why it is more vital than ever to determine an optimal target for medial calcification treatment and that starts with understanding the destruction complex of the WNT-Signaling pathway.

Conclusion

Vascular calcification is a major contributor to the millions of deaths caused by cardiovascular disease worldwide every year. There is a significant gap in the literature understanding of vascular calcification and what can be done to reverse it without the use of invasive surgical procedures. Coming in two major forms like intimal and medial calcification, vascular calcification can strike in a multitude of ways that are often irreversible. A silent killer like

medial calcification targets many at-risk groups like the elderly whose arteries begin stiffening via natural processes, chronic kidney disease patients whose treatment creates high serum phosphate levels, and diabetic patients whose insulin shots inhibit the GSK-3 component of WNT Signaling all leading to progression of medial calcification. Mirroring the complex process of osteogenesis, the initiation and progression of medial calcification is still fogged with uncertainty as understanding the internal cellular factors of vascular smooth muscle cells still defies full scientific understanding. With the help of the WNT-Signaling pathway, vascular smooth muscle cells, when exposed to pro-calcifying conditions like hyperphosphatemia, can defy their current phenotypic state and revert to a synthetic phenotype that allows them to differentiate into osteoblast-like cells. These osteoblast-like cells become capable of depositing hydroxyapatite crystals in the wall of the tunica media and lead to arterial stiffening. To address these hypotheses, the following specific aims have been developed:

Specific Aim 1: Study markers of the WNT Signaling pathway contributing to vascular calcification.

Specific Aim 2: Confirm WNT Signaling and study a target of the pathway as a novel therapy for vascular calcification.

Successful completion of this study will confirm that the LEF/TCF complex is a novel therapeutic target for the treatment of not only the disease of vascular calcification itself but also the consequences that follow its progression. After the identification of this complex as a therapeutic target, manipulation of the WNT-Signaling pathway can be performed by identifying the time at which WNT Signaling begins to progress calcification. This time can be used to

perform an *in vivo* LEF/TCF knockout study to show if suppression of the LEF/TCF complex will inhibit the progression of vascular calcification and prevent fatal cardiovascular events from occurring.

CHAPTER II

AIM I: STUDY MARKERS OF THE WNT SIGNALING PATHWAY CONTRIBUTING TO VASCULAR CALCIFICATION

Introduction

Previously literature from the Simpson lab has shown successful calcification of healthy human aortic smooth muscle cells (HASMCs) when supplemented with Sodium Dibasic Anhydrous. However, since these experiments were run, cells have been frozen for longer periods, so it was important to confirm that all cells were still able to be calcified via supplementation with Sodium Dibasic Anhydrous. Thus, a calcification study will be performed to confirm that the previous *in vitro* model will still be accurate for current studies. After induction of calcification, a Calcium Colorimetric Kit can be used to quantify calcium at various timepoints to determine if calcification is time dependent. For normalization, a study by Holmar et al. confirms that the use of a BCA assay to quantify protein can be then used for comparison to calcium content to study the extent (grade) of calcification within a sample[50]. Calcification can be visualized using Xylenol Orange staining to study the morphology and distribution of calcium deposits. Following staining, ImageJ software can be used to quantify the percentage of area of the stained area covered in calcified nodules and the number of nodules in each image.

It is also important to relate calcification with the changes in cellular markers that may occur due to the role WNT-Signaling plays in vascular calcification. The addition of inorganic phosphate

will induce the phenotypic switch and promote calcification of the HASMCs. Cells will then be cultured for 7 and 14 days (n=4 for each group) then analyzed for phenotypic modulation using immunohistochemistry. Immunohistochemistry will be used to stain both control and calcified cells to show smooth muscle cells losing α-smooth muscle actin and Axin-1. There have been successful immunohistochemistry studies performed for the imaging of α-smooth muscle actin[44-45, 48] and Axin-1[47] so it will be possible to image for these components for the confirmation of their contribution to medial calcification. An upregulation of β-Catenin between control and calcified cell groups can confirm that WNT Signaling is involved in the initiation of calcification in human aortic smooth muscle cells as shown in previous studies[25, 49]. Thus, it is important to not only visualize these cellular markers, but quantify them using enzyme-linked immunosorbent assays (ELISAs) allowing for quantification of the changes in these cellular markers and confirmation that WNT Signaling is involved in the initiation of calcification.

Aim I Specific Objectives

The objectives of these studies were to first determine that calcification could be induced using Sodium Dibasic Anhydrous. Once calcification is induced, calcium can be quantified to determine if calcification is time dependent by completing a calcification study at multiple timepoints. After confirming calcification can be induced, calcium deposition can be visualized in multiple methods to determine morphology and distribution. Lastly, markers of WNT signaling will be studied through visualization and quantification of VSMC contractile phenotype and osteogenic markers at various time points that are hypothesized to be involved in WNT Signaling.

Experimental Section

Materials/Methods

Cell Culture

HASMCs previously frozen were thawed and expanded between passage 5-7. Upon seeding, cells were cultured in a growth medium supplemented with Dulbecco's Modified Eagles Medium (DMEM), 10% Fetal Bovine Serum, and 1% Penicillin every 2-3 days. Light microscopy was used to monitor cell morphology and observe cellular health. After cells reached 100% confluence, they were seeded in 6-well plates in control and calcified groups where both received 3 mLs of growth medium following the same supplementation period of two days. Once cells reached 80% confluence, plates were "serum-starved" for 12 hours using serum-starve media composed of DMEM and 1% Penicillin. Serum-starving was used to prepare cells to receive calcification media. After a starvation period of 12 hours, cells in the control group received normal growth medium while cells in the calcified group received calcification media. Calcification was induced by supplemented cells with calcification media composed of normal growth media with the addition of Sodium Phosphate Dibasic Anhydrous Phosphate at a 3mM concentration. Cells were then calcified between 0-14 days. Both control and calcified cells were fed with their respective medias every 2-3 days.

Calcium Quantification

Cells were cultured and expanded to passage 6 before being seeded in 6-well plates in control (n=6), 7-Day calcified (n=6), and 14-Day calcified (n=6) cellular groups. Following respective periods of calcification, cells were harvested for calcium and protein quantification. First, cells were rinsed in nonsterile PBS and calcium harvested using 0.6M HCl applied to cells for 24

hours with 5 mls of HCl being administered per well. After 24 hours, the HCl samples were collected and analyzed using a Calcium Colorimetric Kit to quantify calcium content for each respective time period and cellular group. To harvest protein samples for normalization, the cell layer was treated with 1 ml of RIPA lysis buffer and placed on ice for 5 minutes. Cells were scraped and removed from the plates into their respective microcentrifuge tubes. Protein concentrations were analyzed using a BCA Protein Kit (Pierce). Calcium samples were then normalized to protein concentrations.

Xylenol Orange Staining

Cells were cultured and expanded to passage 7 before being seeded in 4-well chamber slides in control (n=4) and calcified (n=4) cellular groups. Cells were then fed their respective medias for 7- and 14-Day timepoints. After their respective time periods, negative control images of the cells were taken before staining. Xylenol Orange (Sigma-Aldrich #3618-43-7) powder was dissolved into distilled water to make a 20 mM solution. This solution was then diluted into control growth medium to create a 20 μM dilution. This dilution was added at 1 mL per well in the chamber slides for 24 hours. After 24 hours, the staining solution was removed, and cells were rinsed with PBS. Following rinsing, 300 μLs of 4’,6-diamidino-2-phenylindole (DAPI) was added to each well and incubated for 2-5 minutes. Following incubation, cells were imaged using Brightfield, Texas Red, and DAPI fluorescence microscopy. Following imaging, ImageJ software was used to identify the percentage of area of each Texas Red image covered in calcified nodules and the number of calcified nodules was counted. To find this quantitative data, images were opened in ImageJ software and the Color Threshold was set to highlight the calcified nodules and all background noise was removed from the image. Next, the image was made binary for analysis. Each particle in this binary image was analyzed to determine its area

and counted towards the total population of calcified nodules. From this area of each nodule compared to the empty space of the image where there were no calcified nodules, a percentage of area covered by calcified nodules was determined by ImageJ. This quantitative data was then compared between the 7-Day control and calcified groups in addition to the 14-Day control and calcified groups. This data will show if calcification was induced and if there is any increase in calcification over time.

Immunohistochemistry

Cells were cultured and expanded to passage 7 before being seeded in 4-well chamber slides in control and calcified cellular groups. Cells were then fed their respective medias for 7- and 14-Day timepoints. After their respective time periods, cells were rinsed with PBS and fixed with 400 µLs of 4% Paraformaldehyde for 20 minutes at room temperature. Following fixation, cells were rinsed with PBS and 400 µLs of wash buffer composed of 0.1% bovine serum albumin (BSA) in 1X PBS. Cells were then blocked using a blocking and permeabilization buffer, composed of 10% normal donkey serum and 0.3% Triton X-100 in 1X PBS, for 45 minutes at room temperature. Following blocking, 400 µLs of primary antibodies were added to each of their respective wells for 3 hours at room temperature. The primary antibodies used for staining were α-smooth muscle actin (R&D #MAB1420) and Axin (NOVUS Biological #NBP2-61695). After incubation, primary antibodies were removed, and cells were washed with the wash buffer. Secondary antibodies were then added at 400 µLs per well for 1 hour and incubated at room temperature in the dark. The secondary antibody used for staining was the (Invitrogen #T6390) antibody chosen because its excitation and emission wavelengths were specific to the Texas Red cube in the light microscope used for imaging. After cells were incubated with the final antibodies, secondary antibodies were removed, and the cells were washed with 400 µLs of wash

buffer. To image nuclei of the cells, 300 µLs of DAPI was added to each well and cells were incubated for 2-5 minutes. A final rinse with PBS was performed after incubation and cells were imaged using Brightfield, Texas Red, and DAPI fluorescence microscopy.

Following imaging, ImageJ software was used to identify the percentage of area of each Texas Red image covered in stain for α-smooth muscle actin and Axin. To find this quantitative data, images were opened in ImageJ software and the Color Threshold was set to highlight the area of staining and all background noise was removed from the image. Next, the image was made binary for analysis. The stained area in this binary image was analyzed to determine the percentage of area of the image covered in stain. This quantitative data was then compared between the 7-Day control and calcified groups in addition to the 14-Day control and calcified groups. This data will show if there is a reduction or increase in expression of α-smooth muscle actin and Axin. From this quantification, statistical analysis can be performed to determine if there is any statistical significance in this pattern of staining between images.

ELISA

Cells were cultured and expanded to passage 5-6 before being seeded in 6-well plates in control and calcified cellular groups. Cells were then fed their respective medias for 7 days. After 7 days, cells were rinsed with PBS and solubilized using 1X Cell Extraction Buffer PTR from α-smooth muscle actin (abcam 240678) and β-Catenin (abcam 275100) ELISA kits. The cells were then scraped into microcentrifuge tubes and incubated on ice for 15 minutes. Following incubation, cells were centrifuged at 18,000 xg for 20 minutes at 4˚C and then the supernatant was collected into new microcentrifuge tubes and stored at -80˚C before performing ELISA analysis.

At the time of analysis, ELISA samples were thawed on ice before being used for analysis. Using the protocol provided for each kit, all samples and standards were prepared and added to the microplate strips. After addition of the antibody cocktail to each filled well, the plate was sealed and incubated on a plate shaker at 400 rpm for 1 hour. After 1 hour, wells were washed three times with 300 μL of 1X Wash Buffer PT and wash buffer was left on the cells for 10 seconds. After washing, 100 μL of TMB Development Solution was added to each well and plates were incubated for 10 minutes in the dark on a plate shaker at 400 rpm. After 10 minutes, 100 μL of Stop Solution was added to each well and the plate was placed on a plate shaker for 1 minute. After 1 minute, protein was quantified in a plate reader at 450 nm. Protein measurements for α-smooth muscle actin and β-Catenin were taken and compared between control and calcified groups.

Statistical Analysis

Statistical significance was calculated using one-way ANOVA and two-way ANOVA analysis with a significance level of $\alpha=0.05$.

Results

First, a viable calcification study had to be performed to ensure that all current calcification protocols were able to successfully cause calcification to be induced in HASMCs. Following calcification of cells using 3mM Sodium Dibasic Anhydrous Phosphate, cells were harvested and processed via a Calcium Colorimetric Kit to quantify calcium. This calcium content was then normalized to protein levels indicated by samples processed via a BCA Protein Kit. *Figure 2.1* shows that there was a statistically nonsignificant increase in calcium content between control cells compared to 7-Day calcified cells. It also shows that there was a statistically significant

increase in calcium content between control cells compared to 14-Day calcified cells and that between calcification groups, there was a statistically significant increase in calcium content. This shows that calcification increases in a time dependent manner.

