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Thesis

UNDERSTANDING THE ROLE OF THE TRANSCRIPTION FACTOR *BCL11B* IN VASCULAR SMOOTH MUSCLE CELL PROLIFERATION AND AORTIC ANEURYSM DEVELOPMENT

By

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UNDERSTANDING THE ROLE OF THE TRANSCRIPTION FACTOR *BCL11B* IN VASCULAR SMOOTH MUSCLE CELL PROLIFERATION AND AORTIC ANEURYSM DEVELOPMENT

ABIGAIL LAUREN KUNZE

ABSTRACT

Background: Aortic aneurysms are potentially fatal vascular conditions, with no non-surgical treatments currently approved (Kuivaniemi et al., 2014). Recently, the Seta laboratory discovered that the transcription factor, Bcl11b, is present in the vascular smooth muscle cells that comprise the aorta and has been linked to arterial stiffness. To further investigate the role of Bcl11b in vascular function, a vascular smooth muscle cell-specific Bcl11b knockout (BSMKO) was created and challenged with Angiotensin II (Ang-II), a hypertensive stimulus. Interestingly, it was found that BSMKO mice developed aortic aneurysms while their control littermates did not. This experiment led to the hypothesis that Bcl11b may play a role in the development of aortic aneurysms.

Methods: Using Ingenuity Pathway Analysis (IPA) we compared RNA-sequencing data generated from aortas isolated from wild-type (WT) and BSMKO after Ang-II-treatment and identified differentially expressed genes important in the cell cycle pathway. To test the hypothesis that Bcl11b regulates the VSMC cell cycle, we performed two assays; a propidium iodide uptake and a 5-ethynyl-2′-deoxyuridine (EdU) Incorporation Assay which were both quantified using flow cytometry. An *in vitro* model was used where VSMCs isolated from WT and BSMKO mice were treated with growth

stimulant PDGF. EdU incorporation was also performed *in vivo* in WT and BSMKO mice treated with Ang-II.

Results: Our results indicate that there was a significant increase in the percent of cells in the S phase when stimulated with PDGF in the WT mice, but not in the BSMKO mice in the *in vitro* model. The *in vivo* model did not indicate statistically significant differences between Ang-II-treated WT and BSMKO mice. These results suggest that VSMCs lacking Bcl11b are less proliferative than WT VSMCs when stimulated with PDGF.

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LIST OF ABBREVIATIONS

AA……..Aortic Aneurysm

Ang-II……Angiotensin II

Bcl11b……B-Cell Leukemia 11b

BSMKO……Bcl11b Smooth Muscle Knockout

IL-1β……Interleukin 1 Beta

PDGF……Platelet-Derived Growth Factor

TGFβ……Transforming Growth Factor Beta

VSMC……Vascular Smooth Muscle Cell

WT……Wild-Type

CHAPTER 1: INTRODUCTION

Aortic Aneurysm and the Discovery of Bcl11b in the Vasculature

The increasing prevalence of cardiovascular disease is a growing public health concern in the United States (Kuivaniemi et al., 2014). Among various cardiovascular diseases, aortic aneurysms (AA), a pathological dilation of a segment of the aorta that have an increased risk of further expanding and rupturing, may potentially cause death (Mathur et al., 2016). The structural integrity of the aorta is essential to delivering oxygenated blood throughout the body. The aorta is composed of three layers, the tunica externa, tunica media, and tunica intima. In AA, the muscular tunica media is compromised. This layer is primarily comprised of vascular smooth muscle cells (VSMCs). VSMCs can undergo a unique alteration known as the phenotypic switch, consisting of decreased expression of contractile proteins and increased proliferation. (Petsophonsakul et al., 2019). This is important because VSMCs are highly specialized cells and can undergo this switch during needed repair in response to vascular injury. However, this phenotypic switch, when excessive or uncontrolled, is associated with vascular diseases, including atherosclerosis, hypertension, and AA (Alexander et al., 2011). The pathogenesis of AA is complex and multifactorial, and there are currently no pharmacological treatments for this potentially fatal disease (Ailawadi et al., 2003). Therefore, understanding molecular mechanisms behind AA may lead to the development of needed therapies.

The Seta laboratory recently discovered that the transcription factor B-cell leukemia 11b (Bcl11b) is expressed in VSMC, where it regulates arterial stiffness (Valisno et al., 2021). A recent genome wide association study found that patients with elevated pulse wave velocities (PWV), the gold standard for measuring arterial stiffness, had high rates of single nucleotide mutations in a conserved region upstream of Bcl11b (Mitchell et al., 2011). This region is expected to regulate Bcl11b function, so findings by the Valisno study suggest that increased arterial stiffness due to mutations in this region or to Bcl11b may be due to impaired Bcl11b expression and/or activity in VSMC. Furthermore, the Seta group found that Bcl11b was downregulated in a model of arterial stiffness where mice are fed a diet high in fat and sucrose (Figure 1). The results from a different study also show that a Bcl11b transcript detected in the aorta reflects lymphocyte infiltration in humans and that mutations in Bcl11b gene loci associate with lower Bcl11b levels and aortic stiffness measured *in vitro* (Maskari et al., 2018). This suggests that Bcl11b-dependent immune mechanisms are at play in the development of arterial stiffening. Together these findings indicate Bcl11b may be a potential therapeutic target for vascular stiffness.

To better understand the role of Bcl11b in VSMC, a VSM cell-specific Bcl11b knockout (BSMKO) was developed. There was no change in gross morphology of the aorta in BSMKO vs. WT mice (Valisno et al., 2021). To understand the role of Bcl11b in a model of vascular disease, 1.44 mg/kg/day of the hypertensive stimulus angiotensin II (Ang-II) was administered to WT and BSMKO mice for seven days via Alzet osmotic minipumps. Ang-II is a part of the renin-angiotensin-aldosterone system that is involved

in the regulation of blood volume and systemic vascular resistance (Fountain et al., 2021). The Seta lab found Ang-II increased blood pressure similarly in WT vs. BSMKO mice, however Ang-II-treated BSMKO mice developed AA, compared to Ang-II-treated WT mice, of which none developed AA (Figure 2). This finding led to the hypothesis that Bcl11b may potentially play a crucial role in the development AA.

Understanding Bcl11b-Dependent Mechanisms Linked to AA

Following the discovery of AA in the BSMKO mice, we pursued the question of which molecular pathways involved in the development of these aneurysms were unique to BSMKO mice treated with Ang-II. RNA sequencing was performed on the aortas of WT and BSMKO mice treated with Ang-II. In total, 910 genes were uniquely expressed in BSMKO mouse aortas treated with Ang-II when compared to WT-Ang-II mice. An Ingenuity Pathway Analysis was performed on the RNA Sequencing dataset to identify potential gene networks among these 910 genes differentially expressed genes. From the initial 910 genes expressed in the BSMKO-Ang-II model, unmapped IDs, genes with no cardiovascular function, canonical pathways with p-value > 0.05, and canonical pathways with z-scores between -2 and 2 were excluded. After applying these restrictions, 4 total canonical pathways were identified on the remaining genes: (1) PTEN signaling, (2) cell cycle control of chromosomal replication, (3) kinetochore metaphase signaling pathway, and (4) integrin signaling (Figure 3). Because the cell cycle was involved in 2 of these 4 pathways, we hypothesized that Bcl11b may regulate the VSMC cycle and cell proliferation and this may be causally linked to AA development. This hypothesis was

also consistent with other studies that have analyzed the effect of downregulation of Bcl11b on cell proliferation and found that suppression of Bcl11b inhibits proliferation in human T-cells (Huang et al., 2013). My study aims to understand the differences in the cell cycle pathways between WT and BSMKO mice at baseline and under stimulated conditions to better understand how this pathway can be targeted for the treatment of arterial stiffness and AA.

CHAPTER 2: MATERIALS AND METHODS

Tamoxifen-Inducible VSM-Specific Bcl11b Knockout Mice

At eight weeks of age, *Smmhc*^{CreERT2}/*Bcl11b*$^{flox/flox}$ mice were treated with tamoxifen (200 µL, 2 mg/d, 5 days) or vehicle (200 µL, 5 days) to obtain VSM-specific *Bcl11b*-deficient mice (BSMKO) and WT controls, respectively (Valisno et al., 2021).

Propidium Iodide Staining Technique

One widely used procedure to measure cell cycle is via flow cytometry after propidium iodide (PI)/RNase staining to measure the DNA content of the cells. This technique allows for the differentiation of each phase of the cell cycle as well as the detection of apoptotic cells that have reduced DNA content compared to living cells (Crowley et al., 2016). Crowley et al. describes how the level of PI fluorescence is directly proportional to the DNA content of a cell, so we reasoned that this method would be a simple way of measuring the differences in cell proliferation between our models.

VSMC Culture and Treatments

VSMCs previously isolated from WT and BSMKO mice were thawed and put into culture in p60 plates and grown using DMEM, 1 g/L glucose, and 10% fetal bovine serum (FBS). The characteristics of WT and BSMKO VSMCs I used are listed in Table 1. Once cells reached approximately 80% confluency, they were sub-passaged into p10 plates. Once these plates had again reached 60-80% confluency, cells were starved using

DMEM, 1 g/L glucose, and 0% FBS either 24, 48 or 60 hours prior to either TGFβ (20 ng/mL) or IL-1β (10 ng/mL) stimulation for 24 hours. Because mouse VSM cells do not respond well to Ang-II *in vitro,* other stimuli downstream of Ang-II were initially used to best mimic the *in vivo* studies of Ang-II-treated BSMKO mice, such as TGFβ and IL-1β, two known downstream effectors of Ang-II in VSMC. These are common inflammatory cytokines, which are involved in AA pathogenesis. Cells were then harvested using 2 mL trypsin for 5 minutes in a 37°C incubator and collected by centrifugation for 5 minutes at 1000 rpm. Cells were then re-suspended in 0.5 mL PBS and fixed in 4.5 mL of 70% ethanol before washing with 5 mL FACS buffer solution (1X PBS, 2% FBS, 1mM EDTA). Lastly, cells were re-suspended in 500 µL of the propidium iodide (PI)/RNase staining solution before flow cytometry was performed using a BD LSRII SORP flow cytometer in the BU Flow Cytometry Core.

