Title

The Effect of the 9p21 Locus on Expression of Contractile Versus Synthetic Phenotypic Markers in iPSC-Derived Vascular Smooth Muscle Cells

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The Effect of the 9p21 Locus on Expression of Contractile Versus Synthetic Phenotypic Markers in iPSC-Derived Vascular Smooth Muscle Cells

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Elena Wivina Demeester

Committee in charge:

Professor Adam Engler, Chair
Professor Deborah Yelon, Co-chair
Professor Clara Meaders

2021

The Thesis of Elena Wivina Demeester is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

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Material from this thesis, in part, has been submitted for publication of the material as it may appear in Combining Genetic and Mechanical Factors to Model Disease, 2021, Mayner, Jaimie; Demeester, Elena; Engler, Adam J. The thesis author was a co-author of this chapter.

ABSTRACT OF THE THESIS

The Effect of the 9p21 Locus on Expression of Contractile Versus Synthetic Phenotype of IPSC-Derived Vascular Smooth Muscle Cells

by

Elena Wivina Demeester

Master of Science in Biology

University of California, San Diego, 2021

Professor Adam Engler, Chair
Professor Deborah Yelon, Co-chair

Coronary artery disease (CAD) remains one of the leading causes of death in the US and represents a mechanistically complex disease. GWAS (Genome Wide Association Studies) have identified SNPs (Single Nucleotide Polymorphisms) within the 9p21 locus as having the greatest correlation with increased susceptibility towards CAD (Samani, Schunkert., 2008). However, the 9p21 locus is a non-coding region, making the mechanism through which these SNPs result in CAD less clear. Vascular

smooth muscle cells (VSMCs), which typically function to regulate vascular tone, are known to switch from a contractile to a synthetic phenotype during CAD progression and vascular remodeling.

We hypothesize that the 9p21 risk variants result in a more prominent synthetic population compared to the contractile phenotype and that these two phenotypes can be isolated utilizing a shear assay, which sorts cells based on adhesion strength. Specifically, we aim to show that the weakly adherent is comprised of more synthetic cells and the strongly adherent are comprised of more contractile cells by measuring differences in the expression of contractility and proliferation markers.

Introduction

This project aims to elucidate the disease mechanisms of genetic risk for CAD and its impact on the regulation of vascular smooth muscle cell phenotype. CAD remains the most potent killer amongst the American population and is responsible for one in every five deaths in the developed world (Cassar, 2009). Family history has long been used in medicine to predict CAD, however, the search for associated genes has remained mainly fruitless until the advent of modern sequencing techniques. GWAS studies have not only confirmed genetics account for 50% of susceptibility (Kessler, 2013), but have also identified genetic variants that increase risk (Topol, 2012, Roberts, 2006, Cambien, 2007). At least 10^6 disease-causing single nucleotide polymorphisms (SNPs) have been identified in the genome, but most are located in non-coding regions, making the mechanism through which they enhance disease risk enigmatic (Schaub, 2012).

Three separate GWAS studies in parallel identified variants at the 9p21 locus as having the greatest correlation with CAD, making it the most impactful genomic risk factor (Samani, Shunkert., 2008). Like many other associated SNPs, it does not contain any annotated genes and is in a non-coding region of DNA. Investigating the contribution of these loci could be crucial to understanding not only the genetic component of CAD, but also act as a blueprint for investigating disease associated non-coding loci. In this thesis, I studied this particular locus to determine its role in CAD disease progression.

Specifically, I investigated how this non-coding region may impact the phenotypic regulation of VSMCs. Vasculature consists of multiple layers and VSMCs

are located in the tunica media, contracting and dilating to help regulate the blood vessel diameter in response to changes in hemodynamic forces. During CAD, VSMCs are observed to shift phenotype from a healthy contractile state to a more proliferative and migratory state, a synthetic phenotype (Louis and Zahradka, 2010). Synthetic cells migrate into the intima and proliferate, leading to further arterial narrowing. The narrowing of arteries then leads to the physical symptoms experienced by patients suffering from CAD, such as pain and discomfort in the upper body, shortness of breath, and difficulty in breathing, and eventually myocardial infarction (MI) and death (cdc.gov).

The presence of these different phenotypes result in the inherent heterogeneity in VSMC populations, heightened during inflammation and disease progression. We hypothesize the 9p21 risk variants contribute to phenotypic mis-regulation, promoting a disproportionate number of cells to switch from the more quiescent contractile phenotype to the synthetic phenotype. It is important to have a clear method to identify the two phenotypes and their penetrance across risk and non-risk genotypes. It has previously been shown that integrins, actin and other markers that implicate adhesion and contractility are upregulated in the contractile phenotype (Rensen., 2007), whereas proliferation markers would be assumed to be upregulated in the synthetic phenotype.

To investigate the presence of these VSMC phenotypes from different genetic risk backgrounds, we used a cohort of isogenic IPSC clones derived from CAD patients homozygous for the 9p21 risk variants (R/R) and individuals who were homozygous non-risk (N/N). We also used a risk knockout line, where the entire CAD risk region was excised via TALENs (R/R KO). These cell lines were then differentiated into

VSMC so that we could assess penetrance of the synthetic versus contractile phenotype by analyzing specific markers associated with each phenotype.

First, Western blot was used to compare alpha smooth muscle actin (α-SMA) protein expression levels across genotypes. Expression levels of this cytoskeletal protein are often correlated with contractility. Secondly, flow cytometry was conducted to quantify the percentage of cells positive for α-SMA and Ki67, a proliferation marker that is only expressed in dividing cells. By comparing the percentage of cells that are expressing each marker, the penetrance of the synthetic and contractile phenotypes of was compared across genotypes. Finally, a microfluidic device was used sort VSMCs based on the strength of adhesion to the extra cellular matrix (ECM), specifically collagen, when exposed to acute fluidic shear stress. Cells were seeded into the microfluidic device and then exposed to uniform shear stress, controlled by adjusting the volumetric flow rate of a syringe pump. The cells that detached were collected and defined as the weakly adherent (WA) population. The cells that remained in the microfluidic device were then collected using enzymatic dissociation and are defined as strongly adherent (SA) population. The isolated populations were fixed, stained with fluorescently labeled antibodies for Ki67 and α-SMA, and analyzed using flow cytometry determine the differences in expression of these contractile and synthetic phenotypic markers.

