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Examining the Role of Wnt Signaling in Vascular Calcification

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I. INTRODUCTION

Cardiovascular disease (CVD) is a worldwide epidemic. As the leading cause of death across the world, many researchers are working to better understand the causes and mechanisms resulting in the disease [1]. One of the predictors of and contributors to cardiovascular mortalities is vascular calcification, the buildup of hydroxyapatite deposits within the arterial wall. The mineral deposition can occur in either the intimal or medial layers of the arteries; the location depends upon the causative factors and can create different effects within the body [2]. Conditions conductive of calcification include but are not limited to hypercalcaemia, hyperphosphatemia, and mechanical stress which induce changes to the arteries on a cellular level. Medial calcification, which occurs within the vascular smooth muscle cells (VSMCs) lining the arterial wall, has been linked to hypertension, stiffness, and increased risk of heart failure [3]. The goal of this thesis is to better understand the role that VSMCs play in medial vascular calcification. To accomplish this goal, the first objective is to provide an extensive literature review on the subject. The second objective is to observe these finding firsthand by culturing VSMCs to induce calcification.

II. OBJECTIVE 1: LITERATURE REVIEW

2.1 Background

VSMCs are derived from mesenchymal stem cells and exist primarily as two phenotypes, contractile and synthetic, but they are not terminally differentiated. They are known to maintain their plasticity and can differentiate into other mesenchymal cell derivatives [4]. A depiction of VSMC phenotypes can be seen in Figure 1. During calcification, studies have shown VSMCs undergo a cellular mediated switch into cells resembling bone forming osteoblasts, characterized by a loss of smooth muscle markers and an upregulation of osteogenic markers [5]. Runx2, a

transcription factor necessary for osteoblast differentiation, is upregulated within calcifying VSMC and may be resulting in the transdifferentiation [6]. Runx2 is a target gene of the Wnt signaling cascade, which is known to regulate bone development during embryogenesis, as well as direct bone turnover and remodeling [7]. Because mineral deposition in VSMCs appears very similar to bone formation, many studies have begun investigating Wnt signaling as a possible mechanism and regulator of vascular calcification.

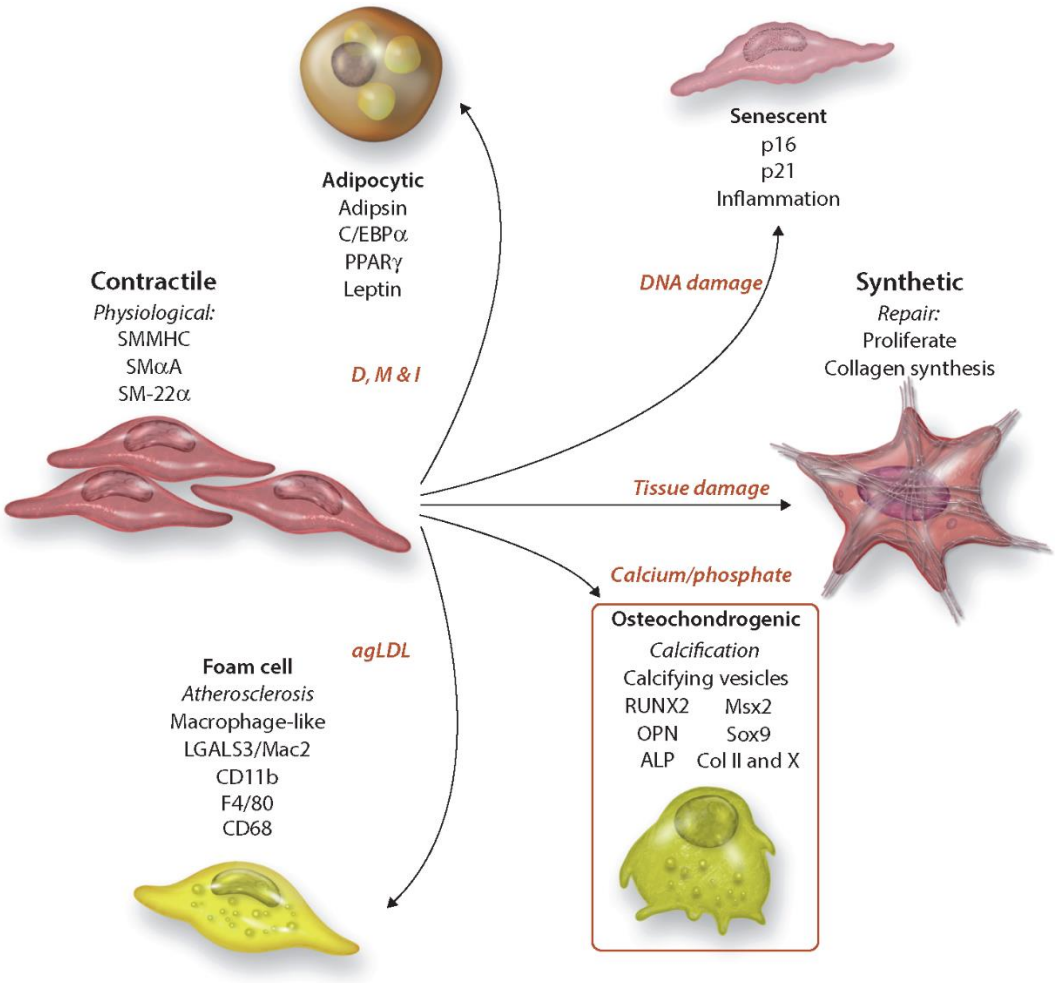


Figure 1. Depiction of a possible range of VSMC phenotypes in response to various environmental stimulus. Abbreviations indicate dexamethasone (D), methylisobutylxanthine (M), and insulin (I). The osteogenic phenotype observed during calcification is boxed in red [4].