No proliferation, as detected by an increase in the number of cells in the S phase, was measured after TGFβ or IL-1β treatment in these cells (Figures 4 and 5). Based on these initial results, it was hypothesized that the DMEM, 1 g/L glucose, and 10% FBS used to grow the cells in culture should be increased to DMEM, 4.5 g/L glucose, and 20% FBS medium to further induce growth and proliferation of these cells. Switching to this medium increased the number of cells in the S phase when compared to the DMEM, 1 g/L glucose, and 10% FBS, however the TGFβ and IL-1β treatments still had no further effect on the percentage of VSMCs in the proliferative phase.

Collagen and Fibronectin

Another method we used to induce growth in cells and mediate differences in growth rates was plating cells onto collagen and fibronectin, two extracellular matrix proteins which should mimic a more physiological milieu for VSMCs. We used 42 µL of fibronectin in 7958 µL PBS, corresponding to a concentration of 0.00525 µg/µL, to cover a p60 plates. The fibronectin solution was then removed and the plate was left to air dry for 45 minutes before VSMCs were then seeded in DMEM, 1 g/L glucose, and 10% FBS.

The collagen solution was made using the same calculations as the fibronectin. The final solution contained 278 µL of collagen in 19.72 mL glacial acetic acid (GAA). This was used to coat the bottom of a p60 plate and left for 1 hour before removing. The plate was then washed four times with PBS before cells were seeded in DMEM, 1 g/L glucose, and 10% FBS.

Platelet-Derived Growth Factor Administration

Finally, based on a lack of response of the VSM cell cycle to Ang-II, TGFβ, or IL-1β treatment, we decided to treat cells with platelet-derived growth factor (PDGF), a well-known proliferative and migratory stimulus for VSMCs. Preliminary flow cytometry trials indicated that starving cells with DMEM, 1 g/L glucose, and 0% FBS 24 hours prior to treatment was the most optimal to synchronize cells into the same cell cycle phase. It was also found that treatment with PDGF (10 ng/mL) 24 hours prior to flow cytometry was the most optimal to induce significant proliferation and movement of cells

from G0/G1 to S phase of the cell cycle. This dose is consistent with the one used by others in previous studies that also analyzed VSMCs *in vitro* (Salabei et al., 2013).

5-ethynyl-2'-deoxyuridine (EdU) Incorporation Assay In Vitro

In addition to the PI staining technique, which produced high variability in the results, a similar, but more sensitive assay, 5-ethynyl-2′-deoxyuridine (EdU) Incorporation Assay, was used to better determine the differences in cell cycle between the WT and BSMKO mice. EdU is a nucleoside analog to thymidine and is incorporated into DNA during active DNA synthesis during the S phase of the cell cycle.

The standard procedure of the EdU Flow Cytometry Kit (Sigma catalog number BCK-FC647-50) was followed as per the manufacturer's recommendations. Similar to PI staining, cells were first starved with DMEM, 1 g/L glucose, 0% FBS 48 hours prior and treated with 10 ng/mL of PDGF 24 hours prior to flow cytometry. A 10 mM EdU stock solution was made using 10 mg of EdU in 4 mL DMSO. This was added to cultured cells in p10 plates for 1 hour at a final concentration of 10 mM. Cells were then collected using 2 mL trypsin for 5 minutes in a 37°C incubator and centrifuged at 1000 rpm for 7 minutes. Then the cells were washed with a 1% BSA in PBS solution and fixed in 4% paraformaldehyde in PBS for 15 minutes. After another washing with 1% BSA and re-suspension in 1x saponin in 1% BSA in PBS, a click chemistry reagent was applied. The reaction cocktail contained PBS, a catalyst solution, a buffer additive and an azide dye that binds ("clicks") to EdU and generates fluorescence at 647 nm wavelength. 500 µL of this solution was added to the cells for 30 minutes before a final washing and re-

suspension in 500 μL of the 1x saponin solution. Then, two drops of a DAPI staining solution, which binds to nuclear DNA, were added and used as a nuclear counterstain for the evaluation of cell death and apoptosis of unfixed cells. Also, one plate of cells was left untreated (i.e., without EdU) and used as a negative control for fluorescence specificity for each experiment (Figure 6).

Aorta Digestion Protocol

To optimize the digestion of aortas to a single cell suspension for EdU/flow cytometry, two solutions were made: (1) 1 mg/mL collagenase type I, 1 mg/mL collagenase type II and 0.5 mg/mL elastase and (2) 2 mg/mL collagenase type II, 1 mg/mL elastase and 10 μg/mL DNase in PBS or HBBS containing calcium and magnesium. These solutions were used to digest the aortas isolated from WT mice, treated with or without Ang-II via osmotic minipump. Aortic solutions were gently pipetted up and down every 15 minutes for approximately 30-45 minutes at 37°C. Once fully dissolved, 1 mL DMEM, 1 g/L glucose, and 20% FBS and 6 mL PBS were added to the solution and filtered through 40-micrometer cell strainers. Cells were then collected by centrifugation at 1250 rpm for 7 minutes before resuspension in 0.5 mL PBS and detection of EdU by flow cytometry, as described for VSMCs above. Preliminary experiments with WT mice with/without Ang-II treatment, indicated that the two digestion solutions tested were equally effective in digesting the aortas to a single cell suspension, therefore solution 1 was used in subsequent studies.

5-ethynyl-2'-deoxyuridine (EdU) Incorporation Assay In Vivo

A literature review was completed in order to determine the best conditions for EdU incorporation to use in our mouse model. We used as reference a study from Clement et al, which analyzed EdU incorporation by flow cytometry in VSMCs from aortas of mice infused with Ang-II, which developed AA, similar to our studies (Clement et al., 2019). Similarly, another study injected mice twice with EdU 24 and 4 hours prior to VSMC extraction and had success in measuring cell proliferation via EdU incorporation (Yu et al., 2019). Therefore, we adapted the protocols from those two studies for our study.

For this method, Alzet osmotic minipumps were implanted subcutaneously into WT and BSMKO mice to administer 2 mg/kg/day Ang-II for 14 days before aortas were isolated and analyzed. 100 μg/g of EdU solution was injected into mice 24 and 4 hours prior to aorta collection. The aortas of these mice were then harvested and digested, as described above. Following the digestion protocol, the cells were then subjected to the same protocol as the *in vitro* assays and received the same click chemistry reaction cocktail in order to analyze cell proliferation and DNA synthesis, as described above.

Flow Cytometry Analysis of VSMC

To analyze the differences in cell cycle and cell proliferation between the WT and BSMKO mice, flow cytometry was used as a quantitative measure of the number of cells in each cell cycle phase. The phases were identified as G0/G1 (quiescent phase), S (DNA synthesis phase) and G2/M (cell growth phase). We were particularly interested in

measuring the changes in the S phase, indicative of cells actively proliferating. This is because this phase consists of the replication of chromosomes and the synthesis of new DNA, indicating that proliferation is either occurring or is blocked (Laskey et al., 1989). We were also interested in the percentage of cells in the G0/G1 phase because this may indicate senescent or apoptotic cells that were not proliferating. The number of cells in G0/G1 phase was also of interest during our optimization process to best determine which length of cell starvation would be needed to synchronize cells into a quiescent phase to serve as our baseline control.

Following cell culture and treatments or aorta digestion, a volume of 500 µL of cells (approximately 10^6 cells per sample) was collected into individual cluster tubes for flow cytometry analysis performed using a BD LSRII SORP flow cytometer in the BU Flow Cytometry Core. No less than 20,000 cells were noted in each analysis. Fluorescence was measured at a wavelength of 488 nm (propidium iodide staining) and 647 nm (EdU incorporation). The percentage of cells in each phase of the cell cycle was estimated using the program of FACSDiva, version 6.2. Apoptotic cells and doublets were excluded via gating on forward scatter vs. side scatter plots using FlowJo software.

Statistical Analysis

To ensure rigor of our studies, the researcher (myself) who conducted the studies was kept blinded to genotypes of mice from which VSMCs and aortas were collected. Sample replicates were classified in group 1 and group 2 and identified as such during data collection and quantifications for the entire study. Data were expressed as means ±

standard error of the mean (SEM). Graphpad Prism (v.9) software was used for statistical analysis and to prepare graphs. One way ANOVA was used to compare the means of group 1 vs. group 2 at baseline and after PDGF treatment for each cell cycle phase separately. Student's t-test was used to compare aortas from *in vivo* studies (n=4 for each group). P values < 0.05 were considered significant.