We hypothesize that VSMCs derived from individuals with alleles containing the 9p21 SNPs associated with CAD will show a higher penetrance of the synthetic phenotype compared to individuals without the 9p21 SNPs. We also hypothesize that the weakly adherent population will be composed of primarily more synthetic cells and

the strongly adherent the contractile phenotype. These hypotheses lead to the following predictions: (1) The risk cell lines will express less contraction and more proliferation markers compared to non-risk cell lines. (2) For a given risk cell line, the WA cells will express less contraction and more proliferation markers compared to the SA population. (3) More cells will detach at a given shear stress for the risk lines compared the non-risk, in other words, the WA population will be greater in the risk cell lines compared to the non-risk. If these hypotheses are true, this would suggest that the non-coding 9p21 locus plays an important role in regulating VSMC phenotype and therefore could increase an individual's susceptibility to developing CAD by increasing the prevalence of the synthetic phenotype independent of outside environmental influence.

Materials and Methods:

iPSC culturing

7 different cell lines and their clones were studied: Risk/Risk from patient 1, R/R from patient 2, Non-Risk/Non-Risk and Risk/Risk Knockout from patient 1. These cell lines were thawed and cultured on Matrigel coated 6 well plates with mTSER stem cell culture media. iPSC's media was changed every day. Once iPSCs reached ~70% confluenty, cells were differentiated into vascular smooth muscle cells using a defined protocol (Cheung et al., 2014) or passaged onto another 6 well plate. To passage cells, Versene and a cell scraper was used to detach cells from plates before cells were resuspended in mTESR and 0.1% ROCK (Rho-Kinase) inhibitor and plated at a lower density.

Table 1: Cell line names and Genotypes

Genotype	Cell Line Names
R/R	1-5 (patient 1), 21.2 (patient 2)
N/N	C151, E14
R/R KO	1-9, 1-15, WB46

Vascular Smooth Muscle Cell Differentiation (Cheung et.al, 2014)

1 day before iPSCs reached 70% confluency, cells were given day -2 media, which consisted of chemically defined media (CDM) with 5mg/ml Bovine Serum Albumin (BSA), 12ng of FGF2 and 10ng/ml of Activin A. On day -1, cells were passaged onto a new plate coated with gelatin with day -1 media consisting of CDM, poly(vinyl alcohol)(PVA), 12ng FGF2 and 10ng/ml of Activin A. On day 0, cell media was changed to CDM PVA with 20ng/ml FGF2, 10uM LY294002 and BMP4. On day 1.5, media was changed to CDM PVA with 20ng/ml FGF2 and 50ng/ml BMP4 (day 1.5/3.5 media). On day 3.5, half of the day 1.5 media was discarded and replaced with day 1.5/3.5 media. On day 5, cells were lifted using TrypleE and passaged at a density of 190,000 cells/ml using CDM PVA with 10ng/ml PDGF and 2ng/ml TGF-β (day 5 media). From day 5 till day 18, day 5 media were used and replaced every 2-3 days. When cells became confluent during this time period, they were passaged to 1/3 density using TrypleE and the same passaging protocol. On day 18, differentiation was complete and media changes were replaced with DMEM/F12 with 10% FBS, 1% Penicillin Streptomycin and 0.2% non-essential amino acids. VSMCs were maintained

by replacing DMEM/F12 media every 2 to 3 days and passaging using TrypleE if confluent. VSMCs used for shear assays did not exceed 10 passages.

IF Imaging

VSMCs were seeded at 30,000 cells/ml onto 18mm coverslips in a 6 well plate. After a day of culturing, cells were fixed using 3.7% formaldehyde in PBS for 10 minutes. Cells were rinsed 3 times with PBS before lysis. 100ul of 10% TritonX was pipetted onto cover slips for 15 minutes to lyse cell membrane. Coverslips were rinsed twice with blocking buffer (10% FBS in PBS) and was left to block for 20 minutes. Coverslip was then stained with α-SMA antibody (1:1000) for 2 hours. Coverslip was rinsed 3 times with Solution A and then stained with a secondary antibody (Goat anti Rabbit 568, 1:5000). Coverslips were rinsed with water 3 times and then stained for DAPI (1:1000) for 3 minutes. Coverslips were then mounted to a glass slide using Fluoromount and after resting overnight, edges were sealed using clear nail varnish.

Western Blot

Cell Lysis: Each well is rinsed twice with pre-chilled PBS. Then 300uL of mRIPA is added per well for 1-2 minutes. Cell scraper is then used to scrape sample from well and lysis is pipetted into a microfuge tube. Samples are then left on ice and vortexed every 5 minutes for 30 minutes. Samples are then centrifuged at 4°C for 15 minutes at 15 000 rpm. Supernatant is then transferred into new microfuge tube. Samples can be kept at -80°C.

BCA (Bicinchoninic Acid) Assay: 10ul of each BCA standard is are loaded into 3 wells of a 96 well plate alongside each sample. Using a multichannel pipette, 200ul of working agent (50:1 BCA agent) into each of the wells. Incubate plate for 30 minutes. Use a plate reader to analyze absorbance of each well and average the 3 trials per standard and sample. Standard curve is generated, and polynomial is used to calculate the protein concentration of each sample.

Gel Electrophoresis: Protein concentrations determined from the BCA assay is then used to prepare western blot samples. 5ug of protein sample was added with specified amounts of 4x loading buffer, DTT, and mRIPA in microfuge tubes. Samples were then heat inactivated for 5 minutes at 95° in a heating block. Samples were then carefully removed by using tweezers to keep microfuge cap closed and place on ice to cool. 4-12% bis tris gels to were allowed to reach room temperature before loading samples. The gel was then rinsed and attached to the assembled the running chamber. Chambers were filled to indicated height with 1X MOPS buffer. Samples were carefully loaded into channels avoiding air bubbles. Chameleon duo was then added to either side of samples to determine orientation of the gel. The gel was run at 140V for 1 hour.

Transfer/Blotting

After gel electrophoresis, the gel was removed from tank and was released from the cassette. The teeth and bottom edge of the gel was removed so that it could lay flat. Transfer system was prepared by placing bottom stack followed by the gel, followed by pre-wetted filter paper and the electrode assembly. Bubbles were rolled out in between

layers. Then the sponge was attached to the transfer system lid and the latch was closed. The transfer system was run for 7 minutes.

Staining

The blot was then removed from transfer system and was blocked using fluorescent blot blocking buffer for 1 hour at room temperature with agitation. Then α-SMA primary antibody (1:2000) was added and blot was left incubate overnight at 4° with agitation. The next morning, the blot was rinsed 3 times for 5 minutes with 1X tris buffered saline + 0.1% Tween (TBST) with agitation. Then the secondary antibody (1:5000) was added for 1 hour at room temperature with agitation. The blot was then rinsing procedure with TBST was repeated before blot was imaged on LICOR imaging system.