2.2 VSMC Phenotypes

Before the 1960s, it was commonly thought that there were two cell types in the arterial media: smooth muscle cells and fibroblasts. The reason for this assumption was the presence of connective tissue within the layer, like that formed by fibroblasts. However, in the early 1960s, studies began providing evidence for only one cell type, smooth muscle cells, within the arterial media [8], [9]. In 1967, Wissler suggested that the medial cells are a multifunctional mesenchyme cell type capable of both contracting and fabricating connective tissue. Wissler was one of the first to realize smooth muscle cell plasticity and that the once thought fibroblasts are really a dedifferentiated smooth muscle cell [10]. Over the next few decades, much more was learned about the range of VSMC phenotypes. The principal function of VSMCs within the body is to maintain blood pressure. To achieve this function, the cells primarily maintain a contractile phenotype that is characterized by slow proliferation, response to neurotransmitters, and expression of cellular markers such as α-smooth muscle actin, smooth muscle myosin heavy chains 1 and 2, calponin, and smoothelin [4]. When in the contractile-state, the cytoplasm of the cell contains primarily myofilaments, with a low number of other organelles such as rough endoplasmic reticulum, Golgi, and free ribosomes [11]. In response to a necessary change in function, such as the need for increased proliferation, VSMCs may display a dedifferentiated phenotype commonly referred to as synthetic. The synthetic state is characterized primarily by proliferation and extracellular matrix production, so the cytoplasm of the cells contains a greater amount of rough endoplasmic reticulum, Golgi, and free ribosomes and a smaller number of myofilaments [11]. The distinctions between the contractile and synthetic phenotypes can be seen in Figure 2. The need for greater proliferation as seen in the synthetic state can arise in various circumstances but has been particularly characterized in response to vascular injury.

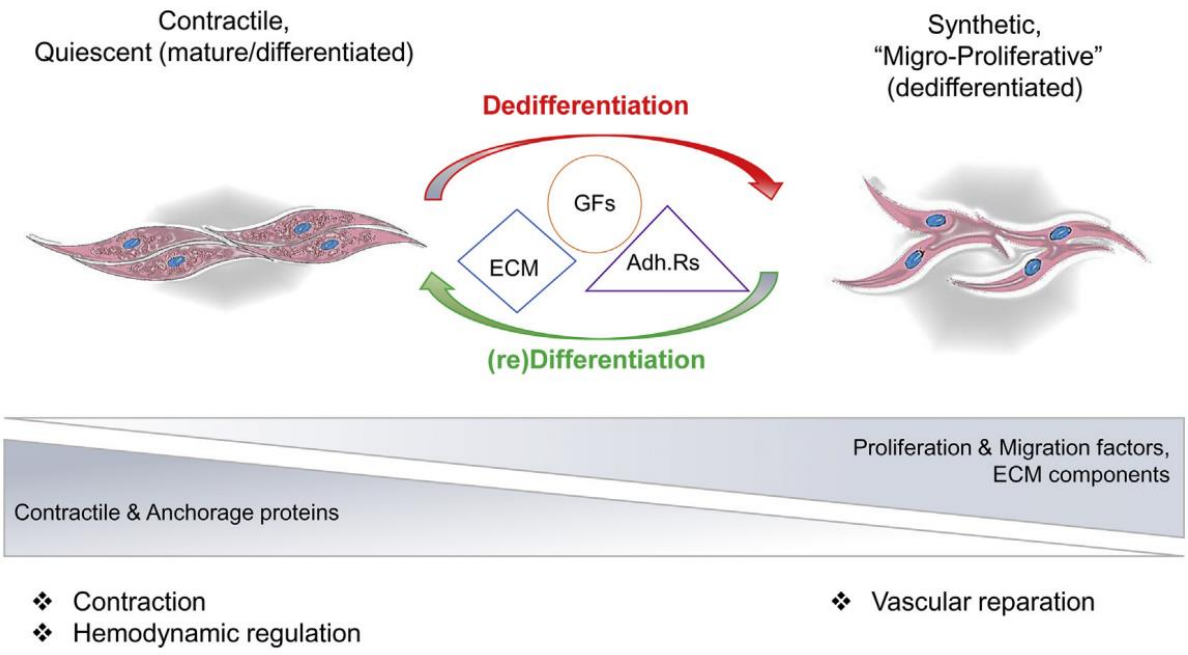


Figure 2. Depiction of the differences between the contractile and synthetic VSMC phenotypes. Most VSMCs exist in the contractile state on the left to maintain blood pressure, but they can dedifferentiate into the synthetic phenotype on the right as necessary for proliferation and tissue repair [12].

2.3 VSMC Transdifferentiation

For vascular repair to occur, the VSMCs revert to the synthetic state explained above. However, as the synthetic phenotype occurs as a dedifferentiated state, the cells can then further differentiate into other cell types depending on environmental cues. VSMCs have been shown to downregulate contractile proteins and display characteristics of other mesenchymal lineage cell types, including those of osteoblasts, chondrocytes, and adipocytes [13]. In pro-calcifying conditions, such as high levels of serum phosphate, VSMCs begin to express osteogenic markers including Runx2, Sp7, osteopontin, osteocalcin, alkaline phosphatase, Sox9, and collagen types II and X [4]. A study done by Patel et al sought to evaluate the similarity between bone formation and calcification by comparing mouse osteoblasts with control and calcifying VSMCs [14]. The quantity of calcium

deposition between osteoblasts and calcifying VSMCs was similar, but osteoblasts formed many large bone nodules whereas calcifying VSMCs formed small discrete regions of calcification, shown in Figure 3.

