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Examination of the Effects of Vitamin D Supplementation on the In Vitro Calcification of Vascular Smooth Muscle Cells

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Examination of the effects of vitamin D supplementation on the in vitro calcification of vascular smooth muscle cells

By

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Examination of the effects of vitamin D supplementation on the in vitro calcification of vascular smooth muscle cells

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Medial calcification refers to mineral deposition in the middle layer of arteries. This mineralization is common in chronic kidney disease patients and causes an increased chance of cardiovascular complications. Calcitriol, the active form of vitamin D, is often administered to these patients to treat an associated condition, secondary hyperparathyroidism. Unfortunately, calcitriol treatment may promote vascular calcification due to increasing serum calcium and phosphate. We examined the effects of calcitriol supplementation on vascular smooth muscle cell (VSMC) calcification, through atomic absorption, scanning electron microscopy, and western blot analysis. Additionally, we examined the effects of the combinations of calcitriol, fibroblast growth factor 23 (FGF-23), and klotho. We determined that calcitriol supplementation alone increased calcification but was not associated with a transition towards an osteoblast-like phenotype. On the other hand, the combination of calcitriol and FGF-23 caused a decrease in calcification, but this decrease was attenuated with the further addition of klotho.

DEDICATION

I would like to dedicate this work to my family. In particular, to my parents, Suzanne and Neil Bennett, and brother, Chad Bennett. Thank you so much for always supporting me and for pushing me to make the most out of life. I hope that you're proud of everything that I've accomplished. I would also like to dedicate this work to Keri Lum. Thank you so much for all the emotional support and encouragement. I don't know how I would have made it through the past two and half years of graduate school without you. Lastly, I would like to dedicate this work to all my friends, who have made my time here at Mississippi State University the most wonderful experience I could imagine. Thank you all!

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Vascular Calcification

Vascular calcification is the pathological deposition of calcium-phosphate bone mineral in arteries. Two forms of vascular calcification exist depending on the location of the mineral deposition within the artery: intimal calcification and medial calcification (also known as Mönckeberg's arteriosclerosis).

As seen in Figure 1.1, the intima layer of arteries refers to the inner layer consisting of a layer of endothelial cells that lines the lumen, or inside, of the blood vessel. Intimal calcification occurs with atherosclerosis, which is the thickening of an artery wall caused by endothelial dysfunction followed by the invasion of white blood cells and the migration of smooth muscle cells. These molecular events lead to the accumulation of cells (living and dead), cellular debris, and lipids, together referred to as plaque (1). Plaque can expand such that it partially or completely occludes the lumen, which can eventually lead to problems if the downstream supply of blood becomes insufficient. Often times, the plaque can also rupture, revealing material that can induce a thrombus, or blood clot, formation in the artery. This thrombus can occlude the artery directly or break off forming a down-stream embolism. Occlusions can become life-threatening when they occur in the coronary arteries or arteries in the brain by causing a myocardial infarction (heart attack) or stroke, respectively. Intimal calcification refers to

the calcification of the plaque; however, research has been inconclusive as to the consequences of intimal calcification in atherosclerosis. Autopsies have revealed a relationship between calcification and plaque burden (2). As for rupturing, some studies suggest that the calcification of the plaque causes locations for high local stresses that can increase the chance for rupture (3). On the other hand, heavily calcified plaque is believed to be less likely to develop a thrombus (4), and finite element analysis suggests calcification may serve to stabilize the plaque, similar to the fibrous cap, by lowering the maximum stresses throughout the structure (5).

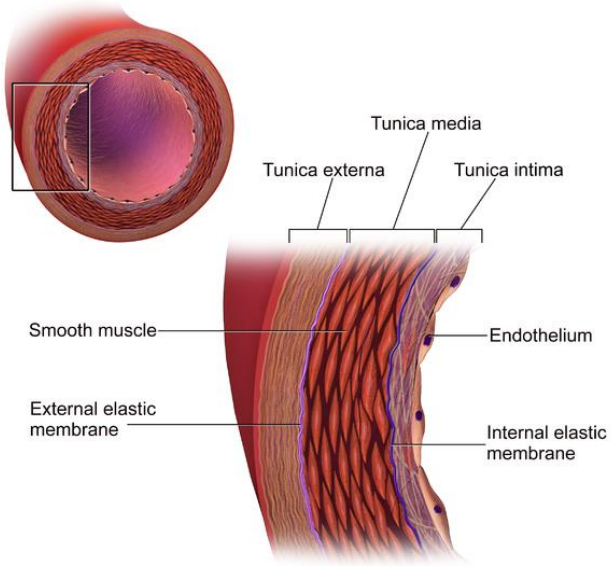

Figure 1.1 Schematic of Artery Structure

Arteries are composed of three distinct layers: tunica intima (inner), tunica media (middle), and tunica externa (outer). From Wikipedia.org

Also seen in Figure 1.1, the tunica media refers to the middle layer of arteries consisting of vascular smooth muscle cells (VSMCs) and elastic fibers composed of the extracellular matrix protein elastin. In medial calcification, mineral deposition occurs

along these elastic fibers. Medial calcification is often associated with chronic kidney disease (CKD), diabetes, and ageing. While there are not traditional symptoms due to this mineral deposition, calcification of elastic fibers causes increased arterial stiffness (measured by pulse wave velocity), which can result in damage to organs, such as the heart and kidneys. As such, research has shown that medial calcification is correlated with an increased risk of total and cardiovascular mortality in type 2 diabetes patients (6) and CKD patients on hemodialysis (7).

Intimal and medial calcification are not entirely exclusive and can be present simultaneously, especially in CKD patients with atherosclerosis. Also, research has suggested that the pathomechanism between intimal and medial calcification may share many similarities (8). However, they remain distinctly different both morphologically and with respect to clinical implications. For these reasons, this project with be solely focused on medial calcification in CKD patients.

Mechanism of Medial Calcification

Initially, medial calcification was considered a passive process, in which the calcium and phosphate in the serum precipitated out and formed minerals along the extracellular matrix. However, it is now widely accepted to be an active cellular-process similar to bone formation. Many factors are believed to be involved in the initiation of this process, such as inflammatory molecules, certain growth factors, and oxidative stresses; however, for CDK patients, the most important factor is high concentrations of calcium and phosphate. Numerous studies have shown that phosphate and calcium are able to increase calcification in a concentration-dependent and synergistic manner (9-13). Some of the key events that are believed to be required for the initiation of vascular

calcification include: 1) the trans-differentiation of VSMCs into osteoblast-like cells, 2) the release of matrix vesicles and apoptotic bodies, 3) the loss of calcification inhibitors, and 4) the degradation of the extracellular matrix.

The resident VSMCs in the medial layer are able to transdifferentiate into osteoblast-like cells due to their shared mesenchymal stem cell lineage. Observed both *in vitro* and *in vivo*, this phenotypic transition is characterized by the loss of smooth muscle lineage markers, such as smooth muscle α-actin (αSMA), smooth muscle myosin heavy chain (SM-MHC) and SM22α, and the increased expression of bone genes and proteins, such as core binding factor α1 (Cbfa1/runx2), osteopontin (OPN), osteocalcin (OCN), and alkaline phosphatase (ALP) (13-16). The produced bone proteins, especially ALP, promotes the deposition of the calcium-phosphate mineral. Intracellular phosphate is directly involved in the phenotypic transition, as the addition of phosphate alone is capable of inducing this phenotypic transition in VSMC cultures (13). Calcium is also involved in the phenotypic change, but it is believed to be an indirect action, as the increase in calcification caused by calcium addition does not involve the upregulation of ALP (17). It is worth noting that VSMCs grown on calcified elastin *in vitro* also undergo this phenotypic switch (16); thus, it is possible for calcium's role in the phenotypic switch to be through increasing the mineral deposition through the other main roles, which in turn, further promotes the differentiation towards osteoblast-like cells.

Two different membrane bound bodies, matrix vesicles (MV) and apoptotic bodies (AB), play large roles in the initiation of mineral formation. MVs are small (20-200 nm) extracellular vesicles formed from budding off of the outer plasma membrane of viable cells. They contain large amount of calcium and phosphate, providing a suitable

microenvironment for the nucleation of hydroxyapatite formation. This occurs naturally in the calcification of bone, cartilage, and dentin (18), but research has shown that they are also involved in pathological conditions, specifically medial calcification (12, 19-21). TEM images of calcified bovine VSMCs grown in the presence of β-glycerophosphate and ascorbic acid revealed membrane-bound MVs associated with calcification (20). They were also found *in vivo* when observing the calcified aortas of matrix gla protein (MGP) deficient mice, which undergo extensive medial calcification (19). The addition of calcium and/or phosphate to VSMC cultures cause increase MV release (12, 21), presumably in an effort to avoid toxicity caused by high intracellular calcium and phosphate. In addition, while MVs are produced naturally, those produced from the addition of phosphate have an increased ability to calcify, associated with increased ALP activity and increased calcium uptake through annexin expression (22). ABs are larger vesicles formed from the fragmentation of cells undergoing apoptosis. *In vitro* studies show that high concentrations of phosphate and calcium increase apoptosis (12) and apoptosis increases calcification of human VSMCs (23, 24). The ABs released from apoptosis can also serve as nucleating sites for mineralization similar to MVs (12, 23). For both vesicles, high extracellular phosphate and calcium increases the tendency of these vesicles to mineralize (12).

In non-pathological conditions, natural inhibitors to calcification are present in various tissues, as well as matrix vesicles produced by VSMCs, in order to prevent ectopic calcification despite relatively high serum levels of phosphate and calcium. One such endogenous inhibitor is matrix Gla protein (MGP), a vitamin-K dependent protein expressed in VSMCs. While MGP binds directly to hydroxyapatite (25), the method of

inhibition is not entirely clear (26). Selective knock-down of MGP causes severe medial calcification (19) and low amounts of activated MGP are found in diabetes patients (27). Another inhibitor that is produced by VSMCs is pyrophosphate, which directly inhibits calcification by binding to calcium phosphate, preventing mineral nucleation and further growth. ALP promotes calcification through hydrolyzing pyrophosphate (28). There are also circulating inhibitors that are not directly produced by VSMCs, such as fetuin-A. Produced predominately in the liver (29), it also binds to calcium phosphate, preventing mineral nucleation, and can be taken up by VSMCs to prevent calcification (30). Expression of these inhibitors are decreased through the phenotypic switch of the VSMCs or through the increased secretion of MVs, allowing the mineralization process to take place.

The role of calcium and phosphate in extracellular matrix degradation (specifically elastin degradation in medial calcification) is not clear; however, research has provided a lot of evidence for involvement of elastin degradation in medial calcification. It was first discovered that matrix metalloproteinase-2 and -9 (MMP-2/-9), the proteins responsible for breaking down elastin, are upregulated in areas of calcification *in vivo* (31-33) and abolishment of this MMP activity prevents the calcification process (34-36). Later, research was able to show that the products of elastin degradation actually accelerate the mineralization process and phenotypic switch of the VSMCs (37). The most recent research has actually shown that the degradation of elastin precedes the deposition of hydroxyapatite mineral and that the mineralization only occurs along the degraded elastin fragments (32). These results, as well as belief that degraded elastin has an increased affinity of calcium (38), have lead researchers to suggest that

extracellular matrix degradation creates sites for the initial nucleation and subsequent growth of the calcium-phosphate mineral in medial calcification.

These events that are involved in medial calcification are tightly connected and are all believed to be affected by high extracellular concentration of calcium and phosphate. Due to this relationship, proper mineral metabolism is important to preventing vascular calcification, and that requires the actions of the molecules vitamin D, parathyroid hormone (PTH), fibroblast growth factor 23 (FGF-23), and klotho.

Vitamin D

Vitamin D and its metabolites are secosteroid hormones that play a key role in calcium homeostasis as well as other important roles in hormone production, immune system regulation, and differentiation of cells. Although the name implies that it is a vitamin, it is technically considered to be hormone as it is predominately produced in the body and doesn't necessarily have to be obtained through dietary intake if enough is produced endogenously. Secosteroids are similar to steroids in both structure and function. The difference in structure is the broken carbon-carbon bond in the B ring of the typical four-ring structure of steroids. Functionally, they are similar in that they both act on their specific nuclear receptor. Two forms of vitamin D exist: vitamin D_3, or cholecalciferol, and vitamin D_2, or ergocalciferol. Vitamin D_3 is produced in the human body, specifically in the skin, in response to ultraviolet (UV) radiation. Vitamin D_2 is produced in plants and fungi also in response to UV radiation and can be obtained through dietary intake. The two forms are structurally and functionally different; however, the extent of the functional differences are still highly debated. Research has shown that a single dose of vitamin D_2 produces less of the circulating metabolite than a

single dose of vitamin D_3 (39, 40); however daily doses of vitamin D_2 and D_3 maintain similar levels of the circulating metabolite (41). Due the potential functional differences as well as the fact that the majority of vitamin D is obtained endogenously, this study will be focused primarily on vitamin D_3 and its metabolites.

