

**NOTCH AND MEDIATOR WORK TOGETHER TO DIRECT HASC
SELF-RENEWAL**

by

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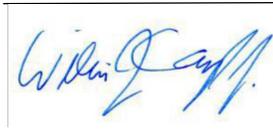
entitled **NOTCH AND MEDIATOR WORK TOGETHER TO DIRECT HASC**
SELF-RENEWAL

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ABSTRACT

Stem cells are unique in that they possess totipotent, pluripotent, or multipotent differentiation capabilities and can also self-renew. Stem cells are in either a state of self-renewal or differentiation, but never both. If we are able to gain a full understanding of the underlying mechanisms that allow stem cells to remain self-renewing and multipotent, we will better be able to control the fate of cells, ultimately allowing stem cells to be used to their full capabilities in regenerative medicine. My research focuses on MED12 and its effects on Notch signaling. Here we describe the expression profile and activity of MED12, Notch1, and Notch3 in self-renewing human adipose stem cells and determine the impact that MED12 knockdown has on Notch1 and Notch3 expression and activity in self-renewing hASC's. We hypothesized that MED12 has a critical role in regulating transcription, while Notch signaling has a role in directing cell fate commitment. We observed that the knockdown of MED12 in hASCs has no effect on the protein expression of Notch1. We also observed that the knockdown of Notch3 does reduce the amount of MED12 transcript expressed in hASCs. To date, our data suggests that MED12 has no effect on Notch1 expression but does affect Notch3 expression. Also, the knockdown of Notch3 directly effects MED12 transcript. This data indicates a unique role for MED12 and a relationship with Notch3 in regulating cell state, leading us one step closer to realizing the clinical potential of these cells and using them in novel cell-based and tissue engineering therapies.

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Author: Jaylen Mumphrey

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DEDICATION

This thesis is dedicated to anyone who has ever doubted themselves, if a normal guy like me can write a Thesis and get a master's degree, you can do anything.

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CHAPTER 1

INTRODUCTION

1.1 Stem Cells

Stem cells are unique in that they possess totipotent, pluripotent, or multipotent differentiation capabilities and can also self-renew (Miana and Prieto González, 2018). Totipotent stem cells (zygote) have the ability to differentiate into any cell in the body as well as extra embryonic tissues, including placenta (Sobhani *et al.*, 2017). Pluripotent stem cells (embryonic stem cells and induced pluripotent stem cells) have the same abilities to form an adult organism but unlike totipotent stem cells, lack the ability to form extra embryonic tissue (Sobhani *et al.*, 2017). Multipotent stem cells (i.e adult stem cells) are the most limited of the three types of stem cells, as they can only differentiate into cells of a defined lineage (Sobhani *et al.*, 2017). Although most limited in their differentiation potential, multipotent adult stem cells possess the most therapeutic potential as they can be used in an autologous manner and offer unique immunomodulatory properties in addition to their differentiation potential.

Mesenchymal stem cells have the ability to differentiate into multiple cell types of the mesodermal lineage such as osteocytes, chondrocytes, myocytes, and adipocytes (Daniel E Shumer, Natalie J Nokoff, 2017) (**Figure 1-1**). Human mesenchymal stem cells are found in bone marrow, adipose tissue, amniotic fluid, and the umbilical cord (NITKIN and BONFIEL, 2014). In addition to the range of tissues from which these cells can be

isolated, they are also not accompanied by the public controversies that are associated with embryonic stem cells, making them a more acceptable source for research and clinical application. When using these cells for regenerative medicine, bone marrow-derived mesenchymal stem cells have been at the forefront of current studies due to their long history of clinical usage in the form of bone marrow transplants (Sobhani *et al.*, 2017). Despite their clinical advantages, in order to obtain human bone marrow-derived mesenchymal stem cells, an invasive and sometimes painful procedure needs to be performed, making them a challenging cell source for additional therapeutic usage.

Human adipose stem cells offer an alternative to bone marrow-derived mesenchymal stem cells that are both easily accessible and abundant using minimally invasive procedures (Miana and Prieto González, 2018). Offering much of the same clinical potential as bone marrow-derived cells, adipose stem cells are derived from adipose tissue through subcutaneous lipoaspiration, a less invasive and less painful harvesting protocol. Human adipose stem cells are currently being used in cancer treatment centers and in multiple clinical trials. The clinical trials include attempts to repopulate areas of the cardiac muscle after a heart attack in both animal models and humans and using the multipotency of hASC's as a regenerative treatment to counteract different conditions such as such as coronary disease, osteoporosis, bone regeneration of the jaw (Miana and Prieto González, 2018).

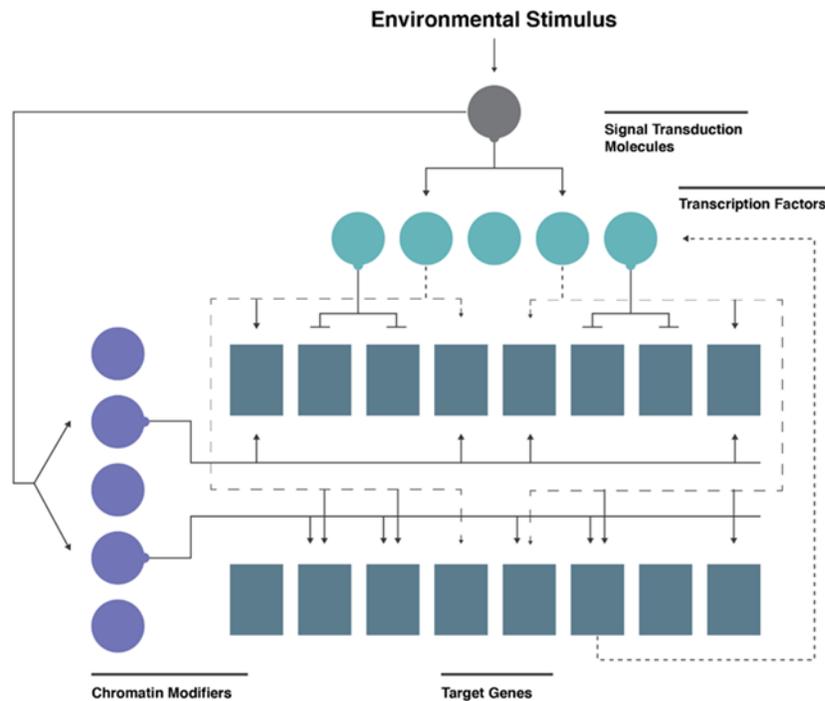


Figure 1-1: Gene expression is controlled by a complex regulatory network. Environmental stimuli initially activate signal transduction pathways. Once signaling components are activated, they can directly affect transcription factors and chromatin modifiers to initiate or inhibit transcription. When 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.

