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THE ROLE OF WNT3a IN BONE-MUSCLE BIOCHEMICAL CROSSTALK AND PROMOTING MYOBLAST DIFFERENTIATION AMONG MOUSE PRIMARY MUSCLE CELLS

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by

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ABSTRACT

THE ROLE OF WNT3a IN BONE-MUSCLE BIOCHEMICAL CROSSTALK
AND PROMOTING MYOBLAST DIFFERENTIATION
AMONG MOUSE PRIMARY MUSCLE CELLS

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The University of Texas at Arlington, 2016

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Bone and muscle crosstalk is a newly emerging field that focuses on the ability of bone cells and muscle cells to communicate with each other through mechanical and biochemical mechanisms. Until recently, the secretory function of both bone and muscle cells has largely been unexplored. My thesis focuses on continuing my faculty mentor's current research on bone-muscle crosstalk by analyzing the effects of WNT3a, an important bone-secreted factor of the Wnt/β-catenin pathway, on myogenesis. Myogenesis is the process of producing muscle cells from stem cells in the embryonic stages of development and the regeneration of muscle tissue after cell injury or atrophy occurs (23). The primary goal was to determine the significance of WNT3a's function in promoting primary myoblast differentiation, building on research from a previous cell line (C2C12). I found

that at concentrations ranging from 10 ng/mL to 20 ng/mL of WNT3a, the myogenic differentiation was increased when compared to the controls. RT-qPCR experiments revealed that several gene expressions were affected, including the increased expression of the Muscle Regulatory Factors (MRF) MyoD and MyoG and the decreased expression of the most potent genetic inhibitor of muscle growth, myostatin.

I discuss the broader implications of our findings in the context of the twin diseases of aging, sarcopenia and osteoporosis, as well as other musculoskeletal diseases such as muscular dystrophy. While our studies are still pilot in nature, molecules secreted by bone cells that can beneficially affect skeletal muscles might have a potential impact on the treatment of sarcopenia, osteoporosis and other musculoskeletal diseases. The basic studies we conducted are needed for the transition from translational, to bench research, to ultimately the bedside, since any treatment that can affect myogenesis could in turn affect how muscles recover from injury and atrophy. Our studies could pave the way for future pharmacological interventions and new diet and exercise modalities that might lead to the elevation of WNT3a in bones and muscles. Therefore, our studies are in agreement with the Strategic Plan of the National Institute of Nursing Research (NINR), Advancing Science: Improving Lives.

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CHAPTER 1

INTRODUCTION

1.1 Skeletal Muscle

Skeletal muscle tissue has many vital roles within the human body, including initiating body movements, stabilizing body positions, transporting substances through the body, and producing thermogenesis (Tortora & Derrickson, 2012). Body movement is a delicate balance of cellular communication between bones, muscles, and joints. Skeletal muscle helps produce varying degrees of body movement, from large energy expenditures such as running or lifting weights, to the small, intricate movements of the diaphragm to help the lungs expand when breathing or the muscles used for facial expressions. Postural skeletal muscle contractions enable remaining in one position for long periods of time without conscious effort (Tortora & Derrickson, 2012). For example, the head can remain upright and the eyes open without consciously being aware of the thousands of muscle contractions simultaneously necessary to do so (Tortora & Derrickson, 2012).

As well as its role in voluntary and involuntary bodily movements, it is also vital to note the storage capacity of muscle tissue. Skeletal muscle contractions promote lymph circulation through the body and aid in the flow of systemic blood returning to the heart to be re-oxygenated by the lungs (Tortora & Derrickson, 2012). Another property of muscle tissue is its ability to generate heat. As skeletal tissue contracts, thermogenesis takes place which enables the body to maintain its homeostatic temperature.

The capabilities of skeletal muscle tissue within the human body are incredible. Four unique properties of skeletal muscle tissue have been identified that enable it to contribute to systemic homeostasis. Firstly, skeletal muscle tissue is able to respond to stimuli in the body by producing muscle action potentials (Tortora & Derrickson, 2012). Muscle action potentials are electrical signals sent by stimuli that excite the muscle cells to elicit a response. The two main types of signals sent by stimuli are electrical and chemical. Electrical signaling is auto-rhythmic, stemming from within the muscle tissue, itself. Chemical signaling is communicated by various stimuli throughout the body. Some examples of chemical stimuli are changes in pH, hormones, and neurotransmitter releases that trigger a chemical action potential the muscle cell then responds to (Tortora & Derrickson, 2012).

Contractility is another property of skeletal muscle tissue that is activated when an action potential occurs. Skeletal muscle tissue is attached to tendons and bones at various anatomical attachment points throughout the body. When muscle tissue contracts, the muscle fibers pull at the attachment points, causing tension, which in turn causes bodily movement (Tortora & Derrickson, 2012). Extensibility is the capability of muscle tissue to stretch to a certain degree without being damaged. Extension and contraction of muscle tissue are the two main properties that allow bodies to move so freely (Tortora & Derrickson, 2012). Elasticity is another important property of muscle tissue that ensures muscles are able to return to a state of rest after extensions and contractions occur

1.2 Myogenesis

Understanding the microscopic anatomy of a skeletal muscle fiber is vital to appreciate the delicacy and intricacy of how muscles work in groups to produce movement

(Frontera, Zayas, & Rodriguez). During embryonic development, progenitor cells receive a signal to specify and commit to becoming satellite stem cells (Verdijk et al., 2014). Myogenesis is the process of producing muscle cells from stem cells in the embryonic stages of development and the regeneration of muscle tissue after cell injury or atrophy occurs (Swatland, 2001). Myogenesis begins with the formation of premyoblast cells, the first specific cells of myogenesis. Premyoblast cells begin to lengthen in response to specific myogenic regulatory factors (MRF), including *MyoD* and *myogenin* and proliferate into myoblasts (Swatland, 2001). Myoblasts synthesize actin and myosin filaments, which are specific muscle proteins (Swatland, 2001). During the synthesizing process, the cells start to divide and myoblasts differentiate into myocytes, which begins the fusion process. The fusion process signals the beginning of the formation of myofibrils, which fuse to produce myotubes (Swatland, 2001). These immature myotubes continue to fuse and differentiate into mature muscle cell fibers (Bentzinger, Wang, & Rudnicki, 2012). Mature muscle cell fibers are fully functional cell units and are the smallest complete contractile units of muscles. Mature muscle fibers are able to participate in chemical messaging, contractility, extensibility, and elasticity.