As alluded to previously, the metabolites of vitamin D_3 are extremely important because vitamin D_3, as produced by the skin, is inactive and requires multiple subsequent enzymatic reactions to be fully active. The structure of vitamin D_3 and its metabolites as well as the sequence of events leading from synthesis to activation can be seen in Figure 1.2. First, 7-dehydrocholesterol, present in the epidermis, is converted into pre-vitamin D_3 with UV light exposure. Pre-vitamin D_3 will then isomerize into vitamin D_3 in a temperature-dependent reaction (42). In order to prevent vitamin D_3 toxicity from prolonged UV exposure, pre-vitamin D_3 can be converted to tachysterol and biologically inactive lumisterol, which can be reversed when pre-vitamin D_3 levels drop (43). To become active, vitamin D_3 is converted into 25-hydroxyvitamin D_3 [$25(OH)D_3$], or calcidiol, through enzymatic reaction by 25-hydroxylase (CYP2R1) in the liver. This reaction is highly dependent on the amount of vitamin D_3 present and is the major form found circulating in the blood, which is why serum levels of $25(OH)D_3$ are usually measured to determine if a patient has sufficient amount of vitamin D (44). To become fully active, $25(OH)D_3$ must be converted to 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], or calcitriol, through enzymatic reaction by 1α-hydroxylase (CYP27B1) in the proximal renal tubule of the kidneys. Other tissues contain the CYP27B1 enzyme; however, they are believed to function to provide local concentration of calcitriol for paracrine or autocrine functions (45). It is also believed that this local production does not affect the

circulating concentration produced from the kidneys. Finally, calcitriol is carried to the target tissue where it exerts effects and deactivated by breaking down it to the water-soluble calcitroic acid by the enzyme 1,25-dihydroxyvitamin D_3 24-hydroxylase (CYP24A1).

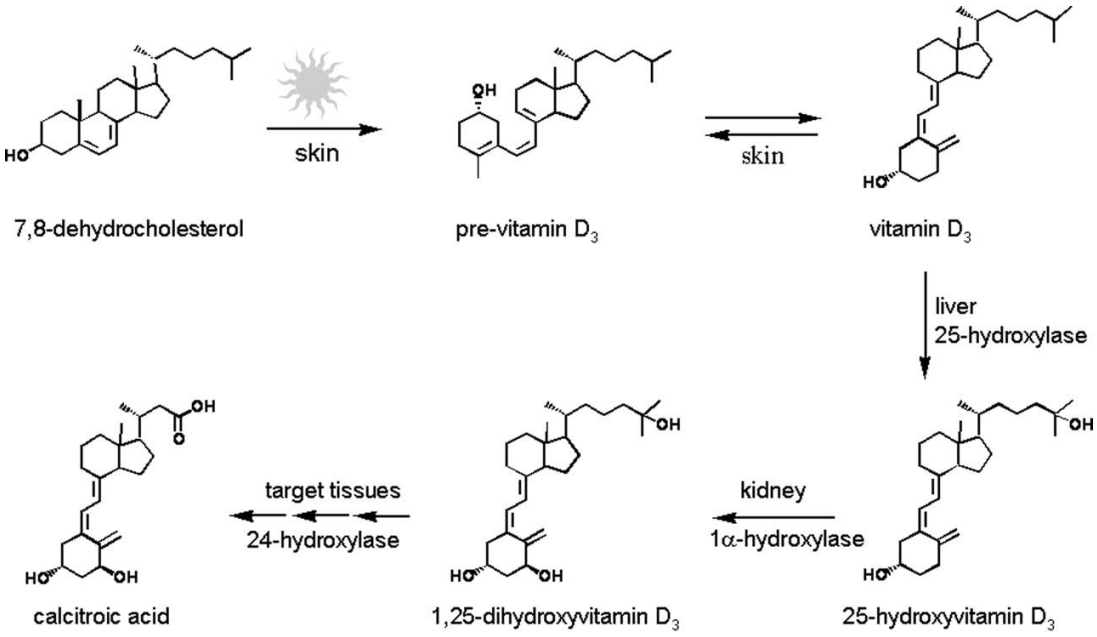

Figure 1.2 Synthesis and Activation of Vitamin D

Synthesis of vitamin D3 begins in the skin from UV radiation and goes through subsequent activation in the liver and kidneys. From (46)

Vitamin D, specifically calcitriol, elicits its response similar to traditional steroids, by acting on specific nuclear receptors. The receptor responsible for vitamin D's response is aptly named vitamin D receptor (VDR). The VDR forms a heterodimer that interacts with specific DNA sequences, causing either expression or repression of transcription. As seen in Table 1.1, there are at least 36 tissues that express the VDR. While many of these tissues are involved in calcium homeostasis and bone remodeling

(which will be discussed in more detail in a later section), the other tissues are involved in vitamin D's many nontraditional roles, including hormone production, regulation of immune system, and differentiation of cells. Research has shown that, while the mechanism isn't well understood, calcitriol stimulates insulin secretion (47) and suggests that vitamin D deficiency increases the risk for type 2 diabetes (48). As for the immune system regulation, calcitriol has a complex role, as it has an inhibitory effect on the proliferation of many cells of the adaptive immune system, such as B cell precursors (49) and T helper-1 cells (50), but is actually able to increase expression of the antimicrobial peptide cathelicidin in the innate immune system (51). Finally, calcitriol is involved in the proliferation and differentiation of cells, such as keratinocytes in the epidermis (52), and may have an inhibitory role on certain cancers (53).

Table 1.1 Tissues that Express VDR

Adipose	Eggshell gland	Muscle, embryonic	Prostate
Adrenal glands	Epididymis	Muscle, smooth	Retina
Bone	Hair follicle	Osteoblast	Skin
Bone marrow	Intestine	Ovary	Stomach
Brain	Kidney	Pancreas β cell	Testis
Breast	Liver (fetal)	Parathyroid glands	Thymus
Cancer cells	Lung	Parotid	Thyroid
Cartilage	Lymphocytes (B and T)	Pituitary glands	Uterus
Colon	Muscle, cardiac	Placenta	Yolk sac (bird)

From (54)

PTH, FGF-23, and Klotho

Regulation of the serum calcium and phosphorus levels is done through strict cooperation between the bone, kidneys, and parathyroid glands. Besides vitamin D (in the form of calcitriol), there are three other key molecules involved in this process:

parathyroid hormone (PTH), fibroblast growth factor 23 (FGF-23), and its coreceptor klotho.

PTH is an 84 amino acid long polypeptide hormone produced and secreted by the parathyroid glands in response to serum calcium (and serum phosphate to a lesser degree). The mature polypeptide is stored in secretory vesicles for release. As a regulatory mechanism, PTH undergoes intracellular degradation also in response to extracellular calcium levels, which is sensed through a G-protein-linked calcium receptor (55). When calcium levels are low (hypocalcemia), PTH secretion increases and intracellular degradation is reduced. When calcium levels are high (hypercalcemia), PTH secretion decreases and intracellular degradation is increased. Once secreted, PTH acts to restore serum calcium levels (a process that will be discussed in more detail in the next section). PTH is quickly cleared ($T_{1/2}$ = 4 minutes) through the kidneys and liver (56).

FGF-23 is a protein produced from osteocytes in bone that respond to serum phosphate levels. FGF-23 belongs to the fibroblast growth factor (FGF) superfamily; however, it differs from the majority of FGF ligands in that it does not contain a heparan-sulfate (HS) binding domain. HS is found as a proteoglycan bound at cell surfaces and in the extracellular matrix. Because FGF-23 lacks a HS binding domain, it is able to avoid matrix binding and able to move into circulation to exert its endocrine function, instead of the paracrine and/or autocrine functions of traditional FGFs (57). Only two other FGFs lack the HS binding domain, FGF-19 and -21, and together they are referred to as endocrine FGFs. FGFs bind to one of four FGF receptors (FGFR1-FGFR4), which consist of 3 extracellular domains, a single-pass transmembrane domain, and a

cytoplasmic tyrosine kinase domain (58). Because HS binding is required for FGF-FGFR binding (59), FGF-23 requires a coreceptor, klotho (60).

Klotho is considered an "anti-aging" protein that is expressed predominately in the distal convoluted tubules in the kidney. It is a type I single-pass transmembrane protein but can also be found in a soluble, secreted form from the proteolytic cleavage, or "shedding", of the transmembrane protein (61). As previously mentioned, the transmembrane klotho acts as a coreceptor for FGF-23 for phosphate regulation. The secreted klotho can be found circulating in the blood, urine, and cerebrospinal fluid (62). While not well understood, its effects are believed to be independent of FGF-23, and may play a role in regulation of calcium and potassium ion channel activity (63, 64). Klotho became known as the "anti-aging" protein because mice with defective klotho gene expression experience phenotypes that resemble accelerated aging, displaying symptoms such as shortened lifespan, infertility, arteriosclerosis, skin atrophy, osteoporosis, and emphysema (65). In addition, overexpression of klotho in mice caused increased life-span compared to wild-type mice (66). The effects of klotho can be explained in part by its role as coreceptor for FGF-23, as FGF-23 knockdown causes a similar phenotypes (67) and excess phosphate itself can causes stresses that may lead to accelerated aging.

Mechanism of Bone/Kidney-Endocrine Axis

The regulation of calcium and phosphate requires the interaction between the bone, kidneys, and pituitary glands, through the actions of PTH, calcitriol, FGF-23, and klotho. A schematic of this process can be seen in Figure 1.3.

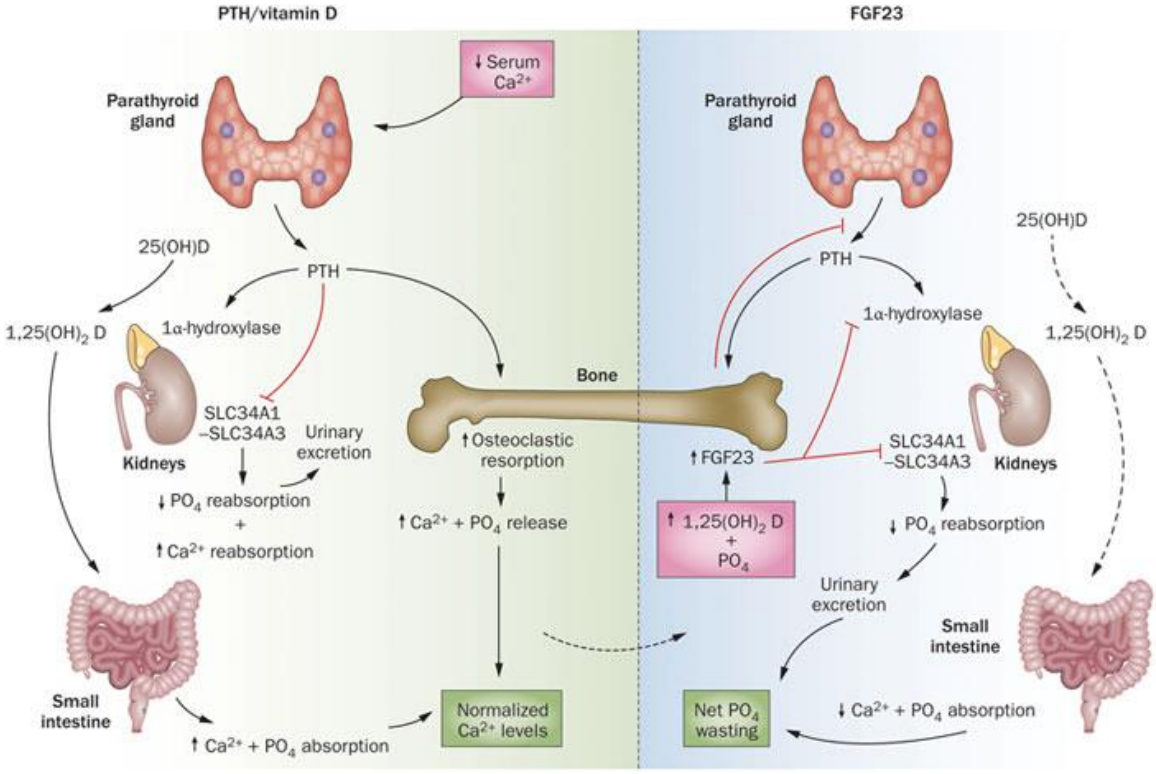

Figure 1.3 Bone/Kidney-Endocrine Axis

Serum calcium and phosphate are regulated by complex interactions between the bone, kidneys, and parathyroid glands. From (68)

When serum calcium is low, the parathyroid glands increases secretion (and decreases intracellular degradation) of PTH. PTH acts to increase calcium production through three separate functions: 1) PTH acts directly on osteoblasts in bone to increase expression of receptor activator of NF-κB ligand (RANKL), causing increased osteoclast activation and subsequent bone resorption and release of calcium and phosphate into the extracellular fluid; 2) PTH acts on the kidneys to increase the reabsorption of calcium by Ca^{2+}-ATPase and a Na^{+}-Ca^{2+}; and 3) PTH acts on the proximal renal tubules in the kidneys to increase CYP27B1 expression, causing increased production of calcitriol (55). Calcitriol acts to increase the active absorption of calcium and phosphate in the intestinal

lumen of the gut. As calcium levels normalize, the parathyroid glands sense the decrease in serum calcium and PTH secretion decreases, ceasing bone resorption. In addition to serum calcium, calcitriol acts directly on the parathyroid glands to decrease PTH secretion (69, 70).