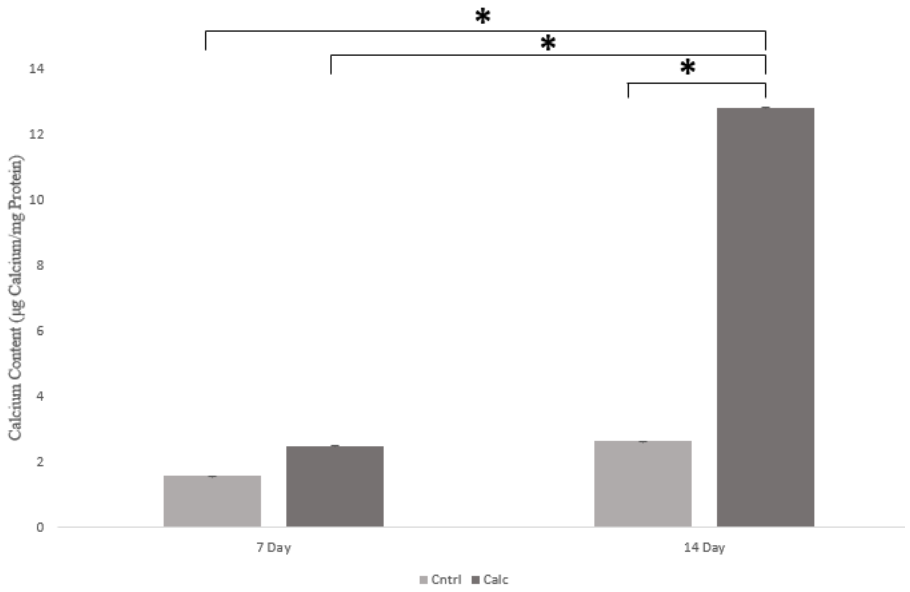

Figure 2.1 The Addition of Inorganic Phosphate Caused a Statistically Nonsignificant Increase in Calcium Content in 7-Day Calcified Cells (α=0.987) Compared to Control Cells. However, there was a Statistically Significant Increase in Calcium Content in 14-Day Calcified Cells (α=0.002) Compared to Control Cells. There was also a Statistically Significant Increase in Calcium Content in 7-Day Calcified Cells Compared to 14-Day Calcified Cells (α=0.002) and Between 7-Day Control Cells and 14-Day Calcified Cells (α<0.001).

Statistical significance was determined using two-way ANOVA analysis at significance level, $p<0.05$ (n=6). Statistically significant values denoted by *. Error bars represent standard deviation.

To visualize calcium content over time and quantitatively determine if there was a time dependent increase in calcification via another quantification method, a xylenol orange study was performed. Cells were seeded and treated with 3mM calcification media for 7 days and 14

days before calcium content was visualized. *Figure 2.2* shows the visualization of calcium content in healthy HASMCs, and *Figure 2.2 (2)* shows that there is no mineralization in control cells that did not receive calcification media. However, in the 7-Day group of cells that were calcified, as shown in *Figure 2.3*, became extremely mineralized throughout the group of cells.

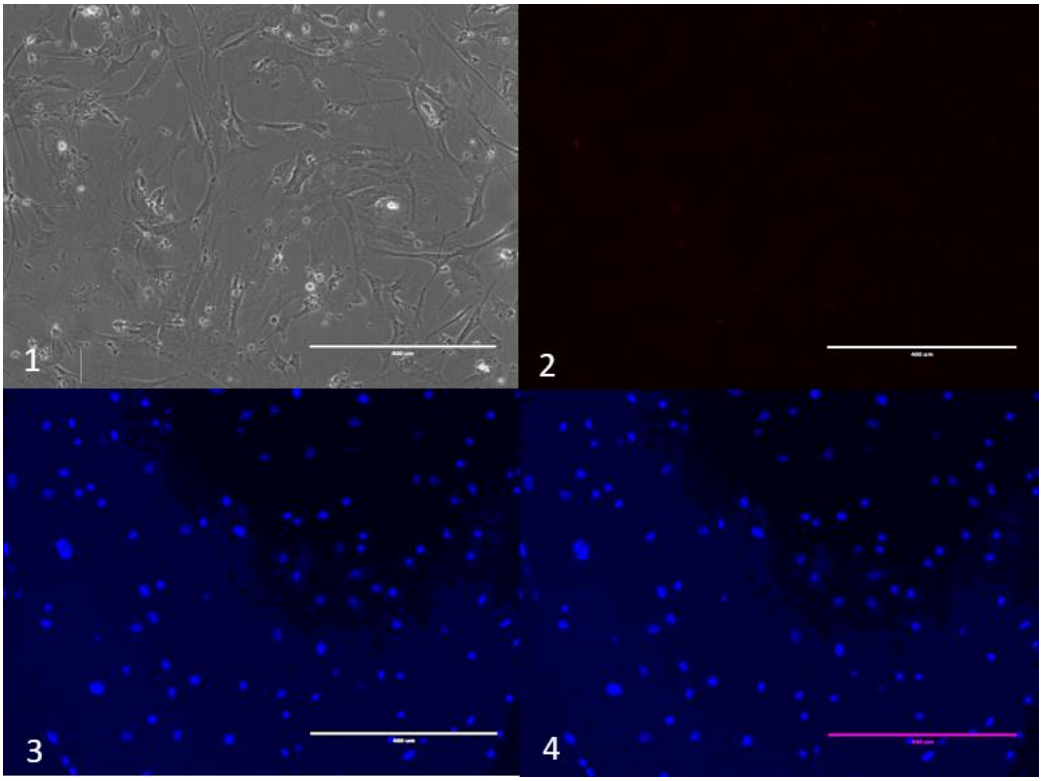

Figure 2.2 Visualization of 7-Day Calcium Content using Xylenol Orange Staining in Control Cells. Control Cells Showed a Lack of Calcium Content as Indicated by Texas Red Imaging.

(1) Light microscopy image. (2) Visualization of calcium content using Texas Red imaging of xylenol orange staining. (3) DAPI staining of nuclei. (4) Merging of Texas Red and DAPI staining.

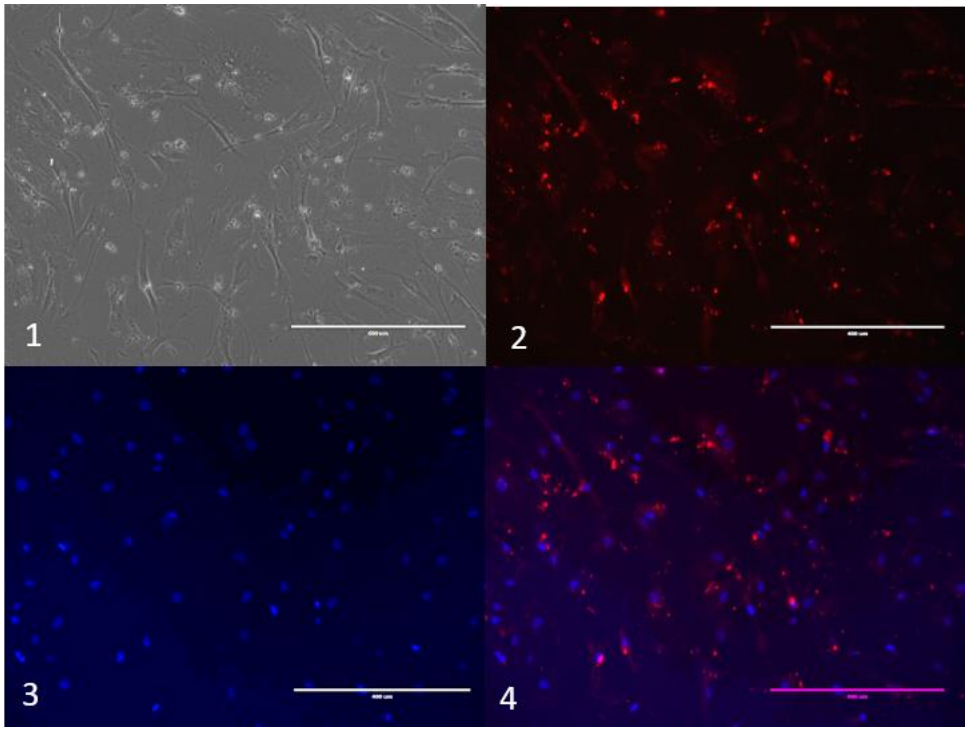

Figure 2.3 Visualization of 7-Day Calcium Content using Xylenol Orange Stain in Calcified Cells Following Addition of Inorganic Phosphate. Calcium Content was Noted to Increase Visually in Texas Red Imaging Compared to Control Cells.

(1) Light microscopy image. (2) Visualization of calcium content using Texas Red imaging of xylenol orange staining. (3) DAPI staining of nuclei. (4) Merging of Texas Red and DAPI staining.

As an extension of this study, another group of cells were calcified for 14 days and then stained with xylenol orange to study if there was an increase in visual mineralization over time. In *Figure 2.4,* cells that were not calcified continued to show no mineralization under Texas Red imaging, as seen in *Figure 2.4 (2),* as with cells from the 7-Day group in *Figure 2.2*. Cells that were calcified for 14 days, as seen in *Figure 2.5,* had an increased amount of mineralization compared with that of the 7-Day group. There were also an increased number of larger deposits of mineralization in the 14-Day group compared to the 7-Day group.

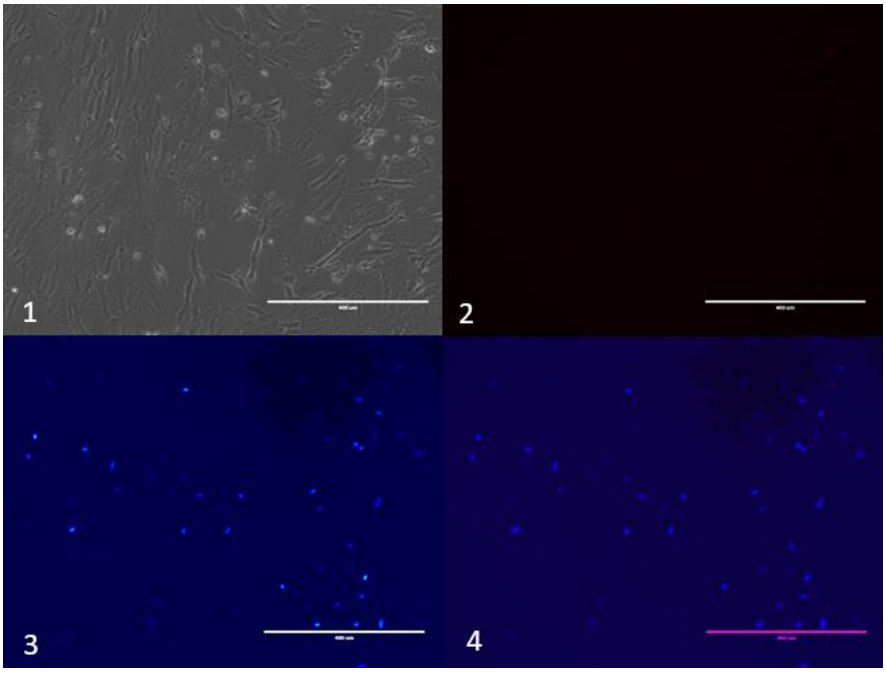

Figure 2.4 Visualization of 14-Day Calcium Content using Xylenol Orange Staining in Control Cells. Control Cells Showed a Lack of Calcium Content as Indicated by Texas Red Imaging.

(1) Light microscopy image. (2) Visualization of calcium content using Texas Red imaging of xylenol orange staining. (3) DAPI staining of nuclei. (4) Merging of Texas Red and DAPI staining.

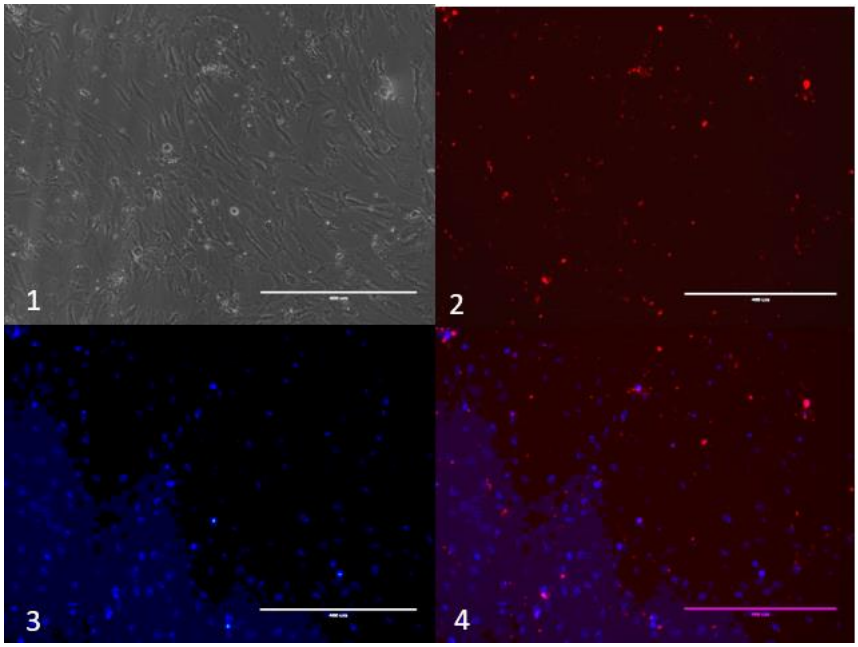

Figure 2.5 Visualization of 14-Day Calcium Content using Xylenol Orange Staining in Calcified Cells Following Addition of Inorganic Phosphate. Calcium Content was Noted to Increase Visually in Texas Red Imaging Compared to Control Cells and 7-Day Calcified Cells.

(1) Light microscopy image. (2) Visualization of calcium content using Texas Red imaging of xylenol orange staining. (3) DAPI staining of nuclei. (4) Merging of Texas Red and DAPI staining.

