Sclerostin as a Potential Therapy for Medial Vascular Calcification through the Inhibition of the Wnt/Beta-catenin Pathway

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Sclerostin as a potential therapy for medial vascular calcification through the inhibition of the Wnt/Beta-catenin pathway

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Mississippi State, Mississippi

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Cardiovascular disease is among the leading causes of death in the US. It stems from the pathological buildup of plaque within the vasculature known as vascular calcification. Medial calcification, or arteriosclerosis is the buildup of plaque within the medial layer of the arteries resulting in artery wall stiffening and reduction of blood flow. Evidence suggests that the vascular smooth muscles cells (VSMCs) that line the medial layer of the arteries, undergo a phenotypic switch to osteoblast-like cells to deposit calcium while in this pathological state. The Wnt/β-catenin pathway could potentially play a role in the phenotypic modulation. Inhibition of the Wnt signaling pathway could be a promising approach to combat vascular calcification.

Sclerostin (SOST) has been shown to be upregulated during arteriosclerosis in a manner that is indicative of the possible therapeutic potential of the protein. Therefore, we propose to confirm the role of Wnt signaling in vascular calcification and investigate the effects of SOST treatment on vascular calcification.
DEDICATION

I would like to dedicate this work to my grandmother, Mary Alice, and my aunts, Cynthia & Nsenga Boone. Although you all left the world far too soon, I will never forget your true exemplification of strength and confidence. Because of you, everyday I’m reminded that I can be determined and work hard while remaining true to myself.

To my family, thank you so much for the encouragement and emotional support. It has always been my intent to be the best role model to my younger relatives and I hope I’ve lived up to that goal. Lastly, I would like to dedicate this work to my aunt Dr. Emma Boone and my mentor, Dr. Angela Verdell. I’m not sure how I would have survived my undergraduate and graduate years at MSU without your constant motivation and guidance. Thank you for not giving up on me and pushing me to make the most of my journey.

I hope I’ve made you all proud with everything that I’ve accomplished. I appreciate and love every one of you!
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CHAPTER I
INTRODUCTION

Background

Cardiovascular disease (CVD) remains among the top 2 leading causes of death in the United States since 1975, with 1 out of 4 deaths being contributed to heart disease [1], [2]. Although, according to the World Health Organization (WHO), CVD still claims the title as being the number 1 cause of death globally, estimating a total of over 17 million deaths in 2015 [1]. Such diseases include myocardial infarction, angina, and stroke [2]. These often-fatal events can result from the underlying pathological condition known as vascular calcification, or the deposition of calcium in the walls of the vasculature that causes a pathological state [2], [3].

Vascular Calcification

Vascular calcification was originally considered a passive, degenerative process, however, is now deemed an active biological process [2] – [4]. Nonetheless, due to the broad nature of vascular calcification, it is important to differentiate between the types of the pathological condition. Intimal and medial are two prominent types of calcification. Intimal calcification, or atherosclerosis, features endothelial dysfunction, inflammation, and intimal thickening usually resulting from endothelial damage due to high blood pressure, smoking, or excess cholesterol, fats, and glucose within the blood [2], [3], [5]. It is characterized by the accumulation of plaque; cholesterol, calcium, fats, and other substance within the blood; along the walls of the endothelium within the arteries [3], [4], [6]. Alternatively, medial
calcification, or arteriosclerosis is characterized by the stiffening of the walls within the medial layer of the arteries due to calcification in the area [3]. As a result of arteriosclerosis, there is a reduction of blood flow which leads to increased cardiovascular mortality with illnesses including chronic kidney disease, diabetes, hypertension, and even coronary heart failure [3], [4]. Withal, intimal and medial calcification should not be considered mutually exclusive events. In theory, stress generated from intimal calcification causes vascular smooth muscle cells (VSMCs) lining the medial layer of the arteries to undergo a phenotypic switch causing them to lose their contractile properties and, in response, induce medial calcification [7]. For this study, however, the primary focus will be calcification of the medial layer.

Medial vascular calcification theoretically mimics the ossification processes of bone remodeling attributing to the active buildup of hydroxyapatite [2], [8]. Osteogenesis, or the formation of bone utilizes mesenchymal stem cells which differentiate into osteoblasts, or bone-forming cells to promote ossification [2], [8]–[10]. This process involves transcription factor, Runx2, which is necessary to promote differentiation to osteoblasts [9], [10]. In the same manner, VSMCs, which are responsible for the physiological functioning of the medial layer of the arteries, undergo a phenotypic switch to osteoblast-like cells to deposit calcium whilst in a pathological state [11]. That is, VSMCs can reversibly switch from a contractile to a synthetic phenotype under stress conditions [12]–[14]. This phenotypic modulation includes deviating from possessing adaptability to the flow of blood, to becoming proliferative, resulting in thickening and stiffness of the arterial wall [14]. Figure 1 contrasts the morphology of contractile and synthetic VSMCs. Moreover, studies have shown an upregulation of osteoblast-like markers and loss of contractile markers within these synthetic
VSMCs, further implicating that the process of ossification serves as a promising model to study the mechanism by which medial calcification occurs [8], [14]. Though, within osteogenesis, there is also regulation that acts in the event of excess and insufficient bone buildup to maintain bone homeostasis. These regulation factors such as osteoblasts or osteoclasts, bone-forming and bone dissolving cells, are not present within the vasculature when there is an accumulation of hydroxyapatite due to calcium deposition [15]. Consequently, the build-up causes turbulent or inappropriate blood flow leading to the different cardiovascular conditions and events.

Figure 1   Contractile vs. Synthetic Vascular Smooth Cells.

The figure highlights the differences between contractile and synthetic phenotypes amongst vascular smooth muscle cells. Adapted from [16].
The exact mechanisms by which vascular calcification occurs are unknown, however, because of its similarities to bone formation, it is appropriate to hypothesize that they occur by the same system. As previously mentioned, Runx2 is a transcription factor necessary for osteoblast differentiation. Studies have shown that Runx2 is in direct association with the Wnt/β-catenin signaling pathway, eluding that the pathway mediates bone formation by activating Runx2 to regulate the differentiation of mesenchymal stem cells to an osteogenic lineage [9], [10]. Thus, evidence suggest that calcification within the arteries potentially forms through the Wnt-signaling pathway.