As for phosphate regulation, an increase in serum phosphate (71), along with direct action by calcitriol (72, 73), causes an increase in FGF-23 production from the osteocytes in the bone. FGF-23 with the coreceptor klotho decreases phosphate reabsorption in the proximal tubules by reducing the expression of sodium-phosphate cotransporter type-2a (NaPi-2A) and type 2c (NaPi-2C) (74). In addition, FGF-23 lowers serum calcitriol levels through down regulation of the CYP271B1 gene and upregulation of the CYP24 gene in the kidneys, simultaneously reducing the amount of calcitriol being produced and increasing the hydrolysis of the calcitriol already in circulation (75). FGF-23 further decreases the serum levels of calcitriol by directly acting on the pituitary glands to reduce PTH secretion (76), preventing PTH from creating more active vitamin D. This action prevents the further absorption of phosphate in the intestines and allows for net phosphate wasting.

Chronic Kidney Disease

CKD is characterized by the gradual loss of kidney function and can be grouped into 5 different states, with stage 4 and 5 known as end stage renal disease (ESRD). As the kidneys stop functioning, the body's method of maintaining proper calcium and phosphate levels also stops functioning.

In advanced CKD patients, when serum calcium levels drop, the calcium receptor in the parathyroid glands respond normally by producing PTH. Also as normal, PTH acts

on the osteoblasts in bone to increase RANKL production. However, because of the impaired kidney function, PTH cannot increase activation of 25(OH)D to calcitriol. Because there is insufficient calcitriol, calcium cannot be absorbed in the gut and the body is forced to increase PTH production further to obtain the calcium from bone. As the PTH production continues, patients can develop secondary hyperparathyroidism, which is the excess production of PTH due to low serum calcium and can result in the weakening of bones and possibly calciphylaxis. As for phosphate, the impaired kidney functions causes a decrease in phosphate excretion through urine and results in high serum phosphate (77). Klotho expression is reduced in the proximal tubules (78) and, as such, FGF-23 cannot act to reduce serum phosphate levels. It is believed that as serum phosphate continues to rise, the body responds by increasing FGF-23 secretion. Research has shown that serum FGF-23 levels are found increasing as CKD progresses, although it did not respond to dietary phosphate intake (79). The high FGF-23 levels also contribute to the secondary hyperparathyroidism problem by decreasing the activation and increasing the hydrolysis of calcitriol (75).

Research has shown that CKD patients have a high prevalence of cardiovascular disease and cardiovascular related mortality (80, 81), and medial calcification often occurs in these patients (7). As previously described, extracellular phosphate and calcium play large roles in the mechanism of medial calcification. With the high serum phosphate levels in CKD patients, the high prevalence of medial calcification is understandable. While serum calcium levels are typically low during CKD, treatments options for secondary hyperparathyroidism and hyperphosphatemia, such as calcium supplementation, calcium-containing phosphate binders, and calcitriol treatment, can

elevate serum calcium levels (82), also promoting medial calcification and cardiovascular complications. While research has shown that calcitriol treatment increased the survival rate of CKD patients (83), the evidence supporting calcium's role in medial calcification has led researchers to study calcitriol's role in the medial calcification in CKD.

Vitamin D and Vascular Calcification

In order to determine if calcitriol treatment promotes VSMC calcification, many *in vitro* and *in vivo* studies have been conducted. However, the results have often been contradictory, and, as such, researchers cannot definitively conclude whether calcitriol treatment increases or decreases medial calcification and whether it acts systemically or locally to produce its effects.

The first study to reveal detrimental effects of calcitriol supplementation on VSMC, published in 1998 by Jono et al., showed that calcitriol treatment on bovine VSMCs *in vitro* concentration dependently increased calcification and ALP activity in the presence of high phosphate in the form of β-glycerophosphate (84). Mizobuchi et al. were later able to show that calcitriol treatment in 5/6 nephrectomized rats resulted in increased serum calcium and phosphate levels, aortic calcification, and runx2 mRNA expression (85). Another study showed that calcitriol supplementation (at dosages of 100 nM and higher) increased calcification *in vitro* rat VSMC grown with β-glycerosphosphate. In addition, they noted that calcitriol treatment caused an increase in RANKL expression, but that the addition of RANKL by itself was not sufficient to increase calcification. They also examined it *in vivo* using 5/6 nephrectomized rats and reached the same conclusions as Mizobuchi et al, except that serum phosphate levels were not upregulated (86). Han et al recently published an article that not only showed

that calcitriol supplementation increased calcification in mouse VSMC cultures in high phosphate conditions, but that it also increased calcification in non-calcifying conditions. *In vivo*, they treated VDR knockout mice with calcitriol and noted no medial calcification occurred (87).

On the other hand, other studies have shown that calcitriol supplementation decreases calcification. *In vivo* experiments using low density lipid receptor-deficient mice with renal ablation and fed a high-fat diet showed decreased calcification with calcitriol treatment. It must be noted that in this model of CKD, only atherosclerotic plaque and intimal calcification was found (88). *In* vitro, calcitriol supplementation on human VSMCs in the presence of high phosphate and tumor necrosis factor-α (an inflammatory cytokine shown to accelerate calcification in high phosphate conditions) resulted in decreased calcification at a concentration of 100 nM (no effect with smaller dosages). Calcitriol did not have an effect on calcification causes by high phosphate alone (89). Because the role of inflammation is not well understood in medial calcification but highly important in intimal calcification, this study may also not apply to medial calcification. In 2012, Lau et al contradicted previous *in vivo* studies when they showed that calcitriol treatment actually decreased arterial calcification in 5/6 nephrectomized mice fed a high phosphate diet. They showed this decrease was correlated with an increase in serum klotho (although kidney expression of transmembrane klotho was not increased) (90). These *in vivo* results tended to agree with the *in vitro* work by Lim et al who were able to show that calcitriol treatment increased transmembrane klotho expression in cultured human VSMCs and able to decrease calcification only with the addition of FGF-23. FGF-23 and calcitriol by themselves did not affect calcification (91).

Finally, many other studies have revealed mixed results that suggest that calcitriol acts systemically to increase calcification through serum phosphate and/or calcium, but do not directly act on VSMCs to produce the effect. Wu-Wong et al found no significant difference in calcification of human VSMC *in vitro* between the control and 100 nM calcitriol treatment at four different concentrations of phosphate (0.9, 1.48, 2.06, and 2.64 mM). However, while they did not test calcitriol *in* vivo, treatment of 5/6 nephrectomized rats with an analog of calcitriol (that also didn't increase *in vitro* calcification) resulted in medial calcification (92). Stubbs et al came across interesting results when they observed FGF-23 null mice with various diets. FGF-23 null mice with a normal diet had accelerated mortality associated with increased calcification, calcitriol, serum calcium, and serum phosphate. Interestingly, low-phosphate dietary restriction in the FGF-23 null mice resulted in no arterial calcification, despite high serum calcitriol, and vitamin D-deficient diet resulted in increased calcification, despite low serum calcium and calcitriol (serum phosphate was still elevated) (93). Very similar results were found when klotho knockout mice were observed. Klotho knockout mice displayed growth retardation, increased calcification, and increased serum calcium, phosphate, FGF-23, and calcitriol. With genetic inactivation of the sodium-dependent phosphate transporter (in addition to the knockout of klotho), serum phosphate levels were normalized and vascular calcification was abolished, despite the continued high serum calcium and calcitriol (94). Lastly, Lomashvili et al took an interesting route of observing the differences between systemic and local reaction to calcitriol treatment by transplanting both VDR deficient and VDR functioning aortas into mice with renal failure (and functioning VDR expression). Calcitriol treatment did increase aortic calcification; however, there was no

difference between the VDR -/- and VDR +/+ allografts nor was there a significant difference between the allografts and the adjacent aortas (95).

Taken together, these results are highly contradictory. This may be explained, in part, by the lack of consistency in testing calcitriol, such as different species of animals and cells, different concentrations of phosphate, and different dosages of calcitriol. However, more evidence is starting to suggest that calcitriol's effect on VSMC calcification may involve the complex interaction with other molecules, such as FGF-23 and klotho, which may also help explain some of the differences found between the *in vitro* and *in vivo* studies.

Specific Aims

With previous studies unable to come to conclusive results on the effects of calcitriol supplementation on VSMC calcification, we wanted to examine its effects on our model of *in vitro* calcification. In addition, we wanted to explore some of the possible interaction effects between calcitriol, FGF-23, and soluble klotho. This project can be broken up into 4 aims:

- Examine the utilization of calcitriol in media of calcifying and non-calcifying VSMCs
- Examine the concentration-dependent effect of calcitriol supplementation on degree of calcification in calcifying and non-calcifying VSMCs
- Examine the effect of 100 nM calcitriol supplementation on calcifying and non-calcifying VSMCs (protein expression and morphological changes)
- Examine the potential interaction effects of calcitriol, FGF-23, and soluble klotho on VSMC calcification

CHAPTER II

MATERIALS AND METHODS

Materials

Calcitriol (catalog no. 2551) was obtained from Tocris Biosciences (Bristol, United Kingdom). Recombinant human klotho (catolog no. 5334-KL-025) and recombinant human FGF-23 (catolog no. 2604-FG) were obtained from R&D Systems (Minneapolis, MN).

For western blot analysis, anti-ALP rabbit monoclonal antibodies (catalog no. Ab108337), anti-αSMA mouse monoclonal antibodies (catalog no. Ab7817), anti-SM-MHC mouse monoclonal antibodies (catalog no. Ab683), and anti-klotho rabbit monoclonal antibodies (catalog no. Ab181373) were obtained from Abcam (Cambridge, MA). Anti-ERK 1 (catalog no. SC93) and anti-ERK 2 (catalog no. SC154) rabbit polyclonal antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX).

For gelatin zymography, 10% Ready Gel Zymogram gel (catalog no. 1611167), 10x Zymogram Renaturing buffer (catalog no. 1610765), 10x Zymogram Development buffer (catalog no. 1610766), Zymogram Sample buffer (catalog no. 1610764), Precision Plus Protein Standards (catalog no. 1610373), and 10x Tris/Glycine/SDS buffer (catalog no. 1610772) were all obtained from Bio-Rad (Hercules, CA).

All other materials were obtained from either Fisher Scientific (Waltham, MA) or Sigma-Aldrich (St. Louis, MO).

Cell Culture

Human primary aortic VSMCs were purchased from ATCC (catalog no. PCS-100-012; Manassas, VA). Cells were grown and maintained using standard growth media, Dulbecco's Modified Eagles Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were incubated at 37^{o}C with 5% CO_2, and media was changed every 2-3 days.

For experiments, to prepare the calcification media, 3 mM inorganic phosphate in the form of dibasic sodium phosphate was added to the cell culture media. For experiments involving the addition of calcitriol, 10% charcoal-stripped FBS was used in place of regular FBS due to FBS containing vitamin D metabolites. Cells were at passage 8 for all experiments.

Calcitriol Utilization: Experimental Design

HVSMCs were seeded in six-well plates and allowed to grow to 80% confluency. Cells were dived into two groups: one group received standard growth media (Cont); the other group received calcification media (Calc).

Once reaching ~80% confluency (Day 0), the cells were given their appropriate media and allowed to grow for 7 or 14 days, with the media being changed every 2-3 days. On day 4 and day 11, for the 7 and 14 day samples respectively, media samples were taken prior to administration on the cells and stored in the -80^{o} C. On day 7 and day 14, media samples were taken from each sample prior to removal and subsequent quantification of calcium deposition. The media samples before and after administration to the cells were examined for calcitriol concentration.

