GENE EXPRESSION IN LONG TERM MYOBLAST /MYOCYTE CULTURES: m RNA expression (Acetylcholine Receptor and Galectin-3 gene)

By

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Signature Page

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Patricia Chemutai

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Abstract

Gene expression is a crucial process in muscle functioning which occurs throughout the skeletal myogenic process. The gene regulatory network involved in the later process could be essential in shedding some light in muscle aging and muscle related diseases. This study employed an experimental design to determine the expression of Acetylcholine receptor and galectin 3 gene in C2C12 cells which are mouse muscle cells that are immortalized and capable of allowing the description of transcriptional steps through myogenesis.

The C2C12 cells were studied over extensive time course of 30 days with the cells being seeded, media changed, and cells pelleted at specific time points for RNA isolation. Following the RNeasy kit protocol the RNA was isolated and quantified by nanodrop spectrophotometry. Analysis of the Acetylcholine receptor and galectin-3 gene was done by quantitative Real-Time Polymerase Chain Reaction(qRT-PCR). The expression of the said genes was measured for each of the three trials that was done and averaged. Inaddition to determining the expression levels of these genes, this level of expression was compared at different time points and correlated with changes in tissue morphology.

During the onset of myogenesis, Acetylcholine receptor was highly expressed compared to Galectin-3. As the muscles mature the expression of Galectin-3 seem to increase as compared to Acetylcholine receptor. This gene expression analysis was normalized to GAPDH. According to this study, it can be concluded that changes in expression of these genes during maturation of muscles are good indicators of aging in muscle cultures.

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TABLE OF CONTENTS

Table of Figures

List of Tables

List of Abbreviations

AChR	Acetylcholine Receptor
AIDS	Acquired Immune deficiency Syndrome.
Chrna1	Cholinergic receptor nicotinic Alpha 1
CRD	Carbohydrate -recognition-binding domain
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase
Gal-3	Galectin-3
Ig	Immunoglobulin
MEF2	Myocyte enhancer factor 2
MyoD	Myoblast determination protein 1
MyoG	Myogenin
MRF4	Myogenic regulatory factor 4
Myf5	Myogenic factor 5
NCBI	National Center for Biotechnology
qRT-PCR	Quantitative Real Time -Polymerase Chain Reaction

CHAPTER ONE

1.0 INTRODUCTION

1.1 Skeletal Myogenesis

Muscle formation is one of the most crucial processes in the body. There are three types of muscles tissues; skeletal, cardiac, and smooth muscles which are formed through the process called myogenesis during embryonic development and can be studied in numerous and diverse stages (Bentzinger et al., 2012). In this study our core focus lies on the skeletal muscles.

Skeletal muscles are the largest and extremely complex tissue that serve a multitude of functions in the body of an organisms. They are vital for support and motion. Skeletal muscles are voluntarily controlled making them distinct from other muscles in the body (Chal & Pourquié, 2017). There are approximately more than 640 of these muscles and the process in which they are generated is referred to as skeletal myogenesis (Guo et al., 2014)

Skeletal myogenesis is a highly organized and sequential process and therefore it is important to understand the origin, its developmental stages and the regulatory network involved. By doing so, these could contribute and shed some light to the mechanisms of muscle diseases and muscle aging.

1.2 Origin of skeletal muscles

Skeletal muscles in vertebrates arise from paraxial mesoderm that constitutes the presomitic mesoderm at the posterior tip of the embryo. This process highly organized phase occurs during embryonic development. The presomitic mesoderm then divides

into two regions, an immature posterior, and a committed anterior region (Chal & Pourquié, 2017). The committed anterior region thereafter segments to form the somites which are blocks of mesoderm that are located on either side of the neural tube in the developing vertebrate embryo and are precursor populations of cells that give rise to the skeletal muscle and other important structures like dermis, cartilage, tendons, and vertebrae associated with the vertebrate body plan.(Grosberg et al., 2011).Skeletal myogenesis is initiated within somites. It begins with the fusion of several different myoblasts which are the muscle precursor cells, that finally mature into myofibers after some developmental stages (Berendse et al., 2003).

For appropriate skeletal muscle development and regeneration, the fusion of muscle precursor cells is a required event. To understand this, muscle embryonic development in invitro have been done to complement in vivo studies. From a previous study done by Nyaboke et al.,2017) myoblasts fuse and differentiate into myocytes, which then adhere and fuse with one another to form nascent myotubes that have few nuclei. To form multinucleated myotubes, nascent myotubes subsequently fuse with myocytes and other myotubes and eventually they mature into myofibers thus called muscle cells.

The muscle cells are specialized cells that can contract, hence causing movement. Several of these cells form a skeletal muscle. They constitute specialized component called the myofibrils. Myofibrils is a bundle of functional units the sarcomere, which are arrangements of the thin and thick filaments. Myofibril's thin and thick filaments enable the muscle movement through the sliding-filament model. This model explains how the thick and thin filaments move relative to each other leading to contraction and relaxation

of whole muscles which thereby causes the movement of the limbs or tissues attached to those muscles. The thick filament lies in the A-band and is bisected by the M-line therefore it also splits the sarcomere in half. Within the area of the thick filaments at an area of the A-band is the H-band. To effectively perform its role each of the thick filament is made up a protein myosin that has elasticity and contractibility properties. The M-line directly interacts with the tail domain of myosin filaments and indirectly with the thin filaments through connecting proteins (Marzuca-Nassr et al,.2018).

The thin filament is made up of the main protein actin, other than that, it contains tropomyosin and troponin. It lies both in A and I band and is separated by a disc of protein called the Z-line. All thin filaments are attached to the Z-line which are the anchoring site of α-actinin, titin and several intermediate filaments, including desmin and vimentin. The intermediate filaments, predominantly the desmin, surrounds the sarcomeres in the Z-line. Titin protein extends from the M- to Z-line (Grosberg et al., 2011).

Myosin is hindered from binding to tropomyosin during relaxation state. For the cross-bridge cycle to be initiated, tropomyosin and actin held together must undergo a conformational change which require calcium. The stimulation of the muscle causes muscle fiber depolarization and is therefore transmitted into the t-tubes. Calcium channels then open in the sarcoplasmic reticulum due to depolarization and calcium is released and diffuses to the myofibril, binds to tropomyosin-actin complex and moving it therefore allowing myosin and actin to bind and contract (Irving, 2017)

During muscle contraction, the electrochemical impulses are sent to the motor neuron which stimulates the release of Acetylcholine at the neuromuscular junction.

Acetylcholine then binds to the receptor on muscle fiber. This then triggers the sodium channels to open causing depolarization of the membrane fiber and the impulses are carried away the t-tube through the muscle fibers. The sarcoplasmic reticulum releases the calcium upon the delivery by the muscle fibers by a process called excitation-contraction coupling mechanism. With this the myofibril contract and shorten and the myofilaments move towards each other through the sliding movement (Crawford & Horowits 2011).

1.3 Sarcomeric contraction

Assembly of the sarcomere contribute at a deeper length the understanding of contraction and relaxation of skeletal muscles. In addition, it is equally significant in understanding why skeletal muscles are striated. Skeletal muscles are made up of cylindrical muscle cells known as the skeletal muscle fibers. Each of these fibers contain several myofibrils.

A sarcomere is a repeating unit that is contractile and makeup the myofibrils. It is located between two Z-discs (Z-lines). Within each sarcomere are the A and I band. Spanning in the middle of the sarcomere is the A band that is composed of the thick filaments and contains the myosin protein complexes which gives the A band its dark color. At the middle of the sarcomere at a region known as the M-line, the thick filaments are anchored by a protein called myomesin while the central region of the A band is the H-zone. The I band is lighter and contain the thin filaments which are attached at the Z-discs the α-actinin protein. These filaments overlap with the thick filaments as they extend into the A band towards the M-line (Moo & Herzog 2020).

When calcium leaks into the cells it migrates down into the sarcomere which then triggers the troponin and tropomyosin to activate muscle contraction and interaction between myosin and the actin filaments. During the skeletal muscle contraction, these filaments do not shorten but instead over each other causing the sarcomere to shorten as shown on figure 1 below. The H-zone which is the region that contains only the thick filaments shorten. Similar observation is made on the I- band while the A band on the other hand remains the same (Squire, 2016).

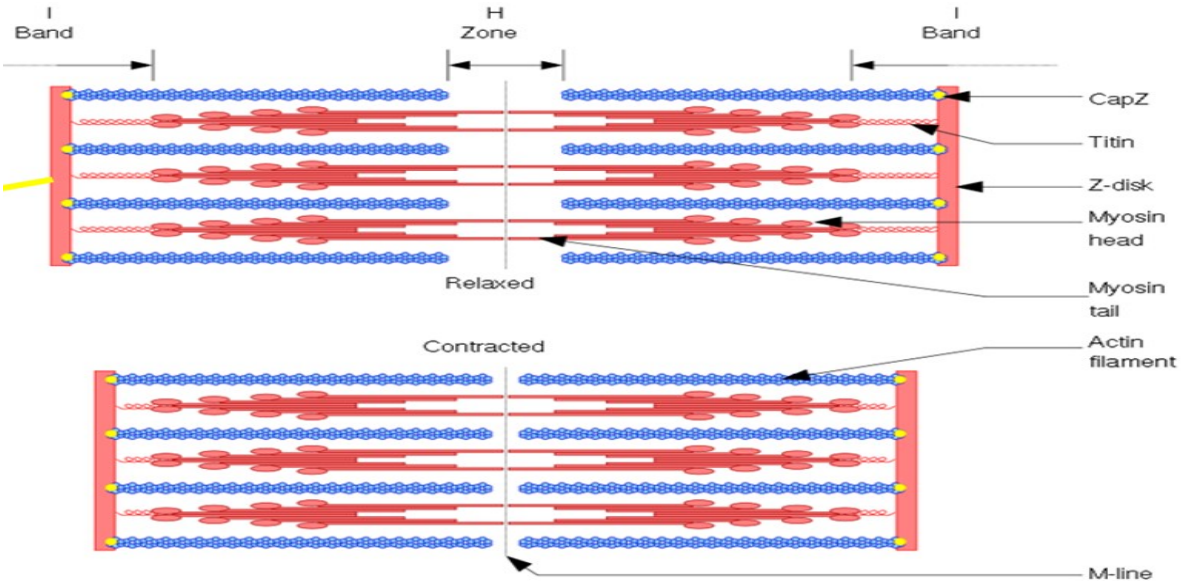

Figure 1: *Relaxation and contraction of the Sarcomere (Chal J & Pourquié, 2017)*

1.4 Regulation of Myogenesis

According to a study done by Wang et al., 2019 myogenesis is considered as complex process and understanding the regulatory network of skeletal myogenesis will contribute to the treatment of human muscle related diseases .Skeletal myogenesis is an orderly process regulated by a series of muscle-specific transcription factors, (Braun &

Gautel, 2011) including myogenic determination protein1 (MyoD), myogenin (MyoG), Myf5, myogenic regulatory factor 4 (MRF4), and myocyte enhancer factor 2 (MEF2) (Buckingham, 2006). For this process to occur successfully it requires these transcription factors to be expressed at the right time and location (Buckingham, 2014), In addition to the above transcription factors, signaling pathways involved are equally important for the embryonic skeletal muscle formation. These pathways include the Shh, Wnts, BMPs, Notch, and FGFs, which are known to function in positive and negative regulatory networks that control Myf5 and MyoD activation in muscle progenitors (Pownall et al., 2002).