Myogenesis is a highly regulated process that can occur in embryonic life stages as well as in the adult when injury or atrophy occurs. When muscle becomes damaged, local satellite cells are activated to repair the tissue (Swatland, 2001). Satellite cells function as myoblast precursors in the regeneration response. However, as we age, the number of available satellite cells in our muscle tissue decreases, which in turn directly impacts the amount of tissue that can be repaired, as well as the quality of the tissue being repaired

(Verdijk et al., 2014). The more muscle mass that is lost, especially due to aging as with sarcopenia, the less satellite cells that are available to repair damaged tissues.

1.3 Sarcopenia

Sarcopenia is a degenerative muscle condition caused by age related muscle tissue loss that results in decreased muscle tone and muscle strength. The term sarcopenia was introduced in 1988 by Dr. Irwin Rosenberg and derived from the Greek word that means "poverty of flesh" (Beres, George, Lougher, Barton, & Verrelli, 2011). Although some muscle tissue loss is considered to be a natural process of aging, sarcopenia refers to a severe loss of muscle that leads to increased frailty, decreased independence, and an increased risk for falls in affected populations (Zeng et al., 2016). When muscle mass is lost, there is a decline in muscle strength and functional ability which leads to loss of independence in older adults (Aleccia, 2014). Sarcopenia is also directly linked to an increase in fatigue, insulin resistance, falls, and mortality (Brotto & Johnson, 2014). Sarcopenia is associated with a decline in number and size of type I and type II myofibers.

In type II skeletal muscle fibers of older adults, satellite cells are significantly reduced which decreases the ability of muscle cells to regenerate and repair after an injury (Pan, Brotto, & Ma, 2014). A fine balance exists between the generation, breakdown, and regeneration of skeletal muscle tissue. The number of skeletal muscle fibers we have is not fixed. It is constantly adjusting to the demands of our bodies and environmental stressors. However, because older adults have a decreased number of type II skeletal muscle fiber cells, even the stress of heavy weight-bearing activities can severely damage muscle function and lead to a loss of independence.

Sarcopenia is a major public health issue, especially in the older adult population, because the loss of functional ability increases dependence on healthcare interventions and hospitalizations. Currently, it's estimated that annual healthcare costs for the treatment and management of sarcopenia exceeds 20 billion dollars annually (Huang et al., 2014). Sarcopenia also increases the risk for hospital and skilled nursing facility admissions. Although sarcopenia is prevalent in the population, there is not yet a clear-cut diagnostic test to definitively prove whether or not an individual is affected. Currently, the lack of a functional definition of sarcopenia is causing the disease to be under-diagnosed and mistreated, or not treated at all (Bentzinger, Wang, & Rudnicki, 2012). Generally speaking, a working definition of sarcopenia is defined as a severe, pathological loss of muscle strength and functional capacity that inhibits older adults from keeping their independence. To date, no medication or accepted treatments have been proven to treat or prevent the effects of sarcopenia.

Currently, nursing goals for the holistic treatment and prevention of sarcopenia include providing adequate nutrition and increasing activity tolerance. There have been studies conducted that show healthy, well-rounded diets with selections rich in fish, fruits, and vegetables lead to greater muscle strength in older adults (Baumgartner, Koehler, & Gallagher, 1998). However, healthy diets cannot reverse the effects of sarcopenia once the muscles have been affected. Resistance exercises have also shown to be effective in maintaining muscle mass and improving muscle functioning in people of all ages, but does not completely reverse the damage already caused by sarcopenia. Therefore, new interventions are needed.

1.4 Osteoporosis

Osteoporosis is a degenerative bone condition commonly found in the aging population. Osteoporosis-related bone degeneration causes frailty and an increased risk of fractures and bone breaks in the affected aging population, thus limiting independence and decreasing ability to perform activities of daily living (Filigheddu, 2007). Osteoporosis is characterized by a systemic loss of bone, leading to increased weakness and frailty in older adults. An estimated 54 million Americans are currently diagnosed and being treated for osteoporosis and it is estimated that osteoporosis costs 19 billion dollars in healthcare related dollars annually (Brotto, 2011).

With osteoporosis, the signs and symptoms can be insidious, going unnoticed until a bone is broken. It is estimated that osteoporosis accounts for two million fractures, annually (Jahn et al., 2012). Unlike sarcopenia, osteoporosis is easily diagnosed with definitive testing. The most common way it is diagnosed is through the use of a Bone Mineral Density Test. A series of x-rays are taken to measure how much calcium, along with other bone density minerals, are in a specific set of bones (Jahn et al., 2012). Usually, the spine, hip, or forearms are the bones of choice for the test.

Osteoporosis and sarcopenia are often found affecting the aging population simultaneously. Osteoporosis leads to increased frailty and decreased independence, which harbors the perfect conditions for sarcopenia to occur (Mayo Clinic Staff, 2016). Symptoms such as cachexia, decreased BMI, and increased fall risk due to frailty are common to both sarcopenia and osteoporosis (Isaacson & Brotto, 2014). Further research needs to be implemented to develop clear-cut treatment plans for those suffering from osteoporosis and sarcopenia.