Determination of Calcitriol Concentration

The change in calcitriol concentration was determined by measuring the calcitriol concentration in the media before and after administration using a calcitriol competitive-enzyme-linked immunosorbent assay (ELISA) kit, purchased from Elabscience (catalog no. E-EL-0016; WuHan, P.R.C), following manufacturer's instructions. Briefly, the wells in the microtiter plate were precoated with calcitriol. Sample was added to each well and "competed" with precoated calcitriol for the biotinylated detection antibodies that were added to each well. After the reaction, each well was washed to remove excess conjugate and the unbound calcitriol from the samples. Next, avidin, horseradish peroxidase conjugate was added to each well and incubated. Then, a tetramethylbenzidine substrate was added to each well. This enzyme-substrate reaction was terminated with the addition of sulphuric acid and the resulting color change was measured spectrophotometrically at 450 nm, using a μQuant microplate spectrophotometer (Bio-Tek Instruments; Winooski, VT).

Calcium Deposition Quantification

Media was removed from the cell cultures, and each well was rinse gently with 1x phosphate buffered saline (PBS) three times in order to remove any residual media and its associated calcium. Next, the cell layer was decalcified by using 5 mL of 0.6 N HCl for 24 hours. HCl supernatants were collected and prepared for atomic absorption (AA) by adding 1 mL of 20,000 μg/mL potassium chloride and 1 mL of 0.1 N nitric acid. The cell layers were then solubilized using 1 mL of 0.1 N NaOH/0.1% sodium dodecyl sulfate (SDS) solution. Six-well plates were observed using a light microscope to ensure complete solubilzation before collection and storage in the -80^{o} C freezer.

Calcium concentration of HCl supernatant was determined with AA. It was conducted using a Shimadzu AA-7000F (Shimadzu Corp; Kyoto, Japan) and Calcium Atomax Hollow Cathode Lamp (catalog no. N2025208; PerkinElmer; Waltham, MA) using a wavelength of 422.7 nM. Standards containing known calcium values were prepared using calcium reference standard solution purchased from Fisher Scientific (catalog SC191-500; Waltham, MA).

Calcium content was normalized to intracellular protein content. Protein concentration of the NaOH/SDS solution was determined using the Pierce bicinchoninic acid (BCA) assay (catalog #: 23225; Thermo Scientific; Waltham, MA) following manufacturer's instructions. Briefly, standards containing known protein values were prepared using a stock solution of bovine serum albumin. Standards and samples were then placed in a non-treated 96-well microplate. An alkaline solution containing BCA and copper (II) sulfate was added to each well and allowed to incubate at 37^{o}C for 30 minutes. The resulting color change was measure spectrophotometrically at 562 nm. Protein concentration of the samples were determined by comparing the absorbance of the samples to the absorbance of the protein standards.

Effects of Calcitriol Concentration: Experimental Design

HVSMCs were seeded in six-well plates and allowed to grow to 80% confluency, using standard growth media. Cells were divided into 8 groups (n=6):

- Standard growth media with 1% ethanol, (Vehicle Cont)
- Standard growth media with 10 nM calctriol (10 nM Cont)
- Standard growth media with 100 nM calcitriol (100 nM Cont)
- Standard growth media with 1000 nM calctriol (1000 nM Cont)

- Calcification media with 1% ethanol (Vehicle Calc)
- Calcification media with 10 nM calcitriol (10 nM Calc)
- Calcification media with 100 nM calcitriol (100 nM Calc)
- Calcification media with 1000 nM calcitriol (1000 nM Calc)

All media was made with 10% charcoal-stripped FBS. Calcitriol was reconstituted in 100% ethanol and added to the media, such that the final concentration of ethanol in the media was 1%.

Once reaching ~80% confluency (day 0), cells were given their appropriate media and allowed to grow for 14 days, with the media being changed every 2-3 days. After 14 days, cells were decalcified and the degree of calcium deposition was quantified as previously mentioned.

In-Depth Examination of Calcitriol Supplementation: Experimental Design

Due to results from previous experiment, supplementation of 100 nM calcitriol was chosen for further in-depth examination. HVSMCs were seeded in six-well plates and allowed to grow to 80% confluency, using standard growth media. Cells were divided into 4 groups:

- Standard growth media with vehicle (Cont)
- Standard growth media with 100 nM calcitriol (100 nM Cont)
- Calcification media with vehicle (Calc)
- Calcification media with 100 nM calcitriol (100 nM Calc)

All media was made with 10% charcoal stripped FBS. Calcitriol was reconstituted in DMEM with 1.25% ethanol and added to the media, such that the final concentration of ethanol in the media was 0.005%.

Once reaching ~80% confluency (day 0), cells were given their appropriate media and allowed to grow for 7 or 14 days, with the media being changed every 2-3 days. Separate samples were prepared for AA (n=6), scanning electron microscopy (n=3), and western blot analysis (n=3). Media was collected from AA samples prior to decalcification for analysis of MMP-2/-9 expression using gelatin zymography.

Visualization of Calcium Deposition

Visualization of the calcium deposition was done using scanning electron microscopy (SEM). Prior to seeding cells in the six well plates, Thermanox plastic coverslips were placed in the wells with the treated side face-up. After receiving the appropriate media for 7 or 14 days, media was removed and cells were fixed in 1/2 Karnovsky's fixative in 0.1 M sodium cacodylate buffer for up to two weeks. The samples were processed for SEM imaging by further fixation using 0.4% osmium tetroxide followed by serial dehydration, using increasing concentrations of ethanol and hexamethyldisilazane (HMDS). Next, samples were allowed to air dry overnight and mounted on stubs using double-sided tape. Lastly, mounted samples were sputtered coated with 15 nm of platinum using an EMS 1150T ES sputter coater.

Samples were imaged using a Carl Zeiss EVO50VP Variable Pressure Scanning Electron Microscope. Working distance of 10 mm and excitation voltage of 10 kV was used.

For quantification of the calcified area, three images (1000x or lower magnification) were randomly selected from each group. Percent area of the calcium deposits in relation to the total area of the image was done using ImageJ software.

Because edges of cells appeared bright in SEM images, they were manually removed from the analysis.

Examination of Protein Expression

Protein was obtained by placing cells in lysis buffer (10 mM Hepes, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 10 mM Na-pyrophosphate, 10 mM NaF, 0.1 mM Na-orthovanadate, 1% Na deoxycholate, 1% Triton x 100, and 0.1% SDS), scraped, sonicated on ice for 5 seconds, and centrifuged at 15000 RPM to remove large cellular debris. Protein concentration was determined using BCA assay and diluted such that 30 μg of protein was loaded in each well. Samples were loaded into the wells of 10% or 8% SDS-Page gel and ran at a constant voltage (120 V) for approximately 2 hours or until tracking blue dye reached the bottom. Once finished, gels were transferred to PDVF membrane overnight.

Membranes were blocked with 5% non-fat dry milk in 1x TTBS w/ Tween-20 buffer for one hour at room temperature. Primary antibodies were added to 5% milk in 1x TTBS w/ Tween-20 buffer, solution was placed on membranes, and membranes were allowed to shake overnight at 4º C. Membranes were rinsed with 1x TTBS w/ Tween-20 buffer. Secondary antibodies were added to 1% milk with 1x TTBS w/ Tween-20 buffer and placed on membranes for 1-2 hours at room temperature. Enhanced chemilumniescence (ECL) solution was added to each membrane and allowed to incubate 5 minutes at room temperature. Membranes were developed on film.

Examination of MMP-2/-9 Expression

Media samples taken prior to decalcification for AA analysis were diluted in order to normalize to intracellular protein content. One part media was added to two parts Zymogram Sample Buffer, and 30 µl of the combined solution was added to each well. Gels were run at constant voltage (125 V) for 90 minutes or until the bromephenol blue tracking dye reached the bottom of the gel. Once completed, gels were removed from the casing and incubated in 1x Zymogram Renaturing buffer for 1 hour with gentle agitation. Gels were then incubated in 1x Zymogram Development buffer for an hour with gentle agitation, replaced with fresh development buffer, and allowed to incubate over night at 37º C. The following day, gels were stained with 0.25% (w/v) Coomassie blue stain (made in 40% methanol, 10% acetic acid, and 50% distilled water) for one hour. Finally, the gels were destained twice, using a solution of 40% methanol, 10% acetic acid, and 50% distilled water and gentle agitation for one hour.

MMP expression was viewed as clear bands on a dark, stained background. Densitometric analysis of the band intensity was done using ImageJ software. Band intensities were normalized to the band for the 7 day control group.

Effects of Calcitriol, FGF-23, and Klotho Supplementation: Experimental Design

HVSMCs were seeded in six-well plates and allowed to grow to 80% confluency, using standard growth media. Samples were divided into 8 groups:

- Standard growth media with vehicle (Cont)
- Calcification media with vehicle (Calc)
- Calcification media with 100 nM calcitriol (Vit D)
- Calcification media with 0.4 nM soluble klotho (Klotho)

- Calcification media with 10 ng/mL FGF-23 (FGF)
- Calcification media with 100 nM calcitriol and 10 ng/mL FGF-23 (Vit D + FGF)
- Calcification media with 100 nM calcitriol and 0.4 nM soluble klotho (Vit D + Klotho),
- Calcification media with 10 ng/mL FGF-23 and 0.4 nM soluble klotho (FGF + Klotho)
- Calcification media with 100 nM calcitriol, 10 ng/mL FGF-23, and 0.4 nM soluble klotho (All)

All media was made with 10% charcoal stripped FBS. Calcitriol was reconstitute in DMEM with 1.25% ethanol. Klotho was provided in a solution containing 20 mM 2-(N-morpholino)ethanesulfonic acid (MES), 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 50% (v/v) glycerol and was diluted using DMEM. FGF-23 was reconstitute per manufacturer's instructions using 1 x PBS with 0.1% BSA and diluted in DMEM. The final concentration of the vehicle in each media was 0.005% (v/v) ethanol, 2.7 nM MES, 135 pM EDTA, 0.000007% (v/v) glycerol, 0.005 % (v/v) PBS, and 0.00005% (w/v) BSA.

Once reaching ~80% confluency (day 0), cells were given their appropriate media and allowed to grow for 14 days, with the media being changed every 2-3 days. After 14 days, cells were decalcified and the degree of calcium deposition was quantified as previously mentioned

Statistical Analysis

Error bars on graphs represent standard deviation. When comparing two groups, student t-test was used ($\alpha = 0.05$). When comparing multiple groups, one way ANOVA with Fisher's Least Significant Difference (LSD) post-hoc analysis was used ($\alpha = 0.05$).

CHAPTER III

RESULTS

Calcitriol Utilization by Calcifying and Non-Calcifying VSMCs

Calcitriol concentration of the cell culture media was measure before and after administration to both control and calcification groups of VSMCs. As seen in Figure 3.1, the calcitriol concentration of the media from both groups increased after administration to the cells at both 7 and 14 days. However, the extent of increase was not significantly different between the two groups at either time point.

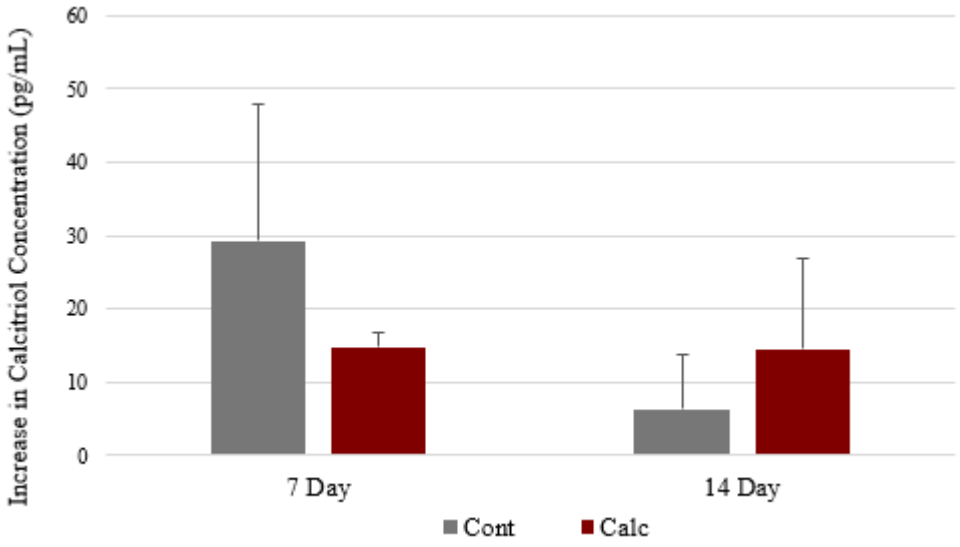

Figure 3.1 Change in Calcitriol Concentration of Media from Calcifying and Non-Calcifying VSMCs

There was no significant difference between the increase of calcitriol concentration of the two groups at either 7 or 14 days

Calcification of the VSMCs receiving the 3 mM inorganic phosphate was verified through AA. As seen in Figure 3.2, the VSMCs receiving the phosphate supplementation had a significant increase in calcium content compared to the control group at both 7 and 14 days.