Flow cytometry

Cells were lifted using TrypLE and resuspended in Live/Dead stain (1ul/ml/million cells) in flow cytometry buffer (5mM EDTA, 2% Goat Serum in PBS). Live/Dead stain covalently binds to intracellular and extracellular amines. In non-viable cells with dissociated membranes, the dye will bind to intracellular and extracellular amines that significantly increases fluorescence intensity compared to viable cells, where the stain only binds to extra cellular amines. Cells were then centrifuged and fixed in 3.7% paraformaldehyde for 10 minutes, followed by permeabilization using 1% TritonX for 15 minutes. Cells were then stained for α-SMA and Ki-67, alongside Live/Dead only control and Fluorescence minus one (FMO) controls (Live/Dead + α-SMA, Live/Dead +Ki67). Cells were incubated with conjugated antibodies for 30 minutes, followed by 2

resuspensions in flow cytometry buffer. Cell samples were then pipetted into pre-wetted flow cytometry tubes.

The Fortessa X-20 was utilized to analyze percentage of live cells positive for α-SMA and Ki-67. BV421 channel was used to gate for live cells. Side scatter and forward scatter was used to gate for single cells. FITC and APC channels were used to detect α-SMA and Ki-67 respectively and were gated using Live/Dead only controls and FMO's.

Microfluidic Device Fabrication

The microfluidic device and shear assay described below was designed and developed by PhD student **Jaimie Mayner.** The microfluidic device was designed with one inlet and outlet with a patterned channel that exposes cells to uniform shear stress throughout (figure 1.1 and 1.2).

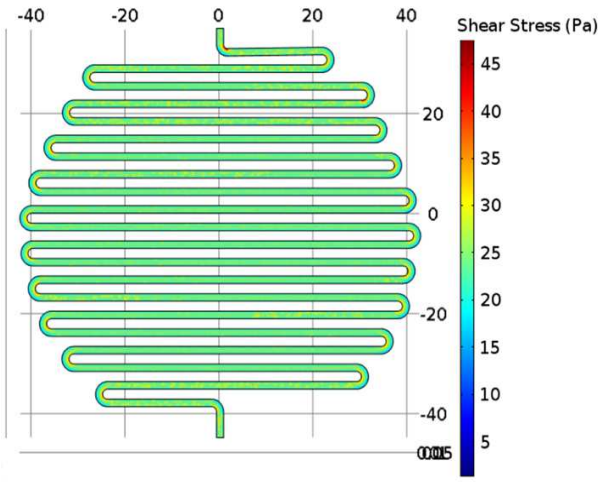


Figure 1.1: Microfluidic Pattern, uniform shear stress across the surface of the channel validated using COSMOL (Design by Jaimie Mayner)

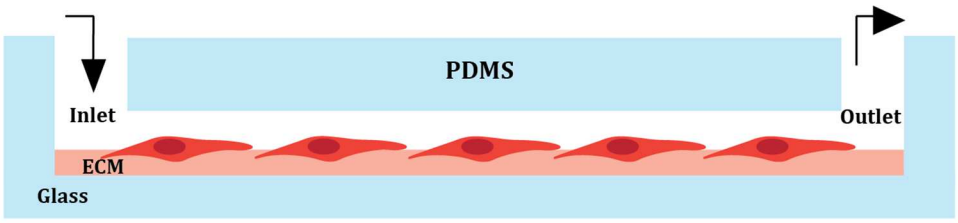


Figure 1.2: Side view of bound microfluidic device

The microfluidic device was fabricated using soft lithography. A silicon wafer with the pattern was lined with aluminum foil to contain the Polydimethlysiloxane (PDMS) mixture. The PDMS mixture was created using a 10:1 ratio of silicone elastomer and curing agent respectively and mixed until uniform. 40g of mixture was used to coat one silicon wafer and was placed in a degassing chamber to remove air bubbles and create a consistent layer. The mixture was then left to cure either overnight or using a hot plate at 40°C. Once the mixture had cured, the PDMS cast was removed from wafer and cleaned with isopropyl alcohol. Inlet and outlet holes were punctured using a biopsy punch and cast was covalently bound to a glass plate using a plasma etch, with each surface exposed to plasma for 30 seconds. Devices were then placed in an 80 °C oven for one hour to reinforce binding.

Shear Assay

Weakly adherent and strongly adherent VSMC populations were distinguished by exposing cells to shear stress within the microfluidic device. A syringe pump was attached to the inlet hole of the device using an inlet needle and plastic tubing. The syringe pump was first set to a flow rate of 0.1ml/min for 0.5 ml with ethanol in order to

sterilize the device, followed by water, and then 0.1% gelatin to functionalize the surface with ECM. Cells were then seeded in the device at a density of 433,000 cells/ml using the same flow rate. Cells were left to adhere to the microfluidic device channel in an incubator (37 C at 5%CO2) overnight. Once cells had adhered to the channel and less than 18 hours later, 3 representative segments of the pattern were marked off, and the number of cells in each segment was counted.

In order to select for the "weakly adherent" (WA) population, cells were sheared at a shear stress of 30 dynes/cm^2 or 90 dynes/cm^2 for Risk/Risk patient 1(R/R P1) cell line and Risk/Risk patient 2 cell line (R/R P2), respectively. The syringe pump was set with flow rates of 1.013mL/min and 3.038 mL/min respectively, calculated from the equation:

$$\tau = \frac{6\mu Q}{w h^2}$$

where τ is shear stress in dynes/cm^2, μ is viscosity (1 mPa*second), w is the width of the channel (1.5mm) and h is the height of the channel (150um). These shear stresses were previously determined to selectively shear VSMC populations with significant morphological differences (Mayner, et al. In preparation).

Once the syringe pump was set and started at their respective flow rates, cells were then collected at the outlet and placed into a conical tube. Cells were spun down and then resuspended in DMEM/F12 media and reseeded onto gelatin plates to be used in either a proliferation assay (flow cytometry) and/or a contractility assay (Traction Force Microscopy). The "strongly adherent" (SA) cells that remained in the device were then counted within the same 3 designated segments. These numbers were then used to calculate the percentage of cells that detached, and these percentages were compared

across different risk and non-risk cell lines.