**Wingless (Wg)/Wnt-Signaling Pathway**

Wnt signals activate transcriptional pathways that may promote developmental processes such as cell proliferation of tissue expansion [2]. There is no limit on the type of process that may be executed by Wnt signaling because the pathway can potentially impact the cell at various points during the life cycle [2], [17], [18]. It has been previously shown that Wnt signals have the ability to shape tissues that are growing while also instructing cells to proliferate and even maintain the tissue architecture in adult life, implying that even fate determination can be influenced by the Wnt signaling pathway [2].

The Wingless family of proteins consists of 19 lipid-modified glycoproteins [2], [17]. The Wnt cascade activates when one of these secreted glycoproteins binds to Frizzled (Fzd) and the coordinating coreceptor complex, depending on which Wnt pathway is being activated. Fzd functions as a signal-transducing protein that is found at the surface of the plasma membrane of Wnt-responsive cells [19]. Once Wnt proteins bind to Fzd and their corresponding co-receptor complex, a signal is transduced into the cell to target the
cytoplasmic protein, Disheveled (Dsh or Dvl), which acts as an interpreter of the signals to guide the activation of the correct pathway [20]. From this point, the signal branches off into one of the three major Wnt signaling pathways: the noncanonical planar cell polarity pathway, the Wnt/Ca\textsuperscript{2+}-pathway, and the canonical Wnt/\(\beta\)catenin pathway [18]. Figure 2 displays the three different pathways, as well as the mechanism by which they are activated.

**Figure 2**  Wnt-Signaling Pathways

Wnt/\(\beta\)-catenin pathway. (B) Planar Cell Polarity Pathway. (C) Wnt/Ca\textsuperscript{2+} pathway. Source: Adapted from [21].

**Non-Canonical (Wnt/Ca\textsuperscript{2+}-) and Planar Cell Polarity Pathway**

The non-canonical pathway consists of two distinct Wnt cascades that are independent of \(\beta\)-catenin. In the Wnt/Ca\textsuperscript{2+}-pathway, Wnt binds to the extracellular cysteine rich domain on the N-terminus of its cognate Fzd receptor, promoting interaction with Dsh and mediating the phosphorylation of the receptor tyrosine [2]. After phosphorylating tyrosine, the signal then
interacts with calcium channels to release Ca\(^{2+}\) [2]. Research has found that the Wnt/Ca\(^{2+}\) pathway functions during embryogenesis in promoting cellular fate, regulate tissue separation, and formation of the heart [22]. The planar cell polarity pathway is assumed to be mediated through Fzd, but independent of a coreceptor complex [18]. Its implication through the Wnt signaling pathway starts with the activation of small GTPases, Rho and Rac, which mediate cytoskeletal rearrangements [2].

**Canonical Pathway (Wnt/β-catenin)**

The canonical (Wnt/β-catenin) pathway is the most studied pathway and is implicated in many developmental processes, including bone metabolism [2], [17], [18]. β-catenin, a cytoplasmic protein, is the central player in the canonical Wnt cascade [10], [18]. Within this pathway, Wnt binds to Fzd and the LRP 5/6 coreceptor complex to activate Dsh, which in response causing the inactivation GSK3, a “cytoplasmic destruction complex” [2], [17]. The complex is responsible for regulating the phosphorylation of the β-catenin protein and targeting it for degradation [23]. Therefore, since the complex is inactive when Wnt is bound, stable β-catenin accumulates in the cytoplasm to eventually translocate into the nucleus resulting in an upregulation of Wnt target gene expression. *Figure 3* below further highlights the variances between active and inactive canonical Wnt signaling.
As previously stated, studies have shown that Runx2 is a target gene of the Wnt/β-catenin pathway, directly influencing osteogenesis [9], [10]. This occurs through the mechanism described above and has been found most prevalent during embryonic development and skeletal maintenance.

**Which Wnt?**

There is a multitude of evidence that suggests the involvement of Wnt in vascular calcification and identifies the specific key players relative to the pathway. It is also likely that there are numerous signaling pathways involved in vascular calcification. However, it is necessary to consider each of these pathways independently to further evaluate their role. More specifically for Wnt-signaling, it is very possible that many of the Wnt glycoproteins may play a role in calcification, however, many researchers have begun to evaluate specific
Wnt ligands to determine if one may contribute more towards calcification. A study performed by Shen et al. confirmed that Wnt16 stimulates bone formation through canonical signaling but also inhibits osteoclast activity in noncanonical signaling [25]. It was also found that Wnt16 is expressed by VSMCs, regulated by arteriosclerotic stimuli and contributes to aortic stiffening [26]. Wnt7b was confirmed to activate canonical signaling within VSMCs that was shown through 8-fold activation of a TOPFLASH reporter assay [27]. However, Wnt3a is the most studied ligand for having the most significance in vascular calcification. Wnt3a has been shown to be significantly increased when vascular calcification has been induced [28]. In the same study, it was also shown that the presence of Wnt3a led to an increase in RUNX2 expression [28]. These studies suggest that the Wnt3a ligand may be the primary focus for future studies involving vascular calcification and potential treatments.

The role of the Wnt pathway in bone formation, in general, leads to the ideal that it could also play a role in vascular calcification due to the phenotypic switch of VSMCs to osteoblast-like cells. If this is the case, an antagonist to the Wnt/B-catenin pathway could be used as a therapy to reverse or limit vascular calcification.