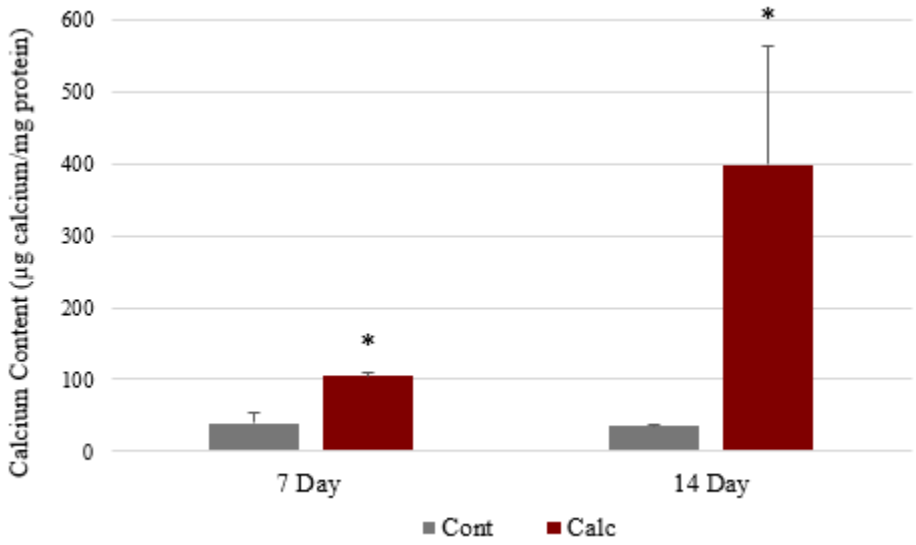

Figure 3.2 Confirmation of Calcification of VSMC with Phosphate Supplementation

* $P < 0.05$ compared to the control group at the same time point

Effects of Calcitriol Concentrations on VSMC Calcification

To examine any concentration dependent effect of calcitriol, three concentrations of calcitriol (10, 100, and 1000 nM) were added to VSMCs with and without the supplementation of 3 mM inorganic phosphate. As seen in Figure 3.3, only 1000 nM calcitriol was able to cause a significant increase in calcium content in VSMCs in non-calcifying conditions. In calcifying conditions, seen in Figure 3.4, supplementation of 100 nM and 1000 nM calcitriol caused a significant increase in calcium content compared to the vehicle. There was no significant difference between the 100 and 1000

nM groups. It is worth noting that while 1000 nM calcitriol supplementation was able to increase calcification in the control group, the calcium content value of 1000 nM control group was still approximately 10 times lower than the vehicle calcification group. Comparisons of the control and calcification groups can be seen in Figure 3.5.

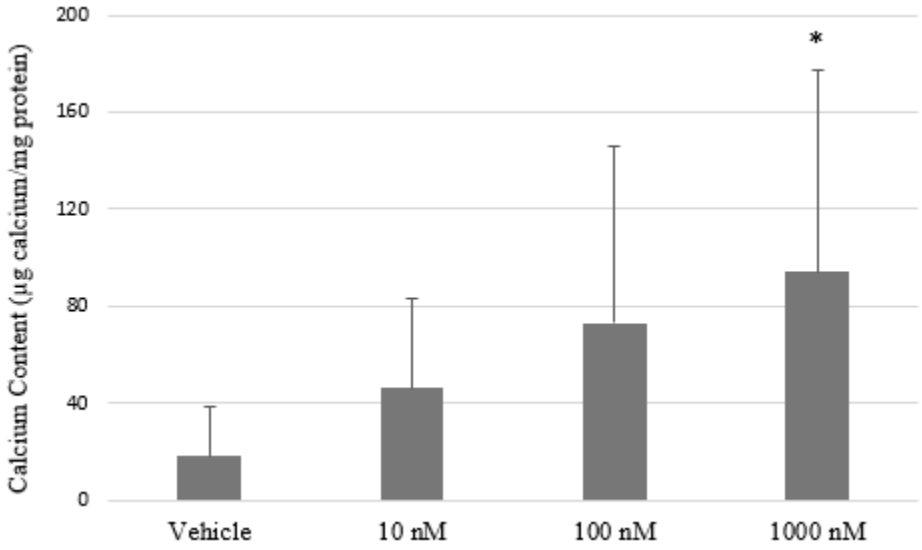

Figure 3.3 Effects of Three Calcitriol Concentrations on Extent of VSMC Calcification in the Absence of Phosphate Supplementation

* $P < 0.05$ compared to vehicle

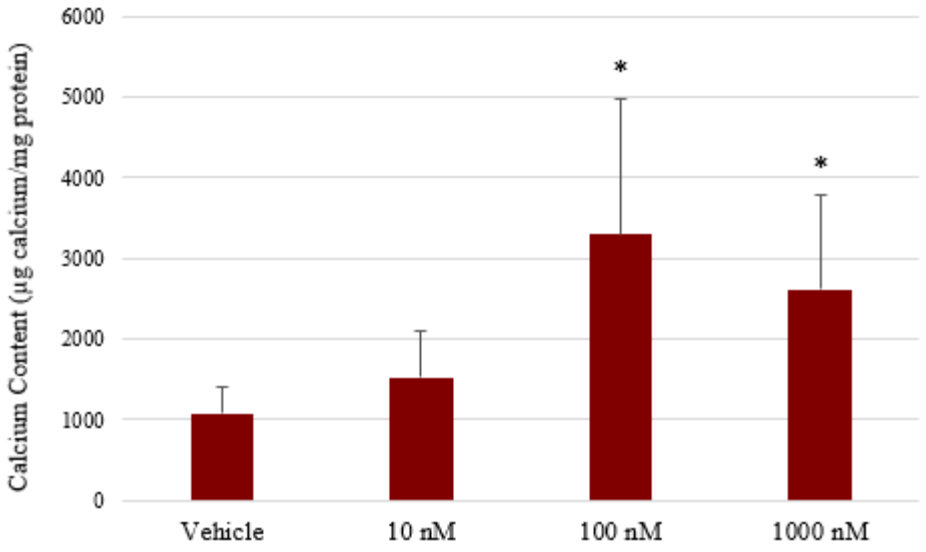

Figure 3.4 Effects of Three Calcitriol Concentrations on Extent of VSMC Calcification in the Presence of Phosphate Supplementation

* $P < 0.05$ compared to vehicle

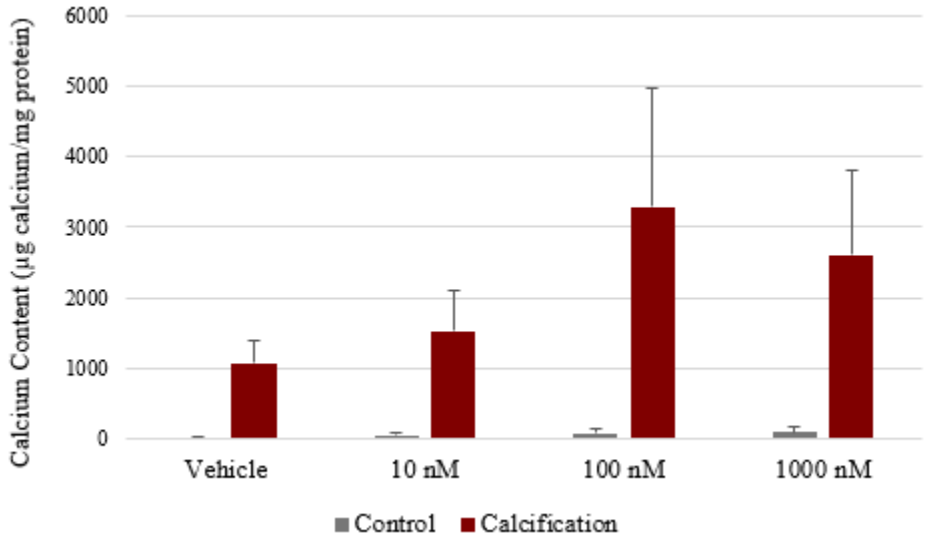

Figure 3.5 Combined Graph of Effects of Three Calcitriol Concentrations on VSMC Calcification

In-Depth Analysis of Calcitriol Supplementation on VSMC Calcification

To further analyze the effects of calcitriol supplementation on VSMC calcification, one concentration of calcitriol was chosen (100 nM). The increase in calcification of VSMCs observed in the previous experiment was confirmed using AA and observed qualitatively using SEM. Protein expression was examined using western blot analysis (for αSMA, ALP, and Klotho) and gelatin zymography (for MMP-2 and -9).

The calcium content of VSMCs can be seen in Figure 3.6. Surprisingly, at 7 days, the supplementation of 100 nM caused a significant increase in calcification in non-calcifying conditions, while there was no effect in the calcification groups. On the contrary, at 14 days, we observed a significant increase in calcium content with the addition of calcitriol in the calcification groups but not the control groups.

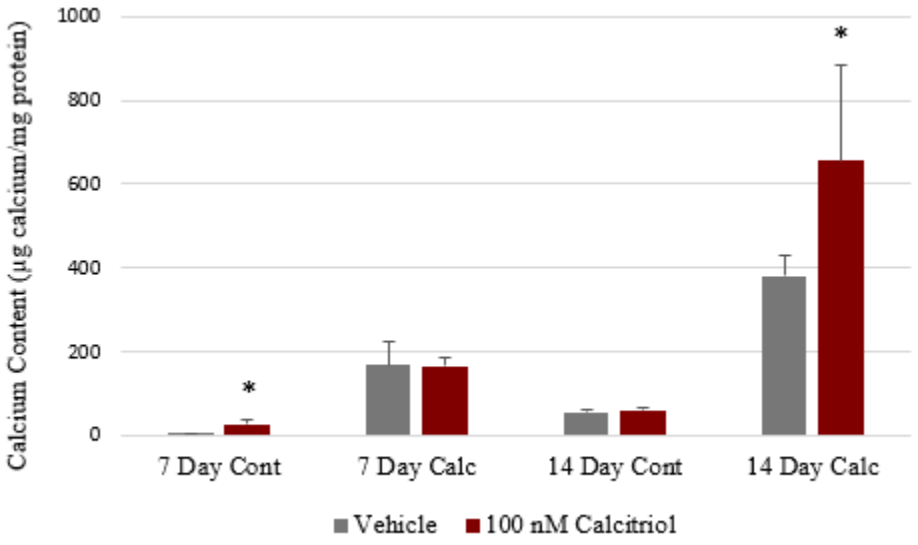

Figure 3.6 Effect of 100 nM Calcitriol Supplementation on VSMC Calcification at 7 and 14 Days

* $P < 0.05$ compared to the vehicle for the same treatment and time point

We imaged the effects of calcitriol supplementation on VSMC calcification through SEM imaging. SEM images (at 1000x magnification) after 7 and 14 days can be seen in Figure 3.7 and Figure 3.8, respectively. SEM images at other magnifications can be seen in Figures A.1, A.2, A.3, and A.4. Comparing the images at day 7, there appears to be more small nodules in the calcification groups compared to the control groups. Although the AA data showed that there was increase in calcification between the 7 day control and 7 day 100 nM control groups, there does not appear to be any distinct morphological differences in the images of these groups. The 14 day images are similar to the 7 day images in that the calcification groups appear to have more nodule formations than the control groups. Also, while AA data showed an increase in calcification with the supplementation of 100 nM calcitriol in the 14 day calcification group, there did not appear to be any distinct morphological differences between the two groups. Quantification of the calcified area, as seen in Figure 3.9, did not show any significant differences in the percent area of calcification with the supplementation of 100 nM calcitriol in neither the control nor calcification groups.

