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How the Notch Signaling Pathway and Mediator Work Together to Regulate hASC Self-Renewal

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HOW THE NOTCH SIGNALING PATHWAY AND MEDIATOR WORK TOGETHER TO REGULATE HASC SELF-RENEWAL

by
Taylor Teach

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ABSTRACT

Obesity is a serious medical condition resulting from excess body fat that triggers changes in both quantity and quality of various cells that reside in adipose tissue, including adipose stem cells. Adipose derived stem cells are multipotent, self-renewing cells that have the ability to differentiate. This process can be controlled by environmental stimuli, transcription factors, and signal cascades that lead to gene transcription and protein expression specific to the cell's fate. The Mediator complex and the Notch signaling pathway are two complexes that allow this to occur. There is still much unknown about the Mediator complex, the Notch signaling pathways, and their interaction, especially during adipogenesis. Here we describe the expression profile and activity of MED12, Notch1, Notch3, Jagged1, and Jagged2 in self-renewing human adipose stem cells and determine the impact each gene has on expression and activity in self-renewing hASC's. We observed a MED12 knockdown leads to decreased expression and activation of Notch3; MED12 may be required to regulate the transcript and expression of Notch3. Notch3 knockdown leads to decrease MED12 transcript and protein; Notch3 may be required to maintain appropriate levels of MED12 expression. Jagged1 knockdown leads to a decrease in MED12 transcript, but has no discernable effects on protein expression. More research is needed to investigate the relationship between Jagged1 and Notch3.

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DEDICATION

This thesis is dedicated to Dr. Newman and all of the members of the Newman Lab. I hope my time in the lab made an impact on someone, whether that be academically or personally, and I hope this research makes even more of one.

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CHAPTER 1

INTRODUCTION

1.1 The Potential of Stem Cells

Stem cells are undifferentiated cells that are characterized by their ability to self-renew and differentiate into a variety of cell types (1). Self-renewal is the process by which stem cells generate undifferentiated daughter cells, and is required to preserve stem cell populations in different tissue that can be called upon to aid in development and tissue repair (2). Stem cells are classified according to their origin and differentiation potential and may be totipotent, pluripotent, or multipotent (Table 1-1). Totipotent stem cells have the ability to differentiate into any cell in the body, including extra embryonic cell types, along with any cells in the three germ layers: endoderm, mesoderm, or ectoderm (1,3). Pluripotent stem cells also have the ability to differentiate into any cell in the body, but unlike totipotent stem cells, lack the ability to form extra embryonic tissue (3). Multipotent stem cells, such as adult stem cells, have the capacity to give rise to

multiple lineages within a defined germ layer, but not all cell lineages in a developing embryo or adult. These cells are capable of activating or inhibiting a sequence of cellular and molecular pathways leading to anti-inflammatory and anti-apoptotic effects both *in vivo* and *in vitro* (3). For example, a recent study demonstrated that bone marrow derived MSCs had the ability to alter the cytokine secretion profile of dendritic cells, effector T cells, and natural killer cells enabling them to induce a more anti-inflammatory or tolerant phenotype (4). Because of their activation and inhibitory effects, multipotent stem cells have gained attention in the scientific community, and been extensively studied in attempts to find treatments for blood diseases like anemia, auto immune diseases like diabetes, and others due to their potential for cellular therapeutic effects.

Type of Stem Cell by Differentiation Potential	Sources	Characteristics	Example(s)
Totipotent	Early embryos 1-3 days old	Have the ability to differentiate into both embryonic and extra-embryonic tissues	Zygote
Pluripotent	Cells of blastocysts 5-14 days old	Can differentiate into all of the three primary germ layers but cannot contribute to extra-embryonic tissues	Embryonic stem cells, induced pluripotent stem cells
Multipotent	Bone marrow, muscle stem cells, adipocytes in fat, nerve cells, and cartilage	Cells that have the capacity to give rise to multiple lineages, but not all cell lineages in the developing embryo or adult	Mesenchymal stem cells, Adipose-derived stem cells, Umbilical cord stem cells

Table 1-1: Comparison of stem cell types.

1.2 Clinical Potential of Stem Cells

Pluripotent stem cells (PSCs) have the most differentiation potential since they have the ability to generate somatic cells of all three germ layers. The original pluripotent stem cells are embryonic stem cells (ESCs) derived from the blastocysts of a 5-14 day old embryo. PSCs have great therapeutic potential both for their nearly infinite replicative potential and limitless differentiation potential, and their applications for regenerative medicine continue to be studied and tested. However, the ethical issues that surround embryonic-derived pluripotent stem cells and induced pluripotent stem cells have resulted in significant controversy, limiting their usage and causing researchers to look for alternatives.

Embryonic stem cells are a type of pluripotent cell derived from the inner cell mass of pre-implantation embryos. Because human embryonic stem cells are extracted from human embryos, the ethics and safety behind embryonic stem cell research has been heavily debated (5). Many people argue that human life begins at conception, therefore an embryo is equivalent to an adult or live-born child. The extraction process of taking a blastocyst and removing the inner cell mass to derive an embryonic stem cell line is considered extremely unethical by some. Aside from this, various privacy concerns are starting to arise. The use of human biological material for cell-based and clinical research creates risks to the privacy of both patients and donors. One of these risks is the re-identification of individuals from anonymized cell lines. Risks will continue to increase as technology and databases used for re-identification become more advanced, affordable, and accessible. Privacy concerns are increased by policies that require linkage of cell lines to donors' clinical information for research and regulatory purposes and

existing practices that limit research participants' ability to control what is done with their genetic data (6).

iPSCs are another type of pluripotent stem cell derived from adult somatic cells that have been genetically reprogrammed to an embryonic stem cell-like state through the forced expression of genes and reprogramming factors (7). Although only discovered in the last two decades, the reprogrammed cells have had much success in clinical trials, and can be used to generate stem cells for disease modeling, drug development, and personalized regenerative stem cell therapy (8). Because of this, iPSCs can be used for modeling monogenic diseases, like Parkinson's Disease (PD), by using directed differentiation to derive disease-affected cell types carrying those genotypes. Patient-specific iPSC-derived dopaminergic neurons have been cultured to allow researchers to study many aspects of the PD phenotypes in a petri-dish, that would otherwise be inaccessible, helping advance our understanding of degenerative disease (9). In addition to disease modeling, researchers utilize these cells to improve drug development. iPSCs allow pharmaceutical companies to test drugs *in vitro* in a cost-effective manner before initiating clinical trials. iPSC-derived hepatocytes and cardiomyocyte cardiotoxicity and hepatotoxicity screens are being used in drug development, with the goal of increasing the accuracy of safety testing (10). Despite their success, concerns still remain about the clinical use of iPSCs. Genetic privacy is a challenge since iPSCs contain the genetic information of the donor. If used carelessly, research and publication could bring about ethical and legal issues regarding the individual donor and their family's privacy. In addition to ethical concerns, uncontrolled proliferation and the potential for unintended

differentiation of transplanted undifferentiated iPSCs has resulted in the generation of tumors, making their clinical usage a potential safety issue (11).

More recently, a class of multipotent adult stem cells was discovered that altered the potential for stem cells in the clinic. Bone marrow-derived mesenchymal stem cells (MSCs) are the most recognized type of multipotent stem cell. They are found in bone marrow, adipose tissue, amniotic fluid, and umbilical cord blood and tissue. Because of their various origins, assigned function in the human body, and multipotent properties, mesenchymal stem cells have the ability to differentiate into multiple cell types of the mesodermal lineage such as osteocytes, chondrocytes, myocytes, and adipocytes (12, 13). Scientists have been studying mechanisms behind MSC proliferation and differentiation in order to utilize the potential of these cells in regenerative medicine.

In addition to their regenerative ability, all MSCs can secrete soluble factors such as the immune secretomes prostaglandin E2 (PGE-2), indoleamine 2,3-dioxygenase (IDO), and nitric oxide (NO) that each contribute towards the inhibition of immune cell migration, proliferation, differentiation, and activation (14). These cells have been shown to aid in the treatment of autoimmune diseases including type I diabetes, systemic lupus erythematosus, and rheumatoid arthritis (15).

Bone marrow derived mesenchymal stem cells (BMCs) have been at the forefront of current studies due to their successful history of clinical usage, including their use in treating patients diagnosed with leukemia (16,17). In the case of leukemia, BMCs are used in the form of bone marrow transplants, a procedure where healthy blood-forming BMSCs are infused into the body to replace damaged or diseased bone marrow (18). They have shown to be a promising option for the treatment of cartilage lesions and

osteoarthritis as well. In a recent study, seventy-two matched patients underwent cartilage repair using chondrocytes or BMSCs. Clinical outcomes were measured pre-operation as well as every three months post-operation for two years. They found that BMSCs that retained their capacity for chondrogenic differentiation could be used to treat cartilage defects better than chondrocytes. Despite their clinical advantages, bone marrow-derived mesenchymal stem cells can be a challenging cell source for therapeutic usage due to the highly invasive and sometimes painful donation procedure, as well as the decline in MSC number and differentiation potential with increasing age (19). Because of this, researchers are looking for alternative sources of mesenchymal stem cells.

1.3 Adipose Derived Stem Cells

Human adipose derived stem cells (hASCs) are abundant and easy to access multipotent stem cells, making them a viable alternative to bone marrow-derived mesenchymal stem cells (20). With the increasing obesity epidemic worldwide, there has been an increased desire to understand adipogenesis, and hASCs provide readily accessible subcutaneous adipose tissue with which to perform critical research (21). Recently, hASCs have become more predominant in the use of clinical research due to their potential in regenerative medicine. Research suggests considerable therapeutic potential for hASCs in tissue engineering, coronary disease, bone regeneration, and osteoporosis (22). Several studies have used hASCs to study osteoporosis in both animals and humans (23). A recent study showed how injection of hASCs to osteoarthritic knees could be used as cell-based therapy, demonstrating that after one year of regular/one time injection, patients saw significant reduction in pain and improvement in knee function (24).

In addition to their potential in regenerative medicine, human adipose stem cells are also currently being used in cancer treatment centers and in multiple clinical trials due to their pro-tumorigenic and anti-tumorigenic roles. Current research suggests that the role of hASCs depends on their origin, the cell line of the cancer being studied, and the cells of the host immune system (25, 26). Human ASCs do possess tumorigenic properties because they secrete cytokines, growth factors like vascular endothelial growth factors (VEGF), and chemokines like platelet-derived growth factors (PDGF), that modulate angiogenesis and immune responses, as well as facilitate the regeneration of damaged tissues (27). VEGF is a homodimeric glycoprotein that is considered to be the key mediator of angiogenesis. In healthy humans, VEGF promotes angiogenesis in embryonic development, and is important in wound healing. It also plays a role in cancer, because angiogenesis is essential for cancer tumor development and growth (28). Similarly, PDGFs are pro-angiogenic factors found in platelets. The PDGF signaling pathway has been extensively studied, and found to regulate several cellular processes such as proliferation, migration, and metastasis (27). Recent studies have shown the PDGF signaling pathway and Notch signaling pathway are linked, and that their synchronization controls vascular differentiation (29).

1.4 Adipogenesis

Adipogenesis is the process of adipocytes developing and accumulating to form adipose tissue at various sites in the body. During this process, the preadipocytes no longer proliferate, and instead begin to accumulate lipid droplets as well as develop characteristics of mature adipocytes like morphological changes, cessation of cell growth, expression of lipogenic enzymes, and the establishment of sensitivity to hormones like

insulin (30). The main role of adipocytes is to store excess energy in the form of fat and, during energy scarcity, be used to meet the energy demand of other organs. Adipocytes arise during late embryonic development, as well as in the developed organism under conditions that promote obesity such as physical inactivity, overeating, or disease (31).