**Sclerostin (SOST)**

Sclerostin (SOST) is a protein primarily expressed and secreted by mature osteocytes functioning as an inhibitor of bone formation [29], [30]. SOST acts as an antagonist to the Wnt/β-catenin pathway by binding to the LRP 5/6 coreceptor complex, which hinders the attachment of Wnt to the Fzd receptor and prevents downstream signaling [29]–[31]. In **Figure 4**, Sclerostin is shown connecting to LRP 4 and 5/6, ceasing the progression of the pathway.
Studies have shown that the absence of SOST expression can lead to high bone mass that features exaggerated bone formation and excess-bone diseases [29]. Conversely, over-expression of SOST has the potential to decrease bone mass which alludes to deficient bone formation and stability [29]. Having said that, although SOST expression is mainly seen through mechanisms and actions of the bone, there is evidence suggesting that it can be detected and correlated to plaque build-up associated with vascular calcification. However, if this is the case, it is unclear whether SOST is a positive/negative inhibitor or simply a biomarker of calcification.
Positive, Negative, or Biomarker

In a study conducted by Qureshi et al, they investigated the association of serum SOST levels in patients with epigastric and coronary artery calcification to evaluate the foretelling potential of the protein [33]. The patients consisted of adult end-stage renal disease (ESRD) patients undergoing living donor renal transplantation. Most of the patients were white and around the age of 46, amounting up to 89 participants total. The study showed evidence of medial layer vascular calcification that was present in 42% of the patients, while also suggesting a positive association between medial vascular calcification and serum SOST levels. It was concluded that serum SOST is associated with a degree of vascular calcification combining various different factors for ESRD patients, however its potential as a biomarker is seemingly limited [33].

A similar study by Gaudio et al. aimed to analyze the relationship between serum SOST levels and Dickkopf 1 (Dkk1), an additional inhibitor of the Wnt/β-catenin pathway, using a Carotid Intima-Media Thickness (CIMT) test and their relationship with arterial stiffness using pulse wave velocity measurements [34]. The intent was to evaluate the possible role that SOST and Dkk1 play as a predictor of arterial stiffness. The 67 participants resided in the urban area of Catania, Italy, and each exhibited either osteoporosis or atherosclerosis. Those with malignant tumors, drug use, chronic kidney disease, and chronic hepatic impairment were excluded. All patients received a carotid artery ultrasonogram in a lying down position to measure CIMT and they were defined according to the ARIC (Atherosclerosis Risk in Communities) clinical study [34]. The pulse wave measurement was used to determine the central aortic pressure. Blood samples were taken after a fasting period and used for measurement of serum albumin, phosphorus, and creatinine. The study yields
that SOST is an independent predictor of arterial stiffness in healthy outpatient subjects. It was also concluded that the pulse wave velocity technique is an effective test of arterial stiffness. Although pulse wave velocity was associated with SOST serum levels, it was not the case for Dkk1, indicating that the degree of the inflammatory response may be in regard to the different clinical characteristics of the patients [34]. The researchers concluded that evidence suggests that serum SOST does seem to correlate with arterial stiffness [34].

The previous studies demonstrate associations of serum SOST with vascular calcification and arterial stiffness. Although, it was not necessarily inferred from these studies whether the association was a result of serum SOST functioning as a defense for vascular calcification, evidence does suggest that the upregulation of the protein is, in part, due to existing as an intervention. Bruzzese et al. sought to study the role of SOST in uremic patients by analyzing its behavior during a hemodialysis session while also evaluating the correlation of its serum levels with regards to markers of uremic osteodystrophy and vascular calcification [35]. The study consisted of 21 adult patients who received intermittent hemodialysis treatment, excluding those diagnosed with cancer or inflammatory disease. The sessions last 3-4 hours, 3 times a week. 20 healthy individuals were also studied as a control group meaning that they had no history of hypertension, diabetes, etc. Blood samples were collected before and after hemodialysis and after a fast for the control group. Interestingly, the study yielded that SOST could be dialyzable or reduced after hemodialysis [35]. It was also found that SOST was not exclusively produced from osteocyte cells but also from the upregulation in VSMCs. This further suggests confirmation of the phenotypic switch of VSMCs in regard to being regulated through the Wnt/β-catenin pathway and the role that SOST could play in serving as a therapy for vascular calcification. Furthermore, high SOST
levels were also linked to inflammation, vascular lesions, and expression of other biomarkers [35]. The study settled on the fact that hemodialysis could reduce SOST levels and that elevated levels of the protein are independently related to vascular calcification.

A different type of study conducted by Bovijn et al. aimed to investigate the effect of factors that increase bone mineral density within the SOST locus on the risk of bone fracture, osteoporosis, cardiovascular events, and whether SOST inhibition therapy has a negative effect on cardiovascular diseases [36]. They found that using a drug to lower SOST levels lifelong does indeed prevent osteoporosis and other bone defects, however it conversely increases the risk of cardiovascular events due to the downregulation of SOST [36]. The researchers also suggested an extensive study into the drug “Romosozumab”, which is a sclerostin inhibitor, to assess its effects on cardiovascular events. Though, still within this study, it suggests that SOST has a positive independent association with vascular calcification which is especially seen through the fact that calcification is more prevalent when SOST is virtually absent.

In any case, it is necessary to evaluate the SOST protein in a different manner. Glucocorticoids (GCs) are synthesized and secreted by the adrenal glands and have an impact on the physiological functioning of several systems within the body including metabolism, adapting to stress environments, and host defense [37]. Signaling components of GCs can be influenced by factors including neuroinflammation, stress, and negative feedback. It has also been shown that GCs play a vital role for bones later on in life. In the study by Beier et al. they investigated the impact of GCs on the Wnt pathway, which is also critical for bone formation. They examined SOST as a facilitator of the inhibitory effects of GCs as well as whether or not the removal of SOST counteracts the inhibitory effects of
bone mineralization [37]. They found that GCs indeed mediate themselves by utilizing SOST to antagonize the Wnt pathway, thus inhibiting osteoblast differentiation. An important association of the study was highlighted when it was confirmed that SOST blocks the Wnt pathway from advancing, which could again yield association of vascular calcification.