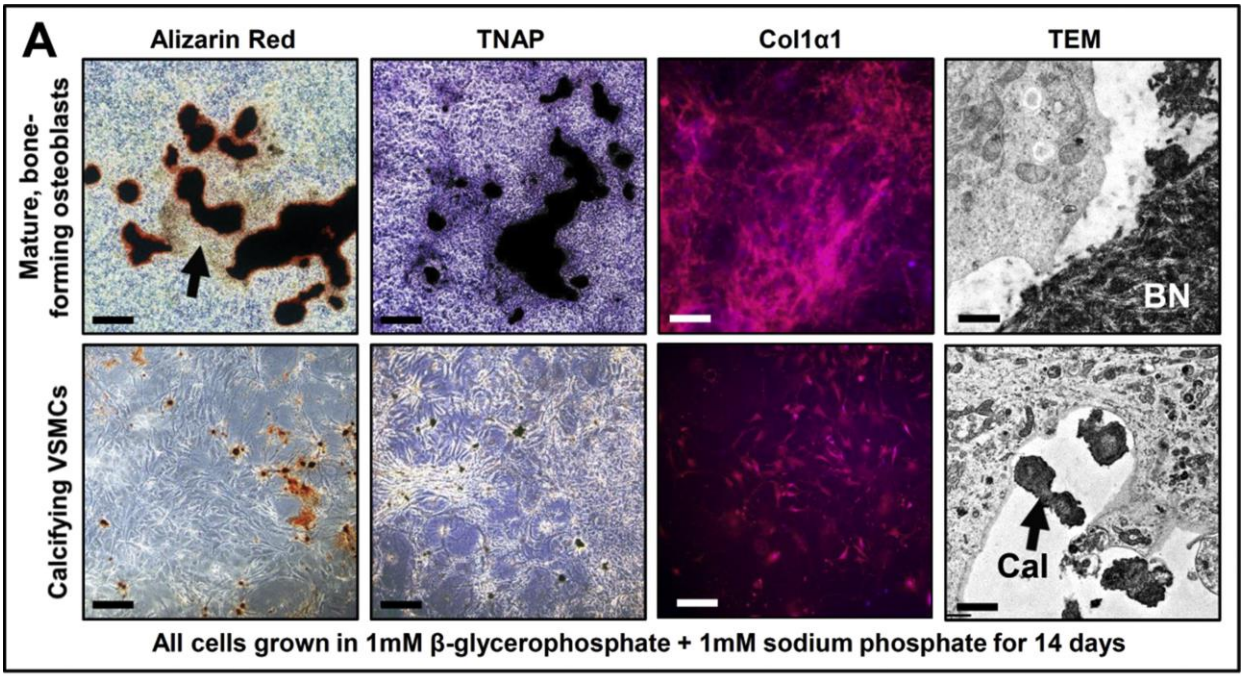


Figure 3. Results from Patel et al [14]. Osteoblasts formed large bone nodules and had more widespread collagen and alkaline phosphatase (TNAP) staining, whereas calcifying VSMCs formed small regions of calcification and had little to no TNAP staining [14].

Calcifying VSMCs saw a 6-fold increase in early osteoblast markers Runx2 and Sp7 compared to control VSMCs but still a 3-fold lower amount compared to the osteoblasts. The study concluded that calcifying VSMCs take on a transitional phenotype between but distinct from that of healthy VSMCs and bone-forming osteoblasts [14]. Many other studies have also noted the increase and possible requirement of Runx2 expression, a transcription factor necessary for osteoblast differentiation, in calcifying VSMCs [15]–[18]. Gaur et al determined the Runx2 gene is directly targeted by the canonical Wnt signaling pathway which activates the gene and regulates bone

production during development and in adults[19]. Because of the governing role of Runx2 in osteoblast differentiation and vascular calcification, studies are investigating Wnt signaling as a possible mechanism of calcification.

2.4 The Wnt Signaling Cascade

A family of 19 secreted glycoproteins, Wnt signaling is conserved among metazoan animals to regulate many cellular functions during development including cell fate determination, migration, polarity, primary axis formation, organogenesis, and stem cell renewal [20]. Wnt ligands each consist of 350-400 amino acids including 22-24 conserved cysteine residues [7]. The signaling cascade is activated when one of the extracellular Wnt ligands binds to a Frizzled (Fz) receptor. Fz is a family of ten different seven-member transmembrane proteins. A Wnt signal binds to the cysteine-rich extracellular N-terminal of a Fz receptor associated with co-receptors such as LRP5 and LRP6 of the low-density lipoprotein receptor family [20]. LRP5 and LRP6 are transmembrane proteins that help Fz to induce the canonical Wnt pathway [7]. After Wnt binds to Fz, the signal recruits the cytoplasmic phosphoprotein Dishevelled (Dsh) to the plasma membrane. At this point, the signaling pathway diverges into three separate branches: Canonical (β-catenin dependent), Planar Cell Polarity, and Wnt/Ca^{2+} [20]. The canonical branch has been most widely studied and is the segment hypothesized to play a role in vascular calcification. Continuing down the canonical branch, Dsh aids in the recruitment of an Axin and GSK3 complex [21]. Under normal, nonactivated Wnt conditions, Axin is the scaffolding protein of a β-catenin destruction complex. The destruction complex is made up of Axin, glycogen synthase kinase 3 (GSK-3), casein kinase 1 (CK1), adenomatous polyposis coli (APC) protein, and the E3-ubiquitin ligase β-TrCP [22]. The complex typically phosphorylates and proteolytically degrades any accumulation of β-catenin in the cytoplasm. When Axin is recruited to the plasma membrane because of Wnt signaling, the

destruction complex is disassembled, resulting in an upregulation of β-catenin [21]. β-catenin then translocates to the nucleus and forms a transcriptional complex with LEF-1/TCF DNA-binding transcription factors. The complex associates to the promoter of Wnt target genes that results in the upregulation of those genes [20]. In summary, during canonical Wnt signaling, the binding of Wnt ligands to the cell membrane inhibits the β-catenin destruction complex resulting in the translocation of β-catenin to the nucleus where the transcription of Wnt target genes is induced, as seen in Figure 4.