CHAPTER 3: RESULTS

After numerous initial optimization trials, I determined that the best technique to use for these experiments was to first place VSMCs into culture on p60 plates and then split them each into 3 p10 plates once they reached 80% confluency. The most optimal growth medium was DMEM, 1 g/L glucose, and 10% FBS. 10 ng/mL of PDGF proved to be the most effective growth stimulus because it increased the percentage of cells in S phase by a significant amount compared to Ang-II, TGFβ and IL-1β, which did not induce any change in cell cycle phases at the conditions tested. The flow cytometry results produced from the cells grown on fibronectin and collagen showed no difference in cell cycle between each other or between cells grown on plastic p60 plates, so plastic plates were used in subsequent experiments (Figures 7, 8, and 9). When measured via flow cytometry, differentiating between each phase of the cell cycle was clear from the graphs produced (Figure 10). Also, because the propidium iodide staining was found to be unreliable and gave inconsistent results, the EdU Incorporation Assay was identified as the more effective and reliable method of analyzing the cell cycle differences between WT and BSMKO VSM cells. Cells that were not given EdU were used as a negative control and showed no EdU incorporation, as expected (Figure 6).

The propidium iodide staining technique showed no significant differences in cell cycle between WT and BSMKO VSMCs at baseline or when treated with PDGF (Figure 11). There were also no statistically significant differences in cell proliferation when analyzing only the S phase or DNA synthesis phase of the cell cycle (Figure 12). The average percentage of cells in the S phase increased from 2.5% ± 0.2% to 5.0% ± 0.4% in

the WT mice and increased from 2.4% ± 0.2% to 3.4% ± 1.0% in the BSMKO mice after PDGF treatment (Tables 2 and 3). A total of three samples were analyzed from each group (WT and BSMKO) in this experiment.

The EdU Incorporation Assay proved to be a more effective method of measuring the cell cycle. At baseline, both groups showed similarities in the percentages of cells in each phase (Figure 13). Statistical analysis showed that the percent of cells in the S phase of the cell cycle was significantly increased in the WT group when stimulated with PDGF. However, in the BSMKO group, there was no significant increase in the percent of cells in S phase after PDGF stimulation when compared to baseline (Figure 14). The average percentage of cells in the S phase increased from 1.1% ± 0.6% to 11.6% ± 4.0% in the WT mice and increased from 1.8% ± 0.4% to 4.7% ± 0.7% in the BSMKO mice after PDGF treatment (Tables 4 and 5). A total of five samples were analyzed from each group (WT and BSMKO) in this experiment.

The characteristics of the mice used in our *in vivo* experiments are listed in Table 6. The results showed the average percentage of cells in the S phase was 1.3% ± 1.0% in WT mice and 1.9% ± 0.6% in the BSMKO mice (Tables 7 and 8). No statistically significant differences were observed in these percentages between WT and BSMKO mice, both of which were treated with Ang-II (Figure 15). A total of four mice per group (WT and BSMKO) were analyzed in this experiment.

CHAPTER 4: DISCUSSION

The Seta laboratory recently discovered that mice lacking the transcription factor Bcl11b (BSMKO) in VSMCs develop aortic aneurysms when stimulated with Ang-II. However, the molecular mechanisms by which the lack of Bcl11b in VSM leads to AA in response to the hypertensive stimulus Ang-II remained to be determined. An IPA analysis of a RNA sequencing dataset conducted in the lab on aortas of Ang-II-treated WT and BSMSKO mice revealed differentially expressed genes that clustered mainly in canonical pathways related to cell cycle and cell proliferation. We, therefore, hypothesize that Bcl11b may play a role in regulating the VSM cell cycle and/or proliferation during AA development.

In order to measure cell cycle and cell proliferation, I used two established techniques: PI staining and EdU incorporation, followed by quantitation with flow cytometry. The results of my study suggest that the BSMKO VSMCs have decreased cell proliferation compared to WT cells, when stimulated with PDGF but not at baseline (non-stimulated) conditions. This is indicated by the percentage of cells in the S phase significantly increasing in the WT mice after PDGF, but not in the BSMKO mice. This impaired cell proliferation may indicate that VSMCs may become apoptotic or senescent and this may play a role in the development of AA in Ang-II-treated BSMKO mice. This is consistent with a previous study finding that, under a proliferative stimulus, lack of Bcl11b in T-cells disrupted the cell cycle and halted them at the synthesis phase, becoming apoptotic (Kamimura et al., 2007). Whether the lack of Bcl11b induces apoptosis (programmed cell death) in VSMCs is yet to be determined.

Some studies indicate that apoptosis of VSMCs is involved in AA development. Recent studies suggest that the elimination of VSMCs via excessive cell death contributes to AA development by depleting a population of cells crucial for vascular repair (Thompson et al., 1997). The elasticity of aortic tunica media is impaired because of VSM cell death and the incidence of AA is increased by the lack of the ability to repair connective tissue damage by VSMCs (Xue et al., 2019). Moreover, there is also evidence that VSM cells isolated from human and porcine abdominal AA can become senescent (Clark et al., 2022).

VSMCs have a unique property known as the phenotypic switch, considered to be a key mechanism in arterial remodeling (Petsophonsakul et al., 2019). VSMCs have a primary function of contracting/relaxing to regulate blood pressure and arterial compliance, but can also display profound changes when a vascular injury occurs, thereby contractile properties are lost and the cells become proliferative (Alexander et al., 2011). This is a complex phenomenon that may vary depending on the type of vascular disease and/or animal model used. The study by Clement et al. showed that VSMC phenotypic switch does play a role in AA when mice are given Ang-II *in vivo* and proliferative VSMC have higher levels of stress and inflammation, which correlate with AA severity (Clement et al., 2019).

Furthermore, AA is characterized by the weakening of the arterial wall and increased risk of rupture, and while the biomechanical stress on the aortic wall greatly contributes to AA development, cell-driven remodeling of the arterial wall is also considered to be a crucial protective mechanism, at least in initial stages of the disease

(Choke et al., 2006). Overall, this indicates the complexity of characterizing the VSM cell phenotype in AA, which may include a mixture of apoptotic, senescent and/or proliferative cells.

Therefore, a potential reason why AA develops in Ang-II-treated BSMKO mice may be that, at least in the initial stages of the disease, BSMKO VSMCs cannot proliferate and remodel to protect the aortic wall against the increased pressure induced by Ang-II leading to thinning of the aortic wall, which bulges into AA. The impaired proliferation in response to PDGF suggests that cell cycle progression in the BSMKO VSMCs cannot be activated as effectively as the WT cells.

A few technical aspects of my study are worth mentioning. First, controlling the growth of the cells in these experiments proved to be challenging because of the high growth variability among different cell lines (i.e., isolated from different mice), even when they were put into culture on the same day or starved for the same duration. To note, cell passage ranged from passage 3 to 8, and higher passage cells showed more variability which potentially contributed to the inconsistency of the results (Table 1). Therefore, preliminary experiments with different growth stimuli as well as multiple starvations and treatment periods were done to a great extent to determine the optimal growth conditions that would provide the most accurate results. A study by Boquest et al. showed that higher amounts of FBS generated a higher percentage of cells in the S and G2/M phases (Boquest et al, 1999). This provided us with further information on how to interpret our flow cytometry results. However, we found that using the DMEM, 4.5 g/L glucose, and 20% FBS did not have a substantial effect in advancing VSMCs from

G0/G1 to S and G2/M phase as the PDGF treatment did, which turned out to be the most effective treatment of all tested.

Secondly, a previous study performed by Joshi, et al. utilized the propidium iodide staining technique to measure cell proliferation and cell cycle in rat VSMCs (Joshi, et al., 2010). However, in our experimental conditions with mouse VSMCs, the propidium iodide staining technique was unreliable and gave inconsistent results. Therefore, we opted for EdU Incorporation Assay, which proved to be a more sensitive and effective method of measuring cell cycle in VSMCs. Compared to 5-bromo-2'-deoxyuridine (BrdU) incorporation, this newer EdU incorporation method overcomes limitations such as the use of specific antibodies and the need of strong DNA denaturation for BrdU detection (Zeng et al., 2010). The EdU Incorporation Assay was also a desirable method because it could be used both *in vitro* and *in vivo*. This was ideal for our experimental conditions because the main goal was to study cell cycle and proliferation in the same *in vivo* model used by Valisno et al. in which they found that aortic aneurysms developed in BSMKO-AngII mice, but not in WT-AngII mice.

In both the *in vitro* and the *in vivo* trials, we carefully performed negative controls such that some mice or VSMCs were not given any EdU in order to ensure that the fluorescence signal detected by flow cytometry was indeed specific for EdU. Therefore, our results accurately represent the cell cycle changes in both *in vitro* and *in vivo* experiments we performed.

One limitation of the *in vivo* trials was that the whole aorta was being analyzed which also includes cell types other than VSMCs, such as endothelial cells and

fibroblasts. Using more samples in future studies will allow for stronger and more definitive conclusions to be drawn about these cell cycle differences between our mouse models.

Despite limitations to the data obtained, this research is still novel and important. Future research should be performed to further investigate the role of Bcl11b in the phenotypic switch in VSMCs to better understand the arterial remodeling that occurs under these conditions. Also, further research is needed to confirm these results using other models and larger sample sizes to focus on understanding the relationship between the impaired proliferation and the observed aortic aneurysm development. This can be done by using different amounts of PDGF and Ang-II treatment as well as attempting higher doses of EdU for longer periods.

In conclusion, the most optimal technique for analyzing cell cycle differences between the WT and BSMKO mouse models proved to be a treatment with PDGF, cell preparation via an EdU Incorporation Assay and analysis via flow cytometry. Results show that the lack of Bcl11b in VSMCs, leads to a decrease in cell proliferation in response to a proliferative stimulus, PDGF. This may contribute to aortic aneurysm development, and future studies are needed to confirm these results and to establish whether Bcl11b or its downstream targets can become potential targets for the prevention of aortic aneurysms.

