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The Role of Notch3 in Human Adipose Stem Cell Fate

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THE ROLE OF NOTCH3 IN HUMAN ADIPOSE STEM CELL FATE

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ABSTRACT

Stem cell-based therapies are the future of medicine, as their natural abilities of self-renewal and differentiation have the potential to treat currently incurable diseases. Stem cells are categorized based on differentiation potential, with multipotent stem cells being the most differentiated while still having the ability to divide down defined lineages. Adipose stem cells (hASCs) are easily derived multipotent, mesenchymal stem cells with already demonstrated therapeutic potential. Despite current success, there are a multitude of factors that regulate the physiology and role of adipose stem cells, including transduction pathways and transcription factors, that remain to be researched and fully understood. One such transduction pathway –the Notch signaling pathway– is known to be crucial in stem cell proliferation, differentiation, and apoptosis in all animals. The focus of my research is on Notch3, one of four Notch transmembrane surface receptors involved in stem cell fate determination, embryonic development, and some forms of cancer. I utilized siRNA-mediated knockdowns of Notch3 to evaluate its role in stem cell proliferation, self-renewal, metabolism, and morphology. Results have indicated that Notch3 plays a highly active role in cellular proliferation and self-renewal, while not eliciting an effect on metabolism and morphology. This data continues to drive the research into stem cells and their regulators forward, bringing us one step closer to finding curative degenerative disease treatments.

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DEDICATION

This thesis is dedicated to all the stressed, confused, and pressured pre-medical students who are in need of some guidance. Hopefully you all find your Dr. Newman.

TABLE OF CONTENTS

ABSTRACT ii

APPROVAL FOR SCHOLARLY DISSEMINATION iii

DEDICATION iv

LIST OF FIGURES viii

LIST OF TABLES x

ACKNOWLEDGMENTS xi

CHAPTER 1 INTRODUCTION 1

1.1 Introduction to Stem Cells 1

1.1.1 Stem Cell Potential 1

1.1.2 Waddington's epigenetic landscape 1

1.1.3 Totipotent Stem Cells 3

1.1.4 Pluripotent Stem Cells 4

1.1.5 Multipotent Stem Cells 7

Mesenchymal Stem Cells 8

Adipose Stem Cells 9

1.2 Factors that Regulate Stem Cell State 10

1.2.1 Cell Regulatory Network 10

1.2.2 Environmental Stimuli 11

1.2.3 Signal Transduction Pathways 12

1.2.4 Transcription Factors 14

1.2.5 Chromatin Modifiers 16

1.3 Notch Signaling and Notch3 17
1.3.1 Introduction to Notch Signaling 17
1.3.2 Role of Notch3 in Stem Cell Fate 19
1.3.3 Role of Notch3 in Embryonic Development 20
1.3.4 Role of Notch3 in Cancer 21
1.4 Conclusion 23
CHAPTER 2 Methods 26
2.1 Cell Culture 26
2.1.1 Thawing cells 26
2.1.2 Passaging cells 27
2.1.3 Transfection 27
2.2 RNA Analysis 29
2.2.1 RNA Collection 29
2.2.2 RNA Extraction 29
2.2.3 RNA Quantification 30
2.2.4 cDNA Synthesis 31
2.2.5 Real-Time PCR 31
2.3 Protein Analysis 32
2.3.1 Protein Collection and Extraction 32
2.3.2 Bradford Assay 33
2.3.3 Western Blot 33
2.4 alamarBlue 34
2.5 Colony-Forming Unit Assay 35
2.6 Phalloidin and DAPI staining 35
CHAPTER 3 Results 37

3.1 Introduction 37

3.2 *notch3* Transcript Levels Decrease Following siRNA-mediated Knockdown. 39

3.3 Notch3 Knockdown can be Sustained Through Passaging 43

3.4 Notch3 Knockdown Leads to a Decrease in *ki67* Expression in hASCs 45

3.5 Notch3 Knockdown Does Not influence Cellular Metabolism 47

3.6 Notch3 Knockdown Results in Decreased Number of Individual Cell Colonies 52

3.7 Notch3 Knockdown Decreased Cell Density but does not Significantly Impact Cytoskeleton Morphology 54

CHAPTER 4 conclusion 57

4.1 Conclusion 57

4.2 Future Directions 59

APPENDIX A Troubleshooting contamination 61

BIBLIOGRAPHY 67

LIST OF FIGURES

Figure 1-1: Waddington's Epigenetic Landscape 3

Figure 1-2: Multipotent Stem Cell Differentiation Potential. 7

Figure 1-3: Regulatory Network of Cell State. 11

Figure 1-4: Positive and Negative Feedback Loops. 13

Figure 1-5: Transcription Factors Affect Process of Transcription. 15

Figure 1-6: Complex Notch Signaling Interactions 18

Figure 1-7: Notch3 Plays an Active Role in Many Aspects of the Human Body 24

Figure 3-1: *Notch3* Knockdown Results in Decrease in Cell Density. 39

Figure 3-2: *Notch3* Transcript Adequately Decreased Following Knockdown in hASCs. 40

Figure 3-3: *Notch3* Expression Averaged Over Three Passages. 41

Figure 3-4: GAPDH Bands Appear Brightly in First Western Blot 42

Figure 3-5: Notch3 Knockdown Resulted in Decrease in Protein Expression 43

Figure 3-6: Knockdown Completed on 10cm plate and then Passaged into Assay-Dependent Plates 44

Figure 3-7: *Notch3* Knockdown can Persist Through Passaging Process 44

Figure 3-8: *Notch3* Expression Following Knockdown and Passage Averaged Over Three Passages 45

Figure 3-9: *Notch3* Knockdown Results in a Significant Decrease in *ki67* Expression .. 46

Figure 3-10: *Ki67* Expression Averaged over Three Passages 47

Figure 3-11: *Notch3* Knockdown has no Effect on hASC Cellular Metabolism at Passage 4. 48

Figure 3-12: *Notch3* Knockdown has no Effect on hASC Cellular Metabolism at Passage 5. 49

Figure 3-13:.Notch3 Knockdown has no Effect on hASC Cellular Metabolism at Passage 6. 50

Figure 3-14: Averaged Values for alamarBlue with Statistics 51

Figure 3-15: Individual Colony Formation Confirmed Using Digitial Inverted Microscope 52

Figure 3-16: More Self-Renewing Colonies were Observed in NC versus KD Group at Passage 4. 53

Figure 3-17: More Self-Renewing Colonies were Observed in NC versus KD Group at Passage 5 53

Figure 3-18: Notch3 Knockdown Decreases Cell Density but does not Significantly Impact Cytoskeleton at Passage 4 54

Figure 3-19: Notch3 Knockdown Decreases Cell Density but does not Significantly Impact Cytoskeleton at Passage 5 55

Figure 3-20: Notch3 Knockdown Decreases Cell Density but does not Significantly Impact Cytoskeleton at Passage 6 56

Figure A-1: Contaminant Could be Visualized by the Naked Eye 62

Figure A-2: Presence of Contaminant Seen on Cell Imager 63

LIST OF TABLES

Table 2-1: Contents of Complete Culture Medium 26

Table 2-2: Reagents Used in Knockdown Protocol 28

Table 2-3: Assays, Plate Types, and Cell Seeding 29

Table 2-4: qRT-PCR Primer List 31

Table 2-5: Antibody List 34

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CHAPTER 1

INTRODUCTION

1.1 Introduction to Stem Cells

1.1.1 Stem Cell Potential

Stem cell research is a new and exciting field, holding implications in human health that could revolutionize medicine forever. Stem cells hold promise in wound healing, degenerative disease, and some forms of cancer (1). Overall, stem cells have great potential to improve modern medicine by providing a natural, curative approach to terminal diseases.

Stem cells have unique properties that make them different from somatic cells, including the ability to self-renew and differentiate (2). Regenerative medicine relies on the ability to more efficiently repair or replace damaged tissue. Stem cells are already involved in the natural process of wound healing, being activated in the body to repair a cut or mediate a broken bone (3). In cases of more extreme injury or degenerative disease, however, the body cannot naturally work fast enough or, in some cases, at all to repair that damage. Identifying ways to activate and utilize stem cells may make currently untreatable conditions, treatable.

1.1.2 Waddington's epigenetic landscape

Stem cells are defined based on differentiation potential as either totipotent, pluripotent or multipotent (4). Stem cell differentiation potential can be best described using Conrad Waddington's epigenetic landscape (Figure 1-1). In 1942, Waddington, a developmental biologist, stated that from the time of conception until development is complete, each individual cell is faced with many decisions that ultimately determine the type of somatic cell it becomes (5). At the top of the hill, cells are considered totipotent, meaning they have the ability to become all cell types (6). As development continues to gastrulation, stem cells roll down the hill, becoming pluripotent and then multipotent as they make more fate-determining decisions. Along the way barriers are established that restrict those fate decisions. Until recently, it was accepted that once a cellular fate was defined, there was no stopping, altering, or reversing differentiation.

Today, we have come to understand that these physical barriers stopping the reversal of differentiation are epigenetic marks across the genome that activate or repress specific gene expression profiles (4). We now know that cancer can promote the dedifferentiation of cells (7), and that cells can be manipulated in culture to direct cell fate by altering the epigenetic landscape (8). With recent scientific advancements, it is now possible for the ball to roll back up the hill (reprogramming), fall down a different path (directed differentiation), or even cross over a hill (transdifferentiation) through genetic manipulation and alteration of a cell's environment. Today, pluripotency can be induced, and cells can be directly differentiated into a selected cell type. For example, skin cells can become muscle cells through exposure to the four reprogramming factors – Oct4, Sox2, Klf4, and c-Myc– (9) and then directed differentiation via the overexpression of MyoD, the master regulator of myogenesis (10). This can also occur directly through

transdifferentiation, in which cells do not have to revert to a stem cell-like state but can be directly differentiated across lineages (11). These new techniques can be utilized in research settings and ultimately the clinic as we learn to manipulate cells and discover the way in which they are therapeutically advantageous.

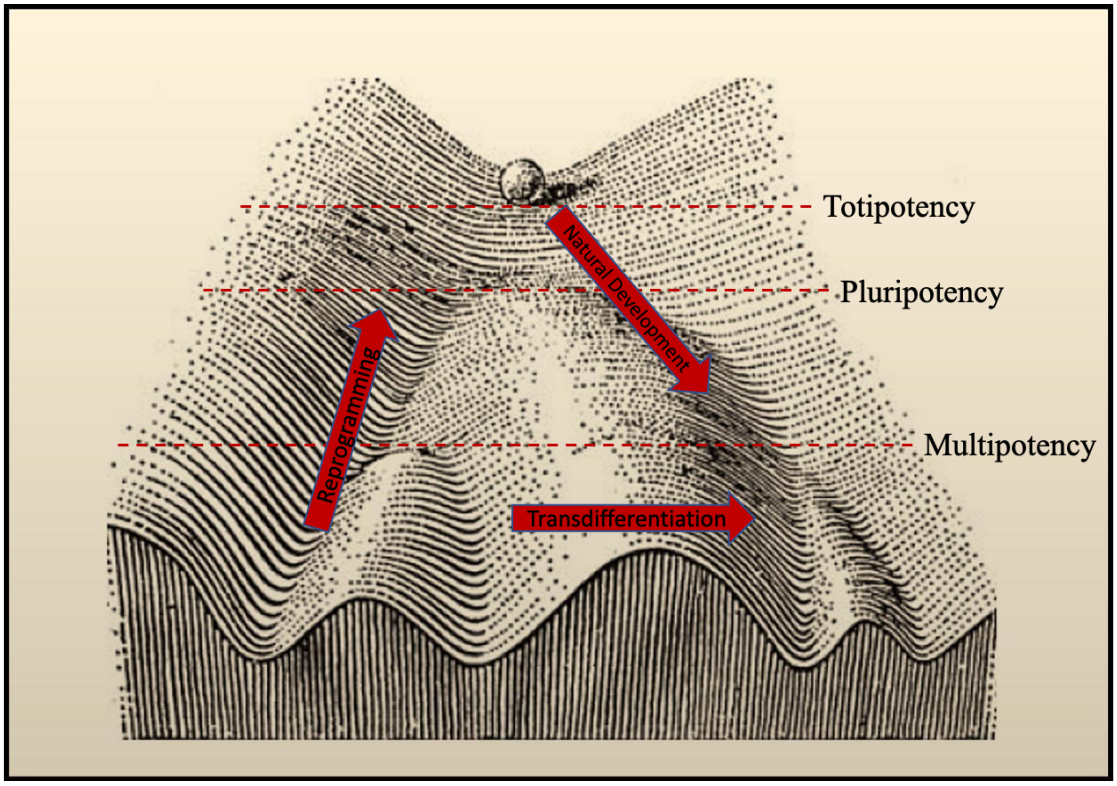

Figure 1-1: Waddington's Epigenetic Landscape. Prior to our current understanding, stem cell differentiation was an unalterable process that could not be stopped or reversed. Outside of a disease state like cancer, once a cell had become fully differentiated, it was not possible to return to a higher differentiation potential or reassign a cell to a different lineage. Today, it is possible to regain differentiation potential in a laboratory setting by overexpressing transcription factors and reprogramming a cell (12).

1.1.3 Totipotent Stem Cells

Each stem cell type has a different potential for clinical application. Totipotent stem cells are considered the most powerful type of stem cell, able to form any cell type

in the human body, including placental cells (13). This type of stem cell has the greatest differentiation potential and is only found in the newly created zygote. Human totipotent stem cells are not currently used in clinical settings due to the complexity involved with harvesting them. They are only present in the early stages of embryonic development– prior to gastrulation– and therefore have to be obtained from an early developing zygote (6). The only advantage totipotent stem cells hold over pluripotent stem cells is that they are capable of differentiating into the cells that makeup the placenta and umbilical cord. Given the source of these cells and the limited gain in clinical application that comes from harvesting them, totipotent cells are rarely used in research or therapeutic applications (6).

1.1.4 Pluripotent Stem Cells

Once a human zygote reaches approximately the 8-cell stage, it begins to generate pluripotent stem cells known as human embryonic stem cells (hESCs). Embryonic stem cells are isolated from the blastocyst of a developing embryo and are capable of self-renewing and differentiating into any cell type of a developing organism, excluding placental cells (14). In the case of human embryonic stem cells, cells are harvested directly from an embryo created and donated through informed consent during the early steps of IVF (15). Typically, patients undergoing IVF elect to freeze any additional embryos that are not implanted in case the implanted embryo does not survive, or they wish to have more children in the future. If a couple does not have further use for their frozen embryos, they may choose to donate these additional embryos to research.

While embryonic stem cell diversity makes them particularly appealing for basic and clinical research, this cell type does face challenges both because of how they are

harvested and because of their robust ability to self-renew. hESCs face the issue of compatibility with the recipient. Human embryonic stem cells can never be obtained directly from the individual who will be receiving them, as they must be taken from a developing embryo that is in turn destroyed by the harvesting of hESCs. The human leukocyte antigen (HLA), an antigen found on most cells used to detect foreign cells, is used to determine how well suited a match is for a recipient (16). If too dissimilar, the recipient's immune system can attack the implanted cells, resulting in rejection of the cells. Symptoms of this can range from mild to severe and can likely become life-threatening (17). Due to this, it is more difficult to identify a perfect match for implantation, leaving open the possibility of rejection and ultimately making hESCs a less clinically viable cell type.

Pluripotent stem cells may also be created from somatic cells and, in this case, are termed human induced pluripotent stem cells (hiPSCs). These cells are created in a laboratory setting through a process called somatic cell nuclear transfer (SCNT) or through the overexpression of transcription factors, in a process known as cellular reprogramming (14). These discoveries, by scientists John B. Gurdon and Shinya Yamanaka respectively, were awarded the Nobel prize in 2012. SCNT can result in a cloned human embryo developed from a somatic cell and an enucleated oocyte, or egg cell (18). Unfortunately, with both SCNT and cellular reprogramming, there remains an increased risk of developmental defects and disease due to incomplete reprogramming leading to imperfect regulation of transcription (19). An example of this is Dolly the Sheep, the first animal cloned utilizing somatic cell nuclear transfer. While the cloning was successful, and Dolly was capable of reproducing, she had unusually short telomeres,

which indicate signs of aging beyond her physical years. For this reason, among developing an aggressive form of lung cancer, she died at an early age of six years (20).

These shortcomings of incomplete reprogramming mean that these embryos are never allowed to develop beyond the blastocyst stage and that the stem cells harvested may have mutations that influence the research being performed (19). Although hiPSCs are derived from a somatic cell and so can be from the recipient themselves, cellular reprogramming still faces similar challenges of sometimes incomplete epigenetic reprogramming.

Both hESCs and hiPSCs can be differentiated directly to any cell within an adult, making them of great interest to those who study regenerative medicine. In fact, a recent study differentiated human induced pluripotent stem cells into functioning microglia which could then be applied to treat various neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (21). Both hESCs and hiPSCs can also be differentiated into organoids, which are smaller versions of necessary organs that are capable of functioning as that organ. An example of this is the creation of a pancreatic organoid from hiPSCs to produce insulin in a diabetic patient without a functioning pancreas (22).