1.1.1 Notch Signaling

The Notch signaling pathway is an evolutionarily conserved cell fate determination pathway that controls cell differentiation, proliferation, and apoptosis (Nueda *et al.*, 2018). The evolutionary conserved Notch signaling pathway functions as a mediator of short-range cell-cell communication (Kopan and Ilagan, 2009). The ectodomain of a Notch receptor can read information about the state of neighboring cells as it recognizes ligands that are expressed at their surface, whereas its intracellular domain, upon activation, acts as a transcriptional regulator that adjusts cell fate according to the state of the neighboring cells (Henrique and Schweisguth, 2019).

The Notch signaling pathway in humans consists of five canonical transmembrane Notch ligands Delta-like ligand 1 (DLL1), DLL3, DLL4, Jagged 1, Jagged 2, and four transmembrane Notch receptors: Notch1, Notch2, Notch3, and Notch4 (Nueda *et al.*, 2018). Each Notch receptor has three functional domains: The Notch extracellular domain, the Notch transmembrane domain, and the Notch intracellular domain (NICD). Finally, there are two forms of the Notch signaling pathway: the NICD /CSL-dependent transcription of Notch target genes known as the canonical Notch signaling cascade, and the CSL-independent cellular responses, referred to as the non-canonical Notch signaling cascade. The multiple components of the pathway and the different signaling mechanisms make this one of the more complex biological pathways (Katoh and Katoh, 2020).

The Notch signaling pathway directly couples 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. Although ligands expressed by neighboring cells have a trans-activation ability, ligands expressed in cis may have an inhibitory function, which acts to maintain the state of the signal-sending cell, keeping them from becoming signal-receiving cells. Once trans-activation is initiated, it leads to a structural change in the Notch receptor that exposes the buried cleavage site (S2) making it available to the metalloproteases of the ADAM/TACE family. Cleavage at this S2 site then generates a membrane-tethered form of Notch that is further cleaved by γ -secretase complex to release the Notch intracellular domain (NICD). The NICD is then translocated to the nucleus to activate gene transcription (**Figure 1-2**). Notch has been

confirmed to have a role in human development and disease making it a significant factor in the study of stem cells and determination of cell fate (Henrique and Schweisguth, 2019).

Stem cells are able to both clone themselves (self-renew) or differentiate into multiple cell types depending on how gene expression is regulated. Stem cell fate can be regulated by altering the function and expression of the general transcription factors (GTFs) that assemble the transcription preinitiation complex (PIC) on gene promoters. GTFs also assemble activators and repressors that bind to gene regulatory elements located 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 (Straub, Venigalla and Newman, 2020).

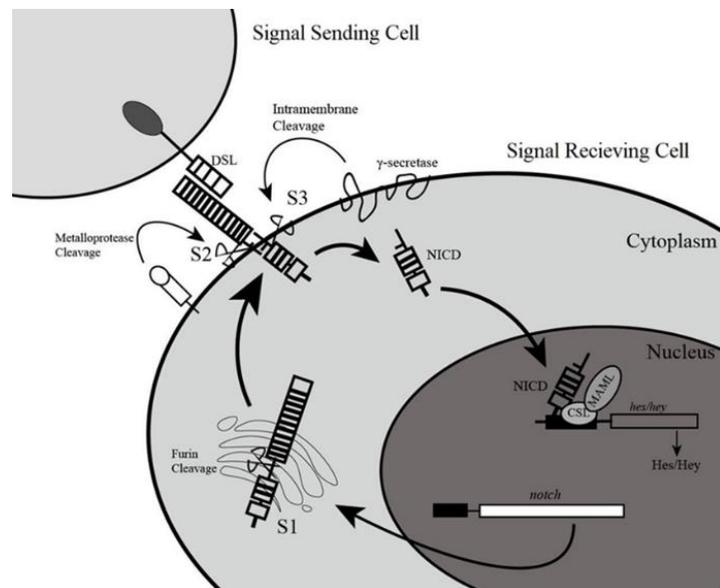


Figure 1-2: Notch signaling pathway

1.1.2 Mediator Complex

The Mediator complex plays a vital role in the regulation of cell-type specific transcription in eukaryotic cells. The Mediator complex is a large protein complex of 30 subunits arranged in four modules: head, middle, tail, and kinase (CDK8) (**Figure 1-3**) (Park *et al.*, 2018). The head and middle modules contain the most highly conserved subunits and maintain cell viability and overall gene expression, while the tail recruit's specific transcription factors to direct and maintain lineage commitment. Indicative of its functional versatility, Mediator is implicated in regulating at least some aspect of many fundamental processes involved in transcription, including transcription initiation, transcription elongation, chromatin architecture and enhancer-promoter gene looping (Allen and Taatjes, 2015). In the Mediator complex, CDK8 attaches to Mediator core complex and activates or suppresses transcription via RNA polymerase II (Park *et al.*, 2018). The kinase module also functions independently of the Mediator complex. RNAPII, TFIIF, histone H3, and MED13 have all been listed as substrates for the CDK8 kinase (Knuesel *et al.*, 2009). CDK8 is considered both an oncogene and a tumor suppressor and promotes cell growth via the serum response pathway (Fant and Taatjes, 2019). In stem cells, Mediator is the factor that controls regulation of transcription and in controlling transcription, Mediator controls the fate of stem cells. CDK8 is a part of the 30 subunit Mediator (MED) complex, which acts as a molecular bridge to mediate transduction of regulatory signals (Leach and Leach, 2017). It completes this task by using a module that consists of Cyclin C, Med12, and Med13. The Mediator complex is composed of multiple important factors that are necessary for the regulation of transcription in eukaryotic cells (**Figure 1-1**).