CHAPTER 2

LITERATURE REVIEW

Bone and muscle crosstalk is a newly emerging field that focuses on the ability of bone cells and muscle cells to communicate with each other through physical and biochemical mechanisms (Brotto, 2011). The traditional nineteenth and twentieth century view between bone and muscle communication was predominantly limited to the mechanical interaction, represented in the mechanostat model. The mechanostat model is an adaptation of Wolff's Law, which strictly focuses on the ability of healthy bone to remodel and adapt to mechanical forces placed upon it (Hinkle & Cheever, 2014). Studies have shown that bone will adapt its mass and structure based on the mechanical load placed against it and the contraction of surrounding skeletal muscle (Brotto, 2011). However, until recently, the secretory function of both bone and muscle cells has largely been unexplored.

Only over the past few decades has research been conducted to show that bone and muscle cells secrete endocrine factors that bidirectionally influence processes such as myogenesis, the molecular signaling involved with motion, stress-related cell changes, and functional degeneration (Hinkle & Cheever, 2014). Recent research actually solidifies the endocrine function of bone and muscle cells based on their ability to produce factors that affects distant tissues (Hinkle & Cheever, 2014). Bone and muscle cell endocrine function has been recently represented through the discovery of many important cell-secreted factors. Osteocalcin, calcium, and prostaglandins (specifically PGE_2) represent a few of the major bone-secreted factors while brain-derived neurotrophic factor, IL-6, and LIF are

important muscle-secreted factors (Brotto, 2011).

The relationship between bones and muscles actually begins during embryonic development, with the rise of the paraxial mesoderm that gives way to the somites that produce specific bone and muscle tissue (Pan, Brotto, & Ma, 2014). As development progresses, bones and muscles are known to communicate with each other mechanically in order to maintain homeostasis. As research related to the degeneration of the bone and muscle function comes into the forefront, especially since such a large quantity of the aging population is affected, the biochemical crosstalk between the two major body systems are now being examined in a much closer light. This new knowledge might provide unrealized opportunities for the development of new therapies that concomitantly address diseases in both bone and muscle tissues. Thus, translational research is required to fill the gaps. These studies are encouraged by the most prominent nursing research entity in the United States, the National Institute of Nursing Research (NINR). The NINR states that its mission "is to promote and improve the health of individuals, families, and communities" (King, 2016). To achieve this mission, "the NINR supports and conducts clinical and basic research and research training on health and illness, research that spans and integrates the behavioral and biological sciences, and that develops the scientific basis for clinical practice" (King, 2016).

2.1 Research of Functions of WNT3a

Dr. Marco Brotto's lab has performed experiments to show that WNT3a affects myogenesis of an established mouse myoblast cell line, known as C2C12 cells (Fisher-Aylor & Williams, 2011). C2C12 cells are a line of mouse skeletal myoblasts established at the Weizmann Institute of Science in Israel in 1977, from the thigh muscle of a C3H

mouse (Fisher-Aylor & Williams, 2011). When C2C12 myoblasts were treated with WNT3a, myogenesis and calcium homeostasis were significantly increased (Fisher-Aylor & Williams, 2011). Another study recently completed highlights the capability of muscle cells to protect against a bone cell-secreted factor that results in apoptosis. Conditioned media from C2C12 myotubes were able to protect the cells from apoptosis, caused by the introduction of MLO-Y4 osteocyte dexamethasone (Brotto, 2012).

Another important research breakthrough is the discovery of bone cell and muscle cell secreted factors that interact between the tissues on a biochemical level. Osteocalcin, calcium, and prostaglandins (specifically PGE_2) represent a few of the major bone cell secreting factors while brain-derived neurotrophic factor, IL-6, and LIF are important muscle cell secreting factors (Brotto, 2011). These factors have the ability to affect distant tissues, which represents the true function of endocrine cells.

WNT3a is an important bone-secreted factor. Wnt3a, Wingless-Type MMTV Integration Site Family, Member 3A, is a gene of the WNT family. The WNT family consists of structurally related genes, which encode secreted signaling proteins (Fisher-Aylor & Williams, 2011). WNT is a merge of two main ideas. The W stands for the wingless in drosophila, the most studied in vivo WNT process and the "NT" stands for the int-1 integration site in mice, which is now called WNT-1 (Fisher-Aylor & Williams, 2011). Mutations in WNT signaling have been shown to result in cancer, human birth defects, and other chronic diseases because of WNT's major role in directing cell proliferation and differentiation during the embryonic development process (Fisher-Aylor & Williams, 2011).

The myogenic effects of WNT3a have previously been studied in the Brotto Muscle-Bone Laboratory. Based on studies using morphologically and immunohistochemical experiments, a concentration of 10 ng/mL of WNT3a has been shown to enhance C2C12 myogenesis by communicating through the expression of muscle regulatory factors (Fisher-Aylor & Williams, 2011). Even WNT3a concentrations as low as 0.5 ng/mL have been shown to increase myoblast differentiation, which was confirmed morphologically and immunohistochemically (Fisher-Aylor & Williams, 2011). Previously, only cell lines were used, and the goal of my studies was to expand and investigate the effects of WNT3a on primary muscle cells because of their higher physiological relevance.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

The materials used in this study were: DMEM high glucose media, penicillin-streptomycin (P/S) 10,000 U/mL each and trypsin-EDTA 1× solution from Mediatech Inc. (Manassas, VA, USA); fetal bovine serum (FBS) and horse serum (HS) from Thermo Fischer Scientific Inc. (Waltham, MA, USA); diamidino-2-phenylindole (DAPI) from Sigma-Aldrich (St Louis, MO, USA); rat tail collagen type I from BD Biosciences (San Jose, CA, USA); Recombinant Mouse Wnt-3a from R&D Systems Inc. (Minneapolis, MN, USA); Tri reagent from Molecular Research Center, Inc. (Cincinnati, OH, USA); ProtoScript First Strand cDNA Synthesis Kit; Direct-zol RNA MiniPrep; RT^2 Real-Time™ SYBR green/Rox PCR master mix from SABiosciences (Valencia, CA, USA) and Anti-human Myosin Heavy Chain-CFS, Carboxyfluorescein (CFS)-conjugated mouse monoclonal anti-human Myosin Heavy Chain antibody from R&D Systems Inc. (Minneapolis, MN, USA).