Despite their potential for use in regenerative medicine, both human embryonic and induced pluripotent stem cells can be considered dangerous when used in clinical situations, as their nearly limitless replicative potential and pluripotency make them tumorigenic (23). If not fully differentiated prior to treating a patient, there is risk that those few undifferentiated cells may improperly differentiate or begin to proliferate uncontrollably. Both cell types also express low levels of *p53*, a tumor suppressor

responsible for regulating the cell cycle, which is downregulated in both cancer and stem cells to promote self-renewal (24). This makes hESCs even more likely to advance into a tumorigenic cell.

1.1.5 Multipotent Stem Cells

Multipotent stem cells, which can be found in a variety of adult tissues, self-renew, although to a more limited extent than pluripotent stem cells, and differentiate down only specific lineages (25) (Figure 1-2). This limitation in differentiation potential is what sets them apart from previous stem cell types. For example, hematopoietic stem cells found in the bone marrow can transition into red or white blood cells as well as platelets; however, they lack the capability to transition to bone, muscle, or fat cells (26). Human adipose-derived stem cells (hADSCs) are another example of multipotent mesenchymal stem cells that naturally give rise to adipocytes, osteocytes, and chondrocytes.

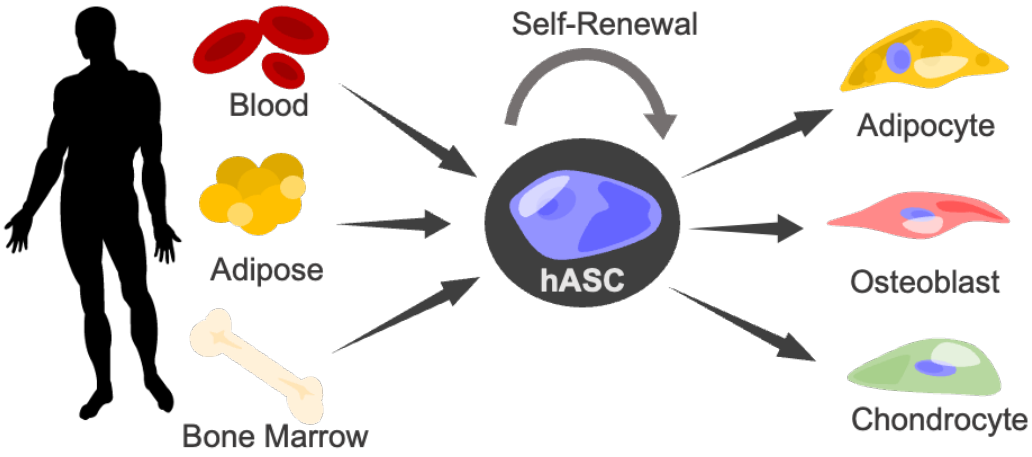

Figure 1-2: Multipotent Stem Cell Differentiation Potential. Human adult stem cells (hASCs) are multipotent and have two properties that make them unique for tissue regenerative treatments. First, they are capable of proliferating while remaining undifferentiated. This is also known as the process of self-renewal. Second, they can differentiate specifically down the mesoderm lineage. Multipotent stem cells can be derived from blood, adipose tissue, and bone marrow, and can transform into adipocytes, osteoblasts, or chondrocytes.

While multipotent stem cells are less diverse in their differentiation potential and have lower replicative potential, they are safer for use in clinical settings. They are less tumorigenic than pluripotent stem cells, and therefore less likely to differentiate into a foreign cell in the body (27). Multipotent stem cells can be taken directly from a patient and reapplied to the patient's own diseased tissue, a perfect match thereby eliminating the possibility of rejection.

Mesenchymal Stem Cells

Mesenchymal stem cells are a particular class of multipotent stem cell which can differentiate into any cell type within the mesoderm lineage (28). This includes adipocytes (fat cells), osteocytes (bone cells), chondrocytes (cartilage), and myocytes (muscle cells). Some examples of mesenchymal stem cells include bone marrow-derived mesenchymal stem cells (BM-MSCs), umbilical cord-derived mesenchymal stem cells (UC-MSCs), and adipose-derived stem cells (ADSCs). BM- and UC-MSCs possess therapeutic potential, but are limited by difficult harvesting methods, with the procedure for each being invasive and complex. Adipose-derived stem cells may be the most interesting source of clinically relevant stem cells, as they can be obtained easily and ethically, directly from a patient's own adipose tissue.

Adipose Stem Cells

Adipose-derived stem cells are a type of mesenchymal, multipotent stem cell that are widely used in clinical research settings. Adipose stem cells are taken directly from the adipose tissue of the recipient, making them easy to obtain and safer for use. Adipose stem cells also grow quickly within a laboratory setting, readily proliferating and differentiating in a cell culture dish, making them ideal for clinical research purposes (29).

Adipose stem cells have additional properties that make them strong candidates for stem cell-based treatments. Adipose stem cells, once implanted in the body, secrete factors that have been shown to influence tissue regeneration, some of which include fibroblast growth factor and hepatocyte growth factor (30). These secretions can also be controlled via directed differentiation, so these cells are easily manipulated to fit their surroundings. Adipose stem cells are also susceptible to different growth factors, and aid in the regeneration of decaying tissue when exposed to these factors. For example, adipose stem cells can enrich scaffolds composed of biomaterials that are to be implanted into the body when exposed to cytokine growth factors. These growth factors stimulate these stem cells to produce other growth factors – such as basic fibroblast growth factor or hepatocyte growth factor– allowing for tissue regeneration (31). For these reasons, adipose stem cells are considered useful when applied to treat degenerative tissue.

Adipose stem cells have previously been used clinically in several instances, including the enrichment of micrografting the skin of patients with system sclerosis, or scleroderma. These patients saw a significant decrease in skin tightening in the area the grafting was placed (32). Another study obtained adipose-derived stromal vascular

fraction taken directly from the recipient's adipose tissue and systematically injected it into the fascia of the recipient's face or intranasally to treat early symptoms of Parkinson's Disease. Following the application of this stem cell treatment, the patients saw a decrease in motor control symptoms as well as a decrease in cognitive decline (33). There are currently over 500 ongoing clinical trials that are using some form of adipose stem cell-based therapy (34).

Stem cells can provide curative approaches to many diseases that degrade the body's tissue. Adipose stem cells can be applied to clinical settings in a uniquely safe way, as they can be attached to an adipose-derived conduit created directly from a recipient's adipose tissue, and then implanted or injected into the body (35). This increases the likelihood that the recipient will biologically accept and respond well to the treatment. Adipose stem cells also have a wide range of applications, from tissue regeneration to decreasing inflammation. This tremendous potential is what drives the research forward (36).

1.2 Factors that Regulate Stem Cell State

1.2.1 Cell Regulatory Network

A stem cell's ability to self-renew and differentiate is complex and influenced by many factors that regulate gene expression and cell behavior. Some of these regulatory factors include environmental stimuli, signal transduction pathways, transcription factors, and chromatin modifiers. All of these can work together, creating a vast network of interconnected proteins working collectively to regulate cell state (Figure 1-3).

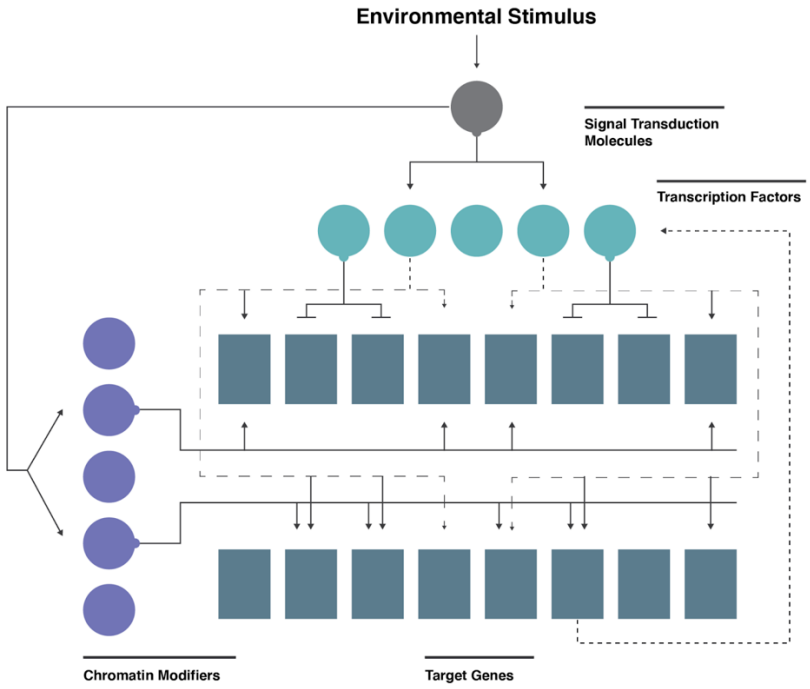

Figure 1-3: Regulatory Network of Cell State. The factors that regulate stem cell state make up a vast network of many interacting proteins and chemical compounds. Environmental stimuli, signal transduction molecules, transcription factors, and chromatin modifiers all work in sync to regulate stem cells and their reaction to the environment. Each component is capable of enacting cellular change directly or via activating or repressing a pathway farther downstream.

1.2.2 Environmental Stimuli

Environmental stimuli, including carcinogens, toxins, pathogens, stress, and drugs can determine the actions of cells. However, environmental stimuli, acting alone, cannot enact lasting transcriptional change (37). The activation of a system further down the complex regulatory network, such as a signal transduction pathway, transcription factor, or chromatin modifier, is required to enact permanent or transcriptional changes. Typically, these systems work in unison, each activating, repressing, or otherwise having

some effect on one another. These components work to change gene expression profiles, ultimately leading to changes in cellular state, differentiation, and could stimulate proliferation or apoptosis.

1.2.3 Signal Transduction Pathways

Signal transduction molecules make up greater and more complex signaling pathways, which work to send information gathered from outside of the cell to the nucleus. Signal transduction can be initiated by hormones, secondary messengers, and the interaction of ligands and cell-bound receptors. Signaling pathways can regulate all aspects of cell fate, including proliferation, differentiation, and apoptosis. Misregulation of these processes, either through overstimulation or inhibition of a signaling cascade, can result in aberrant cell behavior that results in disease, including cancer.

Typically, signal transduction pathways are regulated by either negative or positive feedback loops. Negative feedback loops work to maintain homeostasis by decreasing a response in the presence of a signal. Specifically, when the accumulation of a factor exceeds a cellular limit, the product acts as an inhibitor to the body's response, ultimately decreasing the production of the product itself (Figure 1-4). In contrast, positive feedback loops lead to an increase in a response. In most instances, these processes are working together to regulate cellular pathways to achieve and maintain homeostasis.

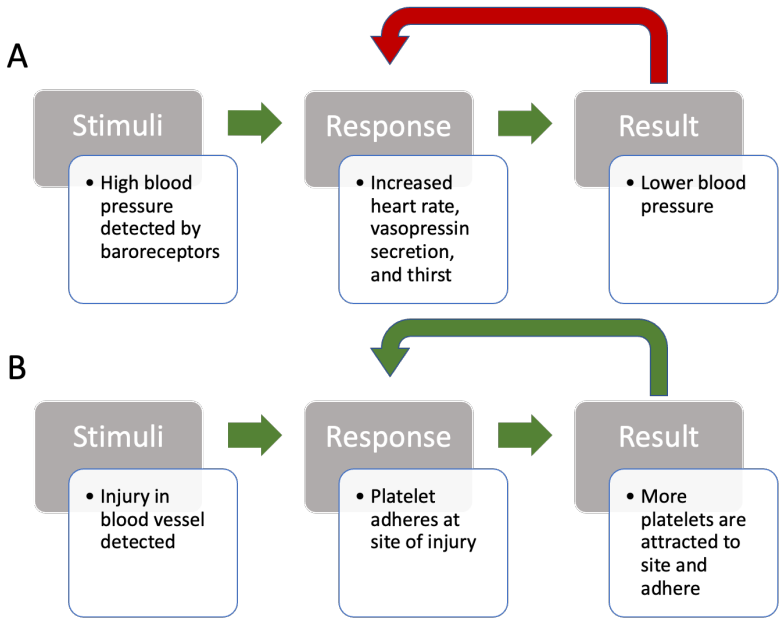

Figure 1-4: Positive and Negative Feedback Loops. It is crucial for signal transduction pathways to be highly regulated. This control occurs through both negative (Panel A) and positive (Panel B) feedback loops. In negative feedback, the result triggers a decrease in the response that initiated its creation. In positive feedback, the result increases the response that initiated its creation.

For example, one pathway comprised of an interconnected group of mitogen-activated protein kinases, termed MAPK signaling, has been shown to aid in regulation of cellular proliferation in stem cells (38). There are currently three known types of MAPK signaling: *p38*-related, extracellular signal-related (ERK), and c-Jun terminal primary amine-related (JNK). These pathways play diverse roles in the cellular control of DNA replication and division as well as apoptosis and are tightly controlled by both positive and negative feedback loops. Misregulation of this pathway has been implicated in cases of several neurodegenerative diseases as well as cancer. Oxidative stress caused by

neurodegenerative diseases –such as Alzheimer's Disease –can directly cause the hyperactivation of the JNK and *p38* MAPK signaling pathways, increasing neural death and leading to the creation of amyloid precursor protein, a key component in the pathology of AD (39). Transcriptional changes in *ras* and *b-raf* lead to the overactivation of ERK MAPK signaling and can promote tumorigenesis in many forms of cancer (40).

1.2.4 Transcription Factors

Transcription factors are another component that contribute to permanent change in cell state. Transcription is the copying of DNA into RNA to create mRNA, which will eventually be utilized in the production of proteins. This process is initiated by RNA polymerase binding to the promoter region of a gene (green region in Figure 1-5) and then transcribing the target genomic region (seen in blue). Transcription factors bind to either enhancer or activator regions located upstream of their targeted gene (Figure 1-5). This binding activates or inhibits transcription of a particular gene, thereby controlling the amount of a target mRNA transcript, as well as the potential for a target protein to be translated and expressed by the cell (41).

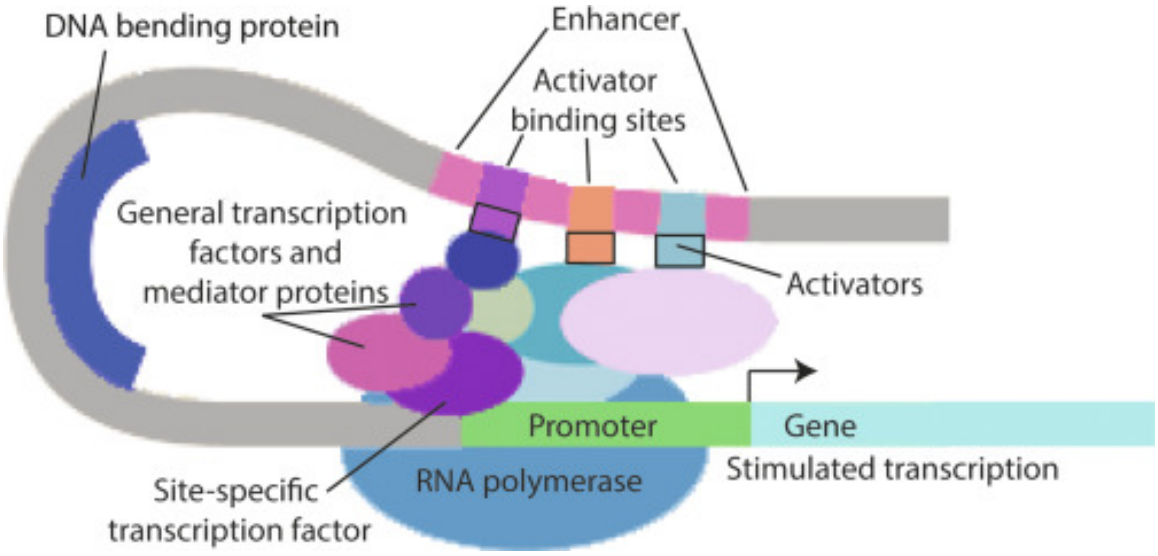

Figure 1-5: Transcription Factors Affect Process of Transcription. Transcription begins when RNA polymerase binds to the promotor region located upstream of a gene. Transcription factors bind to enhancer or activator binding sites and can lead to the under- or overexpression of a target gene. This, in turn, regulates the production of a target protein, leading to changes in cellular state (42).

There are multiple different types of transcription factors, some of which include zinc fingers, β-sheets motifs, and various helices. Transcription factors from each of these families work to regulate cell fate. Two relevant examples of transcription factors are Oct4 and Sox2. These transcription factors are expressed by pluripotent stem cells to maintain stem cell state (43). They can also be overexpressed in differentiated cells to change cellular state by increasing differentiation potential of a somatic cell to that of a pluripotent stem cell, in a process called cellular reprogramming. Oct4 and Sox2 do this by activating protein-coding genes that are necessary for a pluripotent state while also silencing genes involved in differentiation and cellular development (44). Oct4, specifically, plays a role in determining embryonic stem cell fate, as its expression level directly influences the differentiation potential of a cell (9). It is highly expressed in cells

with greater differentiation potential, and its expression is slowly decreased as cells become more and more differentiated (45).