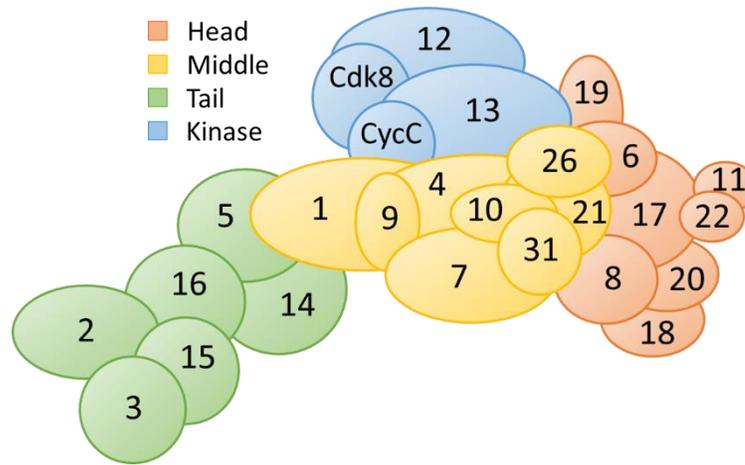


Figure 1-3: *The Mediator complex*

1.1.3 Motivation

Stem cells are in either a state of self-renewal or differentiation, but never at the same time. If we are able to gain a full understanding of the underlying mechanisms that allow stem cells to remain self-renewing and multipotent, we will better be able to control the fate of cells, which is the key to regenerative medicine becoming a reality. With conditions such as degenerative eye disease, stem cell treatment has the potential to replace lost neurons, restore neural circuits, and even induce the growth of new connections in the eye's neural pathways (Mead *et al.*, 2015). If we can extend this to other conditions like Muscular dystrophies (MD) and Parkinson's disease, we will be able to make a significant change to the medical world by not only stopping the effects of these degenerative diseases, but also reversing the damage that the disease has already caused.

The central dogma of molecular biology describes the process of transcribing DNA to RNA and translating RNA to proteins. Transcription is a vital process in all living cells and is ultimately what determines the fate of a cell. If we can gain an understanding of the mechanisms surrounding transcriptional regulation in adipose stem cells, we can control

the fate of the cell to stop stem cells from becoming cancerous and direct their fate for use in cell-based therapies, tissue engineering, and regenerative medicine.

Both Mediator and the Notch signaling pathway work in unison to control the fate of ASCs through the careful regulation of gene expression. Although critical to controlling healthy tissue, the relationship between Mediator and Notch remains poorly defined. A recent study suggests a possible influence of MED12 on Notch signaling in chronic lymphocytic leukemia cells (Wu *et al.*, 2017) found that in order to activate or repress transcription, CDK8 is recruited to the MED12 subunit. 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 aberrant regulation and absence of control leads to the activation of Notch, which causes uncontrolled cell differentiation or proliferation. 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.

CHAPTER 2

METHODS

2.1 Cell culture

2.1.1 Thawing of cells.

Human adipose stem cells (Obatala #70926) were removed from liquid nitrogen and thawed in a 37°C water bath. Cells were transferred into a 15 mL conical containing 4mL of pre-warmed Complete Culture Media (CCM) composed of 203.75mL of Minimum Essential Medium, 41.25mL of Fetal Bovine Serum, 2.5mL of L-Glutamine, and 2.5mL of Penicillin-Streptomycin. Cells were centrifuged at 1500 RPM for 8 minutes. The supernatant was removed, and cells were resuspended in 1mL of CCM. Cells were plated the cells onto 10cm tissue culture plates with 9mL of CCM. Media was changed 24 hours after the initial thaw, and every 48 hours after until cell confluency reached 70-80%.

2.1.2 Passaging of cells.

When the cells reached 70-80% confluence they were rinsed with pre-warmed phosphate buffered saline (PBS). 3mL of Trypsin was added to the plate and cells were incubated for 3 minutes at 37°C. 6mL of CCM (or double the amount of trypsin) was then added to the plate and the cells were collected into a conical tube for centrifugation at 1500 RPM for 10 minutes. Supernatant was aspirated and cells were resuspended in 1mL of pre-warmed CCM. Cells were counted by mixing 20µL of trypan blue and 20µL of the cell solution. Once mixed, 10µL of the cell-trypan solution was added onto both sides of the

FL hemocytometer and the slide was inserted into the cell counter. After cells were counted, they were then passaged onto 6cm plates. (**Table 2-1**)

Table 2-1: *Number of cells plated onto each plate.*

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

2.1.3 Transfection of cells.

When the cells reached 20-40% confluency they were transfected with a control siRNA or a target gene siRNA. 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 changed 24 hours after the transfection using CCM and RNA or protein was collected 72 hours after the transfection.

2.2 RNA

2.2.1 RNA collection.

72 hours after the transfection, plates were rinsed with PBS and RNA was collected in 500µL of Trizol /6cm plate. Plates were gently scraped using a cell scraper and the solution was collected and stored in a -80°C freezer.

2.2.2 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 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.

2.2.3 cDNA synthesis

cDNA was synthesized for each sample using 1 µg of RNA and qScript cDNA supermix following manufacturer protocol.

2.3 RT-PCR

2.3.1 Endpoint RT-PCR.

Primers (**Table 2-2**) and quality of cDNA were confirmed by endpoint RT-PCR using GoTaq following manufacturer's protocols.