Following staining analysis, images were analyzed using ImageJ software to determine the percentage of area covered with calcified nodules following 7 days and 14 days of calcification compared to control groups at both timepoints. Using ImageJ software, the Texas Red images for control cells (n=2), *Figure 2.2(2)*, and 7-Day calcified cells (n=2), *Figure 2.3(2)*, were analyzed in addition to images of other wells not included here to determine the percentage of area of each image covered in calcified nodules. It was found that there was an increase in the percentage of area of the image covered with calcified nodules following 7 days of calcification compared to control cells as shown in *Figure 2.6.* The Texas Red images for control cells (n=2), *Figure*

2.4(2), and 14-Day calcified cells (n=2), *Figure 2.5(2),* were analyzed in addition to images of other wells not included here to determine the percentage of area of each image covered in calcified nodules. As shown in *Figure 2.6,* there was an increase in the percentage of area of the image covered in calcified nodules following 14 days of calcification compared to the control cells. However, following statistical analysis, it was determined that this increase was not statistically significant between the control vs 7-Day calcified cells (α=0.327), control vs 14-Day calcified cells (α=0.129), and 7-Day calcified cells vs 14-Day calcified cells (α=0.215). Two-Way ANOVA statistical analysis confirmed that there was no effect of time on the %Area covered in calcified nodules and that there were no significant differences among groups.

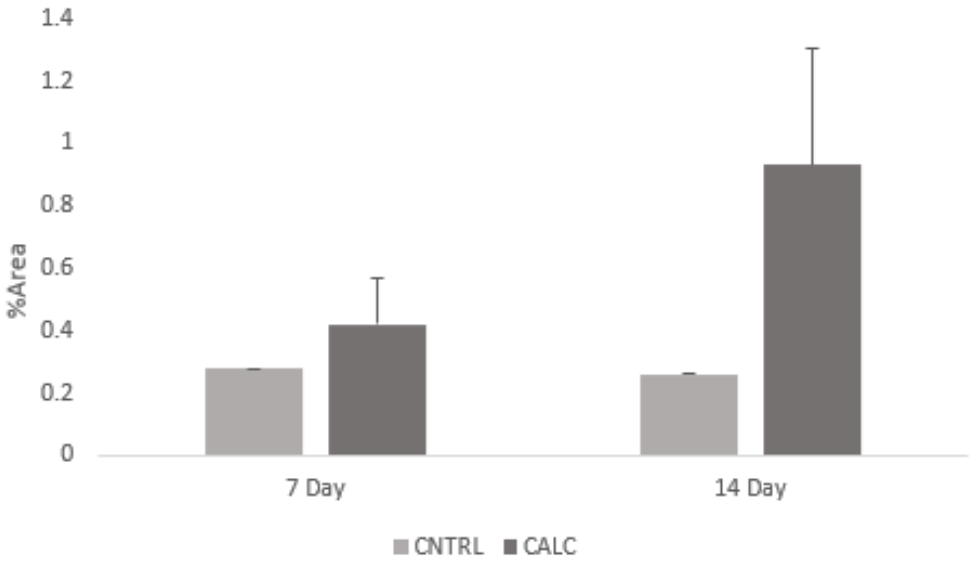

Figure 2.6 Percentage of Area Covered with Calcified Nodules Following 7- and 14-Day Calcification Time Periods Showed Nonsignificant Differences Compared to Control Cells.

Statistical significance was determined using two-way ANOVA analysis at significance level, $p<0.05$ (n=4). Error bars represent standard deviation.

In addition to using ImageJ software to analyze the percentage of area covered in calcified nodules, images were also analyzed to determine the number of nodules in each image captured of the cells. *Figure 2.7* shows the increase in calcified nodules between control and calcified groups over both 7-Day and 14-Day time periods. However, following statistical analysis, it was determined that this increase was also not statistically significant between the control vs 7-Day calcified cells (α=0.242), control vs 14-Day calcified cells (α=0.127), and 7-Day calcified cells vs 14-Day calcified cells (α=0.254).

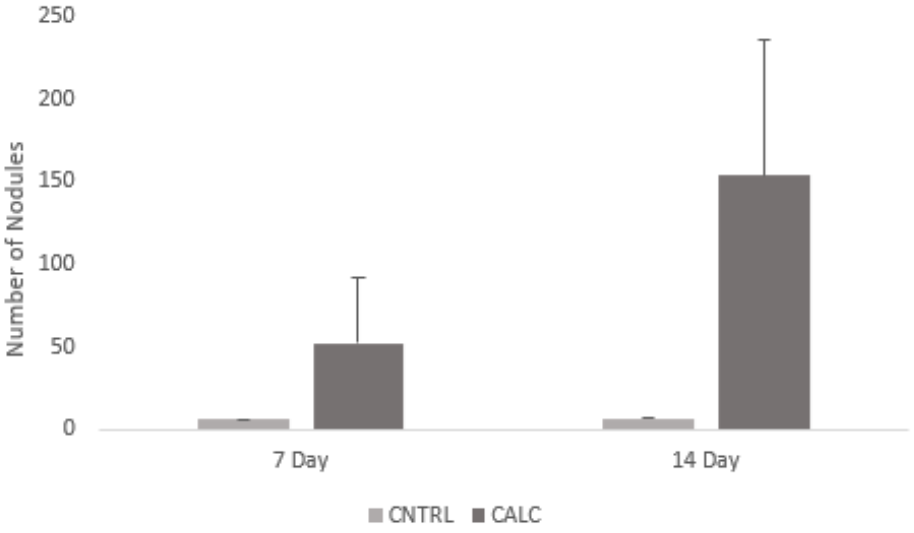

Figure 2.7 Number of Calcified Nodules Increased Following 7- and 14-Day Calcification Time Periods. However, these Differences were Nonsignificant Compared to Control Cells.

Statistical significance was determined using two-way ANOVA analysis at significance level, $p<0.05$ (n=4). Error bars represent standard deviation.

After confirming calcification was induced within HASMCs, WNT signaling markers were observed through immunohistochemical staining and quantified via ELISA kits. For immunohistochemistry, cells were seeded and treated with calcification media for 7 days and 14

days. After their respective time periods, cells were stained for α-smooth muscle actin and Axin and staining differences between control and calcification groups (7 and 14 days) were compared. Following staining, ImageJ analysis was performed to quantify the percentage of area covered in stain for control cells, 7-Day calcified cells, and 14-Day calcified cells. In *Figure 2.8 (2)*, it can be noted that in control cells, there is intensive staining of α-smooth muscle actin, and it can be said that in control cells, there is a high concentration of α-smooth muscle actin present. When in comparison with *Figure 2.9 (2)*, it can be noted that there is less intensive staining and overall, a lower concentration of α-smooth muscle actin present in cells calcified for 7 days. This depicts an image of WNT signaling, noted prior, that upon the initiation of calcification, cells begin to lose their given phenotype and this loss of α-smooth muscle actin is evidence of that phenotypic switch.

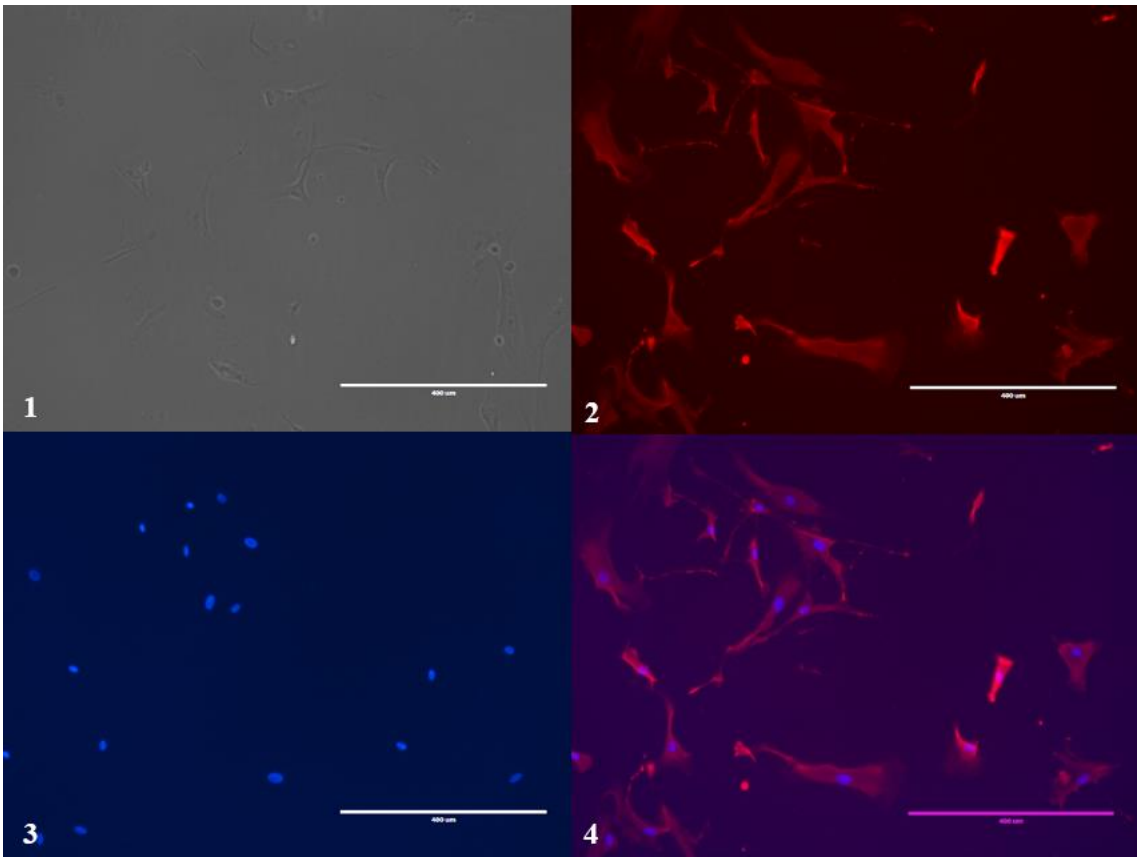

Figure 2.8 Visualization of α-smooth muscle actin in Control Cells. Control Cells Highly Expressed α-smooth muscle actin When Supplemented with Regular DMEM.

(1) Light microscopy image. (2) Visualization of α-smooth muscle actin using Texas Red imaging. (3) DAPI staining of nuclei. (4) Merging of Texas Red and DAPI staining.

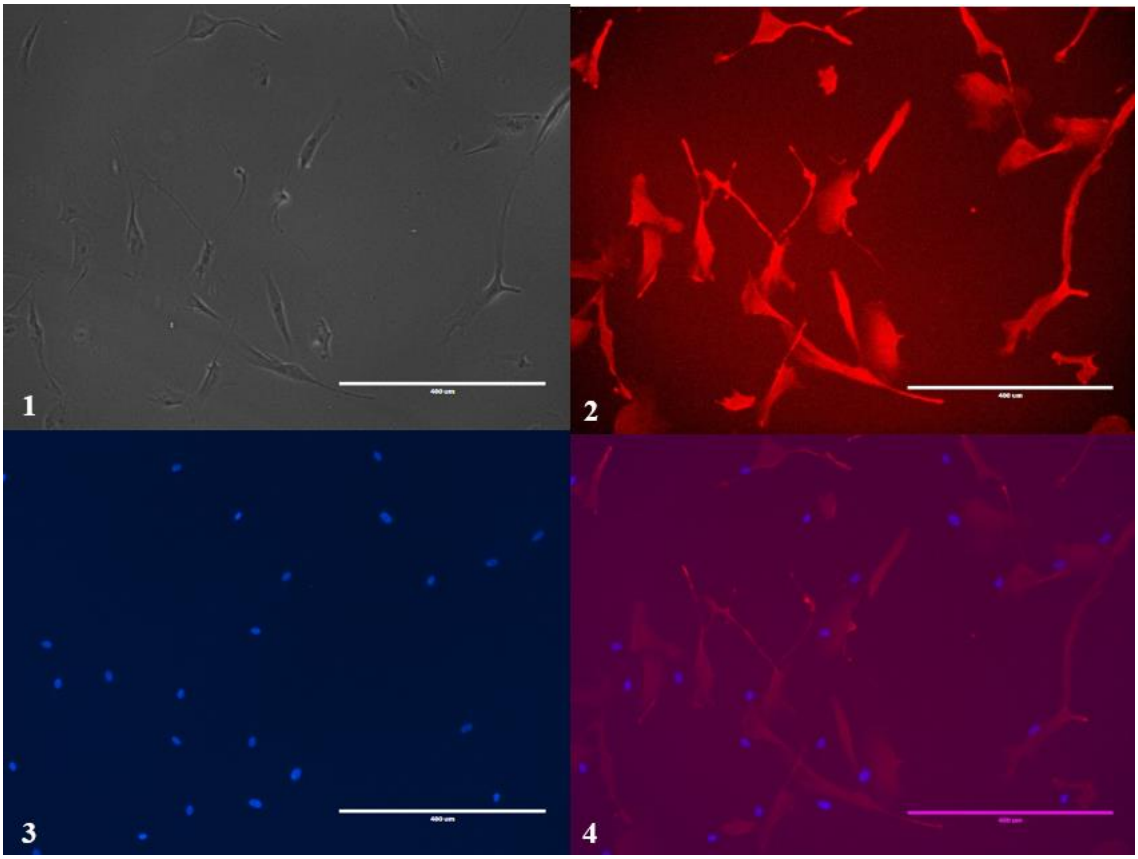

Figure 2.9 Visualization of α-smooth muscle actin in 7-Day Calcified Cells Following Addition of Inorganic Phosphate. Compared to Control Cells, There Appears to be a Visual Reduction in α-smooth muscle actin.