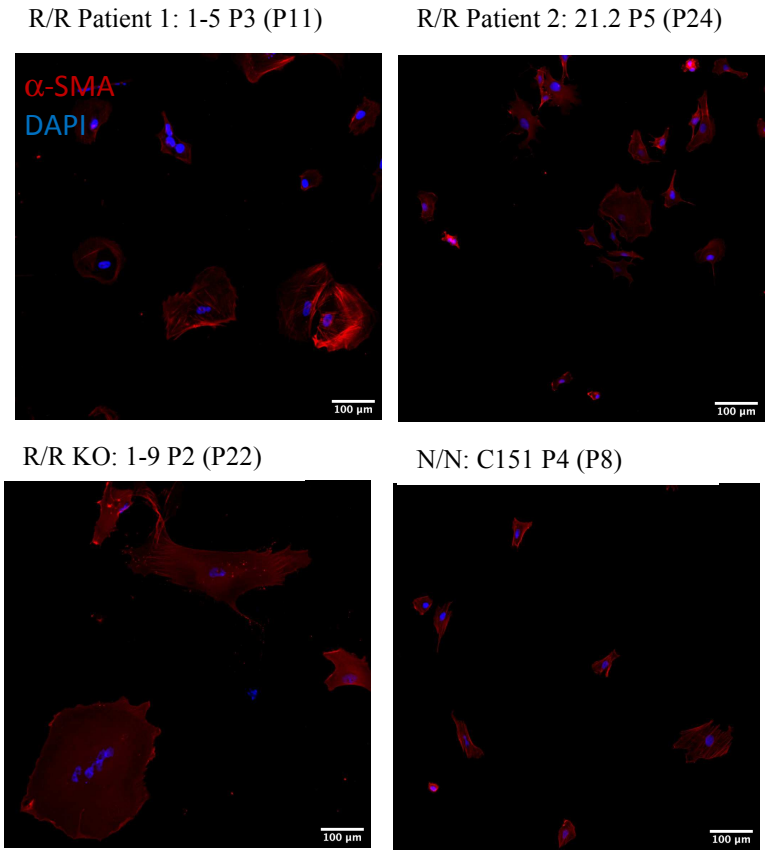
The SA cell population that remained was then lifted using TrypleE to detach the cells from the channel. TrypleE was flowed through the device at a flow rate of 0.1mL/min for 0.5mL. Once the entire pattern was coated, the device was placed in the incubator for 4-6 minutes to allow for TrypleE to cleave adhesion proteins. The device was then checked under the microscope to see if cells had lifted. If not, the device was placed in the incubator for additional 1-2 minutes until all cells were lifted. Media was pumped through the device at a flow rate of 1mL/min and the flow through was collected in a conical tube. The suspension was then centrifuged, and the cells were resuspended in DMEM/F12 media. This "strongly adherent" (SA) population was then replated onto a gelatin coated plate and prepared for either a proliferation assay and/or contractility assay.

Doubling Experiment

Different cell lines in culture were lifted using TrypleE, counted and seeded at 30,000 cells/ml. Cells were then left for 12 hours to reattach. 10 equally sized areas were sectioned off at the bottom of the 6 well plate. Cells were counted in each area for time point t=0. Cells were then counted at 24-hour intervals to analyze growth rate across cell lines.

IV. Results

A)



B)

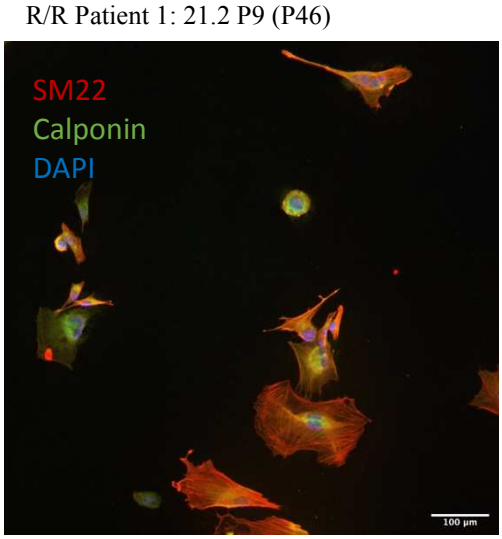


Figure 2: (A) Immunofluorescent staining of iPSC-derived VSMCs for α-SMA and the nucleus (DAPI) across genotypes of. Cell line names, VSMC passage numbers and IPSC passage numbers are shown. (B) R/R iPSC-derived VSMC stained for SM22, Calponin (specific VSMC markers) and DAPI.

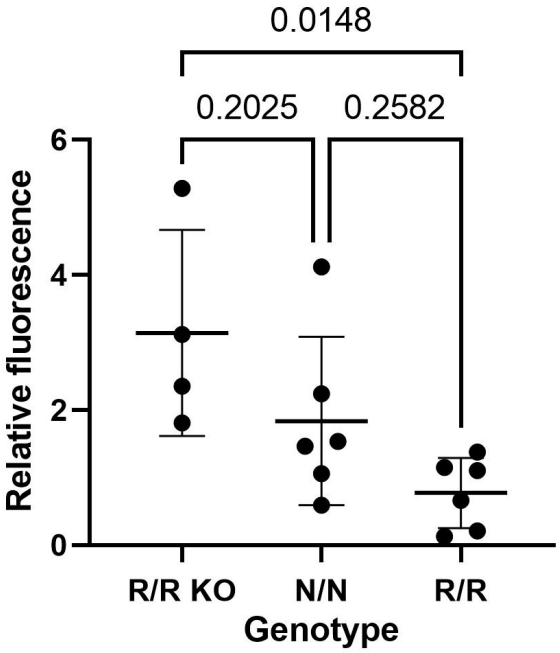


Figure 3.1: Western blot analysis showing relative α-SMA fluorescence normalized to GAPDH across risk, non-risk and risk knock out cell lines. Each point represents a separate de novo differentiation. R/R lines utilized included 21.2 (n=3), 1-5 (n=3); N/N lines included C151 (n=3) E14 (n=3); R/R KO lines included 1-9 (n=2), WB46 (n=1), 1-15 (n=1). P values were calculated using a one-way ANOVA and are shown between sample groups.

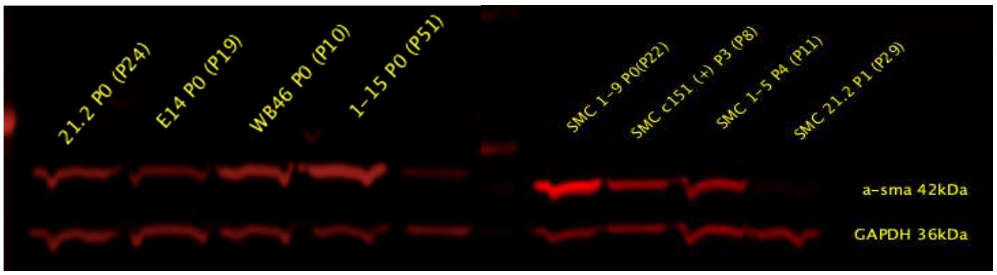


Figure 3.2: Western blot visualization showing bands at 42kDa and 36kDa, for □-SMA and GAPDH, respectively. R/R = 21.2, 1-5. R/R KO = WB46, 1-15, 1-9. N/N = E14, C151. Passage number and passage number when thawed is shown.

