THE ROLE OF NOTCH1 AND NOTCH3 IN ADULT STEM CELL OSTEOGENIC DIFFERENTIATION

by

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ABSTRACT

Human adipose-derived stem cells (hASCs) have significant therapeutic potential due to their ability to self-renew, differentiate down multiple lineages, and modulate the immune system. In addition to these many benefits, hASCs boast a minimally invasive harvesting procedure, making them a readily available cell source for stem cell research and tissue regeneration (Ock, et al. 2016) (Abdi, et al. 2008). Despite their broad use, very little is known about the mechanisms that control cell fate.

One way to enhance our mechanistic understanding of differentiation is through the systematic examination of the signaling pathways. The Notch signaling pathway is a highly conserved, contact dependent, cell-to-cell signaling cascade known to regulate cell state and multipotent differentiation of hASCs. This pathway consists of four unique receptors and five unique ligands (Braune and Lendahl 2016). Two receptors believed to play a significant role in regulating osteogenic differentiation are Notch1 and Notch3.

Here the expression of Notch1 and Notch3 are characterized during osteogenesis and the effect that siRNA-mediated knockdown of each receptor has on osteogenic differentiation is evaluated. By studying changes in osteogenic marker expression following a reduction in Notch expression and activity, we will be able to determine how each receptor individually affects the osteogenic

potential of hASCs and identify potential novel therapeutic targets to treat bone damage and loss.

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DEDICATION

To anyone that experienced hardship in this pandemic, whether through loss or through isolation, that pulled through to do something that they can be proud of.

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CHAPTER 1

INTRODUCTION

1.1 Potential Roles for Manipulated Stem Cell Therapies

1.1.1 The Threat of Degenerative Bone Diseases

In our society, there are rising levels of degenerative bone diseases primarily stemming from an increasingly aging population, but they can also be incited by significant bone trauma. These injuries can result from a multitude of activities such as sports injury, outdoor injury, and motorized vehicle accidents. Direct, physical injury of the bone is not the only cause of damaged bone tissue. With a global trend of an increasingly aging population, the prevalence of diseases such as osteoporosis are continuing to rise (Aspray and Hill 2019). Even with the high prevalence of these injuries and aging population, methods of bone repair have done little to evolve over the past century; mainly relying on pins, screws, and plates to hold damaged and weakend bone together until healed.

As humanity pushes further into deep space, the degradation of bone tissue from exposure to microgravity is also rapidly becoming a significant concern (Axpe, et al. 2020). There is a significant amount of research surrounding the investigation of methods for circumventing bone loss during extended stays in microgravity. These studies tend to focus on providing artificial physical stimuli that simulate the effects of gravity on

weight-bearing bones. Despite the decades of attempts, very little progress has been made on this front. Even with the implementation of very complex and expensive exercise equipment on the International Space Station, astronauts still suffer from debilitating bone degradation (Axpe, et al. 2020) (Beguerisse-Díaz, Desikan and Barahona 2016) (LeBlanc, et al. 2007). These diseases can lead to injuries similar to those seen in physical accidents, but the main component of their pathology is an attack on the bone tissue from a physiological front (Aspray and Hill 2019). The diverse nature of these afflictions poses a significant challenge to doctors and researchers aiming to combat the negative effects of these conditions.

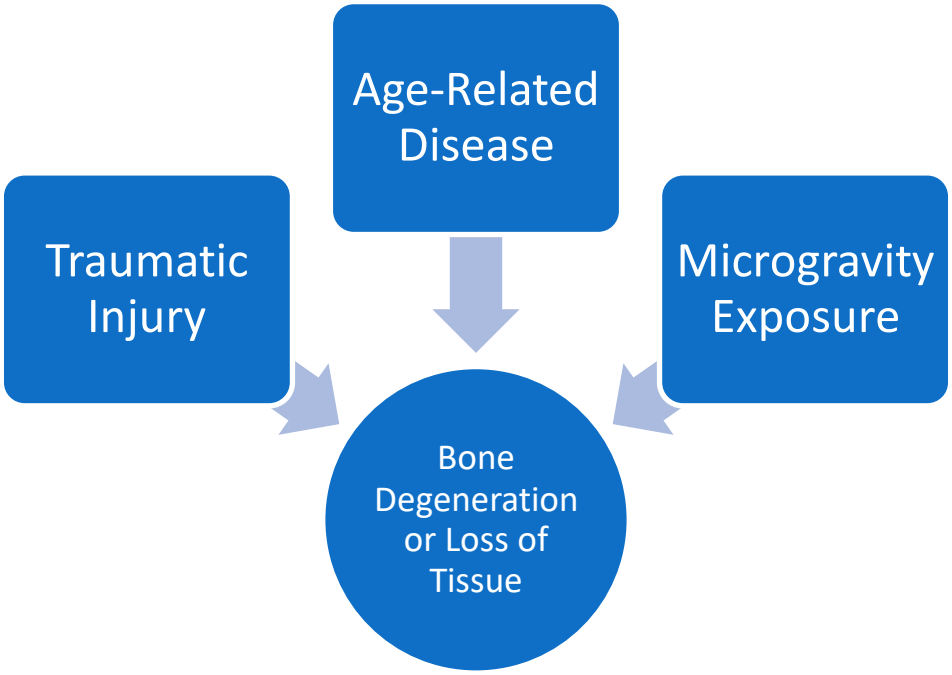


Figure 1-1: Summary of the major factors leading to significant bone degradation.

1.1.2 Current Treatments for Bone Degeneration and Their Limited

While current treatments have produced effective results in the past, they tend to come with some serious limitations. In the case of current polymer biomaterials used to reinforce damaged tissue, these implants tend to be limited in their bio reactive capacity.

The limited ability to interact with the surrounding tissue leaves a significant gap in the potential for more complete healing of the injury (Yuan, et al. 2018). On top of the gap in healing potential seen in both metallic and composite biomaterials, many patients that have been treated with the historically popular metallic implants report pain, increased sensitivity, corrosion, biofilm formation, and potential rejection (Přikrylová, Procházková and Podzimek 2019). While biomaterial implants for major fractures can come with some physical side effects, most pharmaceutical treatments of osteoporosis come with some intense physiological side effects. The most common form of osteoporosis is due to reduced estrogen production in postmenopausal women. This leads to most major treatments being hormone treatments which can lead to various types of cancer as well as producing a series of side effects in the circulatory system (Komm, et al. 2015) (Tella and Gallagher 2014).

The current set of treatments for degenerative bone conditions have been in use for some time now. As their side effects becoming increasingly apparent, researchers have been pushed to develop modern alternatives. For the treatment of major fractures, the use of bone implants is still the main approach but the composition and focus of the implant has shifted. Modern developments in these implants seek to use new and developing ceramic biomaterials that show significant bio reactive properties. Like earlier methods, these implants seek to reinforce the fracture, but they go further and utilize the body's own regenerative capabilities (Ahadian and Khademhosseini 2018). Drug-related therapeutic options will consistently be the most limited form of treatment due to their highly prevalent long-term side effects; however, they can be highly beneficial for patients that are not able to participate in consistent exercise, something proven to play a

major role in maintaining bone mass (Santos, Elliott-Sale and Sale 2017). One of the more novel treatments, and one with significant therapeutic potential, is the use of autologous stem cells for wound healing and curbing the degradation of bone tissue. Enhancing the therapeutic potential of these cells through the selective manipulation of key molecular pathways is currently an active are of research. By using cells directly harvested from the patient, these cells can then be implanted back into the patient after they have been manipulated; thus, allowing the patient's body to heal itself with its own cells and hopefully allow for a restoration of original cellular function (Frese, Dijkman and Hoerstrup 2016). This review will cover some of the current publications and data that are working to develop and further our understanding of these potentially revolutionary new technologies.

1.2 Shortcomings of Drug Therapies for Osteoporosis

1.2.1 Limitations of Bisphosphates and Estrogen Therapies

One of the oldest and most common methods of treating bone degeneration is the use of bisphosphates. These drugs focus on limiting bone resorption in an attempt to reach a balance between the anti-resorption properties of the bisphosphates and the overactive resorption seen in diseases like osteoporosis. Even though this technique has been shown to be effective in the past, this drug does not come without its own side effects. The prolonged use of bisphosphates has been characterized by a substantial increase in the risk of atypical femur fractures (AFFs). AFFs arise in the femurs of patients that experience an overhardening and loss of flexibility in the long bones. This change in the mechanical characteristics of the bone is believed to arise from the prolonged prevention of bone resorption that eventually leads to the characteristic

overhardened bone tissue. The adverse effects of this drug have led to a technique known as “drug holidays” which are temporary suspensions of treatments intended to curb the high risk of AFFs. However, even though these “holidays” do lead to a reduced risk of these fractures, it leaves the patient vulnerable as they are not receiving the treatment that they need (Skjødt, Frost and Abrahamsen 2019).

Even with the significant side effects of bisphosphates, they have proven to be a consistent and effective treatment for osteoporosis. However, some forms of this disease require a more specific pharmacological approach. In the case of postmenopausal osteoporosis, loss in bone mineral density (BMD) primarily arises from the decrease in estrogen production seen in postmenopausal women. The primary avenue of treatment for this disease is the use of hormone replacement therapy, specifically estrogen and progesterone therapy. While this treatment has shown consistent increases in BMD similar to the results seen with bisphosphates, this therapy also comes with a list of side effects. Particularly in older women undergoing this therapy, there has been a measurable increase in risk for breast cancer, stroke, heart attack, and venous thromboembolism (Tella and Gallagher 2014). The inherent risks of utilizing hormone replacement therapy makes it a questionable long-term treatment for osteoporosis, but the shortcomings of these treatments are the driving force behind the development of new drugs.

Table 1-1: Summary of the properties of current treatments for bone degredation and how manipulated stem cells can fill these niches.

	Benefits	**Side Effects**	**Alternatives**
Drugs	Readily available and well documented; easily administered treatment for osteoporosis	Increased risk of breast cancer and potential overhardening of the bone leading to increased fracture risk	Molecularly manipulated stem cells used to bolster existing osteoblasts and rescue bone mass density
Implants	Highly tailorable surfaces and shapes; effective for trauma	Possible physiological rejection of the implant, risk of infection, and lack of biological reactivity	Biomaterials seeded with molecularly manipulated stem cells could lead to increased biological reactivity and patient healing potential

1.2.2 Modern Developments in Pharmaceutical Treatments

With the limitations of these common drug therapies, researchers are pushed to develop alternatives to combat bone degeneration. The need for novel drug therapies has led to the development of several new drugs all currently undergoing animal model testing and clinical trials. Ipriflavone is a drug that is being developed to combat postmenopausal osteoporosis (PMOP), one of the most common and well characterized forms of bone degeneration. In one study seeking to study the effects of this drug in ovariectomized mice (designed to simulate PMOP), it was shown that Ipriflavone treatment led to a significant rescue of bone-mass density (BMD) when compared to control mice (Gao, et al. 2018). Romosozumab is a monoclonal antibody that regulates the resorption and formation of bone tissue. In one clinical trial, 7180 women with PMOP were treated with romosozumab for 12 months and then switched to a denosumab treatment for another 12 months. In this trial, patients treated with romosozumab for 12

months saw a significantly reduced number of new vertebral fractures when compared to placebo trials (Cosman, et al. 2016). Another method seeking to treat PMOP was explored in a clinical trial seeking to determine an effective dosage for a therapy utilizing a combination of denosumab and teriparatide treatment. It was determined that a higher than the previously standard dosage of teriparatide combined with denosumab treatment led to a higher increase in hip and spine BMD when compared to the standard dosage (Tsai, et al. 2019). Even though these novel therapies exhibit promising results, the long-term efficacy of these treatments as well as their long-term side-effects remain to be seen. This still leaves room for potential cell-based therapies to enhance or even replace these novel drug therapies.