TABLES

Table 1. Characteristics of mice from WT and BSMKO groups used in flow cytometry analysis.

Mouse	Group	Passage
X15	WT	8
X18	BSMKO	4
X20	BSMKO	6
X21	BSMKO	5
X23	WT	6
X28	WT	7
X31	WT	6
X32	WT	3
X37	BSMKO	5
X39	BSMKO	3
X40	WT	3
X41	BSMKO	4
X42	WT	7
X43	BSMKO	7
X44	WT	4

Table 2. Average percentages of WT VSMCs in each phase of the cell cycle were measured by PI incorporation via flow cytometry.

Treatment	G0/G1	S	G2/M
5 mL Starvation Media	50.1% ± 0.9%	2.5% ± 0.2%	30.6% ± 2.3%
5 mL Starvation Media + 10 ng/mL PDGF	58.1% ± 7.7%	5.0% ± 0.4%	24.9% ± 2.7%

Table 3. Average percentages of BSMKO VSMCs in each phase of the cell cycle were measured by PI incorporation via flow cytometry.

Treatment	G0/G1	S	G2/M
5 mL Starvation Media	40.6% ± 13.5%	2.4% ± 0.2%	46.5% ± 12.5%
5 mL Starvation Media + 10 ng/mL PDGF	48.8% ± 11.7%	3.4% ± 1.0%	37.3% ± 10.4%

Table 4. Average percentages of WT VSMCs in each phase of the cell cycle were measured by EdU incorporation via flow cytometry.

Treatment	G0/G1	S	G2/M
5 mL Starvation Media	77.5% ± 8.5%	1.1% ± 0.6%	21.8% ± 8.2%
5 mL Starvation Media + 10 ng/mL PDGF	72.8% ± 6.1%	11.6% ± 4.3%	16.8% ± 6.6%

Table 5. Average percentages of BSMKO VSMCs in each phase of the cell cycle were measured by EdU incorporation via flow cytometry.

Treatment	G0/G1	S	G2/M
5 mL Starvation Media	69.8% ± 3.9%	1.8% ± 0.4%	29.5% ± 3.9%
5 mL Starvation Media + 10 ng/mL PDGF	62.6% ± 5.4%	4.7% ± 0.7%	33.4% 5.5%

Table 6. Characteristics of mice from WT and BSMKO groups used in flow cytometry analysis.

Mouse	Group
M5	BSMKO
M6	WT
M7	WT
M8	WT
M9	BSMKO
M10	BSMKO
M11	BSMKO
M12	WT

Table 7. Percentages of WT VSMCs in each phase of the cell cycle were measured by EdU incorporation into aortas extracted from mice via flow cytometry.

Treatment	G0/G1	S	G2/M
2 mg/kg/day Ang-II for 14 days	85.0% ± 2.3%	1.3% ± 0.5%	14.0% ± 2.4%

Table 8. Percentages of BSMKO VSMCs in each phase of the cell cycle were measured by EdU incorporation into aortas extracted from mice via flow cytometry.

Treatment	G0/G1	S	G2/M
2 mg/kg/day Ang-II for 14 days	82.0% ± 5.4%	1.9% ± 0.6%	17.3% ± 5.7%

FIGURES

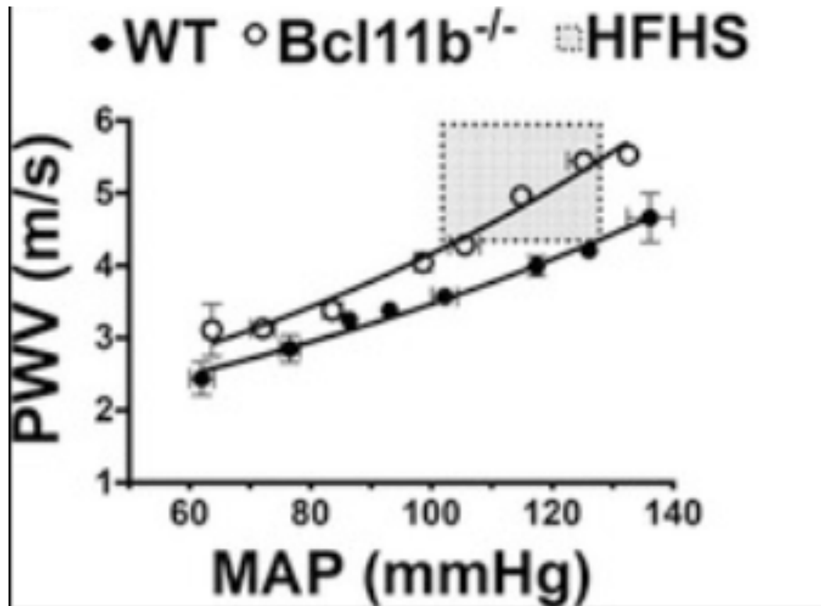


Figure 1. Bcl11b downregulation is associated with increased arterial stiffness. Pulse wave velocity (PWV), the in vivo measure of arterial stiffness, measured over a range of mean arterial pressure (MAP), is increased in 10-month-old mice lacking Bcl11b compared to wild-type (WT) littermates.

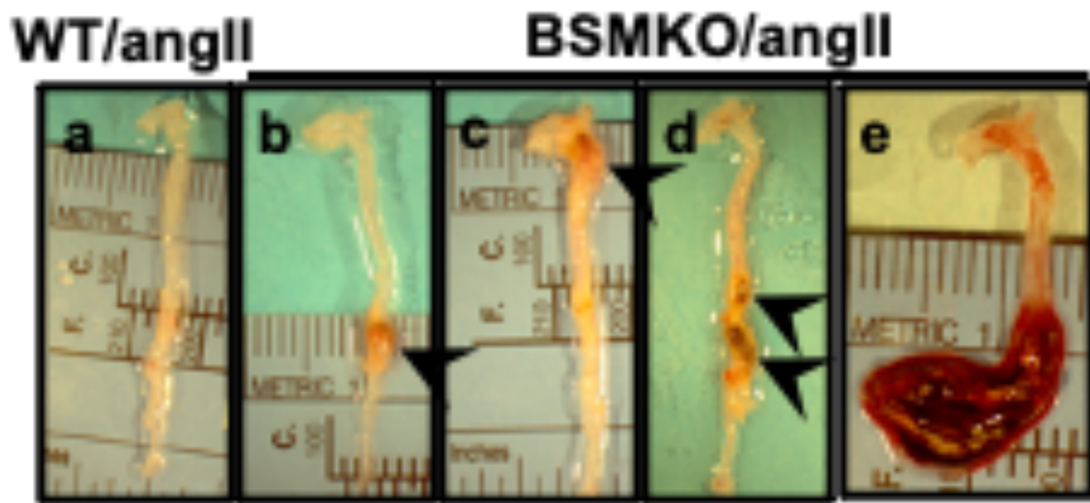


Figure 2. Representative images of WT and BSMKO aortas after 2-week treatment with angII. 70% of BSMKO mice developed aortic aneurysm in the suprarenal (b) or thoracic (c) region or both (d) (indicated by arrowheads), while no aneurysm developed in angII-treated WT mice (a). (e) aorta from an angII-treated BSMKO mouse found dead. Metric ruler showed as reference for microscope magnification. Survival curves in the graph indicate that 100% of BSMKO mice died of aortic aneurysm rupture in response to DOCA/NaCl before the study endpoint (21 days) compared to 50% of WT mice.