1.2.5 Chromatin Modifiers

Finally, chromatin or histone modifiers are ultimately required for transcriptional changes within stem cells. DNA is tightly wound across proteins known as histones, creating a complex of DNA and protein known as chromatin. It is through this interaction that a cell is able to hold the vast amount of genetic material required for life. Histone or chromatin modifiers are enzymes that work by changing the configuration of the chromatin structure to open, increasing access of transcriptional machinery to a gene, or to close, decreasing access of transcriptional machinery to a gene. They can do this directly through the addition of molecular functional groups to amino acid residues that make up the histone protein, or indirectly through the addition of effector proteins which attract enzymes that can make similar configurational alterations (46). Together these modifications create the epigenetic profile of a cell type and can be used to define and understand gene expression profiles.

The most common chromatin modifications in humans are methylation, ubiquitination, phosphorylation, and acetylation. These modifiers are capable of regulating cellular transcriptional state, which can cause changes in stem cell proliferation, differentiation, and even secretions (46). One example of a chromatin modifying complex is the SAGA complex, comprised of many modifiers that regulate cellular response to environmental stimuli, some mRNA processes, and DNA repair following UV exposure. This modifying complex regulates the acetylation and deubiquitylation of the histone protein. This complex also interacts with transcription

factors to regulate gene expression (47). Expression levels of this histone modifier are typically highest in regions of the body where development is occurring the most, such as the brain or within the reproductive organs, as it also plays many roles in human development. Misregulations in this complex has been linked to neurodegenerative disease and incomplete neonatal development. Misregulations in other histone modification complexes has also been linked to cancer, through their regulation of cell cycle progression (48).

Stem cell fate is regulated by a vast array of components, all of which are individually complex. These factors, like signal transduction pathways and chromatin modifiers, can also interact with each other, creating a large network of communicating proteins that work to regulate stem cell proliferation, differentiation, and fate determination. If stem cells are going to be utilized in clinical treatments, it is crucial to thoroughly understand each of the factors that regulate their behavior.

1.3 Notch Signaling and Notch3

1.3.1 Introduction to Notch Signaling

Environmental stimuli, signal transduction pathways, chromatin modifiers, and transcription factors have been shown to work together to regulate cell behavior. The Notch signaling pathway (Figure 1-6) is a complex pathway where receptors and ligands interact to connect the cellular environment to the cell's transcriptional profile and ultimate fate. The Notch signaling pathway regulates cell proliferation, differentiation, fate, and death in all metazoan eukaryotic organisms (49) and has been shown to play a role in determining the fate of adipose stem cells (50).

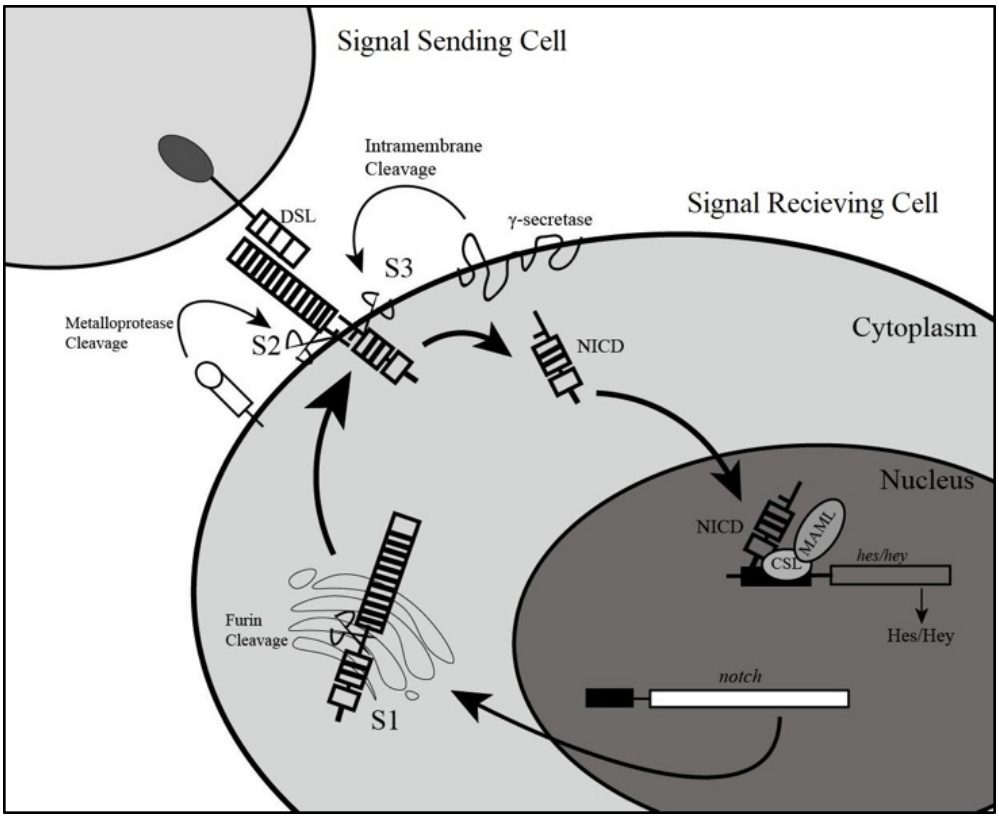

Figure 1-6: Complex Notch Signaling Interactions. The Notch signaling pathway is complex and regulates the properties of stem cells. This diagram provides a simplistic representation of the mechanisms of the Notch signaling pathway between a signal sending cell and signal receiving cell. This pathway provides a means for both intracellular and extracellular signaling.

The Notch signaling pathway consists of four different cell surface receptors, Notches 1-4, as well as two different types of ligands: Delta and Serrate. The Serrate ligands are termed Jagged in humans, and these are Jagged-1 and Jagged-2 ligands. The Delta ligands are termed Delta-like ligands (DLL) in humans, and these are DLL-1, DLL-3, and DLL-4 (51). Of these proteins, the Notch3 receptor has been studied previously in the Newman lab, as it has many implications in human health, with precise roles in stem cells, embryonic development, and cancer.

1.3.2 Role of Notch3 in Stem Cell Fate

Notch3 aids in regulating the differentiation of mesenchymal stem cells where, depending on environmental stimuli, Notch3 contributes to differentiation or self-renewal. Wang et al. inhibited Notch3 using shRNA and induced differentiation of rat bone marrow mesenchymal stem cells. They evaluated expression of osteogenic markers and determined that the decreased expression of Notch3 leads to an increase in osteogenic differentiation (52). It has even been suggested that the loss of Notch3 function may be a causal factor in the overgrowth of bone in patients with mandibular tori, a disease resulting in a benign bony growth located in the jaw that affects 27 out of every 1,000 people (53, 54).

Notch3 also plays a role in adipogenesis, or the generation of fat tissue. Sandel et al transfected human adipose-derived stem cells with Notch3 siRNA and successfully decreased *notch3* genetic expression. They then utilized qRT-PCR to both validate the knockdown of Notch3 and quantify changes in expression of adipogenic markers in adipose stem cells undergoing adipogenic differentiation. They noticed an increase in adipogenesis in cells where Notch3 was decreased, showing that Notch3 has a regulatory role in the process of fat production (55).

With many responsibilities in mesenchymal stem cells, Notch3 has been shown to regulate processes in other stem cell types as well. For example, Kawai et al. noticed an upregulation of *notch3* in quiescent, or not actively dividing, neural stem cells within the subependymal zone of the brain of adult mice. They noticed that knockdown of *notch3* in this zone led to an increase in division of neural stem cells, and complete deletion of the

gene led to a decrease in quiescent neural stem cells (56). This shows that Notch3 plays an active role in cell cycle regulation and proliferation of neural stem cells.

1.3.3 Role of Notch3 in Embryonic Development

Notch3 is necessary for several important processes within early embryonic development in all animals. Proper Notch3 function is crucial for neural and spinal cord development. Rusanescu and Mao generated Notch3 knockout mice and noticed a decrease in mature inhibitory neurons with a correlating increase in immature neurons within the spinal cord. Using behavior testing, they also noticed that the knockout mice had a lower threshold for pain, an indication of chronic pain being experienced by these mice (57). This shows that not only is Notch3 crucial for proper neural development, it may also be a causal factor in instances of chronic pain.

Notch3 is also required for proper development of the vasculature, specifically the smooth muscle cells that make up the circulatory system. The circulatory system of a developing fetus is one of the first aspects of the human body to develop, appearing as early as four weeks gestation. It is crucial that a developing fetus receives healthy and plentiful blood from the parent to aid in development. It was discovered that Notch2 and Notch3 function together in controlling the differentiation of vascular smooth muscle cells in mice during gestation. When both of these receptors were simultaneously knocked-out, mice did not survive to birth due to incomplete development of their arteries (58).

1.3.4 Role of Notch3 in Cancer

Finally, the Notch3 receptor within the Notch signaling pathway plays several roles in the development of various cancers, including breast cancer, cholangiocarcinoma, prostate cancer, and T-cell leukemia. For example, Notch3 underexpression has been linked to uncontrollable cell proliferation in human breast cancer cells. This same study reported that when Notch3 is overexpressed, *Cdh1*– a gene known for regulating entrance of a cell into the stationary phase– is also highly expressed, resulting in cells arresting in the G0 phase of the cell cycle and stopping the growth of a tumor (59). Another study noticed that downregulation of Notch3 resulted in decreased growth in Erb-B2-negative breast cancer cell lines (60). This line of cancer is known to be increasingly difficult to treat, as there are no known treatment targets. These results indicate that Notch3 plays a role in cellular proliferation and will likely affect stem cell self-renewal in a similar way.

Cholangiocarcinoma is an aggressive form of liver cancer, primarily affecting the bile duct. Currently there are no known medical treatments other than surgery to remove the cancerous sections of the duct, which can have drastic lifestyle-altering consequences, including digestion and nutrition problems, infection due to bile leakage, and liver failure. The role of Notch3in the progression of this cancer has been investigated, and it has been found that, in cancerous cells, Notch3 is overexpressed. This upregulation of Notch3 leads to an activation of the P13k-Akt pathway, which directly causes increased tumor cell survival (61). If Notch3 can affect cancerous cells in this way, it is likely that it can also affect the process of apoptosis in stem cells as well.

Prostate cancer is prevalent, especially among the older male population. An estimated 1 in 8 men will develop prostate cancer in their lifetime, while roughly 1 in 41 will die from prostate cancer. The Notch signaling pathway as a whole plays several roles within this form of cancer. Meunier et al. investigated Notch3's specific role within this cancer and found that, when exposed to hypoxic conditions, Notch3 was overexpressed and tumorigenesis occurred. Prostate cancer develops a very solid tumor, which leads to oxygen-depleted conditions. This hypoxic reaction of Notch3 could be a causal factor in the development of prostate cancer. These scientists also discovered that several other components of the Notch signaling pathway were impacted when various prostate cancer cell lines were placed in hypoxic conditions, including an overexpression of Notch1 and a downregulation in DLL-4 (62). This proves that factors within the Notch signaling pathway interact and shows that there can be some functional redundancy within the pathway. This also shows that the Notch signaling pathway is capable of interfering with cellular division and can elicit the same response in stem cells.

Notch3 has some implications in T-cell leukemia. It is known that Notch3 is a causal factor in the development of this disease, but a possible method for utilizing the relationship between Notch3 and T-cell leukemia progression as a means of treatment has recently been investigated. Palermo et al generated several groups of transgenic mice in which various lysine residues within the Notch3 protein were either hyperacetylated or deacetylated. They discovered that when Notch3 was hyperacetylated via histone deacetylase inhibitors, T-cell leukemia and lymphoma growth was halted in mouse models (63). This information can be used to manipulate the genetic expression of

Notch3 and provide a specific treatment target for this individual type of cancer and could even be utilized to control the development and differentiation of stem cells.

Notch3 is responsible for the regulation of many life-altering processes within the human body, including proper development and the accumulation of diseases like cancer. This involvement shows that Notch3 could very well act as a genetic target for the eradication of such ailments. Notch3 expression could potentially be manipulated as a means of direct treatment. This provides a drive to continue the investigation of this receptor.

1.4 Conclusion

Stem cells have vast potential in regenerative medicine both because of their ability to self-renew and to differentiate down specified lineages. Of the various types of stem cells, adipose-derived stem cells offer some of the greatest clinical potential as they are easily and ethically derived with the ability to be used autologously. Adipose stem cells have a unique potential to be applied to treat previously incurable diseases, such as Parkinson's Disease (21) and osteoporosis (64), as they are more prevalent and still hold many regenerative properties.

There is a complex set of factors that regulate stem cells and their fate. These include environmental stimuli, signal transduction pathways, transcription factors, and chromatin or histone modifiers. One such signaling pathway, the Notch signaling pathway, has been previously described as having direct roles in the regulation of stem cell proliferation, differentiation, and death (49). Notch3 is a singular receptor within this

complex regulatory pathway that has been linked to the regulation of stem cell differentiation, different aspects of early development, and various cancers (Figure 1-7).

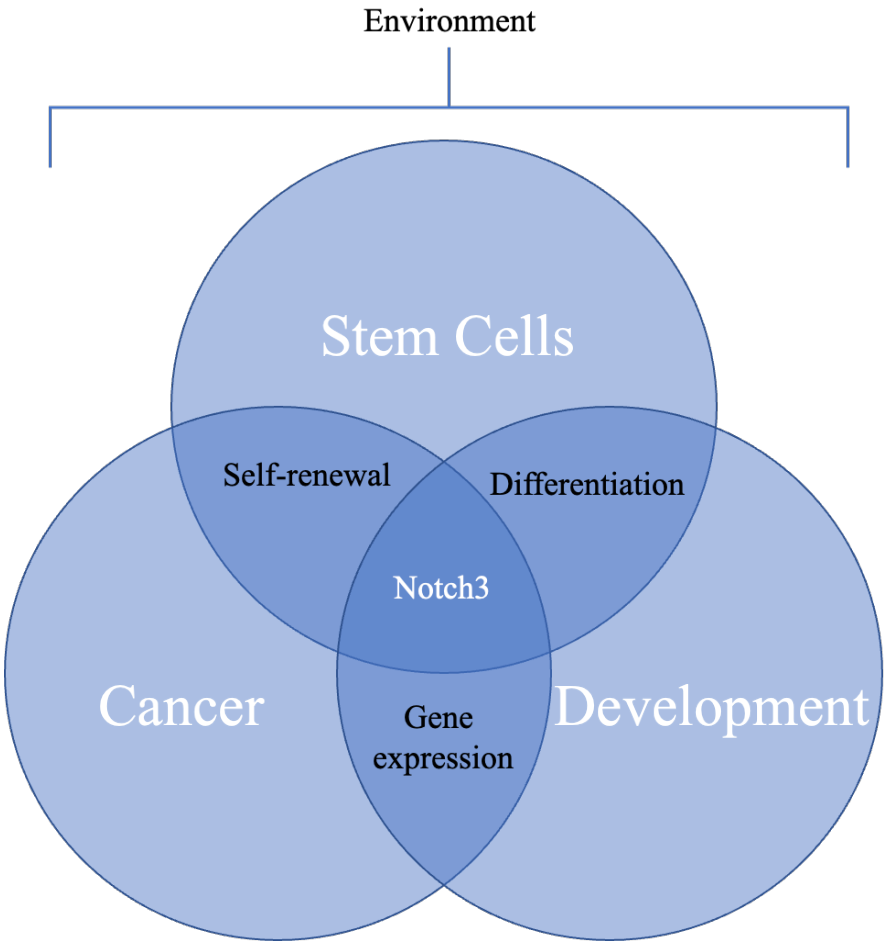

Figure 1-7: Notch3 Plays an Active Role in Many Aspects of the Human Body. Stem cells and cancer are linked by their ability to self-renew, having either positive or negative consequences, respectively. Stem cells and development are correlated by a need for differentiation as a means to continue progression down a predetermined lineage. Finally, genetic expression is essential for both cancer and development, interlinking the two. All three are regulated by Notch3.

Stem cells, development, and cancer are all regulated, in some way, by Notch3 (65). Notch3 is a component of the Notch signaling pathway, which is internally

controlled by a vast regulatory network, the same network that controls all aspects of cellular manipulation. This interconnectedness is what motivates my investigation into this specific receptor. Uncovering the role of Notch3 in stem cell self-renewal could help us to better understand the role of this pathway in cancer and development. This will ultimately lead to therapeutic targets for both the treatment of disease and stimulation of regeneration.

CHAPTER 2

METHODS

2.1 Cell Culture

2.1.1 Thawing cells

Human adipose stem cells (LaCell, LLC #100715) were thawed and transferred to 8mLs of warmed Complete Control Medium (CCM) (Table 2-1).

Table 2-1: Contents of Complete Culture Medium

Media Component	Amount	Vendor	Catalog Number
Minimum Essential Medium Alpha	203.75 mL	Life Technologies	#12561049
Fetal Bovine Serum	41.25 mL	Thermo Scientific	#10437028
Penicillin-Streptomycin	2.5 mL	Life Technologies	#15140122
L-Glutamine 200mM	2.5 mL	Gibco	#25030-081

Cells were then centrifuged at 1500 rpm for 10 minutes, creating a cell pellet. The supernatant (excess media) was removed from the vial, and the cells were resuspended in 1 mL of CCM. This milliliter of cell solution was then transferred to a 10cm plate containing 10mL of CCM. Media was replenished the following 24 hours, and then every 48 hours until the cells reached approximately 80% confluency. At this point, the cells were passaged.

