Gene Expression in Long -term myoblast/myocyte cultures: RNA Analysis

(DYSTROPHIN GENE)

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Annah Oigo

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GENE EXPRESSION IN LONG TERM MYOBLAST /MYOCYTE CULTURES:

RNA expression (Dystrophin Gene)

Annah Oigo

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Annah Oigo, Student Date

Approvals:

_____

Dr. Gary Walker, Thesis Advisor Date

_____

Dr. Jonathan Caguiat, Committee Member Date

_____

Dr. David Asch, Committee member Date

_____

Dr. Sal Sanders, Dean, Graduate Studies Date

ABSTRACT

Myogenesis involves changes in gene expression which is necessary for muscle development and regeneration. This involves the fusion of myoblasts to form myotubes which mature into myofibers to form new skeletal muscles (important in the movement of the body). This process is regulated by Myogenesis Regulatory Factors (MRFs) which include MyoD, Myogenin, Myf5 and MRF4. This study examines how different genes are expressed when myoblast C2C12 cells are grown in long term cultures. We conducted an experimental study design using the immortalized mouse myoblast cell line C2C12. The cells were allowed to differentiate and were harvested at designated time points, seven time points. The focus of this study was to look at expression of dystrophin gene throughout the process of myogenesis. The C2C12 cells were grown and allowed to differentiate over a 30-day time course study with seeding, imaging, growth media change (10% FBS), and pelleting cells at specific time points for RNA isolation. Following the RNeasy kit protocol the RNA was isolated and quantified by nanodrop spectrophotometry. Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR) analysis was carried out to measure expression of dystrophin gene. Gene expression analysis was normalized to GAPDH. Expression of dystrophin gene was measured for the two trials and later the average was done. Dystrophin expression levels are high during activation of satellite cells where it helps to regulate cell polarity and increase number of asymmetric divisions. As the activated cells proliferate, dystrophin levels go down. After the onset of differentiation, increased levels of dystrophin expression are observed which help in plasma membrane stabilization of striated muscle cells. During muscle aging, expression levels slightly decrease to maintain the stability of the cell membrane.

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1.1 Summary of Skeletal Muscles.

Muscle cells are specialized cells that perform contractile functions for organisms. Muscles arise from the embryonic mesoderm during embryogenesis. The body of a human has over 600 muscles (40% of the body mass). The body muscle types include skeletal, smooth, and cardiac. The skeletal muscle is the muscle attached to the skeleton, the smooth muscle is the type of muscle in the viscera as well as blood vessels of the vertebrates and the cardiac muscle is the muscle of the heart. Apart from locomotion, these muscles help in breathing, digestion, stability, posture, childbirth, heartbeat, and blood flow.

Cardiac muscles are quadrangular in shape and have cells in the walls of the heart (they are found only in the heart), they appear striped (striated) and are under involuntary control. Cardiac muscles are the smallest of the three muscle types with a length of between 0.1mm and 0.15mm. Its fibers are short with one nucleus centrally located in the cell. They are surrounded by a thin outer layer, epicardium, and an inner layer endocardium. They have well organized sarcomeres. They possess numerous mitochondria and myoglobin (Anthony S. et al, 2021).

Smooth muscles are fusiform in shape (they are composed of elongated, spindle shaped cells), and are non-striated due to the thick and thin filaments they have that are not arranged into sarcomere. They are 0.5mm in length and have a centrally located nucleus and are under involuntary control. They have a large amount of Actin and Myosin in their cytoplasm which are involved in muscle contraction. These muscles are commonly found in most parts of the body like the stomach and the intestines. (Hafen, B.B and Burns, B. 2018).

Skeletal muscles consist of approximately 90% muscle fibers and 10% connective and fat tissues (Listrat, A. et al, 2016). They are the largest muscle type compared to smooth and cardiac muscles, with a length of 30mm to 40mm. They are multinucleated (each cell has a nucleus that tend to be towards the edge of the cell), and striated muscles which are under voluntary control. Each skeletal muscle is made up of fascicles which are made up thousands of muscle fibers (cells), and these fibers contain arrays of myofibrils containing myofilaments (Dave, H.D, et al,2020). These muscle fibers are individually wrapped and bound together by different layers of fibrous connective tissue. These connective tissue layers are continuous with each other, and they extend past the ends of the muscle fibers to form the tendons that anchor muscles to bone thus facilitating muscle contraction. The layers include the epimysium which is a layer of dense fibrous connective tissue that surround the entire muscle, the perimysium layer that surrounds each fascicle and the endomysium which is the loose connective tissue layer that surrounds each single muscle cell (Howard, J.J and Herzog, W 2021). This study concerns itself with myogenesis in skeletal muscles.

Every skeletal muscle is supplied by blood vessels which supply nutrients for nourishment, deliver oxygen and remove wastes. Every muscle fiber of the skeletal muscle is also supplied by the axon branch of a somatic motor neuron which signals the fiber to contract since contraction of the skeletal muscle is through signaling from the nervous system. The skeletal muscle fibers are long, cylindrical, and large compared to other cells and they contain cellular organelles such as the nuclei, mitochondria, endoplasmic reticulum, Golgi apparatus, cytoplasm, and plasma membrane. They have numerous nuclei which produce large amounts of proteins and enzymes for maintaining normal function of the cell.

They also contain many mitochondria just inside the sarcolemma which are vital organelles that produce energy (ATP) required for muscle function, that is, during contraction and relaxation.

According to Patricia G. 2014, in a normal lifespan, skeletal muscles need regenerative capacities, provided by the satellite cells, thus they have a high energy demand. Skeletal muscle fibers also contain specialized smooth endoplasmic reticulum, sarcoplasmic reticulum, that stores, releases, and retrieves calcium ions. They have Golgi apparatus near the nucleus which help in packaging of proteins into vesicles. They have a cytoplasm called sarcoplasm which helps in PH and ion balance, and it initiates the process of muscle contraction by beginning the process of filament sliding when there is an increase in the levels of Ca2+ in the sarcoplasm. They also have a plasma membrane called sarcolemma which acts as a barrier between the extracellular and intracellular compartments thus defining the individual muscle fibers from their surroundings. Myofibrils are the organelles liable for producing force and motion in skeletal muscles (Garland L. and Robert H. 2011). Myofibrils are composed of rows of sarcomeres lined up end to end along the entire length of the myofiber. The sarcomere is the smallest unit of force generation and contraction.

The sarcomeres are made up of the contractile myofilaments, myosin and actin, and a series of structural proteins, titin, and nebulin (control actin and myosin filament lengths and assembly). Myosin is a bipolar polymer, thick myofilament and actin is a globular monomeric protein, thin myofilament and together, when observed under a light microscope they give skeletal muscles the striated appearance (Moo, E.K et al, 2018).

Sarcomeres also have intermediate filaments, desmin and vimentin, with desmin being the largest, and these filaments interact to act as scaffold and connect the sarcomere to other organelles like the sarcoplasmic reticulum, lysosomes, nucleus, and mitochondria and maintain cellular integrity. Desmin also interacts with keratin protein which links the sarcomere to the sarcolemma thus facilitating interactions with the dystrophin glycoprotein complex (DGC).

Dystrophin is the main component of the DGC, and it interacts with actin, microtubules, and intermediate filament proteins to link the sarcolemma to the extracellular matrix at the costamere. Sarcomeres are arranged between two Z-disks and on either side of these disks is the I-band (region with actin and titin but without myosin filaments). The myosin and actin filaments are aligned at the center of the sarcomere by titin filaments to form the A-band (Henderson, C. A, et al 2011). At the center of the A-band is the M-band which falls within the H-zone. The Z-disks of adjacent sarcomeres are linked by the barbed ends of actin filaments through α-actinin protein.

The costamere links the sarcomere to the plasma membrane (sarcolemma) through the Z-disk and M-band. At the junction of the A-band and I-bands are the T-tubules which are extensions of the sarcolemma, and they penetrate the center of skeletal muscle bringing the sarcolemma close to the sarcoplasmic reticulum thus conducting impulses from the sarcolemma to the sarcoplasmic reticulum. The myotendinous junction transmit force from the sarcomere to the connective tissue of the tendon (Christina A, et al, 2017).

Muscle contraction occurs when a muscle is stimulated (impulse) by a motor neuron. One motor neuron stimulates only a few the muscle fibers within a muscle thus forming a motor unit.

The motor neuron axons and the muscle fiber it stimulates forms the neuromuscular junction. When an impulse reaches the muscle fiber of a motor unit, it stimulates a reaction in each sarcomere between the actin and myosin filaments. Therefore, sarcomere contraction involves the movement of the myosin heads on actin filaments (cross-bridge cycle), which is brought about by a sequence of enzymatic reactions which generate force within each individual myofibril and thus experienced by the muscle. The myosin heads pull the actin filaments towards the center of the sarcomere thus shortening it. At the start of contraction, free cytosolic Ca^{2+} brings a conformational change in troponin, revealing myosin-binding sites on actin filaments.

Myosin heads swings out towards the actin filaments at a 45^{o} angle and is in a stiff state. Available ATP then binds to myosin thus briefly dissociating myosin from actin. This ATPase activity of myosin hydrolyzes ATP to ADP and Pi (free phosphate) causing the myosin filament to weakly rebind actin at the 90^{o} angle relative to the actin filament (cross-bridge). The release of Pi initiates the power stroke and thus the myosin head rotates on its hinge pushing the actin filament past it towards the M-band. The myosin head releases ADP and regains its stiff state at the end of the power stroke (Kavitha M and Shankar S, 2019)

1.2 Sarcomere Cytoskeleton Proteins.

The contractile muscles have three types of proteins, regulatory, motor, and structural. Contraction of these muscles is brought about by the collaboration between the sarcomere, and the cytoskeleton.