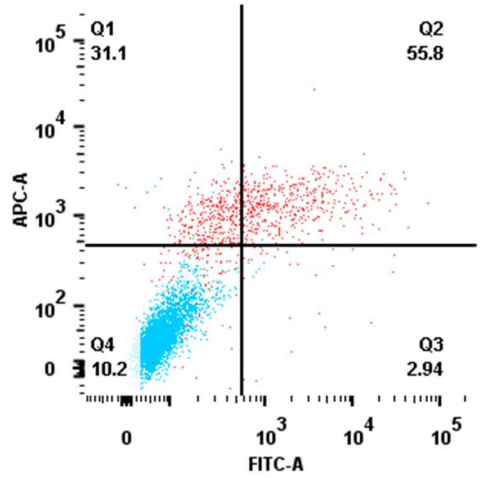


Figure 4.1: Live/Dead only (blue) and experimental sample (red) shown to illustrate quadrant gating.

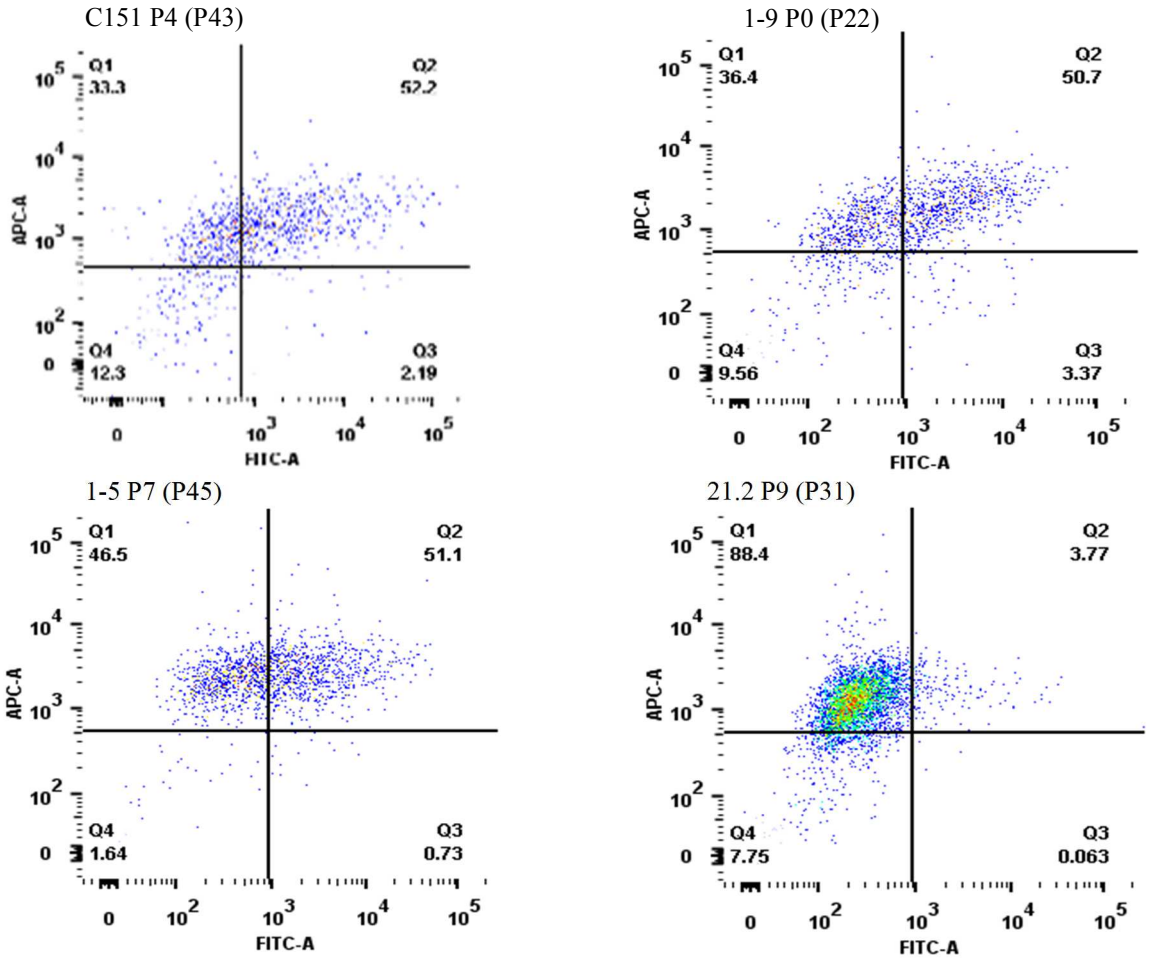


Figure 4.2: Single run flow cytometry run showing percentage of individual cells positive for markers α-SMA and Ki-67 across 4 different genotypes. Cell lines used were C151 (N/N), 1-9 (R/R KO), 1-5 (R/R patient 1), 21.2 (R/R patient 2). FITC-A and APC-A channels were used to detect α-SMA and Ki-67 respectively.

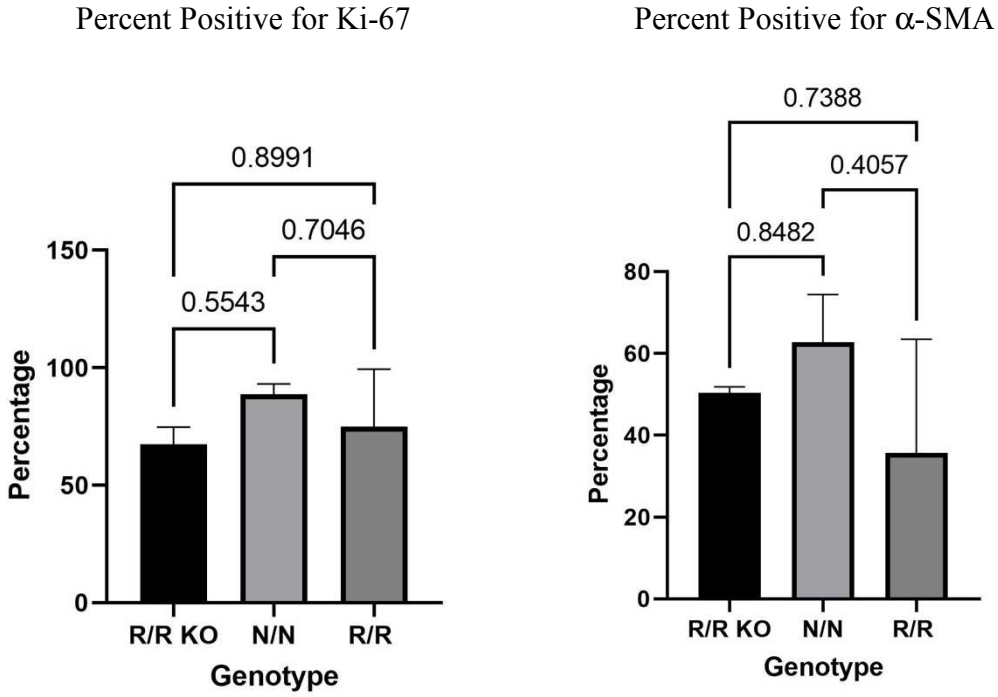


Figure 4.3: Graphs quantifying percent positive for each marker. R/R KO (n=2), N/N (n=2), R/R (n=4).