PX^{3+}/PX^{7+} biomarkers are initially expressed by the myogenic progenitor cells. Myogenesis in the lateral myotome has been studied to be promoted by Pax3.Both biomarkers are significant in maintaining the myogenic program. From a previous study, Myf5 was discovered to be the target of Sonic hedgehog protein (Shh) signaling in epaxial progenitors however, MyoD is not. This pathway is known to initiate myogenesis in the epaxial myotome (Braun & Gautel, 2011). When sufficient myoblasts have been formed, they exit the cell cycle and begin to fuse this occurs in the epaxial myotome and hypaxial myotome and it is the beginning of differentiation. Muscles of the hypaxial requires BMP and WNT to induce myogenesis, however the Bone morphogenetic protein (BMP) impedes myogenesis if it is expressed in the dorsal neural tube (Endo, 2015).

Muscle regulatory factors (MRFs): myogenic determination protein 1 (MyoD) and myogenic factor 5 (Myf-5) are expressed during myoblast proliferation. These factors therefore create a regulatory transcriptional network that is essential to the determination and differentiate of the muscle cell. The proliferation of myoblast is very dependent on

the expression of these factors MRFs as they are transcription factors that activate various downstream genes to initiate muscle cell differentiation (Bentzinger et al., 2012). The embryonic myoblasts fuse to form primary myotubes also called nascent myotubes. Myogenin and myocyte enhancer factor 2 is required for the formation of these primary myotubes requires and it ends when the myotubes begin contracting and are innervated. The secondary fibers which are predominantly mature skeletal muscle are formed through fusion of the myoblasts with each other or with the primary myotube at the onset of contraction. Myogenic regulator factor 4(MRF4) which is also known as Myf-6) and the nuclear factor 1X are required during secondary myogenesis, with MRF4 becoming the prominent MRF expressed in adult skeletal (Taylor & Hughes, 2017).

Previous studies have shown that MyoD over expression converts fibroblasts to myoblasts and leads to the subsequent fusion into myotubes (Guo et al., 2014). Myogenin has also been studied as a sequence that forces the myoblasts to undergo myogenic differentiation which results in myoblasts exiting the cell cycle and fusing to form multinucleated myofibers (Zammit, 2017). A study done on mice by Charge´ and Rudnicki in 2004 resulted in *Myogenin*-deficient embryos dying perinatally due to a deficit in myoblast differentiation. In his study, Zammit in 2017 indicated that MyoG knockdown reverses terminal muscle cell diff erentiation. In other studies, myogenesis is also regulated by epigenetic modification (Wang et al., 2019). For instance, it is accompanied by changes in chromosome modification, especially histone modification in myogenic genes (Jin, Peng & Jiang 2016).

1.5 Actin, Myosin and Titin Proteins and their role in skeletal muscles

Effective performance of muscles relies mostly on actin, myosin, and Titin however there are other muscle proteins that collaboratively function together. Actin and myosin are referred to as contractile proteins and form the thin and thick filaments that controls the skeletal muscle contraction and relaxation (Li et al., 2017). Muscle proteins are categorized into three depending on their function. These are regulatory proteins motor and structural. Troponin and tropomyosin are regulatory proteins with both being components of the thin filaments and responsible for regulation of muscle contraction.

Myosin falls under the motor proteins and its function is to create force and movement with its main function being the generation of force. It is a component of the thick filaments and is also involved in contraction. There are several classes of myosin each made of the heavy and the light chains however myosin I and myosin II are the most abundant. A myosin protein entails a globular head domain which contains actin- and ATP-binding sites and is responsible for generating force; this is the most conserved region among the various myosin's. Next to it, is the α-helical neck region, which is associated with the light chains and regulates the activity of the head domain. The tail domain contains the binding sites that determine the specific activities of a particular myosin (Lordish et al., 2000).

The least type of protein is the structural. Examples of these include desmin, actin and titin. They are responsible for generating force during contraction and providing stability. The reinforcement of the sarcolemma and integration of the sarcolemma, Z disk, and nuclear membrane in the sarcomere is the responsibility of the desmin protein. The importance of desmin was revealed in the early stages of myogenic development study

which was done on knockout mice. The disruption in the organization of muscle fibers, was observed through the loss of desmin with the decreases in the capability of generating force and the efficiency of force transfer therefore making the muscle fibers to become weaker and more susceptible to damage (Lovering et al., 2011). With the core constituent being the thin filaments, actin acts as a static support structure where force is applied. Titin acts a connection between the Z-line to the M-line and helps stabilize the thick filaments position, keeping the thick and thin filaments in alignment. In addition, it is also responsible for the elasticity and extensibility of striated muscles (Crawford & Horowits, R., 2011).

As the largest protein, titin has extensively been studied. It is the third most abundant protein after actin and myosin. With these studies, it has been shown that titin encodes for the TTN gene in humans and that it contains 364 exons and located at the long arm of chromosome 2. Since it is a stretchable protein titin spans along the sarcomere from the Z-line, through the half I-band, over the thick filament, and ends at the M-line. With the same characteristic, this protein provides the passive mechanical tension required by the sarcomere to restore its resting length after contraction. Titin is made up of, 132 fibronectin 3 domains, 14 tetratricopeptide repeat domains ,152 immunoglobulin (Ig) like domains, 19 kelch domains and 15 solenoid domains. It is also divided into regions and bands, that is M-line regions, I-band, A-band, and Z-disk (Wagner, 2020)

According to the National Center for Biotechnology (NCBI) database, there are seven human titin isoforms that have been identified (Nyaboke at al., 2017). The Titin isoforms N2A and N2B arise as a result of alternative splicing. Alternative splicing is a

regulated process that results in a single gene coding for multiple proteins during gene expression (Spletter & Schnorrer, 2013). Both N2A and N2B are found in cardiac muscles while the N2A is found in skeletal muscles. N2A are known to be stiff and shorter while N2B are longer and compliant. In addition to its similarity both have the PEVK and tandem Ig segments. The other difference is that and it has also been discovered that N2A isoform has N2A element (4 Ig domain and 106-residue unique sequence) while the N2B isoform has N2B element (3 Ig domains and a 572-residues unique sequence and is said to contain the Exon 49 (Forbes, (2006).

In 2017, Nyaboke et al sought to examine the expression of the titin isoforms N2A and N2B and their role in C2C12 myoblast cells. The expression of the titin at different time points was an indication that the titin protein is involved in myogenesis. When the expression of the two isoforms was observed and compared to other previous studies, it seemed to differ, N2B tend to be highly expressed in the skeletal muscle compared to N2A. However, it was still determined that they are still involved in sarcomerogenesis.

1.6 Roles of galectin-3 gene and Acetylcholine receptor in skeletal muscles

Expression of genes in skeletal muscles is critical to its development and functioning. Several genes are expressed at specific timeline and in different levels during the skeletal muscle development. With previous studies having focused on expression of MyoD, actin, myosin, and titin in skeletal muscle cell programming, we thought we would look at other genes that contribute to the functioning of skeletal muscles. This study highlights the expression of Acetylcholine receptor gene and galectin

3 gene during the myogenic cell programming. In addition to this, this study will discuss the role of these two genes in skeletal muscle functioning.

1.6.1 Galectin-3

For myogenesis to occur effectively, expression of other genes is important for successful expression and regulation of others. Galectin is known to be one of these gene. Galectin-3 also known as Gal-3 is a protein which is encoded by the LGAL 3 gene in humans. It is grouped under the family of lectins inclusive of other mammalian galectins. Just like any other galectin, Gal-3 contains a carbohydrate -recognition-binding domain (CRD) which is responsible for the specific binding of β-galactosides (Johannes, Jacob & Leffler 2018) This protein is known to be expressed in many tissues inclusive of the muscle tissues (Díaz-Alvarez & Ortega, 2017). Previous studies have shown galectin-3 to be expressed in the cell surface, extracellular space, cytoplasm, nucleus and in the mitochondrion (Cerri et al, 2020). It is known to be involved in many cellular functions.

Several research studies have proven galectin 3 to be involved in multiple biological process. It participates in many functions, both intra- and extracellularly (Díaz-Alvarez & Ortega, 2017). The vital roles demonstrated by this gene includes cell matrix interactions, cell to cell adhesion, activation of macrophages, the formation of new blood vessels through the process of angiogenesis, programed cell death and metastasis (Jia.et al., 2020). Gal-3 also has an essential role in the skeletal muscle repair process. This is due to increasing evidence that some members of the mammalian β-galactoside-binding protein family (galectins) are involved in the muscular repair process, inclusive of galectin-3 (Gal-3) (Cerri, et al,2020).

Whereas galectin 3 has several functions, we have a few the roles that align with development and functioning of the skeletal muscles. In a study that was done by Rancourt et al, 2018 gal-3 was observed to increase the efficiency of myogenesis in a C2C12 cell line during the differentiation stage as it is expressed in both myoblasts and myotubes. A decrease in the level of expression of galectin-3 has shown to also decline the expression of MyoD and myogenin transcription (Cerri et al.,2020).

The structure of galectin-3 is essential to its functioning. It has an N and C domains with the N-domain having the N-terminal which has twelve amino acids and a collagen sequence of 100 amino acids. The intracellular functioning of gal-3 is highly dependent to this N domain through peptide-peptide associations whereas the C-terminal domain contains the Carbohydrate recognition domain of 130 amino acids which aid in coordinating the extracellular functioning of gal 3. The CRD enable this through the specific binding of β-galactosides. At some instances, the CRD is involved in the intracellular functioning. With these structural properties, multiple roles are achieved by gal 3 (Pugliese et al., 2014).

Extracellularly, gal 3 serve as across linkage between adjacent cells through cell-cell interactions and this can be also observed in skeletal muscle development. This function is regulated by its cell adhesion to the extracellular matrix component. Another significant role for an extracellular gal 3is the modulation of the inflammatory functioning of the cells and facilitates tissue repair through the process of fibrogenesis (Henderson & Sethi, 2009).