3.2 Methods

Aim one of my project involved studying the function of WNT3a in mouse primary muscle cell (MPMC) differentiation using morphological, and immunohistochemical studies. To satisfy aim one, I performed myogenesis and Fusion Index (FI) experiments following protocol published by the Brotto Lab (Huang, 2014). Aim two of my project was to investigate the molecular mechanism of WNT3a on MPMC differentiation. Molecular

genetic signaling studies were used to determine WNT3a's mechanism of action on MPMCs. The goal of genetic signaling studies is to discover the mechanisms of WNT3a in myoblast differentiation. These studies are essential in leading to future targets of drug therapies for sarcopenia, osteoporosis, and other musculoskeletal and bone diseases.

3.2.1 Frozen Cell Line Stock

I began my work with readily establish frozen primary muscle cells in the Brotto Lab, using previously published Brotto lab protocols (Huang, 2014). The primary cells were frozen in a medium of 80% FBS + 20% DMSO and stored overnight in -80°C. After 24 hours, the cells were transferred to a liquid nitrogen storage container to be kept for longer periods of time. Primary 6 cells (P6) were used for all portions of the experiment.

3.2.2 Collagen Type – I Coating of Cell Culture Flasks & Plates

To culture MPMC's, T-75 flasks and 6-well plates needed to be coated with a collagen-I coating solution. To coat the flasks and plates, Collagen-I coating solution (rat tail tendon liquid in 0.02 N acetic acid), T-75 flasks and six-well plates were placed inside a cell culture hood. 8 mL to 10 mL of collagen-I coating solution was then placed into each T-75 flask. 2 mL of collagen-I coating solution was used for each well of the six-well plates. Flasks and plates were left to coat for one hour. Then, the collagen solution was removed and the flasks and plates were left in the cell culture hood for an additional hour to dry. The flasks and plates were then stored in the 4°C laboratory refrigerator. The collagen-coated flasks and plates could be stored for use for up to one month.

3.2.3 Cell Culture & Growth of MPMC's

MPMC's had already been established in the Brotto Lab and were cultured using Brotto Lab protocols. Briefly, cells were seeded onto type I collagen-coated T-75 flasks

and grown in a prepared growth medium (F-10 + 20% fetal bovine serum (FBS) + 1% penicillin/streptococcal antibiotics + 5 ng/mL of bFGF). After the cells were seeded onto the collagen-coated flasks, they were cultured at 37°C in a controlled humidified 5% CO_2 cell culture incubator to grow for 48 hours. At 48 hours, the myoblasts proliferated but did not differentiate.

After 48 hours, the cells were removed from the cell culture incubator and the growth medium was removed so that the cells could be split. The cells adhering to the collagen coated T-75 flasks were washed with sterile 10% PBS and trypsinized using 1.5 mL of 0.25% trypsin-EDTA for 3 minutes at room temperature. After 3 minutes, the flask was gently knocked against the edge of a table 10 times to lift the cells from the flask. In the cell culture hood, 4 mL of primary growth medium was then added to the flask to deactivate the effects of the trypsin. Using a pipette, the solution of cells, trypsin, and primary growth medium were transferred into a 15 mL tube and centrifuged at 350 rpm's for five minutes.

After five minutes of centrifuging, the supernatant was disposed of and the cells were suspended with 5 mL of primary growth medium and re-plated onto 2, type-I collagen coated T-75 flasks to proliferate. The cells were stored at 37°C in a controlled humidified 5% CO_2 cell culture incubator for 48 hours.

After 48 hours, the cells underwent the same process of trypsinizing and centrifuging as when they were split. After the cells were centrifuged and the supernatant was removed, the cells were re-suspended in 6 mL of primary growth medium. Then, the cell concentration was determined using a hemocytometer. Using established protocol in

the Brotto lab, the cell concentration was calculated and the cells were divided evenly into two, 6-well plates (300,000/well) and incubated in a cell culture incubator (Huang, 2014).

3.2.4 Cell Differentiation

After 24 hours in the cell culture incubator, to induce differentiation of myoblasts into myotubes, the primary growth medium was removed from the cells and a differentiation medium (2.5 % horse serum in DMEM with 1% penicillin/streptococcal antibiotics) was added along with different concentration of WNT3a (5 ng, 10 ng, and 20 ng/mL), respectively. The cells were then incubated at 37°C in a controlled humidified 5% CO_2 cell culture incubator to differentiate. Fully differentiated, functional, contracting myotubes were observed at 72 hours after differentiation.

3.2.5 Cell Staining with MHC & DAPI

At 24 and 72 hours differentiation, the cells were stained following a protocol already established in the Brotto laboratory. Briefly, the differentiation medium was discarded from the 6-well plates in the chemical hood. Each well was washed with 2 mL of non-sterile PBS for five minutes. This step was repeated three times to ensure all of the media was removed. Cells were then fixed in a Neutral Buffered Formalin (NBF) for 8 minutes at 1 mL per well. After the fixation process was complete, cells were washed with 2 mL of non-sterile PBS for five minutes, three times.