(1) Light microscopy image. (2) Visualization of α-smooth muscle actin using Texas Red imaging. (3) DAPI staining of nuclei. (4) Merging of Texas Red and DAPI staining.

Furthermore, cells were stained for α-smooth muscle actin after 14 days of calcification and the amount of α-smooth muscle actin present was observed. In *Figure 2.10 (2)*, it can be observed that there is less intense staining and overall, a lower concentration of α-smooth muscle actin present in cells calcified with 14 days as compared with control cells and 7-Day calcified cells. This shows that further calcification over time can decrease the presence of the contractile phenotype common to healthy VSMCs.

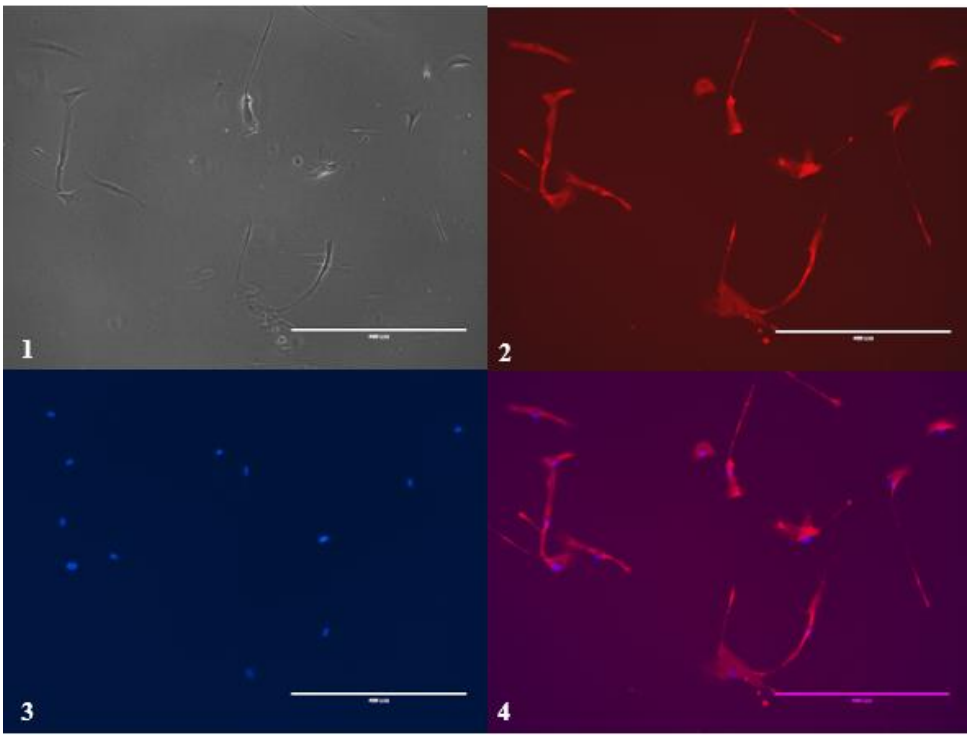

Figure 2.10 Visualization of α-smooth muscle actin in 14-Day Calcified Cells Following Addition of Inorganic Phosphate. Compared to Control Cells and 7-Day Calcified Cells, There Appears to be a Visual Reduction in α-smooth muscle actin.

(1) Light microscopy image. (2) Visualization of α-smooth muscle actin using Texas Red imaging. (3) DAPI staining of nuclei. (4) Merging of Texas Red and DAPI staining.

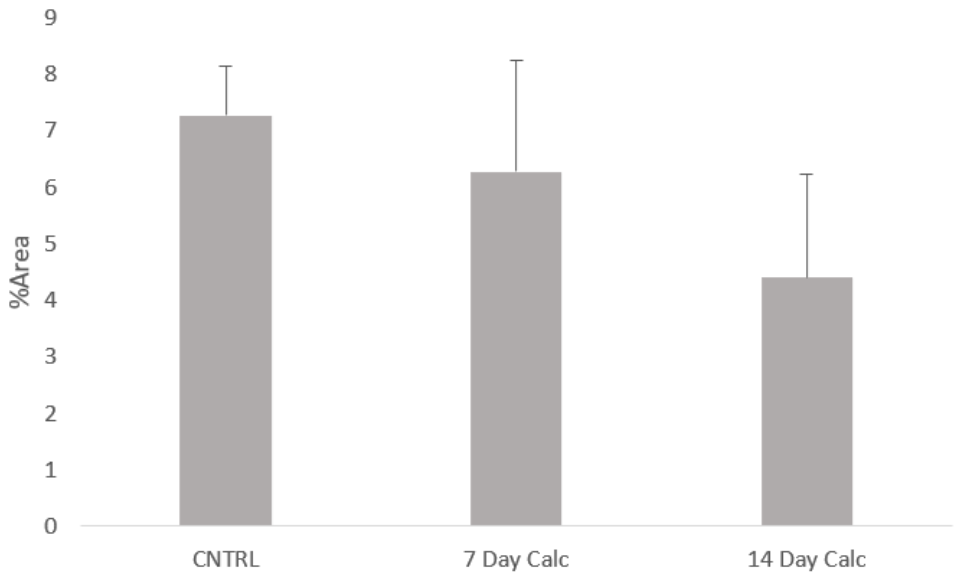

Figure 2.11 Nonsignificant Reduction in α-smooth muscle actin Expression in 7-Day Calcified Cells Compared to Control Cells (α=0.680) and 14-Day Calcified Cells Compared to Control Cells (α=0.080).

Statistical significance was determined using two-way ANOVA analysis at significance level, $p<0.05$ (n=4). Statistically significant values denoted by *. Error bars represent standard deviation.

To confirm if this staining was really decreasing across time periods, ImageJ analysis was performed on *Figure 2.8 (2)*, *Figure 2.9 (2)*, and *Figure 2.10 (2)* in addition to the other three wells stained in a similar manner for each group (not pictured here). *Figure 2.11* shows the nonsignificant reduction in α-smooth muscle actin between control vs 7-Day calcified cells and control vs 14-Day calcified cells. Control cells were harvested and analyzed after 7 days. Between the 7-Day and 14-Day calcified cellular groups, there was also nonsignificant reduction in α-smooth muscle actin expression (α=0.283).

To confirm if the WNT-Signaling pathway was initiated in the induction of calcification, Axin was also stained for as it is a viable component of the destruction complex that keeps β-Catenin levels in check and prevents cells from being calcified. Following staining, ImageJ analysis was performed to quantify the percentage of area covered in stain for control cells, 7-Day calcified cells, and 14-Day calcified cells. In *Figure 2.12 (2)*, control cells were stained for Axin and there is an intense pattern of staining and elevated concentration of Axin present. However, in *Figure 2.13 (2)*, cells that were calcified for 7 days displayed far less intense staining and a decreased concentration of Axin.

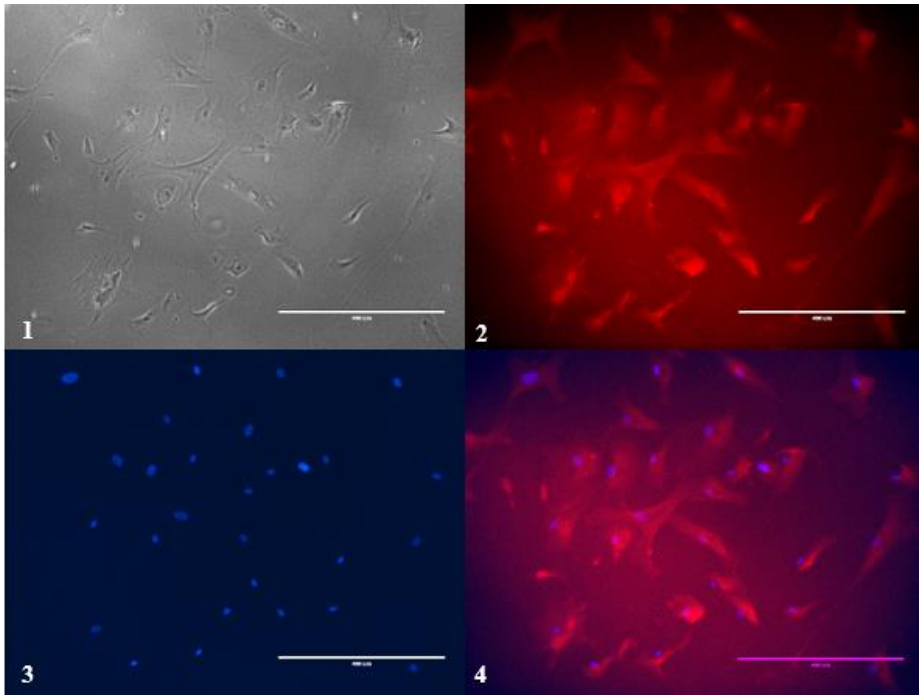

Figure 2.12 Visualization of Axin in Control Cells Following 7 Days of Supplementation. Control Cells Highly Expression Axin When Supplemented with Regular DMEM.

(1) Light microscopy image. (2) Visualization of α-smooth muscle actin using Texas Red imaging. (3) DAPI staining of nuclei. (4) Merging of Texas Red and DAPI staining.

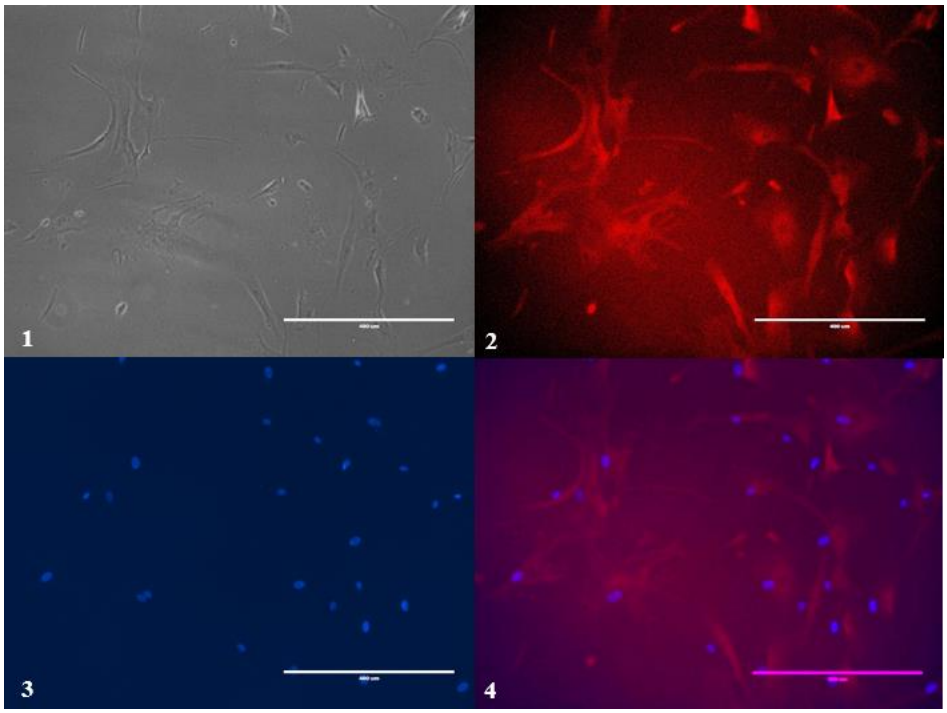

Figure 2.13 Visualization of Axin in 7-Day Calcified Cells Following Addition of Inorganic Phosphate. The 7-Day Calcified Cells Appear to Express Less Axin Compared to Control Cells.

(1) Light microscopy image. (2) Visualization of α-smooth muscle actin using Texas Red imaging. (3) DAPI staining of nuclei. (4) Merging of Texas Red and DAPI staining.

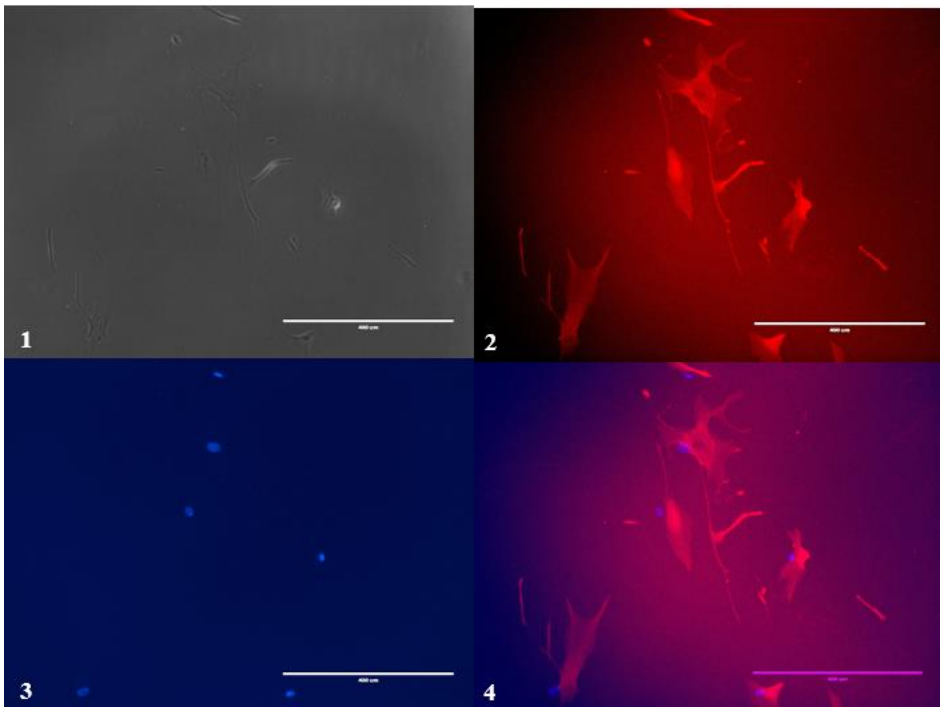

Figure 2.14 Visualization of Axin in 14-Day Calcified Cells Following Addition of Inorganic Phosphate. The 14-Day Calcified Cells Appear to Express Less Axin Compared to Control Cells and 7-Day Calcified Cells.