1.3 Human Adipose Derived Stem Cells

Human adipose-derived stem cells (hASCs) have significant therapeutic potential due to their ability to self-renew, differentiate, and modulate the immune system. In addition to these many benefits, hASCs require a minimally invasive harvesting procedure, making them a readily available cell source for stem cell research and tissue regeneration (Ock, et al. 2016) (Abdi, et al. 2008). hASCs have many properties that make them clinically relevant as a therapeutic cell source for procedures seeking to enhance, repair, or replace damaged tissue. These cells are known to be multipotent, meaning that they have the potential to differentiate from a stem cell into multiple different tissue types of a specified lineage. Human ASCs are known to differentiate into cells of the mesoderm lineage including muscle, bone, fat, cartilage, and nerve tissue indicating strong therapeutic potential for any situation where damaged tissue cannot be repaired on its own (Zuk, et al. 2002). One of the major benefits to using hASCs is that

they can be harvested from the afflicted person using lipo-aspiration from that patient's own fat tissue (Frese, Dijkman and Hoerstrup 2016). Through this procedure, the tissue being used is autologous, meaning it is the patient's own cells and there is no threat of an immune response to the transplanted tissue. Human ASCs are already part of 81 clinical trials and are used in dozens of outpatient treatment centers across the country for both their immunomodulatory and regenerative properties (Search of: Recruiting, Not yet recruiting, Active, not recruiting, Enrolling by invitation Studies | adipose stem cells - List Results - ClinicalTrials.gov n.d.). Despite their broad use, however, the scientific community still knows very little about the mechanisms that control cell fate and how this information might enhance the therapeutic usage of these cells to treat injuries and degenerative health conditions.

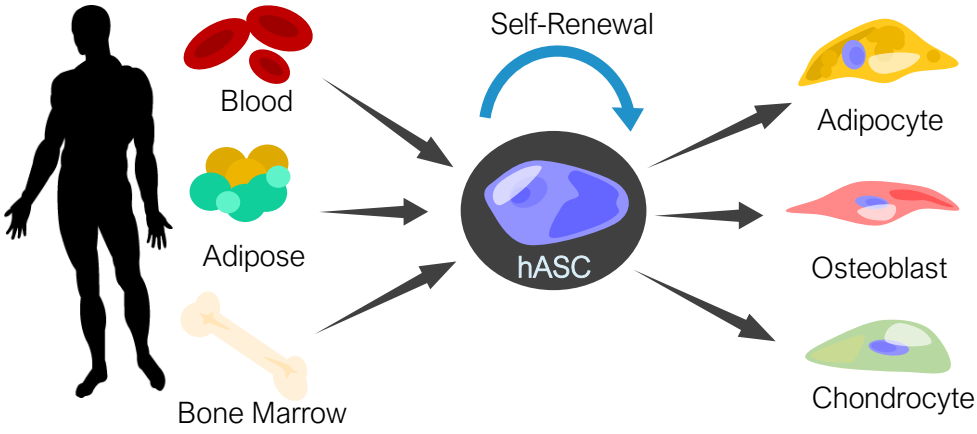


Figure 1-2: Human adipose-derived stem cells (hASCs) have potential to differentiate down multiple different cell lineages depending on a series of environmental stimuli. Image Credit: Rachel Eddy

1.3.1 Wound Healing Properties of hASCs

One of the major challenges to utilizing cell-based therapies is the method of harvesting the stem cells needed for the treatment. In the past, the most commonly used stem cells for these therapies were bone marrow derived stem cells (BMSCs). While

BMSCs do have immense therapeutic potential, they require a highly invasive harvesting procedure that does not yield many viable cells. For this reason, researchers are beginning to turn to adipose derived stem cells (ADSCs) for their ease of harvesting and increased yield. The differentiation potential of the two cell types was explored in a recent study seeking to understand how these cells could be used in a wound healing scenario dealing with diabetic foot ulcers (DFUs). BMSCs were already shown to drastically increase healing time and efficiency so ADSCs were tested in an identical scenario. These researchers found that BMSCs and ADSCs displayed near identical morphology, expression of cellular markers, VEGF secretion, and proliferation rates. With the cells' metabolic characteristics showing similar results, the researchers seeded them onto collagen scaffolds which were grafted into DFUs of diabetic mice. After it was established that BMSCs and ADSCs both showed similar levels of wound healing potential, an identical experiment was established to compare healthy ADSCs and ADSCs from diabetic patients. In this study, the diabetic cells showed almost no decrease in therapeutic potential when compared to non-diabetic cells (Guo, et al. 2018). This experiment demonstrated that there are multiple potential cell sources for use in stem cell therapies with similar therapeutic potential.

1.4 Osteogenesis

Since hASCs differentiate into tissues of the mesoderm layer, they can provide a consistent and effective platform to study the early regulatory processes of osteogenic differentiation. Osteoblasts, the cells responsible for the synthesis and maturation of bone tissue, are derived from mesenchymal stem cells where they are exposed to a microenvironment favorable for osteogenesis (Luo, et al. 2019). However, the formation

of bone tissue is not entirely reliant on osteoblasts. These cells will coordinate with several different cell types such as osteoclasts and chondrocytes; both of which are also derived from mesenchymal stem cells. Bone formation typically takes two different forms: intramembranous ossification and endochondral ossification. While these processes give rise to different types of bones (flat bones and long bones respectively), they both center around the condensation of cells in the mesenchyme into calcified bone tissue. However, it is intramembranous ossification that primarily involves osteoblasts derived directly from mesenchymal stem cells (Qin, et al. 2016).

While osteogenesis shows significant interaction with other cell types derived from the mesoderm, these osteogenic cells also interact with other cells located outside of this shared germ layer. Osteocytes have been shows to have a deep link with endothelial cells during the formation of bone tissue, specifically with cells involved in angiogenesis. This linkage has been shown to produce developmental disorders of the bone tissue like craniosynostosis and midfacial hypoplasia that are caused by dysregulation of angiogenesis (Percival and Richtsmeier 2013). These claims of endothelial cells having strong linkages to osteogenesis are not only seen in medical conditions. Researchers have shown that endothelial cell-specific knockouts of the Notch pathway will significantly inhibit blood vessel formation as well as reducing osteogenesis (Qin, et al. 2016).

1.4.1 Osteoblasts

One of the primary cell types involved in the repair of bone tissue is the osteoblast. Osteoblasts are vital to the growth and formation of new bone tissue while also playing a major role in maintaining existing bone tissue (Lee, et al. 2017). These cells are one of the main cell types that researchers turn to when seeking to study the

regulation of osteogenesis or as a potential cell source for novel regenerative therapies. Primary human osteoblasts that have been either directly extracted or differentiated from stem cells are of particular interest due to their behavior that closely mimics an *in vivo* lineage when they are cultured in an *in vitro* setting. These characteristics have led the osteoblasts, particularly those derived directly from harvested stem cells to the forefront of clinical research in areas of osteogenic regulation and tissue repair (Czekanska, et al. 2012).

Due to osteoblasts having such high levels of clinical relevance, the regulation of osteogenesis within these cells is of particular interest to researchers seeking to unlock their potential. One of the most characterized routes of regulation in osteoblasts and pre-osteoblasts in through the transcription factor Runx2, a component in a family of transcription factors that includes Runx1, Runx2, and Runx3. This transcription factor is primarily found in osteoblasts and chondrocytes throughout most of their development. In the case of osteoblasts, Runx2 sees an upregulation in expression with a peak in the immature osteoblast stage before beginning to drop once the osteoblasts reach maturity. This transcription factor has proven vital to the initiation and maturation of osteoblastic differentiation many times over, but it can be most clearly seen in *Runx2*-deficient mice. In these models, researchers saw that there was a severe lack of bone formation and even an overall lack of osteoblasts in the mice's tissue. Compounding research has shown that Runx2 expression is vital for progenitor cells to commit to the osteoblastic lineage as well as for the transcription of genes dealing with the production of bone matrix proteins (Komori 2019).

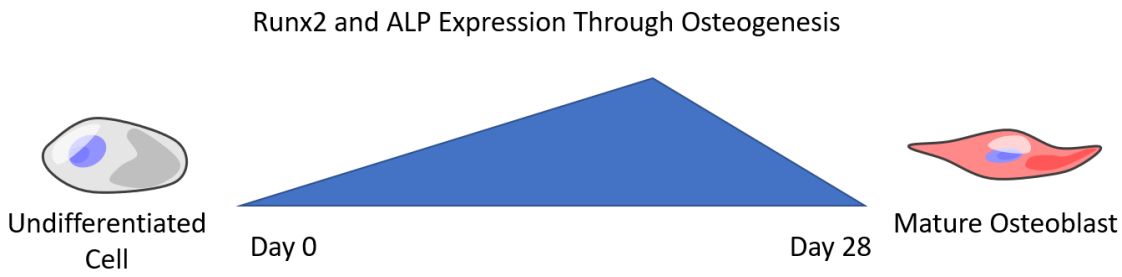


Figure 1-3: Both Runx2 and ALP show a consistent increase in expression levels that peak around the 21 Day mark and begin to decrease until cells have differentiated into mature osteoblasts (Day 28).

1.4.2 Osteoclasts

Osteoclasts are the second type of cell that plays a major role in the proper modelling of bone tissue. These cells are responsible for the resorption of bone tissue, a process vital to the proper formation of bone structure and developmental modification to this bone structure. Examples of this can be seen in mice exhibiting dysregulations such as osteopetrosis that leads to a deficiency in osteoclast activity. These mice tend to lack the ability to produce bone marrow in the long bones while also displaying a lack of tooth eruption. However, when these cells are over-active, they can cause severe damage to bone tissue. These cells are seen to cause destruction of bone tissue in patients with osteoporosis and those with highly metastatic osteosarcomas (Miyamoto 2011).

While osteoblasts and osteoclasts have opposite functions, it does not mean that these cells are mutually exclusive. These two cell types work in tandem to achieve the constant remodeling of the skeleton that is required to maintain proper homeostasis of the tissue. While osteoblasts tend to take the spotlight due to their bone forming properties, the tissue formed by these cells is not immortal. It is the role of the osteoclast to break down the aging bone tissue produced by the osteoblast to recycle components and make room for new bone formation. These two cells also work in tandem to regulate each

other's differentiation and perform general communication through avenues such as the formation of gap junctions between the two cells and cytokine signaling. This communication has been shown to illicit phenomena such as the initiation of one cell type's differentiation and the apoptosis of osteoclasts through osteoblast-derived signaling (Chen, et al. 2018).

1.5 Notch and Osteogenesis

The Notch signaling pathway is a highly evolutionarily conserved, contact-dependent, cell-to-cell signaling pathway that has been shown to play a major role in all stages of development (Braune and Lendahl 2016). While Notch plays many roles in tissue development, perhaps one of the most studied roles of the pathway is its ability to regulate the differentiation and proliferation of mesenchymal stem cells. Notch has primarily been shown to play a role in determining cell fate in differentiating tissue; guiding stem cells along specific differentiation pathways based on specific receptor-ligand interactions (Sandel, et al. 2018). The prevalence of the Notch pathway's role in regulating mesenchymal stem cell differentiation has led to a series of studies aimed at examining this pathway's role in many different forms of differentiation.