Table 2-2: Primer list

Gene	Forward Sequence	Reverse Sequence	Product size (bp)	Temperature	Cycles
MED12	CGAAAAGGGA CAGCAGAAAC	CCCATCCTCCC CACCTAAGA	87	60	30
Notch1-2	CACGCTGACG GAGTACAAGT	GGCACGATTTC CCTGACCA	56	60	35
Notch3	CACCCTTACCT GACCCCATCC	TTCGGACCAGT CTGAGAGGGA	81	60	35
Jagged1	GGCACGCGTC ATTGTGTTAC	TGCGCAGCCTT TTATTCCCT	119	60	35
Jagged2	TGGACGCCAAT GAGTGTGAA	CCCGGGATGC AATCACAGTA	91	60	35
DLL-1	CAGCAAGCGT GACACCAAG	CTTTCAGATGC TTCTCCACCCC	93	60	35
DLL-3	GTCCGAGCTCG TCCGTAGA	AAAAGGGGCG TCGCTACC	109	60	35
DLL-4	GTCCAACGTG GCAAACAGC	TGGCTTTTCAC TGTGTAACCG	148	60	35
GAPDH-2	ACTAGGCGCTC ACTGTTCTCT	CAATACGACC AAATCCGTTGA CT	99	60	30

2.3.2 Quantitative reverse transcriptase PCR (qRT-PCR)

qRT-PCR was performed using Power SYBR® Green PCR Master Mix and run using an Applied Biosystems StepOne Plus machine. Gapdh was used for normalization of qRT-PCR results. Samples were run in triplicate.

2.4 Protein analysis

2.4.1 Protein extraction and collection.

When cells reached 70-80% confluency plates were rinsed with cold PBS. Cells were collected in lysis solution composed of RIPA buffer and protease inhibitor and transferred into 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.

2.4.2 Protein concentration.

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

2.4.3 Western blots

Protein samples were boiled and loaded into a 4-15% polyacrylamide gel based off of the Bradford assay results. 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, then probed overnight with a primary antibody (**Table 2-3**). The membranes were then 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
Notch3	1:1000	Cell signaling	5276S
GAPDH	1:3000	abcam	Ab9485
Goat pAb to Rb IgG (HRP)	1:10000	abcam	Ab6721

CHAPTER 3

RESULTS

3.1 Introduction

Stem cells are unique in that they have the ability to differentiate and self-renew. Human adipose stem cells are a multipotent stem cell that possess therapeutic potential to treat multiple diseases and to help in regenerative medicine. If we are able to gain a better understanding of how we can control the fate of hASCs, this will allow stem cells to be used to their full potential in regenerative medicine. Here we seek to gain an understanding of hASCs by focusing on the Mediator complex and its effects on Notch signaling. Mediator is highly conserved in eukaryotic cells and directly controls transcription by interacting with signaling pathways like Notch.

The Mediator complex plays a vital role in the regulation of cell-type transcription in eukaryotic cells (Straub, Venigalla and Newman, 2020). There are four modules of the Mediator complex: the head, middle, tail, and the kinase. Our focus is on the kinase module that consist of CDK8, Cyclin C, MED12, and MED13 (Park *et al.*, 2018). We are specifically interested in MED12 and its role in regulating and controlling transcription in hASCs as MED12 is a critical part of the Mediator complex implicated in a number of human developmental defects and disease. Similarly, The Notch signaling pathway is an essential regulatory pathway in development, cell differentiation, proliferation, and apoptosis (Nueda *et al.*, 2018).

In this chapter I will discuss how MED12 effects Notch1 and Notch3 in hASCs. We report on the expression and activity of MED12, Notch1, and Notch3 in self-renewing human adipose stem cells and determine the impact that MED12 knockdown has on Notch1 and Notch3 expression and activity in self-renewing hASC's. We hypothesized that MED12 has a critical role in regulating transcription, while Notch signaling has a role in directing cell fate commitment. We observed that the knockdown of MED12 in hASCs has no effect on the protein expression of Notch1. We also observed that the knockdown of Notch3 does reduce the amount of MED12 transcript expressed in hASCs. To date our data suggests that MED12 has no effect on Notch1 expression but does affect Notch3 expression. In addition, the knockdown of Notch3 directly effects MED12 transcript. This data indicates a unique role for MED12 and a relationship with Notch3 in regulating cell state, leading us one step closer to realizing the clinical potential of these cells and using them in novel cell-based and tissue engineering therapies.

3.2 Results

MED12 KD has no effect on Notch1 in self-renewing hASCs

In order to understand the influence of the Mediator complex, specifically MED12 on the highly conserved Notch signaling pathway we transfected hASCs with MED12 siRNA so that we could observe how the transfections affected Notch1. MED12 levels were decreased in cell culture via siRNA transfections and the knockdowns were validated using qRT-PCR and western blot. MED12 transcript was significantly reduced in comparison to negative controls while there was no significant effect on the levels of Notch1 transcript (**Figure 3-1**). In addition, MED12 protein levels were significantly reduced while there was once again no significant effect on the Notch1 protein levels

(Figure 3-2). Although both MED12 and Notch1 have a role in determining cell state, this data suggests that MED12 is not responsible for regulating the transcript and expression of Notch1.