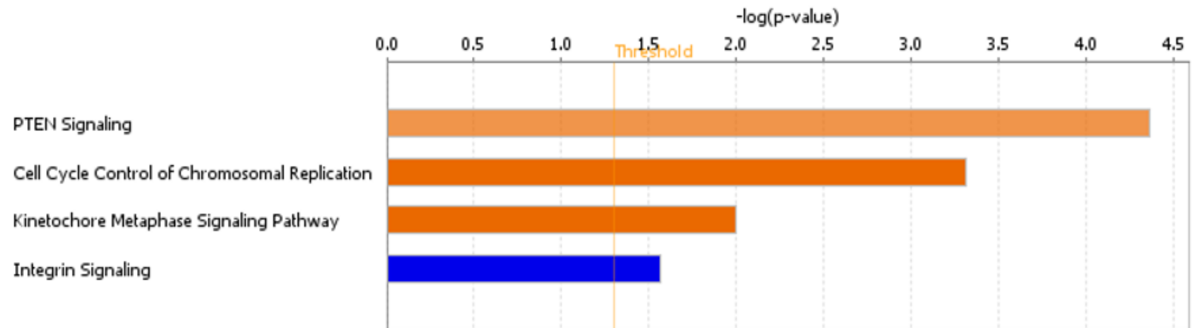
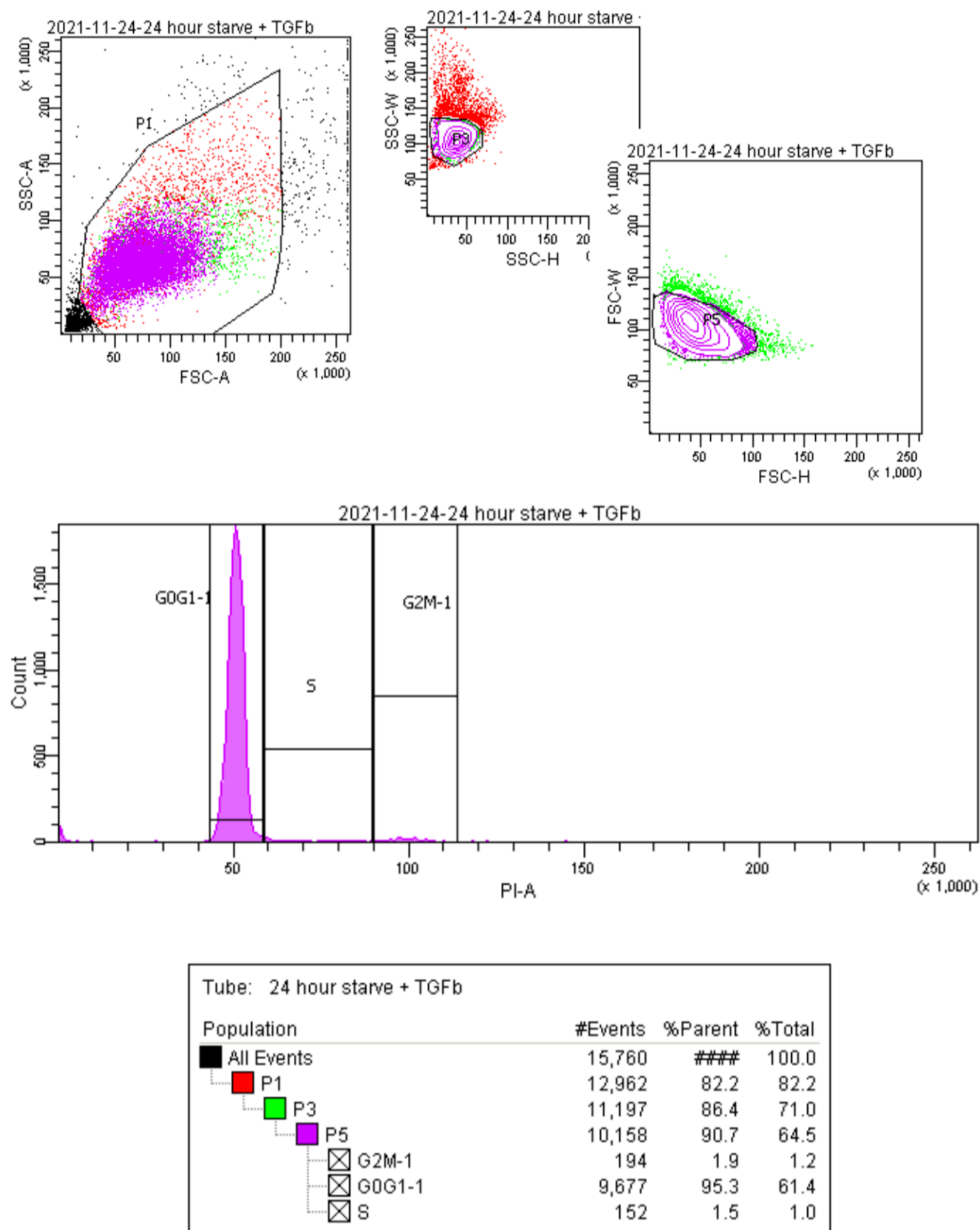


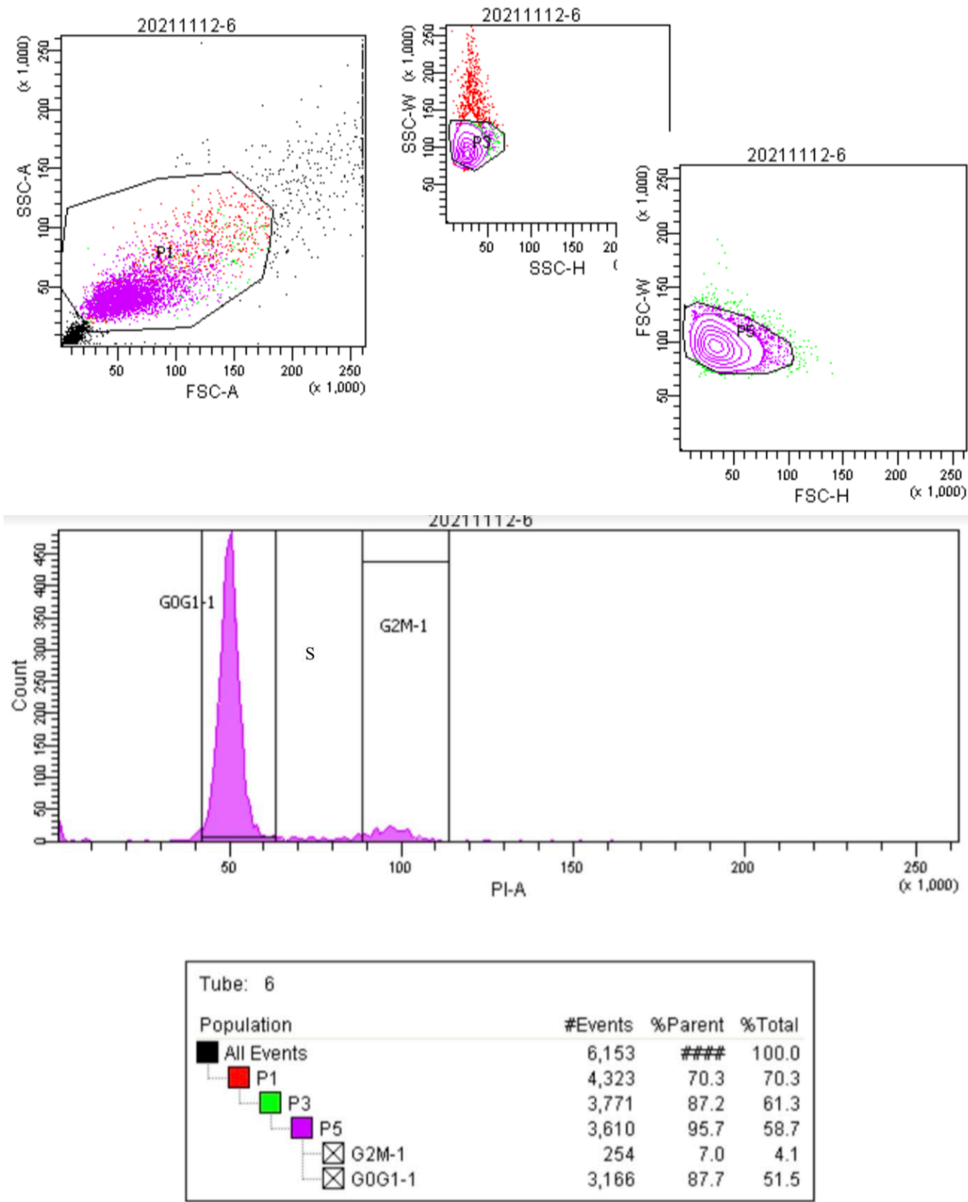
Figure 3. Selected IPA canonical pathway results from WT-AngII vs BSMKO-AngII**.** Four canonical pathways are predicted to be differentially expressed in BSMKO-AngII vs WT-AngII mouse aortas. Orange indicates a positive z-score and blue indicates a negative z-score.



Tube: 24 hour starve + TGFb

Population	#Events	%Parent	%Total
All Events	15,760	####	100.0
P1	12,962	82.2	82.2
P3	11,197	86.4	71.0
P5	10,158	90.7	64.5
G2M-1	194	1.9	1.2
G0G1-1	9,677	95.3	61.4
S	152	1.5	1.0

Figure 4. PI fluorescence of WT VSMCs after 24 hours of 20 ng/mL TGFβ treatment. Dead cells and doublets are excluded by the gating. Cell cycle phases are indicated as G0/G1, S and G2/M. Y-axis represents cell count and X-axis represents PI incorporation.



Tube: 6

Population	#Events	%Parent	%Total
All Events	6,153	####	100.0
P1	4,323	70.3	70.3
P3	3,771	87.2	61.3
P5	3,610	95.7	58.7
G2M-1	254	7.0	4.1
G0G1-1	3,166	87.7	51.5

Figure 5. PI fluorescence of WT VSMCs after 24 hours of 10 ng/mL IL-1β treatment. Dead cells and doublets are excluded by the gating. Cell cycle phases are indicated as G0/G1, S and G2/M. Y-axis represents cell count and X-axis represents PI incorporation.