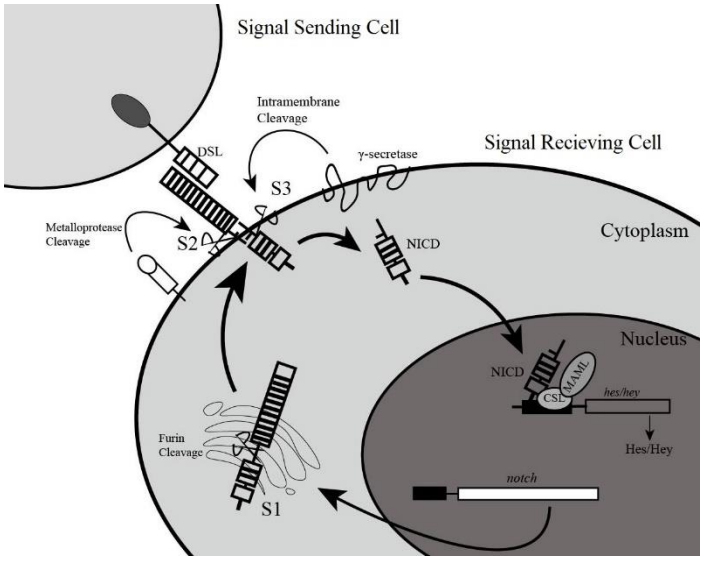


Figure 1-4: The Notch signaling pathway sends and receives signals between neighboring cells in a contact dependent manner. These signals have been shown to play an extensive role in regulating many different types of differentiation in adult stem cells. Figure Credit: Mengcheng Liu

Through the use of transgenic mouse models, Zanotti et al. studied the effects of Notch in scenarios of both over and under expression. It was observed that not only does the Notch pathway play a major role in regulating osteogenesis by inhibiting differentiation, they also concluded that Notch can interact and regulate other regulatory pathways, specifically Wnt and BMP signaling (Zanotti, et al. 2008). In the case of this study, the data shows that Notch inhibition could lead to increased osteogenesis in cells being used to treat bone degradation. Due to Notch's supposedly inhibitory action in osteogenesis, it shows potential to be a significant therapeutic target to induce higher levels of osteogenic differentiation. Studies such as this are vital in establishing a foundation of knowledge from which future studies and therapies can be built upon.

While this study does show that the Notch pathway inhibits osteogenic differentiation, it is somewhat broad and tends to focus on the pathway as a whole rather than the role of individual receptors and ligands. If molecular manipulation of stem cells

is to be used in a clinical setting, then these manipulations need to be highly specific and the potential side effects of these manipulations need to be carefully observed. A study performed by He et al. aimed to define the specific effects that the Notch1 receptor has on osteogenesis. The findings of this research showed that after inhibition at both the protein and transcript level, Notch1 plays a major inhibitory role in regulating osteogenesis. This was observed after there was a sharp increase in osteogenic differentiation following the inhibition of this receptor. The researchers also wanted to study whether or not the inhibition of this pathway could lead to tumorigenesis. They studied this by observing expression levels of the p53 gene, a well-known tumor suppressor, as well as observing any changes in cell viability before and after the inhibition of Notch1. There was both an increase in p53 expression and a decrease in cell viability associated with Notch1 inhibition, thus pointing to the possibility of potential tumors forming if these manipulated cells were to be implanted in a patient (He and Zou 2019).

1.6 Conclusion

Though there are many factors that must be understood in order to develop a cell-based therapy that could be used to combat various forms of bone degeneration, the application of stem cells for regenerative therapies still exhibits promise as a novel treatment for bone degeneration. With an aging population, the possibility of long-term side effects from the current pharmaceutical treatments for bone degeneration continues to be a concern, as the increase in life span is not pushing the onset of these diseases further back. These long-term complications also rule out the possibility of these drugs

being used to combat the bone degeneration seen in microgravity; an environment that will continue be occupied by more people in the coming years.

hASCs have shown their therapeutic potential in the wound healing sector with capabilities very similar to those of the much more costly and painful to extract BMSCs. While the promise of successful wound healing is enlightening, these studies were performed with undifferentiated hASCs. The use of hASCs as a cell source to be used for more tissue type specific therapies utilizing their multipotency is still a relatively new concept that requires the hurdling of multiple obstacles and an expansion of the current knowledgebase. At the heart of the difficulties plaguing the development of this technology is the lack of a thorough understanding of the regulatory mechanisms of these cells. In order to fully unlock the therapeutic potential of hASC multipotency, how these cells handle differentiation must be understood.

In the case of osteogenic differentiation, understanding how this differentiation works and how it is regulated is vital to producing a therapy derived from hASCs. While osteogenesis on a tissue-wide level has been shown to be a complex process that requires many different cell types, it seems that the bone-forming osteoblast boasts the highest potential as a cell lineage target for hASC therapies. The thorough characterization of its transcriptional regulators as well as its defined ability to regulate other cell types, such as the bone-resorbing osteoclast, shows promise as a cell type that could seriously curb the degenerative effects of diseases such as osteoporosis if incorporated into a cell transplantation therapy. However, if this cell-based therapy is to be safely implemented, the methods of molecular regulation must be more deeply explored.

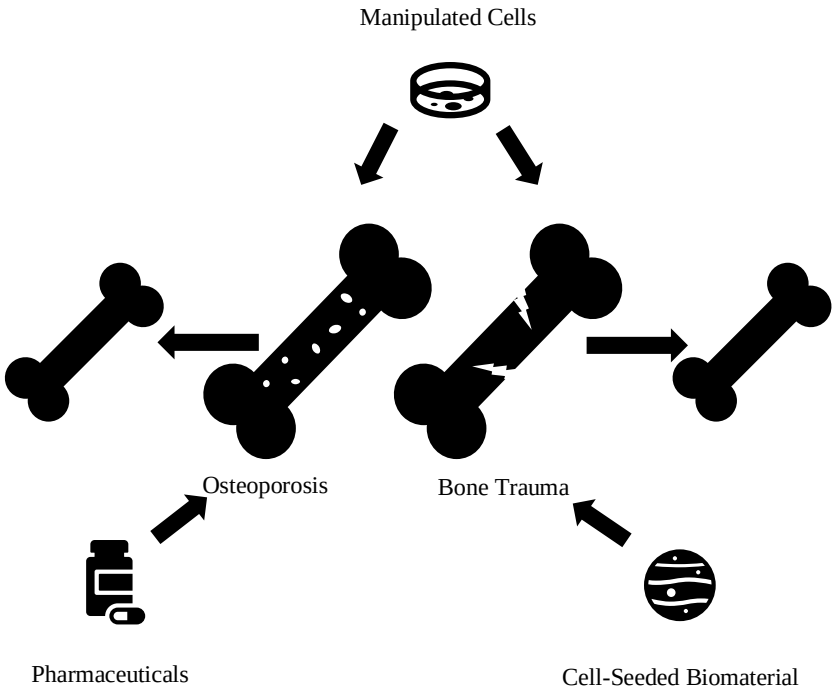


Figure 1-5: The use of manipulated stem cells shows potential as a therapy that can either replace or work in tandem with currently available treatments for both osteoporosis and severe bone trauma.

While it has been shown that there are a series of signaling pathways that work in tandem to produce the complex process of osteogenesis, the Notch signaling pathway is one that seems to show promise as a regulatory target. While targeting the Notch pathway would only cause direct manipulations to Notch itself, these manipulations could have effects on other pathways that also play a role in osteogenesis. Notch has been shown to initiate crosstalk with other pathways such as Wnt and BMP signaling, both of which are known to regulate stem cell differentiation throughout development. The interaction with other pathways is not the only phenomenon that alludes to its promise as a therapeutic target. Notch has been shown to have a direct effect on osteogenic differentiation, especially in the earlier stages of osteoblast formation. However, direct manipulation of the pathway has been shown to cause some adverse side effects. For example, upon

inhibiting the Notch1 receptor, researchers have observed a distinct increase in p53 expression as well as a reduction in cell viability, both of which are factors that point to potential tumorigenesis.

Even though direct manipulation of the Notch pathway poses some obstacles, there is still immense potential for this pathway to develop into a widely used therapeutic target to increase the potential of hASCs as a cell source for novel therapies seeking to combat bone degeneration. These obstacles are the reason that the effects of this pathway on osteogenesis must be further studied and characterized to pave the way for future research to build upon. With a substantial knowledgebase on how this pathway regulates osteogenesis, specifically in osteoblasts, researchers can develop life-saving methods to combat these afflictions while also contributing to the world-wide push to venture deeper into our solar system.

CHAPTER 2
METHODS

2.1 Cell Culture

2.1.1 Culturing hASCs

All cells were initially cultured in a Complete Culture Media (CCM) comprised of 203.75 mL of 1X MEM Alpha (Gibco; 12561-049), 41.25 mL of Fetal Bovine Serum (ATLANTA Biologicals; S11150), 2.5 mL of 200mM L-Glutamine (Gibco; 25030-081), and 2.5 mL of Pen Strep (Gibco; 15140-122). Ingredients were combined into a 250 mL Corning filter system (VWR 28199-770) and filtered under a sterile hood.

2.1.2 Cell Thawing

A cell line (Obatala 70926) of human adipose derived stem cells (hASCs) obtained from Obatala Sciences (New Orleans, Louisiana, United States) were thawed at 37°C and transferred to a conical tube containing warmed CCM. The cells were then isolated by centrifuging them at 1,500 RPM before being resuspended and transferred to a 10 cm tissue culture-treated plate containing CCM. The media on this plate was then changed the following day and then every 48 hours until the cells reached 70-80% confluency.

2.1.3 Passaging Cells

Upon reaching 70-80% confluency, cells were detached from the plate by adding 0.25% Trypsin-EDTA (Life-Technologies 25200-056) and incubating at 37°C for 3 minutes. CCM was then added to the plates to neutralize the trypsin and this solution was collected in a conical tube to be centrifuged at 1,500 RPM. Cells were then isolated and resuspended in CCM where a sample was stained with 0.4% Trypan Blue for cell

counting. The cells were counted on a Countess II FL Hemocytometer and a Countess II FL machine. The live cell count was then used to determine the volume of cells needed to seed at the densities outlined in **Table 2-1**. The media was then changed on the newly seeded plates 24 hours after passaging and then every 48 hours until initiation of differentiation or collection.

Table 2-1: Cell seeding density depenent on culture plate size

Plate Size	# of Cells per Plate/ Well	Total Volume of CCM
10-cm	100,000	10 mL
6-cm	45,000	3 mL
6-well	20,000	2 mL

2.1.4 siRNA Knockdowns

Upon reaching 40-45% confluency, cells were exposed to a transfection solution comprised of 1X Opti-MEM I (Gibco; 31985-062), Lipofectamine RNAiMAX Reagent (Invitrogen; 56531), and an siRNA solution containing negative control, Notch1, or Notch3 siRNA. This transfection solution was then combined with CCM and added to the wells. The day following the siRNA knockdown, the media was changed, and cells were cultured for 14 days in lab-made osteogenic media with media changes every 48 hours.