2.1.2 Passaging cells

Once at 80% confluency, media was gently aspirated. Cells were rinsed with 5mL of PBS (Gibco, #10010023) and then allowed to incubate in 3 mL trypsin (Life Technologies, 25200-056) at 37 °C for approximately five minutes. Cells were observed under a digital inverted microscope at three minutes, and again at one-minute intervals until nearly all the cells were detached from the plate. 6 mL of CCM was added to the plate and this mixture of trypsin and CCM was washed over the plate several times to ensure all cells were collected within the liquid and was then transferred to a 15 mL conical vial and centrifuged at 1500 rpm for 10 minutes.

Once centrifugation was complete, the supernatant was removed from the conical vial, leaving only the cell pellet. This was then thoroughly resuspended in 1 mL of CCM. 20 µL of this concentrated cell solution was added to an Eppendorf tube containing 20 µL of trypan blue stain, and the contents were adequately mixed. 10 µL of this new solution was then added to each side of a hemocytometer which was then placed into a cell counter. Using the estimated number of cells from the cell counter, the concentrated cell solution was diluted with CCM until a concentration of 200,000 cells per mL was reached. Cells were plated on either 10-cm plate for transfection or continuous expansion for future experiments.

2.1.3 Transfection

Once cells reached approximately 50% confluency, cells were transfected with siRNA. To do this, we added Opti-MEM to four 15 mL conical vials, two of which were labelled “lipofectamine”, one labelled “NC”, and the final labelled “KD”. Lipofectamine was added to each vial labelled “lipofectamine”. Negative Control siRNA was added to

the NC vial and an equal amount of Notch3 siRNA was added to the KD vial (Table 2-2). The contents of the lipofectamine vials were then added to the NC and KD vials, which were adequately mixed and incubated at room temperature for 20 minutes.

Table 2-2: Reagents Used in Knockdown Protocol

Reagent	Amount	Vendor	Catalog Number
Opti-MEM	3,208 µL	Gibco	#31985-062
Lipofectamine RNAi Max Reagent	64.2 µL	Invitrogen	#56531
Silencer Select Notch3 siRNA	64.2 µL	Thermo Scientific	#4390843
Silencer Select Negative Control siRNA	64.2 µL	Thermo Scientific	#4392420

The NC solution was then added to two of the 10-cm plates, termed NC plates, at an amount of 1.87 mL per plate. An equal amount of the KD solution was added to the remaining two plates, termed KD plates. Media was replaced 24 hours later and then every 48 hours, until cells in the negative control plate reach 80% confluency. Cells from one NC plate and one KD plate were then passaged onto assay-dependent plates (Table 2-3). RNA was collected from the other two plates, one NC and one KD, to validate the knockdown of Notch3. The expansion plate was also maintained with fresh media every 48 hours until 80% confluency, at which point cells were passaged into 10-cm plates as described in section 3.2 to repeat the experiment.

Table 2-3: Assays, Plate Types, and Cell Seeding

Assay	Plate Type	Amount of Cell Solution	Cells Seeded	Media Volume
Knockdown	10 cm	500 µL	100,000	10 mL
RNA analysis	6 cm	225 µL	45,000	3 mL
Protein analysis	6 cm	225 µL	45,000	3 mL
alamarBlue assay	96-well	5 µL	1,000	200 µL/well
Phalloidin and DAPI staining	24-well	70 µL	4,000	500 µL/well
Colony Forming Unit assay	10 cm	0.5 µL	100	10 mL

2.2 RNA Analysis

2.2.1 RNA Collection

RNA analysis was set up in triplicate, so three 6-cm plates were plated from cells which received negative control siRNA and three from cells which received Notch3 siRNA. Two 10-cm plates were also set up for RNA analysis to verify the knockdown of Notch3. RNA was collected 72 hours after the passage of cells. Cells were cultured until approximately 80% confluency and were then washed with either 3 mL (6 cm) or 5 mL (10 cm) phosphate buffered saline and treated with 500 µL (6 cm) or 1 mL (10 cm) of Trizol reagent (Invitrogen, #15596018). Plates were then scraped with a cell scraper, and the contents of the plates were collected and transferred to 1.5 mL Eppendorf tubes. Samples were stored at -80ºC until extraction.

2.2.2 RNA Extraction

RNA samples were retrieved from -80 ºC storage and allowed to thaw to room temperature. Once fully thawed, chloroform was added to each sample at a volume of 0.2

mL per 1 mL of Trizol used in the collection. The samples were shaken vigorously for 15 seconds and then sat at room temperature for an additional three minutes. Cells were then spun in a pre-cooled centrifuge set at 4 ºC at 12,000 G for 15 minutes.

While samples were in the centrifuge, new Eppendorf tubes were labelled for each sample. Once complete, samples were very carefully removed from the centrifuge. The clear supernatant, which had risen to the top, was separated from the phenol and DNA waste and placed carefully into the new Eppendorf tubes. 1.25 µL of glycogen (Invitrogen, #AM9510) followed by 500 µL of 100% isopropyl alcohol was added to each sample. The samples were inverted a few times and incubated at room temperature for 10 minutes.

Cells were then centrifuged again at 4 ºC and 12,000 G for 10 minutes. Once complete, the liquid supernatant was removed from the tube, leaving only the remaining pellet of RNA. This was resuspended in 1 mL of 75% ethanol with a vortex, and then centrifuged again at 7,500 G for 5 minutes. The supernatant was removed again, and the pellet was allowed to dry at room temperature for approximately 5 minutes, allowing the ethanol to evaporate. Finally, the pellet was resuspended in 30 µL of nuclease-free water.

2.2.3 RNA Quantification

RNA was quantified and analyzed for purity using a BioTEK Cytation 5 plate reader. RNA samples were maintained in a bucket of ice for the entirety of the experiment. To begin, the plate wells were blanked with nuclease-free water. Once blanked, 2 µL of each RNA sample was placed onto the plate in duplicate. The plate was then inserted into the reader and absorbance was measured at 260 nm for RNA and 280

nm for DNA. Results were saved to be analyzed later, and RNA was stored in -80 ºC freezer until it was used to make cDNA.

2.2.4 cDNA Synthesis

Results from RNA spectroscopy were averaged and amounts of RNA and nuclease-free water were calculated to reach a uniformed RNA concentration of one ng/µL. These calculated amounts were carefully pipetted into PCR tubes, and 4 µL of qScript cDNA SuperMix (VWR, #95048-100) was also added to the tubes. The PCR tubes were then centrifuged and placed into a Bio-Rad Thermo Cycler. Samples were maintained at 20 ºC for five minutes, then heated to 42 ºC for 30 minutes, then 85 ºC for five minutes. cDNA was stored at -20 ºC until use.

2.2.5 Real-Time PCR

To quantify changes in transcription of *ki67*, quantitative reverse-transcriptase PCR was performed. cDNA from previous step was removed from -20 ºC freezer and allowed to thaw. Mixes comprised of PowerUp SYBR Green Master Mix (Applied Biosystems, #A25742), nuclease-free water, and gene specific forward and reverse primers (Table 2-4) were created for each gene.

Table 2-4: qRT-PCR Primer List

Gene	Forward Sequence	Reverse Sequence	Product Length (bp)
notch3	CACCCTTACCTGACCCCATCC	TTCGGACCAGTCTGAGAGGGA	81
gapdh	AGGGCTGCTTTTAACTCTGGT	CCCCACTTGATTTTGGAGGGA	216
ki67	CGTCCCAGTGGAAGAGTTGT	CGACCCCGCTCCTTTTGATA	143

A 96-well reaction plate was carefully removed from packaging, avoiding touching the bottom to any surfaces or to gloves. 9 µL of gene-specific master mix was pipetted into each well, followed by 1 µL of cDNA. Samples were plated in triplicates for each gene. Plate was covered and placed into high-speed centrifuge for 20 seconds to remove bubbles and then ran on an Applied Biosystems StepOne Plus machine. Results were normalized to *gapdh*, a standard housekeeping gene. Statistical analysis was performed to obtain significance of results.

2.3 Protein Analysis

2.3.1 Protein Collection and Extraction

Cells were cultured for 72 hours following passaging, then protein was extracted. To do this, cells were washed with 5 mL pre-cooled PBS and then placed onto an ice pack to remain cold. Then, 400 µL of pre-made and cool lysis buffer containing 2,250 µL of DI water, 250 µL Pierce RIPA buffer (Thermo Scientific, #VC297145), and 25 µL Halt protease and phosphatase inhibitor (Thermo Scientific, #UF278477). The plates were then sufficiently scraped with a cell scraper, and the contents of the plate were placed into pre-labelled Eppendorf tubes. These tubes were then placed into a cell rotator, which was placed inside of a refrigerator to remain cool. The tubes were agitated for 30 minutes before being transferred to a centrifuge pre-cooled to 4 ºC, and centrifuged at 12,000 rpm for 20 minutes. Then, the tubes were gently removed, and the supernatant was transferred to a new and labelled Eppendorf tube. The pellet and original tubes were disposed of. Protein samples were stored at -80 ºC until Western blot was performed.

2.3.2 Bradford Assay

To obtain protein concentrations, 250 µL of BioRad Protein Assay Dye Reagent Concentrate (BioRad, #5000006) was added to 12 Eppendorf tubes. Bovine Serum Albumin was utilized as a protein standard and added to six Eppendorf tubes. Protein obtained from extraction was added to the following six tubes in triplicate. Solutions were vortexed and 100 µL of each was transferred to a 96 well plate, each in duplicate. Absorbance was measured at 595 nm using a Biotek Cytation 5 plate reader.

2.3.3 Western Blot

To validate the decrease in Notch3 transcript observed via qRT-PCR correlated to a decrease in the Notch3 receptor itself, 20 µL of 2x Laemmli sample buffer (BioRad, #1610737) was pipetted into six Eppendorf tubes. Amounts of protein and water were added to the Eppendorf tubes, with amounts obtained based on protein concentration determined from Bradford assay. All samples were brought to an equal concentration of protein. Tubes were then placed into a heating block and allowed to boil at 95-100 ºC for 10 minutes. Samples were then cooled on ice for one minute, vortexed, then loaded into a 7.5% polyacrylamide gel (BioRad, #4561024). Samples were loaded at a volume of 35 µL and ladder (BioRad, #1610376) was loaded on each side at a volume of 7 µL. Gel box was placed in refrigerator and was run at 120 V for 105 minutes.

After the gel was run, it was transferred onto a PVDF transfer membrane utilizing the BioRad Trans-Blot Turbo transfer system in buffer composed of methanol and Tris-Glycine buffer for 60 minutes, also in a refrigerator. The membrane was then cut at approximately 60 kD to separate GAPDH and Notch. Membrane fragments were then placed into 15 mL conical vials filled with 7 mL of 5% nonfat dry milk in TBST

(blocking buffer). Vials were placed in a cell rotator located within a refrigerator and allowed to rotate for 2 hours. Membranes were then transferred to fresh 15 mL conical vials containing 7 mL of fresh 5% nonfat dry milk blocking buffer as well as protein-specific primary antibody (Table 2-5). Membranes were probed with this antibody overnight.

The next day, membranes were washed with TBST in 5 five-minute increments. Then, they were placed into fresh 15 mL conical vials filled with 7 mL blocking buffer and 5 µL secondary antibody. These vials were placed in a cell rotator and rotated for one hour at room temperature. The washing process was then repeated. Finally, Clarity western ECL substrate (BioRad, #1705060) was added to each membrane, and they were imaged in a dark room. ImageJ was used for analysis.

Table 2-5: Antibody List

Antibody	Dilution	Manufacturer	Catalog Number
Notch3 (primary)	1:1000	Cell signaling	#2889S
GAPDH (primary)	1:3000	abcam	#ab9485
Goat pAb to Rb IgG (HRP) (Secondary)	1:10000	abcam	#ab6721

2.4 alamarBlue

10 µL of Cell-Quant Alamar Blue cell viability reagent (Gene Copoeia, #A2K1516) was added to each well 24 hours after passaging into 96 well plate. Experiment was run in triplicate, with wells containing no cells used as a blank for the machine. Approximately 100 µL of the cell-media-dye solution was collected from each well and pipetted into a clear bottom, non-treated 96 well plate at one, three, six and 24 hours following the addition of the dye. Absorbance values were measured using a

BioTek Cytation 5 plate reader immediately following collected from the treated plate, and were also measured at both 570 and 600 nm to ensure wavelength did not affect results. Absorbance values for each well were normalized to absorbance values of the blank wells and graphed over time to portray relationship. Statistical analysis was performed to quantify significance.

2.5 Colony-Forming Unit Assay

During passaging following knockdown, 100 cells were plated for both the NC and KD group. Media was changed 24 hours following the passage, and then every 48 hours for 14 days. After two weeks, plates were washed with 5 mL PBS, and then 5 mL of 3% crystal violet dye dissolved in methanol was added to the plate. Plates were incubated at 37 ºC for 20 minutes, and then washed several times with DI water. When plate was relatively clear of dye, it was imaged using a digital inverted microscope to confirm colony formation, and then colonies greater than 1 mm were counted. Average number of colonies for both the NC and KD group over three passages were compared.

2.6 Phalloidin and DAPI staining

Cells were passaged into 24 well plates in triplicate, with three NC and three KD wells. Media was changed 24 hours following passaging. At 72 hours after passaging, all wells were washed with 500 µL PBS and then fixed with 4% formalin for 10 minutes. Cells were then washed three times with PBS and incubated at room temperature in 250 µL 0.2% Triton X-100 for 10 minutes. Cells were washed with PBS thrice more and 250 µL of Alexa Fluor 555 phalloidin stain (Invitrogen, #2089927) was added to each well. The plate was covered in foil to protect it from the light, and then incubated at room temperature for 15 minutes. Next, 250µL of DAPI stain (Thermo Scientific

#UG2803292) was added to each well. The plate was left to incubate for five minutes more before being washed with PBS three more times. After the final wash, 500 µL of PBS was left in each well, and the wells were imaged with a digital inverted microscope in a dark room.

CHAPTER 3

RESULTS

3.1 Introduction

Stem cells hold promise as a revolutionary tool for the treatment of degenerative diseases that are currently incurable. A stem cell is an undifferentiated cell that originates from a single lineage and can self-renew and differentiate into any number of specialized cells (2). Stem cells are identified based on differentiation potential as either pluripotent, multipotent, or totipotent. Multipotent stem cells, which can be found in a variety of adult tissues, can become several different types of cells within a defined lineage. Human adipose stem cells (hASCs) are an example of a multipotent mesenchymal stem cell, meaning they can transform into several different cell lineages, including adipocytes, osteocytes, and chondrocytes (29).

Due to the abundance and physical location of adipose tissue in the human body, hASCs are easily harvested, making them ideal for the study of development and cell fate determination. Additionally, research has shown that hASCs may be effective in regenerative medicine and cancer therapy due to their high differentiation potential and ability to self-renew (66).

There are many factors that regulate the fate of adipose stem cells. These include environmental stimuli, signal transduction pathways, transcription factors, and chromatin modifiers. All of these factors interact with each other, creating a vast network of

components which all cooperate to regulate stem cell differentiation, self-renewal, and programmed cell death. The Notch signaling pathway is a conserved signal transduction pathway that is known to regulate cell proliferation, differentiation, fate, and death across species. The Notch pathway consists of four cell surface receptors (Notch 1-4) and five ligands, termed Jagged-1 and Jagged-2 as well as DLL-1, DLL-3, and DLL-4 (49).

Notch3, a transmembrane cell surface receptor, has been widely researched and implicated in the regulation of proliferation, differentiation, and apoptosis of stem cells (49). Notch3 is also known to have a role in early embryonic development, cell fate determination, and various forms of cancer. Examining the mechanisms behind this receptor to determine what role, if any, Notch 3 plays in a stem cell's ability to self-renew, proliferate, and differentiate could potentially lead to more direct approaches for the creation of stem cell-based therapies for incurable diseases.

In order to evaluate Notch3's role in adipose stem cells, I evaluated the influence of a *notch3* siRNA-mediated knockdown on cellular proliferation, metabolism, self-renewal, and morphology. Efficient knockdown of Notch3 was validated using qRT-PCR and Western blots. Cellular proliferation was assessed by quantifying *ki67* expression and self-renewal by a colony forming unit assays. Finally, changes in cellular metabolic activity were assessed using an alamarBlue assay and changes in cellular morphology monitored by staining actin filaments with Phalloidin stain. All these assays, in summation, allow for the visualization, quantification, and overall understanding of changes in cell state of adipose stem cells, following the decrease of Notch3. To date we have learned that Notch3 plays a vital role in cellular proliferation and self-renewal, while not having an effect on cellular metabolism and morphology.

3.2 *notch3* Transcript Levels Decrease Following siRNA-mediated Knockdown

In order to investigate Notch3's role in adipose stem cell fate, cells first had to be subjected to a decrease in expression of the Notch3 receptor. To do this, cells were transfected with either negative control (NC) or Notch3 (KD) siRNA and incubated for 72 hours. Cells were imaged after 72 hours to document any changes in density and morphology following the knockdown of Notch3 (Figure 3-1). There is a noticeable difference in cell density, with a lower density in the KD group (Figure 3-1B) when compared to the NC (Figure 3-1A). This suggests a decrease of proliferation and self-renewal in the cells transfected with *notch3* siRNA. This qualitative data drove our desire to further investigate this loss in proliferation via RNA and protein analysis.