For adipogenesis to occur, preadipocytes must undergo adipocyte determination and differentiation, which is controlled by a complex regulatory network including a variety of environmental stimuli, transcription factors, and signal cascades that lead to gene transcription and protein expression specific to the cell's fate (Figure 1-1). Both determination and differentiation are tightly regulated, with cross-talk between them that ultimately determines cell type, function, and behavior (32). Many of the molecular details regarding adipogenesis are still unknown, but several factors involved in adipogenesis have been identified.

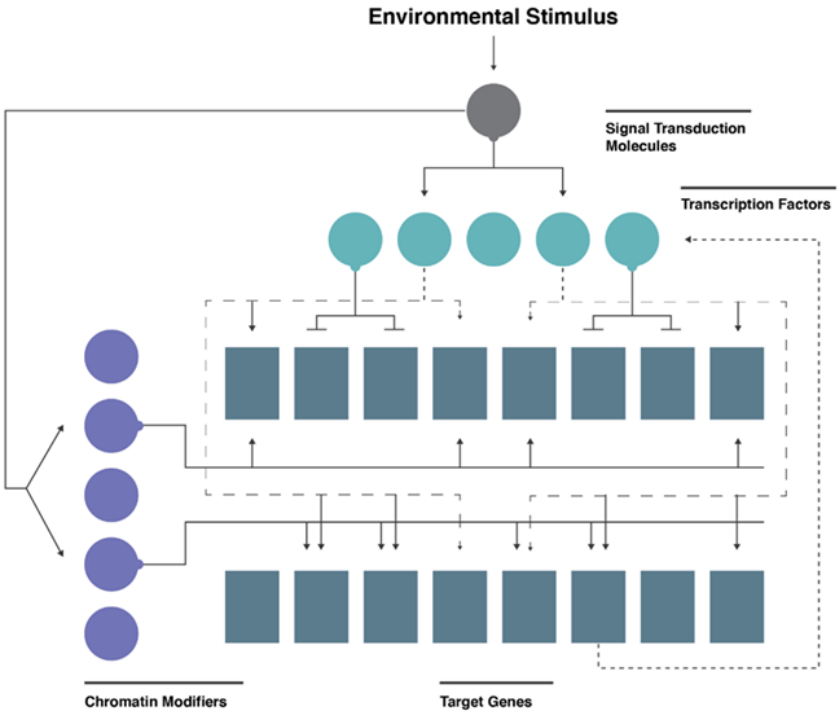


Figure 1-1: Gene Expression. *Gene expression is controlled by a complex regulatory network. Environmental stimuli initially activate signal transduction pathways. Once signaling components are activated, they can directly affect transcription factors and chromatin modifiers to initiate or inhibit transcription. When a gene is transcribed, transcription factors are activated and translocated into the nucleus of a cell to initiate transcription. If a gene target is repressed, chromatin is modified and condensed to prevent transcription.*

The current hypothesis suggests that terminal adipocyte differentiation requires transcription factors peroxisome proliferator-activated receptor gamma (PPARγ), CCAATT enhancer binding proteins (C/EBPs), and the basic helix-loop-helix protein ADD-1/ SREBP-1. PPARγ, controls terminal differentiation of adipocytes, and is required for maintaining their differentiated state (33). Adipogenesis is believed to be controlled by a transcriptional cascade, initiated when chromatin is open by the binding of C/EBPβ. During the initial phases of adipogenesis, C/EBP is expressed in response to adipogenic hormones such as insulin or glucocorticoids, which in turn signals the transcription of PPARγ (32). ADD-1/SREBP-1c is also expressed during terminal adipocyte differentiation, and accelerates adipocyte differentiation, regulates the expression of PPARγ, and provides ligands for this receptor. Finally, terminal adipocyte differentiation requires the concerted action of PPARγ and C/EBP. Those cells re-enter the cell cycle after hormonal induction, arrest proliferation again and undergo terminal adipocyte differentiation (34). Overall, the factors that regulate adipogenesis either promote or block the cascade of transcription factors that coordinate the differentiation

process. Other transcription factors such as insulin-like growth factors (IGF-1) have been shown to be critical for the survival, proliferation, and differentiation of pre-adipocytes (35). In addition to the cellular environmental factors, age, sex, and lifestyle have also been shown to impact adipogenesis (21) (Figure 1-2).

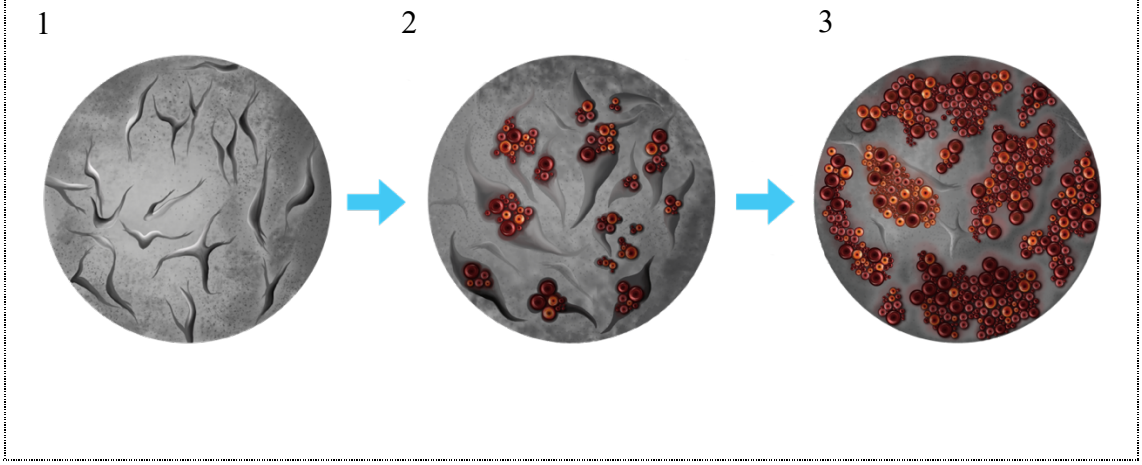


Figure 1-2: Human adipose derived stem cell differentiation. *Mesenchymal stem cells (1) proliferate and develop into preadipocytes (2) after reaching confluency. Preadipocytes become adipocytes through adipogenesis (3). At the molecular level, the core process of adipogenesis is regulated by transcription factors, such as CCAAT/enhancer, binding protein α (C/EBP-α), and peroxisome proliferator activated receptor γ (PPARγ), a heterodimerization partner of Retinoic X receptors (RXRs). The concerted action of these adipogenic transcription factors ultimately drives the expression of adipocyte specific factors such as enzymes responsible for the synthesis and storage of triglycerides in lipid droplets (34).*

1.5 Mediator Complex

Transcription factors bind to DNA directly, but in order for transcription to occur, these factors must be able to communicate across long stretches of the genome from enhancer elements to the promoter where RNA polymerase II is bound (8). The Mediator complex is a multiprotein complex that allows this cell-type specific gene expression communication to occur (36). As mentioned previously, stem cells are able to self-renew or differentiate into multiple cell types depending on how gene expression is regulated (1). Self-renewal and differentiation cannot take place at the same time; therefore, the cell's fate can be regulated by altering the function and expression of the general transcription factors (GTFs). GTFs assemble the transcription preinitiation complex (PIC) on gene promoters, as well as assemble activators and repressors that bind to gene regulatory elements located either upstream or downstream of promoters, and the essential coactivator of cell type-specific genes like the Mediator complex. The fate of any stem cell is ultimately determined by regulating the transcription of specific genes, a feature largely facilitated by the Mediator complex (37).

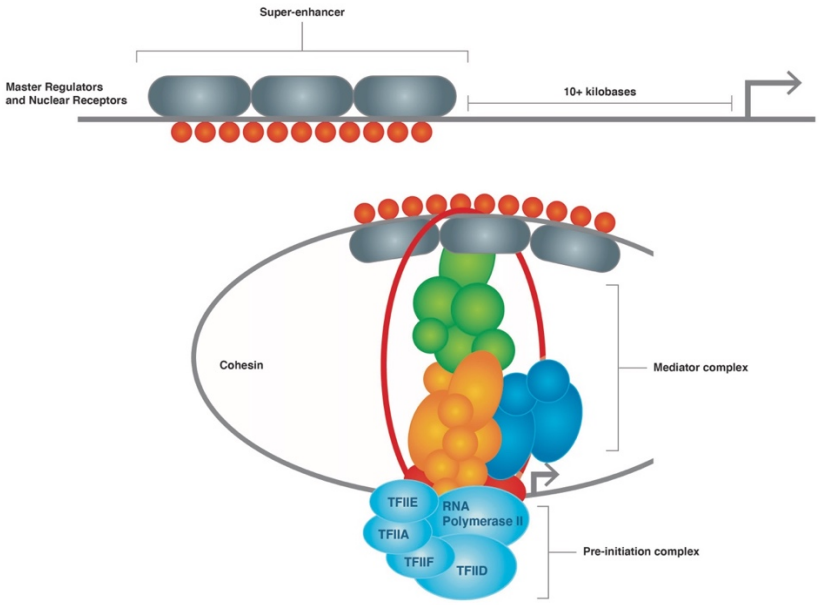


Figure 1-3: Mediator Complex Facilitates Transcription. *Top: linear representation of a super-enhancer. Grey ovals represent DNA-bound transcription factors and orange circles represent the histone mark H3K27Ac, a mark of gene activation, with the gene promoter potentially located over 10 kilobases downstream of the super-enhancer. Bottom: DNA looping mediated by the Mediator complex and Cohesion, which bridge the transcription factor-bound enhancer or super enhancer element to the gene promoter, facilitating expression of the cell-type specific gene (37).*

In order for transcription to occur, transcription factors must be able to communicate across long stretches of the genome from enhancer elements to the promoter where RNA polymerase II is bound (38). The Mediator complex plays a vital role in the regulation of cell-type specific transcription in eukaryotic cells by linking

transcription factors to RNA polymerase II. It is considered to be a global regulator of gene expression, and has an extremely dynamic design (36). Overall, Mediator consists of four modules: a head, a middle, a tail, and a kinase module (Figure 1-4). The head and middle modules contain the most highly conserved subunits, and maintain cell viability and overall gene expression. The tail recruits specific transcription factors to direct and maintain lineage commitment. In the Mediator complex, the kinase module CDK8 attaches to Mediator core complex and either activates or suppresses transcription through RNA polymerase II. The kinase module also functions independently of the Mediator complex. RNAPII, TFIIH, histone H3, and MED13 have all been listed as substrates for the CDK8 kinase (39).

CDK8 is considered both an oncogene and a tumor suppressor, and it promotes cell growth via the serum response pathway. CDK8 is a part of the 30 subunit Mediator (MED) complex, which acts as a molecular bridge to mediate transduction of regulatory signals. It completes this task by using a module that consists of Cyclin C (CCNC), MED12, and MED13 (38). A recent study suggests a relationship between MED12 and Notch signaling in chronic lymphocytic leukemia cells. They found CDK8 is recruited to the MED12 subunit in order to either activate or repress transcription. Once in place, CDK8 interacted with the kinase module, Cyclin C, MED12, and MED13, causing the complex to control transcription by RNA Polymerase II. They found if MED12 was mutated or absent, it did not interact with the CDK8 kinase and the Notch Intracellular Domain (NICD) was not phosphorylated, leading to an increase in Notch activity and transcription activation. Overall, the absence of control leads to the activation of Notch, causing uncontrolled cell differentiation or cell proliferation (40). This recent report

indicates the need for further investigation in order to better understand the interaction and relationship of these proteins in controlling hASC self-renewal and move stem cell research and clinical application forward.

