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Research article

Manipulating the Plasticity of Smooth Muscle Cells to Regulate Vascular Calcification

Kelsey M. McArthur [1], Amber M. Kay [2], Jenna A. Mosier [1], Joshua N. Grant [1], James A. Stewart [2] and C. LaShan Simpson [1,*]

[1] Department of Agricultural and Biological Engineering, Mississippi State University, Mississippi State, MS, United States
[2] Department of Biomolecular Sciences, University of Mississippi School of Pharmacy, University, MS, United States

* **Correspondence:** Email: cls893@msstate.edu; Tel: 662-325-8279.

Abstract: Cardiovascular complications are one of the leading causes of death in patients with kidney disease or diabetes. Vascular calcification (VC) was once considered a passive process resulting from elevated calcium-phosphate interactions, but is now considered an active cell-mediated process. VC can affect quality of life because healthy arteries harden analogously to bone development leading to hypertension and compromised structural integrity. Based on previous literature, the *in vitro* model was developed by culturing human primary aortic smooth muscle cells with 3-mmol inorganic phosphate (Pi) and sodium to induce calcification. The *in vitro* model was then used to prompt VC and promote the genetic switching from healthy smooth muscle cells to osteoblast-like cells through manipulation of the cells' plasticity. The *in vitro* model examined the Wnt signaling pathway in VC and Sclerostin's ability to block activation of the pathway. Atomic absorption spectroscopy, Western blot, and Polymerase chain reaction (PCR) analysis revealed that the model was capable of inducing VC, up-regulating the osteogenic differentiation markers runt-related transcription factor 2 (Runx2) and bone morphogenetic protein 2 (BMP2), and down-regulating α-smooth muscle actin activity. Under the same methods, it was

revealed that Sclerostin was capable of recovering α-smooth muscle actin activity in calcification media and able to down-regulate the osteogenic differentiation marker Runx2. This study proved the effectiveness of the *in vitro* model to induce calcification of healthy vascular smooth muscle cells and Sclerostin's ability to be used as a potential therapeutic target for VC.

Keywords: Smooth muscle cells; vascular calcification; mesenchymal stem cells; plasticity; α-smooth muscle actin

Abbreviations: VC: vascular calcification; Pi: Inorganic Phosphate; Runx2: runt-related transcription factor 2; BMP2: bone morphogenetic protein 2; VSMC: vascular smooth muscle cell; MSC: mesenchymal stem cell; SEM: scanning electron microscopy; EDS: energy dispersive spectroscopy; SDS: sodium dodecyl sulfate; PCR: polymerase chain reaction; LRP: lipoprotein receptor related protein; Pit-1: pituitary-specific positive transcription factor 1; DMEM: Dulbecco's Modified Eagle Medium; BCA assay: bicinchoninic acid assay; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; ANOVA: analysis of variance

1. Introduction

VC has a strong correlation with morbidity and mortality because it reduces aortic and arterial compliance that hinders cardiovascular hemodynamics [1–3]. These cardiovascular complications are the leading cause of death for patients with chronic kidney disease or diabetes [4,5]. The severity of the calcification promotes hypertension, aortic stenosis, cardiac hypertrophy, myocardial and lower-limb ischemia, congestive heart failure, and compromised structural integrity [2,3]. There are two types of vascular calcification each based on their location. The first is atherosclerotic calcification associated with the intimal layer and relates to atherosclerosis [4,6]. The second is known as medial arterial calcification and is more predominant in patients with chronic kidney disease or diabetes [6,7]. Previous studies considered vascular calcification a passive process that resulted from elevated calcium-phosphate interactions [8,9]. However, it is now considered an active cell-mediated process where a phenotypic switch causes vascular smooth muscle cells (VSMCs) to become osteoblast-like cells [1,4]. There are many regulatory bone formation and structural proteins that are expressed in the calcified medial arterial layers and atherosclerotic plaques, which suggest that this is an active process [4].

Unlike other smooth muscle cells, VSMCs are derived from mesenchymal stem cells (MSCs) and can change phenotype due to their plasticity [10,11]. These MSCs possess the ability to differentiate into a specific, single-lineage based on the induction media [12]. For patients with chronic kidney disease or diabetes, the modulatory signals of mesenchymal-derived VSMCs are disrupted and trans-differentiate

into osteoblasts in the presence of calcification media [11,12]. This plasticity disruption can lead to atherosclerosis, restenosis, and hypertension [13].

An important pathway for osteogenic differentiation is through the Wnt signaling pathway [14]. A high-phosphate environment activates this signal transduction pathway, where the Wnt proteins bind to the plasma membrane Frizzled and lipoprotein receptor related protein-5/6 co-receptors (LRP 5/6) [14]. After Wnt attaches to its receptors, it activates a downstream signaling cascade that transports β-catenin into the nucleus to up-regulate Runx2 expression [14,15] (Figure 1). The up-regulation of Runx2 leads to hydroxyapatite deposits. There are some known inhibitors for the VC process such as Matrix Gla Protein, Osteopontin, and Fetuin [7,10,14].

Another protein that is capable of preventing the phenotypic switch is known as Sclerostin. Sclerostin is a protein secreted by osteocytes that is a negative regulator of bone formation [16]. It antagonizes the Wnt Signaling pathway by binding to the LRP 5/6, which prevents Wnt from binding to the co-receptors Frizzled and LRP 5/6 and thereby inhibits the up-regulation of Runx2 [16,17] (Figure 1).