The sarcomere is made up of highly organized filament networks, as part of the motor function and the cytoskeleton has a vast network of interconnected filaments, intermediate filaments, which act as a platform for maintaining cell integrity, as part of the structural functions that need to be in place for integrity and organization. The costamere that binds the sarcomere to the sarcolemma is composed of vinculin-talin-integrin system and the dystrophin glycoprotein complex.

In striated muscles, sarcomere assembly is a systematic process that leads to assembly of individual proteins into Z-line and thick and thin filaments. In this assembling process, specific scaffolding and chaperone proteins are needed. A study conducted by Garland L. and Robert H. show that actin and α-actinin assemble into premyofibril structures, which again gather to form myofibrils with actin filaments attached at the Z-lines and myosin filaments at the center. Speckled α-actinin Z-bodies, muscle tropomyosin and α-actin are the first myofibril precursors.

N-RAP is main scaffolding protein domain in striated muscles which is tightly associated with the myofibril ends and has two isoforms N-RAP-c and N-RAP-s in skeletal muscles. N-RAP is important in that, it connects the terminal actin bundles to the membrane complexes thus conveying tension generated by the myofibrils in the direction of the cell ends (Garland L. and Robert H. 2011).

Titin also known as connectin is the largest known single polypeptide with its gene having 363 exons, in skeletal and cardiac muscles titin is differentially spliced to give rise to specific isoforms found in specific muscle types.

Titin covers half of the sarcomere length from the Z-disk to the M-line and it joins the myosin protein to the Z-line of the striated muscles (Jeffrey G. Forbes et al 2006). The amino-terminal region of Titin is made up Z-line, the I-band and A-band while the carboxy-terminal region is made of the M-line (figure 1).

Molecular and biochemical experiments have shown that during muscle development, Titin helps in assembly and organization of myosin and during contraction of muscles it helps in sarcomere stability and maintenance (Claire Chauveau et al 2014) and regulation of passive stiffness (Siegfried. L et al 2006). Although Titin is the main protein in sarcomere assembly, other proteins like monomers of actin and myosin, nebulin and obscurin are required for that role to be effectively attained (Manuel. N et al 2015) (figure 1). Titin together with nebulin are believed to control actin filament length and assembly (Garland L. and Robert H 2011). The spring-like segment of Titin in skeletal muscles is made up of: PEVK region with residues, proline, glutamic acid, valine and lysine and immunoglobulin-like domains. According to Alkaterini et al., 2009, Titin is highly integrated with nearly 90% of its mass consists of replicating immunoglobulin (Ig) and fibronectin-III (FNIII) domains.

Specifically, it contains 244 recognizable -sheet domains of which 112 have been allocated to the immunoglobulin superfamily and 132 to the fibronectin type III superfamily. The differential splicing of the spring-like segment of titin varies the passive stress of muscles thus maintaining the central position of the A-band in the sarcomere. This is done by deleting some portions of the spring segment like: exon 219-225, which encodes part of the PEVK region, exon 30-38 which encodes 9 of the 15 proximal tandem Ig domains, and exon 49 which encodes the cardiac specific N2B element (Danielle B. et al).

Titin undergoes alternative splicing at the I-band portion of the gene, and gives rise to two different isoforms, N2A and N2B. Both are found in the cardiac muscle where N2B isoforms contains an N2B element, and N2A isoforms are found in skeletal muscle. N2B is short and stiffer, while N2BA is longer and more compliant. N2BA has numerous fetal-neonatal variants but most variation is lost in adult life (Roseline Nyaboke 2016).

Titin isoforms have PEVK and tandem Ig segments, and it has been discovered that N2B isoforms have N2B element (3 Ig domains and a 572-residues unique sequence) and N2A isoforms have N2A element (4 Ig domain and 106-residue unique sequence). Exon 49 contains N2B sequence that is only found in the skeletal titin but also found in all the other cardiac isoforms. Exon 49/50 undergoes splicing to 219 and this produce a small N2B cardiac isoforms, while Exon 102 to 109 codes for N2A element (N2BA isoforms) and this isoform is longer than N2B since it has a longer PEVK segment and contain Ig domains (Wei Guo et al 2010).

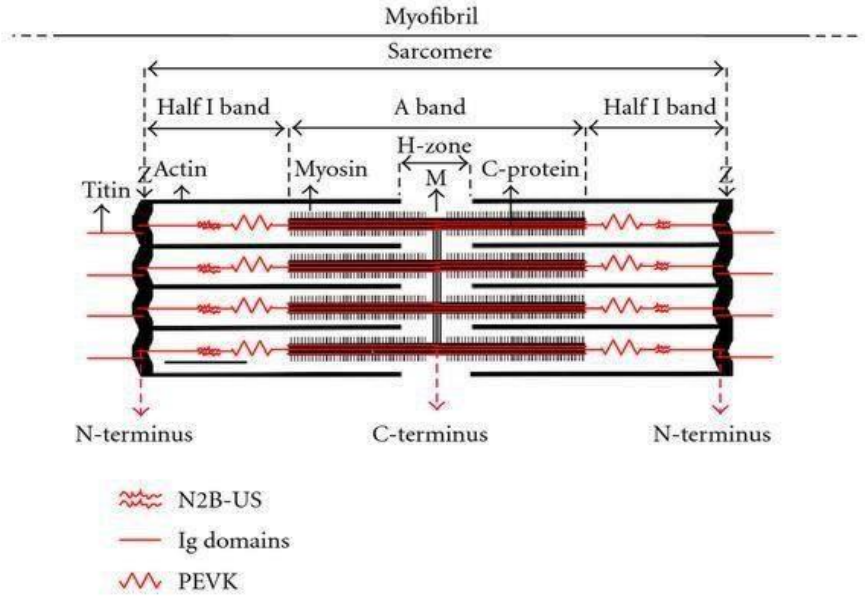

Figure 1: Titin location and arrangement in the sarcomere (Wei Guo et al 2010).

Myosin is a motor protein involved in many motility processes including, muscle contraction, cytokinesis, and vesicular transport in eukaryotic cells. The myosin binding protein and titin protein are elements of myosin. Myosin II (conventional myosin) is the myosin type responsible for producing muscle contraction in muscle cells in most animal cell types. Myosin II consists of two myosin heavy chains (MyHCs) and two myosin light chains. MyHCs are the key determinants of the functional properties of the muscles in which they are expressed, and they are ATP- dependent. The mammalian skeletal muscle has seven MyHC isoforms which include three adult skeletal muscle isoforms, MyHc-IIa, MyHC-IIb, and MyHC-IIx and two developmental isoforms, MyHC-embryonic and MyHC-perinatal.

A study on YAC (yeast artificial chromosome)- based contiguous set of overlapping chromosomes indicated that MyHC genes are on the chromosome 17 in human and chromosome 11 on mice. The MyHC genes are organized in a way that reflect the sequence similarity among its family members (Weiss et al, 1999).

Repair of skeletal muscle injury involves three processes: inflammatory, repair and remodeling phases. The damaged myofibers rupture to release chemokines such as CCL_{17} and CCL_2 and cellular contents and then undergoes necrosis. This initiates the inflammatory phase where fibroadipogenic progenitors are activated thus initiating muscle regeneration. For the repair phase to kick off, the resident mast/immune cells produce neutrophils, clear the damaged tissue, and tumor necrosis factor -α (TNF-α) that induces the quiescent satellite cells to enter the cell cycle. M1 macrophages secrete pro-inflammatory cytokines such as interleukin 1 (IL-1), IL-6, nitric oxide (NO) and reactive oxygen species (ROS) and remove muscle cell debris. TNF-α is believed to be very useful in proliferation, differentiation, and regeneration of skeletal muscles cells.

At this point, the muscle proliferates, and the cell begins to differentiate and fuse into myotubes promoted by the M2 macrophages. M2 macrophages express anti-inflammatory cytokines such as interleukin (IL)-1 receptor antagonist, IL-4, IL-10, IL-11, and IL-13, to decrease the inflammatory response at the site of injury. This helps the muscle to start repairing itself by reinnervation and revascularization. New myotubes fuse with existing myofibers and the gaps between myofibers that are formed during the injury are filled up with extracellular matrix (ECM) by cells. This results in fibrosis and scar formation, the scar is then remodeled and depleted as the muscle fibers repair and contract together (Smoak .M and Mikos A. 2020).

1.3 Muscle Development.

Myogenesis is a complex and tightly regulated process that leads to the formation of skeletal muscular tissue especially during embryonic development (Knight J and Kothary R, 2011). It involves changes in gene expression which is necessary for muscle development and regeneration. Skeletal myogenic molecular pathways are discovered by evaluating the manifestation of growth and transcription factors, and ion channels of calcium and kinases (Twinkle J. et al 2013). This process is regulated by Myogenesis Regulatory Factors (MRFs)[1], a family of transcription factors sharing a common basic helix-loop-helix domain. These MRFs include MyoD, Myogenin, Myf5 and MRF4 (Hernández-Hernández, J et al, 2017). Overexpression of MyoD converts non-muscle cells into myoblast-like cells for example mesenchymal stem cells of the C3H10T1/2 line. Myocyte enhancer factor 2 (MEF2) is a coactivator of the MRF family to activate myogenic gene expression. It has four members: MEF2 A, B, C, and D.