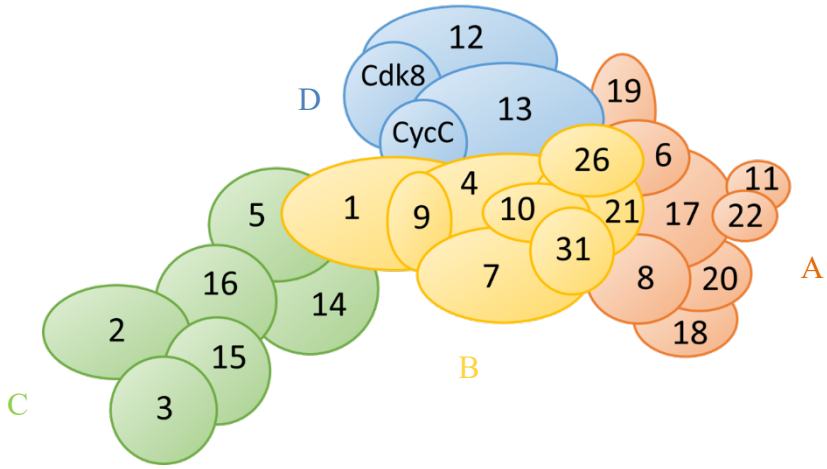


Figure 1-4: The Mediator Complex. *The head (A) and middle (B) modules contain the most highly conserved subunits, and maintain cell viability and overall gene expression. The tail (C) recruits specific transcription factors to direct and maintain lineage commitment. The kinase module (D) CDK8 attaches to Mediator core complex and either activates or suppresses transcription through RNA polymerase II.*

1.6 Notch Signaling

The Notch signaling pathway is an evolutionarily conserved cell fate determination pathway present in all multicellular eukaryotic organisms that controls cell differentiation, proliferation, and apoptosis (41). The pathway consists of four Notch receptors (Notch 1-4) and five canonical ligands (Jag-1, Jag-2, DLL-1, DLL-3, and DLL-4), as well as intracellular proteins that transmit signals to cells (42). The Notch signaling

pathway directly couples events at the cell membrane with the regulation of transcription. Most of the ligands in the Notch signaling pathway are also transmembrane proteins, therefore much of the signaling is restricted to neighboring cells. Through the canonical pathway, receptors on a given cell are activated in a contact dependent manner by cell surface ligands (Jagged1-2, DLL1, DLL3, DLL4) from neighboring cells in a process known as trans-activation. Each Notch receptor has three functional domains: The Notch extracellular domain (NECD) which is present on the outside of the cell, the Notch intracellular domain (NICD) which is present on the inside of the cell, and the Notch transmembrane domain (TM) which connects the NECD and NICD (43). The "sending cell" contains more ligands than Notch receptors, and the "receiving cell" contains more Notch receptors than ligands (44). When the cell-surface Notch receptor interacts with a ligand, the NECD binds to it. In order for the Notch signaling pathway to become activated, the ligands need to become activated (43). This happens by a protein known as "mind bomb" (Mib) in the sending cell, which ubiquitinates the ligand. Once activated, the ligand binds to the NECD, and a protease known as ADAM cleaves the NECD from the receiving cell; this is known as S2 cleavage. Gamma secretase then cleaves the NICD off the TM portion of the Notch receptor in a process known as S3 cleavage. This causes NICD to be free in cytosol, allowing it to bind to a complex of proteins including CSL which further binds to P300 (42). This entire complex translocates into the nucleus of the cell. This can lead to the transcription of Notch target genes such as CyclinD3, which is a critical regulator of the cell cycle in pro and large pre-B cells. Once the Notch target genes have been expressed, NICD is down regulated. In order for trans-activation to occur, the intracellular domain must be cleaved, and travel to the nucleus to regulate

transcription. This is what ultimately influences cell division, fate, and death in metazoans (43).

Because the Notch signaling pathway controls cell differentiation, proliferation, and apoptosis, it is often associated with tissue growth, cell death, tumor suppression, and cancer. In humans, the misregulation or loss of Notch signaling has been proven to be the underlying cause of multiple diseases and cancer (43). Recent evidence shows that germline mutations in *jag1* and *notch2* cause Adams-Oliver syndrome, and mutations in *dll3* cause spondylocostal dysostosis. Mutations in the *notch1* receptor are associated with several types of cardiac disease, and mutations in *notch3* cause the disorder cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) (45). DLL4-Notch3 signaling in human vascular organoids induces basement membrane thickening and drives vasculopathy in the diabetic microenvironment (46). By contrast, somatic alterations in the genes encoding Notch signaling components drive various types of human cancer, such as breast cancer, small-cell lung cancer (SCLC) and T-cell acute lymphoblastic leukemia (T-ALL) (47).

Notch1 signaling is a highly conserved pathway that has been proven to play a pertinent role in stem cell hemostasis and tissue development. In adipocyte progenitor cells, Notch1 signaling regulates the adipogenesis process including proliferation and differentiation of the adipocyte progenitor cells in vitro (48).

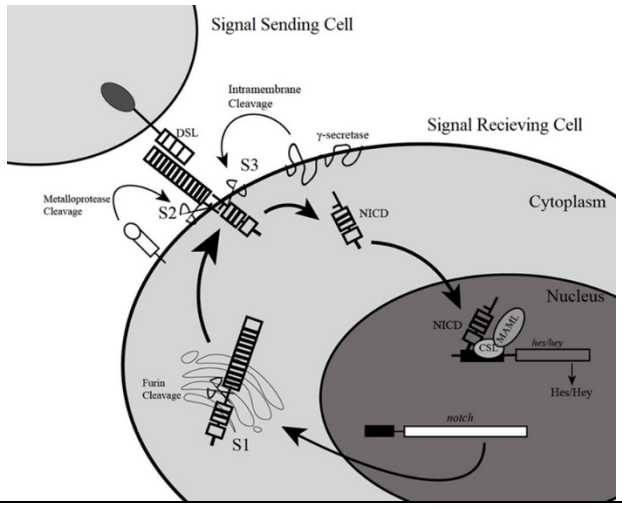


Figure 1-5: The Notch Signaling Pathway. *The Notch Signaling Pathway is an evolutionarily conserved pathway in multicellular organisms that regulates cell-fate determination during development and maintains adult tissue homeostasis. Notch receptors are single-pass transmembrane proteins composed of functional extracellular (NECD), transmembrane (TM), and intracellular (NICD) domains. In mammalian signal-sending cells, members of the Delta-like (DLL1, DLL3, DLL4) and the Jagged (JAG1, JAG2) families serve as ligands for Notch signaling receptors*.

1.7 Motivation

As mentioned previously, stem cells are undifferentiated cells that are characterized by their ability to self-renew and differentiate into a variety of cell types. The process of self-renewal, which generates undifferentiated daughter cells, is required to preserve stem cell populations in different tissue (2). Adult mesenchymal stem cells are found in bone marrow, blood vessels, skeletal muscle, epithelium, adipose tissue, and more (12,13). Human adipose-derived stem cells (hASCs) are a type of multipotent stem cell that are abundant in adipose tissue and easy to access, making them an increasingly

more viable alternative to bone marrow-derived mesenchymal stem cells which are being used in research today (20).

Scientists have been studying the mechanisms behind MSC proliferation and differentiation in order to utilize the potential of these cells in regenerative medicine. They have shown in recent clinical studies to have considerable therapeutic potential in a multitude of areas, including tissue engineering, coronary disease, bone regeneration, and osteoporosis (17,19). If we can extend studies to degenerative diseases like Muscular dystrophies (MD) and Parkinson's disease, we may be able to not only stop these disease's effects, but also reverse the damage these diseases have caused. In order to progress these studies, we must understand the underlying mechanisms that allow stem cells to remain self-renewing and multipotent.

Both the Mediator complex and the Notch signaling pathway work in unison to control the fate of ASCs through the regulation of gene expression. The Mediator complex plays a vital role in the regulation of cell-type specific transcription in eukaryotic cells by linking transcription factors to RNA polymerase II. The Notch signaling pathway is an evolutionarily conserved cell fate determination pathway that controls cell differentiation, proliferation, and apoptosis (41). Although we know interaction between Mediator and Notch is critical to controlling the generation of healthy tissue, the relationship between Mediator and Notch remains poorly defined. Research has been trying to bridge the two in attempt to better understand the underlying mechanisms that allow stem cells to remain self-renewing and multipotent, but more research is needed.

CHAPTER 2

METHODS

2.1 Thawing Human Adipose Stem Cells

Human adipose stem cells (Obatala, #70926) were removed from liquid nitrogen and thawed in a 37˚C water bath. Once thawed, cells were transferred into a 15 mL conical vial containing 4mL of pre-warmed Complete Culture Media (CCM) composed of 203.75mL of Minimum Essential Medium Alpha (Life Technologies, #12561049), 41.25mL of Fetal Bovine Serum (Atlanta Biologicals, #S11550), 2.5mL of L-Glutamine (Gibco, # 25030-081), and 2.5mL of Penicillin-Streptomycin (Life Technologies, #15140122). Cells were centrifuged at 1500 RPM for 10 minutes. The supernatant was removed from the newly formed cell pellet via aspiration, and the cell pellet was resuspended in 1mL of CCM. Media was changed 24 hours after the initial thaw, as well as every 48 hours after until cell confluency reached 70-80% and they were ready to be passaged.

2.2 Passaging

When the cells reached 70-80% confluency, they were rinsed with 5 mL of pre-warmed phosphate buffered saline (PBS) (Life Technologies, #10010023). The PBS was aspirated off the plate and then 3mL of 0.25% Trypsin (Life Technologies, #25200-056) was added to the plate. The cells were incubated for 3 minutes at 37˚C, then checked under a microscope for lifting. 6mL of CCM (or double the amount of trypsin) was then added to the plate and the cells were collected into a conical tube with a pipette for centrifugation at 1500 RPM for 10 minutes. Supernatant was aspirated off and the cells were resuspended in 1mL of pre-warmed CCM. 20µL of the cell solution and 20µL of trypan blue were mixed in an Eppendorf tube. Once mixed, 10µL of the cell-trypan solution was added onto both sides of a FL hemocytometer, and the slide was inserted into a cell counter. After cells were counted, they were then passaged onto 6cm plates and placed in an incubator for 24 hours (Table 2-1).

Table 2-1: Plating table

Plate size	**Number of cells seeded**
10cm plate	100,000 cells
6cm plate	45,000 cells
6-well plate	20,000 cells per well

2.3 Transfection

When the cells reached 50-60% confluency, they were transfected with either a silencer select control No. 1 siRNA (Thermo Scientific, #4390843) or a custom target gene siRNA for Notch1 (Thermo Scientific, #am16708), Notch3 (Thermo Scientific, #4392420), Med12 (Thermo Scientific, #s19364), Jagged1 (Thermo Scientific, #AM16708), or Jagged2 (Thermo Scientific, #4392420). Cells were transfected using RNAi Max Lipofectamine following manufacturer's protocol. Cells were incubated at 37˚C overnight and the next day media was replaced with pre-warmed CCM. Media was re-plated with CCM the next day and either RNA or protein was collected 72 hours after the transfection.

2.4 RNA

2.4.1 RNA Collection

72 hours after the transfection, plates were rinsed with pre warmed PBS and RNA was collected using 500µL of Trizol per 6cm plate. Plates were scraped using a cell scraper for 1 minute and the solution was collected into 1.5 mL Eppendorf tubes and stored in a -80˚C freezer.