Intracellularly, galectin 3 is known to promote cell proliferation which contribute to the cell survival. It regulates the cell cycle by acting as a pre-mRNA splicing factor. Due to it proliferative nature galectin 3 has been implicated in tumor growth inaddition to its protective nature from induced apoptosis. Despite this, it also considered pro-apoptotic as it mediates neutrophil and T cell death (Suthahar et al.,2018)

1.6.2 Acetylcholine Receptor

Muscles are generally under neural transmitter control. A Neurotransmitter is a chemical messenger that transmit a signal from a neuron across the synapse to a target cell that is a muscle cell, gland, or other nerves. Muscles are under the exclusive control of acetylcholine. Skeletal muscles and cardiac muscles are both striated muscles and they respond to acetylcholine but differently. Acetylcholine in skeletal muscles causes contraction by activating the muscles while in the cardiac muscles it gives an opposite effect that is, it relaxes the muscles. The two different responses by these muscles are due to the two different acetylcholine receptors which binds to the acetylcholine which is released at the presynaptic terminal in response to a threshold action potential (Tiwari et al.,2013).

Acetylcholine receptor is an integral protein which is found on the surface of muscle cells, but they are mainly concentrated in the synapse between the nerve cells and the muscle cells. They are categorized into two main types based on their relative sensitivity and their affinities to different molecules. We have the muscarinic acetylcholine receptors and nicotinic acetylcholine receptors. They are both considered cholinergic receptors which means that they are capable of mimicking the function of

acetylcholine. In this case they can selectively bind to muscarine and nicotine respectively (Brown, 2019)

Muscarinic acetylcholine receptor is a G-protein-coupled receptor (GPCRs). They are also called the seven-transmembrane receptor. These receptors are the largest and most diverse group of membrane receptors in eukaryotes and are found in several places in the body which include the smooth muscles, cardiac muscles, blood vessels and the lungs. In cardiac muscles this receptor is commonly known to be responsible with relaxation (Abrams etal.,2006)

There are five subtypes of muscarinic Acetylcholine receptors based on pharmacological activity. These are M1, M2, M3, M4, and M5 receptor subtypes. Each of these subtypes have been studied to be expressed differently at different parts of the body although all the subtypes exist within the central nervous system. Within the gastric glands, salivary glands and cerebral cortex is the M1 receptor as the M2 receptors are sited in smooth muscle and cardiac tissue. In the smooth muscle, salivary and gastric glands is also the M3 receptors. The distribution of the M4 and M5 is observed in the brain within the hippocampus and substantia nigra however it is not well defined (Kruse et al., 2014).

Our focus lies on the Nicotinic Acetylcholine receptor which are the ligand gated ion channels that are present on the neuromuscular junction and is responsible in signaling muscular contraction in skeletal muscles. This means that they are the primary receptors for the nerve-muscle communication (Dani, 2015). The nAChR is known to mediate transduction of electrical signals through neurotransmitter binding which leads to the opening of the pores on the cell membrane therefore enabling the flow of Ca^{2+}, K^{+},

and Na^+ ions across the cell membrane with the Ca^{2+} playing a role in skeletal muscle contraction (Taly et al.,2009).

The nicotinic acetylcholine receptor is further subdivided into two subtypes N1 and N2. The N1 is known as the peripheral or muscle receptor type and is present in the skeletal muscles at the neuromuscular junction whereas the N2 is the central or neuronal receptor subtype and is found is within the central and peripheral nervous systems. The nAChR encodes for the cholinergic receptor nicotinic alpha 1subunit gene as its encoded product being expressed at the neurotransmitter (Brown, 2019).

The successful expression of the nAChR is determined by its structure. This receptor has five subunits, that is two alpha (α) subunits, one of the delta (δ), beta (β) and epsilon (ε) subunits. Each alpha subunit has an acetylcholine binding site where the neurotransmitter acetylcholine binds so at to trigger a conformational change of the ionic channels. This would there after allow calcium ion influx intracellularly and the extracellular outflow of potassium ions.

During muscle contraction, the nervous system first begins by releasing a signal which travels through the motor neurons to the neuromuscular junction where acetylcholine which is released at the synaptic terminal binds to nAChR on the muscle fibers therefore generating an action potential which travels down the T-tubule. This causes the Ca^{2+} channels known as type 1 ryanodine receptors (RyR1) to open and release the calcium ions from the sarcoplasmic reticulum to the cytosol (Santulli, Lewis, & Marks, 2017).

Within the cytosol, the influx of the Ca^{2+} increases the amount of calcium thus binding to troponin which thereafter undergoes a conformational change that result to it moving the actin filaments and as it moves the troponin exposes the myosin binding sites on the actin. The myosin binding sites is then bound to ADP and inorganic phosphate which subsequently causes the formation of a cross bridge as shown in with actin figure 2 and power and force that drives contraction is formed as ADP and inorganic phosphate is release. The muscle is shortened as result of the force causing the thin actin filament to slide past the thick myosin filament. This process is repeated when myosin hydrolyses ATP after binding to it releasing from actin (Kuo & Ehrlich, 2015).

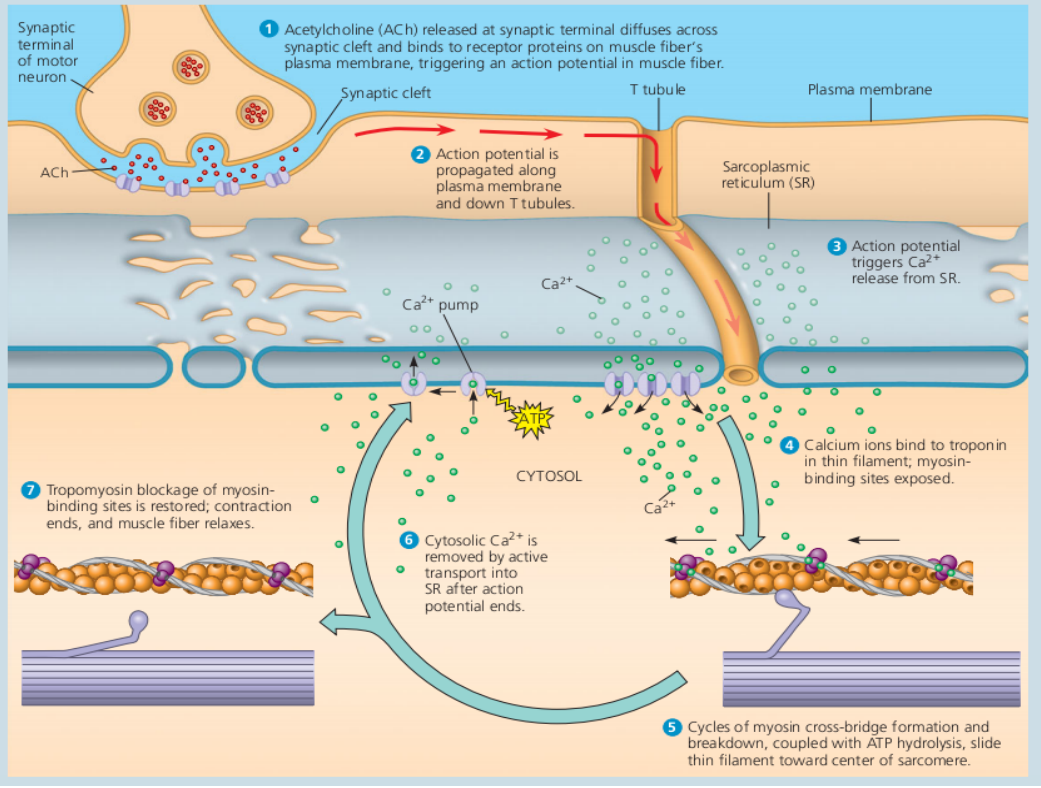

Figure 2: Signaling of muscle contraction by acetylcholine on the acetylcholine receptor (Santulli, Lewis, & Marks, 2017).

In a relaxed state of the skeletal muscle, the Ca^{2+} must be retained in the sarcoplasmic reticulum. This can be achieved if the excess Ca^{2+} is transported back the sarcoplasmic reticulum therefore keeping the sarcoplasmic Ca^{2+} levels low (Allard, 2018)

1.7 Skeletal Muscle aging

One of the most severe risk factors for many diseases and disorders is age (Armanious et al ,2015). Muscle aging can be defined as the decrease in the muscle mass and mitochondrial content coupled with its function. Mitochondrial, myosin heavy chain and muscle protein synthesis declines with age (Johnson et al.2013). Several studies have also shown that aging leads to a declined skeletal muscle function in mammals and is associated with a progressive loss of muscle mass, quality, and strength. For example, in previous studies on diseases and disorders like Kidney disease, heart disease, cancer, dementia and sarcopenia have shown age as the highest risk factor (Egerman & Glass, 2014; Kirkland & Tchkonia 2015). The most common muscle disorder is sarcopenia and is defined as the loss of muscle mass and function as we age. The effects of this disorder observed in individuals, is a decrease of approximately 30–50% in skeletal muscle mass and function by the time individuals reach approximately 80 years of age (Akima et al. 2001) .

As skeletal muscles age it is observed that the general muscle mass decreases, the diameter becomes and their elasticity decreases. The skeletal muscle fibers are seen to also decrease in diameter size as the muscle tolerance for exercise decreases. As for type II fibers it decreases leading to a progressive decrease in the type II-to-type I fiber area ratio. These changes therefore lead to a decrease in the ability to recover from muscular injuries (Churchward- Venne et al 2014).

Numerous diseases, including cancer and AIDS can be a serious consequence of the atrophy of the skeletal muscle. Successful treatments for skeletal muscle atrophy could either block protein degradation pathways activated during atrophy or stimulate protein synthesis pathways induced during skeletal muscle hypertrophy (Lin et al., 2018)

To ensure that skeletal muscles do function effectively and efficiently, enough number of functional mitochondria are needed. These organelles have been referred to as perpetrators of aging. Studies from David Glass and his colleagues in 2013 found markers of mitochondrial metabolism pathway which are down regulated as mice aged and according to them this correlated with the onset of sarcopenia.

Some studies have been done on muscle aging both in in vivo and invitro, however less of this have focused on gene expression in aged skeletal muscles. For example, a study on MyoD and myogenin protein expression in skeletal muscles of senile rats showed an up regulation of both proteins (Dedkov et al.,2003). Actin and myosin did show any change in other study on myosin and actin expression and oxidation in aging muscle. The genes that code for skeletal muscle were also upregulated while those of that encodes for the sarcomere alpha-actinin 1 were down regulated (Lin et al., 2018).