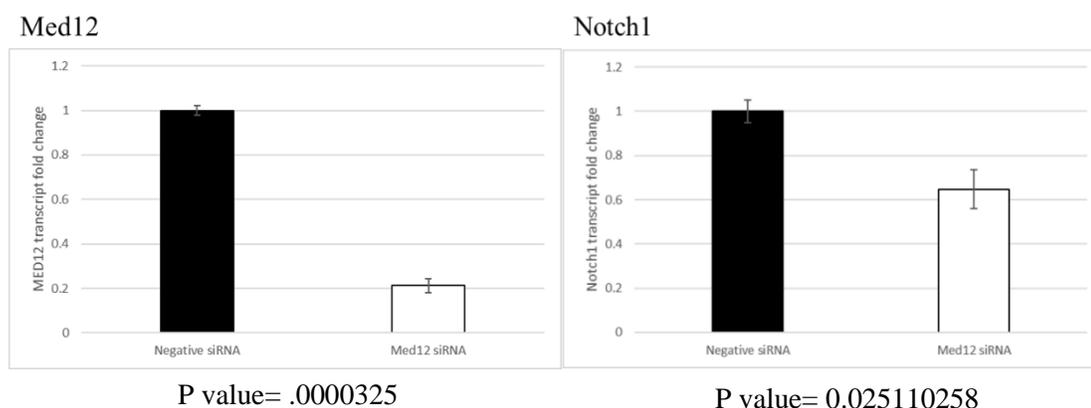


Figure 3-1: MED12 knockdown leads to decreased Notch1 transcript. MED12 siRNA effectively knockdowns MED12 transcript and diminishes expression of Notch1. Transcript levels of MED12 (left) and Notch1 (right) in hASCs transfected with MED12 siRNA analyzed via qRT-PCR. There was not a significant reduction in the Notch1 transcript levels. Data was normalized to GAPDH. P values were calculated with T Test, n=2.

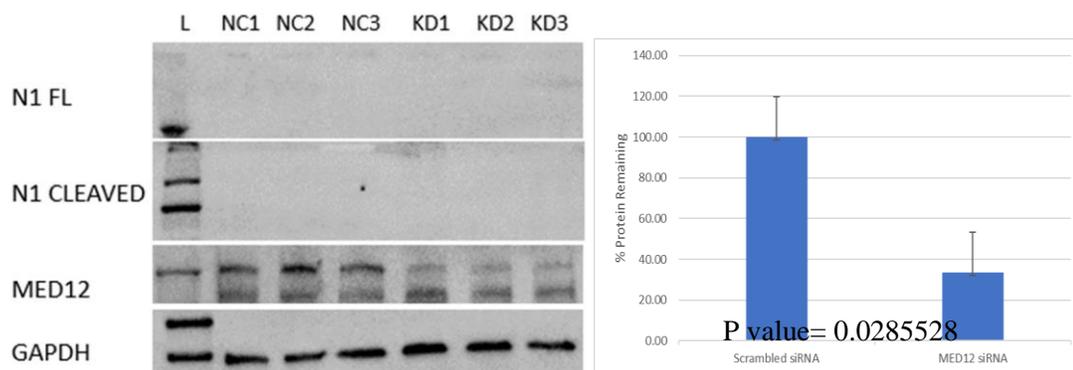


Figure 3-2: MED12 knockdown has no effect on Notch1 protein levels. Protein expression levels of hASCs transfected with MED12 siRNA via Western blot. (Left) The MED12 knockdown was validated while the identification of Notch1 was unsuccessful. ImageJ data showing the MED12 knockdown Western blot quantified using ImageJ software. Western blot was normalized to GAPDH. P values were calculated with T Test, N=2. (Right)

MED12 KD does affect Notch3 expression in self-renewing hASCs

In continuation of trying to understand the influence of the Mediator complex, specifically MED12 on the highly conserved Notch signaling pathway hASCs were transfected with MED12 siRNA so that we could observe how the knockdown of MED12 affected Notch3 expression and activity. MED12 levels were decreased in cell culture via siRNA transfections and the knockdowns were validated using qRT-PCR and western blot. MED12 transcript was significantly reduced in comparison to negative controls and there was also a significant reduction in the Notch3 transcript levels (**Figure 3-3**). The MED12 protein levels were significantly reduced while there was an increase in full length Notch3 protein, and a decrease in cleaved (activated) Notch3 protein (**Figure 3-3**)(**Figure 3-4**). Granted, we know that MED12 and Notch3 have a role in determining cell state, this data suggest that MED12 is responsible for regulating the transcript and expression of Notch3.

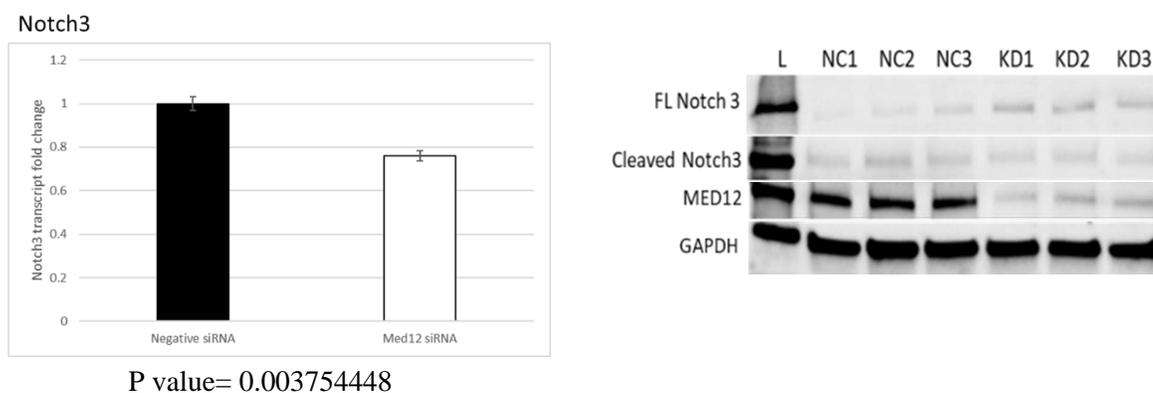


Figure 3-3: MED12 knockdown leads to decreased Notch3 transcript and an increase in full length Notch3 and a decrease in cleaved Notch3. Transcript levels in hASCs transfected with MED12 siRNA analyzed via qRT-PCR. The MED12 knockdown was validated (**Figure 3-1**). There was a significant reduction in the Notch3 transcript levels. Data was normalized to GAPDH. P values were calculated with T Test, N=2. (Left) Protein expression levels of hASCs transfected with MED12 siRNA via Western blot. The MED12

knockdown was validated while the knockdown caused an increase in full length Notch3 and a decrease in cleaved Notch3. (Right)