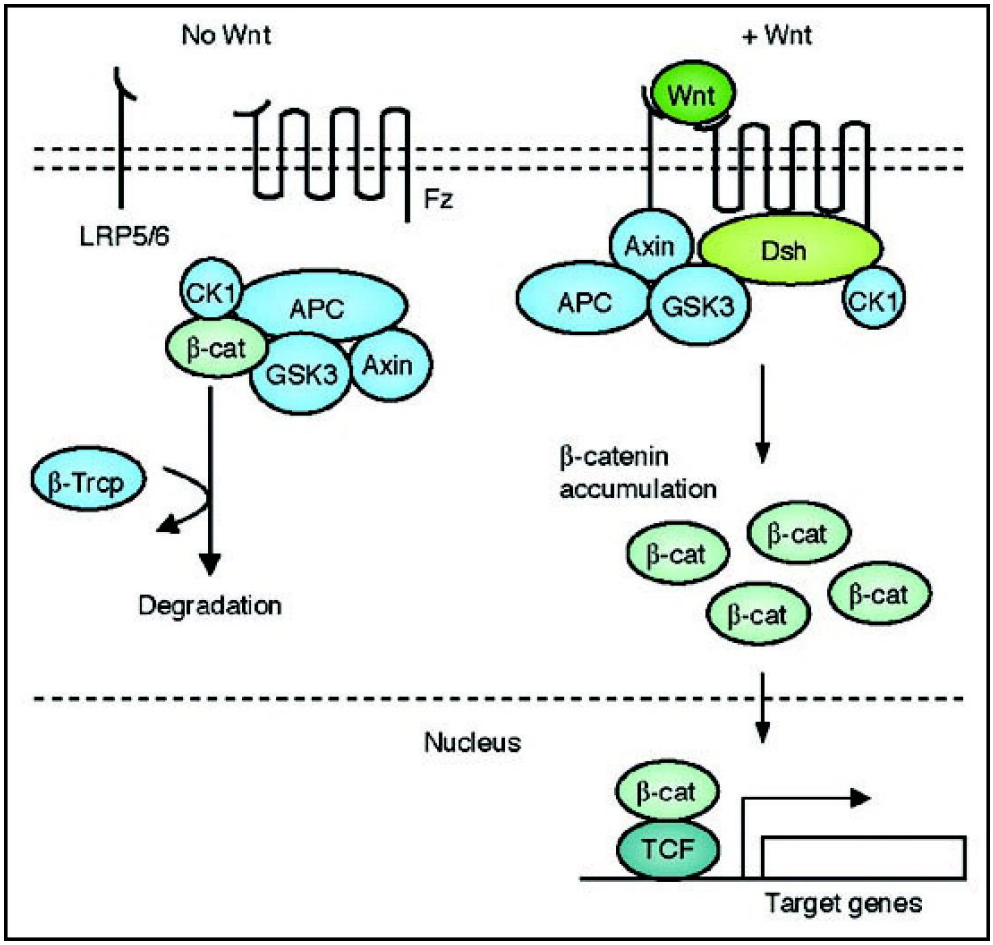


Figure 4. Schematic of the canonical Wnt signaling cascade. Binding of a Wnt ligand to the cell surface causes the translocation of β-catenin to the nucleus and the upregulation of Wnt target genes [20].

2.5 Wnt Signaling in Vascular Smooth Muscle Cells

Because Wnt is involved in bone turnover and calcifying smooth muscle cells resemble osteoblasts, Wnt may play a governing role in calcification [23]. Interestingly, Wnt ligands and signals are found in noncalcifying VSMCs and may contribute to normal regulation of the VSMC phenotype, proliferation, and survival, particularly in response to vascular injury. Studies from the early 2000s indicate the presence of Wnt proteins in VSMCs. A study done in 2004 by Wang X et al used a transfection assay to show that loss of the Wnt coreceptor LRP6 function in VSMCs inhibited cell cycle progression, demonstrating a role for LRP6 and Wnt in VSMC growth and fate [24]. Another study done by Wang Z et al in 2005 noted that Fz1 is highly expressed in VSMC tissues indicating active Wnt signaling, and they suggest a role in development and response to environmental stimulus [25]. In agreement with these studies, in 2014 Wu et al found that a Wnt ligand influences VSMC migration and adhesion to collagen type I [26]. A transwell migration and wound healing assay showed that Wnt3a significantly increased VSMC migration, and an adhesion assay showed that Wnt3a treated VSMCs were able to adhere more to collagen type I, as shown in Figure 5. The study also used Western blot analysis to test for several canonical Wnt components, including β-catenin, GSK-3β, ILK, and β1-integrin. Wnt3a treatment upregulated phosphorylated β-catenin, phosphorylated GSK-3β, and ILK and activated β1-integrin in VSMCs, providing additional evidence of Wnt effecting protein expression and potential therapeutic targets [26].

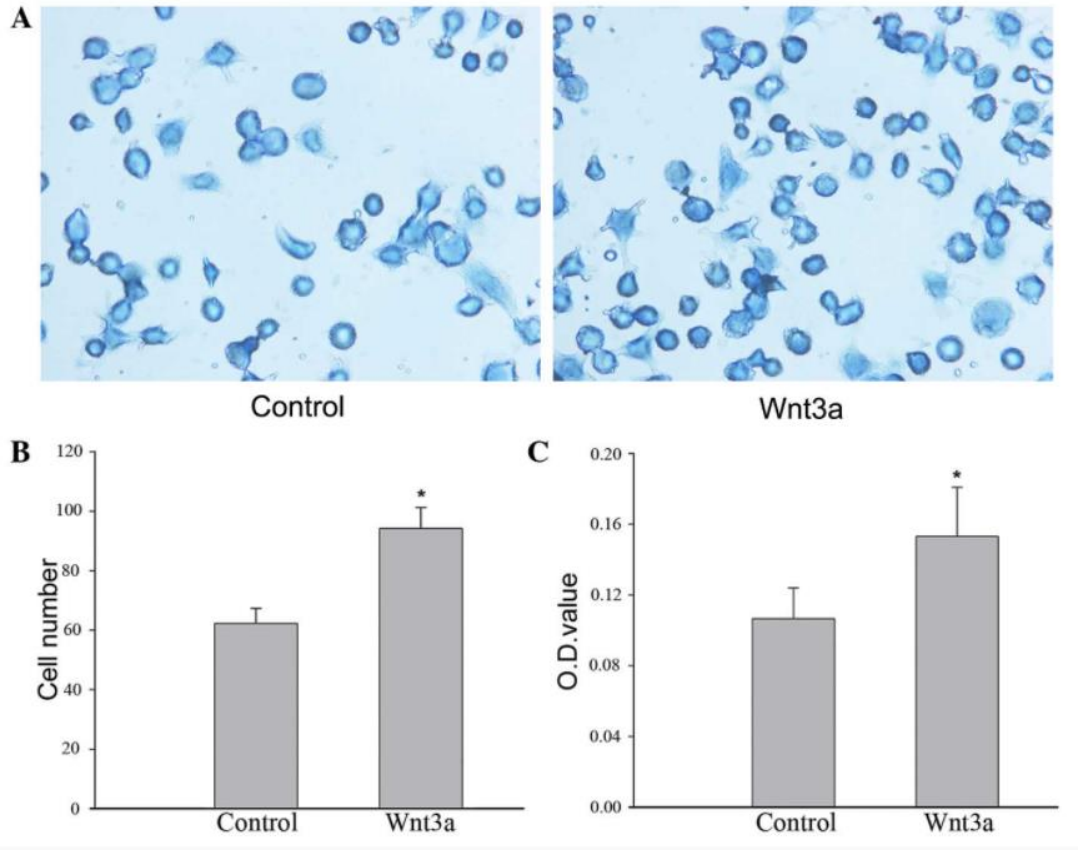


Figure 5. Results from Wu et al [26]. Toluidine blue staining showed VSCM adhesion to collagen type I (A) and showed an increase in adhesion among Wnt3a treated group (B). Optical density was significantly higher for Wnt3a treated group than control group (C) [26].