On the contrary, these associations of SOST with vascular calcification could just be indicative that SOST can be utilized as a biomarker for cardiovascular events as earlier studies sought out. Kalousova et al. aimed to investigate the prognostic significance of SOST in hemodialysis patients. The study was performed in two dialysis centers in the Czech Republic and consisted of 106 hemodialysis patients averaging around the age of 61. The control group consisted of 25 healthy volunteers averaging around the age of 48. Blood samples, vitamin D, and serum SOST was collected from all patients. It was found that SOST concentrations were almost 3 times higher in the hemodialysis patients compared to the controls. Higher cardiovascular risk was correlated with SOST concentrations above the median. Overall, it was inferred that SOST is elevated in hemodialysis patients and linked to higher cardiovascular mortality [38]. Limitations of the study include the fact the study was observational and did not consider various confounding variables when interpreting the results. Moreover, the researchers suggested that the elevated levels of SOST were not necessarily protective agents against calcification but more of an indicator. However, it is feasible to assume that SOST is only needed when levels if vascular calcification is increased, hence, the protein levels are elevated.

In a similar study by Moghazy et al., they investigated the serum levels in both dialyzed and non-dialyzed chronic kidney disease patients as a potential predictor of both vascular and valvular types of calcification [39]. The participants consisted of 20 healthy
volunteers used as a control group and 62 patients with chronic kidney disease explicitly defined. All participants received a clinical examination, radiological investigations, and echocardiographic assessments [39]. Laboratory investigations consist of obtaining blood samples after periods of fasting and before and after hemodialysis sessions. A significantly higher SOST serum level was observed in the dialyzed patients compared to those who were not [39]. It was found that positive levels of SOST were also associated with age and valvular calcification. The study concluded that SOST could be used as a sensitive biomarker or both vascular and valvular calcification in individuals with chronic kidney disease and that addressing the therapeutic potential of SOST could be used in developing treatments for individuals with vascular calcification.

**Conclusion**

There is an abundance of evidence that suggests that SOST has some form of association to vascular calcification. However, it remains unclear whether this association is positive, negative, or simply an indication of a vascular pathological condition. Based on studies discussed within this review, they all yield that SOST is either a positive inhibitor or could potentially be used as a therapeutic agent for vascular calcification. Although, it is not illogical to conclude that naturally occurring SOST should be used as a biomarker of vascular calcification. However, perhaps this can be due to the fact that SOST is elevated in individuals because calcification is present, and the protein is trying to act as a defense against it. Yet, there is not enough natural SOST production to combat the calcification. If this is the case, some studies do suggest that the possible therapeutic potential of SOST may cause a threat to established bone within the body. This implies that while excess SOST may reverse and eliminate the effects of vascular calcification, there may also be adverse outcomes within the bones that could lead to
bone diseases. It is important to note that there is still much that has not yet been discovered regarding SOST, the Wnt-signaling pathways, and overall vascular calcification, which deems necessary further investigation into these proteins and mechanisms. To address a few of these points, the following specific aims have been developed:

**Specific Aim 1**: Characterize in-vitro model of vascular smooth cell calcification & confirm Wnt-signaling.

**Specific Aim 2**: Investigate the dose dependency of Sclerostin as it affects vascular smooth muscle cell calcification.

It is hypothesized that the previously published in-vitro model of vascular calcification from our lab will also prove to be successful within a different lineage of VSMC. Based on previous literature, it is also our belief that the presence of Wnt-signaling will be revealed through the increased presence of “Wnt markers” and the reduction of VSMC markers. Following SOST treatment, it is our belief that there will be a strong correlation between the increasing dosages of SOST with decreasing amounts of SOST. We also hypothesize that there will be recovery of VSMC markers and reduction of “Wnt-markers”.
CHAPTER II
AIM I: CHARACTERIZE IN-VITRO MODEL OF VSMC CALCIFICATION & CONFIRM WNT-SIGNALING

Introduction

Previously reported literature from our lab has shown successful induction of healthy VSMCs when supplemented with inorganic phosphate [40]. However, the VSMCs that were experimented with at that time are of a different cell lineage than the cells being studied in the lab now, although they are VSMCs. For this reason, it is necessary to reaffirm that the previous in-vitro model will be adequate for our current cell line. This would involve determining the best source of inorganic phosphate and the appropriate cell-culture environment.

Inorganic phosphate (Pi) is one of the many options that have been shown to induce calcification in VSMCs. In a study performed by Giachelli et al., they showed that cells that were supplemented with extracellular inorganic phosphate; in levels comparable to that of patients with hyperphosphatemia; tended to have increased levels of calcium deposition with the cell culture [41]. It was also shown from this study that following the addition of inorganic phosphate, cells began to behave with differing mechanical properties, such as expression of osteogenic RUNX2 [41]. A similar study performed by Jono et al., also sought to examine the effects of the addition of inorganic phosphate on culture mineralization [42]. They found that there was a significant increase in mineralization to cell cultures with high
concentrations of inorganic phosphate compared to those that were just supplemented with normal physiological levels of inorganic phosphate [42]. Also like the previous study, there were elevated levels of osteogenic marker expressed by cells with a higher calcium content. Based on these studies, elevated levels of inorganic phosphate can be used as an effective agent to induce calcification.

It is also necessary to relate the calcium content within cells to the markers they may express. Alpha-smooth muscle actin (SMA) is a cytoskeletal actin isoform that is predominately found in VSMCs and plays a role in myofibroblasts differentiation, focal adhesion maturation, and the generation and transmission of mechanical forces [43]–[45]. It is the single most abundant protein within adult VSMCs [43]. It has also been shown to be significantly decreased prior to cells being supplemented with inorganic phosphate [43]. Axin, β-catenin, and RUNX2 on the other hand, are markers indicative of Wnt signaling. Axin is a protein that serves 2 purposes for Wnt signaling; to promote degradation of β-catenin and to interact with LRP5/6 for the recruitment of the GSK3 “destruction complex” to facilitate phosphorylation of LRP5/6 so that Wnt-signaling may occur [46], [47]. Studies have shown that when GSK-3, an inhibitor of Wnt is suppressed, levels of Axin2 were significantly increased [47]. Generally, Axin will be present during active and inactive Wnt signaling. In a similar manner, B-catenin is always also present in some capacity, however it is up regulated during active Wnt-signaling due to the “destruction complex” being inactive [45]. Studies have also shown that β-catenin and RUNX2, as previously mentioned, are both expressed in vascular calcification [45]. Therefore, it is very reasonable to consider visualizing or quantifying these cell markers as a means of evaluating the role of the Wnt-signaling pathway in vascular calcification.
Aim I Specific Objectives

The objectives of these studies were to first determine the appropriate source of inorganic phosphate to promote calcification with the new line of VSMCs, as well as the appropriate culture environment to grow VSMCs. After confirming the appropriate sources, we then want to visualize calcium content and characterize mechanical properties of normal versus calcified cells. Lastly, confirmation of Wnt involvement shall be confirmed through visualizing and quantifying the common VSMC and osteogenic markers at different time points.