With skeletal muscles being under neural control, evidence has shown that age has a profound effect in its functioning. As muscles age, loss of motor units and changes in the neuromuscular junction (NMJ) structure and functional integrity is observed. In addition to this, is the denervation and re-innervation of muscle fibers. The aging muscles switch from type II fast fibers to type I slow fibers and as denervation surpasses re-innervation the fibers begin to degenerate. When muscle fibers degenerates, it leads to

muscle mass loss which therefore contributes to the contractile functioning being compromised (Jang, & Van Remmen, 2011).

The functioning of skeletal muscles is determined by the correct neuromuscular junction morphological which affects the amount of acetylcholine released and binding to acetylcholine receptor.

This study attempted to study C2C12 myoblast cells for a period of 30 days. These cells are originally isolated from mouse muscle. They are the preferred type of cells because they are capable to differentiate thus allowing for description of each of the transcriptional step program. Gene expression during different time points will be analyzed.

1.8 Specific aims

(i) Determine acetylcholine receptor and galectin-3 gene expression their role in long-term myoblast, myogenesis and muscle aging.

(ii) Comparison of the level of gene expression in the long-term myoblast/myocyte at different time points and correlating expression with changes in tissue morphology.

1.9 Hypothesis

Our hypothesis was that when cells age there is a decrease in acetylcholine Receptor expression and galectin-3 gene expression.

1.10 Experimental Design

This study was divided into two phases. During the 1st phase, initial cell culture of the C2C12 mouse myoblast cells was done on a T-75 cell culture flask and there after expansion of the cells done by splitting the cells into two flasks and finally into 10 flasks for the long-term course study of 30 days. The cells were observed using the Olympus LH 50A inverted microscope. For each alternate day the growth media was changed.

They were then harvested at a designated day as indicated on the flask (t(day) =-1, t=0, 4, 8, 12, 25, 30 days) as shown in fig 1 and stored at -80^{0}c.

The 2nd phase of the study entailed RNA isolation and analysis. RNA was isolated from the designated time points following the RNA isolation kit protocols (Qiagen Rneasy and Qiagen Shredder Kits). Before the qRT-PCR is performed, purification and quantification were confirmed using the Nanodrop spectrophotometry. PCR results are thereafter analyzed.

1.11 Summary of the experimental design

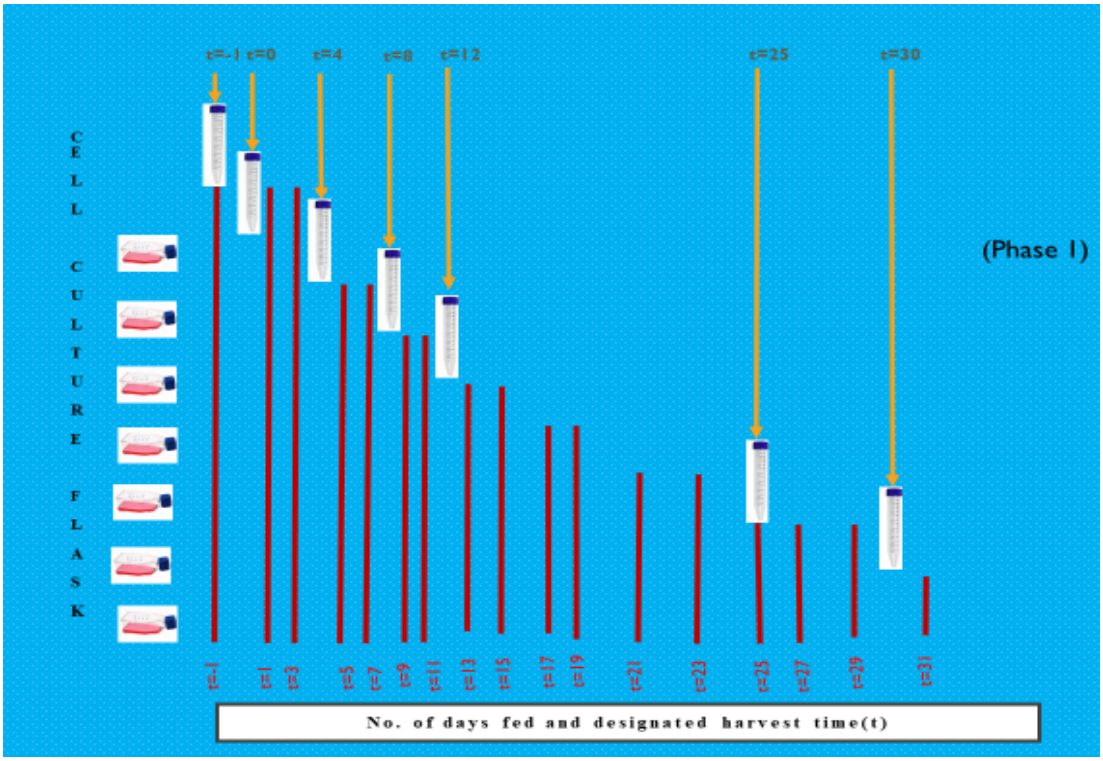

Figure 3: Shows the number the of cell culture flask seeded and the days of feeding and harvesting cells (t, above with yellow arrow)

CHAPTER TWO

2.0 METHODOLOGY

2.1 Culturing of C2C12 Cells

2.1.1 Media preparation (10% Fetal Bovine serum)

Media is first aseptically prepared ahead of starting the experiment. Fetal Bovine serum will be thawed at 37^0 water bath for 10 minutes or until completely thawed. 900ml of Dulbecco's Modified Eagle Medium (DMEM)will be mixed with 100ml of the Fetal bovine serum and pen strap added. After which the mixture is filtered under a hood and labeled. This method is followed for all the media (10%Fefal bovine serum) used in the subsequent procedures in this study.

2.1.2 Initial culturing

C2C12 mouse muscle myoblast cells obtained from ATCC and stored in liquid nitrogen are quickly thawed in a 37^0 water-bath. They are after which transferred to a15ml centrifuge tube. Prewarmed 1ml of the 10% fetal bovine serum (growth medium) is added to the cells in the centrifuge tube and incubated for 10 minutes at room temperature. At a ratio of 1:1 the growth medium is added to make a final volume of 8ml which is then transferred to a T-75 cell culture flask with 17ml of the growth medium. The cultures are incubated at 37^0, 5% CO_2 incubator for 24 hours. Cell attachment to the flask is then confirmed by observing them under an Olympus LH 50A inverted microscope. The culture media is changed a day following the initial culturing by adding 25ml of the 10% fetal bovine serum. This is done until the cells are about 80% confluent and ready to be split.

2.2 Cell splitting (sub culturing)

Once the cells are 80% confluent, they are split by trypsinization method. The medium in the flask is discarded the and the cells are washed with 20ml of PBS for 10 seconds. Following the discarding of the PBS, 15ml trypsin is added to the cells and incubated for 10 minutes in a 37^0 incubator with 5% CO_2. Using an Olympus LH 50A inverted phase contrast microscope, cell detachment is confirmed. Once the cells have completely detached from the flask, 3ml of the trypsin-cell mixture is transferred to five new T-75 flasks with new growth medium. They are then incubated under the same previous conditions for 24 hours and attachment is confirmed by viewing under the Olympus LH 50A inverted phase contrast microscope. The media is changed each alternate day until they are 80% confluent and ready to be split for the two different long-term study on gene expression and proteomics.

2.3 Myoblast long term study and Cell Pelleting (Harvesting)

Following the same procedure as earlier (cell splitting), the cells are split after reaching a confluent level of 80%. With seven time points (-1, 0, 4, 8, 12, 25, 30), the trypsin-cell mixture in one of the T75 flask are split into ten new labeled T75 flasks after the detachment is confirmed. Two Millimeters of the mixture is transferred into the new flasks and 25ml of the growth medium (10% FBS) is added to the flasks and incubated in a 37^0c incubator with 5% carbon dioxide for 24 hours, the cells are observed under Olympus LH 50A inverted microscope to confirm cell attachment. The media is changed every alternate day and the cells are pelleted on every indicated time point. The cells were pelleted by discarding the old media, the flask is then washed with 15ml PBS for 10 seconds and discarded. 15ml of trypsin is added to the flask and incubated for 10 minutes

under the same conditions as used before. The cells are observed for detachment using an inverted microscope. Once they detach, they are transferred into a 15ml centrifuge tube and centrifuged at a full speed for 10 minutes. The excess trypsin is discarded, and the pellets are retained in the centrifuge tube. This continues for one month and all the pelleted cells are stored in a -80^0c freezer.

2.4 RNA Isolation

Cell pellets are removed from the -80^0c storage and thawed. They are then resuspended in 350µL of the lysis RLT buffer, and the lysate is vortexed. The lysate is transferred to a QIAshredder spin column in a 2ml collection tube and centrifuged for 2 minutes at a full speed. 350µL of 70% ethanol is added to the tube and 700µL of the mixture is transferred to a RNeasy spin column a 2ml collection tube. The mixture is then centrifuged at ≥ 8,000g for 30 seconds and 700µL of the RWI buffer is added to the spin column followed by centrifugation for 30 seconds at ≥ 8,000g. The flow through is then discarded and 500µL of RPE buffer is added and centrifuged again at ≥ 8,000g for 30 seconds. The flow through is discarded and centrifugation is done at the same conditions as formerly used. The spin column is then transferred to a new 2ml collection tube and centrifuged at full speed for 1minute to remove any remaining fluid. The flow through in the 2ml collection tube is discarded and the spin column is placed in a new 1.5ml microcentrifuge tube.50µL RNase free water is added to the spin column and centrifuged ≥ 8,000g for 1minute.The spin column was then discarded, and the eluted RNA is retained in the 1.5ml tube and stored at -80^0c.

2.5 RNA Quantification

Once the RNA was isolated, it was then quantified using a NanoDrop 2000 spectrophotometer. The Nanodrop permits for evaluation of the concentration and purity of the RNA. The Nanodrop 2000 software is launched, and the nucleic acid tab is selected from the menu. The Nanodrop is then blanked by loading 2µL of RNase-free water on the lower side of the measurement pedestal and the arm is lowered. The blank button on the computer is then clicked and once the reading is complete a flat spectrum is expected. After the blanking is complete, the RNase-free water on the pedestal is wiped off and 1µL of the RNA sample is loaded on the lower pedestal, the arm is lowered, and the readings are taken. Three readings are taken for each RNA sample corresponding to each of the time points and their average is calculated and recorded in an excel spread sheet. Once complete the pedestal is cleaned by loading 2 µL of the RNase-free water, wiped off and the arm lowered.