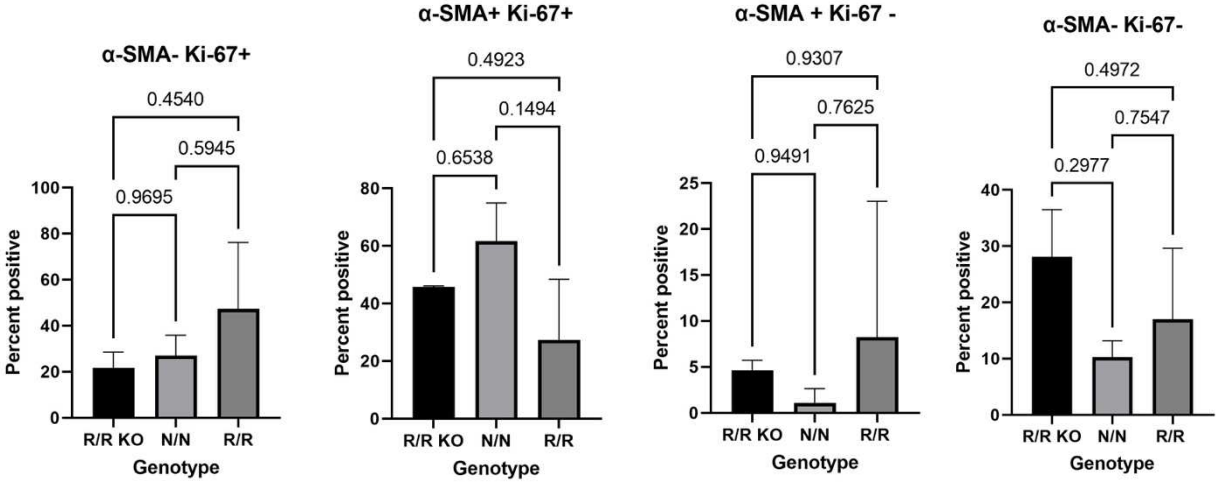


Figure 4.4: Graphs quantifying percent positive for each marker in each quadrant across genotypes. From left to right: Q1,2,3,4.

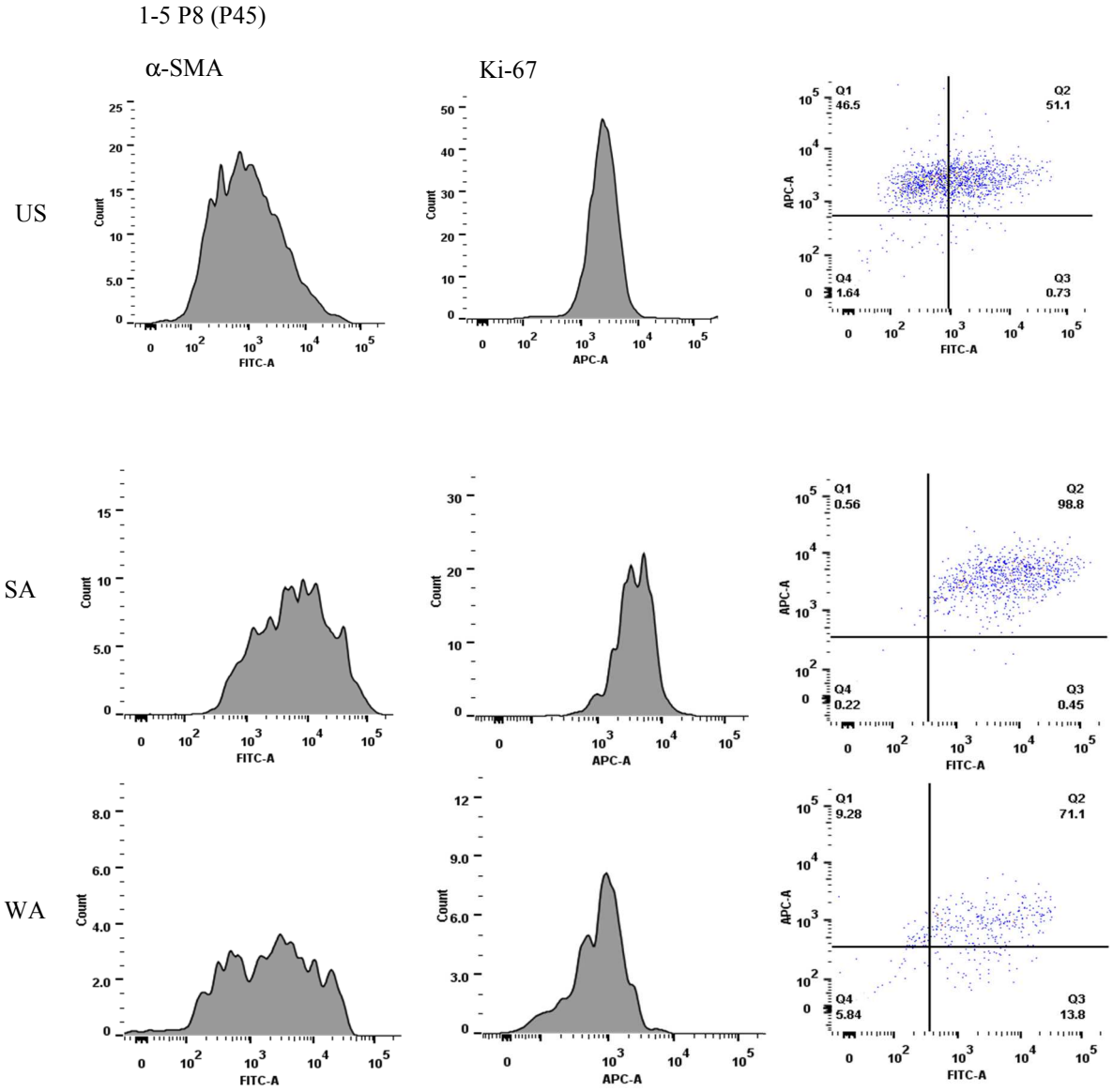


Figure 5: Flow cytometry results from shear assay (n=1). Histograms show marker intensity and cell count positive for α-SMA and ki-67 across samples. Pseudo plots show both channels and intensity. Each row represents a different sample: US (unselected), USID (unselected in device) were used as controls.