Experimental Section

Materials/Methods

*Cell Culture.*

Human Aortic VSMCs (C-007-5C) were purchased and expanded between passage 4-7. Cells were cultured in a standard growth medium containing Dulbecco’s Modified Eagles Medium (DMEM), 10% Fetal Bovine Serum (FBS), and 1% Penicillin. Light microscopy was used to visualize cell morphology. Cells were then seeded in 6-well plates with 3mL of growth medium. Once grown to 80% confluency, plates were then rinsed with sterile Phosphate-buffered saline (PBS) and supplemented with a “serum-starve” media for 12 hours. The “serum starve” media contained the same components of the standard growth medium; however, it lacked the 10% FBS. After the 12 hour “starvation period”, calcification was then induced from either of the following sources: Sodium Phosphate Dibasic Anhydrous (N37595, Mallinckrodt AR), Sodium phosphate dibasic (S5136-500G, Sigma-Aldrich), and Sodium β-glycerophosphate.
(βGP). Cells were calcified for different time points between 0-14 days. They were fed with their experimental media every 2-3 days.

**Determination of Pi Source.**

Human Aortic VSMCs were cultured and expanded to passage 4. Cells were seeded within 6-well plates and supplemented with 3mLs of the standard growth media. After reaching 80% confluency, cells were “serum starved” for 12 hours. Following the starvation period, cells were fed with their conditional media, Sodium Phosphate Dibasic Anhydrous at 3mM, 6mM, &12mM concentrations (n=6 for each); Sodium phosphate dibasic at 3mM, 6mM, & 12mM concentrations (n=6 for each); βGP at a low 5mM & a high 10mM concentration (n=6 for each), and Control with standard growth media (n=6). Cells were cultured for 7 days in their experimental media and fed every 2-3 days.

**Determination of Culture Environment.**

Studies have suggested that certain extracellular matrix molecules, such as collagen, can promote vascular calcification and that some cells preferentially produce these matrix components [48]. Standard glass 6-well plates were coated with Rat-Tail Collagen Coating Solution (122-20, Cell Applications) and incubated at room temperature for 12 hours. After the incubation period, cells were gently rinsed with PBS. Human Aortic VSMCs were cultured and expanded to passage 6. Cells were seeded in the collagen coated 6-well plates and standard glass 6-well plates. They were supplemented with 3mLs of the standard growth media and “serum starved” for 12 hours after reaching 80% confluency. Cells were then fed their conditional media which consisted of either 3mM Sodium Phosphate Dibasic Anhydrous (n=6 for each) or the
standard growth media (n=6 for each). Cells were cultured for 7 and 14 days in their experimental media and fed every 2-3 days.

**Quantification of Mineral Deposition.**

After appropriate time points, cells were rinsed with nonsterile PBS and decalcified in 5mL of HCl for 24 hours. Afterwards, the HCl samples were collected and analyzed via Atomic Absorption Spectroscopy (AAS) for quantification of calcium content. The cell layer was then treated with 1mL of NaOH/0.1% SDS and then scraped and removed from the plates. Protein concentration was then analyzed using a BCA Protein Kit (Pierce, Waltham, MA) Calcium samples were normalized to the protein concentrations.

**Scanning Electron Microscopy.**

Cells were cultured and expanded to passage 7 and seeded onto plastic tissue culture cover slips (60U2612, Sarstedt, Inc.) according to cell culture protocol. After a 7-day treatment period, cells were gently rinsed with PBS and fixed with ½ Karnovsky’s fixative in 0.1 M Sodium Cacodylate buffer for 1-2 hours. Following, cells underwent post fixation in 1% Osmium Tetroxide for an hour and were dehydrated in a graded ethanol series. Imaging was then performed using Field Emission Scanning Electron Microscope at 10kV.

**Atomic Force Microscopy**

Cells were cultured and expanded to passage 7 and seeded onto plastic tissue culture cover slips (60U2612, Sarstedt, Inc.) according to cell culture protocol for 7 days. Cells were gently rinsed in PBS and imaged using Atomic Force Microscopy Bruker Dimension to record average elastic (Young’s) modulus, stiffness, and height. This process involves using a non-coated nitride probe with varying tip radii. The average values of the mechanical properties were
acquired from collecting random points across the surface of each sample. The modulus was calculated via the Hertzian Model\(^1\) shown below, where \(F\) is the force from the force curve, \(E\) is the Young’s Modulus, \(\nu\) is Poisson’s ratio, \(R\) is the tip radius, and \(\delta\) is the indentation. Stiffness is derived based on the linear graph.

\[
F = \frac{4}{3} \frac{E}{(1 - \nu^2)} \sqrt{R\delta^2} \tag{1}
\]

**Staining**

Cells were cultured and expanded to passage 7 and seeded according to cell culture protocol in 4-well chamber slides. Cells were divided into treatment periods of 7 days and 14 days to stain for antibodies. After treatment period, cells were gently rinsed with PBS, fixed with 4\% paraformaldehyde, permeabilized with 0.1\% Triton X, and blocked with 5\% PBSA. Cells were then treated with primary antibodies and incubated in 4°C overnight. After incubation, the primary antibody was removed and replaced with a secondary antibody and left for an hour at room temperature in the dark lighting. After removing the secondary antibody, cells were stained with Dapi and imaged via Light Microscopy. 7 Day cells were also stained with Xylenol Orange to visualize calcium content.