(1) Light microscopy image. (2) Visualization of α-smooth muscle actin using Texas Red imaging. (3) DAPI staining of nuclei. (4) Merging of Texas Red and DAPI staining.

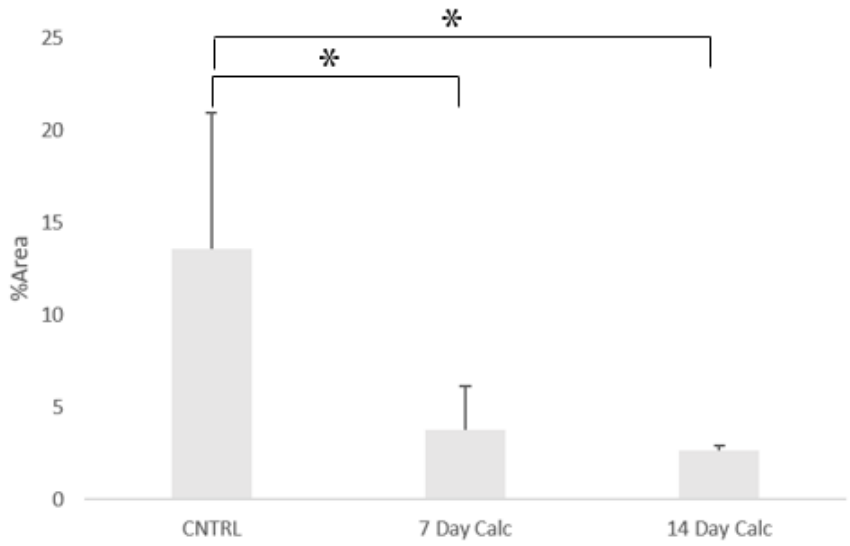

Figure 2.15 Axin Expression Showed a Significant Reduction in 7-Day Calcified Cells Compared to Control Cells (α=0.032). Further Addition of Inorganic Phosphate Caused an Additional Reduction in Axin Expression in 14-Day Calcified Cells Compared to Control Cells (α=0.018).

Statistical significance was determined using two-way ANOVA analysis at significance level, $p<0.05$ (n=4). Statistically significant values denoted by *. Error bars represent standard deviation.

This staining and concentration of Axin can be shown to further decrease in cells calcified for 14 days in *Figure 2.14 (2)*. This indicates that, as expected, cells have Axin in lower concentrations when WNT-Signaling is induced and calcification begins. Over time, this concentration of Axin continues to decrease as the destruction complex is no longer actively inhibiting the release of β-Catenin into the cytosol. To confirm if this staining was really decreasing across time periods, ImageJ analysis was performed on *Figure 2.12 (2)*, *Figure 2.13 (2),* and *Figure 2.14 (2)* in addition to the other three wells stained in a similar manner for each group (not pictured here). *Figure 2.15* shows the statistically significant reduction in Axin between control vs 7-Day calcified cells and between the control vs 14-Day calcified cells. Control cells were

supplemented with regular DMEM for 7 days before staining. However, there was a nonsignificant reduction in Axin expression between the 7-Day and 14-Day calcified cells (α=0.932).

To confirm if markers of WNT-Signaling could be quantified to show the variability in their presence over time between control and calcified groups, ELISA kits were used to quantify markers α-smooth muscle actin and β-Catenin. First, a study was performed to look at their concentrations and the variability between a control cellular group and a 7-Day calcified group with a 3mM concentration of Sodium Dibasic Anhydrous Phosphate. In *Figure 2.16*, it was found that there was a statistically significant decrease in the presence of α-smooth muscle actin in the calcified cellular group after 7 days of calcification compared to the control group.

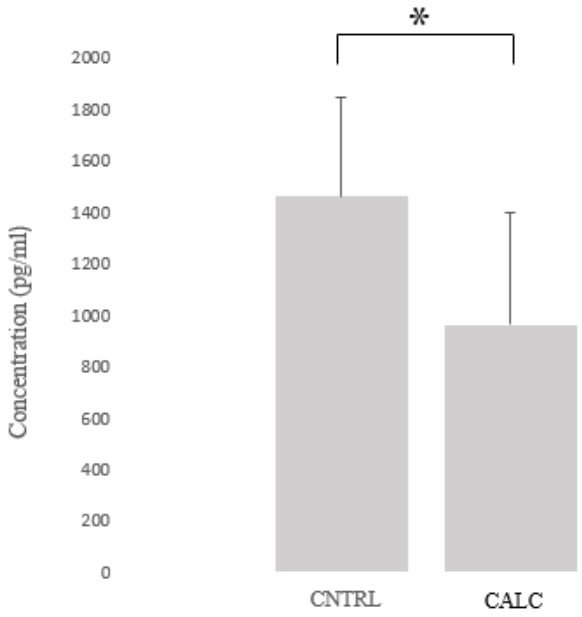

Figure 2.16 α-smooth muscle actin Expression Showed a Statistically Significant Reduction in 7-Day Calcified Cellular Groups Compared to Control Groups Using ELISA Quantification Following Addition of Inorganic Phosphate for 7 Days (α=0.021).

Statistical significance was determined using one-way ANOVA analysis at significance level, $p<0.05$ (n=6). Statistically significant values denoted by *. Error bars represent standard deviation.

In *Figure 2.17*, it was found that there was not a statistically significant difference in expression of β-Catenin in the calcified cellular group after 7 days of calcification compared to the control group.

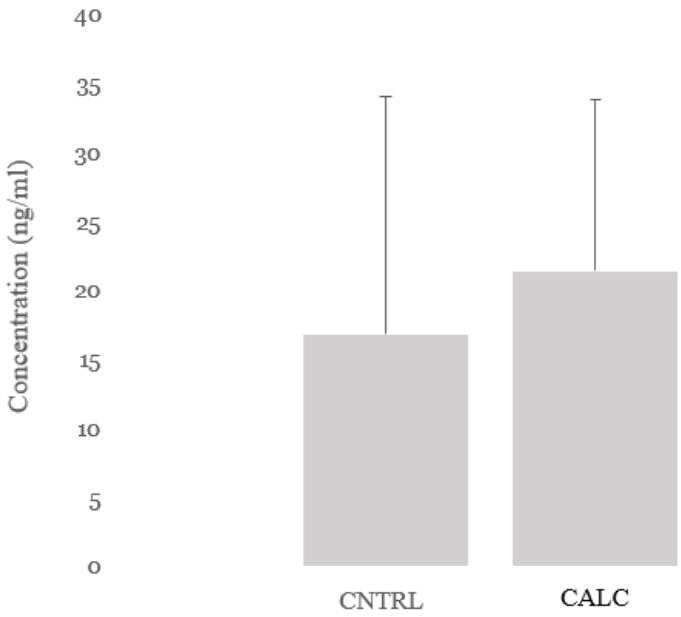

Figure 2.17 β-Catenin Expression Showed Nonsignificant Differences in Calcified and Control Cellular Groups Using ELISA Quantification Following Addition of Inorganic Phosphate for 7 Days (α=0.529).

Statistical significance was determined using one-way ANOVA analysis at significance level, $p<0.05$ (n=6). Error bars represent standard deviation.

Following this study, a full time-course study was performed to study the expression levels of β-Catenin between control and calcified groups at 1-Day, 3-Day, 7-Day, 10-Day, and 14-Day time periods.

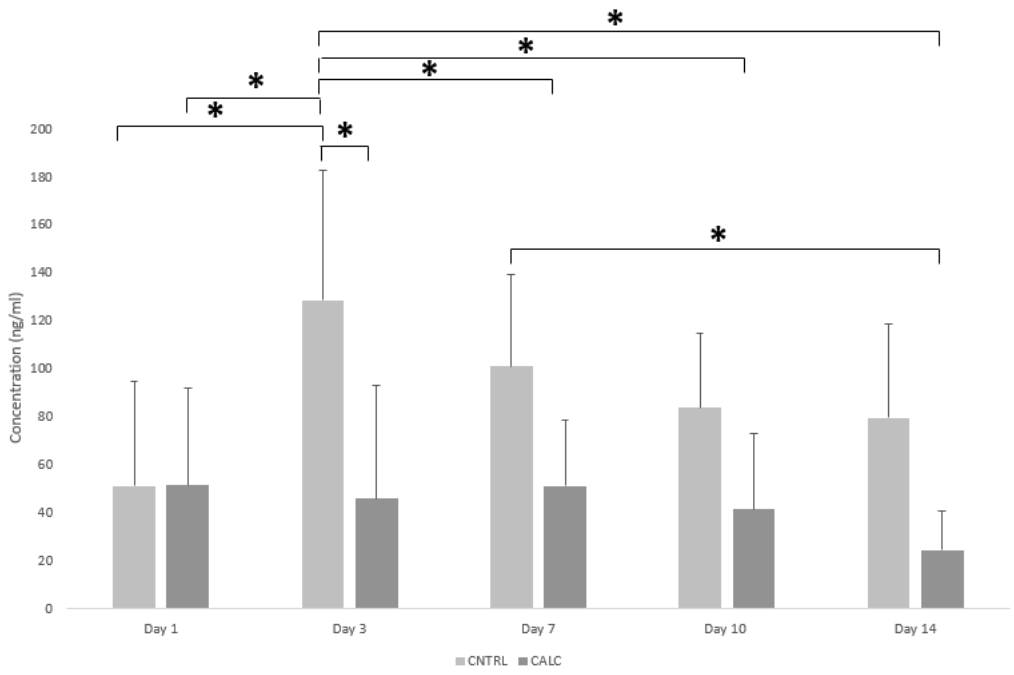

Figure 2.18 β-Catenin Expression Showed a Statistically Significant Reduction in Calcified Cells Supplemented with Inorganic Phosphate Compared to Control Cells Following a 3-Day Time Period. There were Nonsignificant Differences in β-Catenin Expression in Calcified Cells Compared to Control Cells Following a 1-Day, 7-Day, 10-Day, and 14-Day Time Period.

Statistical significance was determined using two-way ANOVA analysis at significance level, $p<0.05$ (n=3). Statistically significant values denoted by *. Error bars represent standard deviation.

As shown in *Figure 2.18*, there were nonsignificant differences ($\alpha=1.000$) in β-Catenin levels following ELISA quantification after 1 day of calcification compared to control cellular groups, in 7-Day calcified cells compared to control cells ($\alpha=0.434$), 10-Day calcified cells compared to control cells ($\alpha=0.657$), and 14-Day calcified cells compared to control cells ($\alpha=0.293$). However, there was a statistically significant reduction in β-Catenin expression in the 3-Day calcified cells compared to control cells ($\alpha=0.016$). There were nonsignificant differences between each control group timepoint and calcified group timepoint apart from the 1-Day control

group and 3-Day control group (α=0.030) and 1-Day calcified group and 3-Day control group (α=0.032) showing a significant increase in β-Catenin expression. There was also a significant decrease in β-Catenin expression shown between the 3-Day control group and 7-Day calcified group (α=0.030), 3-Day control group and 10-Day calcified group (α=0.009), 3-Day control group and 14-Day calcified group (α<0.001), 7-Day control group and 14-Day calcified group (α=0.033).

From these studies, we were able to confirm that calcification could be induced and increased in a time dependent fashion between 7- and 14-Days post-calcification. This calcification was studied in both a quantitative and qualitative manner. Quantitatively, a Calcium Colorimetric Kit to quantify calcium that was normalized to protein levels measured using a BCA Protein Kit. In addition, calcium deposition was studied using Xylenol Orange staining and the images later quantitatively analyzed using ImageJ software to study the percentage of area covered in calcified nodules and the number of calcified nodules present at each respective time period. From these data, we were able to see an increase in calcification over time with statistical significance to the increase using the Calcium Colorimetric Kit. In our analysis of markers of VSMC phenotypic switching and WNT-Signaling, it was determined through immunohistochemistry, coupled with ImageJ software analysis, and ELISA quantification that α-smooth muscle actin expression decreased over time in the presence of calcification. Immunohistochemistry, coupled with ImageJ software analysis, also showed a decrease in Axin-1 expression over time possibly indicating the initiation of the WNT-Signaling pathway. From there, β-Catenin analysis was performed using an ELISA kit which showed in a preliminary study, a nonsignificant increase in calcified cellular groups. However, a further time course study

indicated that following 3 days, 7 days, 10 days, and 14 days of calcification, β-Catenin was reduced in the presence of calcification.