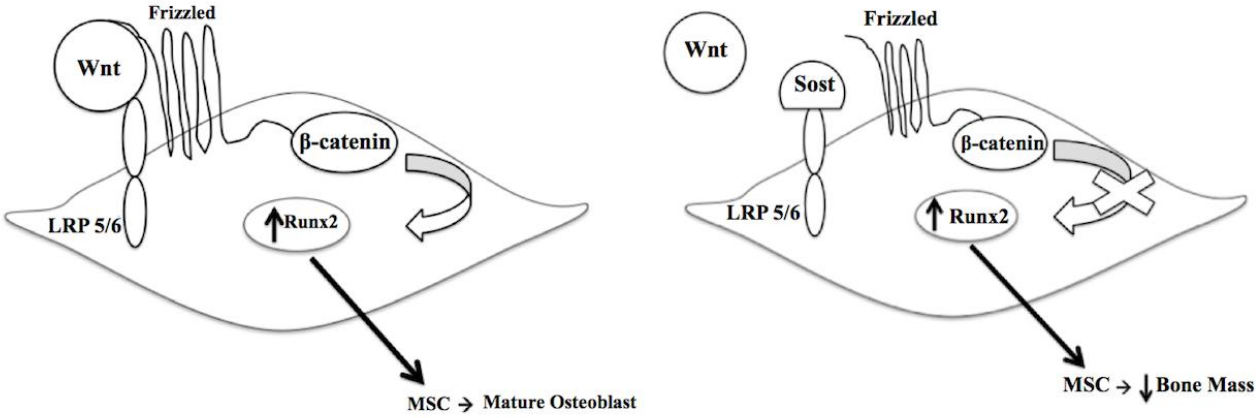

Figure 1. The Wnt-β-catenin signaling pathway and its extracellular regulation. The figure on the left shows how Wnt binds extracellular to the co-receptor Frizzled and LRP5/6, which leads to an intracellular accumulation of β-catenin. β-catenin is translocated into the nucleus leading to an up-regulation of Runx2. This causes mesenchymal stem cells to trans-differentiate from VSMCs into mature osteoblasts. The figure on the right shows how sclerostin is secreted by osteocytes, and it binds to the lipoprotein receptor-related protein 5/6 and inhibits Wnt from binding to the co-receptor complex Frizzled-LRP 5/6. It inhibits the up-regulation of Runx2 and causes a decrease in bone mass.

The purpose of this study was to manipulate the plasticity of VSMCs to treat vascular calcification, and use a developed *in vitro* model to prompt vascular calcification to distinguish the phenotypic switch from healthy smooth muscle cells to osteoblast-like cells. Previous *in vitro* studies concluded that increased serum phosphate and calcium levels contributed to vascular calcification and osteogenic

differentiation [18]. Further, phosphate induced calcification relies on the sodium-dependent pituitary-specific positive transcription factor 1 (Pit-1) channel, which transports phosphate across the membrane to interact with the nucleus and up-regulate osteogenic transcription factors [19,20]. The *in vitro* model was optimized by adding sodium and phosphate to activate the Pit-1 channel. After optimization, the model was able to induce vascular calcification (Figure 2). It was then used to examine the Wnt Signaling pathway, and use Sclerostin to block the activation of the pathway.

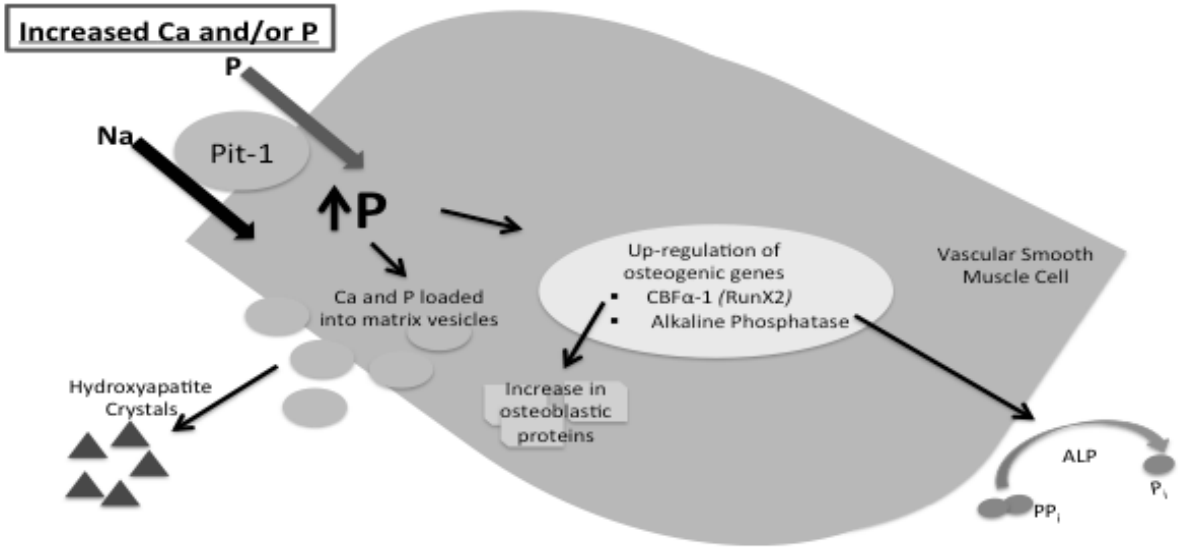

Figure 2. *In vitro* cell culture model schematic. Model was optimized with Sodium and Phosphate to activate Pit-1 channel, which induces calcification. This leads to an up-regulation of osteogenic genes Runx2 and alkaline phosphatase and hydroxyapatite deposits.