Cells were permeabilized on the shaker with 2 mL per well of 0.1% Triton X-100 for ten minutes and then washed with non-sterile PBS. The cells were then moved into the PTI room and stained with 20 µl/mL of conjugated MHC antibody in 1x TBST (PBS with 0.1% Triton and 0.1% Tween) for forty-five minutes. After forty-five minutes, 1 µl of DAPI-1 µg/µl (1:1000) was added to each of the wells and put on the shaker for five

minutes. The cells were then washed with non-sterile PBS and stored in 1 mL of non-sterile PBS to be observed under the microscope.

3.2.6 Fluorescent Imaging & Fusion Index Calculations

After being counterstained, images of the MPMCs were taken using the Olympus IX73 Inverted Research microscopy system set up per Brotto lab protocol (Huang, 2014). Both phase contrast and fluorescent images were captured with this system. To quantify the myogenic differentiation of the MPMCs, the Fusion Index (FI) of the cells in the images were calculated. The Fusion Index is defined as: (nuclei within myosin heavy chain-expressing myotubes/total number of myogenic nuclei) × 100 (Brotto, 2012).

In previous experiments conducted in the Brotto laboratory, the FI of the MPMCs were manually counted from the images. Manually counting cell nuclei is a very time-consuming process that would take several days to complete. Because I would be relying on only the naked eye to count hundreds of cell nuclei, there is potential for error. For my experiment, the FI was calculated using new CellSims software that has an automatization feature. The software counted the total number of individual cell nuclei in each image. To calculate the FI, the total number of nuclei in each myotube was obtained, separately. VSI and TIF images were taken from randomly selected areas of each six-well plate that was imaged and the FI was calculated.

3.2.7 RNA Isolation

Total RNA was isolated from the cells with Tri reagent according to manufacturer's protocol. Briefly, The Tri reagent was added to the cells and sat at room temperature for two minutes. The Tri reagent and cell mixture was transferred to individually labeled 1.5 mL tubes and stored at -80°C. To isolate the total RNA, tubes with Tri reagent were

removed from the freezer and brought to room temperature. Total RNA was isolated by using the Direct-zol RNA MiniPrep kit according to manufacturer's protocol. The concentration and quality of RNA was detected with a Thermo Scientific Nano Drop 1000. The 260/280 and 230/280 ratio needs to be 1.7 or greater to satisfy the requirement for cDNA synthesis. The cDNA was synthesized by using ProtoScript First Strand cDNA Synthesis Kit according to manufacturer's protocol, using 0.5 µg or 1 µg total RNA to synthesize the cDNA of each sample.

3.2.8 Real time PCR (RT-PCR)

RT-PCR was performed using RT^2 Real-Time™ SYBR green/Rox PCR master mix. RT-PCR primers used in this study are summarized below in Table 3.1. RT-PCR was run in a 25 µl reaction volume on 96-well plates using a StepOnePlus instrument (Applied Biosystems, Foster city, CA, USA). Data was analyzed using StepOne Plus Software 2.3. C_t values were normalized to *Gapdh* (Glyceraldehyde-3-phosphate dehydrogenase) as a reference gene. Gene expression was determined by the up or down regulation of each gene, compared to the controls.

Primer	Primer Sequence (5’ to 3’)
GAPDH-F	TGCGATGGGTGTGAACCACGAGAA
GAPDH-R	GAGCCCTTCCACAATGCCAAAGTT
Numb-F	AACCAGCCTTTGTCCCTACC
Numb-R	GGCGACTGATGTGGATGAG
Fhl1-F	CAAGTCATCGGTGTTTGTGG
Fhl1-R	TGTCGTACTTGGGCGGGTTC
MyoD-F	CCCCGGCGGCAGAATGGCTACG
MyoD-R	GGTCTGGGTTCCCTGTTCTGTGT
MyoG-F	TGAGCATTGTCCAGGCCAG
MyoG-R	GCTTCTCCCTCAGTGTGGCT

Table 3.1: RT-PCR primers used in this study

CHAPTER 4

RESULTS

Figures 4.1, 4.2, 4.3, and 4.4 show the images of the primary myotubes taken at differentiation Day 3. Figure 4.1 is a phase contrast image taken using the Olympus microscopy set up. Figure 4.2 is a fluorescent image of nuclei stained with DAPI, a nuclear specific dye, in blue. Figure 4.3 is another fluorescent image taken that focuses on the myotubular expression of the cells, expressing myosin heavy chain (MHC), in green. Figure 4.4 is a merged image of the DAPI and MHC stained image, from which the FI was calculated.

Using the Olympus microscopy system set up per Brotto Lab protocol, Figures 4.5, 4.6, 4.7, and 4.8 qualitatively show mouse primary muscle cells stained without WNT3a (the control), 5 ng/mL, 10 ng/mL and 20 ng/mL WNT3a, respectively. All four images were taken 72 hours after WNT3a was added to the mouse primary muscle cells. Based on observation, it is clear that there are more differentiated myotubes with higher concentrations in Figure 4.7 and Figure 4.8, when compared to Figure 4.5. The FI quantitatively supports an increased number of differentiated myotubes in Figure 4.7 and Figure 4.8, compared to Figure 4.5, as summarized in Table 4.1.

In Table 4.1, averages ± SEs are shown. Table 4.1 shows that the FI calculation for the control was 21.97 and the FI for 5 ng/mL of WNT3a was slightly higher at 36.45 but did not achieve statistical significance ($p=0.44$). However, the Fusion Index for 10 ng/mL

WNT3a was 55.56, which is significantly higher than the control values (p=0.027). This data is also supported visually, as there are significantly more differentiated myotubes in Figure 4.7 there are in Figure 4.5. These observations support the idea that 10 ng/mL of Wnt3a increased mouse primary muscle cell differentiation.