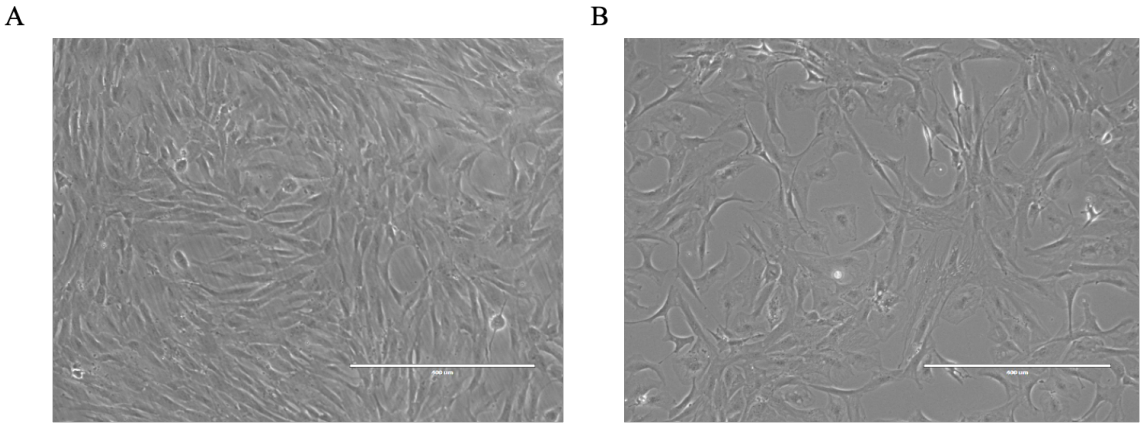

Figure 3-1: *Notch3* Knockdown Results in Decrease in Cell Density. Images taken from cells transfected with scrambled NC siRNA (A) and KD siRNA (B). Images were taken at 10x magnification and from the direct center of the plate to prevent any researcher bias.

In order to confirm that the differences in density were in fact due to a decrease in Notch3 expression, we validated the knockdown using qRT-PCR and western blot. Knockdowns were confirmed for three different passages (p4, p5, p6), with each reaction run in triplicate in order to ensure reliable and reproducible results. *Notch3* transcript was decreased to roughly 15% of its original transcript at p4 (Figure 3-2A), roughly 21% at p5 (Figure 3-2B), and 22% at p6 (Figure 3-2C). Data obtained from all three passages were then averaged, with *notch3* transcript decreased to roughly 19% of its original transcript (Figure 3-3). Results were normalized to *gapdh,* a housekeeping gene unaffected by the knockdown of Notch3.

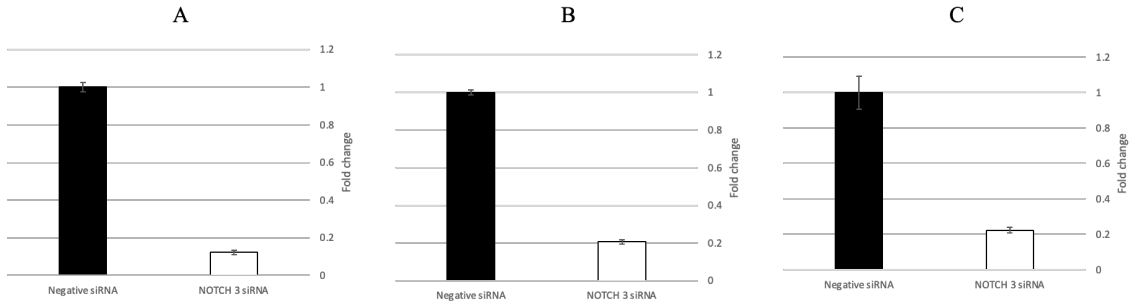

Figure 3-2: *Notch3* Transcript Adequately Decreased Following Knockdown in hASCs. qRT-PCR results of hASCs following the knockdown of *notch3* at passage 4 (A), passage 5 (B), and passage 6 (C). Transcript was significantly decreased over all three passages, normalized to *gapdh* and with scrambled siRNA used as a negative control.

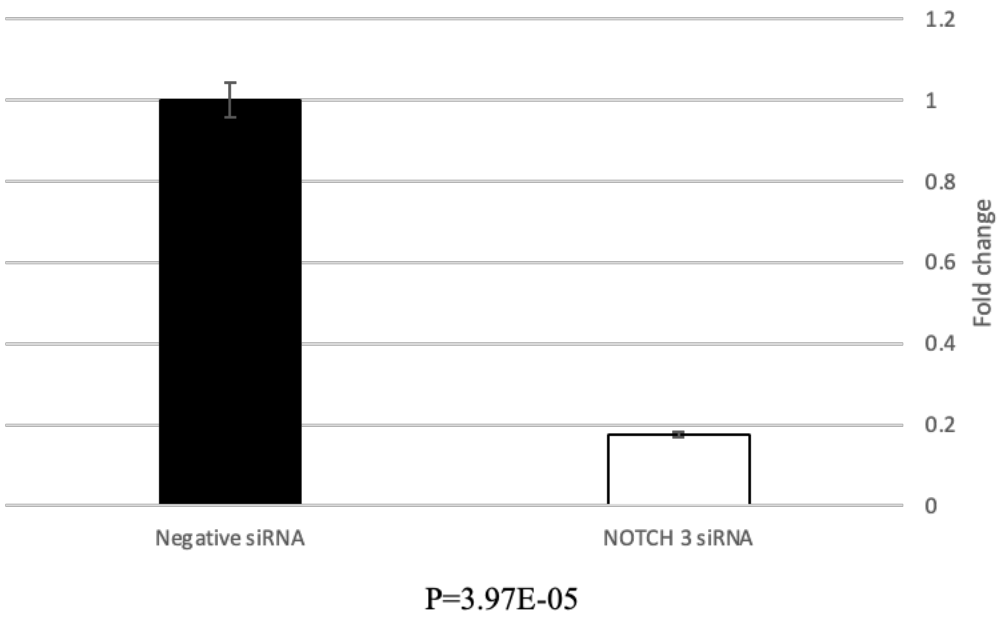

Figure 3-3: *Notch3* Expression Averaged over Three Passages. qRT-PCR results of hASCs following the knockdown of *notch3* over three passages, averaged together. Transcript was significantly decreased over all three passages, normalized to *gapdh* and with scrambled siRNA used as a negative control.

In order to validate that the decrease in *notch3* transcript resulted in a decrease in Notch3 protein, we performed Western blot analysis over three passages. Our first attempt (Figure 3-4) showed consistent and intense signal for GAPDH, which was utilized as a loading control. Unfortunately the first attempt did not indicate successful blotting for Notch3. The detection of GAPDH indicated that we did perform the protocol correctly, but a change had to be made in order to detect Notch3. Notch3 is significantly larger than GAPDH, at approximately 300 kD while GAPDH shows at 37 kD. To account for Notch3's larger size, we decided to run the gel for two hours rather than 90 minutes, providing more time for migration and increased resolution of larger proteins. In addition, we used a 7.5% polyacrylamide gel rather than a 4-10% to further increase the opportunity to resolve Notch3. These protocol modifications did in fact help us to detect

Notch 3 where we are able to see a decrease in protein expression for the Notch3 KD samples compared to the NC (Figure 3-5). Unfortunately, GAPDH did not work as well in this attempt and so additional optimization will continue for these experiments in the future so that we can quantify the differences in signal density for validation of knockdown.

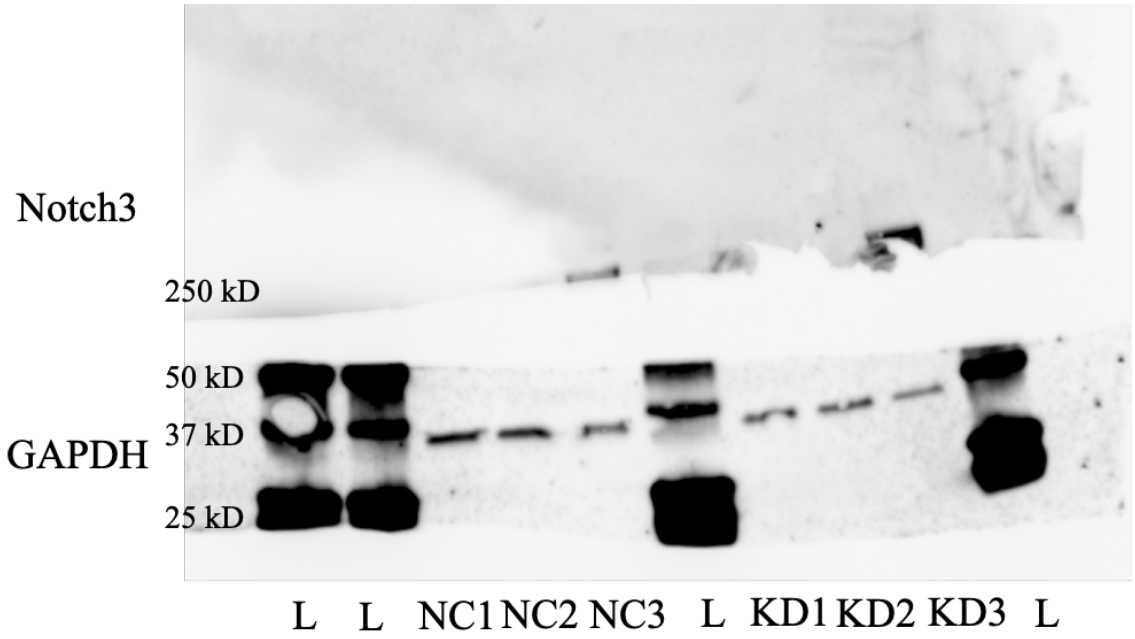

Figure 3-4: GAPDH Bands Appear Brightly in First Western Blot. Western blot analysis which shows consistent band appearance for GAPDH. Notch3 did not have apparent bands. Samples are NC1-3 and KD1-3 from protein collection of first passage. Bands are estimated to be at about 37 kD for GAPDH, and should appear around 300 kD for Notch3.

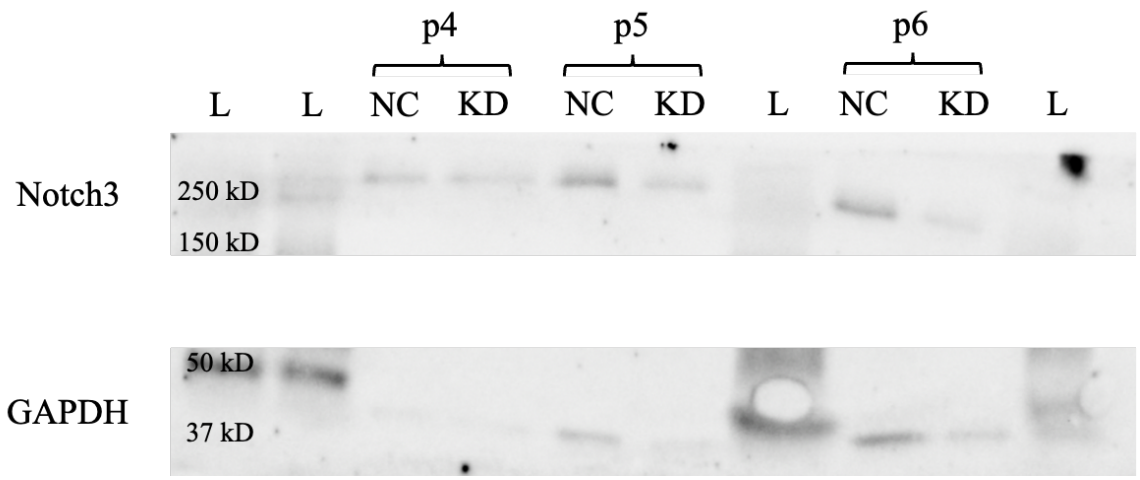

Figure 3-5: Notch3 Knockdown Resulted in Decrease in Protein Expression. Western blot analysis confirming knockdown of Notch3 over three passages (p4-p6). GAPDH was utilized as a loading control, although bands are not consistent or bright. Bands are estimated to appear between 250-300 kD for Notch3 and 37 kD for GAPDH.

3.3 Notch3 Knockdown can be Sustained Through Passaging

Transfection with siRNA at low cell density can be lethal to cells, so in order to perform some of our assays, we had to perform a knockdown under standard conditions and then passage and seed cells at lower densities (Figure 3-6). Since we are using siRNA, a transient knockdown system, we needed to validate that the knockdown persisted through passaging before performing those assays. To do this, we monitored *notch3* transcript levels over time using qRT-PCR. These validation experiments were again performed in triplicate over three passages (p4, p5, and p6). *Notch3* transcript was reduced to approximately 71% of its original transcript at p4 (Figure 3-7A), 46% at p5 (Figure 3-7B), and 61% at p6 (Figure 3-7C). These results were averaged to obtain an average decrease in *notch3* transcript of about 60% over three passages (Figure 3-8).

Western blots will be performed in the future to confirm that there was indeed a loss of protein in these samples and provide further validity to the results.

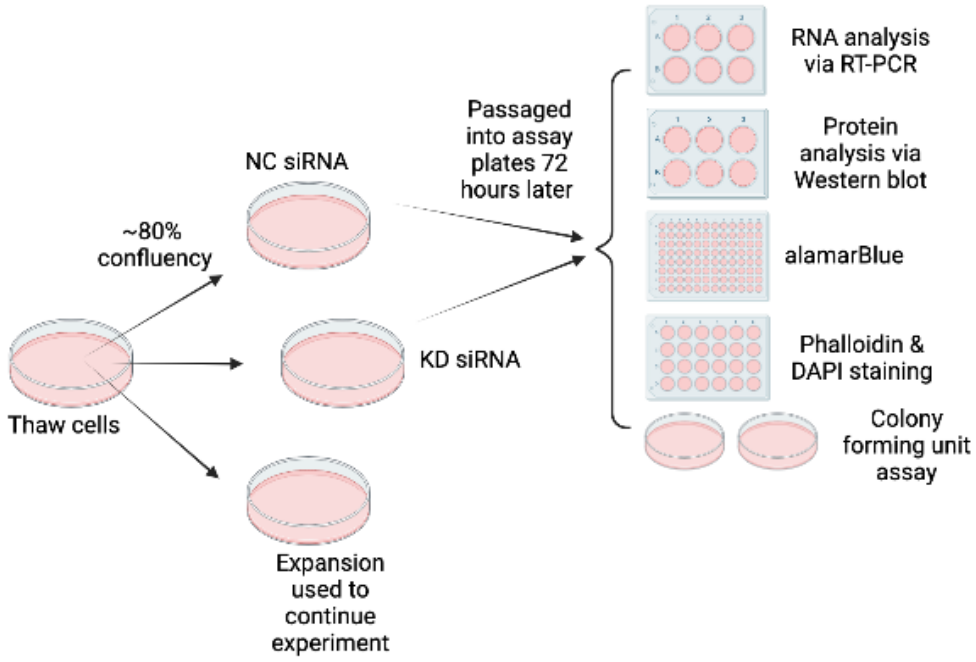

Figure 3-6: Knockdown Completed on 10cm Plate and then Passaged into Assay-Dependent Plates. Cellular characterization assays are performed in plates with wells too small to properly perform knockdown on that plate, so knockdowns were performed on 10 cm plates and then cells were passaged into assay-dependent plates 72 hours after siRNA transfection. qRT-PCR was utilized to validate that the knockdown of Notch3 was sustained throughout the passaging process.

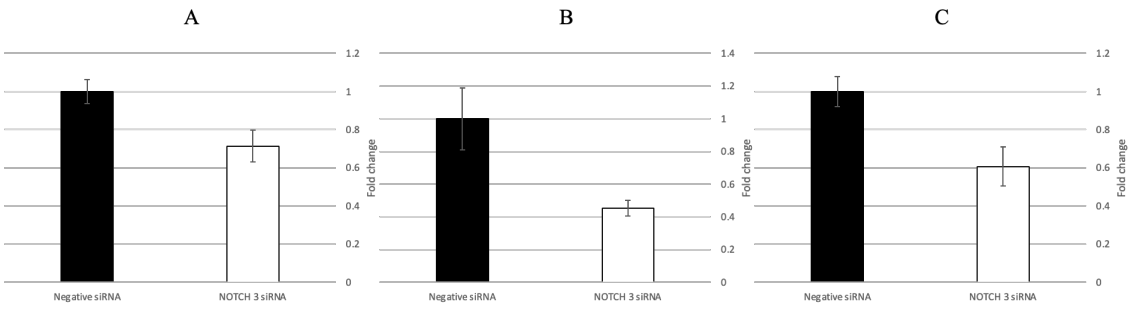

Figure 3-7: *Notch3* Knockdown can Persist Through Passaging Process. qRT-PCR results of hASCs following the knockdown of *notch3* and the passaging process at passage 4 (A), passage 5 (B), and passage 6 (C). *Notch3* transcript was significantly decreased over each passage, though not to the extent of cells immediately following knockdown. Results were normalized to *gapdh* and scrambled siRNA was used as a negative control.