2. Materials and Methods

In Vitro Cell Culture Model. Human primary aortic smooth muscle cells were obtained from ATCC® (PCS-100-012™) and treated with Dulbecco's Modified Eagle Medium (DMEM), 10% Fetal Bovine Serum, and 1% penicillin/streptomycin. The cells were grown to 80% confluence and treated with either a normal or calcification media. The normal media was identical to the growth media; however, the calcification media had an addition of 3-mmol inorganic phosphate. The cells were grown for 7 to 14 days and were maintained by feeding every 2 to 3 days.

Scanning electron microscopy (SEM) and Energy Dispersive X-Ray Spectroscopy (EDS). For SEM, the calcification was viewed on a Zeiss Evo 50 microscope at 10 kV. At day 7 and 14, the cells were fixed in Karnovsky's solution and then placed in storage at 4 ℃. The cells were then removed from storage and processed for imaging at up to 5000× magnification. The same cells for SEM were used for

EDS to determine the elemental composition of a region of interest. The elemental composition was determined by using the Buker EDS system.

Atomic Absorption Spectroscopy. At day 7 and 14, calcium deposition was measured and normalized to protein levels obtained from the Pierce BCA assay. The cells were decalcified for 24 hours with 5 mL 0.6 N HCl. The HCl supernatant was collected. Next, the cell layers were solubilized in 0.1 N NaOH/0.1% sodium dodecyl sulfate (SDS). The cell layers were then collected. To prepare the HCl supernatant samples, 1 mL of 20,000-ppm potassium chloride, 1 mL of nitric acid, and 1 mL of prepared sample was added to each tube. The 1 mL of prepared sample was diluted 1:7 in 4 mL of distilled water to bring a total volume of each sample to 5 mL. The calcium content of the samples was measured via flame atomic absorption spectroscopy. The concentration of each sample was derived from the standard curve.

The cellular protein content of the solubilized cell layers was calculated using the BCA protein assay kit (Pierce). The calcium content was normalized based on cellular protein content.

Western Blot. At day 7 and 14, the cells were lysed with Modified Hunter's buffer and briefly sonicated. To obtain the protein in the supernatant, the cells were centrifuged and the protein concentration was measured with a BCA assay. A protein gel was used to separate the proteins based on molecular weight. Next, the proteins were transferred to a nitrocellulose membrane. The membrane was incubated with primary and secondary antibodies as well as horseradish peroxidase conjugate for enhanced chemiluminescence imaging. The samples were analyzed to determine the amount of α- smooth muscle actin present and the protein content was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

PCR. At day 7 and 14, the cells were trypsinized, extracted from the culture dish, and centrifuged in order to receive a whole cell pellet. The genetic material obtained was tested using the RT^2 Profiler PCR Array from SA Biosciences for human osteogenesis. Gene analysis was performed for Runx2, BMP2, and GAPDH.

Sclerostin Therapy Methods. When cells reached 80% confluence, the medium for the dibasic (calcified) samples was replaced with calcification media containing 3-mmol inorganic phosphate. At day 7, 80 ng/mL concentration of sclerostin was added to the sclerostin normal and calcified groups. This number was derived from previous studies, which used 50 ng/mL and 100 ng/mL, and their data had little variation of sclerostin's effectiveness at these concentrations [22–24]. The sclerostin normal group received calcification media for days 0 through 7 and was replaced with normal media for days 8 through 14. The sclerostin calcification group received calcification media for days 0 through 14. The no treatment group received calcification media for days 0 through 7 and was then replaced with normal media for days 8 through 14. The number of repeats for each experiment performed was n = 4. The calcium content was measured using a calcium kit (O-cresolphtalein method) from Sigma Aldrich.

Statistical Analysis. Statistical analysis was performed with a student's t-test and one-way analysis of variance (ANOVA) tests with software from StatPlus, AnalystSoft Inc. version v6.

3. Results

3.1. In vitro model induces calcium deposition in VSMCs

To identify the ability of our group's *in vitro* model to induce calcification, we studied the calcium content in primary aortic smooth muscle cells. Inorganic phosphate and sodium were used because it is known that they induce calcification in VSMCs [20].

The calcium content was obtained through atomic absorption spectroscopy. As shown in Figure 3, the calcium content for the calcification medium-treated groups was significantly increased compared to the control groups.

To further confirm the model's ability to induce calcification, we performed SEM and EDS. The calcification was increased upon treatment of calcification media. The results from SEM and EDS were visualized and quantified to show the calcification present in the samples (Figure 4).