**Statistical Analysis.**

Statistical significance was determined via one-way Analysis of Variance (ANOVA) and T-Test at the \(\alpha=0.05\) level of significance.
Results

Calcium content was quantified via Atomic Absorption (AA) and normalized to protein concentration after 7 days of treatment of an inorganic phosphate source. As evident from Figure 5, neither the low or high concentration of βGP or the 3mM Sodium Phosphate Dibasic (SPD) significantly induced mineral deposition. However, the 3mM Sodium Phosphate Dibasic Anhydrous (SPDA) was successful in calcifying VSMCs, as it displayed the most mineralization compared to the other concentrations of inorganic phosphate. This also confirmed our previous model of in-vitro calcification of VSMCs. After statistical analysis, it was shown that there was significant difference among the values of calcium content for each phosphate source. However, there was not a significant difference between that of the 3mM SPDA and the 12mM SPDA/SPD. Since this was the case, 3mM SPDA was used for all subsequent studies.

![Figure 5](image)

Figure 5  Calcium quantification of Human Aortic VSMCs treated with Sodium Dibasic Phosphate Anhydrous (3mM, 6mM, 12mM), Sodium phosphate dibasic (3mM, 6mM, 12mM), and BGP (5mM & 10mM) for 7 days. Cells were seeded in standard glass tissue culture plates. *Statistical significance was determined by ANOVA & unpaired t-test (#) at significance level, p<0.05. (n=6)
To determine the best cell culture environment, cells were seeded and calcified in standard glass well plates and collagen coated well plates for 7 and 14 days. Figure 6 shows that there was no significant difference between control nor calcified samples for each time point. However, when comparing 7-day to 14-day samples, there was a significant increase in calcium content. Since, there was no real difference between, standard glass well plates were chosen as the appropriate cell culture environment.

Figure 6  Calcium quantification of Human Aortic VSMCs treated with 3mM Sodium Dibasic Phosphate Anhydrous for 7 and 14 days.

Cells were seeded in standard glass tissue culture plates and collagen-coated well plates. *Statistical significance was determined by ANOVA & unpaired t-test (#) at significance level, p<0.05. (n=6)

Afterwards, VSMCs were treated with 3mM SPDA in standard glass well plates for 7 days to confirm that this was the most efficient in vitro model. As confirmed by t-test and
shown in Figure 7, cells treated with 3mM SPDA had a significant increase in calcium content as compared to healthy control cells.

Figure 7 Calcium quantification of Human Aortic VSMCs treated with 3mM Sodium Dibasic Phosphate Anhydrous for 7 days.

Cells were seeded in standard glass tissue culture plates. Statistical significance was determined unpaired t-test (#) at significance level, p<0.05. (n=6)

Cells were also stained with Xylenol Orange after being treated for 7 days to visualize calcium content. Figure 8 is visualization of a healthy VSMC and as shown by Figure 8 (2), there is virtually no expression of mineralization. However, Figure 9, which displays a calcified cell, shows a definite increase in calcium content.
Figure 8  Visualization of Calcium Content using Xylenol Orange Stain Ctrl.

(1) Standard light microscope image. (2) Visualization of calcium content using xylenol orange. (3) Nuclei staining using Dapi. (4) Merged image with 3 channels.
After confirming the inorganic phosphate source and growth environment and visualizing calcium content, cells were calcified at 3- and 7-day time points to determine when the onset of calcification occurs. As shown by Figure 10 and confirmed through ANOVA, there was a significant difference of calcium content between the timepoints. Also, there was a significant difference between the 3-day healthy cells and the 3-day calcified cells as confirmed through t-test.
Figure 10  Calcium quantification of Human Aortic VSMCs treated with 3mM Sodium Dibasic Phosphate Anhydrous for 3 & 7 days.

Cells were seeded in standard glass tissue culture plates. Statistical significance was determined by ANOVA and unpaired t-test (#) at significance level, p<0.05. (n=6)

Scanning electron microscopy (SEM) was another method used to visualize healthy vs calcified cells after 7 days of treatment. Shown in Figure 11, control cells were frailer and more damaged as compared to calcified cells after undergoing harsh SEM fixation protocols. There is also visible calcium content within the calcified samples as shown in Figure 11 (5-6).
Figure 11  SEM Visualization Control & Calcified Cells.

(1-2) Broad image of cell surface of both healthy and calcification samples. (3-4) Magnified images of fewer cells on surface of healthy vs. calcified cells. (5-6) Single-cell images of health vs calcified cells. Red circle indicated areas of suspected calcium deposition.
Calcified cells were also analyzed via Atomic Force Microscopy (AFM) for mechanical properties. The Young’s (Elastic) Modulus and stiffness of the cells can be determined by using the slope of the force curve after pinpointing different areas on the surface of the cell. The modulus is directly correlated to the Hertzian model, and the stiffness is recorded from a linear graph. *Figure 12* displays the graphs for average modulus and stiffness for healthy and calcified cells. The average modulus for calcified cells is much larger than that of the healthy cells and as confirmed through t-test among stiffness values, calcified cells were significantly harder than healthy cells.

*Figure 12*  AFM Average Modulus & Stiffness of Control vs. Calcified Cells.

Calcified cells exhibited a much larger modulus and stiffness compared to healthy cells as confirmed through t-test (#).
AFM was also used to visualize and determine height differences among healthy and calcified cells. Shown in Figure 13, calcified cells had a higher height peak, 766.4nm, compared to that of healthy cells, 519.6nm. The higher height peak was also associated with more rigidity and stiffness.

Cells were stained at 7- and 14-day timepoints to visualize antibody expression. Figure 14 shows staining for alpha-smooth muscle actin (SMA). The control sample, which is a 14-day healthy cell displays a significantly larger concentration of SMA, as compared to that of the 14-day calcified samples, which shows virtually no SMA expression. Also shown is the gradual decrease of VSMC markers from 7 to 14 days of treatment. Figure 15 visualizes Axin1, which is an indicator of Wnt. Visibility of Axin1 also decreases as calcification increases which is evident from the lack of expression in 14-day calcified samples. Figure 16 shows RUNX2 expression in healthy and calcified cells. RUNX2 is
shown to be increased in the nucleus, as calcification increases and gradually expressed in more cells.

Figure 14  Alpha-Smooth Muscle Actin Staining: 7- vs. 14-day expression of SMA.

Significant decrease in expression of SMA as calcification time increases shown through the red channel.
Figure 15  AXIN1 Antibody Staining: 7- vs. 14-day expression of AXIN1.