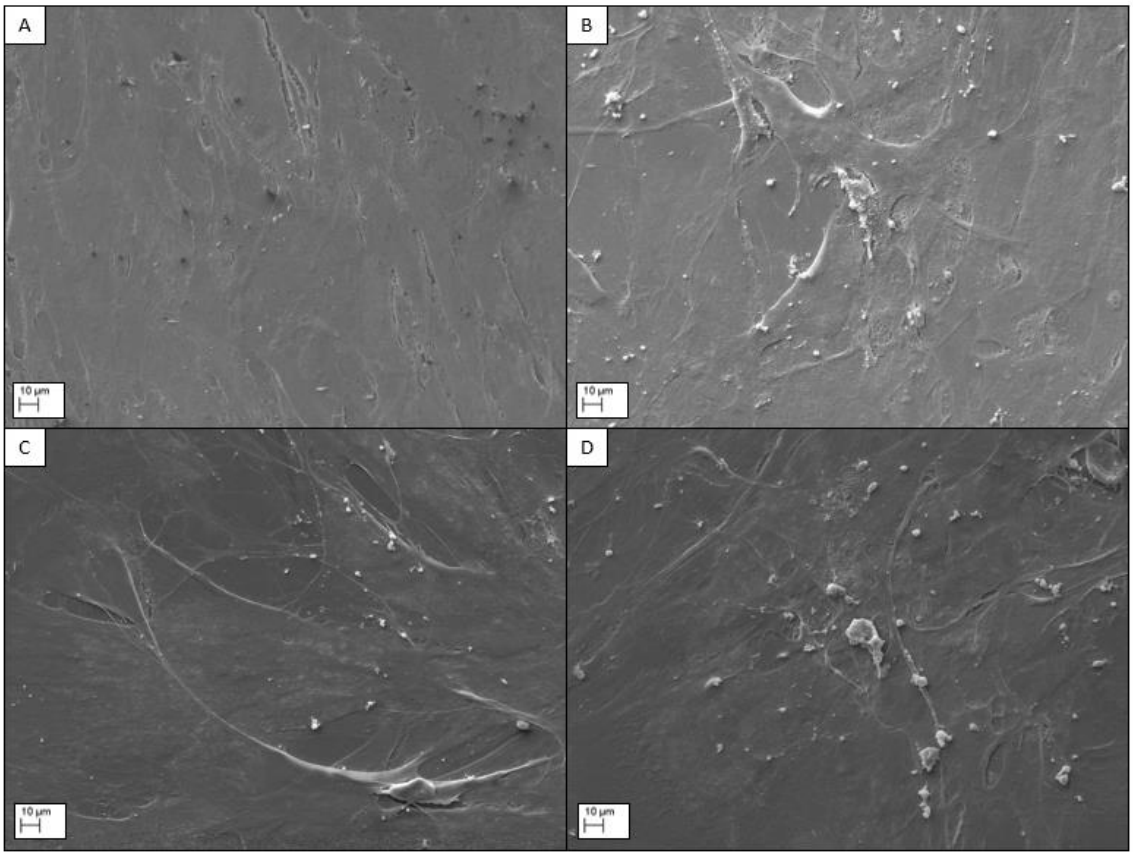

Figure 3.7 SEM Images (1000x Magnification) of Day 7 Samples

SEM images of (A) control, (B) calcification, (C) 100 nM control, and (D) 100 nM calcification after 7 days.

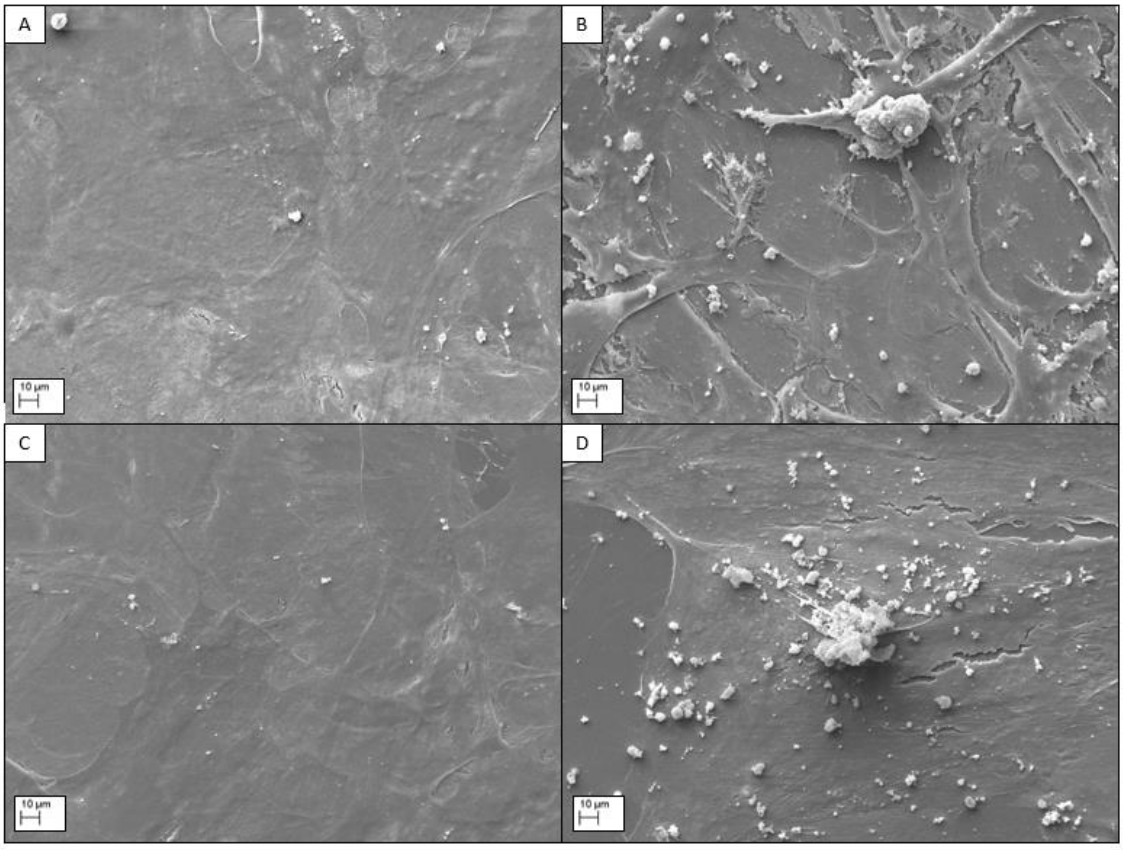

Figure 3.8 SEM Images (1000x Magnification) of Day 14 Samples

SEM images of (A) control, (B) calcification, (C) 100 nM control, and (D) 100 nM calcification after 14 days

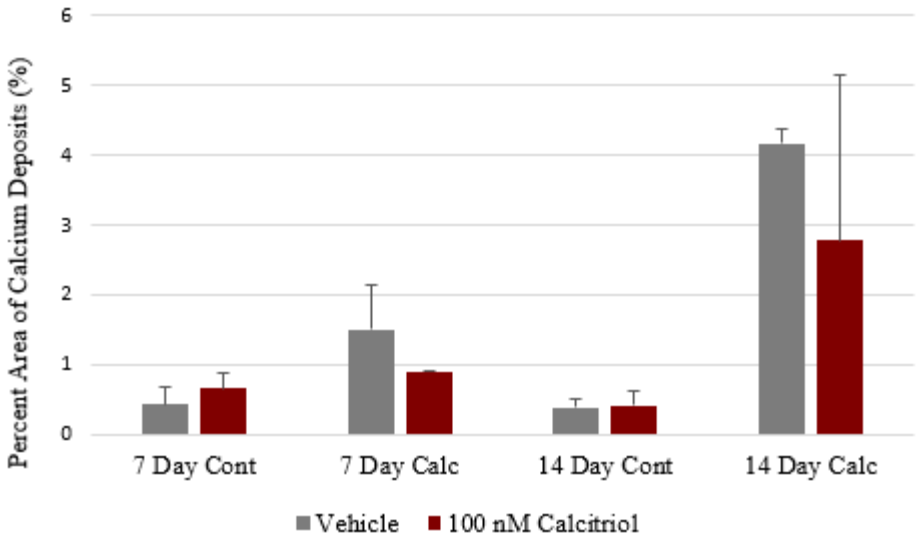

Figure 3.9 Percent Area of Calcium Deposits

There was no significant difference between vehicle group and 100 nM group at either treatment or time point

The protein expression of αSMA, SM-MHC, ALP, and klotho were examined using western blot analysis. The relative protein expression can be found in Figure 3.10. At 7 and 14 days, there was a decrease in αSMA between the control and calcification groups. However, there did not appear to be any effect in αSMA expression caused by the addition of 100 nM in either the control and calcification groups. SM-MHC expression followed a similar trend as αSMA but to a lesser degree. There also did not appear to be any difference ALP expression between the control and calcification groups at 7 or 14 days, and there also did not appear to be any effects from the supplementation of calcitriol. Lastly, there did not appear to be any change to the total klotho expression between any of the groups. Total ERK was used as a loading control. Densitometric analysis could not be done for these proteins because the three replicates were combined to ensure that there was enough protein to load on the gels.

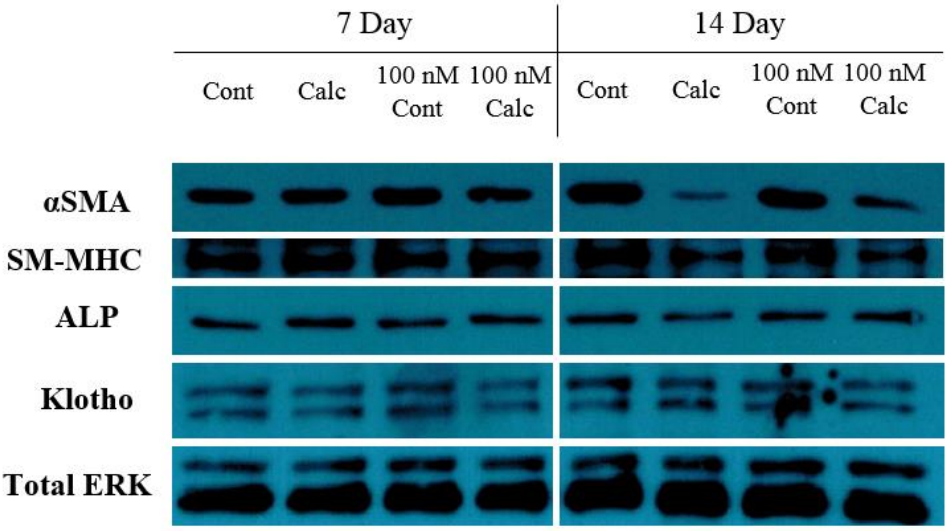

Figure 3.10 Analysis of αSMA, SM-MHC, ALP, and Klotho Protein Expression

The relative expression of MMP-2 and -9 was examined using gelatin zymography and can be seen in Figure 3.11. The presence of what is believed to be proMMP-2 can clearly be seen in all groups at both 7 and 14 days. A much smaller band for proMMP-9 can faintly be seen. According to densitometric analysis, as seen in figure 3.11, there was no difference proMMP-2 expression between the calcification and control groups nor between the vehicle and 100 nM calcitriol groups.

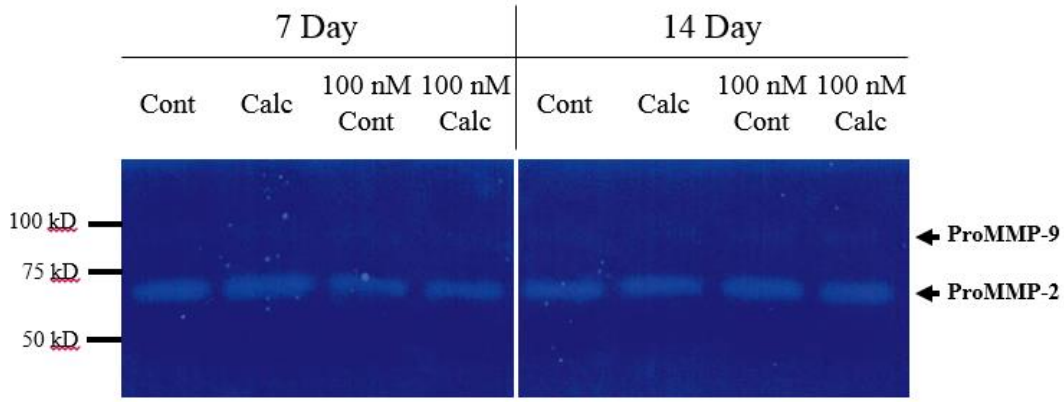

Figure 3.11 Analysis of MMP-2/-9 Expression

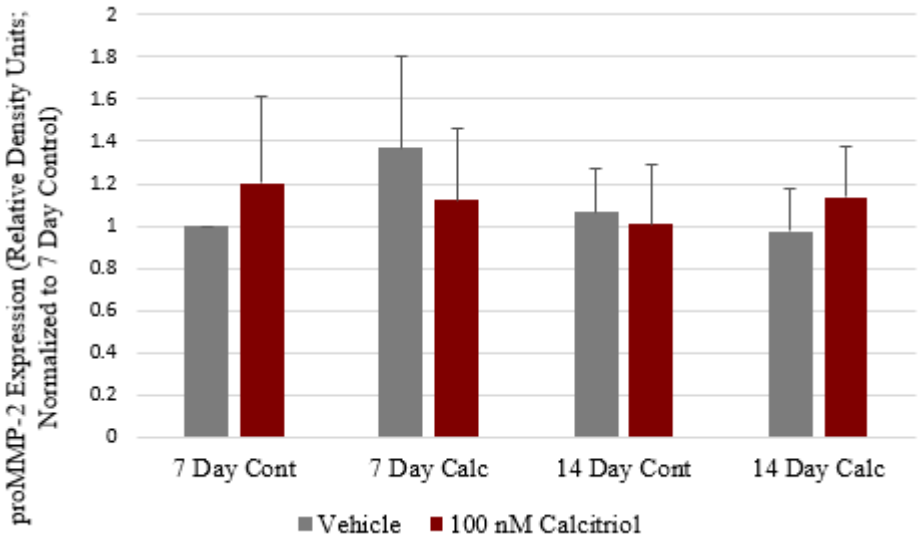

Figure 3.12 Densitometric Analysis of ProMMP-2 Expression

There was no significant difference in proMMP-2 expression between any of the groups

Effects of Calcitriol, FGF-23, and Klotho Supplementation on VSMC Calcification

In order to observe the effects from the interaction of calcitriol, FGF-23, and klotho, various combinations of these molecules were added to VSMCs in calcifying conditions and examined for calcium content using AA. As seen in Figure 3.13, there is a significant decrease in calcium content from the addition of FGF-23 with calcitriol. This combination actually reduced the calcium content to the same level as VSMCs without calcification media. Surprisingly, the only combination to increase calcification was the combination of calcitriol, FGF-23, and klotho, which was significantly higher than all other groups.