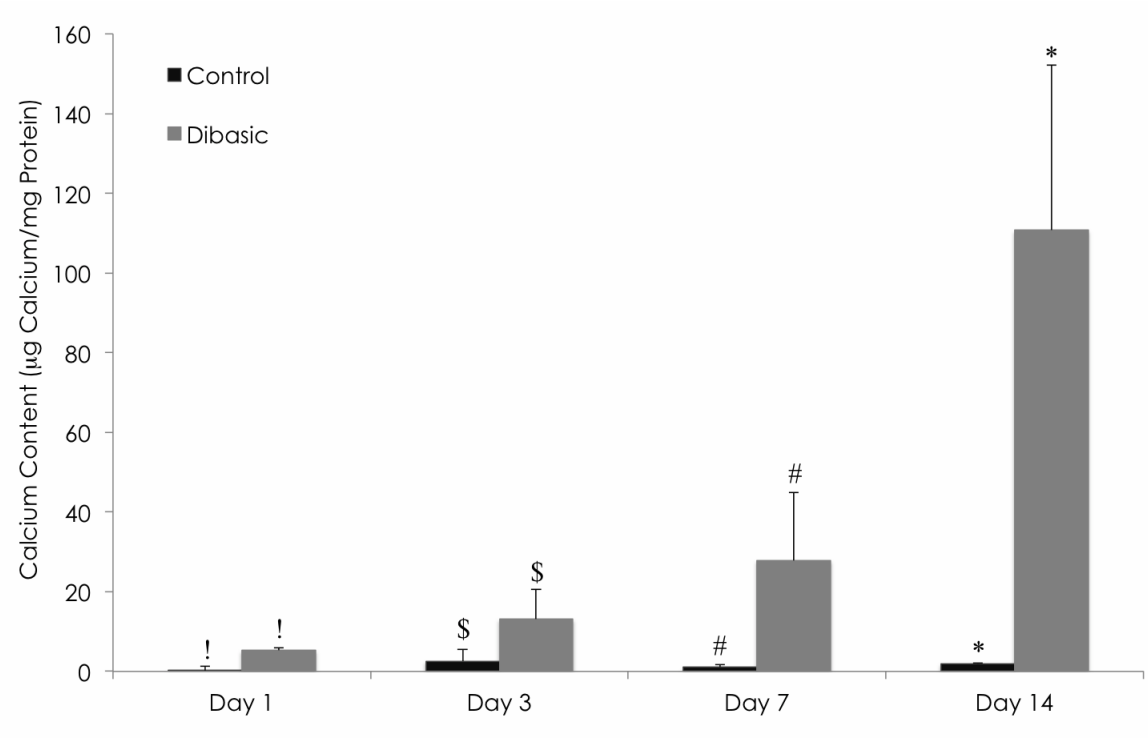

Figure 3. Atomic Absorption of Calcium for *in vitro* cell culture model. The graph represents the calcium content of Day 1 through Day 14 groups for control and dibasic (calcified). A student's t-test was performed to show that the dibasic and control results at each day showed significant variation when $p < 0.05$. [!] Represents significant increase from Day 1 dibasic and control. [$] Represents significant increase from Day 3 dibasic and control. [#] Represents significant increase from Day 7 dibasic and control. [*] Represents significant increase from Day 14 dibasic and control.