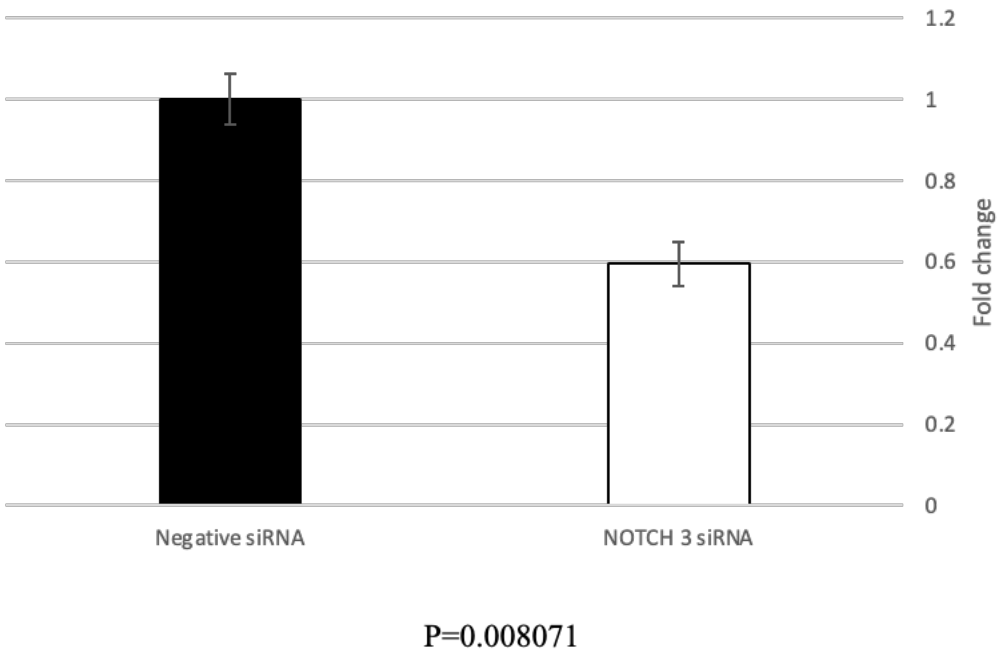

Figure 3-8: *Notch3* Expression Following Knockdown and Passage Averaged over Three Passages. qRT-PCR results of hASCs following the knockdown of *notch3* and the passaging process over three passages, averaged together. Transcript was significantly decreased over all three passages, normalized to *gapdh* and with scrambled siRNA used as a negative control.

3.4 Notch3 Knockdown Leads to a Decrease in *ki67* Expression in hASCs

As initial observations suggested a decrease in self-renewal (Figure 3-1), we went on to more closely evaluate the influence of this knockdown on cellular proliferation by monitoring changes in expression of *ki67*, a gene expressed during DNA replication and

typically used to assess tumor growth, and so a marker for cell division and proliferation (67). This experiment was performed in triplicate over 3 passages (p4, p5, p6), and was performed on samples collected from the initial knockdown. As seen in Figure 3-9, *ki67* expression was decreased by nearly 70% compared to the negative control in passage 4 (Figure 3-9A), 80% at passage 5 (Figure 3-9B), and 65% at passage 6 (Figure 3-9C). Results for all three passages were averaged, and expression of *ki67* was decreased to about 28% of its original transcript (Figure 3-10). These results confirm that Notch3 plays a vital role in cellular proliferation.

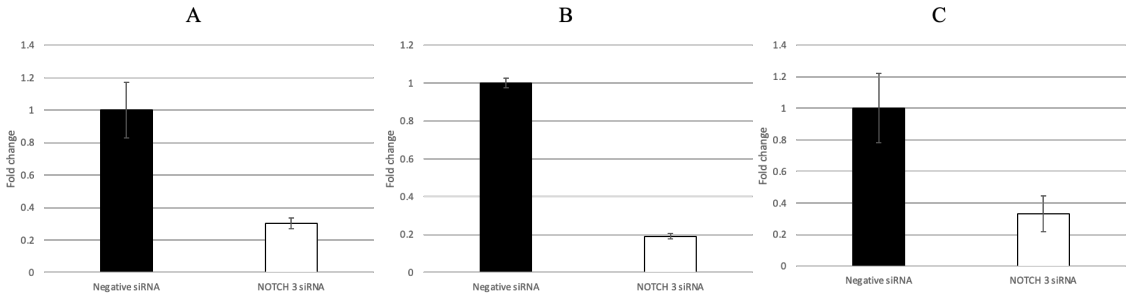

Figure 3-9: *Notch3* Knockdown Results in a Significant Decrease in *ki67* Expression. qRT-PCR results of hASCs following the knockdown of *notch3* over various passages. Expression of *ki67* was significantly decreased. Scrambled siRNA was used as a negative control, the experiment was conducted in triplicate, and results were normalized to *gapdh.* These samples correspond to knockdown validation data presented in Figure 3-2.

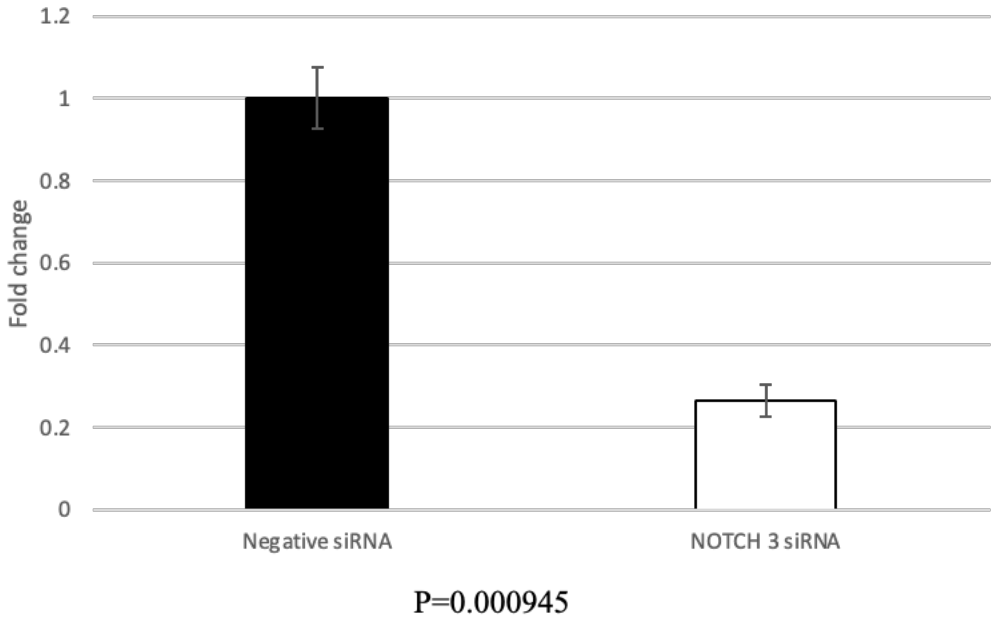

Figure 3-10: *Ki67* Expression Averaged over Three Passages. qRT-PCR results of hASCs following the knockdown of *notch3* averaged over three passages. Expression of *ki67* was significantly decreased. Scrambled siRNA was used as a negative control, the experiment was conducted in triplicate, and results were normalized to *gapdh*. These samples correspond to knockdown validation data presented in Figure 3-2.

3.5 Notch3 Knockdown Does Not influence Cellular Metabolism

In order to determine whether Notch3 has a role in metabolism, we performed alamarBlue analysis. We measured the change in absorbance values over time at both 570nm and 600nm for cells transfected with *notch3* siRNA and those treated with negative control siRNA. At both wavelengths, it was observed that there were no significant differences between NC and KD groups, and it was determined that Notch3 does not affect stem cell metabolic activity. This experiment was performed in triplicate and was replicated three times at passages 4 (Figure 3-11), 5 (Figure 3-12), and 6 (Figure 3-13) to validate accuracy. All data was averaged over three passages to obtain significance (Figure 3-14).

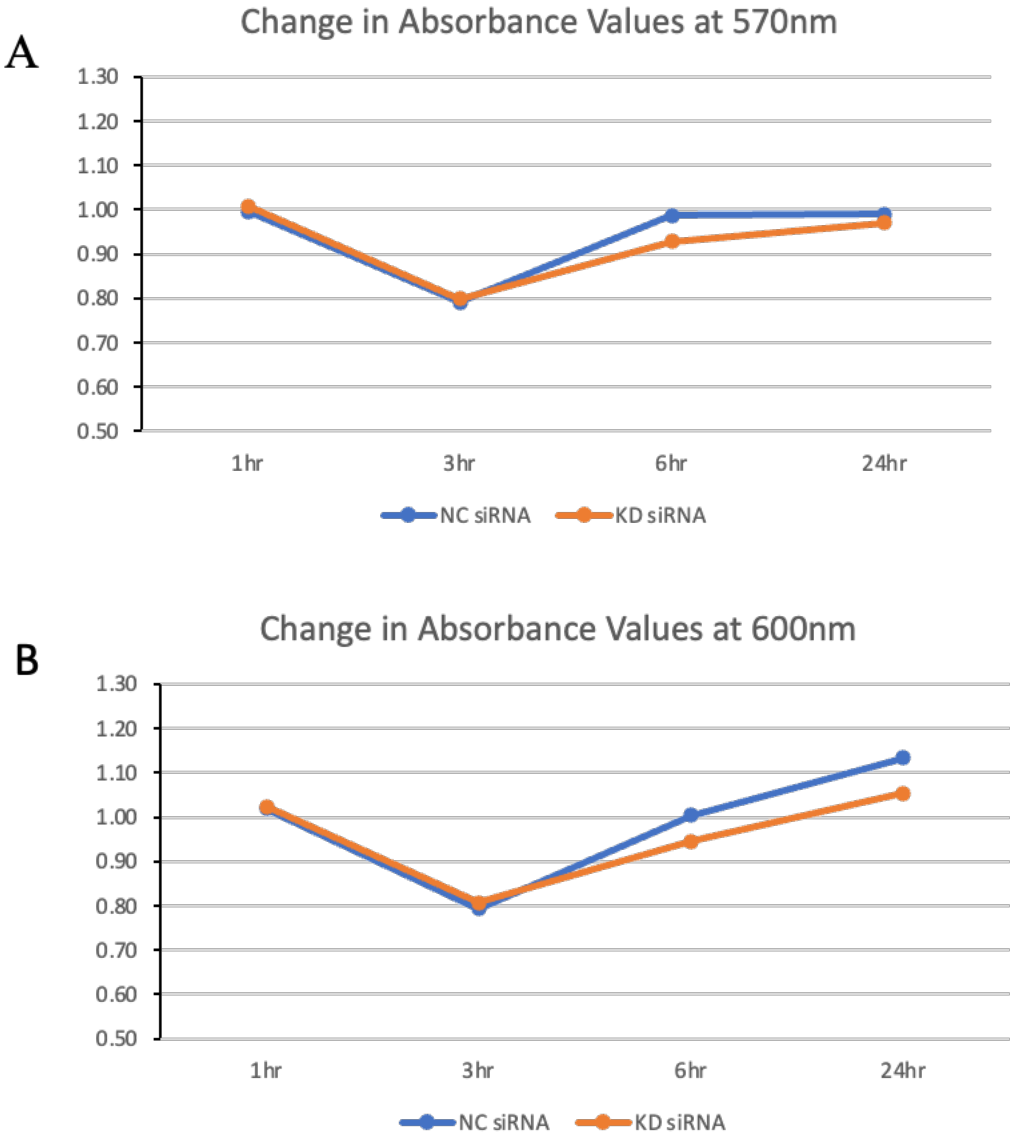

Figure 3-11: *Notch3* Knockdown has no Effect on hASC Cellular Metabolism at Passage 4. alamarBlue results of hASCs following knockdown of Notch3. Absorbance values were measured at one, three, six, and 24 hours following the addition of dye. Absorbance values were recorded for plated media without the addition of cells as a control, and results were normalized to this data. Absorbance values were recorded at both 570nm (Panel A) and 600nm (Panel B) to ensure wavelength did not have an effect on results.

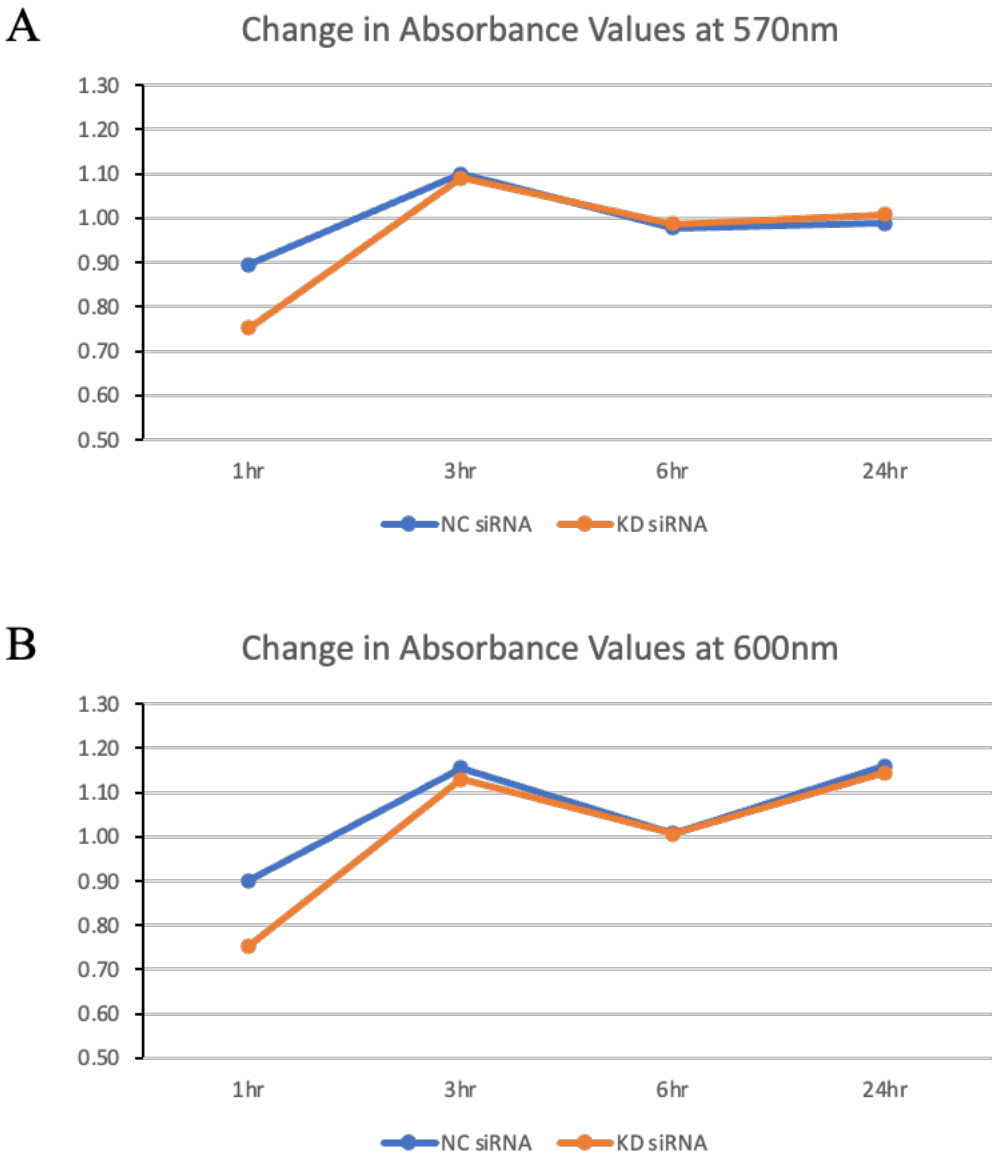

Figure 3-12: *Notch3* Knockdown has no Effect on hASC Cellular Metabolism at Passage 5. Second replicate of alamarBlue study at passage 5, showing similar results to passage 4. Absorbance values were measured at same time points, still utilizing plated media as a control. Values were again measured at two different wavelengths to confirm wavelength does not affect results.