2.4.2 RNA Extraction

Trizol samples was thawed at room temperature and 100µL of chloroform was added to each RNA sample. Each sample was then vortexed for 15 seconds and left to incubate at room temperature for 3 minutes. Once the incubation was complete, the samples were centrifuged at 4˚C at 12000G and the colorless aqueous supernatant was removed and placed into a new Eppendorf tube. 5µL of Thermo Scientific glycogen along with 250µL of 100% isopropyl was added to each sample and each tube was inverted 3 times before it was left to incubate at room temperature for 10 minutes.

Samples were then centrifuged at 4˚C at 12000G for 10 minutes. The liquid supernatant was removed leaving only the RNA pellet. 1mL of 75% ethanol was added to the pellet and then vortexed to wash the pellet. The samples were then centrifuged for 5 minutes at 4˚C at 7500G. After the samples were centrifuged, the liquid supernatant was removed and the pellet was allowed to air dry, before 30µL of nuclease free water was added to RNA pellet. RNA was quantified to assess concentration and purity using the BioTek plate reader. The Gen5 Microplate Reader and Imager Software was used to analyze the data.

2.4.3 cDNA Synthesis

cDNA was synthesized for each sample using 1microliter of RNA and qScript cDNA supermix (VWR, # 95048-100) following the manufacturer's protocol.

2.4.4 Endpoint RT PCR

Primers and quality of cDNA were confirmed by endpoint RT-PCR using GoTaq green mastermix (Promega, #M7122) following manufacturer's protocols.

Table 2-2: Primer list

Gene	Forward Sequence	Reverse Sequence	Product size (bp)	Temperature	Cycles
med12	CGAAAAGGGACAGC AGAAAC	CCCATCCTCCCC ACCTAAGA	87	60	30
notch1-2	CACGCTGACGGAGTA CAAGT	GGCACGATTTCC CTGACCA	56	60	35

notch3	CACCCTTACCTGACC CCATCC	TTCGGACCAGTC TGAGAGGGA	81	60	35
jagged1	GGCACGCGTCATTGT GTTAC	TGCGCAGCCTTT TATTCCCT	119	60	35
jagged2	TGGACGCCAATGAGT GTGAA	CCCGGGATGCAA TCACAGTA	91	60	35
gapdh-2	ACTAGGCGCTCACTG TTCTCT	CAATACGACCAA ATCCGTTGACT	99	60	30

2.5 Real Time

Quantitative reverse transcriptase PCR (qRT-PCR) was performed using Power SYBR® Green PCR Master Mix and run using an Applied Biosystems StepOne Plus machine. GAPDH (Abcam, # ab9485) was used for normalization of qRT-PCR results. Samples were run in triplicate.

2.6 Protein

2.6.1 Extraction and Collection

When cells reached 70-80% confluency, plates were rinsed with cold PBS. Cells were collected in lysis solution composed of Pierce RIPA Buffer (Thermo Fisher Scientific, #89900) and Halt Protease and Phosphate inhibitor (Thermo Fisher Scientific, #78441) and transferred into 1mL Eppendorf tubes where they maintained constant agitation for 30 minutes at 4˚C. They were then centrifuged at 12000 RPM for 20 minutes at 4˚C and the supernatant was transferred into a new Eppendorf tube.

2.6.2 Bradford Assay

Protein concentrations were measured using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, #5000006) following manufacturer's protocol. Absorbance was measured at a wavelength of 595 nm.

2.6.3 Western Blots

Equivalent amounts of protein were used for all samples, samples were boiled with water and 2x laemmli sample buffer (Bio-Rad, #1610737) in a heat block at 95-100 degrees F for 10 minutes before being loaded into the gel. Protein samples ranged from 20-40 microliters with the laemmli buffer amount and water dependent on the protein number calculated from a Bradford Assay. Once boiled, 35 microliters of each sample were loaded along with 7 microliters of ladder on the end into a 4-15% polyacrylamide gel. The gel was run at 120 volts for one and a half hours. Proteins were transferred on a Trans-Blot Turbo Mini PVDF Transfer using the Bio-Rad Trans-Blot Turbo Transfer System. The membranes were then blocked using a 5% blocking buffer composed of nonfat dry milk and TBST, and probed overnight with a primary antibody (Table 2-2). The membranes were washed with TBST and probed with a secondary antibody for 60 minutes. After being washed, the membranes were then imaged with Bio-Rad clarity western ECL substrate and analyzed using ImageJ software.

Table 2-3: Antibody List

Antibody	**Dilution**	**Company**	**Catalog #**
MED12	1:1000	Bethyl	A3000-774A
Notch1	1:1000	Proteintech	20687-I-AP

JAG1	1:1000	Cell signaling	2620S
JAG2	1:1000	Cell signaling	2205S
GAPDH	1:3000	abcam	Ab9485
Goat pAb to Rb IgG (HRP)	1:10000	abcam	Ab6721

CHAPTER 3

THE EFFECT OF NOTCH SIGNALING ON MEDIATOR SUBUNITS

3.1 Introduction

Human adipose stem cells (hASCs) are a type of multipotent stem cell that have recently risen to the forefront of research due to their ability to serve as an alternative to pluripotent embryonic stem cells, induced pluripotent stem cells, or even the more invasively derived bone marrow stem cells (BMSCs). Scientists have been using hASCs to study the mechanisms behind adult stem cell proliferation and differentiation in order to utilize the potential of these cells in regenerative medicine, cell-based therapies, and tissue engineering. If we can gain a better understanding of how to control hASC differentiation and self-renewal, stem cells could be used to their full potential in regenerative medicine.

The Notch signaling pathway is a highly conserved pathway that has several roles in cellular maintenance including proliferation, differentiation, and apoptosis. It has also been found to participate in the conversion of mesenchymal stem cells (MSCs) to mature adipocytes, but its overall role in adipogenesis remains controversial with conflicting

research indicating a context-dependent positive and negative role for Notch signaling. Notch signaling is known to directly couple events at the cell membrane with the regulation of transcription.

Through the canonical pathway, receptors on a given cell are activated in a contact dependent manner by cell surface ligands (Jagged1-2, DLL1, DLL3, DLL4) from neighboring cells in a process known as trans-activation. The canonical ligands are type 1 cell-surface proteins that ultimately control the sending and receiving of signals, and have multiple epidermal growth factor (EGF) repeats in their extracellular domains. Notch receptors undergo conformational changes once trans-activation occurs, allowing for two consecutive proteolytic cleavage events that release the intracellular region of the Notch receptor into the cell's cytoplasm. The EGF in the ligands serves as a protection against proteases, which prevents Notch1 from ligand-independent activation (49). Once in the cytoplasm, the receptor can then travel to the nucleus to induce gene transcription. Because both the Notch receptor and its ligands play crucial roles in gene transcription, dysregulated Notch signaling is associated with developmental abnormalities and cancer. In humans, haploinsufficiency of either Jagged1 or Notch2 is associated with Alagille syndrome, while Notch1 haploinsufficiency is implicated in a subtype of inherited aortic disease (50).

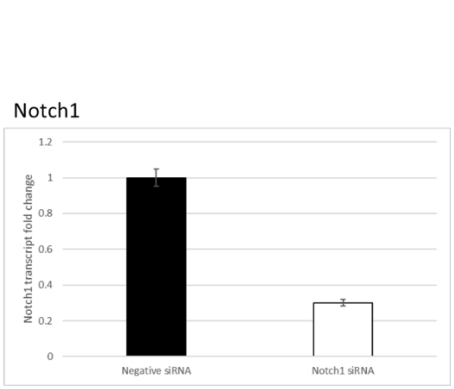
At the center of cell-type specific transcriptional regulation is Mediator, a highly conserved complex that links pathways like Notch to gene promoters. Both Mediator and the Notch signaling pathway work in unison to control the fate of hASCs through the regulation of gene expression, but their relationship remains poorly defined. A recent study found that in order to activate or repress transcription, CDK8 is recruited to the

MED12 subunit, which suggests a possible influence of MED12 on Notch signaling in chronic lymphocytic leukemia cells (51). Once in place, CDK8 interacts with the rest of the kinase module, Cyclin C, MED12, and MED13, causing the complex to activate or suppress transcription by RNA Polymerase II. In this study, MED12 effects Notch signaling by functioning as an activator of Cyclin C/CDK8. If MED12 is mutated or absent, it does not interact with the CDK8 kinase and the Notch Intracellular Domain (NICD) is not phosphorylated, leading to an increase in Notch activity and transcription activation. This recent report indicates the need for further investigation in order to better understand the interaction and relationship of these proteins in controlling hASC self-renewal.

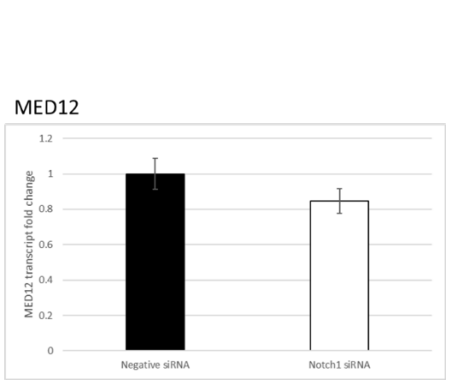
Although there are four modules of the Mediator complex, the kinase module consisting of CDK8, Cyclin C, MED12, and MED13 remains of particular interest given its unique role both as part of the core complex and in its ability to act independently. MED12 in particular is under investigation as it is believed to regulate and control transcription in hASCs. Studies have shown that MED12 has a role in pluripotent stem cell self-renewal, could be involved in several human developmental defects, and may be responsible for many diseases, including Lujan syndrome (52).

Previous data from the Newman lab investigated the relationship between MED12 and Notch1. The results indicated that Notch1 does not have an active role hASC self-renewal. *Med12* transcript was not significantly affected by the knockdown of *notch1*, and protein expression of MED12 was not affected by a Notch1 knockdown (Figure 3-1 and Figure 3-2). In addition, transcript levels of *notch1* decreased after the knockdown of *med12*, but protein levels of Notch1 were not affected by the MED12 knockdown (Figure

3-3 and Figure 3-4), suggesting a minimal role if any for this receptor in hASCs cultured under standard conditions (Figure 3-3 and Figure 3-4) (53).