2.6 Gene expression by Quantitative RT-PCR

After RNA was isolated from the C2C12 mouse myoblast cells, the RNA was then analyzed for gene expression by qRT-PCR. Forward and reverse primers targeting the genes of interest will be designed by using a primer design software. To determine if the primers were working, they were run through a standard PCR and gel electrophoresis. Following this step, a reaction setup will be made by mixing all the components required. A reaction mix of 2µL of the forward primer, 2µL reverse primer, 0.25µL reverse transcriptase, 10µL of the iTaq universal SYBR® Green and 3.75µL of nuclease free water. Since there are 10 different tubes of RNA isolated for different time points, 11 tubes of the mix will be made including one of the control tubes. Once the mix has been

made, they are dispensed into the PCR wells and the RNA is added. qRT-PCR was then run. It begins by reverse transcription of the RNA into the cDNA by the reverse transcriptase for 10minutes at 50^0c. Denaturation of the double stranded DNA to single stranded DNA is followed at 95^0c for 1 minutes. Amplification then occurs at 95^0c for 5 seconds. Annealing/extension are the final steps at 60^0c for 30 seconds. The previous three reaction makes a complete cycle therefore 40 cycles are made for a complete PCR reaction. Data obtained was then be used for analysis.

2.7 Designing Primers.

Primers to the genes of interest were designed using the NCBI primer blast data base some the primers were also confirmed using the primer 3 design tool. The forward and reverse primers to the glyceraldehyde 3-phosphate dehydrogenase (Gapdh) transcript variant 1, Galactose binding soluble3 and Cholinergic receptor nicotinic (Chrna1) gene s were designed. Three possible sets of each were first selected and after which used the elimination method by considering the characteristics of a good primer.

CHAPTER THREE

3.0 RESULTS

3.1 Quantification of RNA

From the C2C12 cell pellets that were harvested, RNA was isolated then quantified using the nanodrop spectrophotometry. The purity and concentration of the RNA was evaluated by measuring the absorbance and absorbance ration for the nucleic acids and the proteins as indicated in table 1,2 and 3. Nucleic acids are known to absorb UV light at a wavelength of 260nm while the proteins absorb at 280nm. Several organic compounds also depict a strong absorbance at 230nm.

A A260/280 ratio is commonly used to determine protein contamination of a nucleic acid sample and in this case the RNA whereas the A260/230 ratio indicates the presence of organic contaminants in the RNA sample.

The other measurement from the Nanodrop is the concentration of the RNA sample in ng/uL which is recommended that the samples between 10 ng/uL and 3700 ng/uL can be measured. While the later statement is the case, the sample out of these range can be diluted or dried down respectively to give more accurate spectrophotometry results.

3.1.1 Trial 1 data

Sample Name	Nucleic Acid(ng/uL)	A260/A280	A260/A230	A260	A280
T=1(P1)	159.095	2.12	0.204	3.977	1.876
T=1(P2)	30.734	2.061	0.447	0.768	0.373
T=0	86.663	2.084	0.973	2.167	1.04
T=4	112.276	2.122	0.463	2.807	1.323

T=8	301.748	2.125	1.419	7.544	3.549
T=12	81.711	2.11	0.921	2.043	0.968
T=20	260.201	2.119	1.681	6.505	3.069
T= 25	264.865	2.12	1.684	6.622	3.124
T=30(P1)	122.929	2.082	1.046	3.073	1.476
T=30(P2)	105.447	2.122	0.931	2.636	1.242

Table 1: Amount of RNA isolated in ng/µL and concentration of the RNA for trial 1.

3.1.2 Trial 2 data

Sample Name	Nucleic Acid(ng/uL)	A260/A280	A260/A230	A260	A280
T=-1	48.808	2.192	0.055	1.22	0.557
T=0	480.2	2.14	0.437	12.005	5.61
T=4	98.313	2.169	0.092	2.458	1.133
T=8	23.426	2.742	0.025	0.586	0.214
T=12	30.598	2.605	0.031	0.765	0.294
T=25	14.083	2.746	0.015	0.352	0.128
T=30	85.478	2.506	0.172	2.137	0.853

Table 2: Amount of RNA isolated in ng/µL and concentration of the RNA for trial 2.

3.1.3 Trial 3 data

Sample Name	Nucleic Acid(ng/uL)	A260/A280	A260/A230	A260	A280
T=-1	48.808	2.192	0.055	1.22	0.557
T=0	480.2	2.14	0.437	12.005	5.61
T=4	98.313	2.169	0.092	2.458	1.133
T=8	23.426	2.742	0.025	0.586	0.214
T=12	30.598	2.605	0.031	0.765	0.294
T=25	14.083	2.746	0.015	0.352	0.128
T=30	85.478	2.506	0.172	2.137	0.853

Table 3: Amount of RNA isolated in ng/µL and concentration of the RNA for trial 3.

3.2 Designed primers

Having designed primers targeted to AChR and galectin-3 genes, using NCBI primer blast and confirming the same using primer 3 designing tool, they were then commercially obtained from integrated DNA technologies. Since the two genes were normalized using the house keeping gene Gapdh its primers were also designed and obtained. Table 4 below shows the sequences of the primers obtained.

Sequence Description	**Sequence**
Galactose binding, soluble 3 (Lgals3) F	AGCGGCACAGAGAGCACTAC
Galactose binding, soluble 3 (Lgals3) R	GGTAGGCCCCAGGATAAGCAG
Chrna1F	GCACCCTGAGGTGAAAAGCG
Chrna1R	TCCCGATGAGACACACCAGC
Gapdh1F	AAGAGGGATGCTGCCCTTACC
Gapdh1R	CGGCCAAATCCGTTCACACC

Table 4: Forward and reverse primer sequences of the genes of interest

3.3 C2C12 cell Morphology

To study the cell morphology in this experiment, the C2C12 cells were grown in 10% fetal bovine serum and images taken using the Cytation™ 5 cell imaging multi-mode reader at each designated time points. From the images taken the cells showed a normal growth. At the earlier time points t= -1 and t=0 the myoblast cells are observed they begin to proliferate fuse and become 100% confluent at t=0 while as the cells mature, they begin to differentiate the formation of myotubes are revealed as in t=4. As the cells continue to fuse, they become multinucleated as seen on t=8. At t=12 the cells are cylindrical and multinucleated. This is the maturation stage. As these cells continue to 'age' as in t=25 and t=30 more myoblasts are observed to form underneath the myotubes.

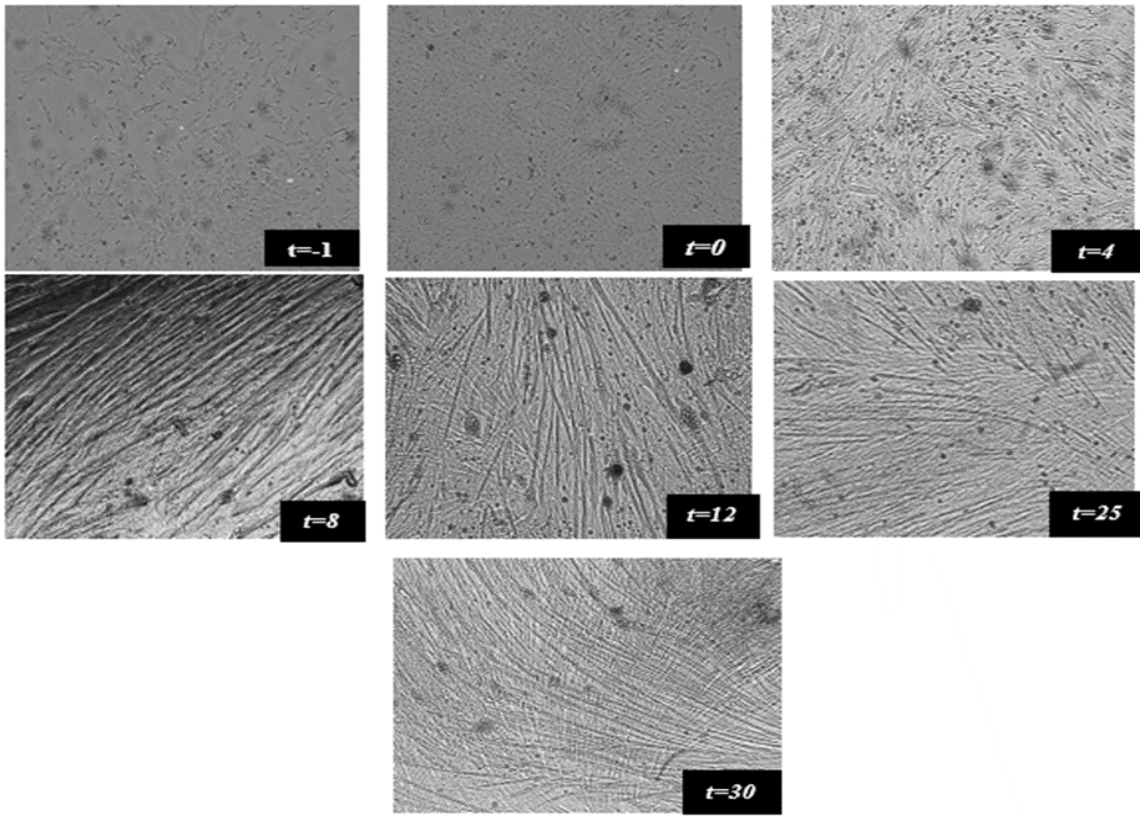

Figure 4: C2C12 cell images at designated timepoints

3.4 Gene Expression Analysis

3.4.1 Galectin-3 Expression

Once RNA was isolated and quantified, qRT-PCR was utilized to analyze the expression of galectin-3 gene. Its expression was normalized to Gapdh which is the housing gene for each of the three trials. Each trial showed high expression at the determination and proliferating stages that is at timepoints t=- and t=0. When the cells reached confluency and past confluency as they exit the cell cycle the expression of this gene seem to be declining. This is seen at timepoint t=4 through t=12. As the cells 'age'

at timepoints t=25 and t=30 the expression of galectin-3 is seen to increase again. Some variation in the expression was also observed in the first trial as shown in figure 3 compared to trial two and trial 3 in figure 4 and figure 5 respectively. However, when the average of the three trials was done it exhibited a high expression at timepoints t=-1 and t=0, a decline at t=4 to t=12 and increase at timepoints t=25 and t=30 as represented in figure 6.