The calculated Fusion Index of 20 ng/mL of WNT3a was 81.12, which is also significantly higher than the FI of the control values and any other WNT3a value (p=0.001). Based on the data collected in Table 4.1 and the qualitative data in Figure 4.8, compared to the control in Figure 4.5, both 10 ng/mL and 20 ng/mL WNT3a significantly increased the myogenic differentiation of MPMC's.

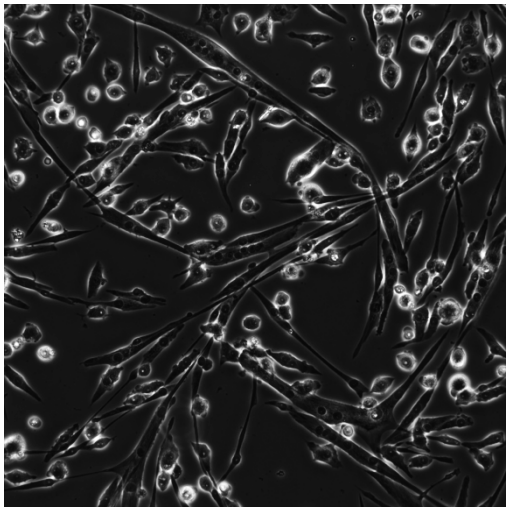

Figure 4.1: Phase Contrast image of primary myotubes

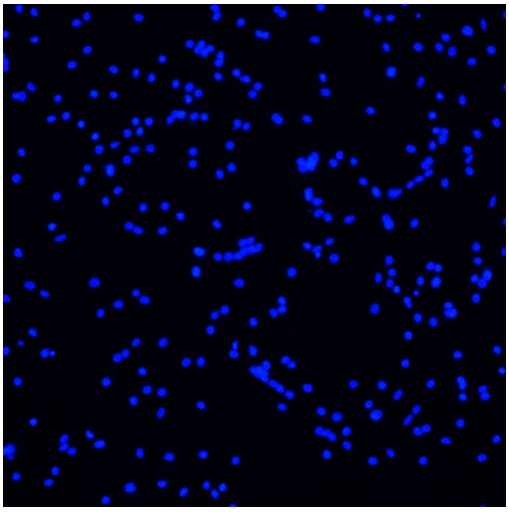

Figure 4.2: Fluorescent image of nuclei stained with DAPI, in blue

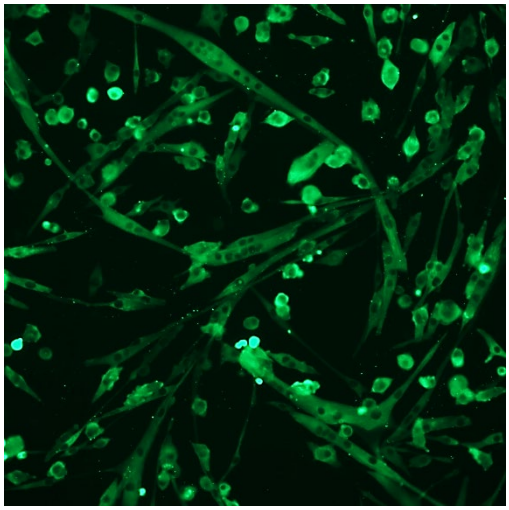

Figure 4.3: Fluorescent image of primary myotubes stained with myosin heavy chain (MHC), in green

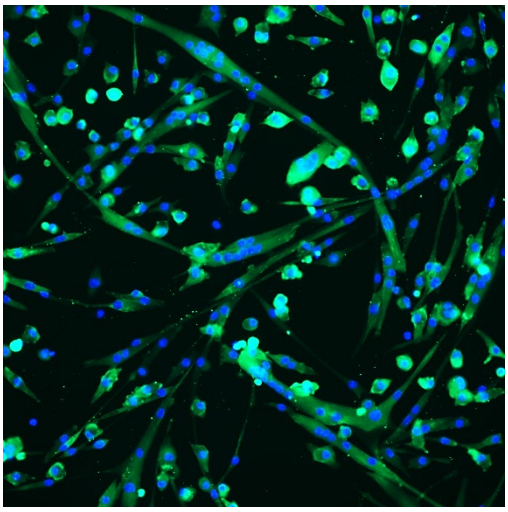

Figure 4.4: Fluorescent image of nuclei stained with DAPI in blue and primary myotubes stained with myosin heavy chain (MHC), in green

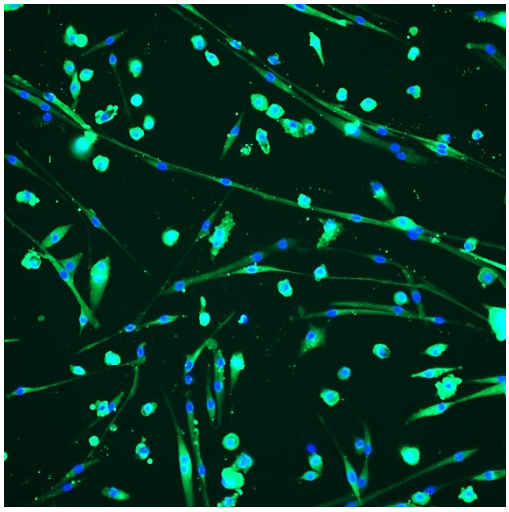

Figure 4.5: Fluorescent image of nuclei stained with DAPI in blue and primary myotubes stained with myosin heavy chain (MHC) in green, Control