2.1.5 Osteogenesis

Cells were seeded onto 6-well plates containing CCM according to **Table 2-1** with media being changed every 48 hours until cells reached 55-65% confluency. The media was then switched to a purchased osteogenic media (Thermo Scientific A1007201) or a lab-made osteogenic media comprised of 89% DMEM/F-12 (Gibco 10565018), 10%

Fetal Bovine Serum (ATLANTA Biologicals; S11150), and 1% Pen Strep (Gibco; 15140-122) with the addition of 1 nM Dexamethasone, 10 mM Beta-Glycerophosphate, and 50 uM L-Ascorbic Acid 2-Phosphate. The cells were cultured for up to 14 days with media being changed every 48 hours.

2.2 Gene Expression Analysis

2.2.1 RNA Collection and Extraction

Cellular RNA was collected using a Trizol reagent (Thermo Scientific 15596018) according to the manufacturer's protocol where the lysate was then stored at -80°C.

Prior to the extraction process, the collected lysate was allowed to thaw at room temperature. Chloroform was then added to the samples where they were agitated and allowed to incubate at room temperature. The samples were then centrifuged at 4°C and 12,000x G. After centrifugation, the clear supernatant was collected and the remaining phenol waste was discarded. 5mg/mL glycogen (ThermoFisher Scientific R0551) was then added to the samples and followed by 100% isopropanol where they were then incubated at room temperature. After another round of centrifugation at 4°C and 12,000x G, an RNA pellet was isolated and the remaining isopropanol was removed. The RNA pellet was then washed with 75% ethanol and centrifuged at 4°C and 7,500x G. The ethanol was then removed and the pellet was allowed to dry until resuspension in nuclease-free water (VWR 10220-404) and stored at -80°C. The RNA would later be quantified using a spectroscopy program on a Cytation 5 BioTek Plate Reader.

2.2.2 cDNA Synthesis

1 ug of extracted RNA, nuclease-free water, and qSctript™ cDNA SuperMix (Quantabio 95048-100) were used to synthesize cDNA according to the manufacturer's protocol.

2.2.3 RT-qPCR

(Reverse Transcription-Qualitative Polymerase Chain Reaction) RT-qPCR was performed using a technical triplicate by combining PowerUp SYBR Green Master Mix (Applied Biosystems A25742), nuclease-free water, forward and reverse primers (**Table 2-2**), and 1 uL of CDA per reaction in a 96-well plate. The reaction was then run using a StepOnePlus™ Applied Biosystems machine standard quantitation experiment. The data from this experiment was then plotted on a Comparative C_T ($\Delta\Delta$ C_T) curve.

Table 2-2: Forward and reverse primers used for RT-qPCR

Gene	Forward Sequence	Reverse Sequence	Product Size (bp)
gapdh	ACTAGGCGCTC ACTGTTCTCT	CAATACGACCAA ATCCGTTGACT	99
notch1	CACGCTGACG GAGTACAAGT	GGCACGATTT CCCTGACCA	56
notch3	CACCCTTACCT GACCCCATCC	TTCGGACCAGT CTGAGAGGGA	81
runx2	CTCACTACCAC ACCTACCTG	TCAATATGGTCG CCAAACAGATTC	320
alp	CTAACTCCTTA GTGCCAGAG	CATGATGACAT TCTTAGCCAC	125

2.3 Protein Expression Analysis

2.3.1 Protein Extraction

6 cm plates were rinsed with PBS (ThermoFisher Scientific 10010023) before the addition of 1X RIPA lysis buffer containing a protease and phosphatase inhibitor. The cells were then scraped and the lysate was collected in tubes for a 30 minute rotation at 4°C. The samples were then centrifuged at 12,000 RPM at 4°C for 20 minutes where the resulting supernatant was removed and stored at -80°C.

2.3.2 Bradford Assay

A protein dye and distilled water were combined to form a 20% Bradford buffer prior to the creation of samples. BSA, water, and buffer were mixed to form 6 standards of increasing protein concentration. Protein samples with unknown concentrations were mixed with Bradford buffer and distilled water. All samples were pipetted in duplicate into a clear-bottom 96-well plate before determining protein concentration in a Cytation 5 BioTek plate reader.

2.3.3 Western Blot

Prior to the experiment, a 10% running buffer and a 10% Tris/Glycine buffer were prepared and stored in a refrigerator. A Laemmli buffer (Biorad 1610737), water, and protein solution was prepared based on the protein concentrations provided by Bradford assay. Samples were then boiled at 100°C and spun down before being loaded into an electrophoresis gel (Biorad 456-1084) alongside a protein standard (Biorad 1610376). This gel was then run in an electrophoresis apparatus containing to previously prepared 10% running buffer for 90 minutes at 120V.

Following electrophoresis, the gel was transferred onto a PVDF membrane (Biorad 1704156) in an electrophoresis apparatus run in the previously prepared 10% Tris/Glycine transfer buffer for 60 minutes at 100V. After the transfer process, the membrane was cut into sections dependent on the location of the protein of interest based on size in kDa. The membrane sections were then blocked in a non-fat milk blocking buffer (Cell Signaling Technology 99995) for 120 minutes. During the blocking process, primary antibody solutions were prepared using the non-fat milk buffer. After blocking, the membranes were probed in the primary antibody solution overnight in a refrigerator.

Table 2-3: Primary and Seconday Antibodies Used in Wester Blot Analysis

Antibody	Company	Call Number	Concentration
Anti-GAPDH Rabbit pAb	Abcam	ab9485	1:3000 Dilution
Anti-Notch3 Rabbit mAb	Cell Signaling	5276s	1:1000 Dilution
Goat Anti-Rabbit IgG H&L	Abcam	ab150077	1:1000 Dilution

2.4 Osteogenic Characterization

2.4.1 Alizarin Red Stain

Media was removed from the 6-well plates after a 7 or 14-day osteogenic differentiation period and the wells were washed with 150 mM NaCl solution. An ice-cold 70% ethanol solution was then added to the wells and the cells were allowed to fix for one hour. The ethanol was then removed, and the cells were washed with DI water before adding a 2% alizarin red solution (Lifeline Cell Technology CM 0058). After 15 minutes of staining the alizarin solution was removed and the cells were washed with DI water before being imaged in a Cytation 5 BioTek plate reader.

CHAPTER 3

RESULTS

3.1 Knowledge of Notch Signaling is Vital to Understanding of Osteogenesis

The differentiation of stem cells is a highly regulated process that involves many mechanisms working together to attain the desired lineage. One of the most potent methods to increase cellular differentiation is the selective inhibition or activation of signaling pathways that carry out this regulation. Before manipulations such as this can occur, researchers must fully define the role and mechanism of action for a given pathway. One of the most actively researched of these regulatory pathways is the Notch signaling pathway. This pathway is well known for its significant role in guiding the differentiation of stem cells at all levels of development, but the exact role of its components in specific types of differentiation remains unknown.

One way to enhance our mechanistic understanding of differentiation is through the systematic examination of signaling pathways that control cell fate. The Notch signaling pathway is a highly conserved signaling cascade known to regulate cell state and multipotent differentiation of hASCs. The Notch pathway is involved in the regulation of many differentiation processes where it plays a key role in initiating and maintaining the cellular modification of these processes. The Notch pathway is a cell-to-cell contact-dependent signaling pathway; meaning that the pathway is activated when a receptor on the surface of the signal-receiving cell encounters a specific ligand on the surface of a signal-presenting cell. The pathway consists of four unique receptors and five unique ligands. These receptors are Notch1, Notch2, Notch3, and Notch4 while the

receptors are DLL-1, DLL-3, DLL-4, Jagged1, and Jagged2 (Braune and Lendahl 2016). The gene expression profile and subsequent phenotype that a cell adopts depends on which receptor binds to which ligand. This is crucial during tissue development because it establishes coordination between cells during the phases of proliferation (cell division), differentiation, and maturation of differentiated cells (Braune and Lendahl 2016).

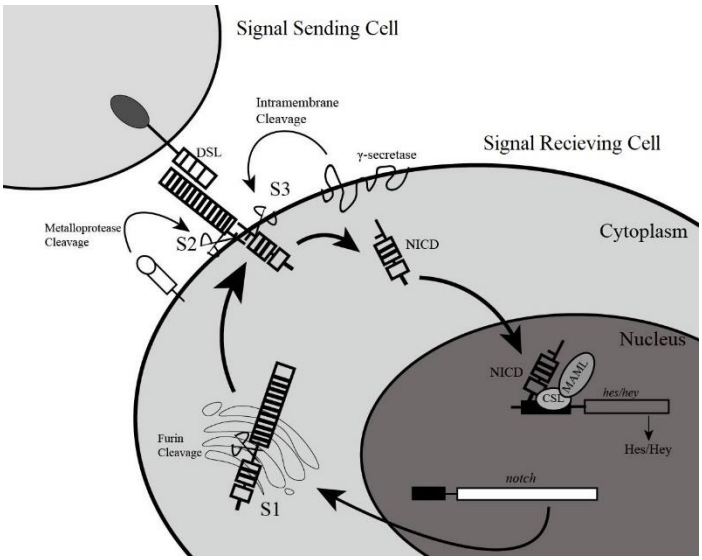


Figure 3-1: Diagram representing the mechanism of the Notch signaling pathway. Figure Credit: Mengcheng Liu

With the knowledge that osteogenesis is a large-scale process involving interactions between multiple cell types, studying a cell-to-cell signaling pathway like Notch has potential to elicit valuable insight into the molecular regulation of this process. By characterizing how the Notch pathway changes during hASC osteogenesis, valuable knowledge of the role of Notch1 and 3 in this process stands to be gained. Through the use of siRNA knockdowns, the effects of these receptors can be more precisely determined while also gaining insight into how molecular manipulation of this pathway could be used to enhance the osteogenic potential of hASCs for therapeutic applications. Through these assays, we have been able to determine that Notch3 plays an active role in

initiating and maintaining osteogenic differentiation in hASCs as seen by its increase in expression and negative impact on osteogenesis following siRNA-mediated knockdown. Notch1 appears to not have a role in promoting osteogenesis; however, the decrease in expression seen at the 14-day time point seems to allude to a potential role in pre-osteoblast proliferation which would echo findings seen in other literature (He and Zou 2019). The opposing effects seen in these two receptors suggests that the Notch signaling pathway can take on many different roles in regulating differentiation through the use of multiple receptor interactions.

3.1.1 Proper Cell Culture Conditions Are Needed for Osteogenesis

Prior to characterization of Notch signaling, culture conditions for efficient and reproducible osteogenic differentiation needed to be optimized. This included examining cell density prior to inducing differentiation and duration of differentiation.

Cell Density.

In order to produce proper *in vitro* testing, it is vital that the cells form a uniform layer across the bottom of the culture plate. This ensures that any cell-to-cell communication mechanisms necessary for proper differentiation can interact. Initially, osteogenesis was initiated by adding the Purchased Media the day after cells were passaged onto 6-well plates. This yielded a "clumping" phenomenon that was deemed to be due to low density and the increased tension associated with osteocyte calcification [**Figure 3-2**]. We repeated differentiation by seeding cells and waiting for them to reach a confluency of 55-60%, at which point they were in a a uniform and stable "cell sheet." This ensured more consistent cell-cell contact across the plate rather than in concentrated areas.