Other studies have looked at the involvement of Wnt particularly in calcifying VSMCs. Mikhaylova et al found that Wnt3a induced a 3.5-fold increase in mineralization when used with hypertrophic chondrocyte conditioned media, suggesting Wnt paired with other chondrocyte-derived factors, such as VEGF, may be a positive regulator of calcification [27]. Rong et al used phosphate and BMP-2 to induce calcification in VSMCs, and then tested the cultures for various cellular markers [5]. They were able to observe a phenotypic change as SM 22α and α-SMA were downregulated and osteogenic markers including Msx2, RunX2, Pit1, and β-catenin were upregulated. When β-catenin was knocked down using a transfection assay, these results were

reversed, indicating a dependent role of β-catenin and Wnt signaling in VSMC transdifferentiation [5]. In 2016, Cai et al also provided strong evidence for the involvement of Wnt signaling in VSMC transdifferentiation and mineralization, as seen in Figure 6 [6]. In a pro-calcifying high phosphate environment, RunX2 expression was induced in a time-dependent manner, observed by Westem blot analysis. To determine if the increase in RunX2 expression was caused by Wnt signaling, they used Western blots to track β-catenin activity and found that both dephosphorylated and phosphorylated β-catenin was upregulated. Immunostaining showed the high phosphate treatment also promoted β-catenin translocation to the nucleus, a typical cellular response to Wnt signaling, and they were able to use luciferase reporter assays to identify two specific TCF binding elements that mediate the interaction with TCF in response to β-catenin translocation. Further evidence of Wnt included a positive result for phosphorylation of LRP6, a Wnt dependent reaction. To confirm these results, a Wnt inhibitor was used that abolished RunX2 induction during high phosphate treatment. VSMCs were then treated with Wnt3a, which successfully induced calcium deposition and osteocalcin induction. The results from Cai et al suggest a specific pathway in which Wnt3a can activate β-catenin and induce RunX2 and osteocalcin expression and promote calcification of VSMCs [6].