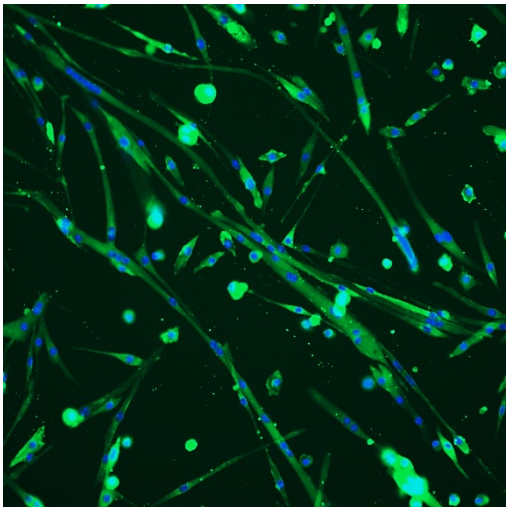

Figure 4.6: Fluorescent image of nuclei stained with DAPI in blue and primary myotubes stained with myosin heavy chain (MHC) in green, 5 ng/mL WNT3a

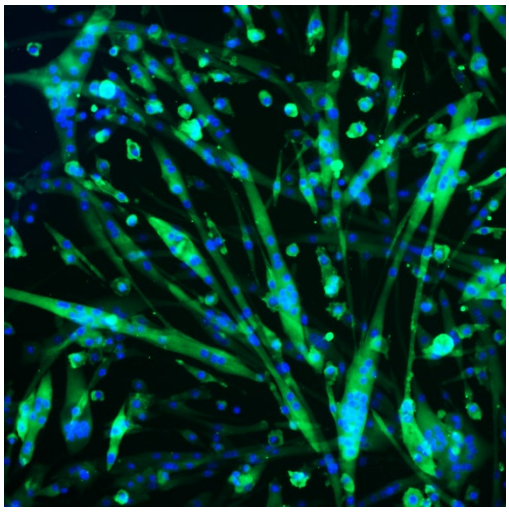

Figure 4.7: Fluorescent image of nuclei stained with DAPI in blue and primary myotubes stained with myosin heavy chain (MHC) in green, 10 ng/mL WNT3a

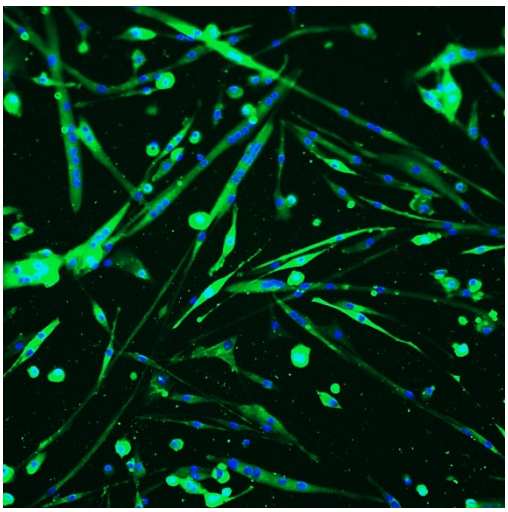

Figure 4.8: Fluorescent image of nuclei stained with DAPI in blue and primary myotubes stained with myosin heavy chain (MHC) in green, 20 ng/mL WNT3a

Treatment/Group	Fusion Index	SE
Control (no WNT3a)	21.97	± 6.58
WNT3a 5 ng/mL	36.46	± 6.59
WNT3a 10 ng/mL	55.57	± 4.98
WNT3a 20 ng/mL	81.12	± 7.65

Table 4.1: MPMC summary data of Fusion Index (FI) averages

WNT3a	RNA 260/280	RNA 260/230
C1	2	2.13
C2	2.14	1.7
C5	2.18	1.75
10	1.98	2.15

Table 4.2: MPMC RNA extraction for Control and 10 ng/mL of WNT3a

Genes	Gene Expression (10 ng/mL WNT3a)
MyoD	Increased
MyoG	Increased
Myostatin	Decreased
Numb	Increased

Table 4.3: RT-PCR gene expression of 10 ng/mLWNT3a

Total RNA was isolated from the cells with Tri reagent based on Direct-zol RNA MiniPrep protocol. RNA was then prepared for cDNA synthesis using ProtoScript First Strand cDNA Synthesis Kit. Based on manufactures protocol, RNA ratios of 1.7 or greater are needed to produce consistently accurate results. The RNA 260/280 and RNA 260/230 ratios 1.7 and greater for concentrations of 10 ng/mL WNT3a are listed in Table 4.2. After RNA was calculated and cDNA was synthesized, a RT-PCR experiment was performed and replicated, and the results are listed in Table 4.3.

CHAPTER 5

DISCUSSION

The morphological and immunohistochemical studies showed that WNT3a increases the myogenesis of mouse primary muscle cells. This is an important finding because myogenesis does not only occur in embryonic life, but also continues into adulthood when muscles become damaged or injured. These findings can also be implemented in disease states where the cycles of injury and repair are altered, such as in muscular dystrophies. Therefore, for translational nursing research, it is imperative that we find new ways to treat these diseases and new ways to modulate with the process of myogenesis.

Qualitative and quantitative data both express that added concentrations of WNT3a, ranging from 10 ng/mL to 20 ng/mL increase myogenic differentiation. Based on previous studies in cell lines, we expected to see that any concentration of added WNT3a would increase FI when compared to the controls. However, based on the calculated FI of these experiments, the WNT3a concentration of 5 ng/mL produced very similar FI as the controls. The FI steadily increased with concentrations of 10 ng/mL and significantly increased with concentrations of 20 ng/mL of WNT3a. The significant increase in FI at 20 ng/mL suggests that this concentration significantly increased the differentiation of mouse primary muscle cells when compared to the control values.

With the RNA, cDNA, and RT-qPCR experiments, we were expecting the following results: *MyoD* increased, *MyoG* increased, and *Myostatin* decreased. *Numb* and

FH1-1 are recently discovered genes of the Wnt pathway, which we predicted would increase. *GAPDH* was predicted to show no change. As shown in Table 4.3, the results from our RT-PCR analyses confirmed our predictions, confirming at the molecular level the enhanced differentiation induced by WNT3a.