Triple knock-out of Mef2 a, c and d impairs muscle regeneration in satellite cells. Knock-out of Mef2c causes defects in skeletal muscle development for example sarcomere disorganization. Mef2a knock-out delays muscle regeneration in C2C12 cells. The upregulation of MEF2 can be triggered by hyper-polarization of the potassium channel Kir 2.1.

To get more insight of mammalian myogenesis at the molecular level, Stk40 has been identified to be a putative serine/threonine kinase which activates the Erk/MAPK pathway to induce mouse embryonic stem cells differentiation into the extraembryonic endoderm and muscle regeneration. It is also important for skeletal myogenesis in vitro and for proper working of MEF2 (He K, et al 2017). Stk40 expression is regulated by a microRNA, miR-31. Down-regulation of Stk40 decreases myogenic differentiation of C2C12 myoblasts and its overexpression increases myogenic differentiation. According to Colin Fennelly et al 2016, long term expansion of murine progenitor cells (MPCs) in vitro demand that the use of extracellular matrix (ECM) substratum and myogenic cell medium for the cells to maintain their capacity to self-renew and revitalize myogenically.

Myogenesis in mESCs is initiated with mesoderm induction (induction factors include Mox2, MyoD, Myf5, and PAX3)[2] followed by specification of the progenitor cells according to myogenic lineage, then proliferation and migration to appropriate positions within the embryo occurs. Differentiation of muscle cells begins with cell-cycle exit, and the process is wrapped up by differentiation and fusion. Proliferation, migration, and differentiation are controlled by cell signaling molecules called growth factors.

These growth factors are introduced to cells in a spatially controlled way through either covalent binding or non-covalent binding state. The latter is commonly preferred, and biomaterials made of glycosaminoglycans, fibrin or collagen have been successfully developed to present the growth factors to cells. Bone morphological proteins, BMP-2 and BMP-7 are growth factors commonly used because of their osteogenic inductive properties and are delivered to cells by collagen sponge and paste matrices. Slow release of high doses of BMP-2 promotes increased bone formation whereas spatial presentation of it determines the bone generated location. When BMP-7 is presented to C2C12 cells they act differently compared to their response to BMP-2 (Almodóvar J, et al 2014). Jorge and coworkers found out that BMP-2 induced expression of alkaline phosphate (ALP) in C2C12 cells thus highest osteogenic inductive activity compared to the other BMP's.

The resting membrane potential (Vmem) is also another key factor in cell proliferation, migration, and differentiation. Vmem is an ion gradient across the plasma membrane and is maintained by specific Na+/K+ ion channels and pumps moving these ions back and forth across the cellular membrane. Sustained depolarization of the Vmem regulates MPCs growth and upholds stem cell properties in Vitro. Colin Fennelly and his mates examined this by treating cultures with either potassium gluconate or the sodium -potassium pump blocker ouabain. Pax activity is essential for myogenesis to occur normally. Stem cells which are tissue-specific usually differentiate towards the tissues from which they were derived, and this makes them more productive. By being tissue-specific stem cells means they are multipotent and self-renewing cells with endogenous functions for tissue renewal and repair at their respective resident tissues. Based on lineage specification, muscle-derived stem cells (MDSCs) more effectively undergo myogenesis compared to other types.

Pax3/7-positive progenitor population once formed replenishes the satellite cell niche. Pax7 is required for PW1+/Pax7- interstitial cells (PICs) for myogenic specification. Cultures treated with potassium gluconate maintain higher populations of cells expressing muscle stem cell marker Pax 7. Multipotent and mononucleated satellite cells are found between the basement membrane and the sarcolemma of matured skeletal muscle fibers. In their quiescent state, they are triggered to multiply in reply to injury to refill the satellite cell niche (Smoak .M and Mikos A. 2020) and some of these muscle-derived side populations can form new myotubes or fuse to existing myofibers and help in muscle repair and regeneration (Tyler Pizzute et al 2014).

Proliferating satellite cells require activation of a Notch signaling pathway while, when differentiating along a specific myogenic lineage they require activation of Wnt signaling pathways which is usually upregulated in skeletal muscle during aging (Colin Fennelly et al 2016). Notch signaling also regulates stem cell quiescence whereby it returns Pax7+MyoD- cells to quiescence and inhibit their differentiation. Studies by Henriette Henze et al 2020 show that Notch signaling regulates quiescence of muscle stem cells declining with age by inducing collagen V connecting it to calcitonin receptor signaling and inactivation of this Notch signaling resulted into spontaneous differentiation of quiescent muscle stem cells and depletion of the stem cell pool.

According to Christopher M. et al 2016, for muscle regeneration to occur these satellite cells are the major effectors. Satellite cells are regulated by the transcription factor Pax7, and they up-regulate the transcription factor MyoD and increase expression of myogenin.

Pax 3 protein when expressed in a dominant-negative nature it causes abnormal expression of MyoD and myogenin in cultured satellite cells and when forced-expressed it induces MRF expression and embryonic tissue myogenesis. Therefore, for skeletal myogenesis to occur before MRFs expression, Pax 3 is required. (Tammy Ryan et al 2011).

In mESCs, skeletal myogenesis is stimulated by Retinoic Acid (RA). RA acts early in differentiation to enhance the expression of the precursor genes Pax 3 and Meox 1 by directly binding the retinoic acid receptors (RARs) to regulatory regions of this genes. Presence of Pax 3 +ve MyoD +ve cells at certain time points help muscle progenitor cells to differentiate into skeletal myoblasts. The low concentrations of RA used to induce skeletal myogenesis can also promotes cardiomyogenesis in mESCs though the timing of treatment differ.

Skeletal muscle differentiation is enhanced by early exposure of embryonic stem cells to RA at the onset of embryoid body formation while early exposure to RA inhibits cardiomyogenesis and is enhanced by later treatment. During C2C12 skeletal muscle differentiation, assessment of gene expression changes is very important, and it is accomplished by Real-time quantitative PCR (qPCR). During qPCR, RNA integrity is determined by gel electrophoresis or using a bioanalyzer and purity is assessed using a spectrophotometer.

Gene expression normalization in mouse studies uses two genes: β-actin (Actb), which encodes a cytoskeletal protein which helps in cell movement and transport of organelles and chromosomes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which encodes a metabolic enzyme that aids in processes like transcription activation. GAPDH is used in this study for gene expression normalization.

Mouse reference genes used include hypoxanthine phosphoribosyl transferase (Hprt) which encodes a transferase enzyme involved in generation of purine nucleotides, ribosomal protein S12 (Rps12) which produces the ribosomal subunit 40S, TATA binding protein (Tbp) which encodes a transcription factor that binds to the TATA box and help in the three RNA polymerases initiation (Masilamani, T. J, et al 2014).

1.4 Role of Dystrophin in Myogenesis and Cell Membrane Stabilization

Dystrophin is a rod shape elongated 427 kDa sub-membrane cytoskeletal protein that links the subsarcolemmal cytoskeleton to a large macromolecular complex of proteins in the surface membrane, dystrophin-associated protein complex (DAPC)/ dystrophin glycoprotein complex (DGC) and then connects to other extracellular matrix (ECM) proteins through laminin (figure 2). It also has a major role in regulating signaling pathways especially those that activate reactive oxygen species (ROS) and nitric oxide (NO) production, and Ca^{2+} entry (David G et al, 2016).

Dystrophin is encoded by the DMD gene that maps the short arm of chromosome X at locus Xp21.2 and represents about 0.002% of the total muscle protein and it measures 2.4mb that accounts for nearly 0.07% of the total human genome. Dystrophin protein is located primarily in skeletal muscles and cardiac muscles and small amounts are also found in nerve cells (Hina F et al, 2017). The transcription of the DMD gene is controlled by three different independent promoters, the brain, muscle, and Purkinje which reflect the tissue distribution of expression of dystrophin. The muscle promoter drives high level of expression in striated skeletal and cardiac muscles (Bruno Constantin, 2013).

According to Sandra M and Kay O in 2015, in skeletal muscles dystrophin forms tight associations with a variety of sarcolemmal, cytosolic and extracellular proteins such as, dystroglycans, sarcoglycans, syntrophins, dystrobrevin, sarcospan, laminin and cortical actin which also associates with key components of the ECM and the intracellular cytoskeleton thus stabilizing muscle fibers during the physical tension excitation-contractionrelaxation cycles. Dystrophin protein has four main functional binding domains based on sequence homology; actin-binding amino terminal domain (ABD1).