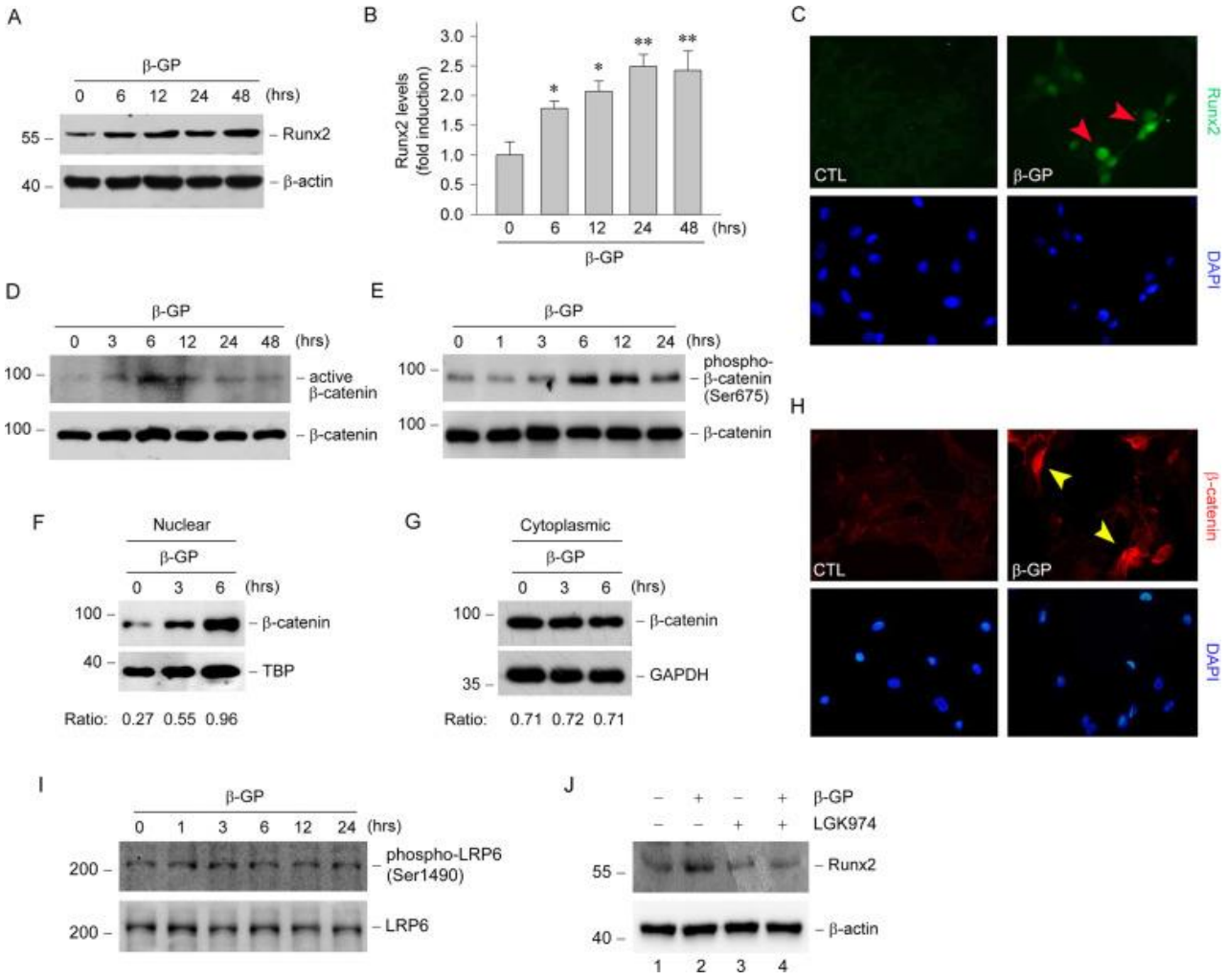


Figure 6. Results from Cai et al [6]. Western blots showed β-glycerophosphoricacid (β-GP) induced RunX2 expression in VSMCS (A, B). Immunostaining showed a positive result for localization of RunX2, as indicated by red arrows (C). Western blots showed β-GP induced β-catenin activation in VSMCs (D,E). Western blots and immunostaining showed β-GP induced β-catenin nuclear translocation, indicated by yellow arrows (F-H). Western blots showed β-GP induced LRP6 phosphorylation (I). Western blots showed inhibition of Wnt decreased RunX2 induction (J) [6].

2.6 Which Wnt?

Though there is surmounting evidence of the involvement of Wnt in vascular calcification, each study identifies different inducers, inhibitors, and regulatory proteins relative to Wnt and even

other signaling pathways, such as VEGF and BMPs mentioned previously. Though must be studied independently, it is likely that each of these different processes may contribute and work together to create an environment conducive to VSMC transdifferentiation and thus calcification. As Wnt has been more heavily investigated as a possible mechanism, some studies have begun testing specific Wnt ligands to determine if one contributes most significantly toward calcification. Wnt7b has been shown to play a role in vascular development and can activate canonical Wnt signaling [25]. Wnt16 is also expressed in VSMCs. In a recent study by Behrmann et al, in vitro and in vivo studies showed that Wnt16 suppressed the contractile phenotype, supported osteofibrogenic matrix metabolism, and contributed to aortic stiffening [28]. However, the most studied Wnt ligand in vascular calcification is Wnt3a. Some of these studies were referenced in the previous section, including those by Wu et al, Mikhaylova et al, and Cai et al [6], [26], [27]. These studies provide some of the most convincing support for the Wnt signaling cascade in calcification, so Wnt3a may be the focus of future studies working toward a treatment.

2.7 Literature Review Results

Many key points were identified when searching for the role of VSMCs in calcification throughout literature. One primary conclusion was that VSMCs display a range of phenotypes within two key states: contractile and synthetic. When VSMCs exist in the synthetic state, they exhibit a less differentiated phenotype and can be directed down different cell lineages in response to abnormal environmental cues. Another key point was that a high phosphate environment can induce calcification in VSMCs and direct them toward an osteogenic phenotype. This transdifferentiation is characterized by a loss of muscle markers and an increase in osteogenic markers, most notably RunX2. Because of the upregulation of RunX2, many believe the canonical Wnt signaling pathway may be the cellular mechanism resulting in the osteogenic switch of VSMCs. RunX2 is a target

gene of Wnt, which also involves many other proteins, including β-catenin, LRPs, Fzd, Dsh, and Axin. Wnt has been found in to regulate normal VSMC phenotype and proliferation, and much data has been elucidated for a role in VSMC transdifferentiation during calcification. Many studies have used Western blots, PCR, and luciferase assays to identify various Wnt proteins, notably β-catenin, and track the Wnt cascade to determine its involvement in the initiation and regulation of medial vascular calcification. This literature review has broadened the understanding of the role of VSMCs in calcification and helped to identify ways in which to further study the mechanism of the disease.

III. OBJECTIVE 2: CELL CULTURE STUDIES

3.1 Materials and Methods

Purpose

The purpose of the cell culture studies was to develop an *in vitro* cell culture calcification model that could then be used to observe changes within VSMCs during calcification.

Cell Culture

A concentration study was performed to determine an optimal cell culture calcification model. Human aortic smooth muscle cells (HASMCs) were cultured in six well plates. Passage number four was used for this experiment. Cells were cultured in a control media, consisting of 1X Dulbecco's Modification of Eagle's Medium with 4.5 g/L glucose, L-glutamine, and sodium pyruvate supplemented with 1% penicillin and 10% fetal bovine serum, until 80% confluence. Calcification media was then used to induce calcification in experimental groups. Calcification media consisted of control media with either (a) sodium phosphate dibasic anhydrous (DA) (Sigma), (b) sodium phosphate dibasic (D) (Mallinckrodt) or (c) sodium β-glycerophosphate

pentahydrate (βGP) (Alfa Aesar) and ascorbic acid (Fisher). Concentrations of dibasic sodium phosphate tested include 3 mM, 6mM, and 12 mM as found in other studies [16], [29], [30]. Concentrations of β-glycerophosphate tested included 5 and 10 mM with 50 ug/mL of ascorbic acid as used in other studies [31]–[35]. HASMCs were cultured in calcification media for five to seven days, and then the protein and calcium content were extracted for analysis.

Atomic Absorption Spectroscopy

At the end of the trail period, 0.6 N HCl was used to decalcify the cells for 24 hours. The HCl supernatant was collected, and 0.1 N NaOH/.1% SDS was used to solubilize the cell layers. The calcium deposition of each sample was determined by measuring the HCl supernatant with flame atomic absorption spectroscopy. The calcium content was then normalized with the cellular protein content for each sample, determined by measuring the solubilized cell layers with a BCA protein assay kit (Pierce).

Statistical Analysis

Statistical analysis was performed on the normalized calcium content results with a student's t-test. P-values less than 0.05 were considered significant.

3.2 Cell Culture Results

To determine an appropriate model to induce calcification, the calcium content of human aortic smooth muscle cell samples with various concentrations and sources of phosphate was determined. The concentration of calcium was determined using atomic absorption and a BCA assay as outlined above. The results can be seen below in Figure 7.