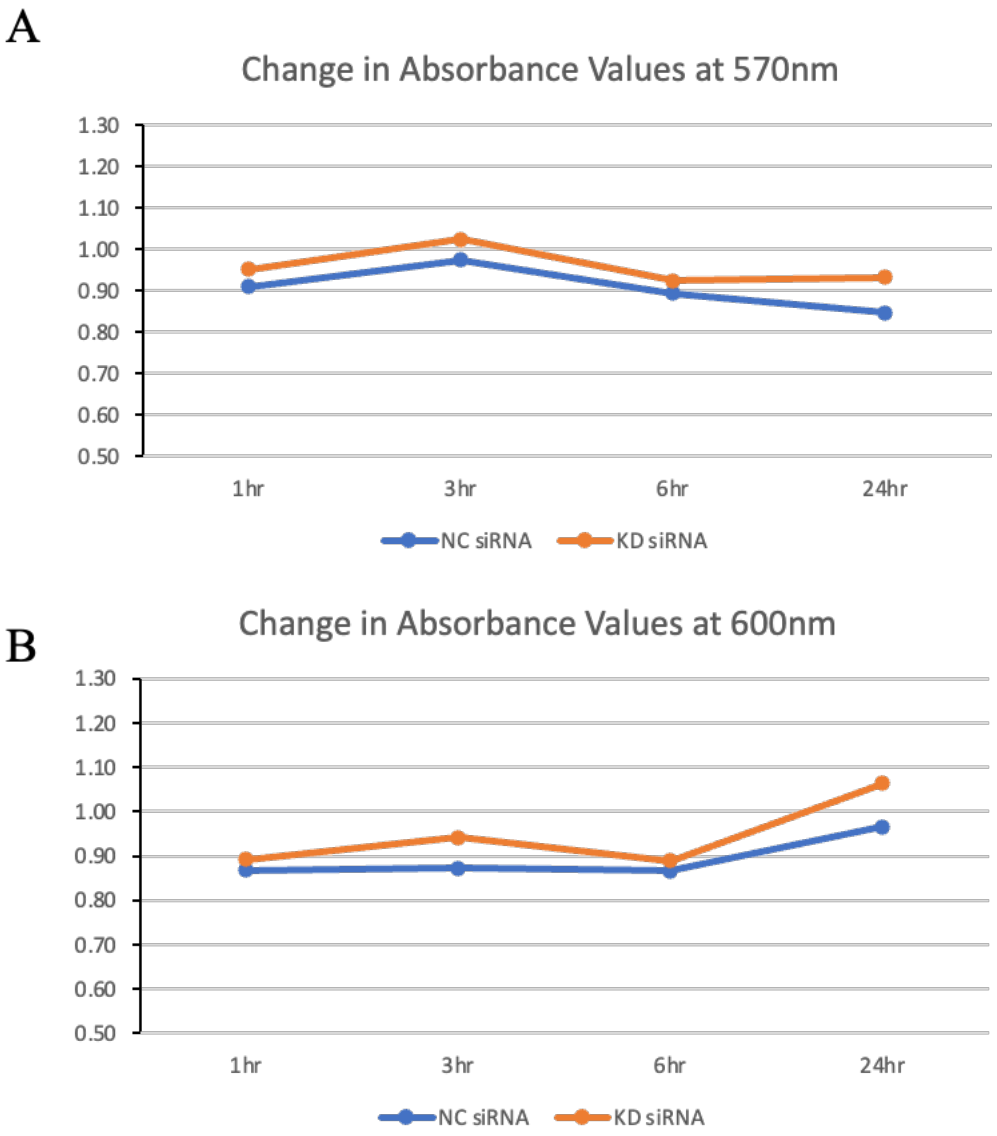

Figure 3-13: N*otch3* Knockdown has no Effect on hASC Cellular Metabolism at Passage 6. Third replicate of alamarBlue study at passage 6, showing similar results to passage 4 and 5. Absorbance values were measured at same time points, still utilizing plated media as a control. Values were again measured at two different wavelengths to confirm wavelength does not affect results.

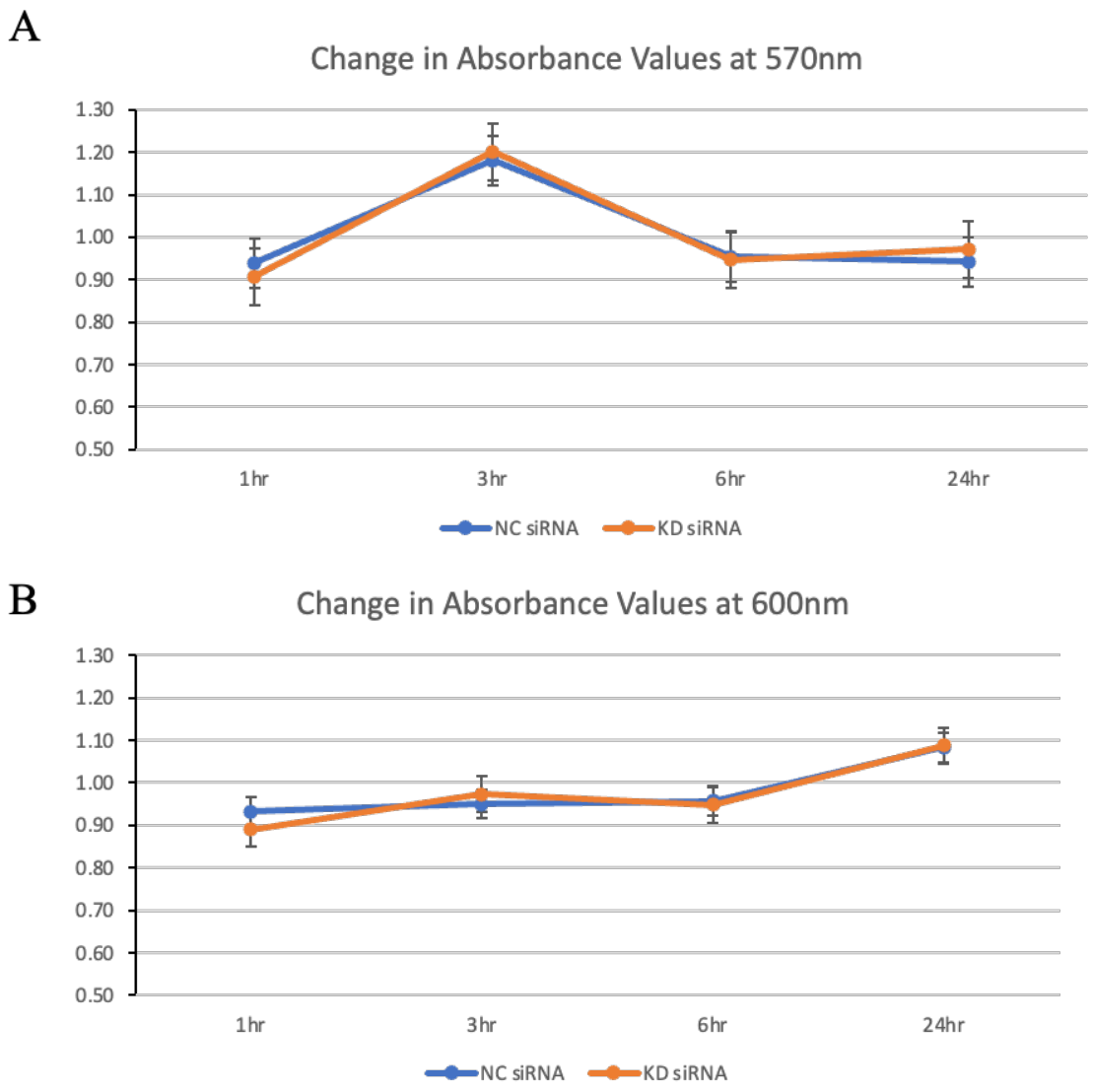

Figure 3-14: Averaged Values for alamarBlue with Statistics. Data taken from alamarBlue results over three passages were averaged, and statistical analysis was done. An unpaired t-test was performed to obtain p-values. Results were not significantly different at 570 nm (P=0.9756) or 600 nm (P=0.9188).

3.6 Notch3 Knockdown Results in Decreased Number of Individual Cell Colonies

To further investigate Notch3's role in cellular self-renewal, 100 cells were plated for both the NC and KD group, and then incubated for two weeks. Cells were then stained with crystal violet, and colony formation was confirmed using a digital inverted microscope. For two passages (p4 and p5), colonies were seen clearly in the NC group (Figure 3-15) but did not appear in the KD group. Colonies greater than 1mm were then counted, which passage 4 having approximately 72 individual colonies (Figure 3-16) and passage 5 having approximately 63 individual colonies (Figure 3-17). Experiment should be performed again at passage 6 to validate reproducibility.

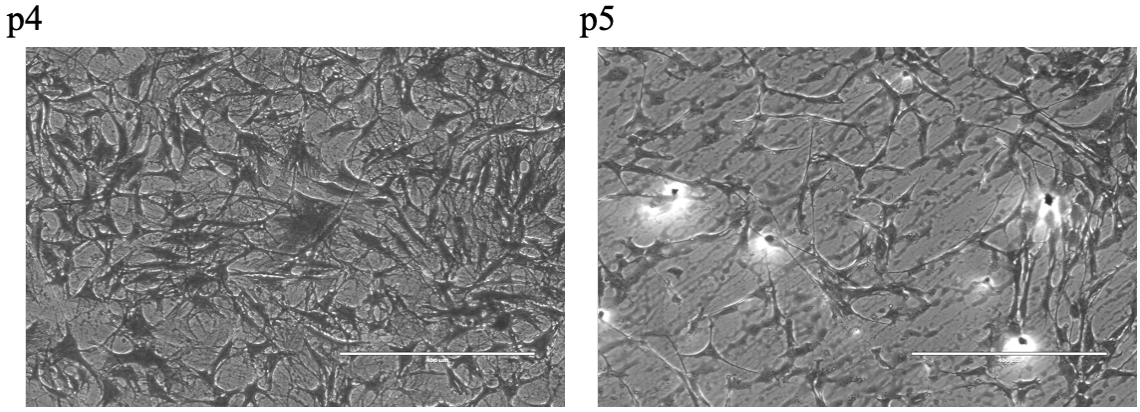

Figure 3-15: Individual Colony Formation Confirmed using Digital Inverted Microscope. To ensure that the crystal violet dye was staining cells rather than anything else, cells were imaged using a digital inverted microscope. The darker color of each individual cell confirms that the staining process was successful. The colonies seen by the naked eye can then be assumed to be true cell colonies.

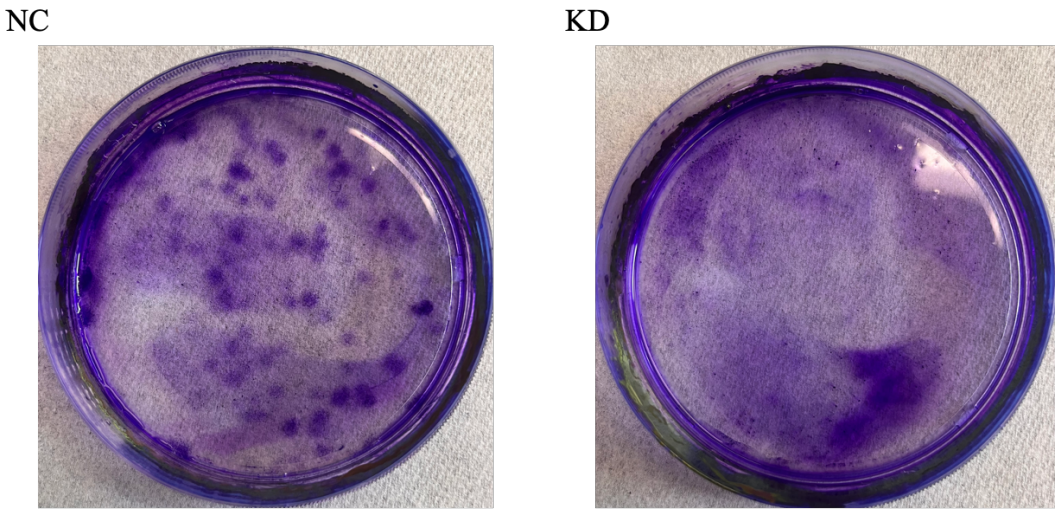

Figure 3-16: More Self-renewing Colonies were Observed in NC versus KD Group at Passage 4. Following several washes with PBS, no individual cell colonies could be visualized in the KD group. However, approximately 72 self-renewing colonies greater than 1 mm were counted in the NC group, indicating these cells are actively proliferating.

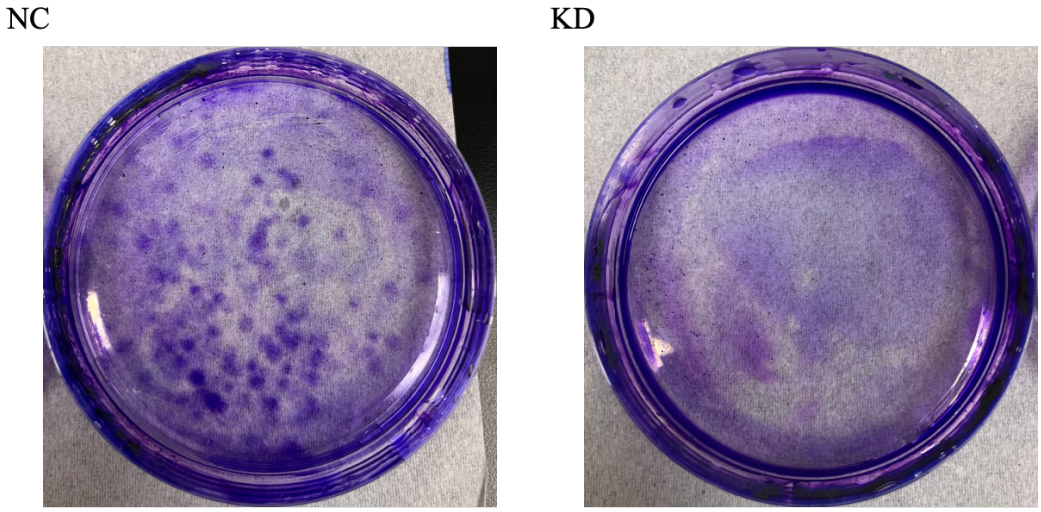

Figure 3-17: More Self-renewing Colonies were Observed in NC versus KD Group at Passage 5. Following several washes with PBS, no individual cell colonies could be visualized in the KD group. However, approximately 63 self-renewing colonies greater than 1 mm were counted in the NC group, indicating these cells are actively proliferating.

3.7 Notch3 Knockdown Decreased Cell Density but does not Significantly Impact Cytoskeleton Morphology

To more closely examine the influence of decreased Notch3 expression on cellular morphology, Phalloidin and DAPI stain was utilized. Phalloidin stains actin filaments while DAPI stains DNA. Stains were performed in triplicate at passage 4 (Figure 3-18), passage 5 (Figure 3-19), and passage 6 (Figure 3-20). Qualitative analysis showed that while there was a decrease in cell density, there are no distinct changes in the cytoskeleton of adipose stem cells.

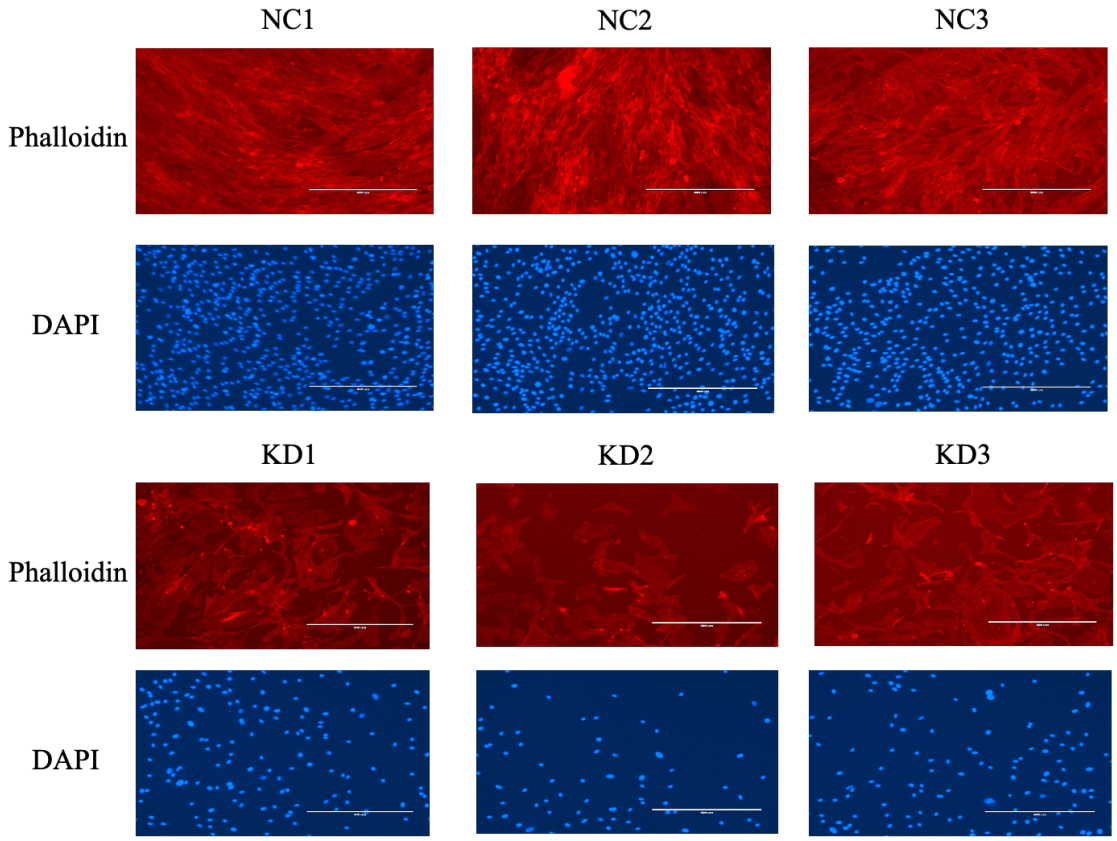

Figure 3-18: Notch3 Knockdown Decreases Cell Density but does not Significantly Impact Cytoskeleton at Passage 4. Results from Phalloidin and DAPI staining of adipose stem cells. Experiment was performed in triplicate and images were taken from

center of well and in exact arrangement to prevent bias. Images were taken at 10x magnification.