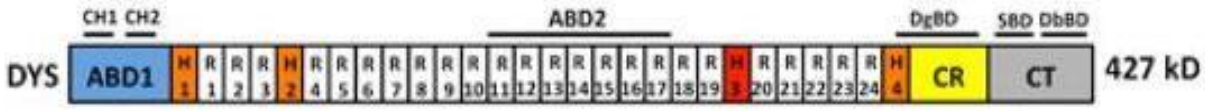

Figure 2: Dystrophin functional domains. In blue is ABD1-the N-terminal actinbinding domain with two calponin-homology motifs (CH1 and CH2), in white is the central rod domain with 24R's (spectrin-like repeats) with the second actin-binding domain (ABD2) spanning from R11-R17and in between it has 4 proline-rich hinges (H1-H4),3 in orange and 1 in red, in yellow is the Cysteine-rich domain (CR) and grey is the C-terminus (*Quan G and Elizabeth M 2015).*

According to Quan G and Elizabeth M in 2015, this domain has the calponin homology domains (CH1 and CH2) which binds directly to F actin thus linking dystrophin to the subsarcolemmal actin network. Dystrophin together with the dystrophin complex serve as cytoskeletal integrator for muscle membrane stability whereby ABD1 binds to the costamereenriched intermediate filament protein cytokeratin 19 (K19) connecting dystrophin to the contractile apparatus in skeletal muscle cells (figure 2). These conventional CH domains have actin binding properties like those present in α-actinin and β-spectrin (Hina F et al, 2017).

Adjacent to ABD1 is the central rod-domain which is composed of more than 2800 amino acids building the 24 slightly flexible but sufficiently rigid homologous triple helical spectrin repeats which provide structural integrity to muscle fibers and helps in actin binding and four hinge domains responsible for the central rod domain flexibility (Hina F et al, 2017; Bruno Constantin, 2013). The rod domain also harbors the second actin-binding amino terminus domain (ABD2) which spans in the middle of the rod from repeat 11-17 where it collaborates with ABD1 to form a lateral association with actin filaments (figure 2). This rod domain also mediates dystrophin interaction with microtubules through repeats 20-23 which is essential for the microtubule lattice organization in skeletal muscle cells (Quan G and Elizabeth M, 2015).

The third domain is the Cysteine rich domain with 2 Ca^{2+} binding motifs, WW, and ZZ binding domains (figure 2). The WW domain is followed by the 2 EF-hand motifs which binds with β-dystroglycan and is usually involved in intracellular signaling anchoring the dystrophin at the sarcolemma. and the ZZ domain (zinc finger) binds to Calmodulin and ankyrin-B which are adaptor proteins required for retaining dystrophin at the sarcolemma (Bruno Constantin, 2013) and it stabilize the interaction between dystrophin (WW domain) and dystroglycan in the dystrophin glycoprotein complex (Hina F et al, 2017).

The last domain is the Carboxyl-terminus domain which contains 2 polypeptides that fold into αhelical coiled coils which help in protein- protein interactions. This domain provides binding site for dystrobrevin and syntrophins mediating their sarcolemma localization (Karen A et al, 2004).

The Dystrophin-associated protein complex (DAPC) also called dystrophin-glycoprotein complex (DGC) connects the sarcolemma to the extracellular matrix and is the second major component of the costamere after the first major component which is integrin-vinculin-talin complex and its primary component is dystrophin. Dystrophin links the intracellular cytoskeleton network to the transmembrane components, also it interacts with F actin, microtubules, and intermediate filaments to form a link to the extracellular matrix at the costamere. Dystrophin associated proteins can be divided into sarcolemma proteins: β-dystroglycan, α-sarcoglycan, γsarcoglycan, δ-sarcoglycan, sarcospan, cytosolic proteins: dystrobrevins, syntrophins, nNOS and extracellular proteins α-dystroglycan and laminin (Sandra M and Kay O, 2015).

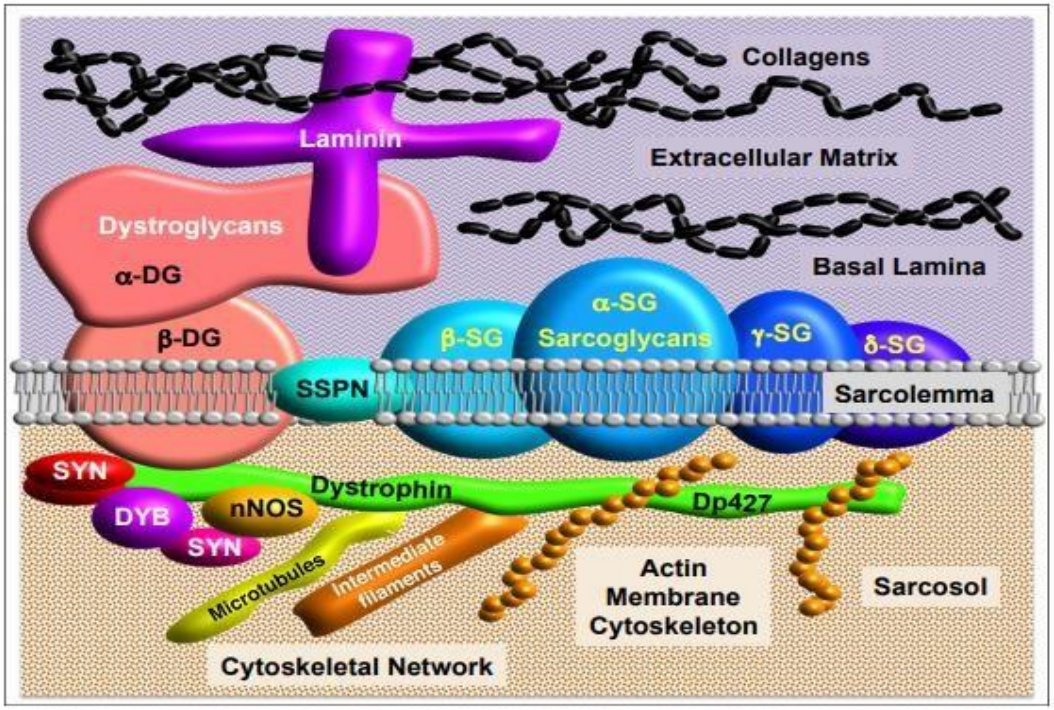

Figure 3: Dystrophin-glycoprotein complex (DGC). Shows how dystrophin links the intracellular cytoskeleton network to the transmembrane components of the DGC and to the extracellular matrix through laminin (Sandra M and Kay O, 2015).

Dystroglycan is an integral transmembrane component of the DGC, and it undergoes posttranslational proteolytic cleavage into 2 peptide subunits whereby the N-terminal produces αdystroglycan (figure 3). Dystroglycan is an extensively O-glycosylated extracellular peripheral membrane protein with 2 globular domains connected by an extensible portion (central mucin domain), thus mediating the binding of extracellular matrix components such as laminin and the C-terminal produces the β-dystroglycan (figure 3).

Dystroglycan is a single-pass transmembrane protein which lies in the cytosol and contains N-glycosylation sites that bind to the laminin G domain in the extracellular matrix components such as laminin, agrin, and perlecan thus binding dystrophin in striated muscles. Caveolin-3, scaffolding protein, binds directly to the C-terminals of β-dystroglycan and is seen to compete in dystrophin recruitment to the plasma membrane and supporting sarcolemma localization (Quan G and Elizabeth M, 2017). The C-terminal cytoplasmic domain of β-dystroglycan binds to the WW, EF and ZZ modules in dystrophin within the DGC and several signaling molecules like rapsyn, Grb2 which implicates its role in some crucial signal transduction pathways within the cell (Hina F et al, 2017).

Sarcoglycans (SGC) are transmembrane glycosylated proteins that form a tight tetrameric complex with the DGC whereby the sarcospan strengthens the connection between α and βdystroglycans thus increasing muscle membrane integrity and stability of the sarcospan (figure 3). The sarcoglycans also helps to facilitate interactions with the cytoskeleton and the extracellular matrix ensuring proper assembly and functioning within the DGC and its stability.

The family members of sarcoglycans include alpha (α-), beta (β-), gamma (γ-), delta (δ-), epsilon (ε-), and zeta (ζ-) where the first four interact to form sarcoglycan sub complex of DGC in skeletal muscles (Hina F et al, 2017) (figure 3). Sarcoglycans mediates signaling of the DPC by directly interacting with some of the core signaling proteins such as biglycan, α-dystrobrevin and sarcospan and with other proteins such as filamin 2. The entire sarcoglycan complex is lost in absence of one of the sarcoglycans but sarcolemmal dystrophin remains largely unaffected (David G et al, 2016).

Sarcospan (SSPN) is an integral transmembrane protein composed of 4-transmembrane domains with a large extracellular loop between domains 3 and 4 which forms the main binding site for the sarcoglycans. It is tightly associated with the sarcoglycan complex and serve as a chaperone in the assembly of DGC. Sarcospan can form homo oligomers by associating with one another at the membranes and thus help keep various cellular components of the DGC clustered in close complexity and partial control of several signaling pathways like those involving laminin and integrins at the cell membrane (Hina F et al, 2017). Recent emerging roles of sarcospan have been associated with improving cell surface expression of the dystrophin, utrophin glycoprotein and integrin complexes, muscle cell survival and regeneration by modulating the abundance of laminin-binding complexes (Bruno Constantin, 2013).

Dystrobrevin is a dystrophin related protein encoded by 2 distinct genes α- and β- DB. αdystrobrevin -1 and -2 are known to participate in skeletal muscles dystrophin glycoprotein complex where they link other cytoskeletal proteins, intermediate filaments proteins and some more times nitric oxide thus signaling through interactions with syntrophin to the dystrophin glycoprotein complex.