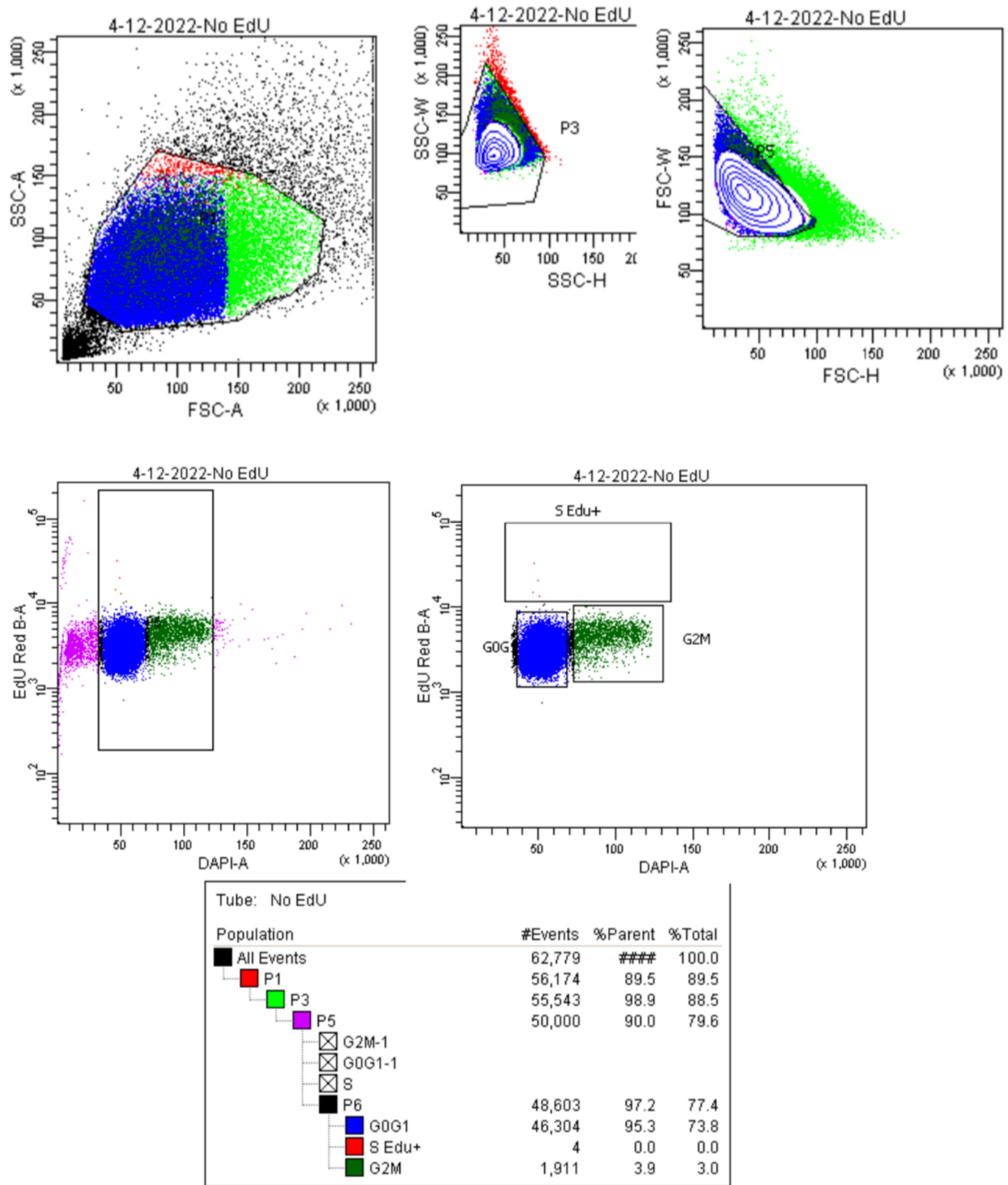
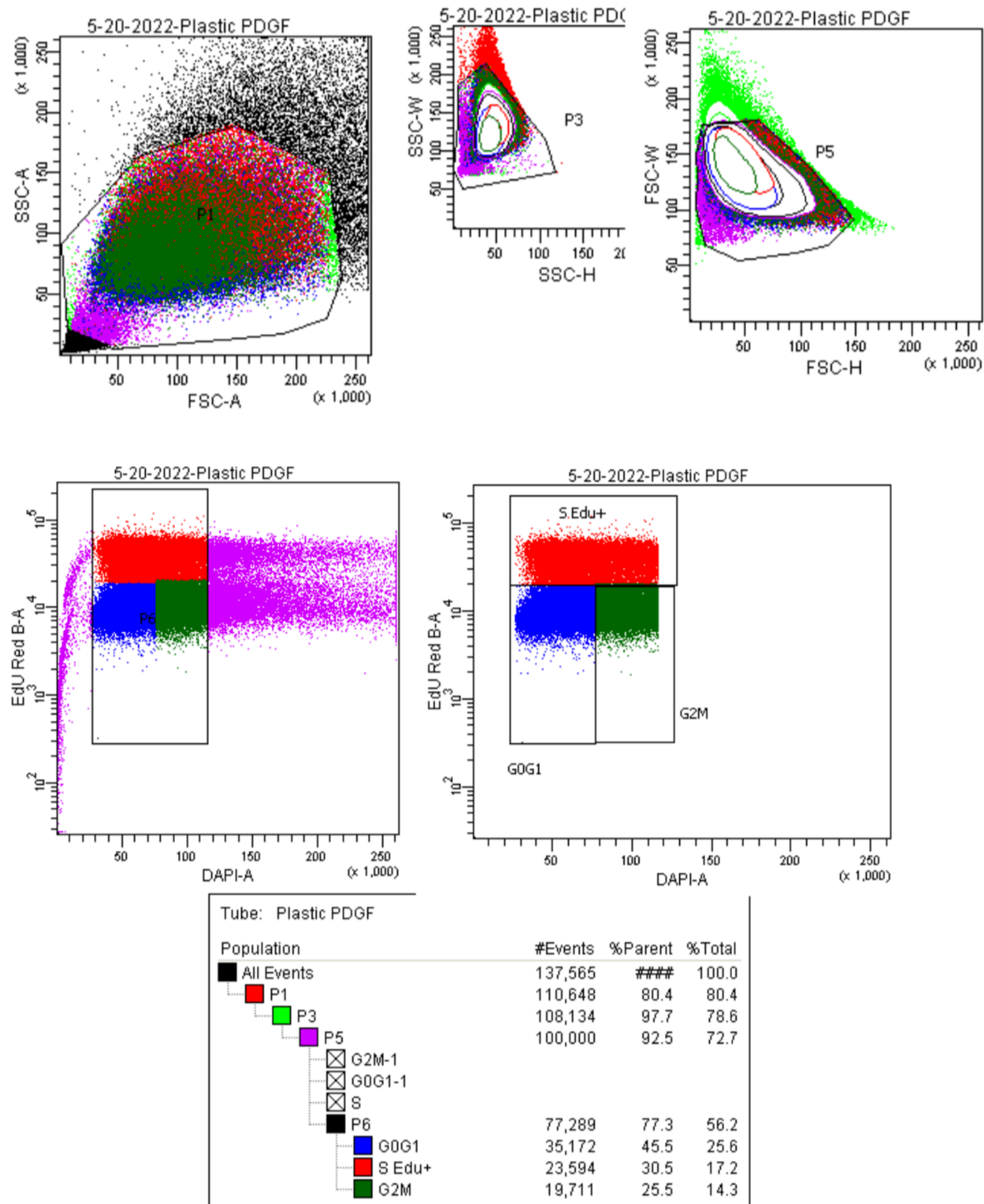


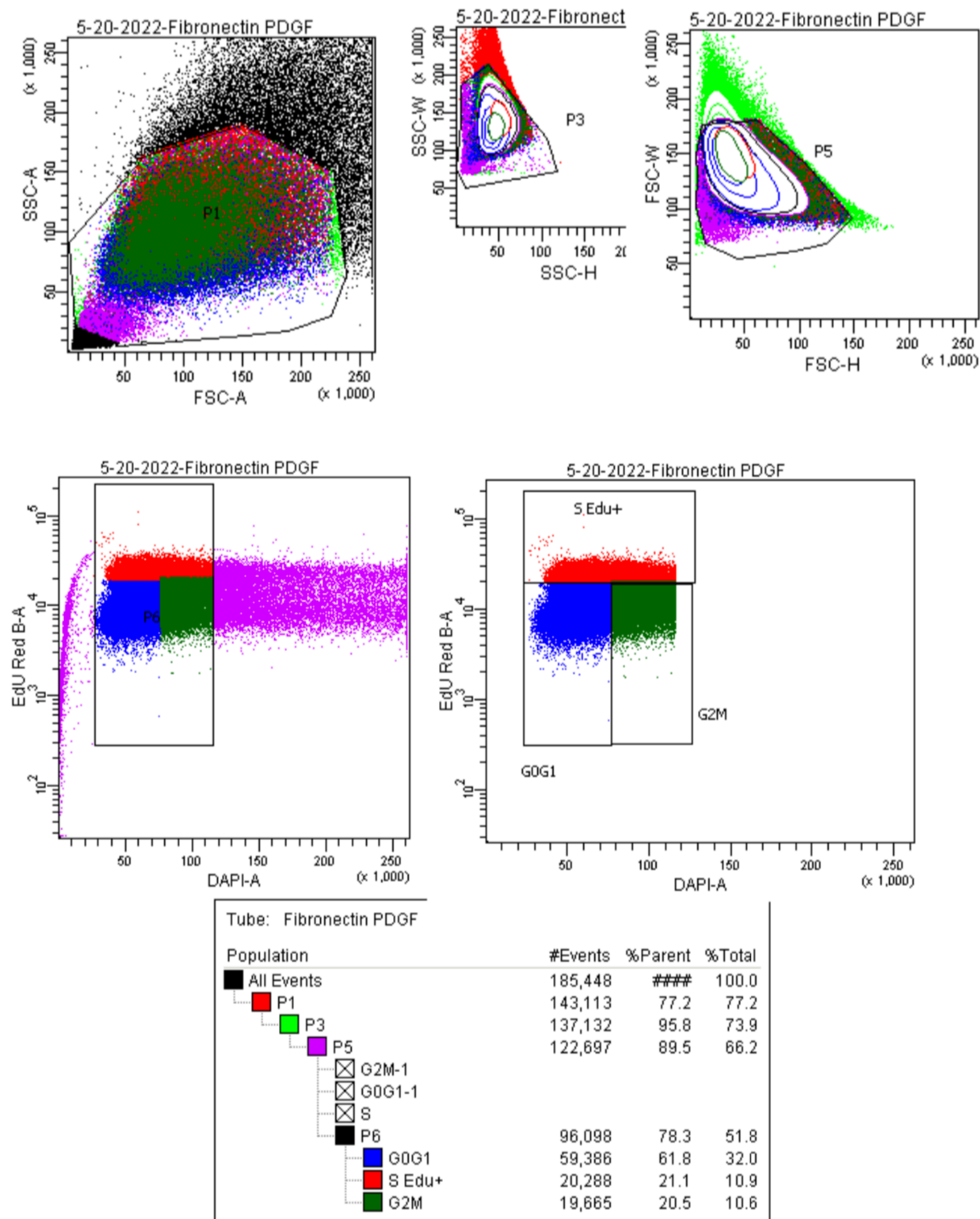
Figure 6. EdU incorporation of WT VSMC treated with no EdU measured via flow cytometry analysis. Dead cells and doublets are excluded by the gating. Cell cycle phases are indicated as G0/G1, S (EdU+) and G2/M.