CHAPTER III

AIM II: CONFIRM WNT SIGNALING AND STUDY A TARGET OF THE PATHWAY AS A NOVEL THERAPY FOR VASCULAR CALCIFICATION

Introduction

Another method by which to determine this optimal target for the inhibition of medial vascular calcification is the use of a dual-luciferase assay system. In recent years, the luciferase assay has become more widely used because of its cost and time efficiency. The luciferase assay is a bioluminescent assay that can be useful in studying how a protein regulates a gene of interest[32]. By applying this concept, a dual-luciferase assay system can be used to study the LEF/TCF complex of the canonical WNT-Signaling pathway and its regulation of RUNX2 levels within the nucleus of the cell. Before the use of a dual-luciferase assay system, cells must be transfected with two DNA constructs: one to act as a control and another as an experimental construct. One construct will contain coding for the protein of interest while the other will contain the gene's promoter and the coding region for the luciferase reporter that will be read via the luminometer[32]. Once the cells undergo a period of transfection, they can be analyzed via a luminometer, which measures the amount of luciferase enzyme that when quantified can be used to infer the strength of the promoter equivalent to the strength of the luciferase signal. The study of the canonical WNT-Signaling pathway will be conducted using human aortic smooth muscle cells since smooth muscle cells make up the greater portion of the tunica media. Smooth muscle cells, a primary cell type, are considered difficult to transfect with foreign DNA. However, a previous

study conducted by Zheng, et al. used a luciferase assay to successfully detect transcription factor activity in YAP1 overexpressing 293T cells, smooth muscle cells of the kidneys[29]. Another study by Zhang, et al. used human aortic smooth muscle cells with a luciferase assay system to study cell percentage and the relationship between H19, miR-148b, and WNT1[30]. He, et al. studied abdominal aortic aneurysms with long noncoding RNAs as a specific target to determine specific interactions using human aortic smooth muscle cells[31]. These studies, each conducted less than 5 years previous, show it is possible to successfully transfect smooth muscle cells, specifically human aortic smooth muscle cells, with foreign DNA sequences. A study by Li et al. showed that upon successful transfection of vascular smooth muscle cells with a RUNX2-vector, levels of RUNX2 can be analyzed between cellular subsets[48]. These previous studies conclude that it should be possible to transfect HASMCs with the appropriate Renilla control plasmid and the specific vector that corresponds to the LEF/TCF complex to study if the LEF/TCF complex is a viable target for the treatment of vascular calcification.

Aim II Specific Objectives

The specific objective of this study is to analyze the LEF/TCF complex of the WNT-Signaling pathway and determine if it is a viable target for a novel vascular calcification therapy by confirming that WNT-Signaling is involved in the induction of calcification. This can be confirmed by an upregulation of LEF/TCF complex activity in calcified cellular groups compared to control cellular groups. This will confirm that the WNT-Signaling pathway is directly involved in the initiation and progression of vascular calcification as it is such a late target of the WNT-Signaling pathway in the nuclei of cells. Study of the LEF/TCF complex at 7-Day and 14-Day timepoints will also confirm if there is an uptick in WNT-Signaling activity as calcification progresses or if it is a steady stream of activity that furthers the calcification present

at a steady pace. One unknown within the study is if cells will be able to be transfected following 7-Day and 14-Day calcification periods using inorganic phosphate. A preliminary study will be conducted to determine if the human aortic smooth muscle cells are successfully transfected with both plasmids using two different methods of transfection. A measure of the presence of Renilla will confirm that the cells are successfully transfected.

Experimental Section

Materials/Methods

Cell Culture

HASMCs were cultured to passage 5-7 and 2.0 x10^5 cells were seeded into 96-well plates. First, cells were experimentally transfected with Fugene 6 to determine if it was a viable transfection reagent for HASMCs. Fugene 6 was not a viable transfection agent, so Lipofectamine-3000 was utilized to transfect HASMCs. Once a viable method of transfection was determined, cells were seeded into 96-well plates with both a control and calcified group for 7- and 14-Day timepoints. After their respective 7 or 14 days of supplementation, cells were transfected with their respective plasmids and analyzed. To the knowledge of this author to date, this luciferase reaction has never been performed using HASMCs that have been calcified, so cell culture methods may have to be altered for this novel experiment.

Luciferase Reactions

The specific vector that was used to study the regulation of RUNX2 via the LEF/TCF complex will be pGL4.49[*luc2P*/TCF-LEF RE/Hygro] (Promega #E4611) which contains a LEF/TCF response element upstream of its luciferase reporter gene. pGL4.49 will be referred to as the Luciferase plasmid in the rest of this document. A Renilla plasmid (Promega #E6931) was also

transfected with the Luciferase plasmid to act as a control when luminescence was measured. For transfection to occur, a specified quantity of DNA plasmids and transfection reagent were added to Opti-MEM medium (Gibco #31985-070) in a 1.5-mL micro-centrifuge tube and mixed. Preparation following this mixing of DNA and transfection reagent is dependent upon the reagent used.

To avoid the waste of materials, the first experiment performed was a transfection experiment using the transfection reagent Fugene 6. Fugene 6 was a transfection reagent that an assisting lab had been successful in using for transfection with their cell type. Cells were grown to confluence in a T-75 flask before being seeded into 96-well plates and treated with regular DMEM as control cells in previous experiments received. To act as a control, one column of cells was treated with the transfection reagent, Fugene 6, but no plasmids. A column between samples was left empty to obtain readings that would result from empty wells. Finally, the last column of cells was treated with the transfection reagent, Fugene 6, in addition to the plasmids to see if transfection occurred. For the transfection reagent Fugene 6 (Promega #E2691), the optimal amount of DNA per sample is 0.08-µgs of each plasmid. Both plasmids, Luciferase and Renilla, are diluted in a 1:3 ratio in Fugene 6. From there, the DNA mixture was diluted further into Opti-MEM medium, a reduced-serum medium used to keep mammalian cells stable during the transfection period. This diluted plasmid and Fugene 6 mixture was then added to cells by pipetting 10 µls to each well of cells. In addition, 100 µls of Opti-MEM was added to each well and cells were incubated overnight. The next morning, the mixture was aspirated off cells and replaced with regular media and calcification media, respectively. After allowing cells to return to equilibrium for 5 hours, a dual-luciferase assay (Promega #E1910) was run on cells using the

system's protocol that measured both Renilla and LEF/TCF activity using a luminometer. Following measurements, analysis was done to determine if cells were able to be transfected, indicated by Renilla activity, and if the LEF/TCF complex was activated in the control or calcified cellular groups.

Transfection was unsuccessful using Fugene 6, so Lipofectamine-3000 (Invitrogen #L3000-001) was used to transfect cells using the following method. On the day that calcification ends, Lipoefectamine-3000 was used to transfect both plasmids into the cells. First, Lipofectamine-3000 was diluted in Opti-MEM using the Lipofectamine-3000 protocol which calls for 5 µls of Opti-MEM per well and 0.15 µls of Lipofectamine-3000 per well to be transfected. Based on the number of wells to be transfected, Opti-MEM and Lipofectamine-3000 were combined in a sterile microcentrifuge tube. Next, the master DNA mix was created by diluting the P3000 reagent of the Lipofectamine-3000 system into Opti-MEM. For every well to be transfected, 10 µls of Opti-MEM were added to a sterile microcentrifuge tube. Then 0.08 µgs of each plasmid was multiplied by the number of wells to be transfected and this amount of each plasmid was added to Opti-MEM in a sterile tube. To create the final diluted plasmid mixture for transfection, 5 µls of the diluted DNA mix was multiplied by the number of wells to be transfected and this amount of the master DNA mix was added to a new sterile microcentrifuge tube. Finally, 5 µls of the diluted Lipofectamine-3000 mix was multiplied by the number of wells to be transfected and this amount of the diluted Lipofectamine-3000 was added to the new sterile tube. To each well, 10 µls of this new DNA-lipid complex and 100 µls of each respective media was added to the transfected wells. Two concentrations of Lipofectamine-3000 were tested to determine if at a certain concentration, Lipofectamine-3000 is toxic to the cells. Thus, this process was repeated

where the only variable that changes is the amount of Lipofectamine-3000 diluted in the first Lipofectamine-3000: Opti-MEM mixture. In this new mix, 0.30 μls of Lipofectamine-3000 was diluted per well transfected instead of 0.15 μls.

The next morning, the mixture was aspirated off cells and replaced with regular media and calcification media, respectively. After allowing cells to return to equilibrium for 5 hours, a dual-luciferase assay (Promega #E1910) was run on cells using the system's protocol that measured both Renilla and LEF/TCF activity using a luminometer. Following measurements, analysis was done to determine if cells were able to be transfected, indicated by Renilla activity, and if the LEF/TCF complex response element was activated in the control or calcified cellular groups.

Once a viable transfection reagent was determined, a study comparing control and calcified cellular groups was seeded. For each group, control and calcified, the total number of wells of cells was n=6. However, to control for the possibility that the transfection reagent is causing the readings coinciding with successful transfection, there were n=3 control cells transfected with plasmids and n=3 cells that did not have plasmids transfected but were introduced to the transfection reagent.

Statistical Analysis

Statistical significance was calculated using one-way ANOVA and two-way ANOVA analysis with a significance level of $\alpha=0.05$.

Results

The first major challenge was to achieve successful transfection of HASMCs, a primary cell line. Following experimental transfection with Fugene 6, both Renilla and LEF/TCF activity were negligible (not reported graphically) and did not result in successful transfection due to a luminescence reading less than 500. Thus, cells were seeded into another 96-well plate to study transfection with Fugene 6 in comparison with another transfection reagent, Lipofectamine-3000. From *Figure 3.1*, plasmid transfection was successful in wells where plasmids were transfected with 0.15-µl Lipofectamine-3000. There were significant differences in cells transfected with 0.15-µl Lipofectamine-3000 compared to Fugene 6 (α=0.034) and 0.15-µl Lipofectamine-3000 compared to 0.30-µl Lipofectamine-3000 (α=0.034) because 0.15-µl Lipofectamine-3000 was the only successful transfection agent. There were nonsignificant differences in cells transfected with Fugene 6 compared to 0.30-µl Lipofectamine-3000 (α=1.000) because cells were unsuccessfully transfected with these two agents and therefore had very similar Renilla expression readings. Cells that received a higher concentration (0.30-µl) of Lipofectamine-3000 were not able to be successfully transfected indicating that most likely the transfection reagent was too strong at this concentration and may have been toxic to the cells. In a comparison of Renilla expression between control cells, successful transfection is indicated by smaller fractions of expression because Renilla expression levels exceeded 500 and Luciferase activity (indicative of LEF/TCF activity) was negligible resulting in a smaller fraction of Luciferase/Renilla expression in *Figure 3.2*. The Luciferase plasmid was not activated so the WNT-Signaling pathway was not activated in cells receiving regular DMEM just like control cells in previous calcification experiments.

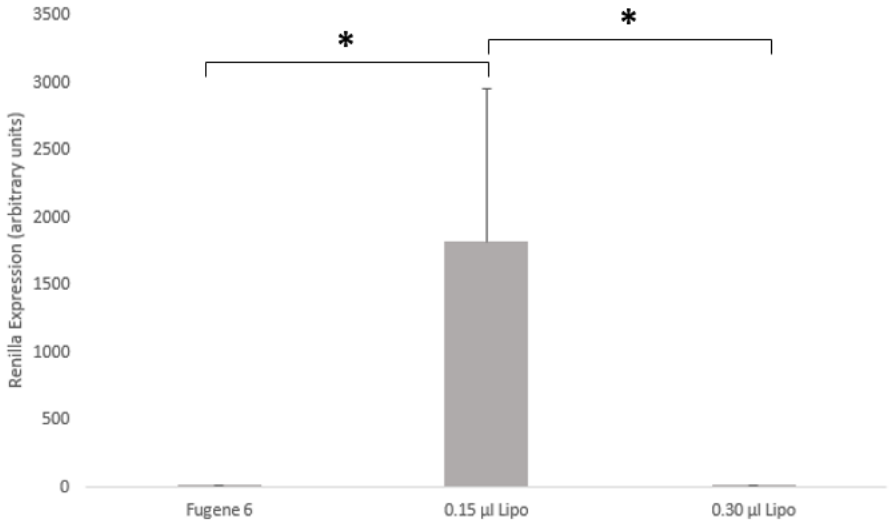

Figure 3.1 Renilla Expression in Fugene 6, 0.15-µl Lipofectamine-3000, and 0.30-µl Lipofectamine-3000 Indicating Successful Transfection Using 0.15-µl Lipofectamine-3000 Only. Successful Transfection Denoted by Renilla Expression Exceeding 500.

Statistical significance was determined using one-way ANOVA analysis at significance level, $p<0.05$ ($n=3$) and denoted by *. Error bars represent standard deviation.

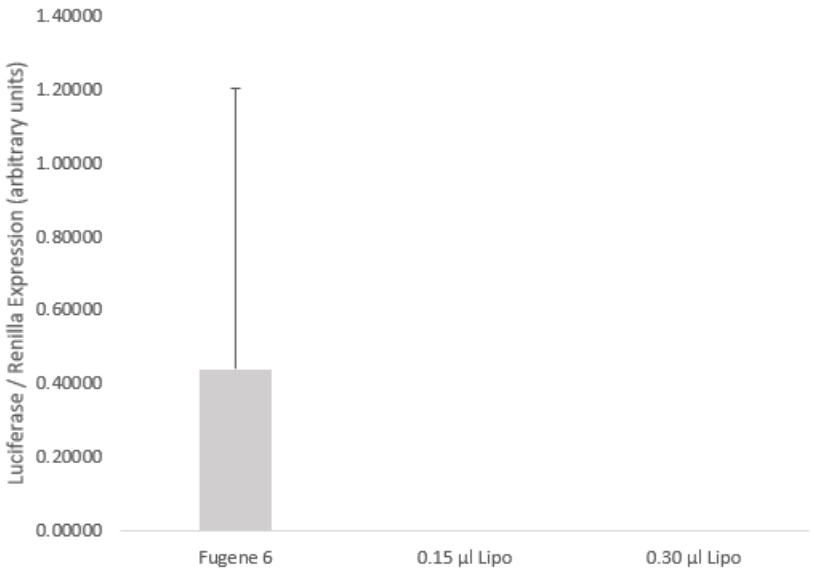

Figure 3.2 Successful Transfection Using 0.15-µl Lipofectamine-3000 Compared to Fugene 6 and 0.30-µl Lipofectamine-3000. There were Nonsignificant Differences in Luciferase Expression Between Cells Transfected with Fugene 6 Compared to 0.15-µl Lipofectamine-3000 (α=0.483), Fugene 6 Compared to 0.30-µl Lipofectamine-3000(α=0.483), and 0.15-µl Lipofectamine-3000 Compared to 0.30-µl Lipofectamine-3000 (α=1.000) Due to a Lack of LEF/TCF Expression in all Cells.