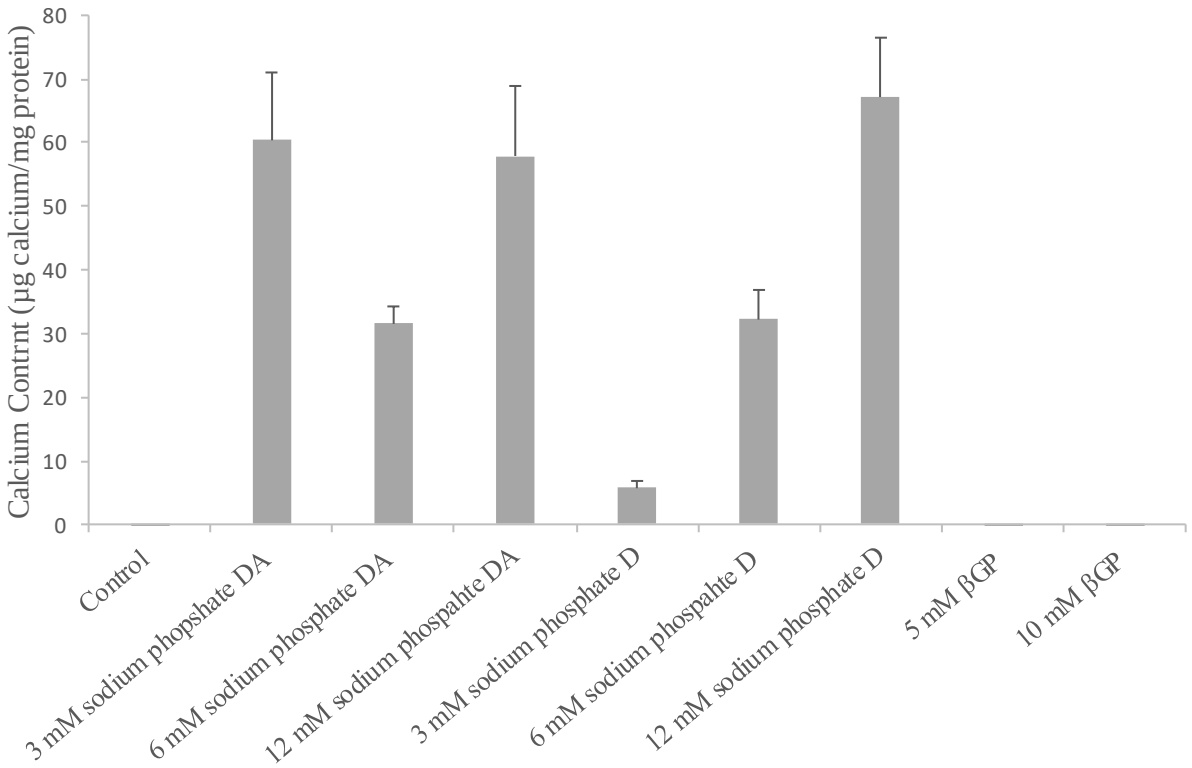


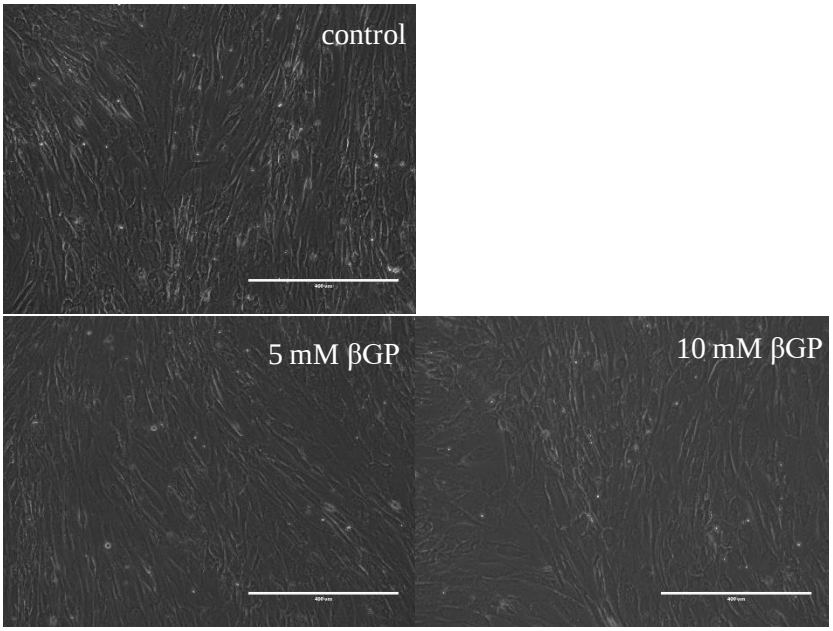
Figure 7. Calcium content of HASMCs treated with various sources and concentration of phosphate for five to seven days.

A student's t-test was performed between the experimental groups to determine significant changes in calcium content between groups. Results from the statistical analysis cam be seen in Table 1.

Table 1. P-values from student's T test. Green cells indicate $p<0.05$.

	Control	3 mM DA	3 mM D	5 mM βGP	10 mM βGP	6 mM DA	6 mM D	12 mM DA	12 mM D
Control	-	4.2E-07	1.82E-08	5.54E-02	1.87E-01	4.37E-11	5.84E-09	2.75E-07	5.42E-09
3 mM DA	-	-	1.28E-06	4.45E-07	4.47E-07	1.90E-04	3.97E-04	7.46E-01	3.17E-01
3 mM D	-	-	-	2.74E-08	5.05E-08	1.20E-09	9.34E-08	9.74E-07	1.88E-08
5 βGP	-	-	-	-	8.14E-01	4.85E-11	6.61E-09	2.94E-07	5.79E-09
10 βGP	-	-	-	-	-	5.48E-11	6.81E-09	2.94E-07	5.80E-09
6 mM DA	-	-	-	-	-	-	7.64E-01	2.87E-04	4.28E-06
6 mM D	-	-	-	-	-	-	-	5.42E-04	9.43E-06
12 mM DA	-	-	-	-	-	-	-	-	1.65E-01
12 mM D	-	-	-	-	-	-	-	-	-

All the experimental groups except for those treated with βGP had a significant increase in calcium content compared to the control group. 6 mM sodium phosphate DA had a significant decrease in calcium when compared to 3 mM sodium phosphate DA. However, 6 mM and 12 mM sodium phosphate D had a significant increase in calcium compared to 3 mM sodium phosphate D. 12 mM sodium phosphate D had the highest calcium content, but it was not significantly more than the 3 mM and 12 mM sodium phosphate. Images of the HASMCs from each treatment group on day five of culture can be seen below in Figure 8.