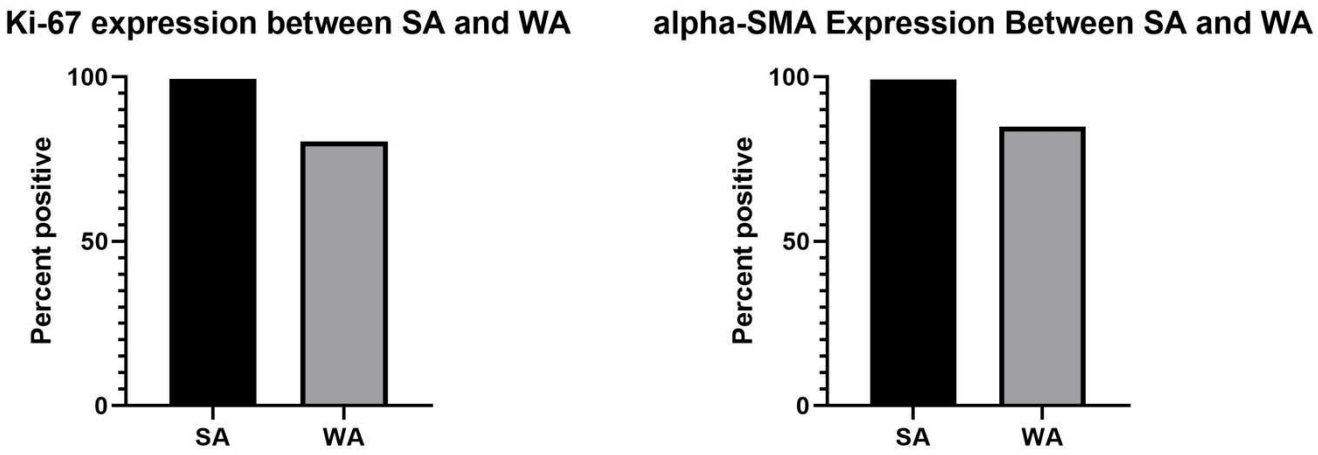


Figure 6: Percent of cells positive for proliferation marker Ki-67 and contractility marker α-SMA between sorted SA and WA cell populations (n=1)

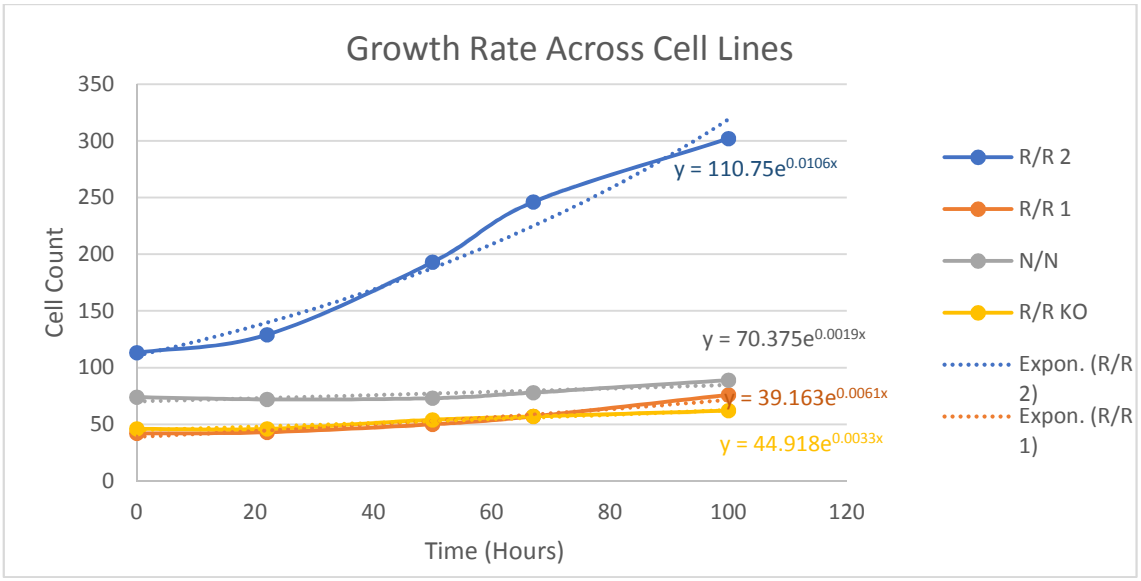


Figure 7: Growth rate differences between IPSC-dervied vascular smooth muscle cell genotypes (n=1).

Table 2: Doubling times across genoypescalculated from exponential regressions calculated in Figure 7.

Genotype	Doubling time (hrs)
R/R	89
N/N	365
R/R KO	210

V. Discussion

This project aimed to show that the 9p21 non-coding locus affects phenotypic regulation of vascular smooth muscle cells. We hypothesized that SNPs in the 9p21 locus associated with CAD lead to a higher penetrance of the synthetic, or more proliferative VSMC phenotype, and therefore, could lead to a higher susceptibility of developing CAD. Therefore, knockout of this CAD risk region was hypothesized to rescue the contractile phenotype. It was also hypothesized that VSMCs could be sorted using a shear assay into two distinct populations which would show inherent differences in contractility and proliferation rates.

IF Staining

IF staining was carried out to assess the success of the iPSC differentiation into VSMCs and the presence of SM22 and Calponin suggests an effective differentiation protocol (figure 2). From the IF images, we are also able to make observations about cell size and abundance of contractile marker α-SMA. It has been shown that large cell area is indicative of a contractile phenotype and small cell area is indicative of the synthetic phenotype. Although cell area was not quantified here, IF imaging seems to show the R/R KO lines to be the largest cells, followed by R/R patient 1, N/N and R/R

patient 2. Though we would hypothesize that the N/N to be larger than the R/R lines, there is variation batch to batch across differentiations that is not accounted for in these images. When cell area is compared using multiple differentiation batches, R/R KO and N/N cells are larger than both R/R lines (Mayner, et al. In preparation). This supports the previous hypothesis that KO lines can rescue the contractile phenotype, and that the presence of the CAD risk locus increases the expression of the synthetic phenotype

IF staining for contractility marker α-SMA was used as a metric to assess contractility of cells. The increased abundance of α-SMA would suggest upregulation of the contractile phenotype. IF imaging does allow us to visualize presence of α-SMA, but it does not allow us to quantify the expression of this contractility marker. In order to quantify the expression of α-SMA across genotypes, I shifted my focus onto western blots.