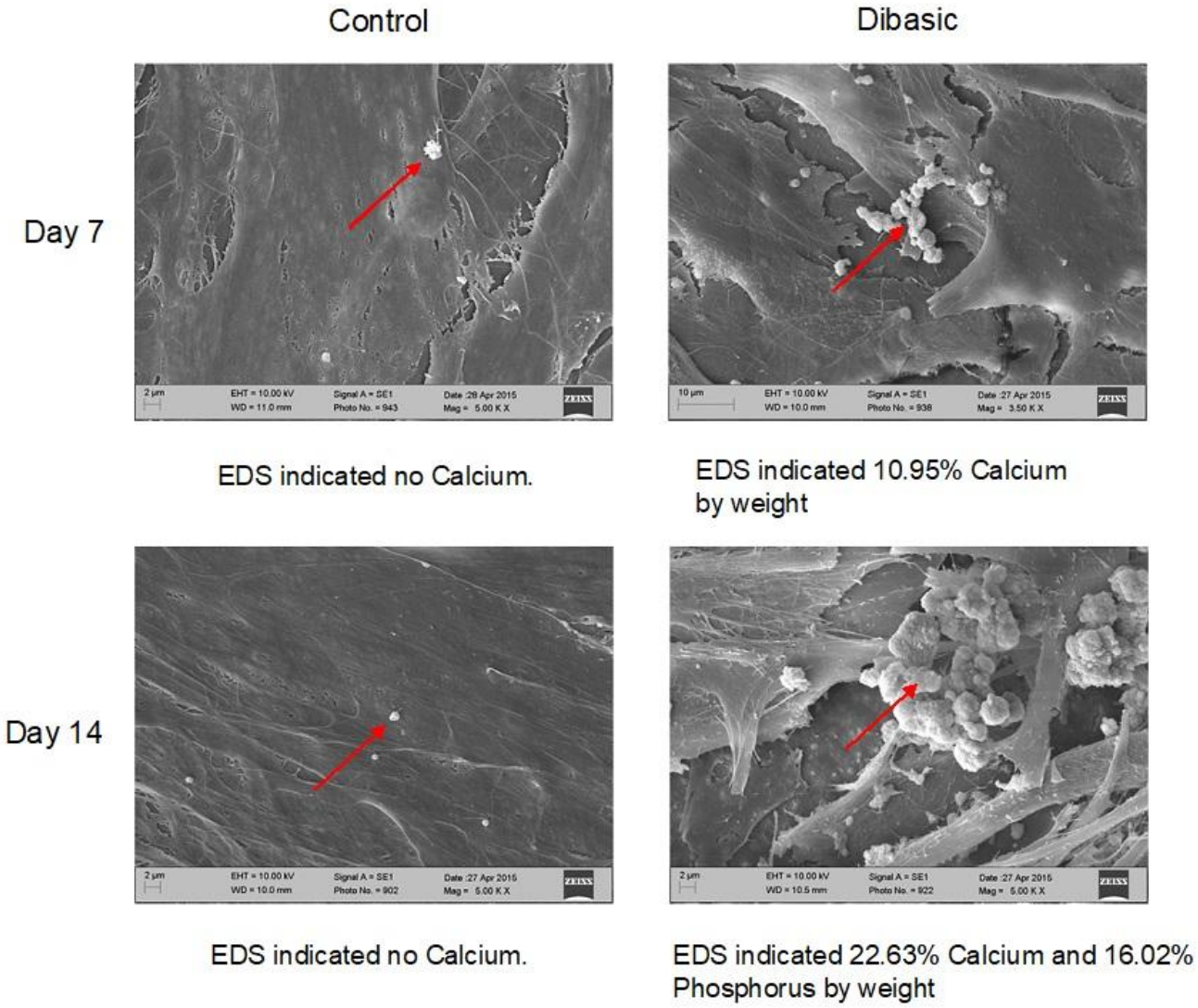

Figure 4. Scanning Electron Microscopy (SEM) and Energy Dispersive X-Ray Spectroscopy (EDS) for *in vitro* cell culture model. The SEM images represent the calcification present within the *in vitro* model of Human Vascular Smooth Muscle cells. The red arrows indicate where the calcium content was measured with EDS. A represents the control for Day 7 and indicated no calcium; B represents the calcified for Day 7 and indicated 10.95% calcium by weight; C represents the control for Day 14 and indicated no calcium; D represents the calcified for Day 14 and indicated 22.63% Calcium and 16.02% Phosphorus by weight.

3.2. Calcified smooth muscle cells exhibit osteogenic markers

MSCs differentiate into osteogenic lineages in the presence of calcification media. MSCs osteogenic differentiation markers such as Runx2 and BMP2 were examined and compared to GAPDH, and the presence was confirmed by real-time PCR analysis (Figure 5). Runx2 and BMP2 were examined because they are earlier markers of osteogenic differentiation and more specific to osteogenic lineages. The results for Runx2 expressed a significant up-regulation between days 7 and 14 when the groups were treated with inorganic phosphate. BMP2 was also up-regulated between day 7 and 14. The up-regulation of these osteogenic markers coincides with a phenotypic switch.

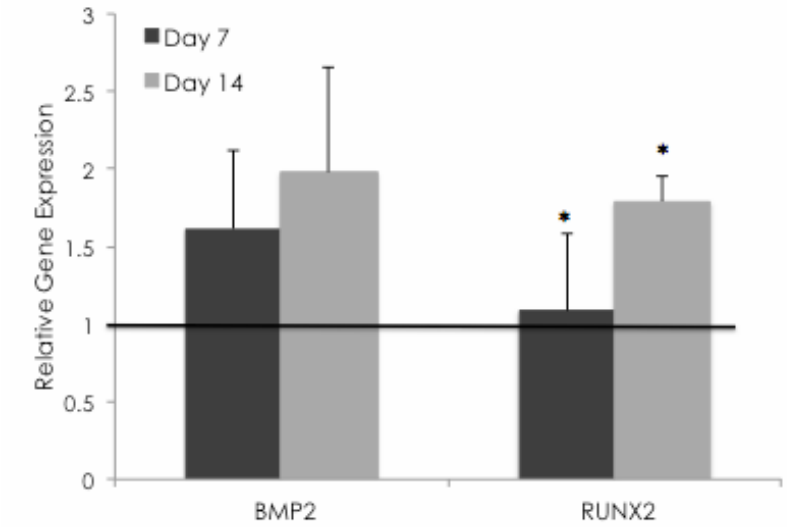

Figure 5. Polymerase Chain Reaction (PCR) for Runx2 and BMP2. The graph shows the results for two osteogenic markers. For relative gene expression levels analyzed by the ΔΔ Ct method, the housekeeping gene was beta-2-microglobulin (B2MG), which is represented by the baseline at 1. * Represents Runx2 is significantly up-regulated between Day 7 and Day 14 when $p < 0.05$. Also, BMP2 is up-regulated in calcified cells. These results suggest that there is a phenotypic switch present within the *in vitro* model for vascular calcification.

3.3. In vitro model causes down-regulation of α-smooth muscle actin in calcified cells

To confirm the differentiation in VSMCs, the α-smooth muscle actin activity was observed. This actin isoform is prevalent in VSMCs and acts a differentiation marker for this cell type [21]. A western blot was used to examine the difference in expression levels for α-smooth muscle actin in the day 7 and 14 control and dibasic samples and was compared to GAPDH. Our results confirmed a down-regulation of α-smooth muscle actin in calcified cells, signifying an osteogenic switch (Figure 6).