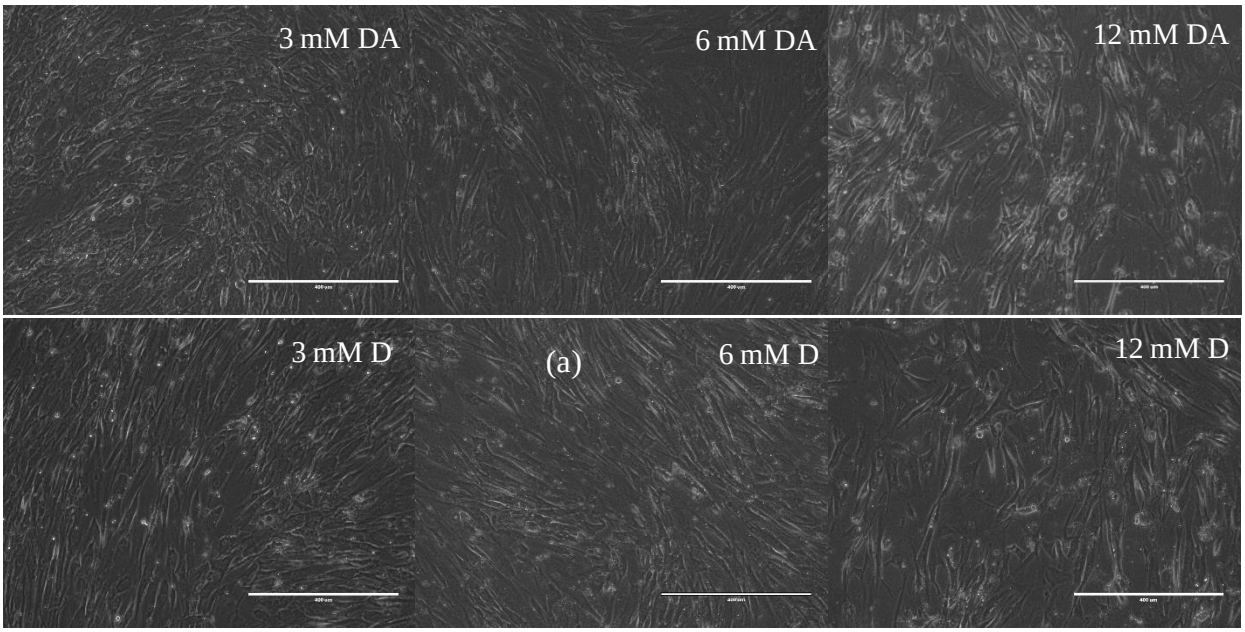


Figure 8. Microscopic images of HASMCs on day five of culture. Experimental group labeled on figure.

IV. DISCUSSION

4.1 Analysis

During the literature review, many studies used high levels of inorganic phosphate to induce calcification of VSMCs. Based on these studies, several sources and concentrations of phosphate were chosen including 3 mM, 6 mM, and 12 mM sodium phosphate dibasic anhydrous (DA), sodium phosphate dibasic (D), and sodium β-glycerophosphate pentahydrate (βGP). Interestingly, β-glycerophosphate pentahydrate showed no increase in calcium concentration compared to the control. Some previous studies that have successfully induced calcification with β-glycerophosphate also supplemented the media with insulin, so it is possible that this component may be necessary to induce calcification [31], [34]. However, all the sodium phosphate groups did have a significant increase in calcium content; each were determined to have successfully induced calcification in the HASMCs. Calcium content within the sodium phosphate D group did significantly increase with an increase in concentration, but this was not true within the sodium

phosphate DA group. This discrepancy could possibly be caused if apoptosis had been induced in some groups before calcification was able to occur. Though 12 mM sodium phosphate D had the highest calcium content, it was not significantly more than the 3 mM and 12 mM sodium phosphate DA. Based on these results, 12 mM sodium phosphate D and 3 mM and 12 mM sodium phosphate DA provided the best model of vascular calcification in HASMCs. It is also important to consider the limitations of how a 2D *in vtiro* culture can independently affect the phenotype of cells. Campbell and Campbell observed that when SMCs were seeded at a high density of 10^6 cells/ml, they maintained their contractile phenotype [36]. However, when seeded at a lower density between $5x10^4$ to $1xl0^5$ cells/ml, the cells transitioned to the synthetic state after a few days and returned to the contractive state once confluence is achieved. When seeded very sparsely at $1x10^3$ to $5x10^3$ cells/ml, SMCs appeared to remain permanently in the synthetic state. Similar results were seen upon subculture with comparable seeding densities [36]. As the literature review indicated that VSMCs may need to first be in a synthetic state to undergo transdifferentiation, seeding density may be an important factor to take into consideration during future studies. Another limitation to this study would be the five to seven days trial period. Some other studies have observed cells needed 10-14 days to induce calcification, or even longer [31]–[34]. If the current study had been done for an extended period, it is possible that more of the experimental groups may have induced calcification in the HASMCs.

4.2 Conclusion

Based on the results of this study, sodium phosphate dibasic supplemented media can be successfully used to induce calcification in HASMCs. This cell culture model could be used in the future to observe the transdifferentiation of VSMCs during calcification and to study the signaling pathways, such as canonical Wnt, which may be involved. These studies will hopefully

provide greater insight into the mechanism behind medial vascular calcification. If the primary signaling cascades and regulatory proteins can be identified, manipulation of these mediators may allow for the creation of a target treatment or preventative for the disease.

4.3 Future Works

To confirm the results, this study should be repeated to see if the results are reproducible. Another study may help to clarify whether calcification increases with an increase in sodium phosphate concentration and to determine which concentration produces the best model. When repeated, it would be beneficial to consider the seeding density and phenotype of the cells before calcification is induced and to extend the study to at least 14 days. Upon confirmation of the sodium phosphate dibasic supplemented media model, the model could then be used to quantity VSMC phenotypic change. This could be done using PCR, immunohistochemistry, and Western blots at various time points to test for cellular markers relevant to SMCs, osteoblasts, and Wnt signaling. Luciferase reporter assays could also be used to track Wnt signaling within the cells.

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