Significant decrease in expression of AXIN1 as calcification time increases.
Figure 16  RUNX2 Antibody Staining: 7- vs. 14-day expression of RUNX2.

Significant increase in expression of RUNX2 as calcification time increases.
CHAPTER III

AIM II: INVESTIGATE THE DOSE DEPENDENCY OF SCLEROSTIN AS IT AFFECTS VSMC CALCIFICATION

Introduction

A previous study performed by our lab had the primary focus manipulating the plasticity of VSMCs to treat vascular calcification using SOST. Until this study, SOST had not yet been evaluated as a treatment for calcification but revealed itself as a promising therapeutic agent. It was shown that SOST inhibited phosphate-induced calcification, down-regulated the osteogenic marker RUNX2, and even recovered a percentage of the vascular smooth muscle cell marker, α-SMA within the cell culture [40]. However, the study did not evaluate the effects of SOST at different dosages and needs further examination to fully determine the effects of SOST as a treatment.

Aim II Specific Objectives

The objectives of this study were to examine the effects of low and high dosages of SOST treatment on calcified cells by quantifying and visualizing calcium content. We also want to evaluate the effectiveness of the SOST treatments to halt and/or reverse the expression of osteogenic markers in calcified cells and restore VSMC markers. As previously mentioned, we believe that there will be strong correlation of increasing SOST treatment with the reduction in calcium content and the recovery of VSMC markers.
Experimental Section

Materials/Methods

Cell Culture.

Human Aortic VSMCs were cultured and expanded to passage 7. Cells were then seeded in 6-well plates with 3mL of growth medium. Once grown to 80% confluency, plates were rinsed with sterile PBS and supplemented with a “serum-starve” media for 12 hours. After the 12 hour “starvation period”, calcification was then induced with Sodium Phosphate Dibasic Anhydrous and calcified in time periods of 7 and 14 days. They were fed with their experimental media every 2-3 days.

SOST Treatment.

Once plates reached day 7, cells were treated with SOST at 80ng/mL, our previous treatment dosage, and 160ng/mL, doubling the original dosage. Cells treated with SOST supplement continued to receive calcification media. After the end of each appropriate time-period, cells were rinsed with non-sterile PBS and decalcified in 5mL of HCl for 24 hours. HCl samples were collected and analyzed via Atomic Absorption Spectroscopy (AAS) for quantification of calcium content and normalized to protein concentration via BCA kit.

Staining.

Cells were cultured and expanded to passage 7 and seeded according to cell culture protocol in 4-well chamber slides. Cells were divided into treatments of low SOST concentration (80ng/mL) and high SOST concentration (160ng/mL). Slides were given their experimental media throughout the entire duration of the study and were supplemented with SOST treatment at day 7. At the study’s end, cells were gently rinsed with PBS, fixed with 4% paraformaldehyde,
permeabilized with 0.1% Triton X, and blocked with 5% PBSA. Cells were then treated with primary antibodies and incubated in 4°C overnight. After incubation, the primary antibody was removed and replaced with a secondary antibody and left for an hour at room temperature in dark lighting. After removing the secondary antibody, cells were stained with Dapi and imaged via Light Microscopy. Plates were also stained with Xylenol Orange to visualize calcium content.

**Results**

Calcium content was quantified via Atomic Absorption (AA) and normalized to protein concentration after 7 and 14 days of treatment of an inorganic phosphate source and SOST supplementation. As evident from Figure 17 and confirmed via ANOVA, there was a significant decrease in calcium content after being treated with both low and high concentrations of SOST. There was also a significant difference and large decrease in calcium content among low and high SOST treated samples. Cells treated with the high concentration of SOST also showed lower calcium content than that of the 7-day calcified cells.
Cells were seeded in standard glass tissue culture plates and collagen-coated well plates. Statistical significance was determined by ANOVA & post-hoc pairwise test at significance level, p<0.05. Symbols indicate values that are statistically varying. (n=6)

Figure 18 shows calcium visualization in healthy, calcified, and SOST treated samples. As seen by the second row or the red channels, there is no visible calcification in control cells as compared to the remaining three calcification samples. However, there is a gradual decrease in visual mineralization as the SOST treatment concentration increases.
The first row represents the standard light microscope image. The second row is visualization of calcium content using xylenol orange on a Texas Red channel. The third row is nuclei staining using Dapi. The fourth row is a merged image with red and blue channels.

*Figure 18* Visualization of Calcium Content using Xylenol Orange Stain after SOST Treatment

*Figure 19* and *Figure 20* below aim to visualize markers associated with VSMC and the Wnt-signaling pathway. It is clearly shown in *Figure 19* that SMA was recovered after treatment with SOST. It is also shown that the higher dosage of SOST recovered a greater amount of SMA as opposed to the lower dosage of SOST treatment. *Figure 20* shows AXIN1 expression in healthy, calcified, and SOST treated samples. As seen by the second row or the red channels, there is no visible expression in control in any of the samples.

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Figure 19  Alpha-Smooth Muscle Actin Staining: Expression of SMA after SOST Treatment.

Significant recovery of expression of SMA as SOST dosage increases. The first row represents the standard light microscope image. The second row is visualization of calcium content using xylenol orange. The third row is nuclei staining using Dapi. The fourth row is a merged image with the red and blue channels.
Figure 20  AXIN1 Antibody Staining: Expression of AXIN1 after SOST Treatment.

No recovery of expression of AXIN1 as SOST dosage increases. The first row represents the standard light microscope image. The second row is visualization of calcium content using xylenol orange. The third row is nuclei staining using Dapi. The fourth row is a merged image with the red and blue channels.
CHAPTER IV
DISCUSSION

Our original goals were to first characterize and confirm Wnt-signaling within in-vitro vascular calcification of VSMCs and investigate the dose dependency of SOST as a treatment for vascular calcification. In our first experiment, we wanted to determine the best inorganic phosphate source to induce calcification in VSMCs. Our previous research confirmed that 3mM Sodium Phosphate Dibasic Anhydrous (SPDA) successfully induce significant calcification, however, within a different VSMC lineage. Therefore, we wanted to confirm our model still works for our current cell lineage. We saw an increase in calcium content within cells that were treated with 3mM SPDA, 6mM SPDA and SPD, and 12mM SPDA and SPD. However, the 3mM inorganic phosphate yielded the most calcium content among the inorganic phosphate sources. This confirms our initial hypothesis and further proves our previous research that 3mM SPDA successfully induces calcification and significantly increases calcium content.