The longer tail of α-dystrobrevin-1 contains numerous serine, threonine, and tyrosine phosphorylation sites, suggesting that kinases may regulate α-dystrobrevin- mediated interactions of signaling proteins with the DPC (David G et al, 2016). Syntrophins are a heterogenous family of intracellular membrane-associated adaptor proteins which plays a key role in coordination of intracellular signaling molecules such as calmodulin, stress-activated protein kinase-3, Grb2, and nNOS (Quan G and Elizabeth M, 2017). The syntrophin family comprises of five known subtypes, α-1, β-1, β-2, γ-2, and γ-1.

The first four subtypes are expressed in skeletal muscle, whereby the first three are part of the DPC on the sarcolemma and the fourth is situated on the sarcoplasmic / endoplasmic reticulum (David G et al, 2016). α-1 facilitates the attaching of nNOS to the sarcolemma and the dystrophin complex. Syntrophins have a N-terminal PH-1 domain with a PDZ domain embedded within it, a central PH-2 domain, and a C-terminus domain unique to syntrophins (SU). SU is very crucial in interactions with dystrophin, dystrobrevin or utrophin within the DGC. The presence of PH, PDZ and SU domains allow syntrophins to interact with several other proteins and lipids leading to the building of multi-protein/lipid signaling complexes (Bruno Constantin, 2013).

Some cellular and extracellular molecules associate with the DGC complex to help it in executing its roles. Caveolin-3 is an integral membrane protein in skeletal muscles that form the structural and regulatory component of the cell sarcolemma where it helps in DGC mediated signaling. nNOS (neuronal nitric oxide synthase) is a cellular enzyme anchored at the sarcolemma by the DGC which synthesizes NO, an endogenous signaling molecule. nNOS controls blood flow through vasoconstriction.

A summary of the activities of the DGC on the interactions between the cytoskeleton/ intracellular matrix and the extracellular matrix (ECM) can be seen in figure 4. Interaction patterns between tubulins, vimentin, desmin, annexins and collagens and the DGC as part of the intracellular matrix-sarcolemma-extracellular matrix has been shown. The bioinformatics database STRING (Search Tool for the Retrieval of Interacting Genes/proteins) was used to predict the protein-protein interactions as shown in the central panel on the proteomic map.

The flow charts surrounding the proteomic map are pointing up the major changes in DP427-deficient muscle fibres, reactive myofibrosis caused by a hyperactive connective tissue, a compensatory up-regulation of microtubules and intermediate filaments, and a loss of sarcolemmal integrity due to loss of the dystrophin complex.

Dystrophin is expressed in activated muscle stem cells (satellite cells) and together with Ser/Thr kinase MARK2 (Par 1 b) they help in regulating cell polarity. Dystrophin also regulates the number of asymmetric division where it increases the number of myogenic progenitors required for proper muscle regeneration. Reduced expression of dystrophin or its absence and many of its components usually lead to muscular dystrophies, which involves muscle damage leading to muscle degeneration (Nicolas Dumont et al 2015). According to a study conducted by Natasha Chang et al 2016, satellite cells with dystrophin deficiency had reduced expression of MARK2, loss of PARD3 polarization and reduction in asymmetric stem cell divisions which resulted in progressive loss of myogenic progenitors and impaired muscle regeneration.

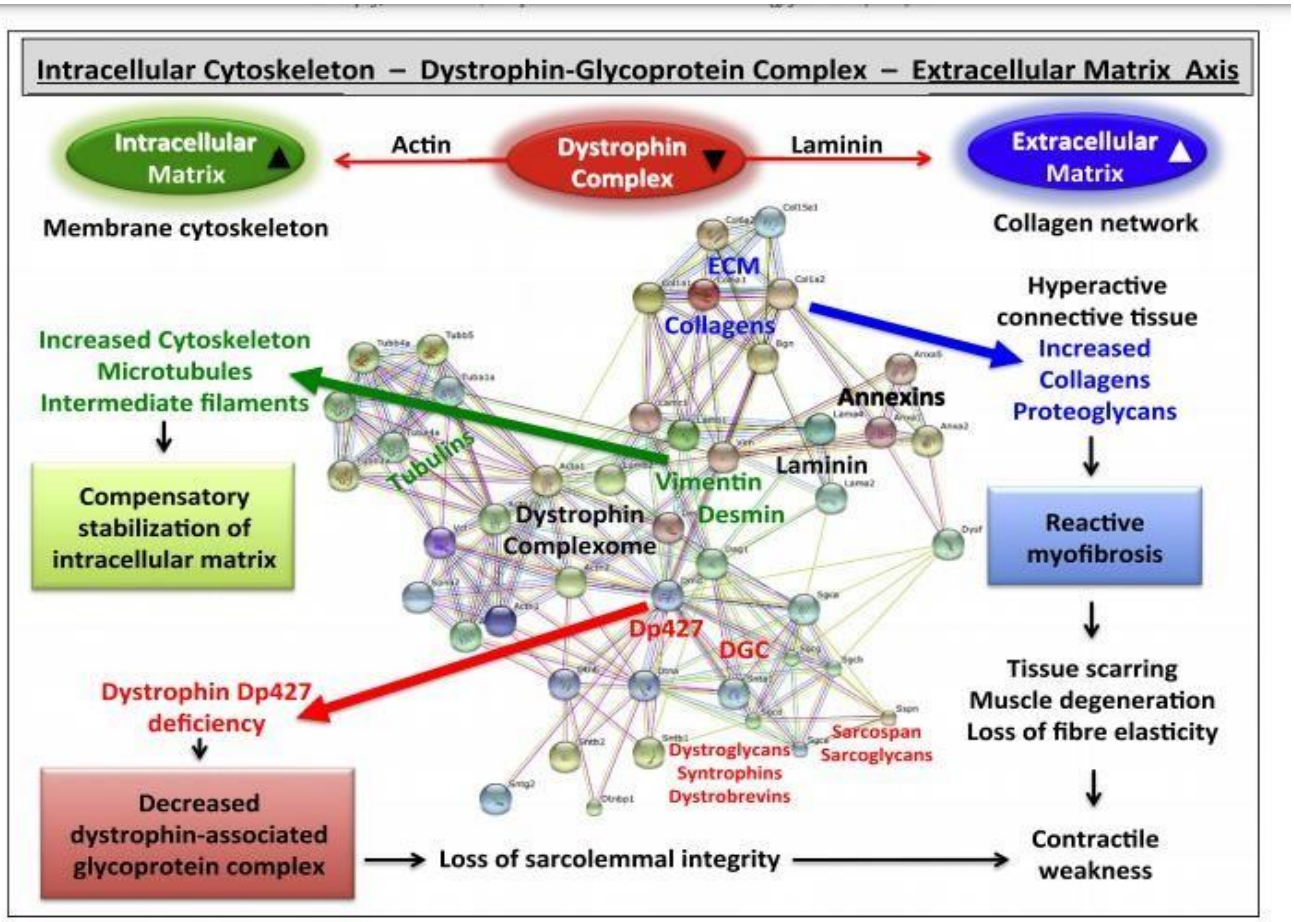

Figure 4: Bioinformatics STRING analysis of Dystrophin complexome (Sandra M and Kay

O, 2015).

1.5 Muscle Aging.

Sarcopenia has been defined by Jeremy D. Walston in 2012 as 'an age related, involuntary loss of skeletal muscle mass and strength'. Skeletal muscle is very useful in the human body as it controls voluntary movement. Aging process leads to decrease in muscle mass and strength. Reduction in number of muscle fibers, muscle fiber size and motor units result in a loss of muscle mass. According to a study conducted by Karsten K. and Martin E. in 2013, accelerated muscle mass and strength loss starts earlier in the 40^{th} life year. Aging leads to decline in the ability of the resident stem cells in individual tissues to give rise efficiently and effectively to new parenchymal cells thus leading to impairment in tissue homeostasis, repair, and regeneration (Suchitra D and Thomas A, 2008). During aging, the ECM is one of the components of the muscle stem cell niche that is mostly affected. Aging also increases the stiffness of the MuSC niche thus impairing the proliferation and fibrogenic conversion of MuSCs.

Skeletal muscle is known to be a high energy consuming tissue, this means it entirely depends on the mitochondrial organelle for its energy requirements. In older people, the mitochondria are thought to decrease in number thus restricting the amount of energy produced for the skeletal muscles to work effectively. Mitochondrial mass reduction could be possibly due to dysfunction of major regulators of mitochondrial biogenesis including AMPK and PGC-1α. To preserve a healthy mitochondrial pool, mitophagy (mitochondrial autophagy) is necessary which helps to sustain muscle performance. To curb this problem, compounds that are natural with strong anti-aging effects have been evaluated for anti-sarcopenic properties.

These compounds regulate mitochondrial health by aiming at the NAD+/SIRT1 pathway, mitochondrial stress pathways, or upregulating mitophagy (Tyler B. et al 2018). During muscle repair, some defects associated with age are more likely to slow down healing of muscle function and on the other hand they stimulate replacement of damaged myofibers with connective tissue and fat rather than newly formed muscle. An example of such a factor is pro-inflammatory microenvironments which reduces restoration potentials and impairs physiological functions. In aged muscles, the frequency of satellite cells is reduced, and myogenic activities are reduced. Nuclear factor kappa B (NF-κB) is the transcription regulator of inflammation and as one ages, its activity is upregulated.