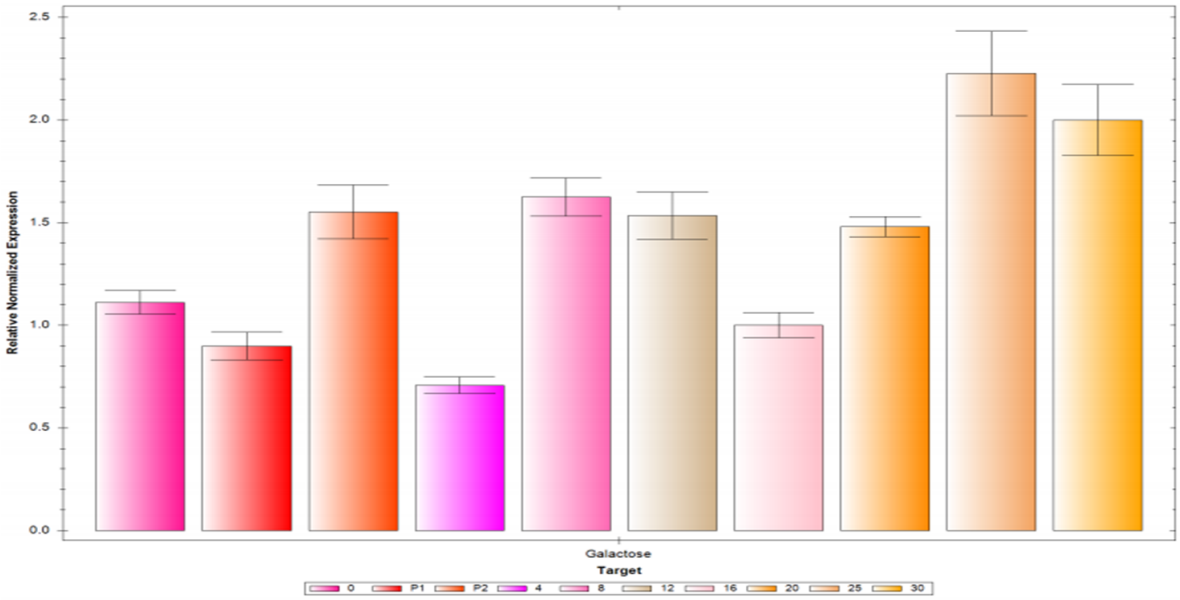

Figure 5: Galectin -3 gene expression levels (Normalized to Gadph) for trial 1.

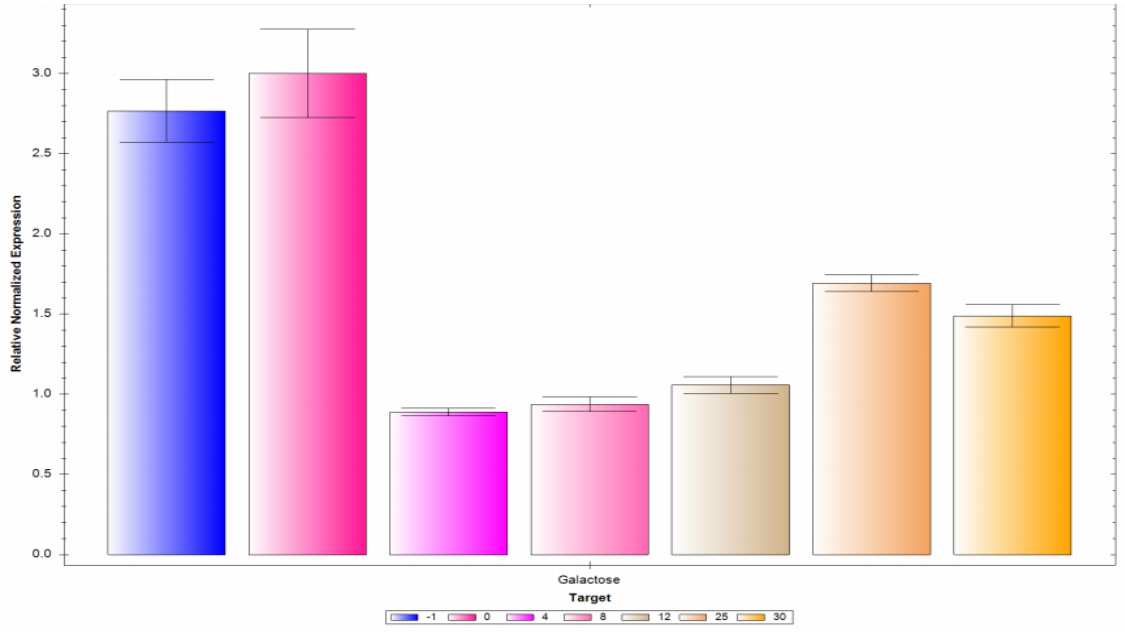

Figure 6: Galactin -3 gene expression levels (Normalized to Gadph) for trial 2.

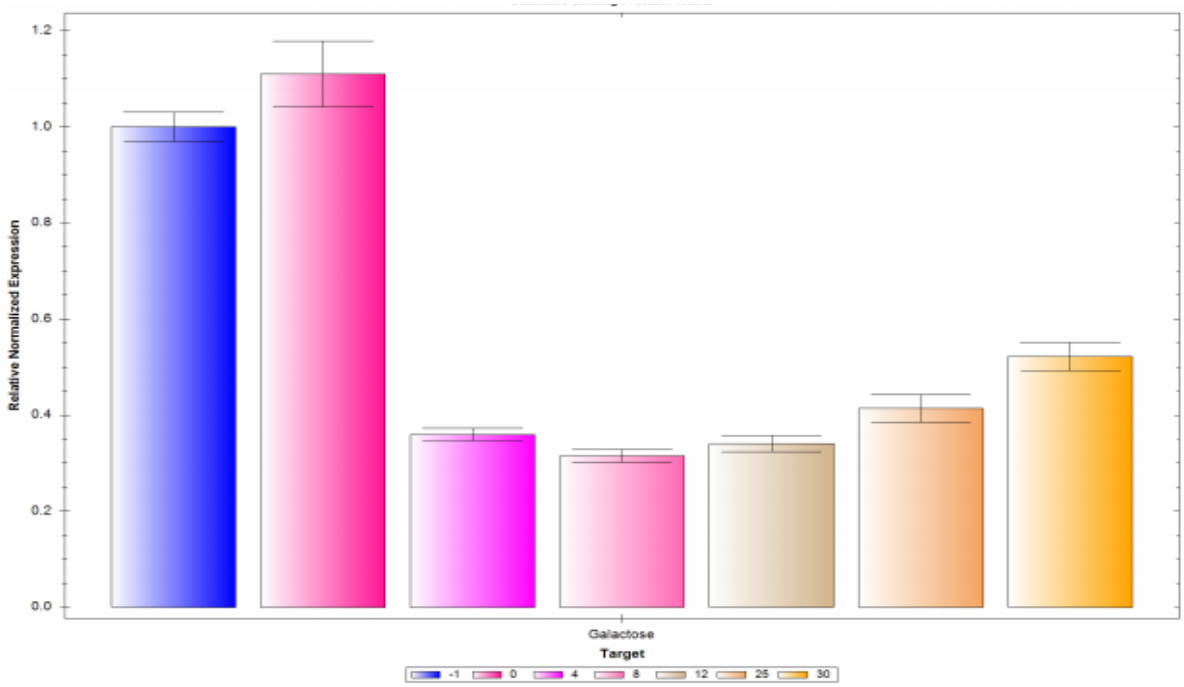

Figure 7: Galactin -3 gene expression levels (Normalized to Gadph) for trial 3.

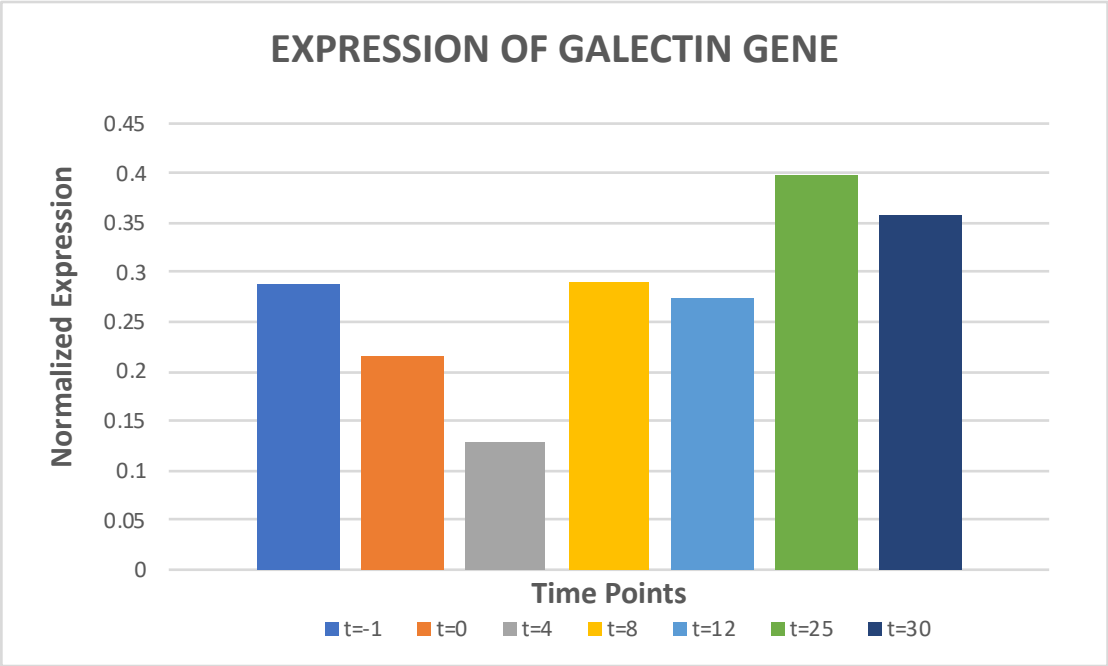

Figure 8: Graph representing the average results for the three trials for galectin-3 expression levels.