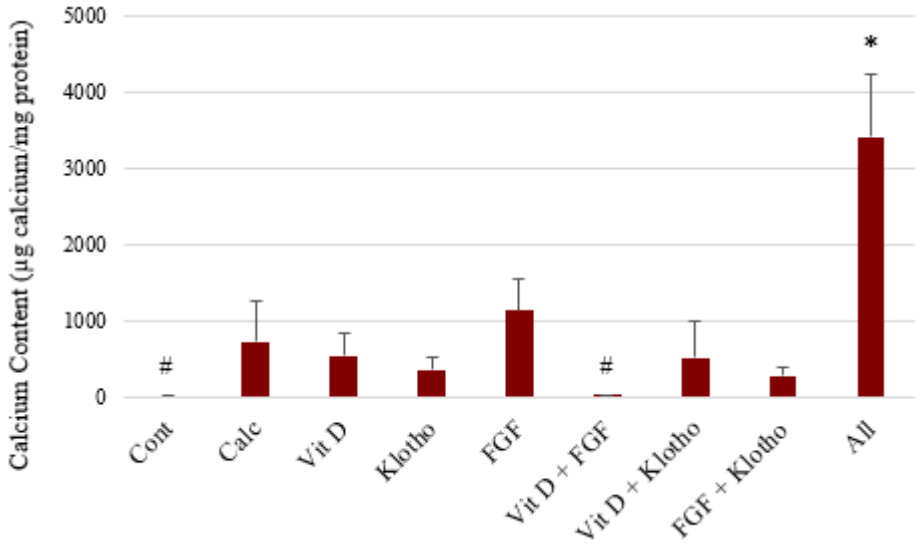

Figure 3.13 Effect of Calcitriol, FGF-23, and Klotho Supplementation on VSMC Calcification

* $P < 0.05$ compared to all groups; # $P < 0.05$ compared to calcification group

CHAPTER IV

DISCUSSION

In the first experiment, we wanted to determine if there was any difference in the utilization of calcitriol between VSMCs that received 3 mM inorganic phosphate and VSMCs that did not receive any inorganic phosphate. We saw an increase in calcitriol concentration in the cell culture media from before administration to after 3 days of administration on the VSMCs in both groups. Calcitriol is found in serum, but it is only present in very small amounts (~1000x less) compared to the metabolite 25(OH)D (54). While the majority of calcitriol activation occurs in the kidneys, various tissues can express the CYP27B1 enzyme, including VSMCs (96). Thus, this increase in both groups was expected. While the calcification of VSMCs is believed to involve the transition to an osteoblast-like phenotype, osteoblasts are also capable of expressing the CYP27B1 enzyme (97); thus, it is not entirely surprising that there was not a difference in the degree of increase in calcitriol concentration between the calcifying and non-calcifying VSMCs. We conducted AA to ensure that our VSMCs were increasing their mineral deposition in response to the 3 mM inorganic phosphate. Our calcifying VSMCs showed increased calcification, consistent with previous studies (9, 11-13).

When looking at the concentration-dependent effects of calcitriol on VSMC calcification, we saw that 100 nM and 1000 nM calcitriol supplementation was able to increase the calcification in the presence of high phosphate and only 1000 nM calcitriol

supplementation was able to increase the calcification without the presence of high phosphate. These results generally agree with many previously published *in vitro* studies (84, 86, 87). Jono et al were able to show that concentrations as low as 10 nM calcitriol was able to increase calcification in the presence of high phosphate; however in this study, they did not use charcoal stripped FBS (84), which may be the reason our 10 nM group did not show a significant increase in calcium content. To the best of our knowledge, we are the first group to examine concentrations as high as 1000 nM calcitriol. Cardus et al examined 300 nM calcitriol and showed there was no difference in calcium content compared to the 100 nM group (86), similar to our 100 and 1000 nM groups.

When we further examined the effects of 100 nM calcitriol supplementation on the VSMCs, we first verified our findings from the previous experiment. Interestingly, at 7 days, 100 nM calcitriol supplementation did not increase calcification in the presence of high phosphate but did in the absence of high phosphate. After 14 days, the results were the same as the experiment using the three calcitriol concentrations, in that 100 nM calcitriol supplementation increased calcification in the presence of high phosphate but did not in the absence of high phosphate after 14 days. No one has previously noted a time dependent effect of calcitriol on VSMC calcification; however, it is worth noting that calcitriol's effects on osteoblast differentiation is highly dependent on the stage of maturation of the cell (97). In fact, recent research has shown that calcitriol accelerates matrix vesicle formation in osteoblasts but only during the early phase of differentiation (98). Thus, it is possible that calcitriol has a similar time-dependent effect on VSMCs.

Still, it is surprising that only the control showed increased calcification at day 7 and not both groups.

Looking at the SEM images, there appears to mineral formation in the calcification groups. Nodule formations appear in large number in the calcification groups but not the control groups, suggesting that these are the mineral deposits. Comparing the vehicle groups to the 100 nM calcitriol groups, there do not appear to be any distinct morphological differences to suggest a cause for the increase in calcification caused by the calcitriol supplementation. To the best of our knowledge, we are the first group to observe calcifying VSMCs using SEM. Schembri et al observed calcification of a carotid artery section that had atherosclerotic plaque (99), but these morphologies are very different.

Looking at the protein expression, we saw a loss of αSMA and SM-MHC expression in the calcification groups compared to the control groups at 7 and 14 day. This is consistent with previous studies that show that VSMCs undergo transition to an osteoblast-like phenotype with the addition of high phosphate (13). On the other hand, there did not appear to be a difference in αSMA and SM-MHC expression between the vehicle and 100 nM calcitriol groups at either 7 or 14 days. Han et al showed an increase in runx2 expression after the supplementation of 100 nM calcitriol (87). While we were unable to detect runx2 expression in our cells (data not shown), upregulation of runx2 is typically coupled with the loss of smooth muscle markers, so it is surprising that we did not see a difference in αSMA or SM-MHC. In our study, we saw that total klotho expression was unaffected by both calcitriol and phosphate supplementation. Lim et al actually showed an increase in transmembrane klotho with calcitriol supplementation,

which allowed the combination of calcitriol and FGF-23 to decrease VSMC calcification (91). We did not detect any transmembrane klotho expression, but because we also saw a similar decrease in calcification with the combination of calcitriol and FGF-23, it is possible that our sample preparation prevented us from detecting the transmembrane form and only total klotho. The last protein expression we examined was for MMP-2 and -9. As mentioned earlier, the degradation of elastin plays a large role in the pathology of medial calcification. Rahman et al examined the MMP expression of heart tissue from VDR knockout and wild type mice. They showed that MMP activity and expression was higher in the knockout mice than wild type mice (100). Looking at our gelatin zymography results, we see a large band in each group, which we believe to be proMMP-2. We can also see a faint band for what appears to be proMMP-9 in each group. These are the inactive forms of MMP-2 and -9, respectively, and the expression of these proteins do not appear to be effected by the addition of calcitriol or phosphate. MMP-2 and -9 activity is upregulated with medial calcification; however, it has only been shown *in vivo* (31-33) and *in vitro* when elastin peptides were added to the VSMCs (37). It is possible that increased MMP-2 and -9 expression and activity requires the interaction with elastin in the extracellular matrix and not just high extracellular phosphate. For this reason, we cannot conclude that calcitriol does not have an effect on MMP expression.

Lastly, we examined the effects of combinations of 100 nM calcitriol, 10 ng/mL FGF-23, and 0.4 nM soluble klotho. As mentioned earlier, we saw a significant decrease in calcification with the combination of calcitriol and FGF-23, agreeing with the findings of Lim et al. They were able to show that this decrease is due to the increased expression of transmembrane klotho, as klotho siRNA was able to abolish this decrease in their

study (91). It is believed that the increased expression of transmembrane klotho allows FGF-23 to react with its receptor and exert a beneficial effect. Hu et al showed that the addition of 0.4 nM soluble klotho by itself decreased VSMC calcification *in vitro* (101). In our study, 0.4 nM soluble klotho by itself appeared to cause a small decrease in calcification; however, statistical analysis concluded that it was not significant. In their experiment, they only added 2 mM inorganic phosphate to the VSMCs, so it is possible that 0.4 nM was too low of a concentration to elicit a similar, beneficial effect on the VSMC calcification caused by 3 mM inorganic phosphate in our study. The most surprising result was that the addition of all three, calcitriol, FGF-23, and soluble klotho, caused an extreme increase in VSMC calcification. This is the first time this combination has been added to VSMCs in the presence of high phosphate *in vitro*. Research has shown that soluble klotho can still act as a coreceptor for FGF-23 signaling (102), although it is believed to not be as active as the membrane form. Because the FGF-23 + calcitriol group and the FGF-23 + klotho group do not experience an increase in calcification, it may be possible that the combination of calcitriol, FGF-23, and klotho causes an overexpression of klotho and allows for excess FGF-23 activity. Supporting this idea, Jimbo et al found that FGF-23 causes concentration-dependent increase of calcification in klotho-overexpressing VSMCs in the presence of high phosphate (103).

CHAPTER V

FUTURE WORKS

The initial motivation for this project was to examine the feasibility of a site-specific treatment of calcitriol to reduce vascular calcification. While there are treatments for the plaque buildup associated with atherosclerosis and intimal calcification, such as balloon angioplasty and stents, there are not any clinically available treatment for medial calcification.

While our study, combined with previously published studies, do not particularly support a calcitriol treatment for vascular calcification, they also do not rule out the possibility for it. Future studies should be aimed at further examining the interaction between calcitriol, FGF-23, and klotho in calcifying conditions. The mechanisms behind the decrease or increase needs to be elucidated in order to ensure the safety of calcitriol supplementation. In addition, because treatments are often started after symptoms appear, the possibility of the combination of calcitriol and FGF-23 to reverse pre-existing calcification needs to be examined.

If the research still supports the creation of a site specific treatment using calcitriol, the delivery vehicle needs to be created. In particular, this could be done utilizing nanoparticles, due to their ability to be functionalized with various molecules, ability to avoid clearance by the immune system, and ability to reach the medial layer of arteries. The nanoparticles would need to be functionalized with a targeting agent in order

to deliver calcitriol locally and avoid the increase in serum calcium and phosphate caused by systemic administration. In particular, modification with an antibody specific for elastin has shown promise in targeting the sites of vascular calcification specifically by binding to the degraded elastin that is thought to be the sites of initial mineral nucleation (104). These nanoparticles would need to be examined for their ability to load calcitriol, release calcitriol, and accumulate in the sites of vascular calcification.