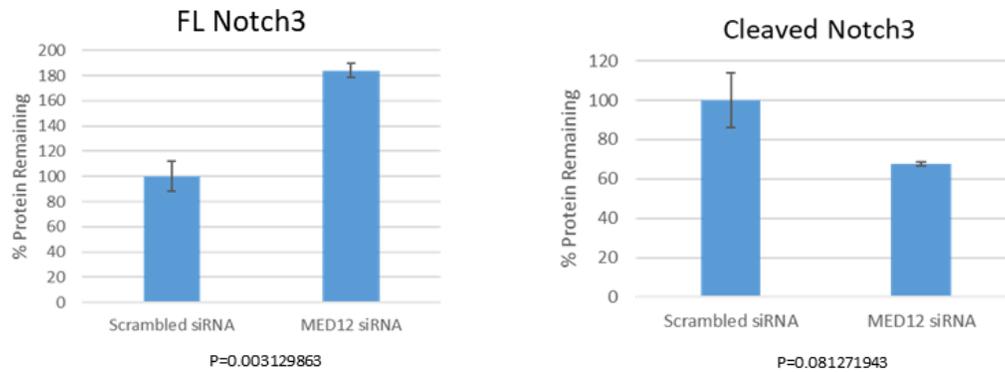


Figure 3-4: MED12 knockdown effects on Notch3 protein levels. ImageJ analysis of MED12 knockdown Western blot effects on Notch3. Western blot quantified using ImageJ software. Western blot was normalized to GAPDH. P values were calculated with T Test, N=2.

Notch1 KD has no effect on MED12 in self-renewing hASCs

After it was determined that the knockdown of MED12 did not have any significant effect on Notch1 transcript or protein, results were confirmed by transfecting cells with Notch1 siRNA to determine if it had any effect on MED12 transcript or protein. Notch1 levels were decreased in cell culture via siRNA transfections and the knockdowns were validated using qRT-PCR and western blot. Notch1 transcript was significantly reduced in comparison to negative controls and there was not a significant reduction in the MED12 transcript levels nor protein levels (**Figure 3-5**)(**Figure 3-6**). This data is confirmation of what we had already observed, that although both MED12 and Notch1 have a role in determining cell state, Notch1 is not responsible for the regulation of MED12 transcript and protein levels.

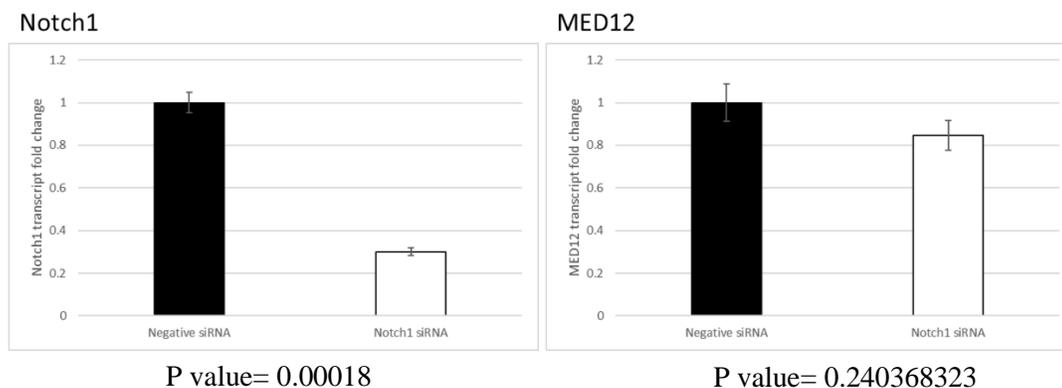


Figure 3-5: Notch1 knockdown has no effect on MED12 transcript. The Notch1 knockdown was validated. Transcript levels in hASCs transfected with Notch1 siRNA analyzed via qRT-PCR. There was not a significant reduction in the MED12 transcript levels. Data was normalized to GAPDH. P values were calculated with T Test, N=2.

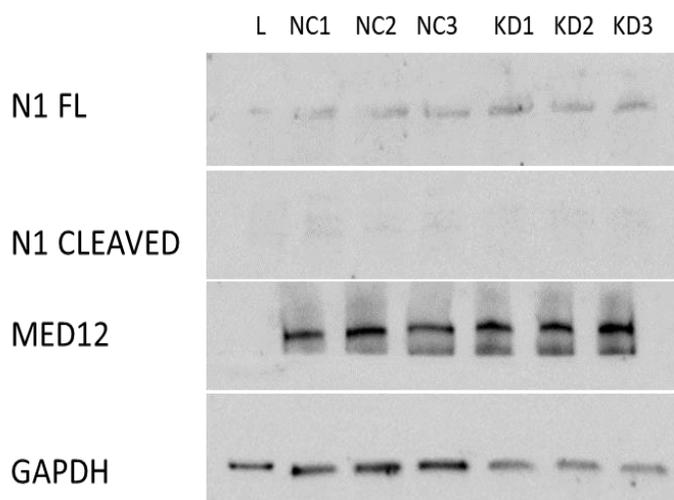


Figure 3-6: Notch1 knockdown has no effect on MED12 protein levels. Protein expression levels of hASCs transfected with Notch1 siRNA via Western blot. MED12 was not affected by the Notch1 knockdown, while the identification of Notch1 was unsuccessful

Notch3 KD does affect MED12 in self-renewing hASCs

Since it was determined that the knockdown of MED12 did significantly affect Notch3 transcript and protein, we completed the transfection of Notch3 to determine if it had any effect on MED12 transcript or protein. Notch3 levels were decreased in cell culture

via siRNA transfections and the knockdowns were validated using qRT-PCR and western blot. Notch3 transcript was significantly reduced in comparison to negative controls and there was also a significant reduction in the MED12 transcript levels (**Figure 3-7**). The Notch3 protein levels were significantly reduced while there was a decrease in MED12 protein levels (**Figure 3-8**)(**Figure 3-9**). The data observed shows that Notch3 regulates MED12 transcript and expression. This data combined with the data found in relation to MED12 and how it regulates Notch3 transcript and expression shows that MED12 and Notch3 work together to determine cell fate.