P value= 0.00018



P value= 0.240368323

Figure 3-1: *Notch1* knockdown has no effect on *med12* transcript. *A. The* notch1 *knockdown was validated by qRT-PCR. B.* med12 *transcript was measured following the siRNA-mediated knockdown of n*otch1. *No significant reduction in the* med12 *transcript level was observed. Data was normalized to* gapdh. *P values were calculated with T Test, N=3. Image courtesy of Jaylen Mumphrey.*

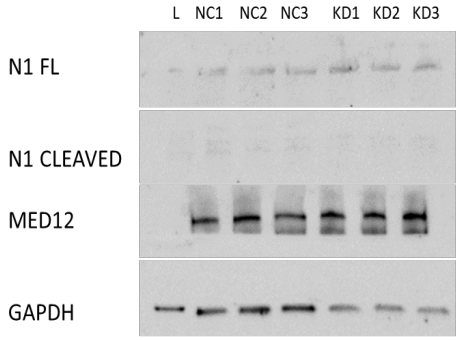
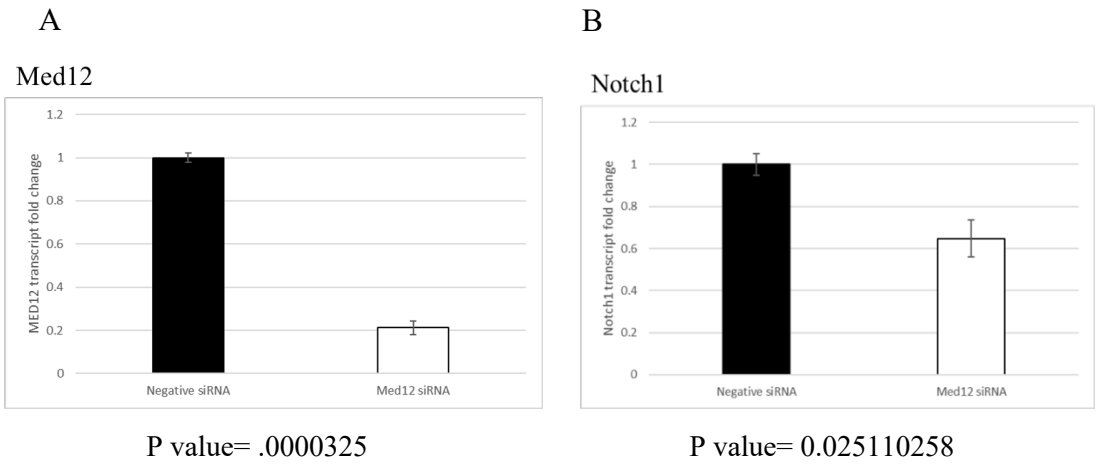


Figure 3-2: **Notch1 knockdown has no effect on MED12 protein levels.** *Protein expression levels of hASCs transfected with* notch1 *siRNA were assessed via Western blot. GAPDH was used as a loading control and unfortunately showed decreased*

expression in the Notch1 knockdown samples. As it is a loading control for normalization, it's levels should not have change. Therefore, the protein levels also do not clearly indicate sufficient KD of Notch1. This suggests possible inconsistency in loading and requires reexamination. MED12 did not appear to be affected by the Notch1 knockdown, and active Notch1 (N1 Cleaved) was undectable despite a slight signal in full length (FL) Notch1. Image courtesy of Jaylen Mumphrey. NC Negative control, KD knockdown, N1 Notch1, FL Full Length, MED12 Mediator12.



P value= .0000325

P value= 0.025110258

Figure 3-3: *MED12* knockdown leads to decreased *Notch1* transcript. *MED12 siRNA effectively knockdowns* med12 *transcript and diminishes expression of Notch1. Transcript levels of* med12 *(A) and* notch1 *(B) in hASCs transfected with* med12 *siRNA analyzed via qRT-PCR. There was not a significant reduction in the* notch1 *transcript levels. Data was normalized to* gapdh*. P values were calculated with T Test, N=3. Image courtesy of Jaylen Mumphrey.*

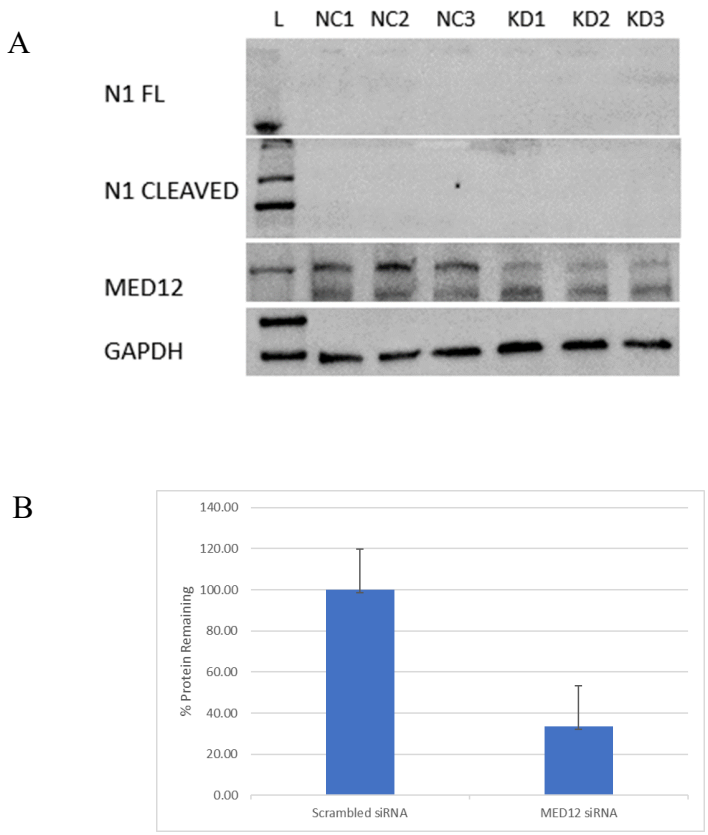


Figure 3-4: **MED12 knockdown has no effect on Notch1 protein levels.** *A. Protein expression levels of hASCs transfected with MED12 siRNA via Western blot. The MED12 knockdown was validated while the identification of Notch1 was unsuccessful. B. ImageJ data showing the MED12 knockdown Western blot quantified using ImageJ software. Western blot was normalized to GAPDH. P values were calculated with T Test. N=3. Image courtesy of Jaylen Mumphrey. NC Negative control, KD knockdown, N1 Notch1, FL Full Length, MED12 Mediator12.*

To validate and expand on these results, I investigated the relationship between Notch1, Notch3, the Mediator kinase subunit MED12, and Notch ligands during hASC self-renewal.

3.2 Results

Notch1 KD leads to a decrease in med12 *transcript in self-renewing hASCs*

In order to further investigate the relationship between Notch and Mediator and confirm previous studies in the Newman lab, we analyzed the influence of Notch1 on MED12. Human adipose stem cells were transfected with *notch1*-specific siRNA. The knockdown of *notch1* was validated using qRT-PCR. *Notch1* transcript was reduced in comparison to negative controls, with less than 80% transcript remaining after knockdown (Figure 3-5). The expression of *med12* was evaluated using qRT-PCR and showed a slight decrease in transcript compared to negative controls, with less than 40% remaining after knockdown, but the data was not significant (Figure 3-6).

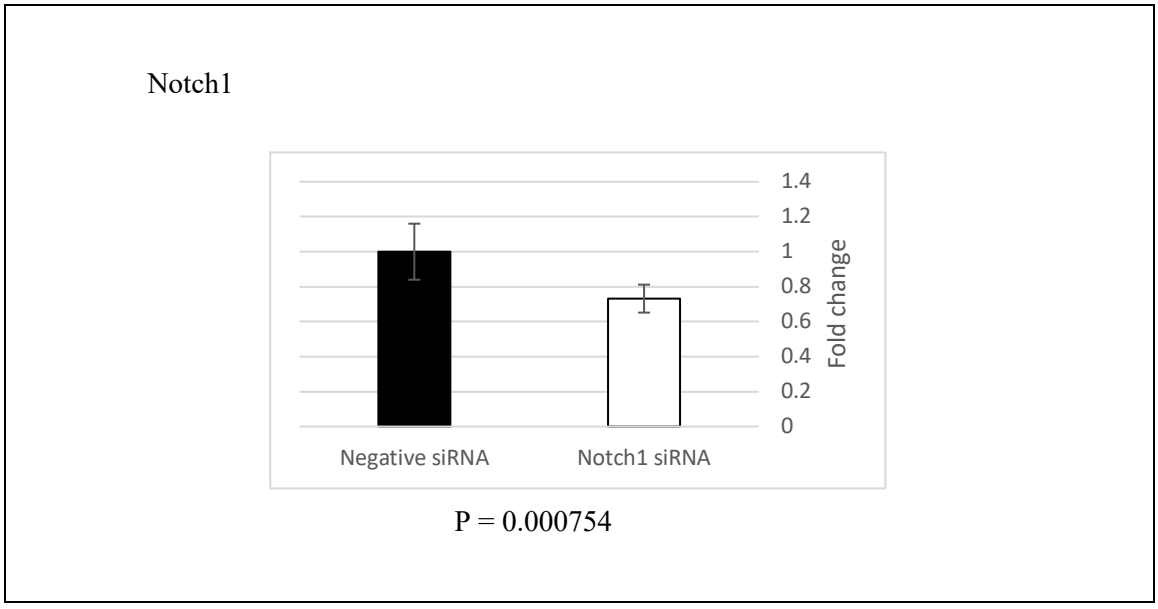
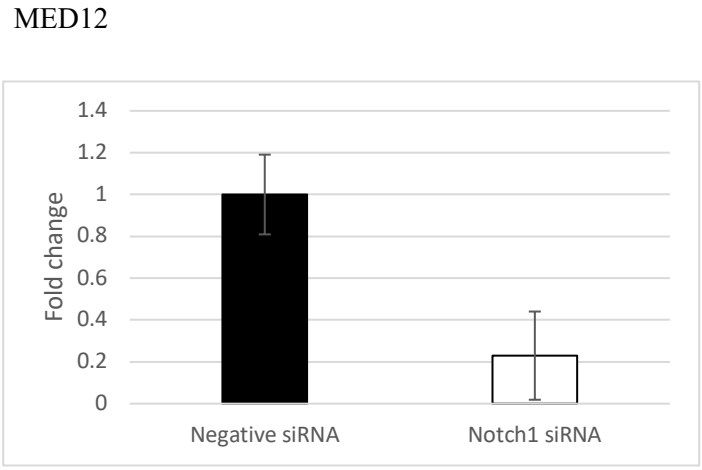


Figure 3-5: *Notch1* knockdown leads to a decrease in *notch1* transcript. *Transcript expression levels of* notch1 *in hASCs transfected with* notch1 *siRNA for 72 hours analyzed via qRT-PCR. Data was normalized to GAPDH. N=3.*



P = 0.642208

Figure 3-6: *Notch1* knockdown leads to a decrease in *med12* transcript. *Transcript expression levels of* med12 *hASCs transfected with* notch1 *siRNA for 72 hours analyzed via qRT-PCR. Data was normalized to GAPDH. N=3.*

Notch1 KD leads to an increase in notch3 *transcript in self-renewing hASCs*

In order to further investigate the relationship between the Notch receptors, we analyzed the influence of Notch1 on Notch3. Human adipose stem cells were transfected with *notch1*-specific siRNA. The knockdown of *notch1* was validated using qRT-PCR. *Notch1* transcript was reduced in comparison to negative controls, with less than 80% transcript remaining after knockdown (Figure 3-5). The

expression of *notch3* was evaluated using qRT-PCR and showed an increase in transcript compared to negative controls, but the data was not significant (Figure 3-7).