Tube: Plastic PDGF

Population	#Events	%Parent	%Total
All Events	137,565	####	100.0
P1	110,648	80.4	80.4
P3	108,134	97.7	78.6
P5	100,000	92.5	72.7
G2M-1			
G0G1-1			
S			
P6	77,289	77.3	56.2
G0G1	35,172	45.5	25.6
S Edu+	23,594	30.5	17.2
G2M	19,711	25.5	14.3

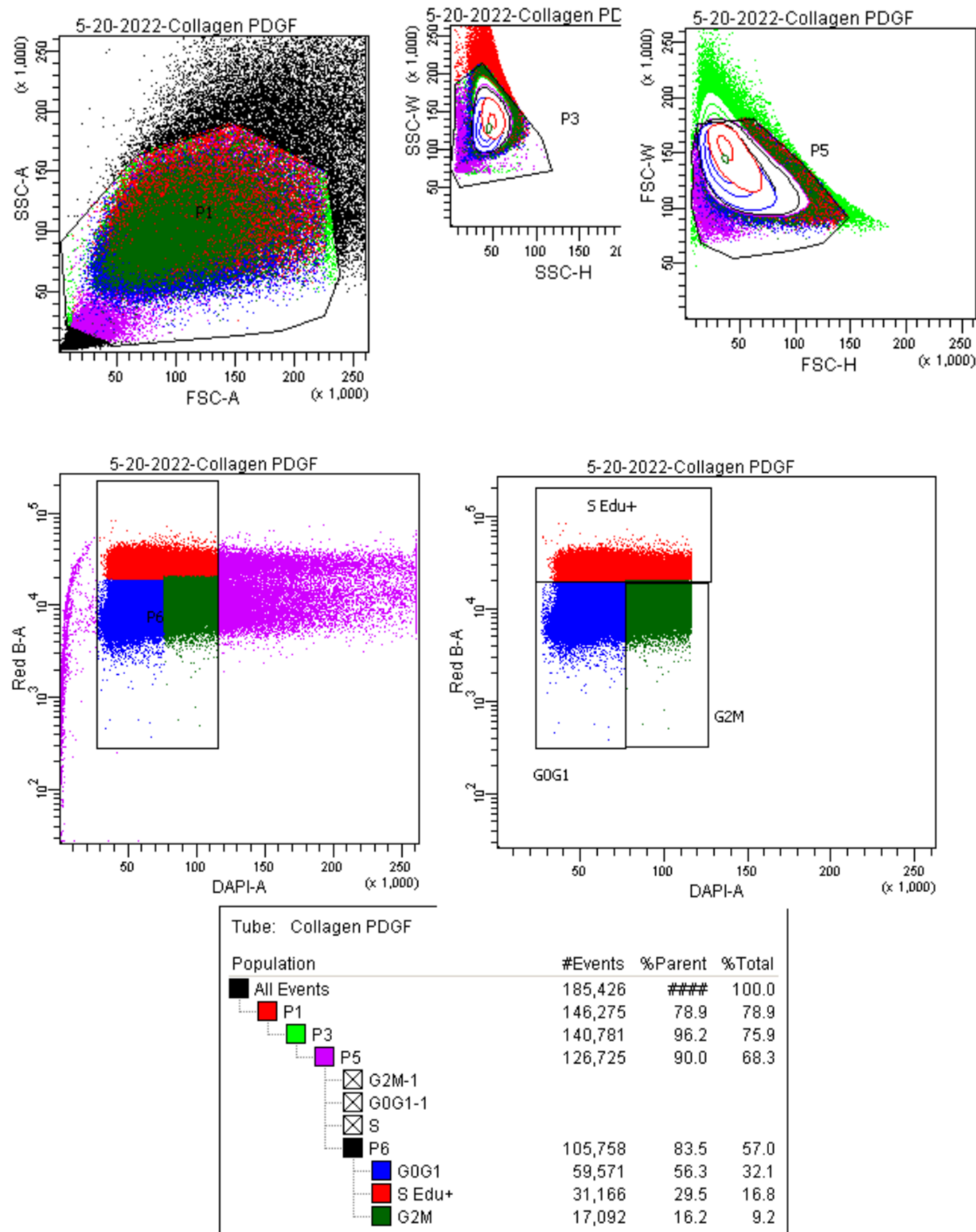
Figure 7. EdU incorporation of WT VSCMs treated with 10 ng/mL PDGF for 24 hours on plastic p60 plates. Dead cells and doublets are excluded by the gating. Cell cycle phases are indicated as G0/G1, S (EdU+) and G2/M.



Tube: Fibronectin PDGF

Population	#Events	%Parent	%Total
All Events	185,448	####	100.0
P1	143,113	77.2	77.2
P3	137,132	95.8	73.9
P5	122,697	89.5	66.2
G2M-1			
G0G1-1			
S			
P6	96,098	78.3	51.8
G0G1	59,386	61.8	32.0
S Edu+	20,288	21.1	10.9
G2M	19,665	20.5	10.6

Figure 8. EdU incorporation of WT VSCMs treated with 10 ng/mL PDGF for 24 hours on fibronectin-coated p60 plates. Dead cells and doublets are excluded by the gating. Cell cycle phases are indicated as G0/G1, S (EdU+) and G2/M.



Tube: Collagen PDGF

Population	#Events	%Parent	%Total
All Events	185,426	####	100.0
P1	146,275	78.9	78.9
P3	140,781	96.2	75.9
P5	126,725	90.0	68.3
G2M-1			
G0G1-1			
S			
P6	105,758	83.5	57.0
G0G1	59,571	56.3	32.1
S Edu+	31,166	29.5	16.8
G2M	17,092	16.2	9.2

Figure 9. EdU incorporation of WT VSCMs treated with 10 ng/mL PDGF for 24 hours on collagen-coated p60 plates. Dead cells and doublets are excluded by the gating. Cell cycle phases are indicated as G0/G1, S (EdU+) and G2/M.

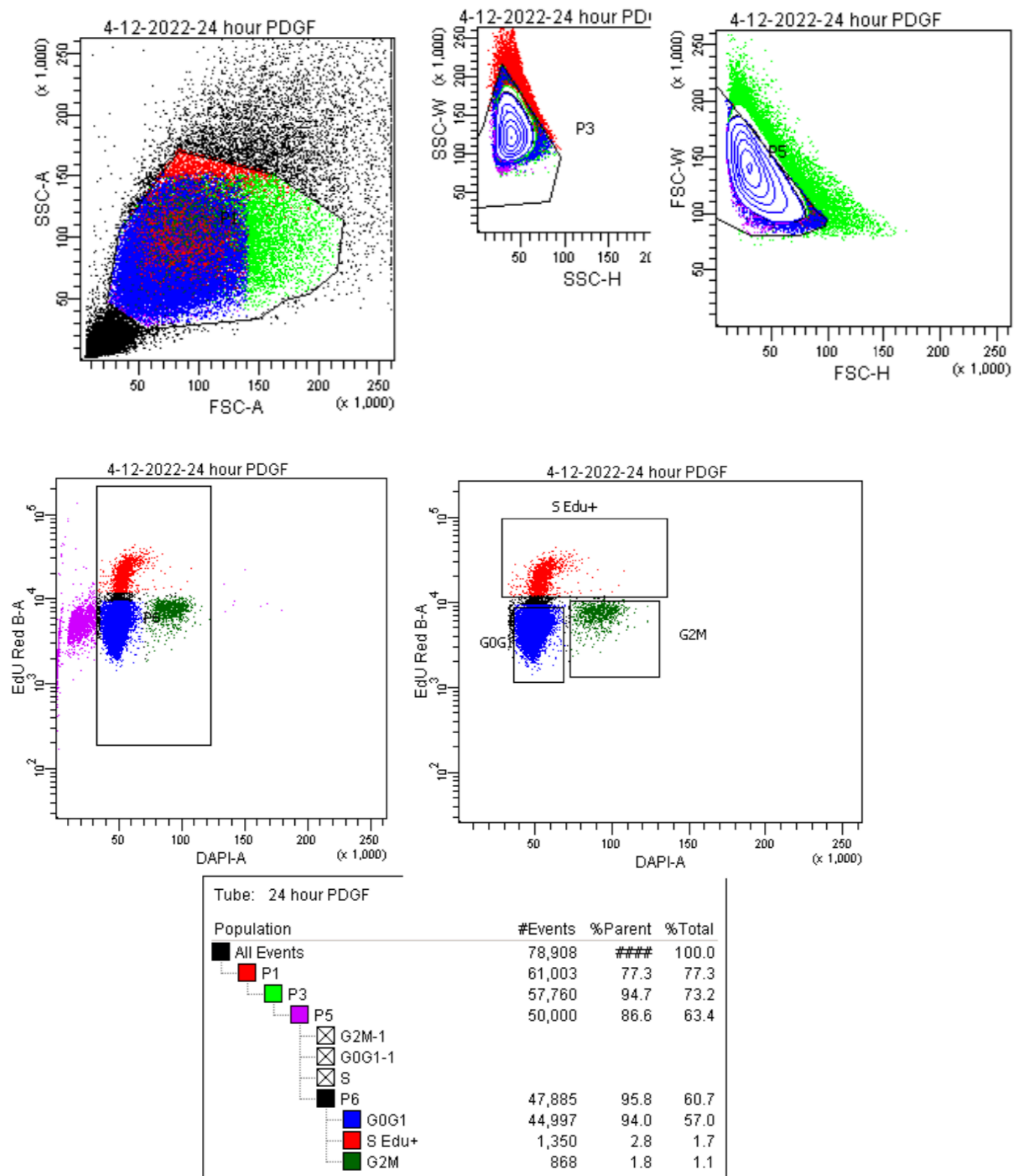


Figure 10. EdU incorporation of VSMC treated for 24 hours with 10 ng/mL PDGF and given 10 mM EdU measured via flow cytometry analysis. Dead cells and doublets are excluded by the gating. Cell cycle phases are indicated as G0/G1, S (EdU+) and G2/M.