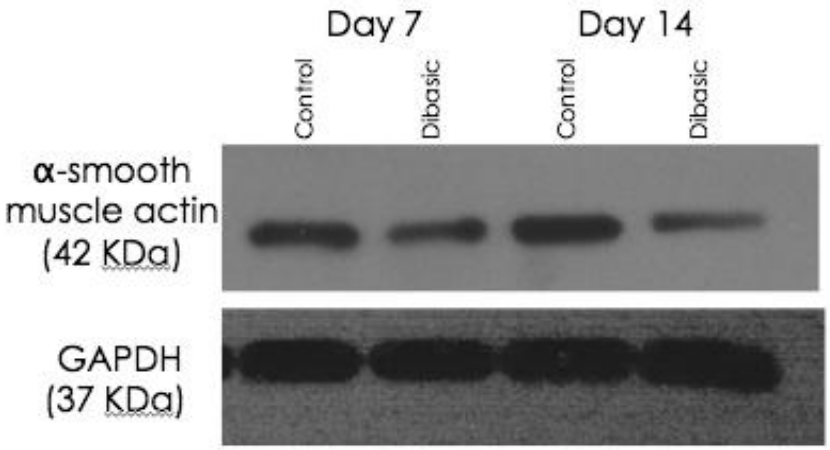

Figure 6. Western Blot for *in vitro* cell culture model. The western blot data shows the difference in expression levels in α- smooth muscle actin between the control and dibasic samples in Day 7 and Day 14. This difference suggests that the α- smooth muscle actin is down regulated in calcified cells. Further, this suggests the presence of an osteogenic switch.

3.4. Sclerostin inhibits Pi-induced calcification in VSMCs

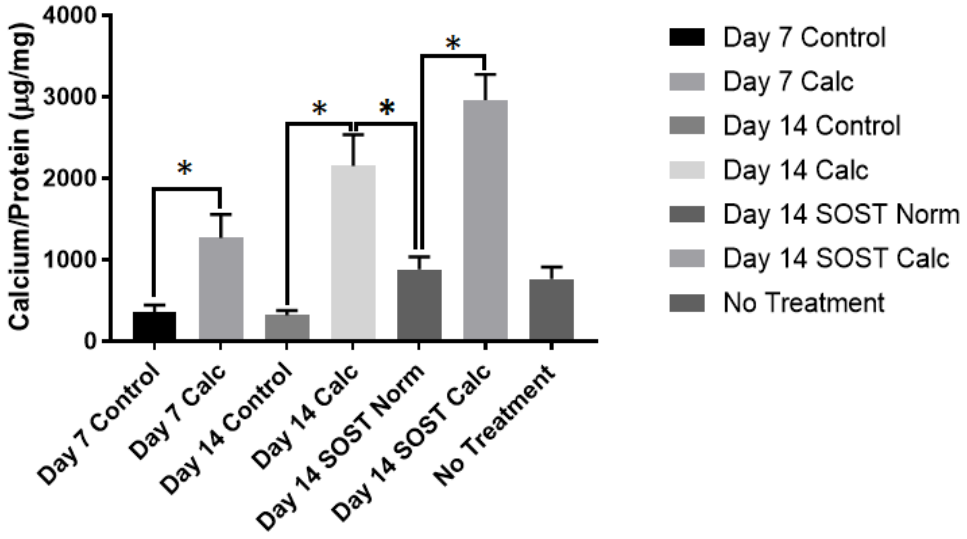

Figure 7. Atomic Absorption of Calcium Content for Sclerostin Study. The graph shows the calcium content for Day 7, Day 14, sclerostin, and no treatment groups for control and calcified and the normal and calcified groups. A one-way ANOVA test was performed to show that the calcified and control/normal results at each day showed significant variation when $p < 0.05$.

To identify the effect of sclerostin on vascular calcification, we studied the calcium content in primary aortic smooth muscle cells. The calcium content was obtained through atomic absorption spectroscopy. As shown in Figure 7, the calcium content for the calcification media-treated groups was significantly increased compared to the control groups. Further, the sclerostin calcification group exhibited a decrease in calcium content in the presence of Sclerostin as compared to the day 14 calcification group.

3.5. Sclerostin down-regulates the osteogenic marker Runx2

The presence of osteogenic marker Runx2 was determined through real-time PCR analysis and compared to GAPDH. The results show that treatment with inorganic phosphate led to an up-regulation of Runx2 for the day 14 calcification group and the sclerostin calcification treatment group compared to the no treatment group. However, sclerostin was able to down-regulate Runx2 in the presence of calcification media as compared to the day 14 calcification group. Further, Runx2 was down-regulated in the sclerostin normal treatment group compared to the no treatment group (Figure 8).