We also wanted to determine the best cell culture environment that would promote growth and calcification. As previously mentioned, collagen has been shown to promote calcification of VSMC, as some cells have been shown to produce collagen whilst in a calcification state. To study this, we compared VSMCs seeded and calcified within standard glass well plates to VSMCs seeded and calcified into collagen-coated well plates. Although we saw an increase in calcium content in the cells grown in the collagen-coated plates, the
difference was not significantly different from that of the cells grown in standard glass well plates. We concluded that this is possibly due to the fact that the cells are already producing collagen while they are calcifying, so adding them to a collagen environment does not entirely create a different environment.

After confirming successful induction of calcification, we wanted to characterize in-vitro calcification. SEM imaging displayed the properties of the cells from their intactness after the fixation protocol. Sample processing for SEM imaging involves a series of fixations in Karnovsky’s fixative and Osmium Tetroxide and dehydration within a graded series of ethanol. This prove to be a harsh environment for VSMCs, as shown by the damaged membranes and visibility of the nucleus. However, calcified cells were seemingly not as impaired compared to the control cells. To further confirm the mechanical properties of the healthy versus calcified cells, we performed AFM. Imaging using AFM displayed a significantly higher elastic modulus, stiffness, and peak height for cells treated with inorganic phosphate. Therefore, we concluded that calcified cells are much harder, rigid, and not as flexible as healthy cells. This further suggest that VSMCs in vascular calcification are indeed undergoing a phenotypic modulation into osteoblast-like cells from their healthy contractile phenotype.

When visualizing protein expression, there was a loss of SMA and AXIN and an increase in RUNX2 expression in calcified cells. The loss of SMA and the gain of RUNX2 expression is consistent with previous literature and studies regarding the switch to osteoblast-like cells and the involvement of Wnt-signaling. AXIN showed decreasing expression in increasing calcification, although it is usually always present in some capacity. As previously mentioned, the role of AXIN is to promote degradation of β-catenin to prevent
Wnt-signaling. Therefore, the decrease of AXIN could be due to suppression because of the accumulation of β-catenin. We can, however, conclude that Wnt-signaling is involved based on the increasing expression of RUNX2 and the early expression of AXIN.

Lastly, we examined the effects of SOST treatment on calcification. When investigating dosage dependency, we saw a decrease in calcium content of cells treated with a low and high concentration of SOST. Moreover, VSMCs treated with high concentrations of SOST showed a significantly lower calcium content than those treated with the low concentrations SOST treatment. This confirms that supplementation of SOST has an inhibitory effect on calcification, as well as confirms our hypothesis that it can be used as a therapeutic agent. This was also confirmed through the visualization of calcium content and antibodies after staining, where it is shown that calcium content decreases as the SOST dosage increases. α-SMA was also successfully recovered after SOST treatment from both low and high dosages. On the other hand, AXIN1 was not present in VSMC treated with SOST. It is unclear why AXIN1 was not recovered after treatment, but it is possibly due to the fact that as times proceeds, there is a gradual reduction in AXIN1 expression. This is also in support of the concept that aging plays a role in the modulation of VSMCs to a synthetic phenotype.

Concluding, our study was effective in characterizing and confirming the role of Wnt-signaling. We were also able to identify VSMC and Wnt-signaling markers and track their expression or lack-there-of during vascular calcification and determine mechanical properties in correlation with healthy vs calcified cells. Most importantly, we were able to confirm that SOST can be an effective method of combating calcification, further implicating progression towards a treatment.
CHAPTER V
FUTURE WORKS

The study proves that inorganic phosphate is effective with inducing calcification and stimulating the phenotypic modulation of VSMCs. It was also further confirmed that our previous in-vitro model of supplementing VSMCs with 3mM Sodium Dibasic Phosphate Anhydrous is still successful with significant mineral deposition within a different cell line. Moreover, SOST proves to be an effective therapy against calcification. These conclusions are supported by quantification and visualization of calcium content in experimental cell-culture plates at various time points. We also identified and characterized mechanical properties of calcified/non-calcified VSMCs, as well as, stained for antibodies within calcified cells and SOST-treated calcified cells. However, to ensure the true effectiveness of the in-vitro model of calcification and SOST as a treatment, the study must be expanded to include the following objectives:

1. Expand calcification studies to include examination of contractile and synthetic cells to assess differences among cell phenotypes as it relates to calcium content.

2. Incorporate co-culture calcification studies with endothelial cells to create a better model to represent the structure of an artery.

3. Perform ELISA to detect and quantify β-catenin in calcified cells after varying time points, as well as, after SOST treatment.
4. Perform Luciferase Reporter assays to quantify Wnt within cells that have been supplemented with inorganic phosphate before and after they are treated with SOST to assess the true effectiveness of SOST treatment on halting and/or reversing the osteogenic phenotype.

5. Further expansion on the in-vitro model to include:
   a. Treatment times extended beyond 14 days.
   b. Antibody analyses at earlier timepoints to further pinpoint the initiation of calcification.
   c. Quantify protein expression in healthy vs. calcified cells.
   d. Evaluate the mechanical properties of calcified cells at varying time points and before and after SOST treatment.

In order for our model to be further evaluated in vivo, it is imperative to incorporate the recommendations previously mentioned. It is also necessary to consider intimal vascular calcification to determine the effectiveness of Sclerostin treatment. Although, our studies prove to be promising and reliable in efforts to examine calcification and SOST.

SOST has evident potential in acting as treatment against calcification, however, its mechanism by which it occurs also needs to be better understood. This should involve determining the way by which its inhibition occurs and considering other key players that may be involved. If these suggestions are to be applied, this would implicate a promising progression towards a localized treatment for vascular calcification.
REFERENCES