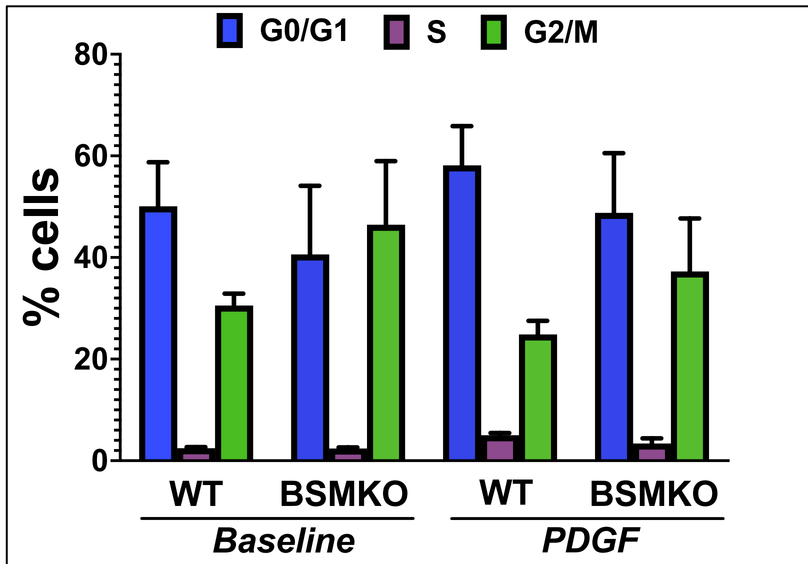


Figure 11. Propidium iodide incorporation into VSMC extracted from mice treated with 10 ng/mL PDGF for 24 hours and analyzed by flow cytometry shows no statistically significant difference in cell cycle phases between WT and BSMKO mice.

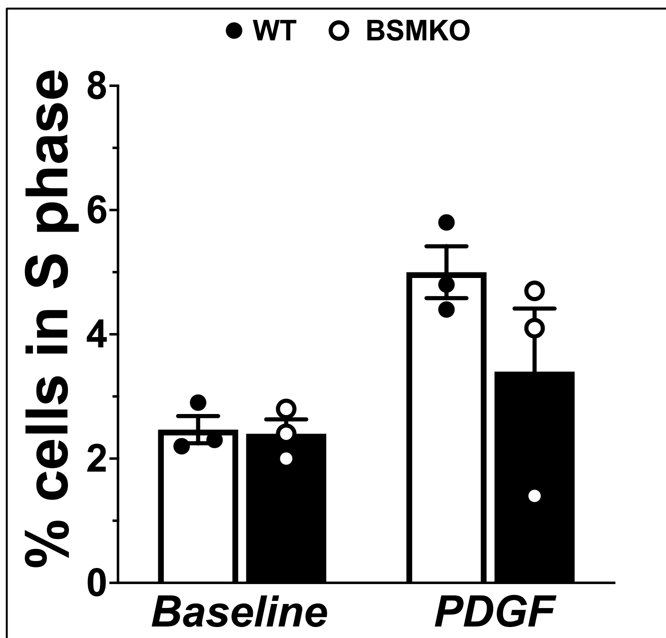


Figure 12. Propidium iodide incorporation into VSMC extracted from mice treated with 10 ng/mL PDGF for 24 hours and analyzed by flow cytometry: S phase shows no statistically significant difference between WT and BSMKO mice.

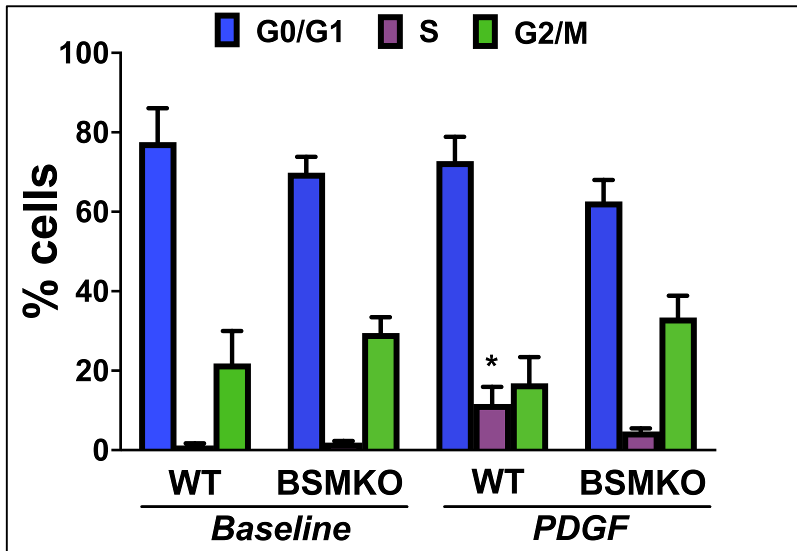


Figure 13. EdU incorporation into VSMC extracted from mice treated with 10 ng/mL PDGF for 24 hours and analyzed by flow cytometry shows a significant increase in the S phase in WT VSMC compared to baseline, but no significant increase in BSKMO mice.

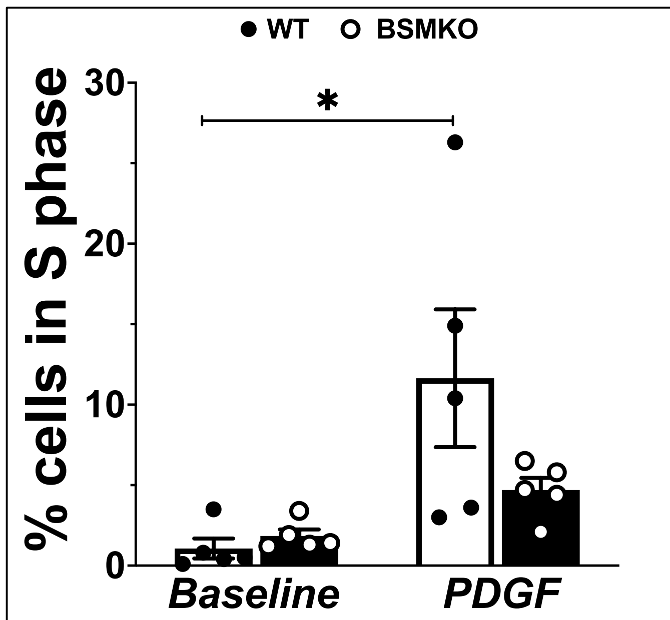


Figure 14. EdU incorporation into VSMC extracted from mice treated with 10 ng/mL PDGF for 24 hours and analyzed by flow cytometry: S phase only indicated a statistically significant increase in number of cells in S phase in WT cells treated with PDGF but not in BSMKO VSMCs.

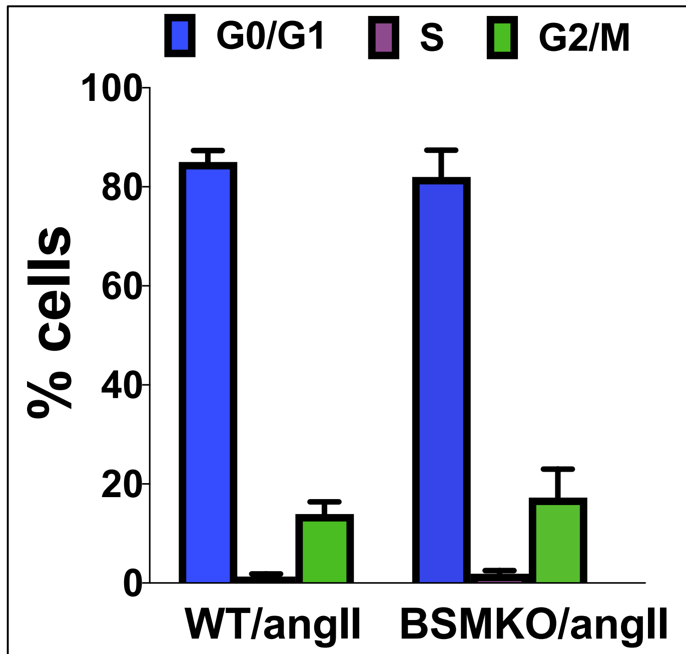


Figure 15. EdU incorporation into aortas extracted from mice treated with 14 days of 2 mg/kg/day angiotensin II and analyzed by flow cytometry.

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CURRICULUM VITAE