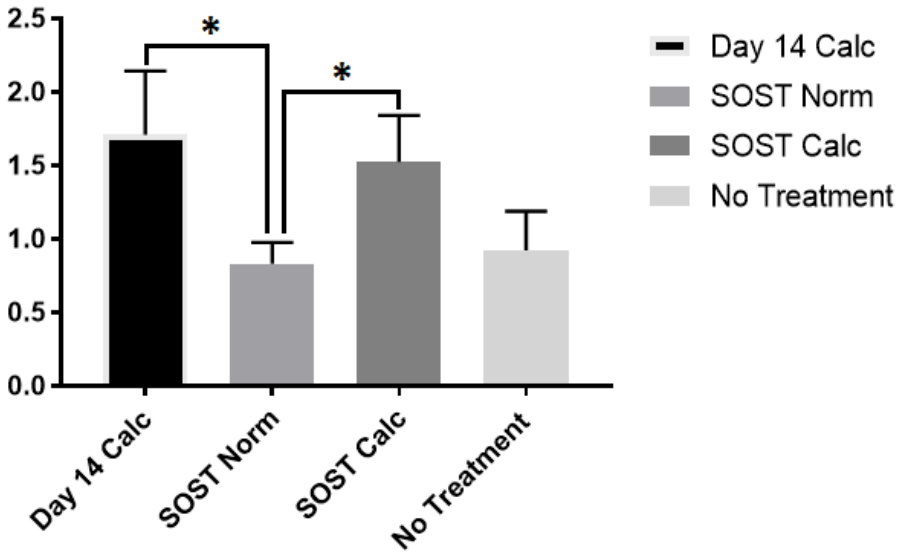

Figure 8. Polymerase Chain Reaction (PCR) for Runx2 for sclerostin study. The graph shows the results for the osteogenic marker, Runx2. For relative expression levels analyzed by the ΔΔ Ct method, the housekeeping gene was GAPDH. The data shows an up-regulation of Runx2 for Day 14 calcification group and the sclerostin calcification treatment group compared to the no treatment group. There is also a down-regulation of Runx2 in the sclerostin normal treatment group. Further, it shows a down-regulation of Runx2 in the sclerostin calcification group compared to the day 14 calcification group. This suggests that sclerostin is capable of preventing vascular smooth muscle cells from a phenotypic switch to osteoblast-like cells.

3.6. Sclerostin recovers α-smooth muscle actin activity in calcified cells

In the *in vitro* cell culture study, calcified cells exhibit a down-regulation of α-smooth muscle actin activity. The α-smooth muscle actin activity was examined for each group and compared to GAPDH. Consistent with the previous western blot results, the day 14 calcification group experienced a decrease in α-smooth muscle actin activity. However, the sclerostin was able to recover α-smooth muscle actin activity by 45.8% in the presence of calcification media (Figures 9, 10).

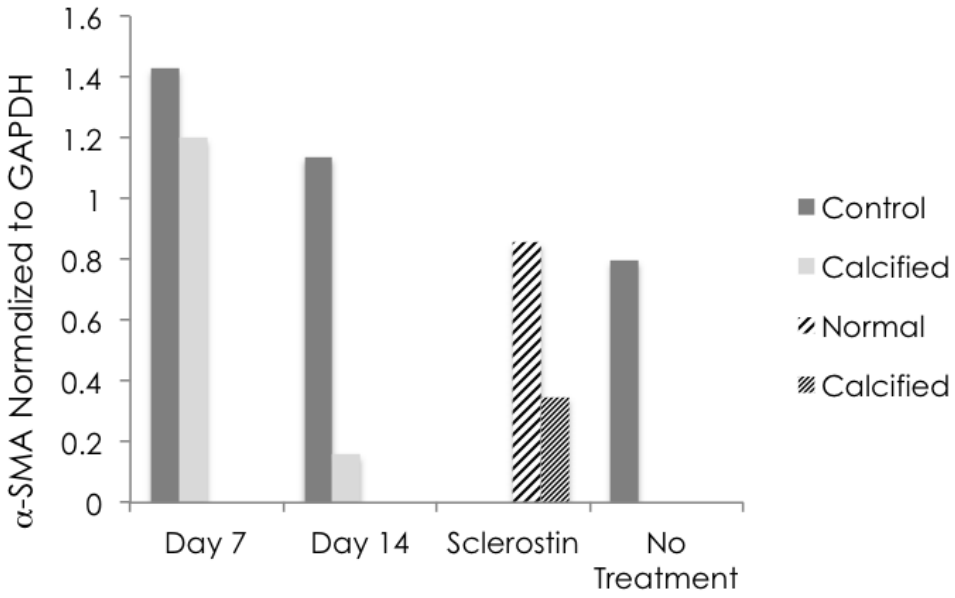

Figure 9. α-SM actin normalized to GAPDH. The graph represents the amount of the α- smooth muscle actin normalized to GAPDH for day 7, day 14, sclerostin, and no treatment groups. Cells supplemented with sclerostin after 7 days of calcification showed a recovery of α- smooth muscle actin, which suggests it is capable of preventing phenotypic switch in VSMCs. The graph was obtained by performing densitometric analysis with NIH Image J software to generate arbitrary numbers for the values of α- smooth muscle actin expression and the expression of the loading control, GAPDH. The values for alpha-smooth muscle actin were normalized to the expression of GAPDH. This experiment was completed with an n = 1 with no other experimental repeats to compare to this experiment, and therefore did not generate any error bars.