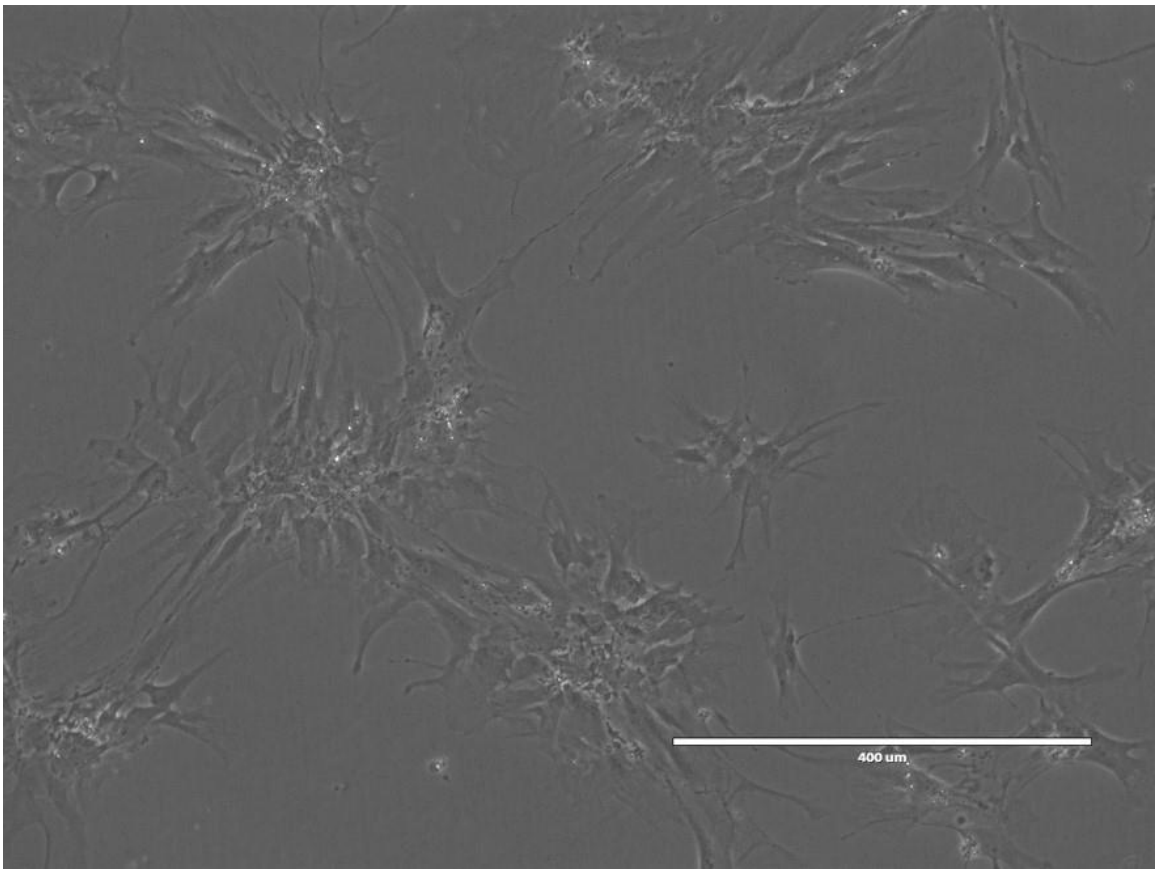


Figure 3-2: Phase contrast image displaying the "clumping" phenomenon seen from initiating osteogenesis too early. Image taken at 10x magnification.

Duration of Differentiation.

Once we determined proper density for inducing differentiation, cells we attempted to culture cells for up to 21 days under osteogenic conditions. The cells were very inconsistent over this period of time, with most wells not able to get past 17-19 days of differentiation before we noticed dramatic changes in cell behavior. Between 17 and 19 days after inducing differentiation, the cells would begin to lift off of the plate due to the increased tension associated with osteogenesis. Due to this phenomenon, the final timepoint for collection was determined to be 14 days into differentiation.

3.1.2 Lab-Made Media Displays Highest Osteogenic Potential

To ensure the reproducible and efficient initiation of osteogenesis, two different media recipes were tested. We compared purchased media, which offered consistency in manufacturing but came with the need to frequently order, wait on delivery, and an additional expense to the lab. We also looked through the literature and identified a recipe we could make in the lab that would save the lab money and ensure fresh media generation for repeated experiments. The testing was conducted by culturing cells in parallel the Lab-Made Media and the Purchased Media for up to 14 days. qRT-PCR assessment of *runx2*, a master regulator of osteogenesis, showed that the Lab-Made Media displayed higher levels of *runx2* expression at the Day 7 timepoint when compared to the Purchased Media [**Figure 3-3**]. This was repeated several times and demonstrated the ability to reproducibly and efficiently initiate osteogenic differentiation of hASCs. Once the Lab-Made Media was determined to produce higher levels of differentiation, effects of the media on differentiation were examined more closely. The expression of *runx2* and an additional osteogenic marker, *alp*, were evaluated to ensure consistent differentiation [**Figure 3-4**]. The observed increase in expression of both *runx2* and *alp* shows that this media is initiating consistent osteogenesis for a prolonged period of time. With verification that the Lab-Made Media produces high quality levels of osteogenesis compounded with the logistical and financial benefits, this was the media that was chosen to be used in further experiments.

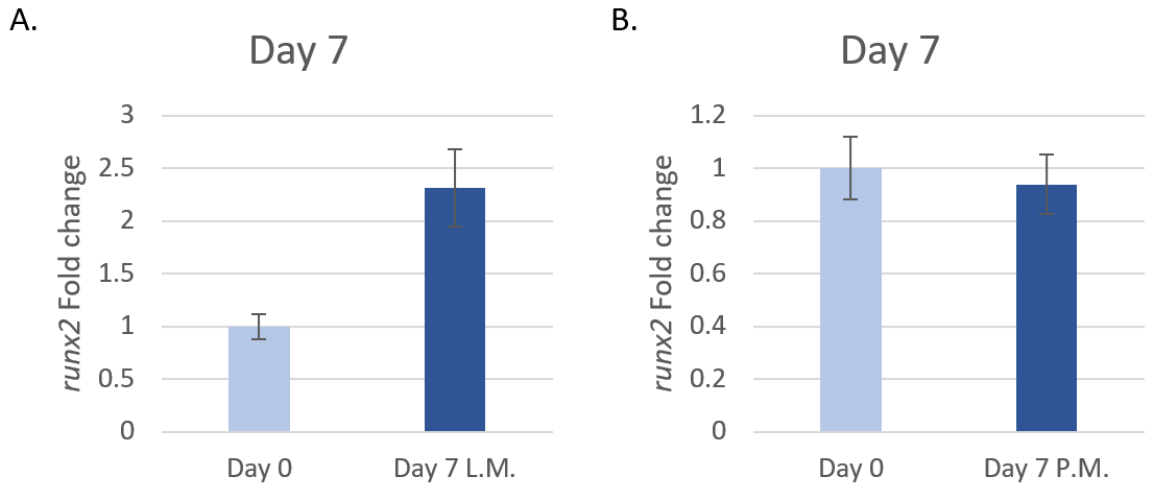


Figure 3-3: Characterization of *runx2* expression in Purchased Media (P.M.) and Lab-Made Media (L.M.) samples. **A** qRT-PCR observing the difference in *runx2* expression between Day 0 and Day 7 samples cultured in Lab-Made Media (p=0.025564). **B** qRT-PCR observing the difference in *runx2* expression between Day 0 and Day 7 samples cultured in Purchased Media (p=0.726747). Experiments were run in biological triplicate with n=3.

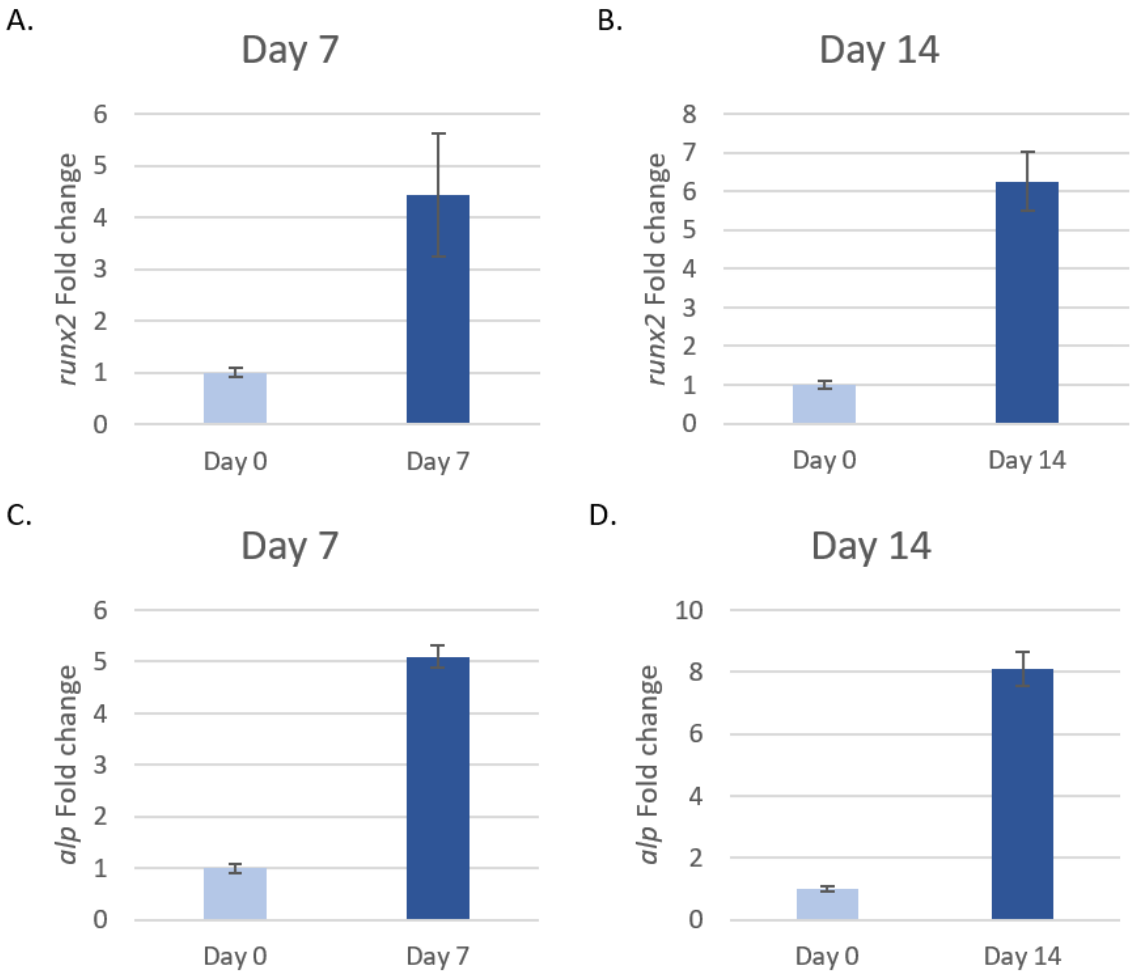


Figure 3-4: Characterization of *runx2* and *alp* expression in samples cultured in the Lab-Made Media. **A** qRT-PCR observing the difference in *runx2* expression between Day 0 and Day 7 samples (p=0.044647). **B** qRT-PCR observing the difference in *runx2* expression between Day 0 and Day 14 samples (p=0.002263). **C** qRT-PCR observing the difference in *alp* expression between Day 0 and Day 7 samples (p=0.0000625). **D** qRT-PCR observing the difference in *alp* expression between Day 0 and Day 14 samples (p=0.000194). Experiments were run in biological triplicate with n=3.