Tumor necrosis factor-alpha (TNF-α) is a primary cytokine that affects muscle mass and function and is chronically elevated in the circulation of aged populations (Adam Philip et al 2011). It reduces myogenic differentiation through transcriptional activation of NF-κB and decreases protein stability of myoD which is a transcriptional factor that helps in regulation of myoblast transition from proliferation to differentiation. After multiple divisions of aging human myoblast, it is suggested that there is reduced Mrf5, myoD and myogenin thus reduced differentiation capacity. The p57 cyclin dependent kinase inhibitor also reduces which in turn reduces expression of both myoD and myogenin thus the cells are unable to exit the cell cycle with age, for them to differentiate, (Ying Wang et al 2018).

1.6 Proteomics.

The specific protein composition found in cells, tissues or biofluids can be identified, quantified, and modified on large scale using the concept of proteomics.

Mark Wilkins in 1994 defined the term proteome as the plasma proteins manufactured by the genome of an organism (Lindoso, R.S et al 2019). Simply stated the proteome is the entire protein population expressed in a biological compartment, a cell, a tissue, organ, etc.

Generally, proteomics is experimental analysis of proteins and proteomes but more specifically it involves protein purification and mass spectrometry. Proteomics confirms the presence of the protein and provides a direct measure of the quantity present. Identification can be done through gel electrophoresis followed by mass spectroscopy. Since this method is long running, the number of proteins and samples analyzed in a study is limited. Due to this, liquid chromatography followed by mass spectroscopy is applied which mainly involves nanoflow separation chromatography.

Due to the need to generate numerous mass spectra with a large amount of information, bioinformatics tool is used whereby algorithms such as Mascot and SEQUEST search engines are used. Label-free or isotopically labeled quantification methods are used in quantitative analysis. In Label-free the peptide precursors in the mass spectra and spectra counting are chosen to be used for bioinformatics algorithms while in isotopically labeled the peptide areas are used for quantification since the isotopes for the peptide labeling are known. Most accurate method is by use of high purity heavy labeled peptides. Posttranslational modification gives insight into the function of the proteins.

1.7 Muscle model systems

The mouse model is commonly and widely used**.** Mouse C2C12 myoblast cells are secondary cell-line based, well-established in vitro model that is used for the study of gene expression during the differentiation phase of myogenesis. An evaluation of changes in gene expression is done through Real-time quantitative PCR (qPCR) (Twinkle J. et al 2013).

Skeletal muscle myoblasts readily multiply and can easily be triggered to differentiate and fuse to form myotubes, closely resembling events seen in vivo. The gene expression profile during differentiation in C2 cells summarizes that seen in skeletal myogenesis in vivo, as expression of cytoskeletal components modifications are from non-muscle-specific to mainly muscle-specific isoforms (Berendse et al., 2003).

The study of muscular function has been mostly based on human muscle biopsies (taken for diagnostic purposes) and analyses of murine models, both in healthy and genetically modified animals that recapitulate myopathic diseases. However, muscle biopsies are progressively being replaced in clinical practice by less invasive diagnostic methods, such as diagnostic radiology and subsequent molecular profiling, which have quickly become the clinical gold standard. On the other hand, animal models do not always reflect all aspects of human pathology. For these reasons, the ability to generate a human cell-based, tissue-engineered system to study muscle physiology and pathology constitutes a major scientific goal (Patricia G. et al 2014).

1.8 Research Aims

The overall aim of this study is to help us know and understand how different genes are expressed when myoblast C2C12 cells are grown in long term cultures. This research specifically aims at expression of Dystrophin considering its various roles in skeletal myogenesis and cell membrane stabilization.

1.9 Hypothesis

Myoblasts cultured for a long time (30 days), is a good indication of muscle aging in more specifically skeletal muscles. We hypothesize that Dystrophin expression will increase as the myoblast continue to differentiate, and mature, but slightly decrease later as they age.

1.10 Basic Experimental Design.

The cells were cultured in one T-75 flask in the biosafety cabinet and later divided into 7 flasks of 25ml as summarized in the figure 4 below. The 7 flasks contained 25ml of growth medium and was incubated under 37^0 C with 5% CO_2 and the cells were viewed every day under the phase contrast microscope and the medium was changed after every 48 hrs. After the cells attained 80% confluency as shown below in the experimental design, they were harvested at different times at T=-1 Day, T=0 Days, T=4 Days, T=8 Days, T=12 Days, T=25 Days and T=30 Days. The day in the experiments shows the appropriate time that the cells were harvested.

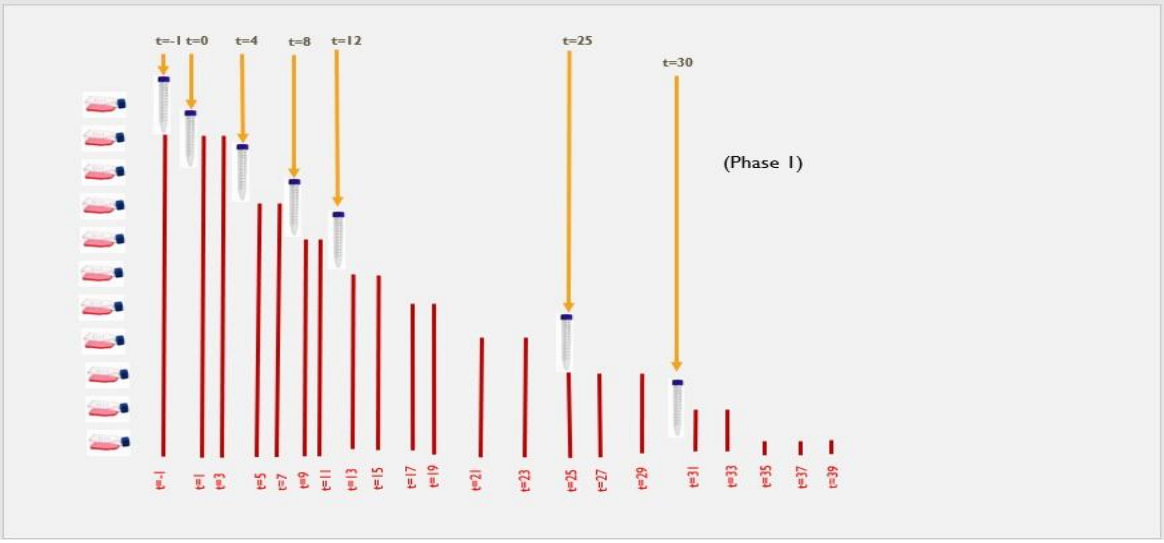

Figure 5: graphic representation of the experimental media and the specific harvesting time points.

Chapter 2 Methods

2.1 Cell Culture

2.1.1 Media preparation.

Media is prepared using DMEM (1X) (Dulbecco's Modified Eagle Medium) with a composition of 4.5g/L D-Glucose, L-Glutamine, and 110mg/L Sodium Pyruvate and (state the percent FBS) HI FBS (Heat Inactivated Fetal Bovine Serum) of Qualified Standard. Both DMEM and HI FBS are from Gibco company. FBS is thawed in a 37^{o}C water bath until it feels warm on touch on the skin. In a hood, 55Ml of FBS is added to a 500 ML bottle of DMEM. This makes 555 ML of media. Then using a Stericup (filtration sterilization), the medium is filtered using a quick release filtration system. The medium is then stored in the refrigerator, 4˚C. Media is warmed to 37˚C before using on cultures

2.1.2 Initial Cell Culture.

The C2C12 cells were purchased from the American Type Culture Collection (ATCC) Manassas, VA, and stored in the liquid nitrogen container, DMSO is in the frozen cultures. These are the cells used throughout the experiment. The cells are removed from the liquid nitrogen and quickly thawed in a 37^{0}C water bath and then transferred into a 10mL sterile conical centrifuge tube. Growth media called Dulbecco's Modified Eagle Medium (DMEM) that was supplemented with 10% Fetal Bovine Serum (FBS) and filtered is also warmed in a 37^{0}C water bath. The cells are thawed, then subjected to serial dilution with fresh growth medium, thus diluting out the DMSO and thus re-establishing normal cellular membrane properties. Into the 10mL tube with 1ml cells (thawed cells), 1ml of the growth medium is added and cells are incubated at room temperature (25^{0}C) for 10 minutes.

Next, 2ml of the growth medium is added and again allowed to stand for 10 minutes. Lastly, 4ml of the growth medium is added and allowed to stand for 10 minutes. This final volume, 8ml, is then transferred to a 75cm^2 flask with 17ml of additional C2C12 growth media (10% FBS) to make a total of 25ml. Cultures are incubated at 37^{0}C and 5% CO_2 for 24 hours. The following day cultures are viewed using an Olympus LH 50A inverted phase contrast microscope to confirm cell attachment. Every other day following initial culturing, media is removed and 25ml of 10% FBS is added. Cultures remain at 37^{0}C and 5% CO_2.

2.1.3 Passaging of cells.

After every 48 hours, the medium is changed. Cells are split when they are 80% confluent which took approximately 6 days to achieve. 10% FBS and Trypsin are warmed in a 37^{0}C water bath. Used media is discarded and the cells are washed with 20ml sterile PBS for 15 seconds which is also discarded. The cells are then treated with 15ml of trypsin and allowed to incubate at 37^{0}C and 5% CO_2 for 10 minutes. The Olympus LH 50A inverted phase contrast microscope is used to view the cells to confirm cell detachment. The 75cm^2 flask is gently tapped on the sides for 10 seconds to ensure complete cell detachment. Once cell detachment is confirmed, the trypsin-cell mixture is transferred into a fresh 15ml conical centrifuge tube. Then 2ml of the trypsin/cell mixture is transferred to 7 new 75cm^2 flasks. 23ml of fresh growth media (10% FBS) is added to the new flasks to make a total of 25ml in each new 75cm^2 flasks. Cells are incubated for 24 hours at 37^{0}C and 5% CO_2. Cell attachment is confirmed by viewing the cells under the Olympus LH 50A inverted phase contrast microscope. Used media is removed and 25ml of fresh 10% FBS media is added every other day following splitting. Cultures remain at 37^{0}C and 5% CO_2.