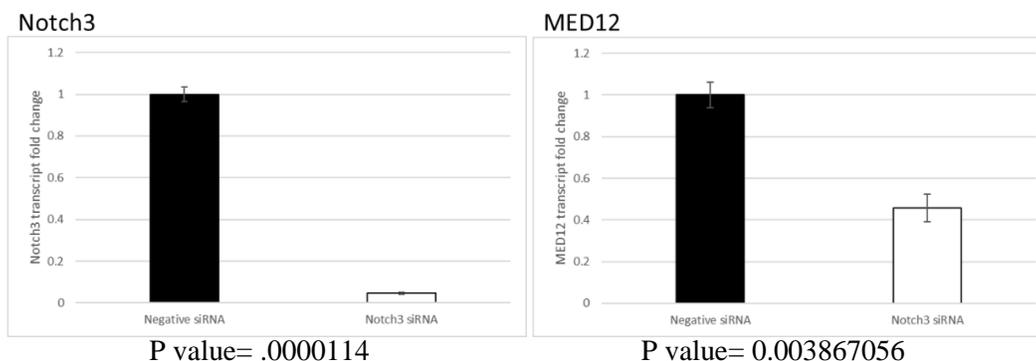


Figure 3-7: Notch3 knockdown leads to reduction in MED12 transcript. Transcript levels in hASCs transfected with Notch3 siRNA analyzed via qRT-PCR. The Notch3 knockdown was validated. There was a significant reduction in the MED12 transcript levels. Data was normalized to GAPDH. P values were calculated with T Test, N =2.

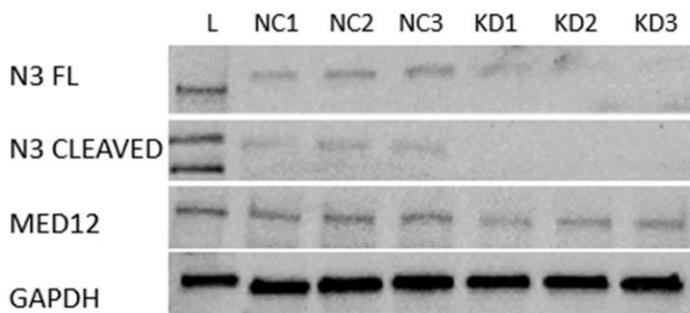


Figure 3-8: Notch3 knockdown leads to reduction in MED12 protein levels. Protein expression levels of hASCs transfected with Notch3 siRNA via Western blot. The Notch3 knockdown was validated while the Notch3 knockdown had a slight effect on MED12 protein levels causing MED12 protein levels to decrease.

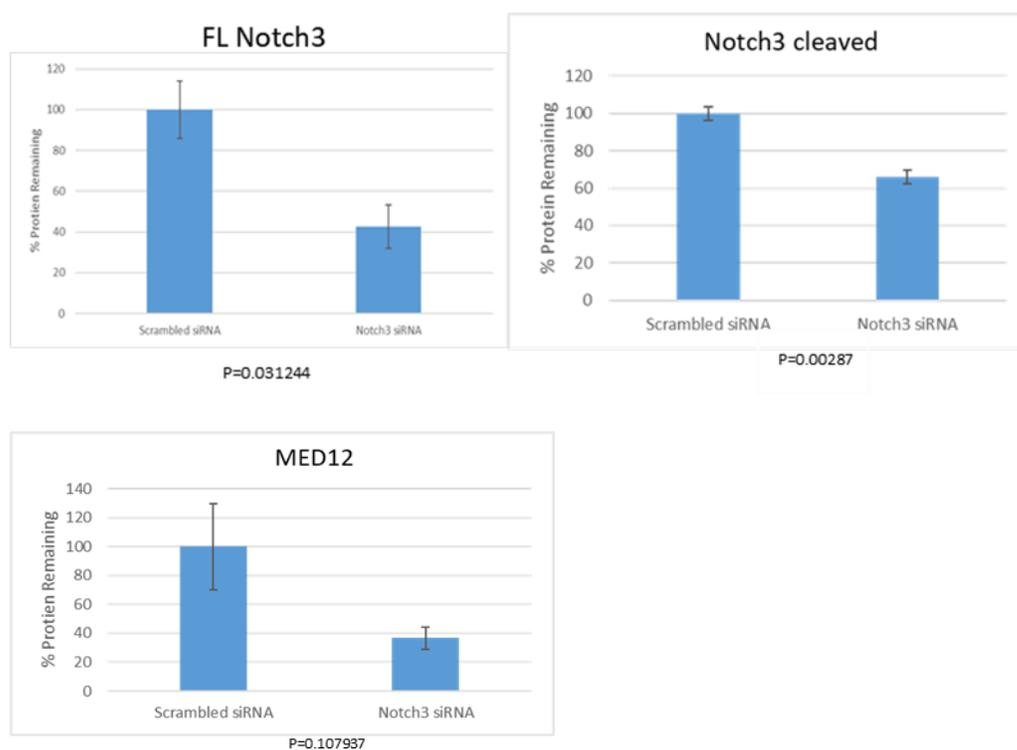


Figure 3-9: Notch3 knockdown leads to reduction in MED12 protein levels. ImageJ analysis of a Notch3 knockdown effects on MED12. Western blot quantified using ImageJ software. Western blot was normalized to GAPDH. P values were calculated with T Test, N=2.