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APPENDIX A

ADDITIONAL SEM IMAGES

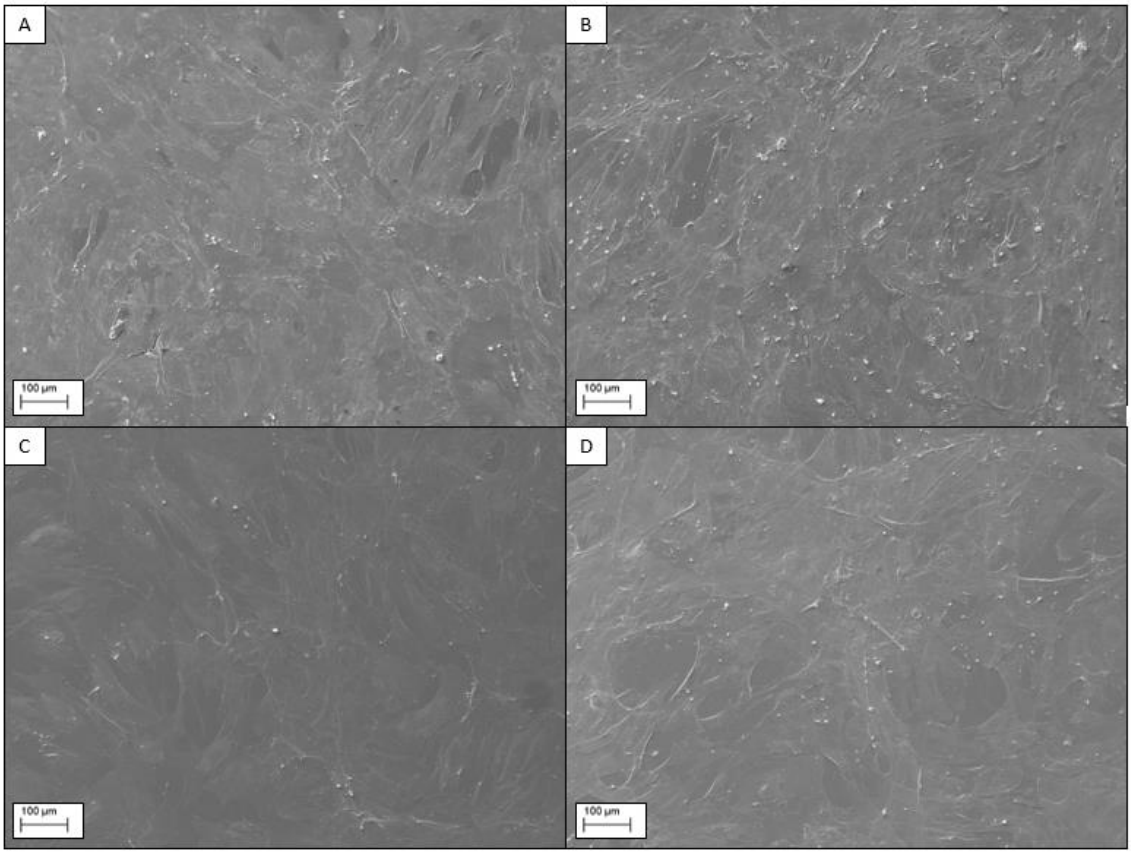

Figure A.1 SEM Images (250x) of Day 7 Samples

SEM images of (A) control, (B) calcification, (C) 100 nM control, and (D) 100 nM calcification after 7 days

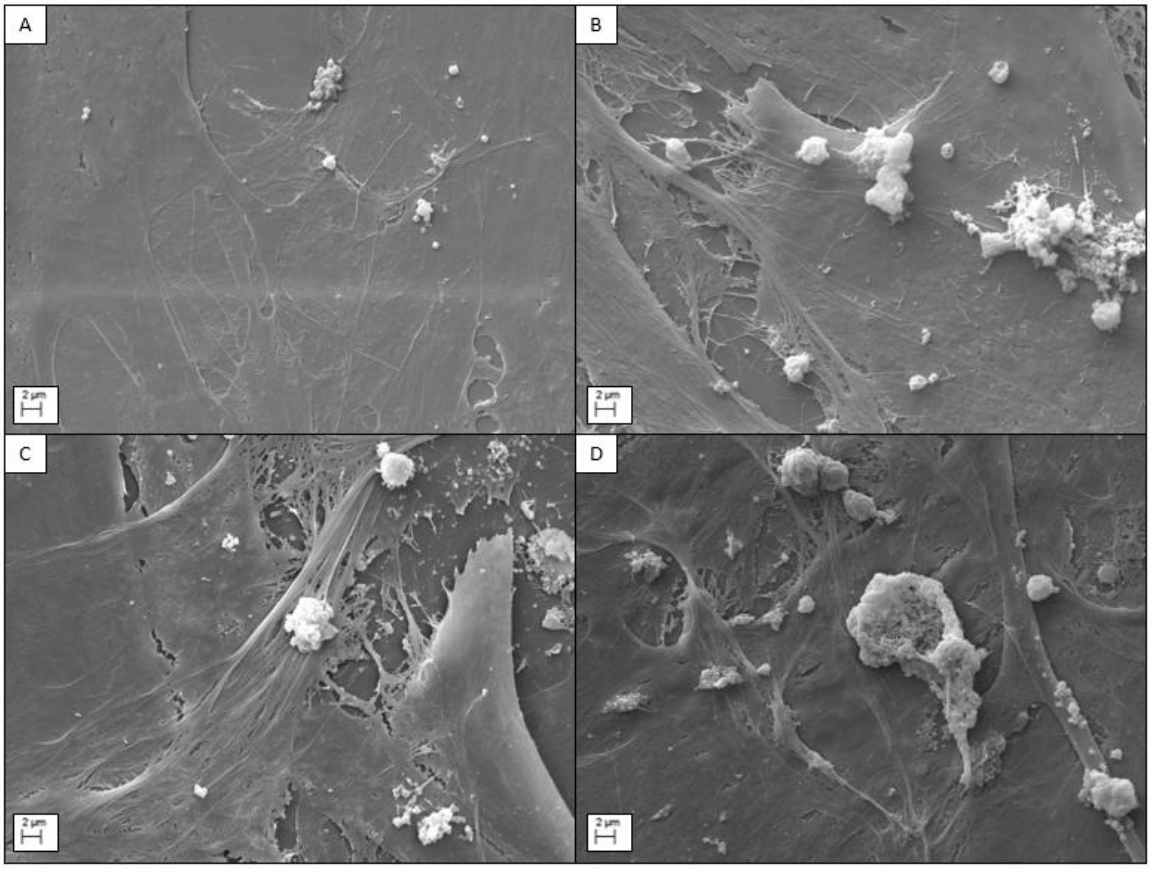

Figure A.2 SEM Images (5000x) of Day 7 Samples

SEM images of (A) control, (B) calcification, (C) 100 nM control, and (D) 100 nM calcification after 7 days

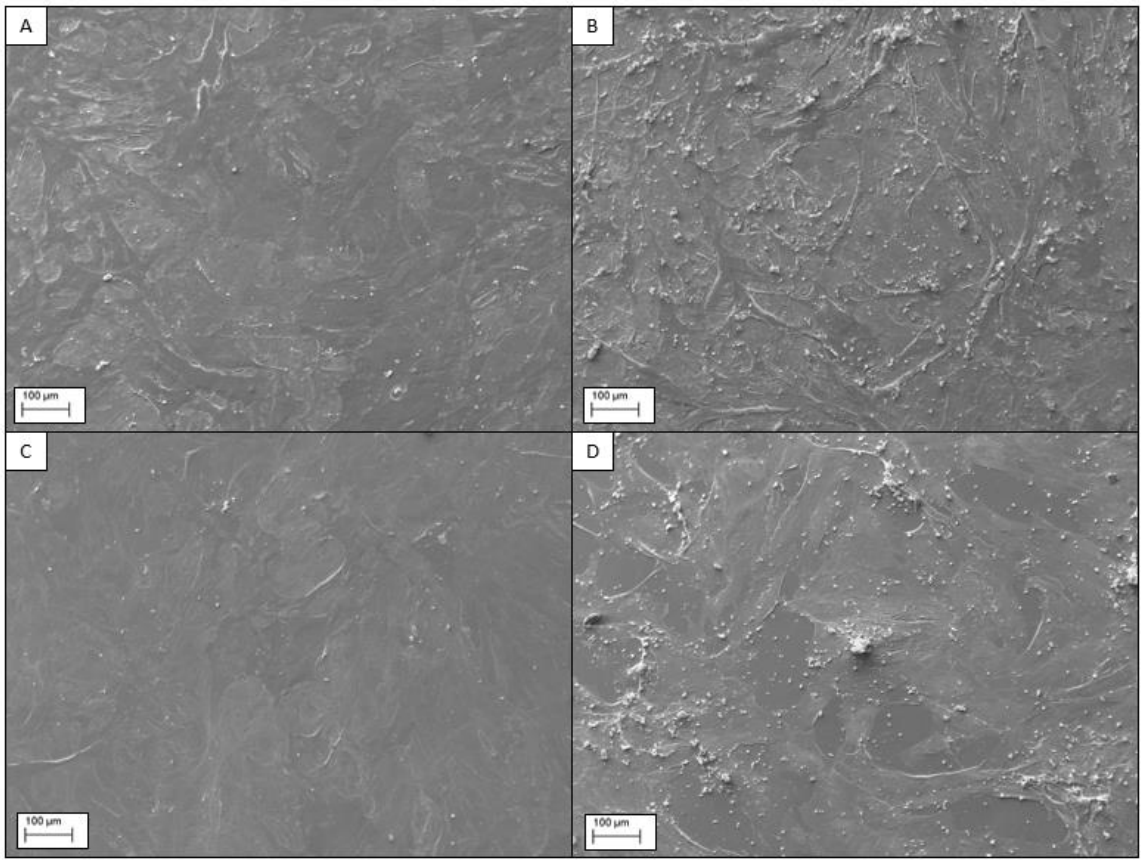

Figure A.3 SEM Images (250x Magnification) of Day 14 Samples

SEM images of (A) control, (B) calcification, (C) 100 nM control, and (D) 100 nM calcification after 14 days

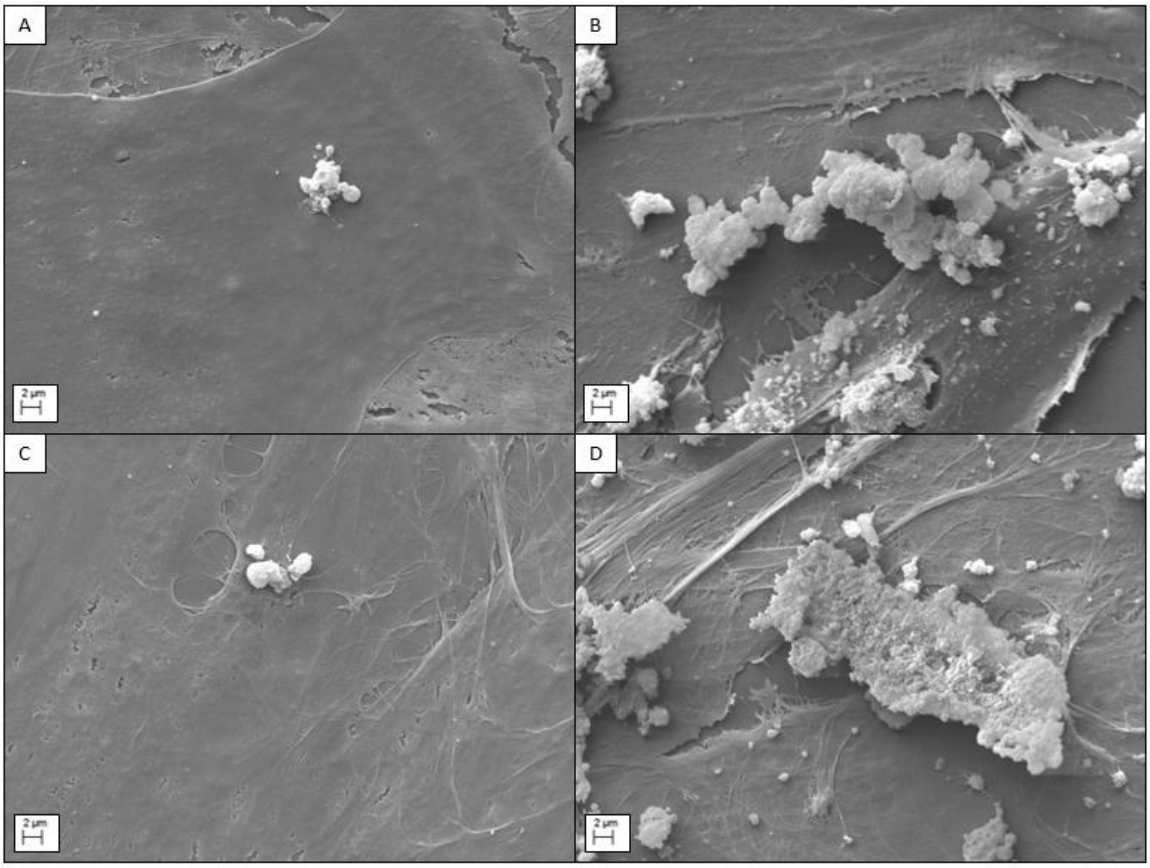

Figure A.4 SEM Images (5000x) of Day 14 Samples

SEM images of (A) control, (B) calcification, (C) 100 nM control, and (D) 100 nM calcification after 14 days