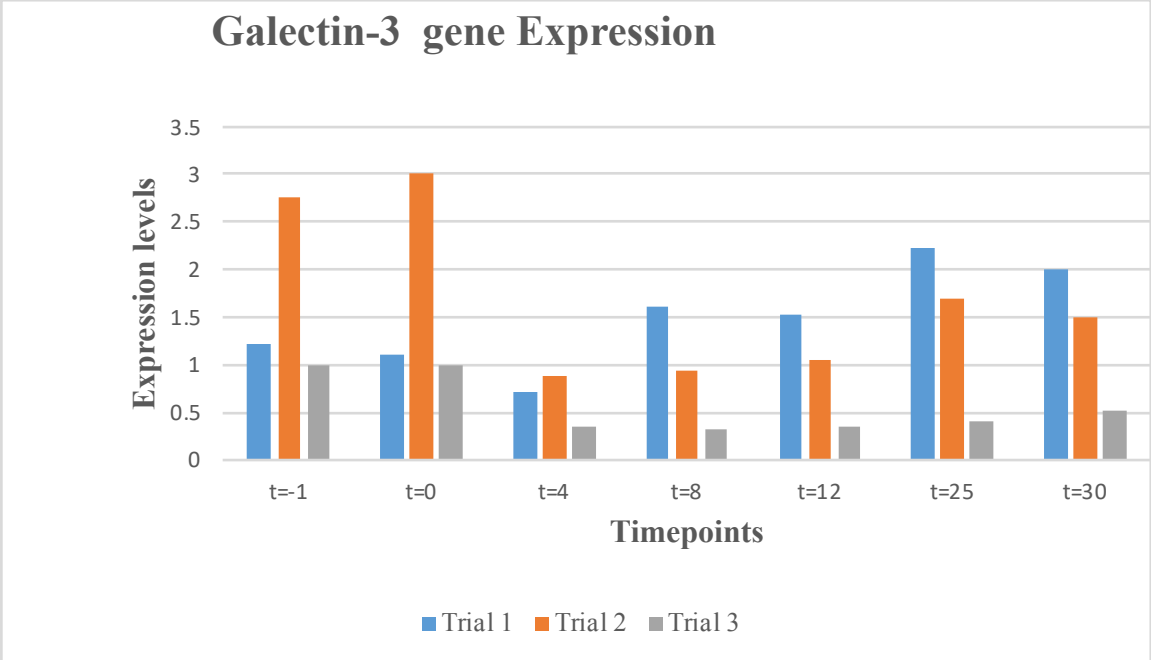

Figure 9: *Expression of galectin 3 in three separate trials.*

3.3.2 Acetylcholine Receptor Expression

qRT-PCR was used to analyze the expression of AChR following the quantification of RNA for all the three trials performed. A general observation for the three trials as seen in figure 7 ,8 and 9 is higher expression at the onset of the myogenesis process which is the cells are at the determination stage and proliferating stage during timepoints t=-1, t=0, t=4. The same observation could be made at timepoint t=8 which could mean that this gene could be involved at the initial initiation of the differentiation step. At timepoints t=25 and t=30 all the three trails portrayed a decline in the expression. When compared, a variation was observed for the first trial (figure 7) in comparison with the other two trials that is figure 8 and 9, respectively. To try and solve the variation, the average expression of the three was done as seen in figure 10. This also displayed the same trend as observed in all the trials earlier.

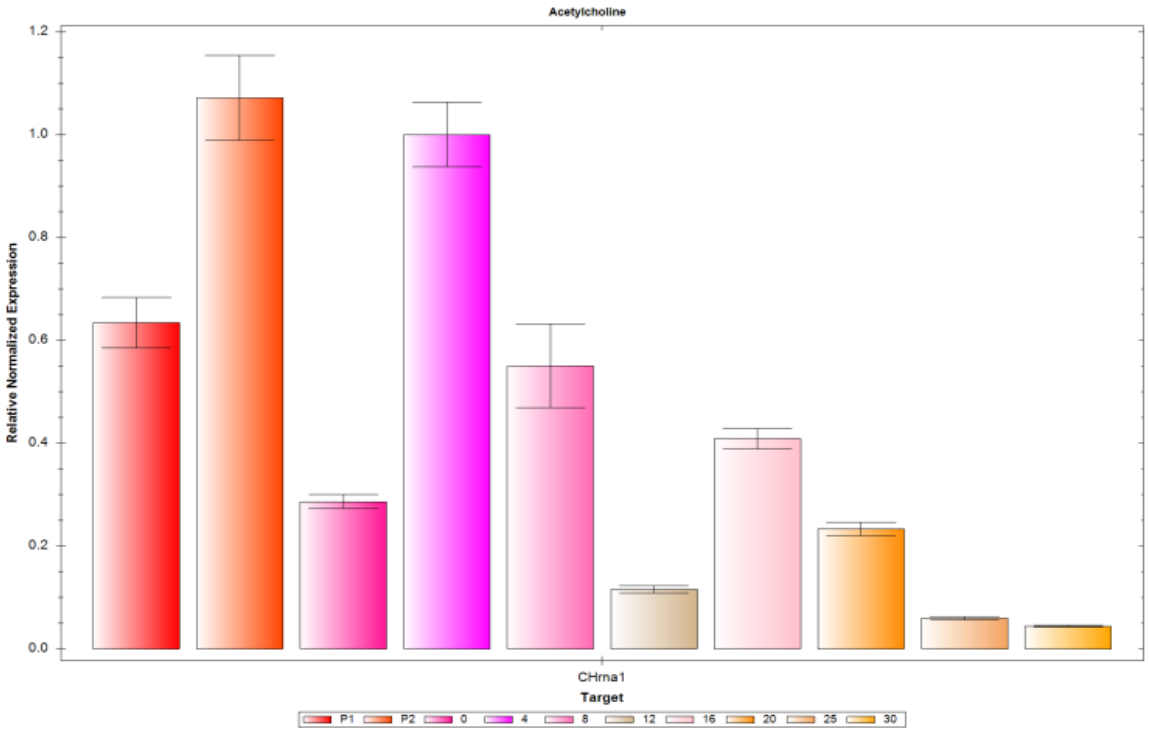

Figure 10: Acetylcholine Receptor expression levels (Normalized to Gadph) for trial 1.

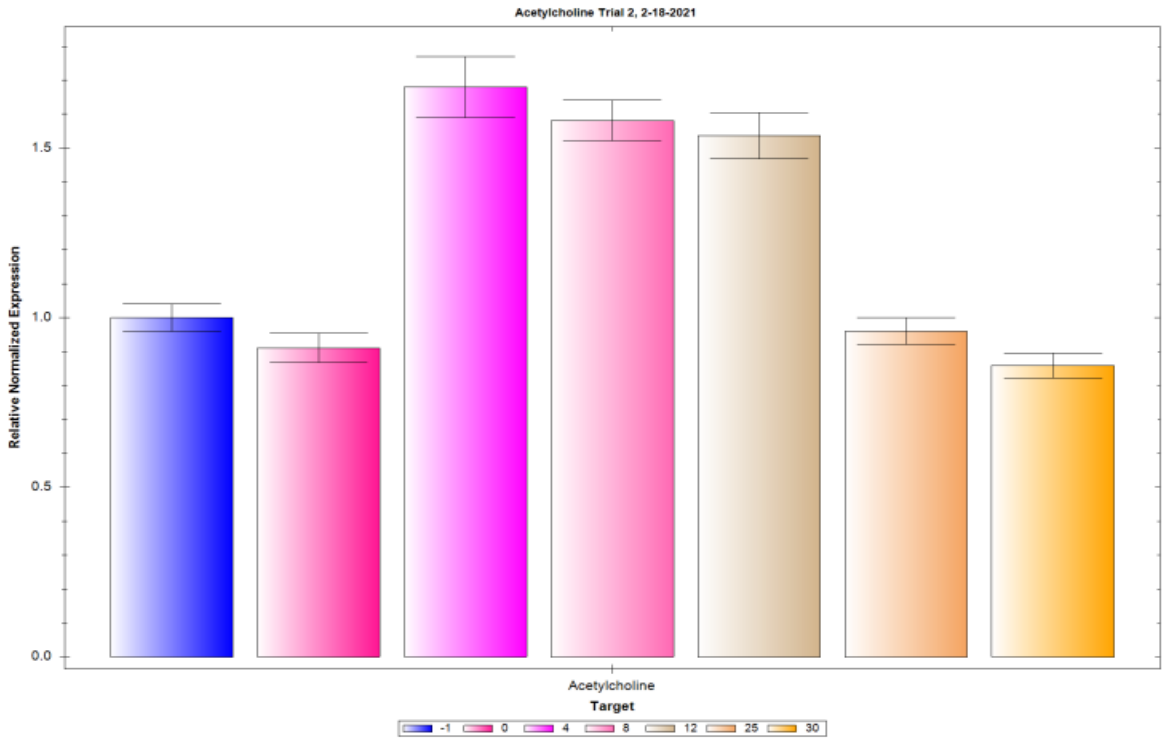

Figure 11: Acetylcholine Receptor expression levels (Normalized to Gadph) for trial 2.

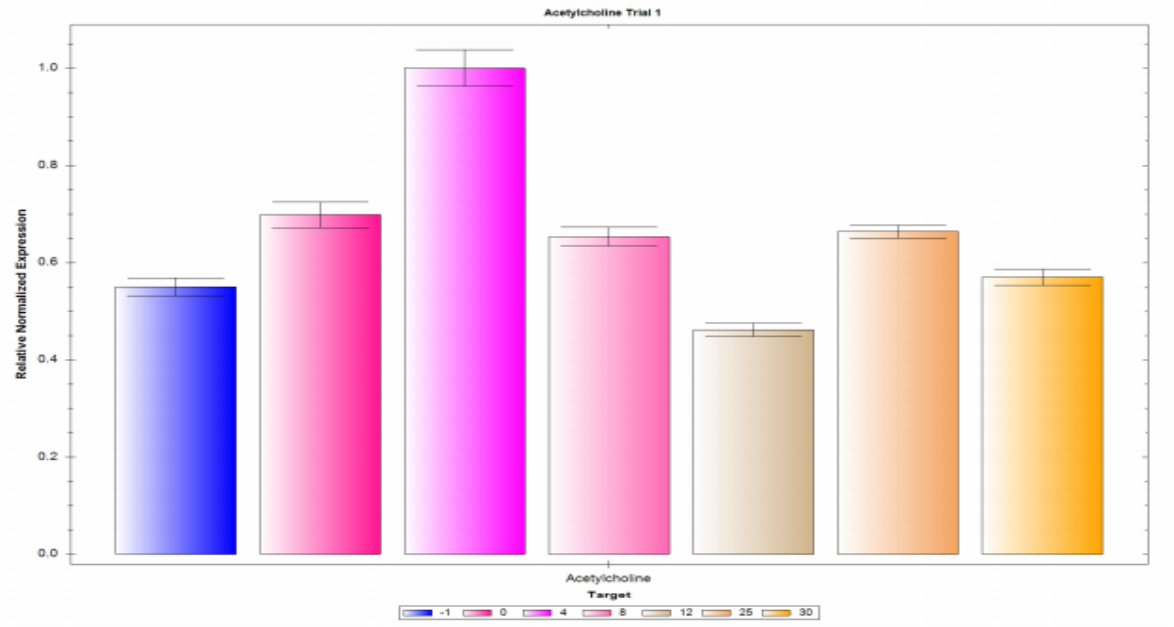

Figure 12: Acetylcholine Receptor expression levels (Normalized to Gadph) for trial 3.