MyoD and *MyoG* are essential muscle-specific transcription factors that can induce myogenesis (Yusuf, 2012). *MyoD* and *MyoG* are vital proteins to the development of muscle tissues. *Myostatin* is a protein produced by muscle cells that acts to inhibit muscle growth and differentiation (Yusuf, 2012). It is the most potent muscle inhibitor known, and there are several new medications being developed in clinical trials targeting this pathway to increase muscle mass in humans. Therefore, we expect to see a decreased in *Myostatin* production when WNT3a is added to cells, which was what happened in our experiments. *Numb* is a protein that regulates *Notch1* during myogenesis (Beres, George, Lougher, Barton, & Verrelli, 2011). *Numb* inhibits the degradation function of *Notch1* during myogenesis. Therefore, we expect to see an increased about of *Numb* during myogenesis because the more *Numb* that is present, the less degradation takes place (Beres, George, Lougher, Barton, & Verrelli, 2011). *FHl-1*is a downstream gene in the Wnt/β-catenin pathway that is involved in inducing myogenesis. Therefore, we expect to see *FHl-1* to increase with added concentration of WNT3a.

CHAPTER 6

FUTURE DIRECTIONS

The nursing implications for the biochemical crosstalk between muscle and bone cells are phenomenal. The newly emerging field opens many doors in research that could lead to groundbreaking therapeutic treatments for prevalent diseases such as Duchene's Muscular Dystrophy (DMD), sarcopenia, and osteoporosis. Nursing care will be directly affected by drug therapies, dietary changes, and lifestyle changes that emerge from the results of WNT3a research.

There are many examples of the power of translational research. This includes vaccines, treatments for diabetes, hypertension, cancers, and new health care delivery systems. Some of the most modern treatments for hypertension translated directly from cells to humans in only a few years. One recent example of translational research is: Tumor Paint. Scorpions are rarely considered in medical research, but for Dr. James Olson of the Fred Hutchinson Cancer Research Center and his colleagues, the scorpion offers a solution to a challenging problem for surgeons: differentiating normal cells from cancer cells (Aleccia, 2014).

Tumor Paint consists of chlorotoxin, a component of scorpion venom, coupled with Cy5.5, a fluorescent dye (Aleccia, 2014). Using magnetic resonance imaging (MRI), Tumor Paint lights up brain-cancer cells during surgery, allowing surgeons to spare the maximum amount of normal tissue. Tumor Paint was developed in collaboration with researchers at the University of Washington, School of Medicine and Seattle Children's

Research Institute with support from the National Institutes of Health and the Hutchinson Center Synergy Fund (Aleccia, 2014). Dr. Olson's research required the convergence of disparate disciplines, such as chemistry, biology, physics and radiology, and the combined work of neurosurgeons, engineers and biologists. Independent research institutes like the Hutchinson Center foster a collaborative spirit that breaks down barriers between disciplines and accelerates the development of innovative tools for nursing and medicine like Tumor Paint.

Because we know that WNT3a increases the differentiation of mouse primary muscle cells, next, we need to explore the mechanism. The next step in this research process, based on my experiment results, is to investigate the cellular and molecular mechanisms whereby WNT3a is able to enhance the differentiation of primary muscle myoblasts. Future experiments will use the RT-PCR pathway finder gene array to detect changes in gene expression of multiple signaling pathways. The University of Texas at Arlington also has a sophisticated Proteomics Core and Proteomics and lipodomics approaches could complement the gene PCR experimentation in determining mechanistic insights.

Calcium homeostasis is very import to muscle function and is a surrogate of muscle contraction (Frontera, Zayas & Rodriguez, 2012). Calcium imaging is a functional biochemical study of calcium homeostasis used to represent muscle function and the skeletal muscle maturation process. Future experiment will be conducted using the Photon Technology International (PTI) imaging system to detect potential changes in intracellular calcium homeostasis in myotubes treated with WNT3a. Recent studies have demonstrated improved treatment outcomes for muscular dystrophy when intracellular calcium

homeostasis is normalized in dystrophic muscles. Therefore, it will be important to conduct these studies to determine if bone-secreted factors such as WNT3a have the capability to modulate muscle function. The implications of our findings could be very important for nursing research as nurses are at the forefront of bedside care and are part of the multidisciplinary approach to healthcare. WNT3a could be a target not only for new pharmacological agents but for new diets and exercise regimes. These new targets could help elevate the levels of WNT3a in both bone and muscle tissue, leading to a healthier musculoskeletal system and new ways to combat osteoporosis, sarcopenia, and other musculoskeletal diseases. In fact, there are registered ongoing clinical trials with WNT3a that further validate the importance of nursing translational research.

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BIOGRAPHICAL INFORMATION

Sarah was born and raised in Arlington, TX with her mother, father, and younger brother. From an early age, she became interested in the health sciences and set goals to be involved in the healthcare field. At the age of 17, Sarah enrolled in the University of Texas at Arlington, and shortly thereafter, she was accepted into the Honors College at UTA. Sarah will be graduating with an Honors Cum Laude Bachelor of Science in Nursing, with a Psychology Minor in December of 2016.

Recently, Sarah accepted a Graduate Nurse Residency position through Texas Health Harris Methodist Fort Worth Hospital, as a Neuro-Surgical Intensive Care Unit Registered Nurse. She plans to pursue a nursing career in critical care through Texas Health Resources. Sarah also desires a graduate degree in health sciences and plans to continue her education through to the doctorate level.

Researching in the Brotto Muscle Research Lab at the University of Texas at Arlington was Sarah's first opportunity in non-clinical based research. Her experience in the Brotto laboratory has ignited her passion for evidence-based researching practice and Sarah desires to extend her research into her profession as a Registered Nurse.