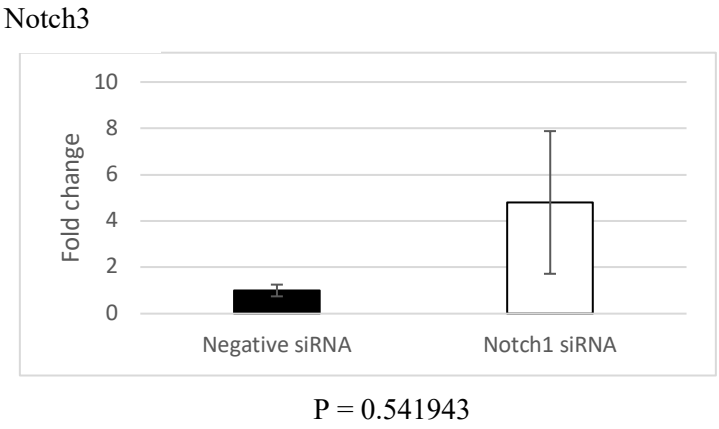
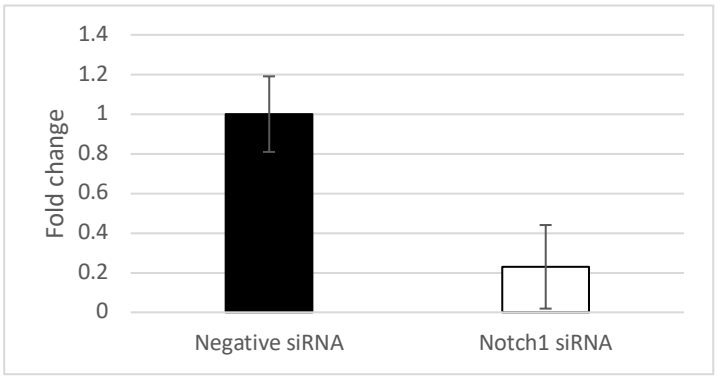


Figure 3-7: *Notch1* knockdown leads to an increase in *notch3* transcript. *Transcript expression levels of* notch3 *hASCs transfected with* notch1 *siRNA for 72 hours analyzed via qRT-PCR. Data was normalized to GAPDH. N=3.*

Notch1 KD leads to a decrease in jagged1 *transcript in self-renewing hASCs*

In order to further investigate the relationship between Notch and Mediator, we analyzed the influence of Notch1 on Jagged1. Human adipose stem cells were transfected with *notch1*-specific siRNA. The knockdown of *notch1* was validated using qRT-PCR. *Notch1* transcript was reduced in comparison to negative controls, with less than 40% transcript remaining after knockdown (Figure 3-8). The expression of *jagged1* was evaluated using qRT-PCR and showed a slight decrease in transcript compared to negative controls, with less than 80% remaining after knockdown (Figure 3-9).

Notch1

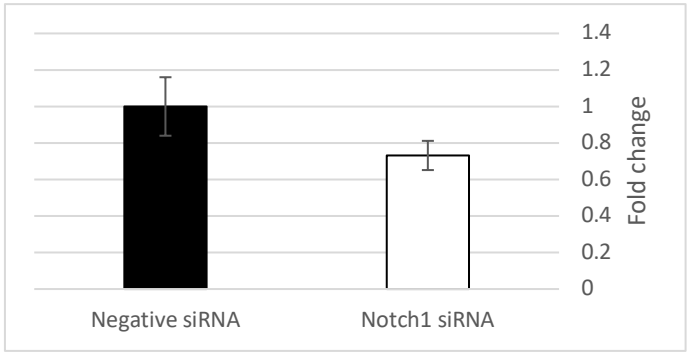


P = 0.01652

Figure 3-8: *Notch1* knockdown leads to a decrease in *notch1* transcript.

Transcript expression levels of notch1 *hASCs transfected with* notch1 *siRNA for 72 hours analyzed via qRT-PCR. Data was normalized to GAPDH. N=3.*

Jagged1



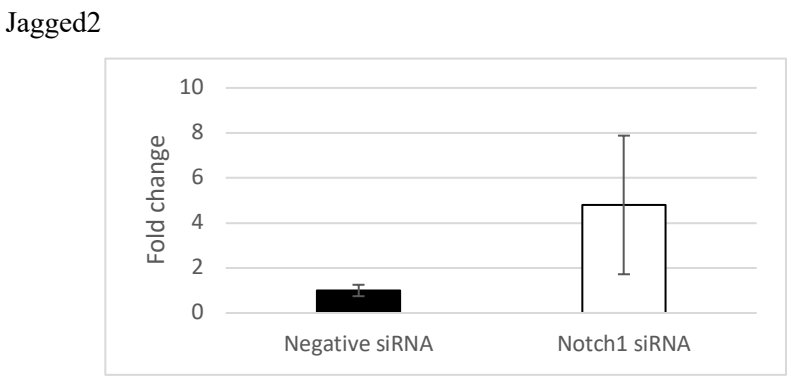
P = 0.208

Figure 3-9: *Notch1* knockdown leads to a decrease in *jagged1* transcript.

Transcript expression levels of jagged1 *hASCs transfected with* notch1 *siRNA for 72 hours analyzed via qRT-PCR. Data was normalized to GAPDH. N=3.*

Notch1 KD leads to an increase in jagged2 *transcript in self-renewing hASCs*

Since the knockdown of *notch1* appeared to result in a decrease in transcription of *jagged1*, the relationship between Notch1 and Jagged2 needed to be investigated. hASCs were transfected with *notch1* siRNA to determine if it had any effect on *jagged2* transcript. Human adipose stem cells were transfected with *notch1*- specific siRNA. The knockdown of *notch1* was validated using qRT-PCR. *Notch1* transcript was reduced in comparison to negative controls, with less than 40% transcript remaining after knockdown (Figure 3-8). The expression of *jagged2* was evaluated using qRT-PCR and showed a slight decrease in transcript compared to negative controls, with less than 80% remaining after knockdown, but the data was not significant (Figure 3-10).

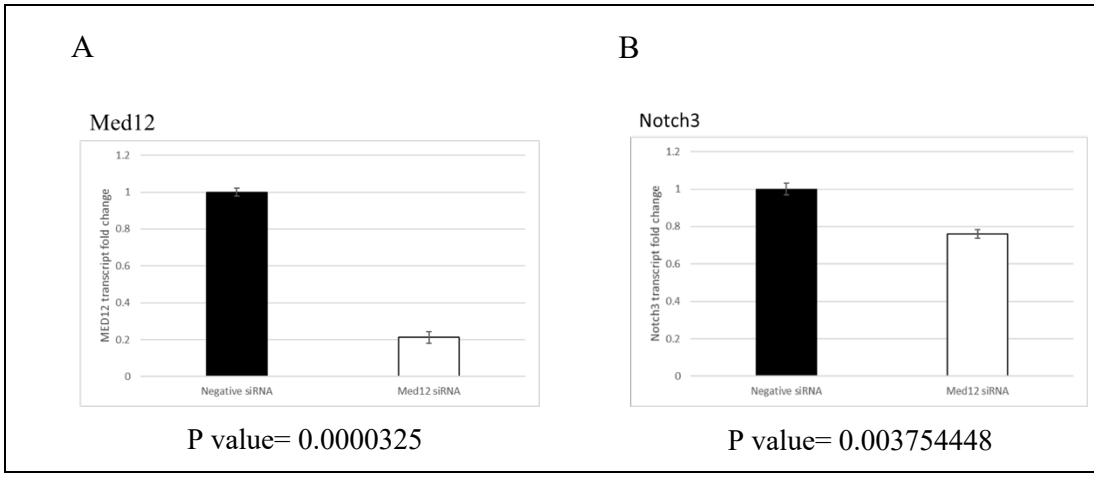


P = 0.286154

Figure 3-10: *Notch1* knockdown leads to an increase in *jagged2* transcript. *Transcript expression levels of* jagged2 *hASCs transfected with* notch1 *siRNA for 72 hours analyzed via qRT-PCR. Data was normalized to GAPDH. N=3.*

MED12 KD leads to decreased expression and activation of Notch3 in self-renewing hASCs

In order to further investigate the relationship between Notch and Mediator, we analyzed the influence of MED12's effect on Notch3. Human adipose stem cells were transfected with *med12*-specific siRNA. The knockdown of *med12* was validated using qRT-PCR and Western Blot. *Med12* transcript was reduced in comparison to negative controls, with less than 40% transcript remaining after knockdown (Figure 3-11A). The expression of *notch3* was evaluated using qRT-PCR and showed a slight decrease in transcript compared to negative controls, with less than 80% remaining after knockdown (Figure 3-11B). The knockdown of MED12 was further validated through Western Blot (Figure 3-12A and B). Notch3 was affected by the knockdown of MED12, where the knockdown caused an increase in full length (FL) Notch3 and a decrease in cleaved Notch3 (53) (Figure 3-12C and D).



P value= 0.0000325

P value= 0.003754448

Figure 3-11: *Med12* knockdown leads to reduction in *notch3* transcript. *A. The* med12 *knockdown was validated by qRT-PCR.* B. A*nalysis of* notch3 *transcript following the knockdown of MED12. Data was normalized to* gapdh. N = 3. *Image courtesy of Jaylen Mumphrey.*

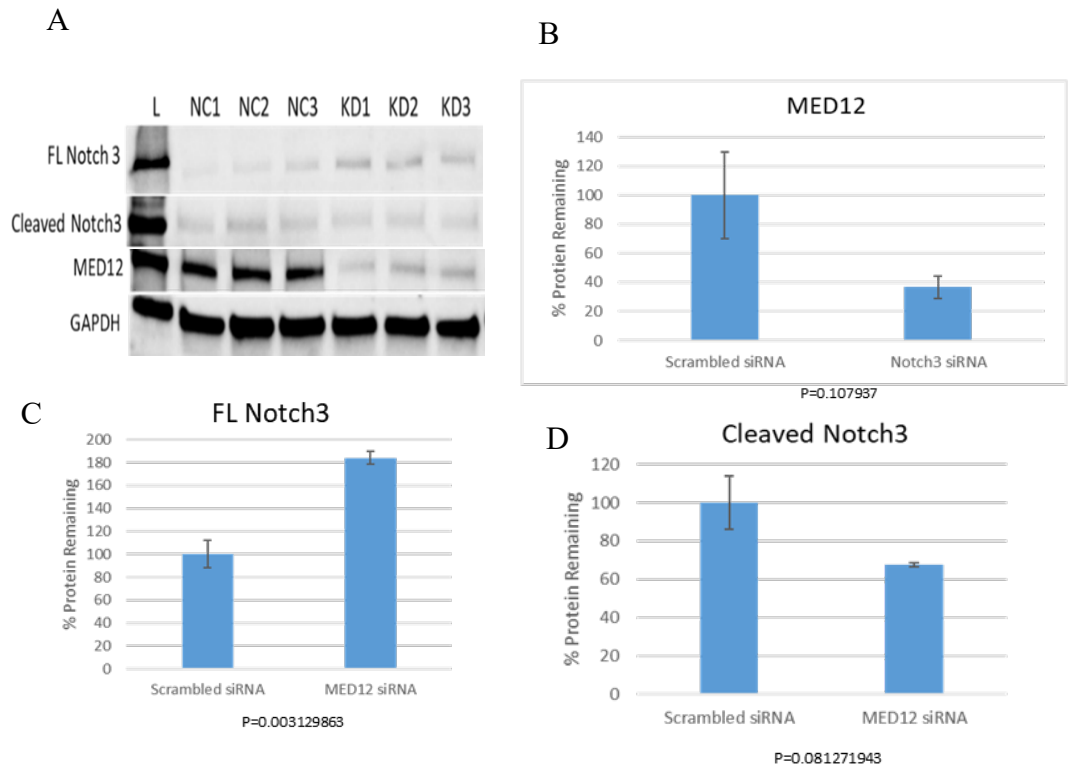
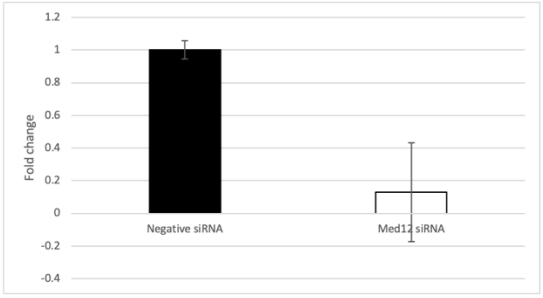


Figure 3-12: MED12 knockdown leads to reduction in Notch3 protein levels. *A. Effect of MED12 knockdown on full length (FL) Notch3, cleaved Notch3, and MED12. GAPDH was used as a loading control. B. ImageJ analysis of a Notch3 knockdown effects on Med12. P values were calculated with T Test, N=3. C. Effect of MED12 knockdown on full length (FL) Notch3. P values were calculated with T Test, N=3. D. Effect of MED12 knockdown on cleaved Notch3. P values were calculated with T Test, N=3. Images courtesy of Jaylen Mumphrey. NC Negative control, KD knockdown, N3 Notch3, FL Full Length, MED12 Mediator12.*

MED12 KD does not affect Jagged *ligand expression in self renewing hASCs*

In order to further study the relationship between Mediator and Notch, we monitored changes in the Notch signaling canonical pathway ligands Jagged1 and Jagged2. Human ASCs were again transfected with *med12*-specific siRNA, and *jagged1* and *jagged2* transcript were evaluated using qRT-PCR. *Med12* knockdown was validated using qRT-PCR and was significantly reduced in comparison to negative controls, with only 10% transcript remaining after knockdown (Figure 3-13). A slight increase in *jagged1* and *jagged2* transcript was observed following the knockdown of *med12*, but neither were significantly affected. These data suggests that neither *jagged1* nor *jagged2* transcript is affected by MED12 (Figure 3-14). Unfortunately, the effects of a MED12 knockdown on JAG and JAG2 protein expression was unable to be confirmed through Western Blot. MED12 was shown to have a decrease in expression confirming the knockdown, but JAG1 and JAG2 did not show up and were unable to be analyzed (Figure 3-15 and Figure 3-16).