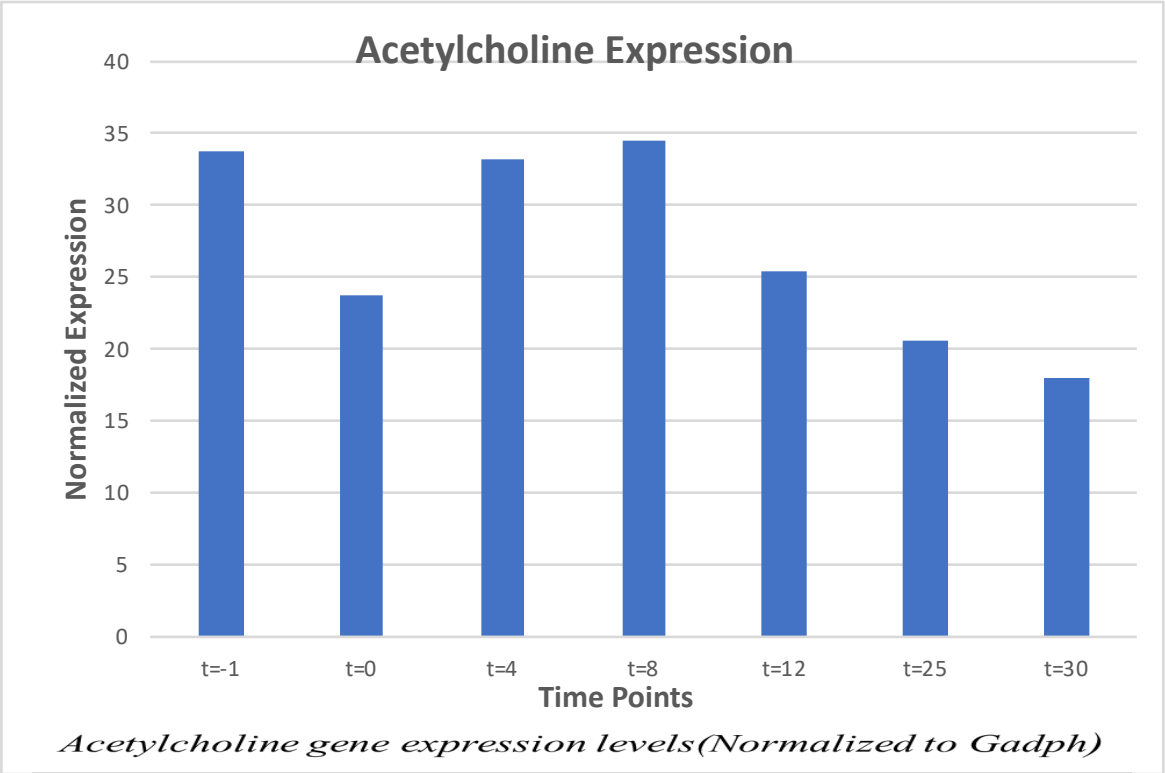

Figure 13: A graph representing the average results for the three trials for Acetylcholine receptor expression levels.

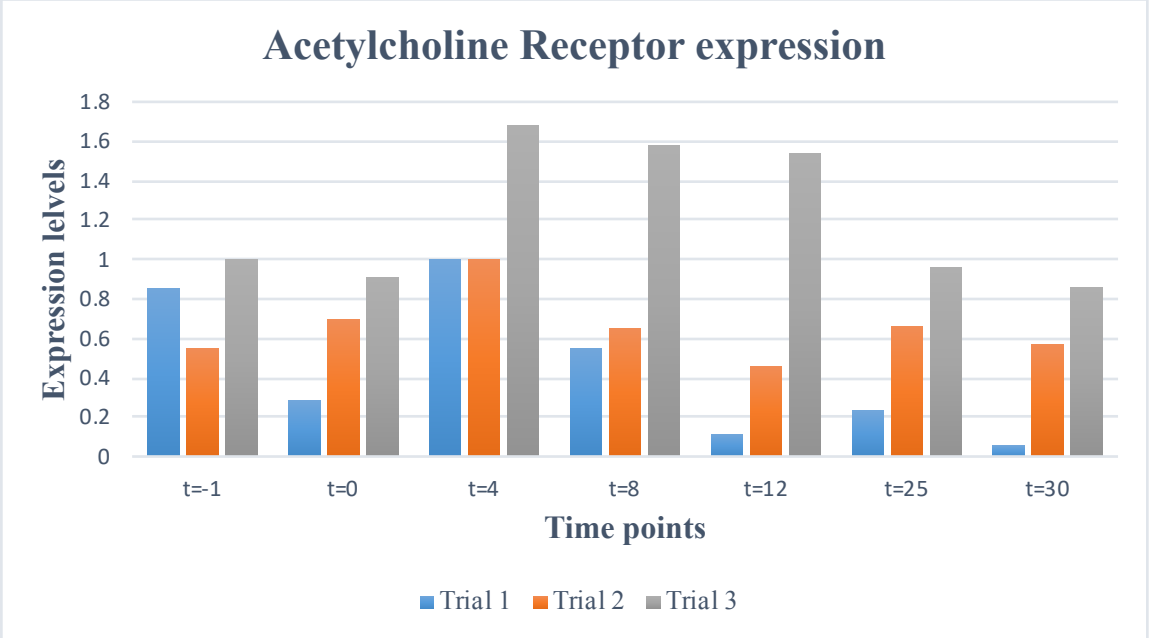

Figure 14: Expression of Acetylcholine receptor in three separate trials.

CHAPTER FOUR

4.0 DISCUSSION

4.1 Quantification of RNA

Determination of the purity and the amount of RNA is an important step in determining the amount used reverse transcription PCR (RT-PCR). NanoDrop Spectrophotometer was used for assessing RNA quantity and quality. A pure RNA is expected to have a A260/280 ratio of 2.0. A ratio of 1.8 is also acceptable however any value < 1.8 indicates potential protein and DNA contamination of the RNA sample. In this study, data from table1shows the RNA sample with a ratio of 2.0 and above which indicated that sample is pure however the A260/A230 ratio is below 2.0 which could be due to contaminations with the washing buffers.

4.2 C2C12 cell Morphology

C2C12 cells are immortalized myoblast cells obtained from mouse muscle. They are the most preferred type cells because they are capable of differentiating and therefore allowing the description of each transcriptional step program during the myogenesis process.

The cellular morphology analysis was done using the Cytation™ 5 cell imaging multi-mode reader to take images of the cells. The 10% fetal bovine serum cells images were thereafter observed for normal growth for each designated timepoints. At t=-1 the myoblast cells were about 70% confluent and therefore what was observed is a change in the shape and size of the cells. The cells seem to appear as star shaped at t=0 they changed to round shape and became denser as a result of proliferation. As they begin to differentiate, elongated tubular shapes called myotubes containing more of two nuclei are

seen due to fusion of cells. This is observed at timepoint=4. During the differentiation stage cells align with the neighboring cells to form tubular shapes. These results agree with the study that was done by Niioka et al.,2018 and Wagner, 2020.

At the late differentiation stage, which is at timepoint t=8, the cells continue to fuse forming myotubes which are multinucleated. At timepoint t=12 the cells are termed as fully mature myofibers which are multinucleated single muscle cell. The same observations were made by Ferri et al., 2009, Nyaboke et al., 2014 and Wagner, 2020. At the later timepoints t=25 and t=30 as the cells begin to 'age' myoblast is seen beneath the myotubes. This could be due the self-renewal mechanism that the skeletal muscles possess.

4.3 Gene expression Analysis

4.3.1 Galectin-3 Expression

From this study it is observed that at earlier stages of myogenesis, the expression of galaectin-3 gene is high because it is needed for the cell adhesion. When the cell culture reaches confluency levels, its level of expression begins to decline. This is attributed to the maximum cell to cell contact that the cells have achieved as they begin to exit the cell cycle which could mean that this gene is no longer needed by the cell for cell adhesion. My observation in this study agrees with the previous study done by Jia.et al., 2020 on the importance of galectin-3 in myogenesis.

At later stages of the myogenesis process which indicates muscle aging at time points t=25 and t=30, galectin-3 expression levels are seen to be increasing. This is because as muscles age, it wears off and there is need for muscle to be repair. According to Cerri et al.,2020 in their research, galectin-3 is essential in skeletal muscle repair. In

their study which they used a mouse model they demonstrated that the endogenous galectin-3 is involved in the myogenic programing and that it is required by the skeletal muscles for self-repair. They later proved this with Gal-3 knock out gene which exhibited persistent inflammation and a decline in the expression of MyoD and myogenin regulatory factors. MyoD is the first marker for myogenic commitment and differentiation while myogenin is responsible for terminal differentiation of the myoblast therefore this shows how galectin-3 is expression is these markers even though its exact mechanism is not yet known. This study also agrees with another study done by Rancourt et al.,2018.

4.3.2 Acetylcholine Expression

Unlike the Galectin-3 gene, Acetylcholine in this study was expressed more at earlier stages of the myogenesis process that is at timepoints t=-1, t=0, t=4 and t=8. During muscle building, more expression of Acetylcholine receptor could be needed especially during the embryonic development. This gene is expressed on the muscle cell surface and it changes during myogenesis. Previous research has shown accumulation of postsynaptic AChRs at sites of nerve-muscle contact during muscle development (Liu et al., 2008). Lui et al., 2008 further demonstrated the importance of the alpha subunit in nicotinic AChRs in proper muscle development and contraction which they concluded that the absence the alpha subunit could contribute to abnormal localization of the receptors.

As muscles matures or ages, which in my study is during the later timepoints t=25 and t=30, AChR seem to be decreasing. This could be because this gene is not very essential in muscle aging but rather is maintained due to its importance in muscle contraction and normal protein turn over.

CHAPTER FIVE

5.0 CONCLUSION

Both galectin-3 gene and acetylcholine receptor are always expressed throughout the process of myogenesis. Importance of Galectin-3 in expression in regulation of other myogenic factors involved in skeletal muscle development for example the MyoD and myogenin could be an essential factor for this gene to be expressed during the entire myogenic process. As a channel that signals muscular contraction at the neuro muscular junction, Acetylcholine's receptor expression is necessitated throughout the myogenesis process. This is because it is the primary receptor for the nerve -muscle communication that leads to the general functioning of skeletal muscles.

Finally changes in Acetylcholine receptors and galectin-3 gene expression during maturation of muscles are good indicators of aging in muscle cultures.

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