Statistical significance was determined using one-way ANOVA analysis at significance level, $p<0.05$ (n=3). Error bars represent standard deviation.

From this data, a full calcification experiment was begun where one 96-well plate were seeded in control (n=6) and 7-Day calcification groups (n=6). From there, a 7-Day calcification period began where one group received regular DMEM and calcification media for 7 days. Then, transfection was performed using the successful 0.15-µl Lipofectamine-3000 with both the Luciferase and Renilla plasmids in each of the groups calling for plasmid transfection. Half of each group (n=3) was not transfected with plasmids, acting as a negative control, to remove background expression from the Luciferase and Renilla expression levels recorded.

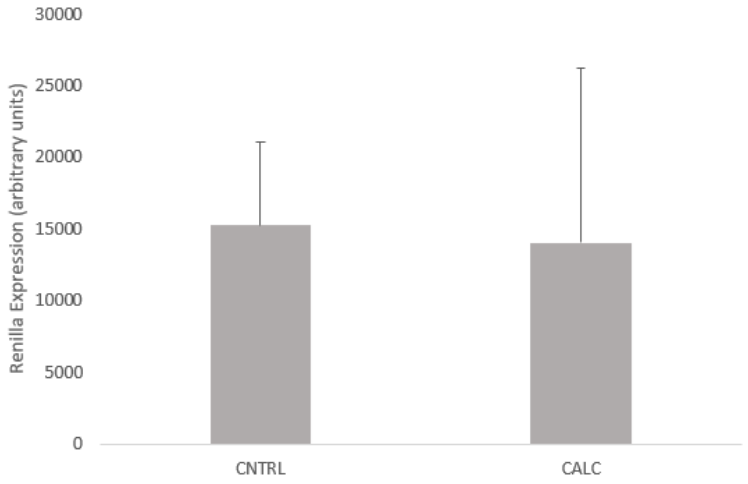

Figure 3.3 7-Day Control and Calcified Cells Renilla Expression Showing Successful Transfection Given Luminescence Readings Greater Than 500. There were Nonsignificant Differences in Renilla Expression Between Control and Calcified Cells (α=0.880).

Statistical significance was determined using one-way ANOVA analysis at significance level, $p<0.05$ (n=3). Error bars represent standard deviation.

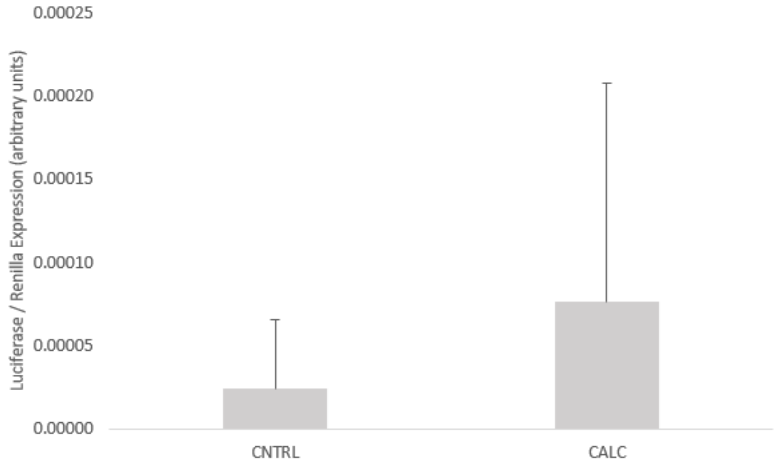

Figure 3.4 Luciferase Expression Showed Nonsignificant Differences in Control and 7-Day Calcified Cellular Groups (α=0.549).

Statistical significance was determined using one-way ANOVA analysis at significance level, $p<0.05$ (n=3). Error bars represent standard deviation.

From *Figure 3.4*, it was found that there were nonsignificant differences in expression between control and 7-Day calcified cellular groups. From raw data shown in *Figure 3.3*, it was found that transfection was successful due to the readings of Renilla plasmid activity. However, even after 7 days of calcification, there was no recorded LEF/TCF activity. This was a step forward in determining that calcified cells could be transfected in addition to control cells. Following successful transfection in both control and calcified groups, another calcification experiment was seeded where two 96-well plates were seeded to compare different time periods of calcification. From there a 7- and 14-Day calcification period began where one group received regular DMEM and calcification media for 7 and 14 days, respectively, at a concentration of 3 mM Sodium Dibasic Anhydrous for calcification media. Then, transfection was performed using the 0.15-µl concentration of Lipofectamine-3000.

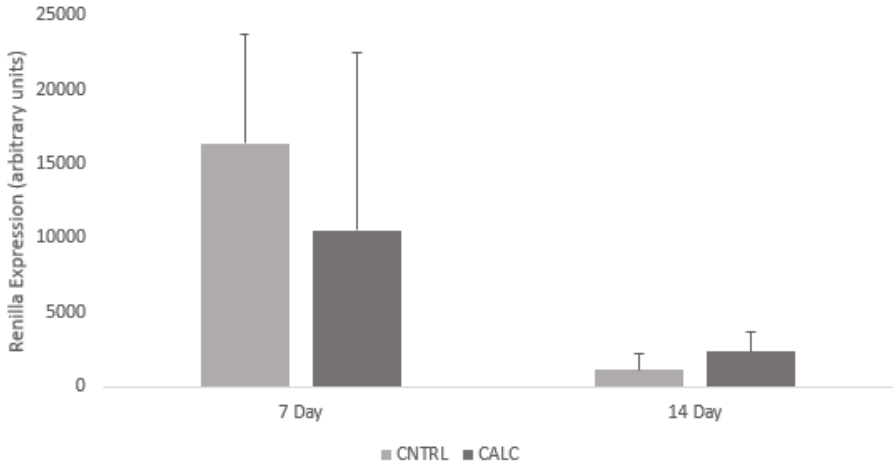

Figure 3.5 Comparison of 7-Day and 14-Day Renilla Expression in Control and Calcified Cells Where Transfection was Successful in All Groups Given Luminescence Readings Over 500. There were Nonsignificant Differences in Renilla Expression Between 7-Day Control Cells and 7-Day Calcified Cells (α=0.749), 14-Day Control Cells and 14-Day Calcified Cells (α=0.996), 7-Day Control Cells and 14-Day Control Cells (α=0.109), and 7-Day Calcified Cells and 14-Day Calcified Cells (α=0.522).

Statistical significance was determined using two-way ANOVA analysis at significance level, $p<0.05$ (n=3). Error bars represent standard deviation.

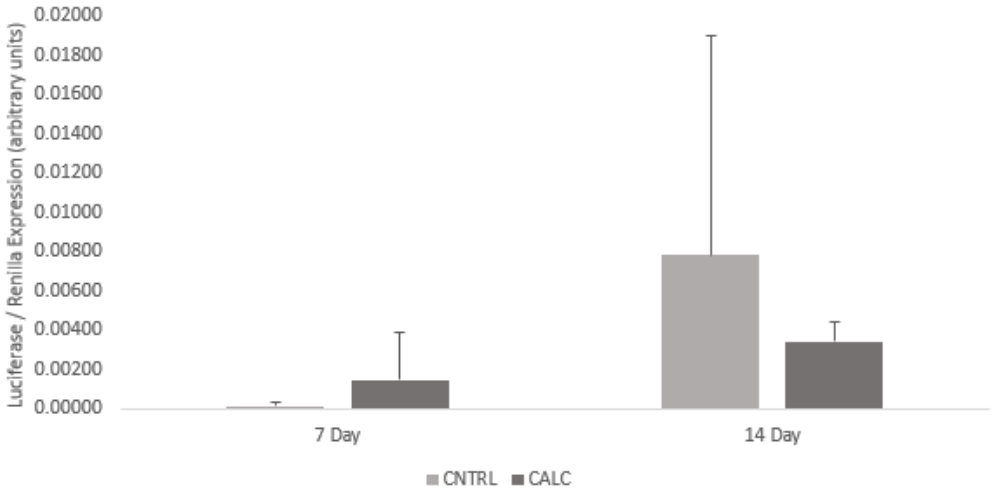

Figure 3.6 When Compared to Control Cells, 7-Day Calcified Cells Showed Nonsignificant Differences in Luciferase Expression Following Addition of Inorganic Phosphate (α=0.991). In 14-Day Calcified Cells, there were also Nonsignificant Differences in Luciferase Expression Compared to Control Cells (α=0.785).

Statistical significance was determined using two-way ANOVA analysis at significance level, $p<0.05$ (n=3). Error bars represent standard deviation.

From *Figure 3.5*, it was found that plasmids were able to be successfully transfected into cells following both 7-Day and 14-Day calcification periods. However, in both instances no LEF/TCF activity could be read using the luminometer. There was also a nonsignificant increase in Luciferase expression from 7-Day control cells vs 14-Day control cells (α=0.416) and 7-Day calcified cells vs 14-Day calcified cells (α=0.976) as shown in *Figure 3.6.* All the previous studies using this luciferase reaction were performed with calcification media at a 3mM concentration of Sodium Dibasic Anhydrous Phosphate. To determine if a higher concentration of Sodium Dibasic Anhydrous Phosphate could mimic a more severely calcified environment, another luciferase study was performed where two calcified groups, one using a calcification media supplemented with 6mM Sodium Dibasic Anhydrous Phosphate and the other

supplemented with 12mM Sodium Dibasic Anhydrous Phosphate, were studied in comparison with the control group after 7 days of treatment.

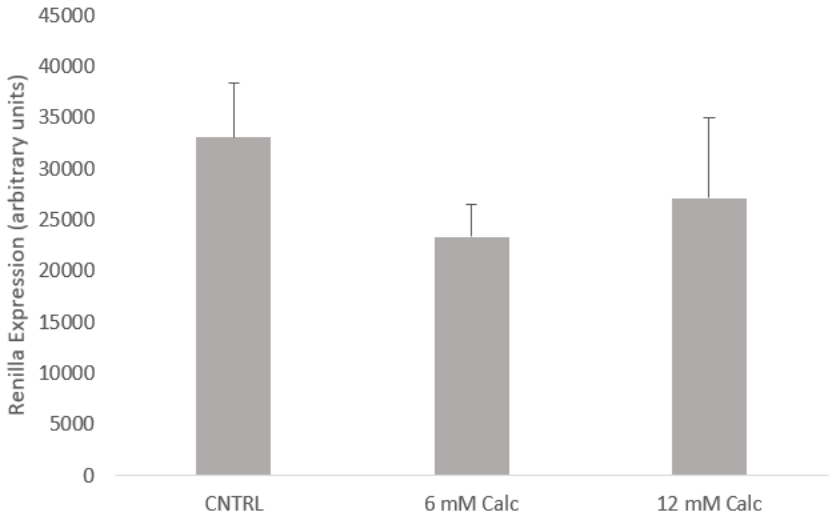

Figure 3.7 Following an Increase in Calcification Media Concentration, Renilla Expression was Compared Across 7-Day Groups to Confirm Transfection was Successful. Luminescence Readings Over 500 Support that Transfection was Successful in Every Group. Nonsignificant Differences in Renilla Expression Between Control Cells and 6mM Calcified Cells (α=0.177), Control Cells and 12mM Calcified Cells (α=0.464), and 6mM Calcified Cells and 12mM Calcified Cells (α=0.714).

Statistical significance was determined using one-way ANOVA analysis at significance level, $p<0.05$ (n=3). Error bars represent standard deviation.

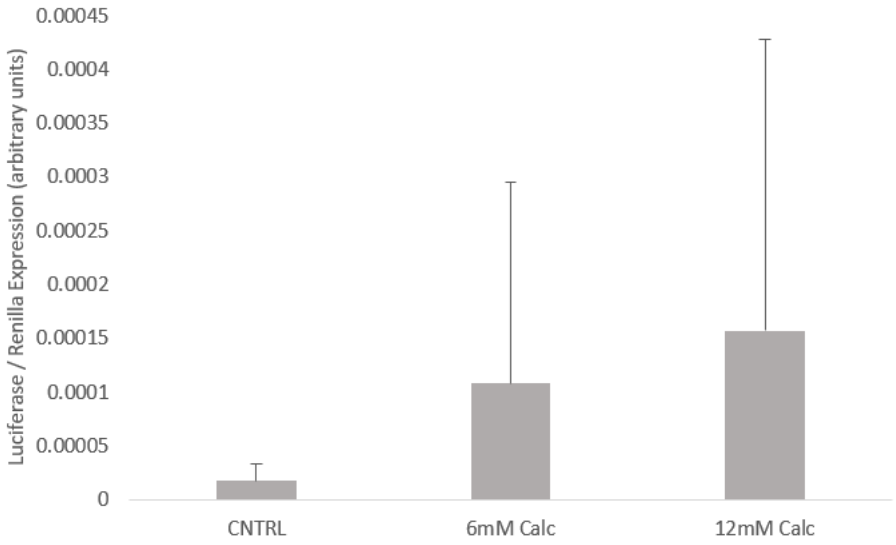

Figure 3.8 Following Addition of 6mM Inorganic Phosphate, 7-Day Calcified Cells Showed Nonsignificant Differences in Luciferase Expression Compared to Control Cells (α=0.834). Addition of 12mM Inorganic Phosphate also Caused Nonsignificant Differences in Luciferase Expression in 7-Day Calcified Cells Compared to Control Cells (α=0.663).