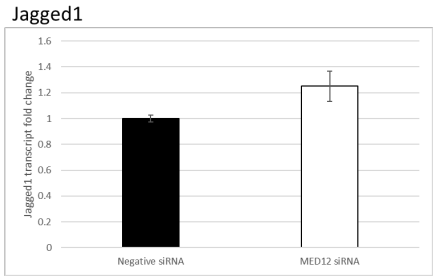
MED12



P value= 0.000114

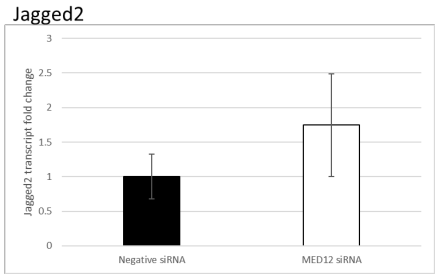
Figure 3-13: *Med12* knockdown leads to a decrease in *med12* transcript. *Transcript expression levels of* med12 *hASCs transfected with* med12 *siRNA for 72 hours analyzed via qRT-PCR. Data was normalized to GAPDH. N=3.*

A



P value= 0.106797

B



P value= 0.371344063

Figure 3-14: *Med12* knockdown does not significantly affect *jagged1* and *jagged2* transcript. *Transcript expression levels of hASCs transfected with* med12 *siRNA for 72 hours analyzed via qRT-PCR.* Jagged1 *(A) and* jagged2 *(B) expression levels slightly increased but neither were significant. Data was normalized to GAPDH. N=3.*

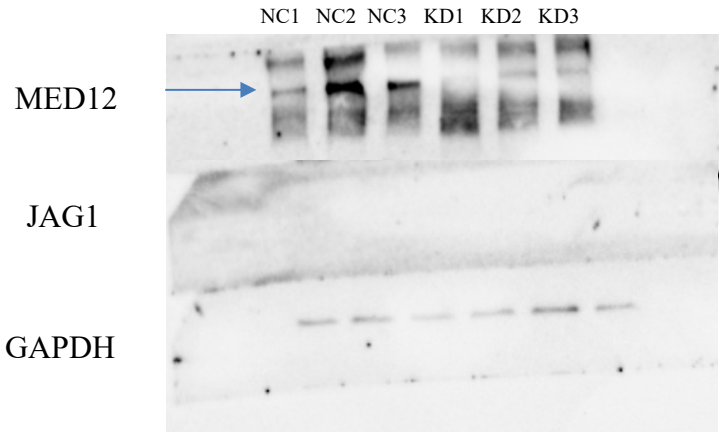


Figure 3-15: Identification of MED12 KD effect on JAG1 protein levels was unsuccessful. *Protein expression levels of hASCs transfected with* MED12 *siRNA via Western blot. Knockdown of* MED12 *was successful but identification of* Jagged1 *(*JAG1) *protein was unsuccessful. NC Negative control, KD knockdown, MED12 Mediator12, JAG1 Jagged1. N=3.*

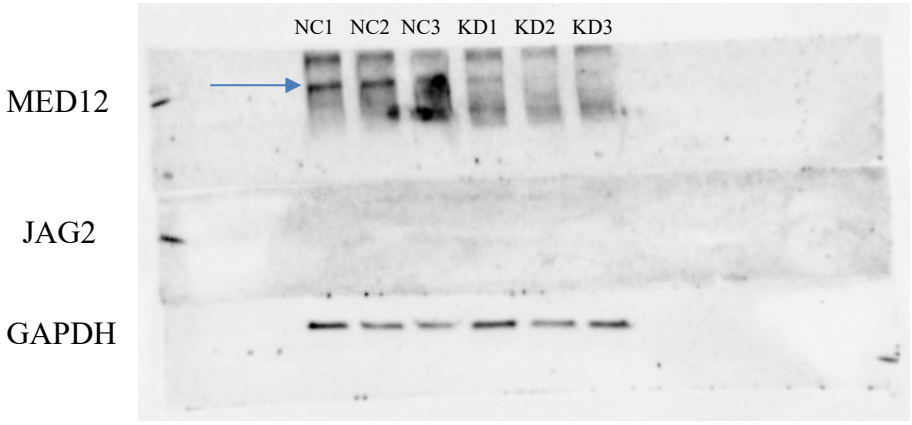
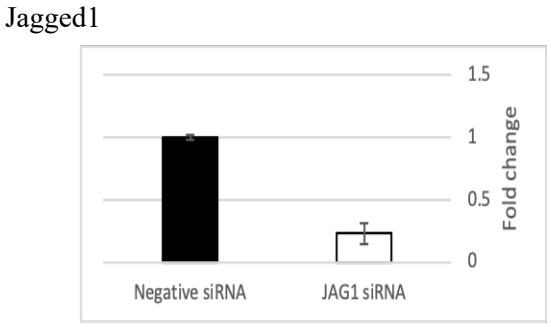


Figure 3-16: Identification of MED12 KD effect on JAG2 protein levels was unsuccessful. *Protein expression levels of hASCs transfected with* MED12 *siRNA via Western blot.* MED12 *knockdown was successful, but identification of* Jagged2 (JAG2) *protein was unsuccessful. NC Negative control, KD knockdown, MED12 Mediator12, JAG2 Jagged2. N=3.*

Jagged1 KD leads to a decrease in med12 *transcription in self renewing hASCs*

Since the knockdown of *med12* appeared to result in an increase in transcription of *jagged1*, hASCs were transfected with *jagged1*-specific siRNA to determine if it had any effect on MED12 transcript or protein. *Jagged1* levels were decreased in cell culture via siRNA transfections, and the knockdowns were validated using qRT-PCR. *Jagged1* transcript was significantly reduced in comparison to negative controls, confirming the knockdown (Figure 3-17). *Med12* transcript appeared to decrease significantly, suggesting a possible relationship between Notch signaling and *med12* expression (Figure 3-18).

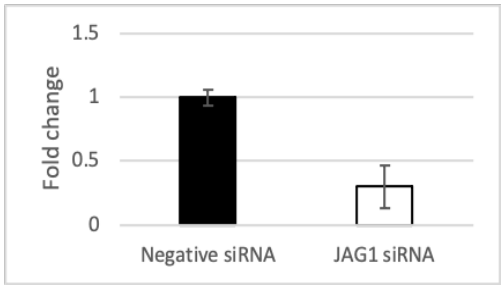


P value= 0.00092

Figure 3-17: *Jagged1* knockdown leads to a decrease in *jagged1* transcript.

Transcript expression levels of hASCs transfected with jagged1 *siRNA analyzed via qRT-PCR. Data was normalized to GAPDH. N=3.*

MED12



P value= 0.008868

Figure 3-18: *Jagged1* knockdown leads to decrease in *med12* transcript.

Transcript expression levels of hASCs transfected with jagged1 *siRNA analyzed via qRT-PCR.* Med12 *expression levels decreased significantly. Data was normalized to* gapdh*. N=3.*

Jagged1 KD leads to an increase in jagged2 *transcription in self-renewing hASCs*

Once both *jagged1* and *jagged2* expression levels were assessed following the knockdown of *med12*, we decided to investigate the relationship between the Jagged1 and Jagged2 ligands. *Jagged1* was once again knocked down using siRNA and levels of *jagged 2* were evaluated. *Jagged1* levels were decreased in cell culture via siRNA transfections, and the knockdowns were validated using qRT-PCR (3-17). *Jagged1* transcript was significantly reduced in comparison to negative controls, confirming the

knockdown. Conversely, *jagged2* transcript levels increased (Figure 3-19). These data suggests that Jagged1 may have an inverse relationship with Jagged2.

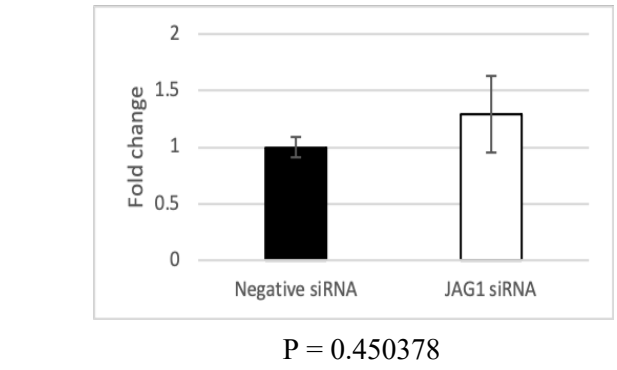
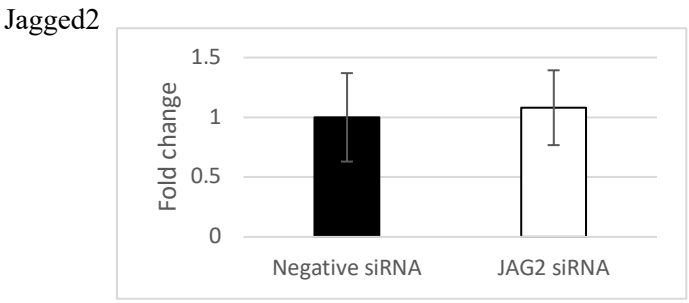


Figure 3-19: *Jagged1* knockdown leads to an increase in *jagged2* transcript. *Transcript expression levels of* jagged2 *hASCs transfected with* jagged1 *siRNA analyzed via qRT-PCR.* Jagged2 *expression levels increased, but not significant. Data was normalized to* gapdh*. N=3.*

Influence of Jagged2 KD on med12 *transcription in self renewing hASCs could not be determined*

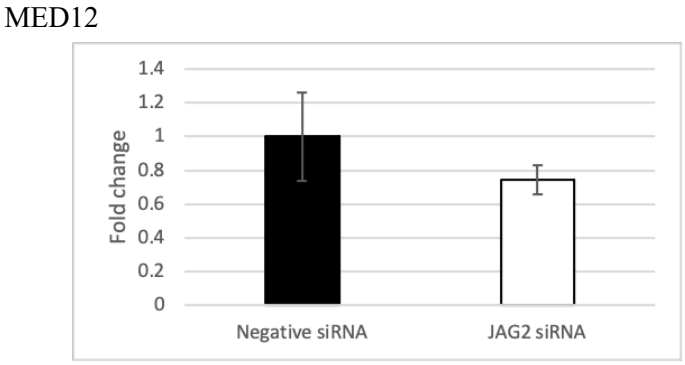
Since the data suggested the knockdown of *med12* increases transcription of *jagged2*, the trend was further investigated by transfecting hASCs with *jagged2* siRNA to determine if it had any effect on MED12 transcript or protein. *Jagged2* levels were decreased in cell culture via siRNA transfections, but the knockdowns were unable to be validated using qRT-PCR, as *jagged2* transcript increased following siRNA transfection (Figure 3-20). Although *med12* transcript decreased in comparison to negative controls, it's relationship with Jagged2 remains undetermined since the *jagged2* knockdown was

not able to be validated (Figure 3-21). Because of this, Jagged2 assays need to be optimized and repeated. There are plans to continue working with Jagged2 to further analyze its role in Notch and Mediator interactions, but it may not be expressed at high levels.