To further characterize the difference in osteogenic potential between the Lab-Made and Purchase Media, a series of alizarin stains was performed [**Figure 3-5**]. Alizarin stains stain the calcium deposits that develop in the extracellular matrix excreted by developing osteoblasts. The staining of these deposits give a very good qualitative measure of osteogenic potential of the differentiating cells. Mixed results were seen in the

Purchased Media samples which displayed bright red staining but the characteristic "film" that grows over differentiating osteocytes would consistently detach after adding the stain. In Lab-Made Media samples, a different brand of alizarin solution was used due to limited stock of the previous stain used on the Purchased Media samples. With the stain used on the Lab-Made Media samples, there were many challenges with getting the stain to produce the characteristic red color. Different fixing and rinsing reagents were tried including fixing the cells with 70% ethanol instead of formalin and rinsing the cells with saline instead of PBS. When the fixing reagent was switched to ethanol and the rinsing reagent was switched to saline, the red color was still not present, but staining quality was much higher [**Figure 3-6**]. After consulting with Dr. Jeffrey Gimble's lab at Obatala Sciences, we determined that our issue was most likely due to the pH of our alizarin solution. However, difficulties with equipment used to determine the solution's pH meant that the pH could not be properly adjusted. This issue will continue to be explored in the future.

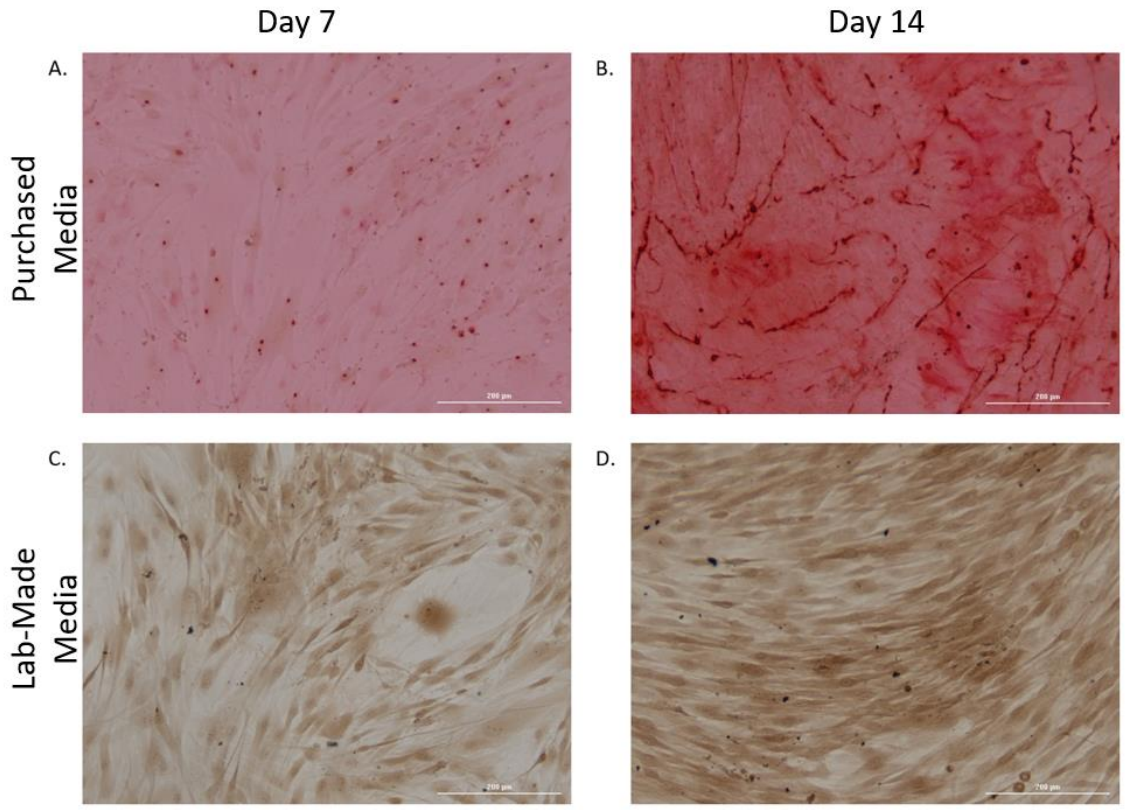


Figure 3-5: Alizarin red stain which stains calcium deposits in differentiating osteocytes, giving a qualitative measure of osteogenic activity. **A.** Purchased Media: 7 days of osteogenesis. **B.** Purchased Media: 14 days of osteogenesis. **C.** Lab-Made Media: 7 days of osteogenesis. **D.** Lab-Made Media: 14 days of osteogenesis.

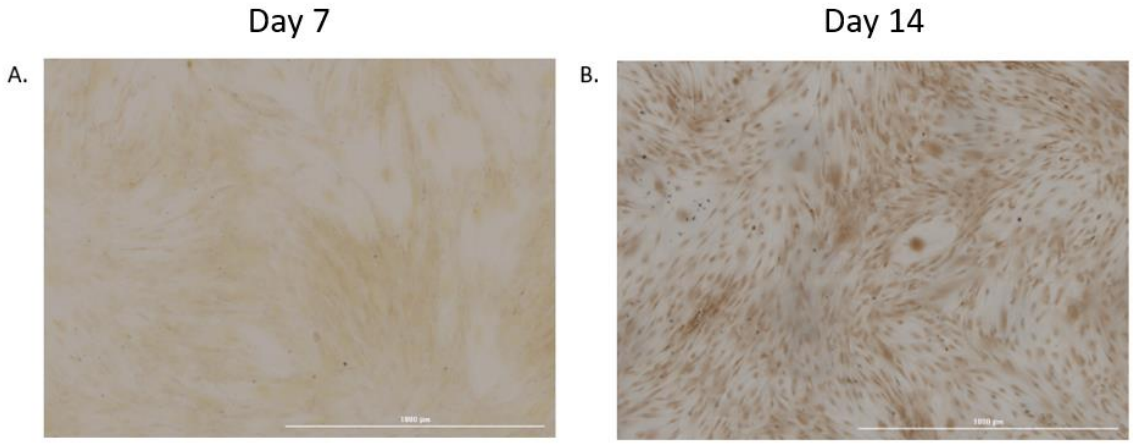


Figure 3-6: Fixing cells with 70% ethanol and rinsing with saline yielded better staining even though it did not get the characteristic color of alizarin staining. **A.** Alizarin stain performed using formalin for fixing and PBS for rinsing. **B.** Alizarin stain performed using 70% ethanol for fixing and saline solution for rinsing.

3.1.3 Notch Gene Expression Profile Changes During Osteogenesis

Once we determined optimal conditions for osteogenesis and had some tools to assess differentiation, we wanted to understand how the Notch pathway regulates osteogenesis. The first step was to profile Notch receptor expression during differentiation. By observing the expression changes that the Notch pathway experiences throughout osteogenesis, we can gain greater insight into the role that individual receptors have in this process. After cells were exposed to osteogenic media for a total of 14 days, qRT-PCR results show that *notch1* gene transcription remained unchanged at the Day 7 timepoint while it decreased at Day 14. Conversely, *notch3* appears to show elevated expression levels at both Day 7 and 14 with a peak at the Day 7 timepoint [**Figure 3-7**].

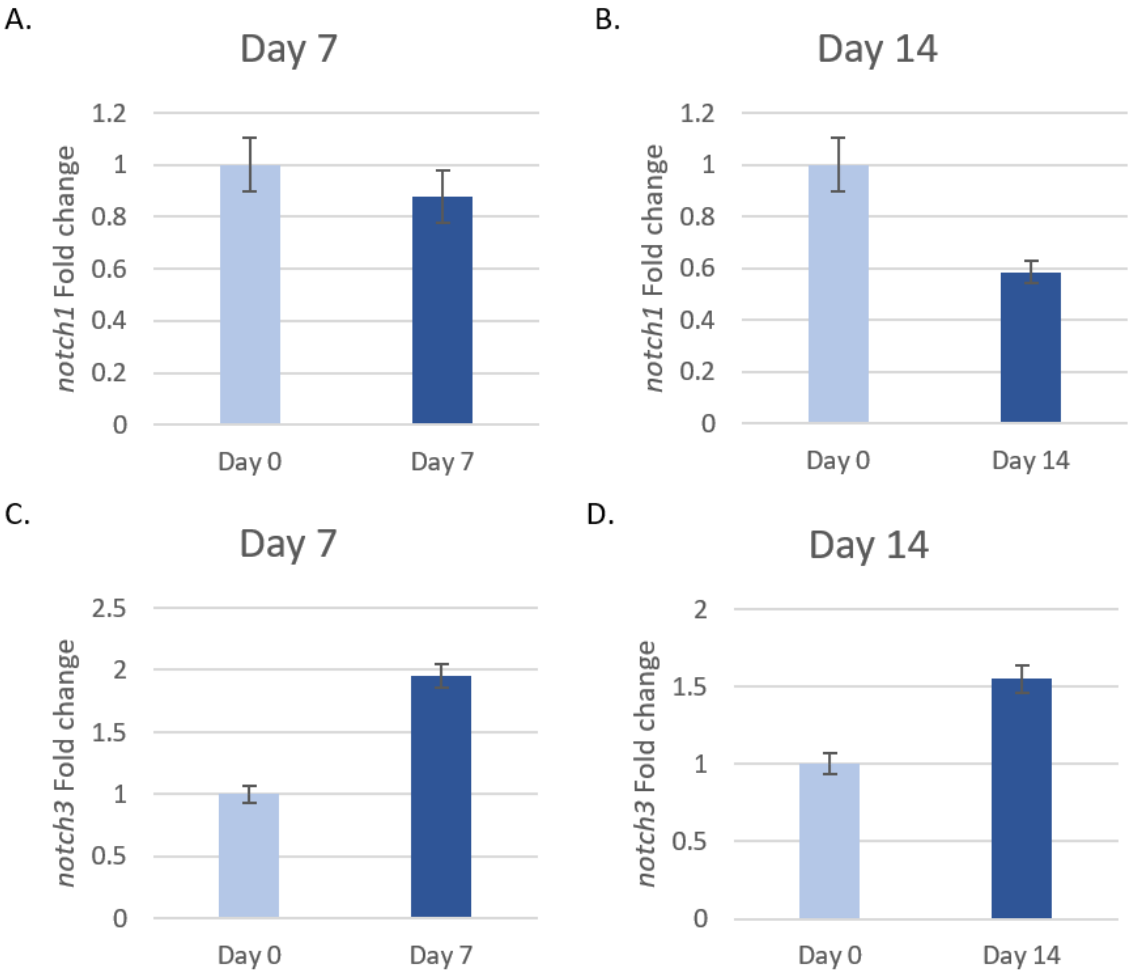


Figure 3-7: Characterization of *notch1* and *notch3* expression in samples cultured in the Lab-Made Media. **A** qRT-PCR observing the difference in *notch1* expression between Day 0 and Day 7 samples (p=0.441873). **B** qRT-PCR observing the difference in *notch1* expression between Day 0 and Day 14 samples (p=0.019794). **C** qRT-PCR observing the difference in *notch3* expression between Day 0 and Day 7 samples (p=0.001278). **D** qRT-PCR observing the difference in *notch3* expression between Day 0 and Day 14 samples (p=0.007865). Experiments were run in biological triplicate with n=3.