Statistical significance was determined using one-way ANOVA analysis at significance level, $p<0.05$ (n=3). Error bars represent standard deviation.

From *Figure 3.8*, it was found that following calcification for 7 days even at an increased calcification media concentration of 6mM and 12mM Sodium Dibasic Anhydrous Phosphate, there was still no LEF/TCF activity recorded. However, it is worth noting that there was not a debilitation in transfection even at increased levels of Sodium Dibasic Anhydrous Phosphate being administered to the cells as shown in *Figure 3.7*. Although there appears to be an increase in luciferase expression in *Figure 3.8* between control cells compared to 6mM calcified cells and 12mM calcified cells, statistical analysis showed this to be nonsignificant. Further analysis comparing 6mM calcified cells to 12mM calcified cells also showed nonsignificant differences in expression (α=0.948).

The use of a dual luciferase assay was a new method initiated as a part of our research for the analysis of the LEF/TCF complex that is part of the later stages of the WNT-Signaling pathway. As part of using this new method of analysis, the proper transfection agent for the transfection of plasmids into this primary cell type had to first be determined. Once the proper transfection agent and concentration of that agent was determined, calcification was induced at varying timepoint with varying concentrations of Sodium Dibasic Anhydrous to study the LEF/TCF complex and its activity. Following analysis, it was determined that our primary cell type, HASMCs, could be transfected and survived transfection even after being calcified. However, there was no indication of LEF/TCF activity following calcification at any of the tested timepoints or concentrations.

CHAPTER IV

DISCUSSION

The initial goals of these experiments were to first study the canonical WNT-Signaling pathway and its markers to confirm that canonical WNT-Signaling was responsible for the progression of calcification in an *in vitro* environment. First, calcification had to be induced. From our previous research, it was confirmed that calcification could be induced using 3mM Sodium Dibasic Anhydrous Phosphate in this cellular lineage. However, due to the age of cells and materials, we wanted to confirm that this procedure would still work for our cells. Following calcification at 7- and 14-Day time points, it was confirmed through calcium quantification via a Calcium Colorimetric Kit and BCA Protein Kit that calcification was induced and significantly increased over time between 7 and 14 days. To visualize this calcification, Xylenol Orange staining was performed to visualize the morphology of calcified nodules. Visualization of calcium content also showed an increase in calcification between 7- and 14-Day timepoints, consistent with previous literature from the Simpson lab. To confirm this observation, ImageJ software was employed as a secondary quantification tool to study the percentage of area of each Texas Red image covered in calcified nodules and quantify the number of nodules in each image. ImageJ analysis found that there was an increase in the number of calcified nodules observed between 7- and 14-Day timepoints. It also confirmed that there was an increase in the percentage of area of each image covered with calcified nodules also confirming that calcification spreads over time.

This confirmed that our calcification model was still accurate and could be used to study changes in expression of WNT-Signaling markers and VSMC markers over time.

To study markers of WNT-Signaling and VSMC lineage, immunohistochemistry was employed to stain for and study α-smooth muscle actin. Immunohistochemistry was used to determine if there was an increase or decrease in the α-smooth muscle actin when calcification was induced and if it increased or decreased in a time dependent fashion. Following calcification for 7 and 14 days, respectively, cells were staining for these markers and analysis was done observationally. Initial observations of staining patterns indicated that there was a decrease in α-smooth muscle actin expression following 7 days of calcification. It also appeared that α-smooth muscle actin expression decreased following 14 days of calcification. To confirm these observations were correct, two methods of quantification were performed. First, ImageJ analysis was employed to analyze these images and determine if there was a decrease in α-smooth muscle actin. ImageJ analysis confirmed that when compared to control cells, there was a decrease in the α-smooth muscle actin content after 7 days of calcification and 14 days of calcification however statistically nonsignificant. ImageJ analysis only studies a subset of images, so ELISA analysis was performed to quantify α-smooth muscle actin expression levels following 7 days of calcification. ELISA analysis determined that following 7 days of calcification, there was a statistically significant decrease in α-smooth muscle actin expression between control and calcified cellular groups. This confirmed that when calcified, VSMCs begin to lose their native contractile phenotype as a reduction in α-smooth muscle actin indicates a phenotypic switch to a synthetic phenotype that can potentially become an osteoblast-like cellular line. This decrease in

α-smooth muscle actin expression is consistent with literature sources that note that α-smooth muscle actin as a viable marker for studying the loss of the contractile phenotype of VSMCs[13, 34, 58].

Another marker that was studied was Axin-1. Axin-1 was studied visually via immunohistochemistry before ImageJ analysis was performed to quantify the percentage of area of each image with Axin-1 staining. Following staining, it was observationally determined that there was a decrease in the amount of Axin-1 expressed in both 7-Day and 14-Day calcified cells compared to control groups. To confirm that these observations were correct, ImageJ analysis was performed on Texas Red images to confirm that there was a decrease in Axin-1. From this analysis, it was determined that there was a statistically significant decrease in Axin-1 in control vs 7-Day calcified cells and in control vs 14-Day calcified cells. This statistically significant decrease in Axin-1 seemed to point in the direction of canonical WNT-Signaling being an instigator of calcification. A decrease in Axin-1 content should have indicated that the destruction complex of the canonical WNT-Signaling pathway was no longer being utilized and β-Catenin levels were being allowed to rise in the cytosol as noted by literature [2, 57].

To see how this increase in calcification and decrease in Axin-1 content was affecting β-Catenin levels, ELISA analysis was performed to quantify the amount of β-Catenin present in cells over varied time periods of calcification. A preliminary study of β-Catenin levels between control cells and cells calcified for 7 days showed that there was a nonsignificant and only slight increase in β-Catenin levels following 7 days of calcification. To study this further, a full time-course study was seeded to study β-Catenin levels following calcification periods of 1 day, 3

days, 7 days, 10 days, and 14 days. From this time-course study, it was determined that after the 1-Day calcification time point, there was a decrease in the amount of β-Catenin expressed between each respective control and calcified group. However, there was not a consecutive increase or decrease in β-Catenin across timepoints. If canonical WNT-Signaling was being induced and the destruction complex hindered from operation, there should have been an increase in β-Catenin levels as calcification proceeded over time as noted by literature[2, 24-25, 58]. Inconsistent with literature, our study of β-Catenin expression showed a decrease over time which could be due to a lack of calcification induction, or it could point to a possible inconsistency in the literature. Perhaps, another pathway was induced in this round of calcification that led to an increase in the degree of calcification but did not require the use of β-Catenin to cause the progression of this condition. This could point to our induction mechanism inducing a non-canonical WNT Signaling pathway and that our induction mechanism must be modified to include induction via specific canonical WNT Signaling proteins in order to observe true canonical WNT Signaling.

To further study the canonical WNT-Signaling pathway, a potential therapeutic target along this pathway was studied using a dual-luciferase assay. Two plasmids were transfected into both control and calcified cells where one was used to monitor LEF/TCF activity and the other was used as a control to confirm transfection was successful. To the knowledge of this author to date, this luciferase reaction has never been performed using HASMCs that have been calcified. However, following the study of different methods of transfection, it was determined that HASMCs, both control and calcified, could be transfected using a 0.15-μl concentration of Lipofectamine-3000. After a successful method of transfection was determined, the activity of

the LEF/TCF complex was studied. A preliminary study was performed where cells were calcified for 7 days at a 3mM concentration of Sodium Dibasic Anhydrous Phosphate. However, there was no activity recorded for the LEF/TCF complex. The study was expanded to calcify cells for 7 and 14 days at a 3mM concentration of Sodium Dibasic Anhydrous Phosphate and still no activity was recorded for the LEF/TCF complex. To determine if a higher degree of calcification could induce activity of the LEF/TCF complex and indicate that this complex does not become active until later in the calcification process, another study was performed where two cellular groups were administered two different calcification medias: one prepared with a 6mM concentration of Sodium Dibasic Anhydrous Phosphate and the other with a 12mM concentration. However, even with these drastically calcified cells being studied, there was still no recorded activity for the LEF/TCF complex. This was inconsistent with literature which notes that upon induction of calcification and initiation of the WNT-Signaling pathway, the destruction complex is deactivated, and β-Catenin levels are allowed to rise in the cytosol and cross into the nucleus of cells where it initiates LEF/TCF activity. However, our previous ELISA results indicated that β-Catenin levels did not rise even when treated in the exact manner that was shown to induce calcification. If β-Catenin levels were not rising, that could explain why there was no LEF/TCF activity. To determine if these results are pointing to the possible initiation of another pathway that promotes the progression of WNT-Signaling, components of the destruction complex that are hypothesized to ubiquitinate β-Catenin could be studied in HASMCs receiving regular DMEM to determine if they are active. If components of the destruction complex are active, then this could point to inconsistencies in the literature that suggest that β-Catenin must rise to initiate activity of the LEF/TCF complex and promote calcification[2, 24-26, 59].

Concluding, our study was effective in inducing calcification in HASMCs to study markers of WNT-Signaling and markers of the phenotypic switch of VSMCs. A reduction in α-smooth muscle actin indicated a loss of the native contractile phenotype of VSMCs undergoing calcification as literature suggests[13, 34]. A decrease in Axin-1 expression seemed to point to an induction of the canonical WNT-Signaling pathway, but a reduction over time of β-Catenin levels and a lack of LEF/TCF activity could point to another pathway being responsible for the induction and progression of vascular calcification. However, this study was also successful in determining a viable method of transfecting the primary cell type of HASMCs that have been supplemented both with regular DMEM and calcification media indicating that a dual luciferase assay can be used to study other markers of the canonical WNT-Signaling pathway to determine if it truly is the culprit driving the progression of vascular calcification or if another pathway should be studied to find a viable therapeutic target for the treatment of calcification.

CHAPTER V

FUTURE WORKS

This study confirms that calcification can be induced using 3mM Sodium Dibasic Anhydrous Phosphate and that calcification proceeds in a time dependent manner. It also confirmed that upon induction of calcification, VSMCs lose their contractile phenotype as evidenced by the reduction in α-smooth muscle actin over time. Further, the induction of calcification caused a reduction of Axin-1 that points to WNT-Signaling as the underlying cellular mechanism for the progression of vascular calcification. These conclusions are supported by the quantification and visualization of these components and calcification. Even though we were not able to confirm canonical WNT-Signaling due to a reduction in β-Catenin over time under calcifying conditions and a lack of activity of the LEF/TCF activity when cells are calcified, we were able to create a new protocol for transfecting plasmids into cells to study activity levels of various components of the WNT-Signaling pathway. Given these conclusions, we can expand this study to include the following objectives:

1. Expand ELISA and Immunohistochemistry studies to quantify and stain for markers of the specific contractile and synthetic phenotype of HASMCs to determine that with their contractile phenotype, cells are returning to a synthetic phenotype instead of only losing the markers of their given phenotype.

2. Perform luciferase studies using a new plasmid to study LEF/TCF activity to determine if the previously used plasmid was viable for this cellular group and there was truly no LEF/TCF activity reported under calcifying conditions.
3. Perform luciferase studies using plasmids for LRP 5/6 activity to determine if the canonical WNT Signaling pathway is initiated under calcifying conditions or if another pathway may be responsible.
4. Study markers of the non-canonical WNT-Signaling pathway using ELISA and Immunohistochemistry alongside canonical WNT-Signaling markers to determine if there is any indication of both pathways being involved in calcification simultaneously.
5. Use Alizarin Red staining to visualize calcified nodules as a new method for visualizing calcification.

These expansions would allow us to confirm that when VSMCs lose their contractile phenotype, as evidenced by the reduction of α-smooth muscle actin, they are in fact returning to a synthetic phenotype before transitioning to an osteoblast-like cell type. To determine if the LEF/TCF complex was truly not activated following induction of calcification, another plasmid can be purchased and transfected to study LEF/TCF activity and confirm that this complex is not activated. In addition, other plasmids can be purchased to study complexes further up the canonical WNT-Signaling pathway to determine if they are activated and show that WNT-Signaling does contribute to calcification. If that proves to be the case, then it is possible that WNT-Signaling follows a different path than previously suggested by literature. To determine if another pathway may be involved in the progression of calcification, markers of non-canonical WNT-Signaling can be studied to determine if this pathway works alongside the canonical WNT-Signaling to progress calcification or if it is responsible for calcification and canonical

WNT-Signaling is not. Together, this would create a more cohesive study of calcification. Once a pathway is confirmed to be the main contributor to the progression of calcification, a therapeutic target along that pathway can be chosen and targeted using inhibitor complexes to develop a treatment for vascular calcification.

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