2.1.4 Imaging of Cell Morphology.

The Olympus LH 50A inverted phase contrast microscope is used to view cells at different time points following myogenesis process initiation. The images are taken from the lowest to the highest magnifications. The cells are captured using the SPOT imaging software and microscope camera at 10x and 1.0x objectives and are saved on the computer to be used for comparison.

2.1.5 Harvesting of cells.

When cells reach 80% confluency they are split as previously done. Once cells detachment is confirmed cells are transferred into 7 new $75cm^2$ flasks. Each flask is for each time point (t=-1 ,0,2,4,8,12,25,30 days). T= minus 1 is defined when the cultures are about ≥ 50% confluent. 25mL of C2C12 media (10% FBS) is added to each flask. All flasks are incubated at 37^0C & 5% CO_2 for 24hrs. Cells are viewed using the Olympus LH 50A inverted phase contrast microscope to confirm cell attachment. 25ml of media,10% FBS, is changed every other day. Cells are imaged and harvested at their respective time points.

2.2 RNA Isolation and Quantitation.

2.2.1 Pelleting of Cells.

Cell pelleting is done at respective time points. Old media is discarded, and the cells are washed with 20ml sterile PBS for 10 seconds. PBS is discarded and the cells are treated with 15ml trypsin and allowed to incubate at 37^0C and 5% CO_2 for 10 minutes. After that, cells are viewed using the Olympus LH 50A inverted phase contrast microscope to confirm cell detachment. If cells are not completely detached, the flask is gently tapped at the sides to ensure complete detachment.

The trypsin-cell mixture is transferred to 15ml conical tubes once cell detachment is confirmed. The cells are then centrifuged at the highest speed for 10 minutes. A compact pellet is achieved, the supernatant is discarded, and the pellet is stored in -80^{0}C freezer, if RNA isolation is not occurring immediately.

2.2.2 RNA Isolation

RNA isolation is done for all the time points, t=-1, t=0, t=4, t=8, t=12, t=25 and t=30. Isolation of RNA was done on a RNase-free bench. Isolation followed the manufacturer's protocol provided by QIAGEN.

2.2.3 RNA Isolation Protocol

RNA extractions are performed for all the 7 time points. Cell pellets are removed from 80^{0}C freezer and thawed in ice. Once thawed, the cell pellets are resuspended in 700 µL of Lysis RLT buffer. The lysate is then vortexed and transferred to a QIAshredder spin column (QIAGEN) in a 2ml collection tube. The column is then centrifuged at full speed for 2 minutes. 700 µL of 70% ethanol is added to the tube via pipetting. 700µL of the mixture is transferred to a RNeasy spin column in a 2mL collection tube (RNA binding matrix). The mixture is centrifuged at ≥ 8,000g for 30 seconds and the flow through is discarded. 700µL of RW1 buffer is added to the spin column and it is centrifuged again at ≥8,000g for 30 seconds. The flow through is discarded and 500µL of RPE buffer is added and the tube centrifuged again at ≥8,000g for 30 seconds. Flow through is discarded and the previous step is repeated. The spin column is then transferred to a new 2mL collection tube. The new tube and spin column are centrifuged at full speed for one minute to remove any residual fluid.

The flow through and 2mL collection tube are discarded and the spin column is placed in a new 1.5mL centrifuge tube. 50µL of RNase free water is added to the column and centrifuged at ≥8,000g for 1 minute, the released RNA is collected. The spin column is discarded, and the eluted RNA is capped and frozen at -80˚C.

2.2.4 Spectrophotometric Quantitation of RNA

RNA concentration and quality are read using the Nanodrop 2000 spectrophotometer and its corresponding computer program. The program is opened and set to the Nucleic Acid Tab. The machine is blanked with 2µL of RNase-free H_2O prior to RNA concentration readings. Once blanking is completed 2µL of each RNA sample is analyzed, and the readings are recorded in an excel spreadsheet.

2.2.5 Primer Sequences

A free online primer 3 tool was used to design and analyze the primers to be used. The primers were specifically designed to target dystrophin gene against GADPH gene. GADPH is commonly used in comparisons of gene expression data. The table below shows the primers.

Sequence Description	Sequence
Dystrophin 3 F	GGCAAGCTTACTCCTCCGCT
Dystrophin 3 R	TGGGGTGGTCCCAACAAGTG
Gapdh1F	AAGAGGGATGCTGCCCTTACC
Gapdh1R	CGGCCAAATCCGTTCACACC

Table 1: Forward and reverse primer sequences of the gene of interest.

2.3 Analysis of gene expression in myoblast

2.3.1 Quantitative Analysis Of gene expression (qPCR)

RT-qPCR is utilized in conjunction with RT-PCR to get a quantifiable analysis of gene expression. On ice the reaction is setup for all reactions by adding all required components except RNA to the reaction mix. Each reaction requires a 20µL volume; 10µL of iTaq universal SYBR® Green reaction mix (2x), 0.25µL of iScript reverse transcriptase and 2µL of each forward and reverse primer (if at 10nM). Mix reaction thoroughly to ensure homogeneity and dispense into wells PCR plate. In each well add 2µL RNA (if at 100ng/µL concentration) and 3.75µL of nuclease-free H_2O. Place plate onto CFX connect^{TM} systems real-time PCR instrument, a Bio-Rad CXF-96, and start the RT-qPCR run. Protocol for RT-qPCR run is reverse transcription 21 reaction, 10 min at 50˚C, polymerase activation and DNA denaturation, 1 min at 95˚C, Amplification, denaturation, 10 sec at 95˚C, Annealing/Extension and plate read 30 sec at 60˚C, for 40 cycles and melt-curve analysis 2-5 sec/step (or use instrument default setting) at 65-95˚C. Once completed, data analysis is performed.

Chapter 3: Results.

3.1 Cell Morphology

C2C12 cells can divide indefinitely to form normal part of the development of a multicellular organism. These cells proliferate to form myoblasts which differentiate to form myocytes which in turn fuse to form myotubes which finally mature to myofibers. These cells eventually lead to formation of skeletal muscles. In this study, cell morphology was studied by growing the C2C12 cells to confluency in 10% FBS for the 30-day time course study. The Cytation^{TM} 5 cell imaging multi-mode reader was used to take images at time points t=-1, t=0, t=4, t=8, t=12, t=25 and t=30. The images obtained exhibited normal growth throughout the time course study. The cells changed their phenotype in the process of differentiation. During proliferation, initial stage of myogenesis, at time points t=-1 and t=0, the myoblast cells appear round shaped, mononucleated and they start fusing and thus appear star-shaped and attain 100% confluency at t=0 where they start to differentiate. At time point t=4, the cells are still in the differentiating phase, and they form myocytes. At t=8, the myocytes are fully differentiated forming elongated tubular shaped cells, earlier myotubes, due to fusion of cells. At time point t=12, matured differentiated myotubes with multiple nuclei resulting from cellular fusion are seen. At time points t=25 and t=30, the cells are in the aging phase and need for regeneration thus more myoblast form in the myotubes.

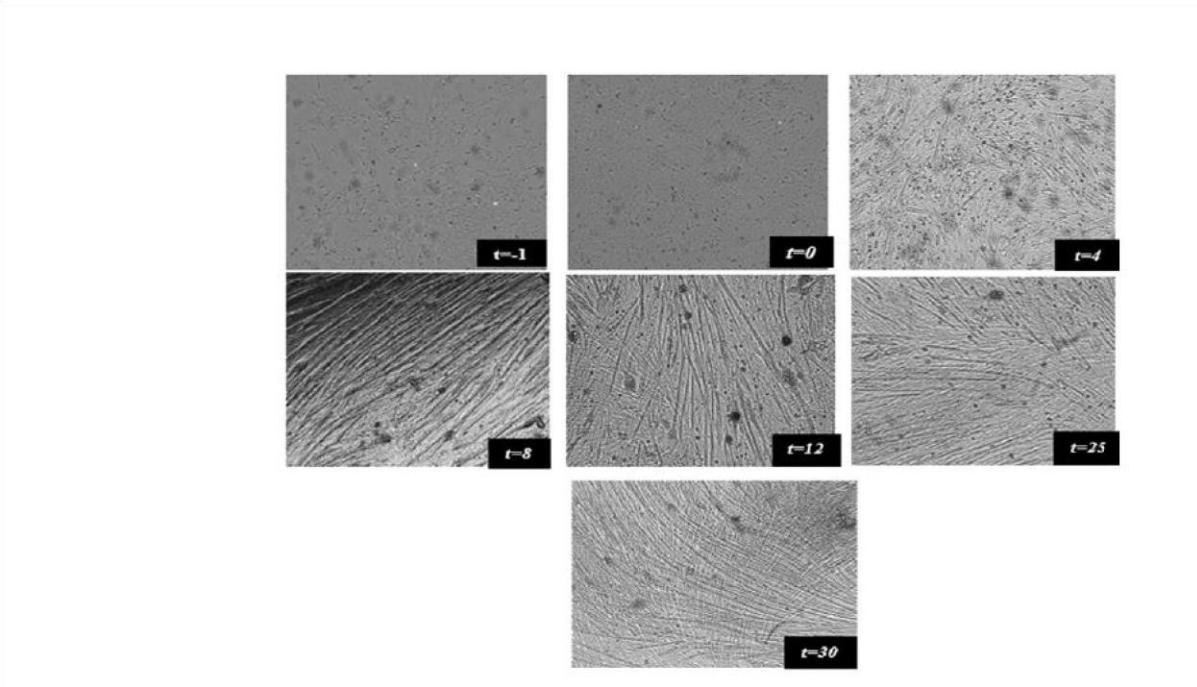

Figure 6: Cell Morphology at the different time points.