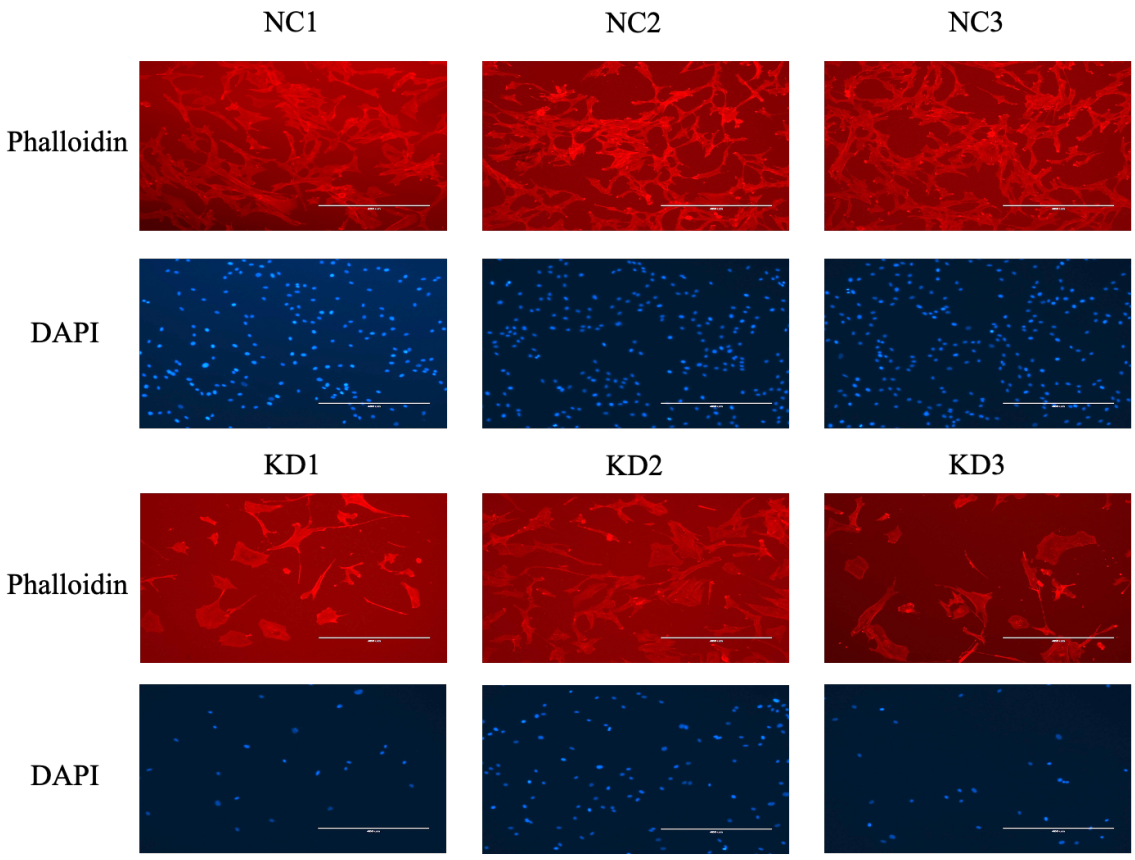

Figure 3-19: Notch3 Knockdown Decreases Cell Density but does not Significantly Impact Cytoskeleton at Passage 5. Results from Phalloidin and DAPI staining of adipose stem cells. Experiment was performed in triplicate and images were taken from center of well and in exact arrangement to prevent bias. Images were taken at 10x magnification.

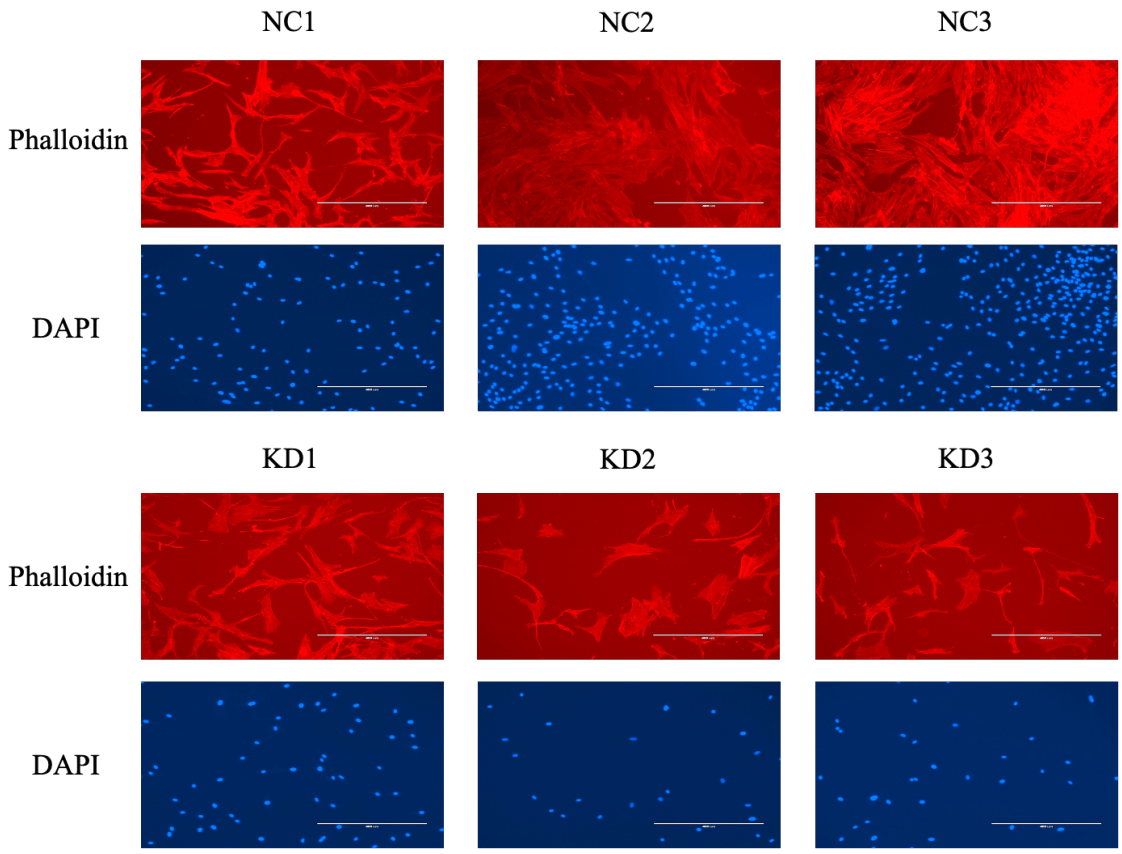

Figure 3-20: Notch3 Knockdown Decreases Cell Density but does not Significantly Impact Cytoskeleton at Passage 6. Results from Phalloidin and DAPI staining of adipose stem cells. Experiment was performed in triplicate and images were taken from center of well and in exact arrangement to prevent bias. Images were taken at 10x magnification.

CHAPTER 4

CONCLUSION

4.1 Conclusion

Stem cells hold promise in revolutionizing the way previously incurable, degenerative diseases are treated due to their properties of self-renewal and differentiation (1). There is a plethora of factors that work together to control these stem cell properties –such as signaling pathways and modifiers of DNA– and full understanding of these factors is required for proper manipulation of stem cells in stem cell-based therapies.

A stem cell signaling pathway that has previously been linked to cell state change in all animals is the Notch signaling pathway. This pathway is comprised of four transmembrane cell surface receptors and five canonical ligands (49). Of these receptors, Notch3 has been abundantly researched and has been shown to be necessary for proper stem cell differentiation (52), neonatal development (56), and has been linked to a multitude of cancers, some of which include breast (58), prostate (61), and T-cell leukemia (63). This direct relationship between Notch3 and human health creates a drive for further research into – and a deeper understanding of – this receptor's role in stem cells and the human body.

Here we investigated the role Notch3 plays in adipose stem cell fate determination, with special interest in cellular proliferation, self-renewal, metabolism, and morphology. We utilized siRNA-mediated knockdowns to decrease levels of Notch3 mRNA. We utilized quantitative reverse transcription PCR and western blots to validate that the knockdown of Notch3 was successful and to ensure that this knockdown could be sustained throughout the passaging process. We found that Notch3 persisted, although to a lesser degree, throughout the passaging process. This loss of knockdown is likely due to the transient nature of the technique used. As siRNAs are transfected at a given concentration, the amount of available siRNA decreased with each passage and so does not produce a stable knockdown the way a viral transformation does. However, the amount of Notch3 transcript was still significantly decreased as compared to the negative control, allowing us to continue our experiments and monitor the influence of the knockdown on hASC self-renewal, metabolism, and morphology.

We also utilized qRT-PCR to investigate the role of Notch3 in cellular proliferation by quantifying changes in expression of *ki67.* We found that levels of *ki67* transcript were significantly decreased in cells that underwent a knockdown of Notch3 as compared to the control, implying that a loss in Notch3 function leads to a decrease in cellular proliferation. To coincide with these findings, we performed colony-forming unit assays to assess self-renewal capabilities. We found that cells that underwent a knockdown of Notch3 did not have any self-renewing colonies as compared to those in the negative control group having 60-80. Both tests imply that Notch3 plays an active role in cellular proliferation and, specifically, self-renewal of adipose stem cells.

The effect of Notch3 on cellular metabolic activity was investigated using an alamarBlue assay, where changes in absorbance measurements correspond to the rate at which cells can metabolize the reagent dye, with an increase in absorbance indicative of an increase in metabolism. We found, over two passages, that a Notch3 knockdown did not have a significant effect on cellular metabolism. We measured absorbance at two different wavelengths to ensure that wavelength did not affect results. This suggests that, while Notch3 has many important roles in various aspects of cellular state, it does not directly alter cellular metabolism. To complete this idea, the experiment should be replicated again at passage 6 to confirm reproducibility.

Finally, Notch3's role in cellular morphology was observed utilizing both Phalloidin and DAPI staining. Phalloidin stains actin filaments within the cell, allowing us to visualize changes in the cytoskeleton of adipose stem cells. DAPI stains DNA within the nucleolus and allows for visualization of changes in cell density. We found that cell density was decreased in cells following a knockdown of Notch3 across three passages. However, there were no distinct visual changes in the cytoskeleton of Notch3 knockdown cells. This indicates that Notch3 does not affect the development and structure of actin within adipose stem cells. It does, however, provide further evidence that Notch3 is significantly affecting cellular proliferation in adipose stem cells.

4.2 Future Directions

This research revealed the vital role Notch3 has in cellular proliferation within human adipose stem cells. The lab is continuing this work and utilizing RNA sequencing methods to pinpoint exact transcriptional changes within cells following a decrease in *notch3* expression. Understanding which genes are affected by this knockdown can help

to provide cellular targets for manipulation of the Notch signaling pathway. These methods will also be utilized to investigate transcriptional changes following the knockdown of other genes within the Notch pathway.

The responsibility of Notch3 as a regulator of cellular proliferation should be further investigated in other cell types within the human body. As Notch3 is directly linked to some cancer forms, there is a theoretical effect of decreasing Notch3 as a means to decrease cellular proliferation in cancerous cells. This idea could be further investigated and could provide novel cancer treatments. Studies in which Notch3 is overexpressed to see if this results in cancer-like cell growth could also be helpful.

APPENDIX A

TROUBLESHOOTING CONTAMINATION

Work in the Newman lab was drastically affected this year by bacterial contamination. It was first noticed in the media (Figure A-1) as well as on a digital inverted microscope (Figure A-2) around February of 2022 and was not entirely eradicated from the lab until October of 2022. This near year-long series of events drastically set back the timeline for my thesis research. I did, however, learn a great deal about what to do in instances of contamination in cell culture. I detail some steps to take to investigate the cause of contamination and hopefully eliminate it from a laboratory setting.

Figure A-1: Contaminant Could be Visualized by the Naked eye. The contaminant was able to be seen by the naked eye and appeared as a cloudiness within the cell media. Contaminant was fast-growing and appeared overnight. Bacterial density was inferred from the level of opaqueness observed in the media.

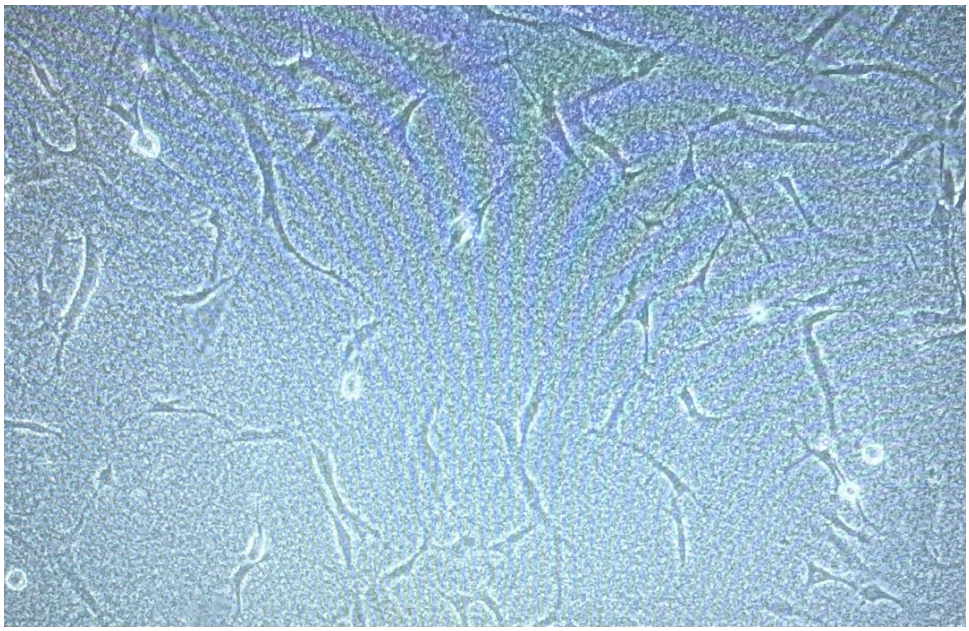

Figure A-2: Presence of Contaminant seen on Cell Imager. Contaminant was seen on inverted microscope at 10x magnification. It appeared as small and moving bacteria within the media, covering the surface of the plate in which cells were adhered. It eventually affected the growth of the cells and resulted in cell death.

Troubleshooting to isolate the source.

When cells are first found to be contaminated, the most crucial step is to properly dispose of any contaminated plates by first bleaching the plate and then disposing of them in a in biohazard bag. This should be followed by a very thorough cleaning of any lab areas where the cells were kept or manipulated. This includes incubators, biological safety cabinets, imaging surfaces, and the benchtop. 75% ethanol can be used for each of these surfaces. There is always a chance that the contamination occurred as an isolated event due to an error, such as accidentally lifting the lid of a cell plate outside of the incubator or safety cabinet or accidentally touching the end of a pipette to an unclean surface prior to using it within a cell plate.

If contamination persists, then it becomes critical to identify the source of the contamination. To do this you need to isolate as many factors as possible and evaluate them individually as sources of contamination. One of the first things to look at is culture media. This can be evaluated by plating only media and then allowing it to incubate, seeing if the contaminant grows. If the media appears clean then look at other reagents used during cell culture, which for us included lipofectamine, both negative control and Notch3 siRNA, and Opti-MEM. Allow these plates to incubate to see if they grow the contaminant. In addition, all of the reagents used for passaging could be tested. To do this, you should plate each reagent used and allow them to incubate. Isolating each factor allows for the identification of a source so that it can be removed from the lab and replaced with a new, sterile reagent.

Since our contamination coincided with an unfortunate loss of our freezer stocks from an empty liquid nitrogen storage dewar, we examined this as a potential source of contamination. Some contaminants can exist in conditions present in a liquid nitrogen dewar and so it is advised that cells be stored in the gas phase rather than the liquid phase (68). It is advised, if contamination persists, to empty your dewar, thoroughly clean it with diluted bleach, and refill with liquid nitrogen. While this was not the source of contamination within our lab, it is a possible occurrence. To eliminate this possibility, we began storing our cell aliquots in an -80 ºC freezer.

During our battle with contamination, there was an incident in which bacterial agar was overflowed into the small autoclave around a similar time that we used the autoclave to sanitize self-filled containers of both plastic pipette tips and glass aspirator tips. Plates of media were plated and the tips from each type were swirled around the plate. Nothing

grew from these plates. Despite this, we began to use filtered plastic pipette tips and eliminated the use of an aspirator and autoclaved Pasteur pipets from our protocols.

Cleaning Protocols

Having a clean and sterile environment prior to any experimentation is crucial in preventing contamination. You should clean with a reagent that is both strong enough to effectively destroy contaminants but not so strong that it evaporates prior to cleansing. Make sure that the reagent you are utilizing to clean your surfaces is capable of destroying contaminants. It is recommended to use 70% ethanol or reagent alcohol. Reagents should not be overdiluted, as this can decrease their effectiveness. Make sure that you dilute any cleanser (ethanol, reagent alcohol, bleach, etc.) with DI water free from contaminants. Contaminants can exist within DI water, so thoroughly clean any DI water storage or obtain fresh DI water prior to any experiment in which it is required.

Finally, a major source of contamination can come from a water bath. If you have a water bath and utilize it to warm reagents prior to placing them on cells, ensure you are cleaning it weekly with diluted bleach and refilling it with contaminant-free DI water. It is also beneficial to introduce a water bath cleansing reagent, such as Clear Bath (Spectrum, #88691-1), into your water bath. These work to eliminate contaminants from your water bath and keep the water clean for longer periods of time. In addition, whenever items are removed from the waterbath, be sure to spray them with 70% ethanol and wipe them down thoroughly.

We are still not 100% sure what directly lead to our contamination problem, or if it was a combination of several compounding factors. After enacting these changes, we

eventually stopped seeing instances of contamination. All members of the lab were taught new methods of preventing contamination, such as keeping containers closed in the hood and avoiding opening cell culture plates outside of the incubator or hood. While this did decrease the amount of time I had to perform the experiments necessary for my thesis research, I do feel like troubleshooting this contamination aided in my knowledge of science and helped me to grasp a deeper understanding of cell culture methods.

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