Western blots were used to ensure that changes in *notch* transcript were echoed in Notch protein expression [**Figure 3-8**]. We examined both full length and cleaved version of Notch3 in order to study the activation of the receptor. There is the possibility that Notch3 shows elevated expression but if that receptor is not being activated then it will not have an effect on osteogenesis. This data shows that activated (cleaved) Notch3 visually suggests an increase in protein expression through 14 days of osteogenesis. The

consistent rise in GAPDH expression during osteogenesis brings questions to GAPDH's effectiveness as a loading control. While it does not maintain a consistent level of expression, housekeeping genes such as GAPDH tend to go through variations in expression as differentiation progresses. Given this, ImageJ analysis was performed to normalize the Notch3 data to the GAPDH data. The analysis of this preliminary western blot indicates that there is little change in cleaved Notch3 7 days into differentiation while the cleaved levels of Notch3 14 days into differentiation are elevated. This suggests that there is increased activation of the Notch3 receptor as osteogenesis progresses. As this was the first western blot performed for this series of experiments, it will be repeated to confirm reproducibility of results.

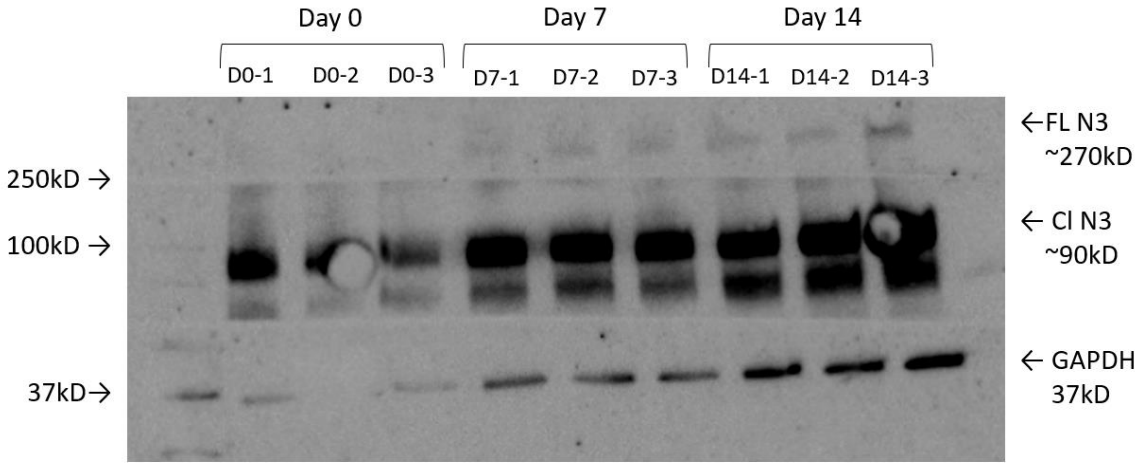
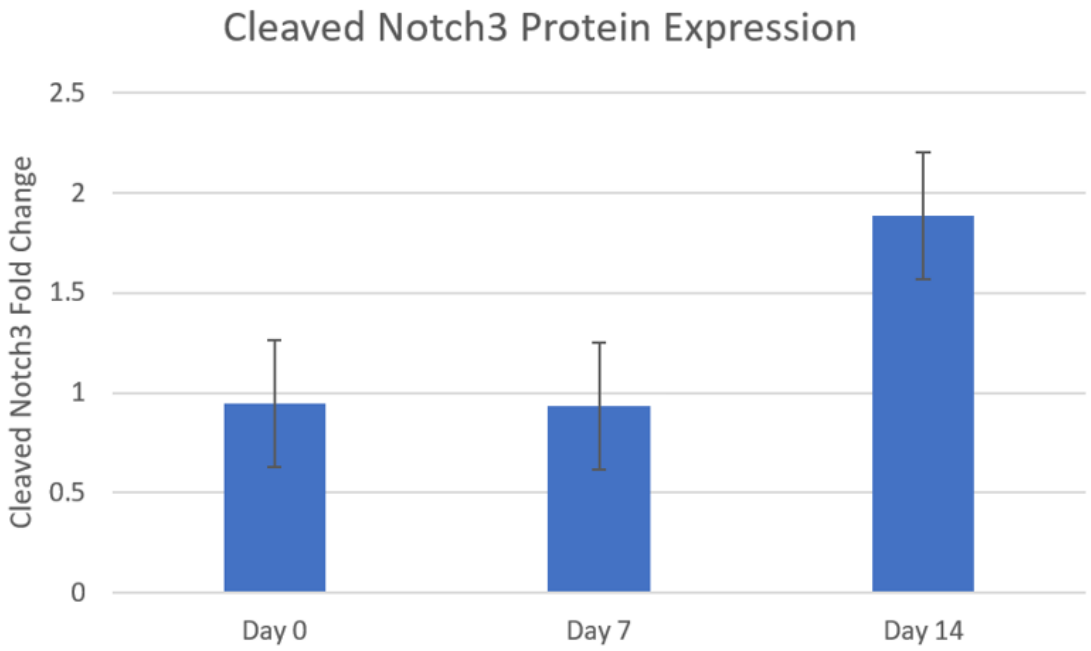


Figure 3-8: Western blots depicting the expression level change of the full length Notch3 receptor, the cleaved Notch3 receptor, and GAPDH at 0,7, and 14 days of osteogenesis. n=3

A.



B.

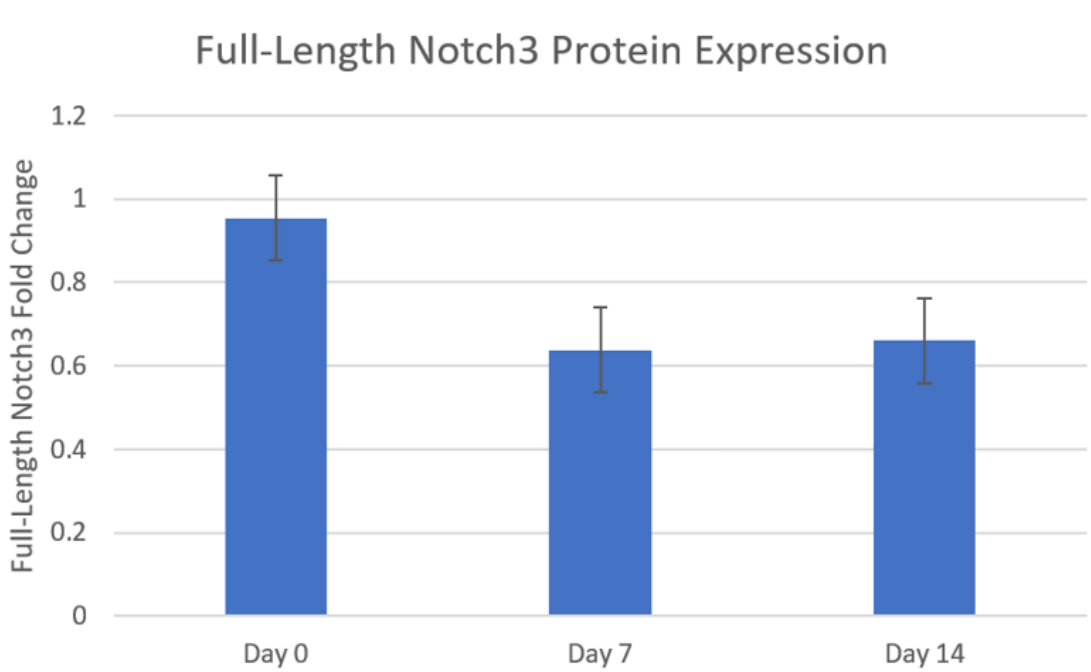


Figure 3-9: ImageJ analysis displaying the increase in cleaved Notch3 expression and a decrease in full-length Notch3 expression after normalization to GAPDH. Data displayed is the average value for each timepoint.

3.1.4 Notch3 siRNA Knockdowns Suggests That Notch3 Promotes Osteogenesis

Based on the expression data for Notch1 and Notch3 during the 14 days of osteogenesis, it was determined that the Notch1 receptor most likely plays a role in stem cell proliferation and maintaining stemness while the Notch3 receptor seems to play a role in promoting osteogenesis. To confirm these roles, siRNA knockdowns of each receptor were performed and their effects on osteogenesis were observed. The siRNA knockdown of *notch3* yielded a significant decrease in *alp* expression when compared to negative control samples for both day 7 and 14 of osteogenesis. However, the knockdown seems to have only affected *runx2* expression around the day 7 timepoint with day 14 samples showing transcript levels similar levels to those observed in the negative control [**Figure 3-10**]. The lack of impact of the *notch3* knockdown on *runx2* could allude to Notch3 only impacting Runx2 expression in the earliest stages of osteogenesis. The *notch1* knockdown was performed, but after using qRT-PCR to confirm the effects of the knockdown, it appeared that the initial knockdown was unsuccessful and so will be repeated for future studies [**Figure 3-11**].