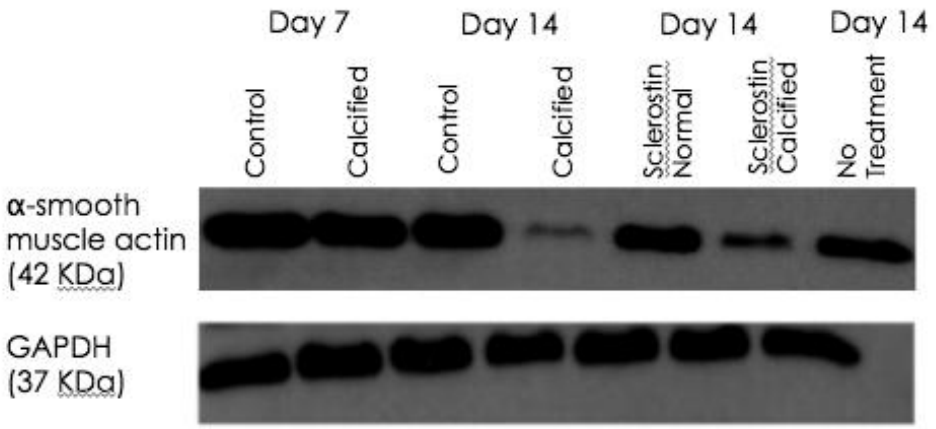

Figure 10. Western Blot Data for α-smooth muscle actin and GAPDH for Sclerostin Study. The results from the western blot of the control/normal and calcified samples suggests that the α- smooth muscle actin is down regulated in calcified cells. Further, cells supplemented with sclerostin after 7 days of calcification showed a recovery of α- smooth muscle actin. This suggests that sclerostin is capable of preventing VSMCs from a phenotypic switch to osteoblast-like cells.

4. Discussion

In this study, we found that our *in vitro* model successfully induced calcification of healthy VSMCs upon the addition of inorganic phosphate and that calcified cells exhibit a loss of α-smooth muscle actin activity. We also revealed that calcified smooth muscle cells exhibit osteogenic markers, such as Runx2 and BMP2. Further, we showed that sclerostin could be used in the future as a potential therapeutic target to treat vascular calcification.

Previous studies showed that increased sodium and phosphate levels successfully induce calcification [18–20]. This is hypothesized to activate the Pit-1 channel, which in turn leads to an up-regulation of Runx2 [18,19]. This up-regulation causes an osteogenic switch where smooth muscle cells change their phenotype to osteoblast-like cells thus confirming their plasticity [18–20]. Our study coincided with previous research that vascular calcification expresses osteogenic markers such as Runx2 and BMP2 [8,10,14]. These osteogenic markers are up-regulated under calcifying conditions that lead to the phenotypic switch of VSMCs to osteoblast-like cells [8,11,18–20].

Recent studies have demonstrated that sclerostin is a therapeutic target for treating osteoporosis and osteoporotic fractures [22,23]. Previous groups have utilized sclerostin monoclonal antibody in rodents and primates to increase bone formation and strength due to the increase in osteoblasts and bone formation markers as well as a decrease in osteoclasts [16]. Other studies have discovered that sclerostin plays an important role in tooth movement [24]. The study observed the effects of compression and tension stress on sclerostin expression with mice studies [24]. It was reported that compression and tension have the same effect on sclerostin expression, but in hypoxia conditions for compression sites,

the amount of sclerostin is increased. Further, it was discovered that sclerostin knockout mice have increased osteoblastic activity suggesting that sclerostin plays an important role in bone remodeling and osteoclastic activity [24].

Sclerostin is an important factor for regulating bone formation [16,17,22–24], but until our study it has not been used as a treatment to decrease vascular calcification. In the past, many researchers have determined there is a correlation between serum sclerostin levels and vascular calcification especially in patients with chronic kidney disease [25–27]. Their previous research has shown that increased serum sclerostin levels are heavily associated with patients that have chronic kidney disease and aortic calcifications, but the use of sclerostin as treatment had not been tested [25–28]. To our knowledge, we for the first time revealed that sclerostin is capable of regulating inorganic phosphate induced VSMC calcification. In our study, we concluded that treatment with sclerostin regulated calcification and down-regulated the osteogenic marker, Runx2, *in vitro* by preventing Wnt proteins from attaching the Frizzled and LRP 5/6 co-receptors and thus causing a downstream effect that up-regulates Runx2 activity. In our study, sclerostin was capable of reducing inorganic phosphate calcification when compared with the day 14 calcification group.

VMSCs are derived from mesenchymal stem cells and can be differentiated into a single-lineage based on the induction media because of their plasticity [10–13]. One of the most significant findings from this study was sclerostin's ability to manipulate the plasticity of smooth muscle cells in calcification media to recover α-smooth muscle actin activity. It was able to reverse the effects of lost α-smooth muscle actin activity by the day 14 calcification group by 45.8%.

In conclusion, we have shown that our *in vitro* model is capable of inducing calcification and that calcified cells express osteogenic markers. Further, our results show that sclerostin can act as potential therapeutic target to treat vascular calcification because of its ability to reduce the calcium content *in vitro*, to reduce the expression of the osteogenic marker Runx2, and to recover α-smooth muscle actin activity.

Acknowledgments

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Conflict of Interest

All authors declare no conflicts of interest.

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