P = 0.875875

Figure 3-20: *Jagged2* knockdown was unable to be confirmed. *Transcript expression levels of* jagged2 *hASCs transfected with* jagged2 *siRNA analyzed via qRT-PCR. Data was normalized to* gapdh. *N=3.*



P = 0.487484

Figure 3-21: *Jagged2* knockdown was unsuccessful, therefore its effect on *med12* transcript is undetermined. *Transcript expression levels of* med12 *hASCs transfected with* jagged2 *siRNA analyzed via qRT-PCR. Data was normalized to* gapdh*. N=3.*

The influence of Jagged2 KD on jagged1 transcription in self renewing hASCs could not be determined

Finally, the relationship between Jagged1 and Jagged2 needed to be investigated. This was done by first transfecting hASCs with *jagged2*-specific siRNA to determine if it had any effect on Jagged1 transcript or protein. Jagged*2* levels were decreased in cell culture via siRNA transfections, but the knockdowns were unable to be validated using qRT-PCR and western blot. The *jagged2* knockdown was unable to be confirmed, as *jagged2* transcription increased following transfection of the siRNA (Figure 3-20). *Jagged1* transcript did not change in comparison to negative controls, which was to be expected since the knockdown was not able to be validated (Figure 3-22). These data prevented us from further investigating the relationship of the Jagged ligands with each other. Because of this, Jagged2 assays needs to be optimized and repeated.

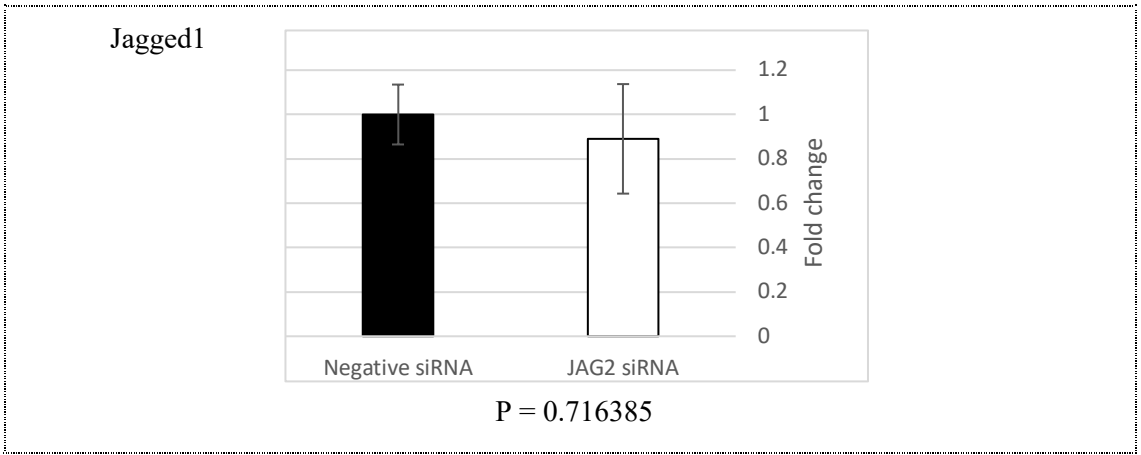


Figure 3-22: *Jagged2* knockdown was unable to be confirmed, therefore its effect on *jagged1* transcript is undetermined. *Transcript expression levels of* jagged1 *hASCs transfected with* jagged2 *siRNA analyzed via qRT-PCR. Data was normalized to* gapdh*. N=3.*

CHAPTER 4

CONCLUSION AND FUTURE WORK

4.1 Conclusion

Stem cells have tremendous potential to aid in regenerative medicine and the treatment of injury and degenerative disease. In order to realize this potential, we must first understand the factors that regulate cell state. Here we continued work to understand the relationship between Notch signaling pathway and the Mediator complex in attempt to uncover the mechanisms responsible for regulating stem cell fate.

In order to study the influence of specific factors on human adipose-derived stem cells, we used target-specific siRNA-mediated knockdowns. These knockdowns target mRNA to temporarily diminish expression of the targeted gene transcripts and ultimately

decrease protein expression, allowing researchers to determine the function or role of a specific gene and its protein product. I used knockdowns to isolate and study MED12, Notch1, Notch3, Jagged1, and Jagged2 and their influence in human adipose stem cells.

The relationship between *notch1* and *notch3* was explored via qRT-PCR. We observed that the knockdown of *notch1* in hASCs results in increased *notch3* transcription. Although the data was not significant, it indicates a potential inverse relationship between the Notch receptors. These experiments need to be repeated for further validation using Western blot and analysis of protein expression. In addition, there are plans to continue working with Notch3 to further analyze its role in Notch and Mediator interactions as well.

The relationship between *notch1* and the *jagged* ligands was also explored via qRT-PCR. The knockdown of *notch1* decreased *jagged1* transcription but increased *jagged2* transcription, indicating the Jagged ligands have an inverse relationship with each other, and encouraging us to further investigate their relationship.

The relationship between *notch1* and *med12* was explored via qRT-PCR. The knockdown of *notch1* led to a decrease in *med12* transcript, but the data was not significant. Previous data in lab showed similar results, but more experiments need to be run to confirm these data, as well as analysis of protein expression through Western blot.

We observed that the knockdown of MED12 in hASCs results in diminished Notch3 expression. We also observed that the knockdown of *notch3* reduced the amount of *med12* transcript in hASCs. These data indicate a potentially significant relationship between MED12 and Notch3 that may have a role in regulating cell state.

The relationship between *med12*, *jagged1*, and *jagged2* was also explored via qRT-PCR. We found that *med12* knockdown does not affect *jagged1 or jagged2* ligand transcription in self-renewing hASCs. Although there was a slight increase in *jagged1* and *jagged2* transcript observed following the knockdown of *med12*, neither were significantly affected.

Jagged1 knockdowns were performed to evaluate the influence of this ligand on *med12* and *jagged2* transcription. After the knockdown of *jagged1* was confirmed by qRT-PCR, *med12* transcript appeared to decrease significantly. This suggests a possible relationship between Notch signaling and *med12* expression. We also found that the knockdown of *jagged1* leads to an increase in *jagged2* transcription in self-renewing hASCs, suggesting an inverse relationship between the two ligands. It is possible that MED12 expression is affected by JAG1 but not by JAG2, however, more experiments need to be run to confirm this (Figure 4-1)

There are plans to continue working with Jagged2 to further analyze its role in Notch and Mediator interactions, but I believe the *jagged2* knockdown may not have worked because Jagged2 is not highly expressed in hASCs. If Jagged2 is already minimally present to start, isolating it becomes a challenge. It may be beneficial in the future to see if it is present in a higher volume in other cell types, such as BMSCs.

Gene	NOTCH1	MED12 KD	NOTCH3 KD	JAG1 KD	JAG2 KD
Notch1	↓	↓	TBD	Inconclusive	TBD
Notch3	↑	↓	↓	Inconclusive	TBD
MED12	No Effect	↓	↓	↓	TBD
Jagged1	↓	No Effect	TBD	↓	TBD
Jagged2	↑	No Effect	TBD	↑	↓

B

Gene	MED12 KD	NOTCH3 KD	JAG1 KD	JAG2 KD
FL Notch3	↑	↓	TBD	TBD
Cleaved Notch3	↓	↓	TBD	TBD
MED12	↓	↓	Inconclusive	Inconclusive
Jagged1	↑	↓	↓	TBD
Jagged2	↑	↓	TBD	↓

Figure 4-1: Summary figure that shows the relationships of MED12, Notch1, Notch3, Jagged1, and Jagged2. *A. Transcript summary figure. B. Protein expression summary figure. MED12 knockdown leads to decreased expression and activation of Notch3; MED12 may be required to regulate the transcript and expression of Notch3. Notch3 knockdown leads to decrease MED12 transcript and*

protein; Notch3 may be required to maintain appropriate levels of MED12 expression. Jagged1 knockdown leads to a decrease in MED12 transcript, but has no discernable effects on protein expression. More research is needed to investigate the relationship between Jagged1 and Notch3.

Unfortunately, not all of my work was able to be confirmed by Western Blot and analysis of protein expression. Specifically, there were challenges in assays related to both Jagged 1 and Jagged2, as well as Notch3. There were several adjustments made in order to optimize the Western blot protocol, including altering the PAGE concentration from a 4-15% gel to a 7.5% gel. The 4-15% gels are standard in our lab, and detect 15-250kD. Jagged1 bands show at 180kD, and Jagged2 bands show at 150kD, so in theory they should be detectable by our standard gels. Notch3 is expressed at 300kD, so by switching gels we were hoping to be able to capture all of these gene's expressions, especially since they are on the higher end. We also hoped switching to a gel with a lower PAGE concentration would allow our gels to run for longer than the standard 90 minutes, losing the lower molecular weight proteins while allowing resolution and visualization of higher molecular weight proteins. In addition, we also adjusted sample boiling time both increasing time from the standard 10-minute period to 15 and 20 minutes, and decreasing time to 8, 5, and 3 minutes. In a final attempt to see Jagged1 and Jagged2, the concentration of antibody used was increased. Despite these protocol modifications, we continued to be unable to detect Jagged2 protein and suspect that expression might just be too low in hASCs to detect or necessarily warrant further investigation.

4.2 Future Directions

Work in the Newman lab continues to be performed to validate these results and look further into the role of these proteins in self-renewal. Specifically, since preliminary data shows an influence of MED12 on Jagged1 and Jagged2 transcription, we need to validate changes at protein level. We are also planning to examine MED12 knockdown influence on other Notch ligands such as DLL1, DLL3, and DLL4. If we determine that one of the five Notch ligands is significantly affected by the MED12 knockdown, either increased or decreased, the next objective will be to knockdown that specific ligand and work to determine the influence of that ligand on MED12, Notch1, or Notch3 expression and hASC self-renewal. We can also observe the non-canonical Notch signaling pathway by knocking down MED12 and determining the potential role of non-canonical ligands, specifically DLK1 and DLK2, in controlling hASC self-renewal. Finally, in order to determine the global changes in gene expression following the knockdown of any one of these critical transcriptional regulatory, we could perform microarray analysis or RNA-Seq. Understanding of how we can control the fate of stem cells, will allow manipulation so that stem cells may be used to their full potential in regenerative medicine.

4.3 Significance

Researchers have been looking for an alternative to bone marrow-derived mesenchymal stem cells for therapeutic usage due to the highly invasive donation procedure, as well as the decline in MSC number and differentiation potential with increasing age. Because of this, human adipose derived stem cells are being used in clinical trials and research, and are proven to work as alternative sources of mesenchymal

stem cells. Human ASCs have only recently become more common in clinics and research labs, meaning that their self-renewal and differentiation mechanisms are not yet well understood. Our work seeks to uncover mechanisms of cell state regulation that will allow for greater clinical application of these cells in the future.

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