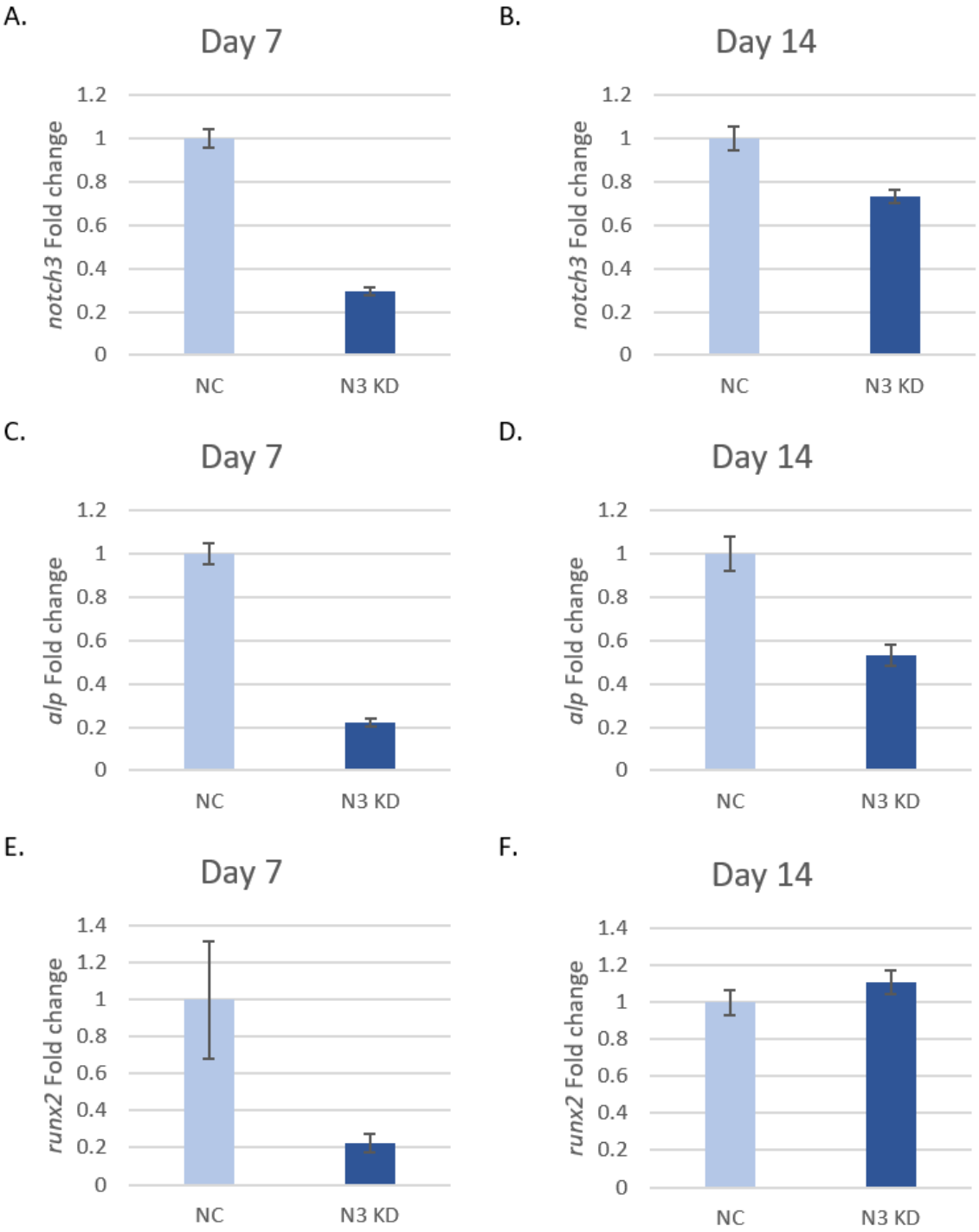


Figure 3-10: qRT-PCR data showing the change in gene expression of *notch3*, *alp*, and *runx2* after a *notch3* siRNA knockdown when compared to a negative control. **A.** *notch3* at day 7 of osteogenesis: p=0.0001. **B.** *notch3* at day 14 of osteogenesis: p=0.014621. **C.** *alp* at day 7 of osteogenesis: p=0.000113. **D.** *alp* at day 14 of osteogenesis: p=0.006541. **E.** *runx2* at day 7 of osteogenesis: p=0.058104. **F.** *runx2* at day 14 of osteogenesis: p=0.881895. Experiments were run in biological triplicate with n=3.

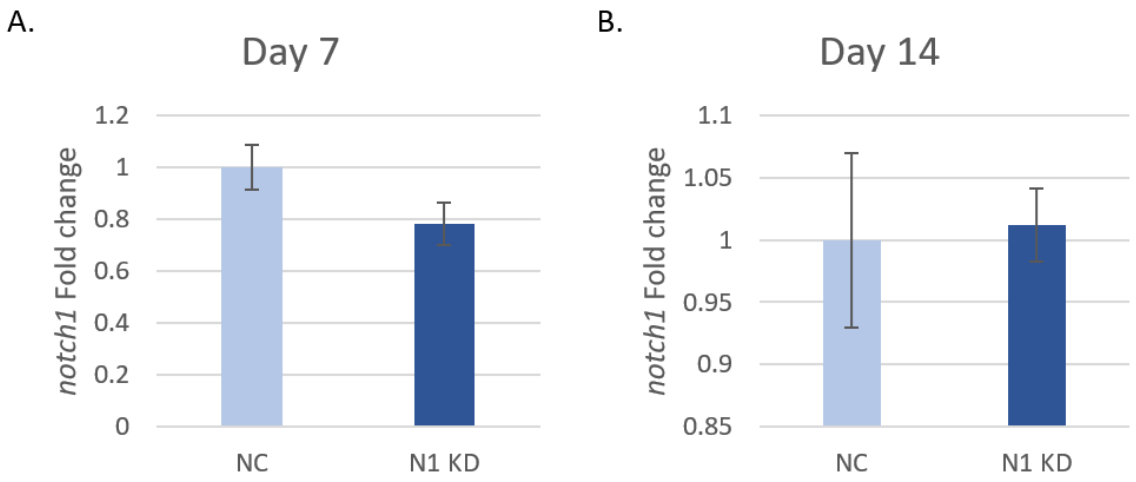


Figure 3-11: qRT-PCR data showing the change in *notch1* gene expression after a *notch1* knockdown when compared to a negative control. **A.** *notch1* expression at day 7 of osteogenesis: p=0.13783. **B.** *notch1* expression at day 14 of osteogenesis: p=0.881895. Experiments were run in biological triplicate with n=3.

CHAPTER 4

CONCLUSION AND FUTURE DIRECTIONS

4.1 Notch3 Play a Role in Regulating Adult Stem Cell Osteogenesis

4.1.1 Notch3 Initiated Differentiation While Notch1 Maintains Stemness

This research sought to investigate the role of the Notch1 and the Notch3 receptor in adult stem cell osteogenesis. Through these experiments, the trends observed suggest that Notch3 plays a role in initiating and potentially maintaining early osteogenesis due to its increase in expression during differentiation and a decrease in differentiation following the knockdown of Notch3. Conversely, Notch1 shows a decrease in expression as osteogenesis progresses, suggesting that Notch1 may have a less significant role in initiating osteogenic differentiation.

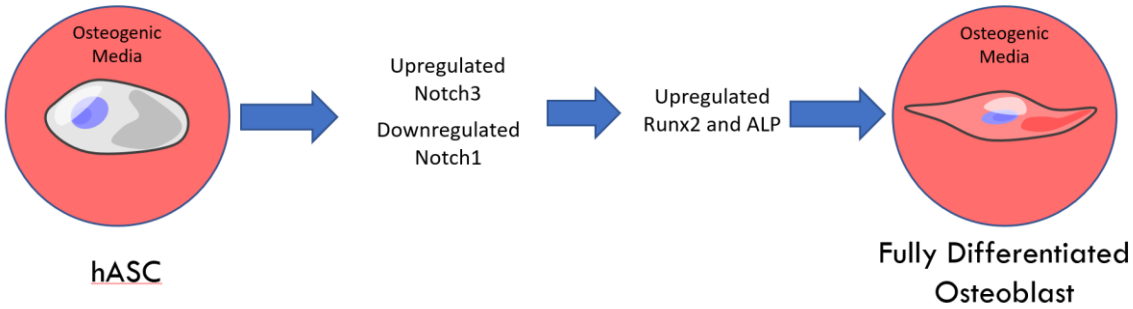


Figure 4-1: Notch1 and Notch3 both play a role in molecularly regulating early osteogenesis in adult stem cells.

Though the data gathered from both protein and genetic data echoed the same trend, there were some discrepancies around whether Notch3 expression continued increasing after day 7. Even though Notch3 expression remained elevated at day 14 of osteogenesis when compared to day 0 samples, genetic data showed these expression levels drop lower than day 7 while protein data showed expression higher at day 14 than day 7. This discrepancy highlights the importance of observing expression of both transcript and protein when studying drives of cell fate.

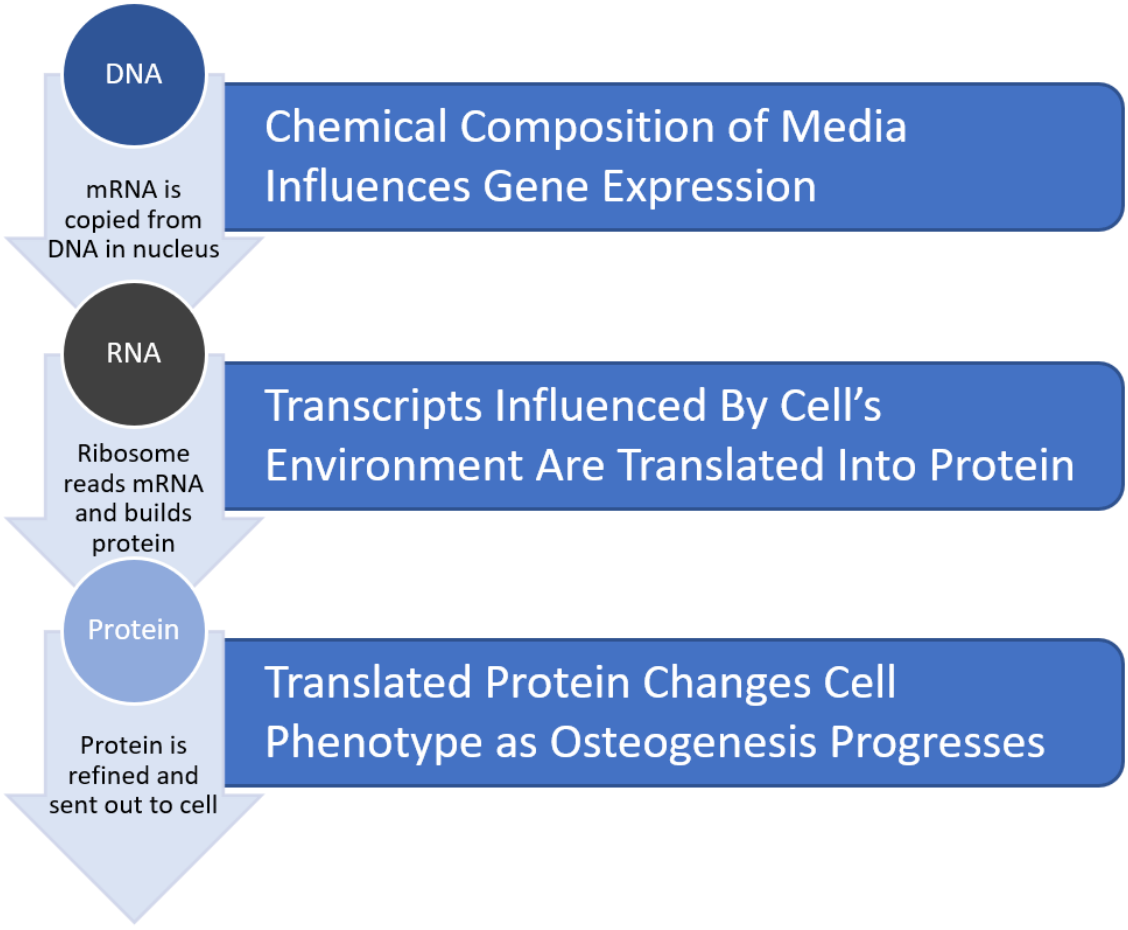


Figure 4-2: Osteogenesis is a process that affects transcription and translation and so requires analysis at each level.

This research takes a more systematic and detailed approach to studying Notch in osteogenesis., when compared to the literature which often relies on global pathway inhibitors to investigate the role of Notch signaling. By investigating the specific roles that individual Notch receptors have in the process of osteogenesis, this research provides a knowledge base that can be used to identify targets for therapeutic techniques seeking to utilize manipulated stem cells to curb bone degeneration. With the knowledge that Notch3 appears to initiate osteogenesis, researchers could develop cell lines that have a natural upregulation in Notch3 expression or even develop biomaterials that can activate Notch3 to prime hASCs for transplantation into patients suffering from bone degradation. Even if this method of Notch3 upregulation could not be developed or if it is not effective, the knowledge that Notch3 plays such a role in osteogenesis gives researchers identifying the roots of bone degradation a target to investigate in bone degradation models.

In the future, the focus of this project will be to gather more data on the effects of Notch1 on osteogenesis by repeating siRNA knockdowns to gather definitive data on this receptor's role. Also, the data taken from this project will be compared to ongoing studies seeking to investigate any potential changes to the Notch pathway in osteogenic hASCs experiencing simulated microgravity. Bone degradation is a significant side effect experienced by astronauts spending prolonged amounts of time in microgravity. With NASA and other organizations preparing for longer manned missions to mars and other celestial bodies, this phenomenon is becoming a growing concern and must be addressed. By establishing Notch's role in osteogenesis, it provides a research target that can be explored in this microgravity environment to see if the pathway is affected by these

conditions. This could provide deeper insight into the mechanism of microgravity-related bone degradation which would lead to a potential solution to this growing issue.

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