Western Blot Analysis of α-SMA

Western blots quantifying the levels of α-SMA were also used to further asses the expression of the synthetic versus contractile phenotype. The expression of α-SMA is implicated in contraction and increased in the contractile phenotype relative to the synthetic. Western blot assays aimed to illustrate expression differences in α-SMA across genotypes, hypothesizing that R/R KO and N/N would show higher expression levels of α-SMA compared to R/R lines. Results here showed that risk knock out lines (1-9, WB86, 1-15) had the highest average expression levels of α-SMA, followed by non-risk lines (C151, E14), and then risk lines (1-5, 21.2). This finding also supports the hypothesis that CAD associated SNPs in the 9p21 locus are involved in upregulating

the synthetic phenotype in VSMCs which decreases the expression of contractility markers such as α-SMA.

α-SMA levels were shown to be statistically significantly different between R/R KO and R/R ($p=0.0148$) lines but insignificant between the N/N and the R/R lines ($p=0.2582$). These findings could be due to small sample size or the use of GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) as a housekeeping gene. GAPDH is a key enzyme in glycolysis and is a frequently used for normalization of protein expression. However, since our hypothesis looks at differences in proliferation, certain cell lines may be more metabolically active compared to others and so GAPDH may not be suitable for normalization in this context. The research group that had created these iPSC lines used PPIA (cyclophilin A) to normalize for polymerase chain reaction experiments. However, PPIA is transcribed but not translated in VSMCs and is therefore unsuitable for Western blots. Although GAPDH bands show similar fluorescence across genotypes (Figure 3), further research into a more suitable housekeeping gene could be important for future experiments.

Flow cytometry to Assess Expression of Proliferation and Contractility markers

Flow cytometry results illustrate that the R/R patient 2 cell line shows an enhanced population of Ki67 only positive cells, suggestive of a more synthetic phenotype. Cell population is minimally spread across Q1, Q2 and Q4 but are largely concentrated in Q1(Ki67 positive only). R/R patient 1, N/N and R/R KO lines show more significant heterogeneity, with a large spread primarily across Q1 and Q2 (Ki67 and α-SMA positive). The large number of Ki-67 positive cells was unexpected in the

N/N and R/R KO genotypes, as while culturing these cell lines it was observed that they were not exceptionally proliferative. Ki-67 is a proliferation marker that is only present in dividing cells, but the relationship between Ki-67 and the rate of proliferation in VSMCs was not investigated here. It is possible that the intensity of Ki-67 is more indicative of proliferation rather than just the presence of the marker itself.

Shear Assay

The shear assay was carried out using the R/R patient 1 cell line (1-5) was used as it was shown to be the most heterogenous of the two R/R cell lines in terms of Ki-67 and α-SMA expression. The increased heterogeneity should allow us to more effectively sort cells into a SA (α-SMA$^{+/hi}$, Ki -67$^{-/low}$) and WA (α-SMA$^{-/low}$, Ki -67$^{+/hi}$) population.

Flow cytometry sorting results suggested that our understanding of the relationship between the expression of proliferation markers (Ki-67) and contractility markers (α-SMA) was incorrect. We had previously hypothesized an inverse relationship between Ki-67 and α-SMA that capitulates the synthetic and contractile phenotype respectively. Though, flow cytometry results from the shear assay do corroborate the α-SMA trend (figure 6), illustrating that the strongly adherent population had a higher concentration of α-SMA^{hi}, whereas the weakly adherent population had a higher concentration of α-SMA$^{low.}$, it does not suggest this inverse trend with Ki-67 (Figure 6). Instead, we see from the sort that there is a lower percentage of the WA cells that are Ki-67$^{+/hi}$ compared to the SA cells.

These results indicate that while the SA express more of the contractile phenotype marker α-SMA relative to the WA, suggesting the SA contains more of the contractile phenotype relative to the WA, the expression levels of the proliferation marker Ki67 does not support that the WA contain more of the synthetic phenotype relative the SA. The relationship between Ki-67 and α-SMA is not inversely correlated and there are likely many other factors that contribute to the dynamic between proliferation and contractility seen in the synthetic and contractile phenotype.

The conclusions that are drawn from flow cytometry results are dependent on how effective these specific markers capture the property/behavior of cells we are trying to study. In this case, there could also be alternatives to Ki-67 that better measure the presence of the synthetic phenotype, and more accurately describe proliferation differences observed in culture.

Proliferation differences observed in culture was quantified by performing a doubling experiment. Cell counts at different time points were recorded for the different genotypes. Their growth rate was then plotted and doubling times calculated. It was shown that the average doubling times across the R/R patient cell lines, the R/R KO and N/N cell lines was 89 hours, 210 hours, and 365 hours respectively (figure 7).

This result does show inherent proliferation differences across genotypes and suggests that the Ki-67 marker does not fully capture proliferation trends as suggested by the doubling times. Ki-67 is commonly used as a proliferation marker but is actually present during all phases of the cell cycle except for G0 (Kaufman, 2018), when cells leave the cell cycle and become quiescent. It may be more relevant to assess the intensity of Ki-67 rather than use it as a binary marker for proliferation (Miller, 2018).

It is possible that the N/N, R/R KO cells are not quiescent, but rather have a longer cell cycle which explains the need to passage the R/R lines more frequently.

Finally, the flow cytometry sort was only carried out once. The small sample size does not allow us to capture trends between differentiation batches, shear assays or even passage numbers. In the future multiple sorts should be carried out to further investigate the relationship between contractility and proliferation between the WA and SA cell population.

Future Directions

The assays performed here are dependent on how effective the selected markers can capture the cellular characteristic of interest. To address the concerns raised about the effectiveness of Ki-67 as a proliferation marker, I would suggest performing an EdU (thymine analogue) that selectively identifies cells that are in S phase (synthesizing new DNA). This would be a binary marker to identify cells that are actively replicating and may be able to capture difference in cell cycle length observed in culture. This assay may more accurately highlight the presence of the synthetic versus contractile phenotype observed in iPSC-derived VSMCs.

The 9p21 locus seems to play an important role in VSMC plasticity, but the exact molecular mechanism is still unknown. There is long non-coding RNA that is expressed within the 9p21 region, ANRIL (associated non-coding RNA in the INK4 locus) that is suspected to play a role in the development of CAD and other diseases of the vasculature (Holdt et al.). Future research could explore the effects of ANRIL and whether the overexpression or under expression of this long non-coding RNA can

impact the phenotypic switching observed in VSMCs.

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