MED12 KD effects on Notch signaling conical pathway ligands in self renewing hASCs

The data showed that after a MED12 knockdown in hASCs Notch1 transcript and protein levels were not affected, while Notch3 transcript and protein levels were significantly affected. The next step was to take a more focused look at the Notch signaling pathway and to see which of the Notch signaling conical pathway ligands, specifically Jagged1 and Jagged2, were affected by a MED12 knockdown. MED12 levels were decreased in cell culture via siRNA transfections and the knockdowns were validated using qRT-PCR. MED12 transcript was significantly reduced in comparison to negative controls and there was a slight increase in Jagged1 and Jagged2 transcript, but neither were significantly affected (**Figure 3-10**). This data suggests that neither Jagged1 nor Jagged2 contributes to MED12 role in determining cell state.

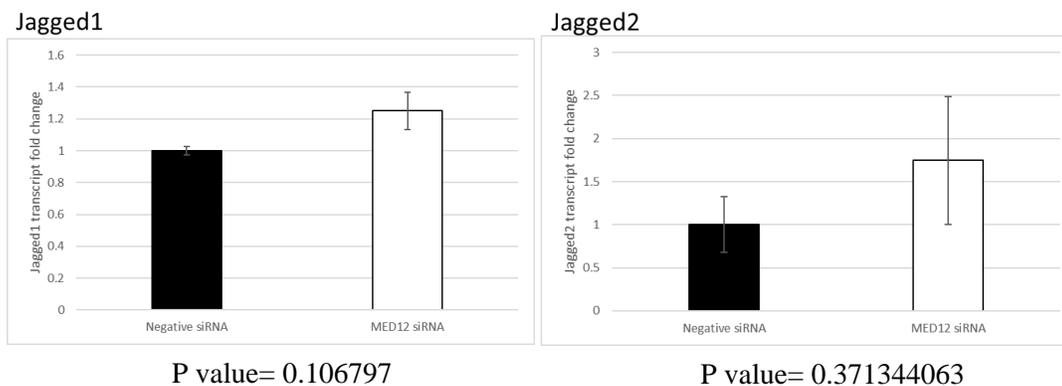


Figure 3-10: MED12 knockdown leads to increase in both Jagged1 and Jagged2 transcript. Transcript expression levels of hASCs transfected with MED12 siRNA analyzed via qRT-PCR. The MED12 knockdown was validated while Jagged1 and Jagged2 expression levels slightly increased but neither were significant. Data was normalized to GAPDH.

3.3 Conclusion

After completing the knockdown of MED12 in hASCs and observing both Notch1 and Notch3 transcript expression and protein levels, the results indicate that Notch1 does not have an active role in keeping the cells in the self-renewing state. The transcript levels and protein levels were low in the negative control samples and were not significantly affected by the MED12 knockdown. However, the data suggest that after the MED12 knockdown, Notch3 transcript and protein levels were significantly affected. The transcript expression data shows that Notch3 was reduced after the MED12 knockdown. The protein levels of Notch3 after the MED12 knockdown showed that full length Notch3 increased while cleaved or activated Notch3 levels decreased. When observing how the canonical pathway, specifically Jagged1 and Jagged2, of the Notch signaling pathway was affected by the MED12 knockdown, the data shows that neither Jagged1 nor Jagged2 transcript was significantly affected although we did see increases in the transcript.

CHAPTER 4

CONCLUSIONS AND FUTURE WORK

4.1 Conclusions

At the start of our research, we hypothesized that MED12 has a critical role in regulating transcription, and directly effects the Notch signaling pathway and therefore cell fate commitment. After completing countless experiments where either siRNAs targeting MED12, Notch1, or Notch3 were transfected into hASCs, the data suggest that Notch1 does not have an active role in directing cell fate. Both Notch1 transcript (**Figure 3-1**) and protein levels (**Figure 3-2**) were unaffected by the MED12 knockdown and when Notch1 was knocked down, neither MED12 transcript (**Figure 3-5**) nor protein levels (**Figure 3-6**

Figure 3-6) were affected. This suggest that Notch1 does not have an active role in regulating these mechanisms and may not have a role in hASC self-renewal. Next, we observed how Notch3 was affected by the MED12 knockdown. We were able to show that Notch3 transcript levels (**Figure 3-3**) were significantly reduced, while the levels of full length Notch3 protein (**Figure 3-4**) increased and the cleaved (activated) form of Notch3 decreased. This observation suggests that MED12 has an active role in the regulation and activation of Notch3 in hASCs. The influence of diminished expression of MED12 on Notch3 lead us to knockdown Notch3 to determine what impact that might have on MED12 expression. These results showed that after a Notch3 knockdown in hASCs, MED12 transcript were significantly reduced (**Figure 3-7**) and protein levels appeared to follow a

similar trend (**Figure 3-8, Figure 3-9**). This data does not completely support our initial hypothesis that that MED12 has a critical role in regulating transcription by directly influencing the Notch signaling pathway, but it does suggest that MED12 and Notch3 work together in some way to regulate hASCs self-renewal.

With the data that we have accumulated through multiple assays, we have contributed critical information that will aid in moving closer to an ability to control and manipulate hASCs for research and clinical applications. To date there is little literature on the relationship between Notch signaling and the Mediator complex. Some more recently studies did observe the effects on Notch1 after MED12 was mutated in chronic lymphocytic leukemia cells but other than that one instance, there is no literature linking the two (Wu *et al.*, 2017). What I have completed while working in the lab starts to fill a much-needed gap in the literature that links Notch signaling and the Mediator complex in regulating hASC self-renewal.

We are currently in the process of furthering this research by investigating how the canonical Notch transmembrane Delta-like ligands are affected by the MED12 knockdown. We have started this work and have qRT-PCR data where we observe changes in Jagged1 and Jagged 2 expression following a MED12 knockdown (**Figure 3-10**), but we have yet to observe how DLL1, DLL3, and DLL4 transcript are affected by the MED12 knockdown. 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.

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