3.2 RNA Quantification

At each time point, t=-1 to t=30, C2C12 cells pellets were harvested. RNA isolation was done using the Qiagen RNeasy kit and nanodrop spectrophotometry was used to quantify the RNA. RNA quantification was performed to determine the average concentration in the samples prior to gene expression analysis. To calculate the accurate amount of RNA in a sample, sample purity was highly considered. Very pure RNA is expected to have an A260/A280 ratio of ~2.1 and values higher than 1.8 have acceptable purity. The constant used to calculate concentration of RNA is 40. The concentration of nucleic acids is directly calculated from their measured absorbance values at 260nm. The common source of contamination in the nucleic acid isolation was column extraction, specifically residual guanidine.

Based on RNA purity, all the samples in the two trials had the acceptable purity, that is, more than 1.8 and some of the samples had values ~2.1 which indicates pure RNA was obtained. For trial 1, at time point t=8, the highest amount of 271.871 ng/ uL of RNA was obtained with a 2.099 A260/A280 ratio. Time points, t=0, t=25 and t=4 showed medium amount of RNA compared to the other time points and their A260/A280 ratio was above 2.0. Time points t=-1, t=12, and t=30 had the lowest RNA amount but still had a good A260/A280 ratio of above 2.0. Generally, trial 2 samples had low concentrations of RNA compared to trail 1. The highest amount of RNA obtained in trial 2 was at time point t=4 which was 112.792 ng/ uL. All the samples had a A260/A280 ratio above 2.0. Time points t=30, and t=8 had the lowest amounts of RNA but gave the highest A260/A280 ratio of 3.003 and 2.3 respectively.

3.2.1 Trial 1data

Sample Name	**Nucleic Acid(ng/uL)**	**A260/A280**	**A260/A230**	**A260**	**A280**
t=-1	61.47	2.084	0.296	1.537	0.737
t=0	94.917	2.063	0.931	2.373	1.15
t=4	175.24	2.086	0.466	4.381	2.1
t=8	271.871	2.099	1.483	6.797	3.238
t=12	26.949	2.127	0.061	0.674	0.317
t=25	103.476	2.08	0.611	2.587	1.244
t=30	24.525	2.052	0.075	0.613	0.299

Table 2: Amount of RNA isolated in ng/uL and concentration of the RNA for trial 1.

3.2.2 Trial 2 data

Sample Name	Nucleic Acid(ng/uL)	A260/A280	A260/A230	A260	A280
t=-1	101.549	2.08	0.903	2.539	1.221
t=0	50.657	2.072	0.285	1.266	0.611
t=4	112.792	2.083	0.693	2.82	1.354
t=8	11.438	2.3	0.025	0.286	0.124
t=12	65.927	2.086	0.356	1.648	0.79
t=25	57.679	2.072	0.287	1.442	0.696
t=30	21.762	3.003	0.023	0.544	0.181

Table 3: Amount of RNA isolated in ng/uL and concentration of the RNA for trial 2.

3.3 Primer Designing

Primers to be used for the PCR analysis of the main gene, dystrophin gene, were selected and then designed using a program called Primer 3 tool. These primers were then procured from IDT (Integrated DNA Technologies). Dystrophin gene was normalized by GAPDH, whose primers were too designed and obtained. GAPDH is frequently considered as a constitutive housekeeping gene used to normalize changes in specific gene expression. It is assumed that in the use of housekeeping genes, their expression remains constant in the cells or tissues under investigation though there are some exceptions. GADPH is used as a control RNA in RT-PCR analysis and of late real time RT-PCR (Barber, R.D., et al 2005).

PCR (RT-PCR) is rated as the gold standard for quantification of mRNA and thus is commonly used for gene expression evaluation in biological research and other fields of research. This analysis involves very critical steps which must be carefully undertaken to realize reliable and reproducible data whereby the key step here is data normalization. In this study, GADPH was used to normalize the quantitative gene expression of dystrophin.

3.4 Analysis of Gene Expression

3.4.1 Dystrophin Expression

In activated muscle stem cells (satellite cells) dystrophin is expressed as is it is required for cell polarity regulation and asymmetric cell division. As the cells enter the cell cycle and proliferate, they generate myogenic progenitors, myoblasts, that differentiate to form myocytes which fuse together to form myofibers. Dystrophin gene expression increases at differentiation stage and plays a vital role in sarcolemmal integrity where it links the extracellular matrix to the subsarcolemmal actin cytoskeleton. (Figures 7 & 8)

In trial 1 timepoints t=-1 and t=0, dystrophin is expressed in low levels because it is not required in myoblasts. For time points t=4, t=8, and t=12 dystrophin is upregulated in the differentiated myotubes where it functions to connect the sarcolemma to the extracellular matrix and thus stabilizing the plasma membrane of striated muscle cells (Figures 7). The last two-time points t=25 and t=30, the cells are aging, and dystrophin expression levels slightly decrease because it is required to maintain the stability of the cell membrane (Figure 7).

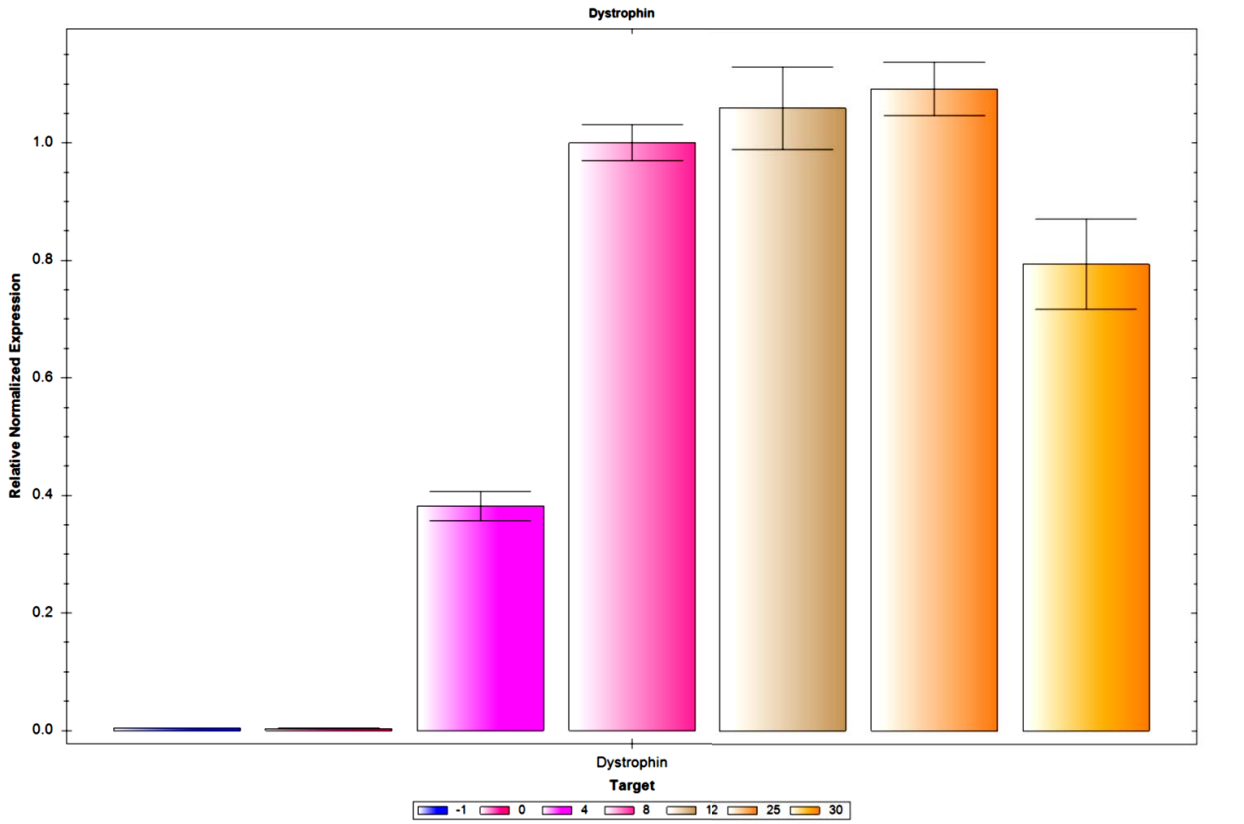

Figure 7: Dystrophin Normalized Expression levels for trial 1

In trial 2, the expression levels are almost the same with trial 1 except for some small deviations at time points t=4, t=25, and t=30 as shown in the figure below (Figure 8).

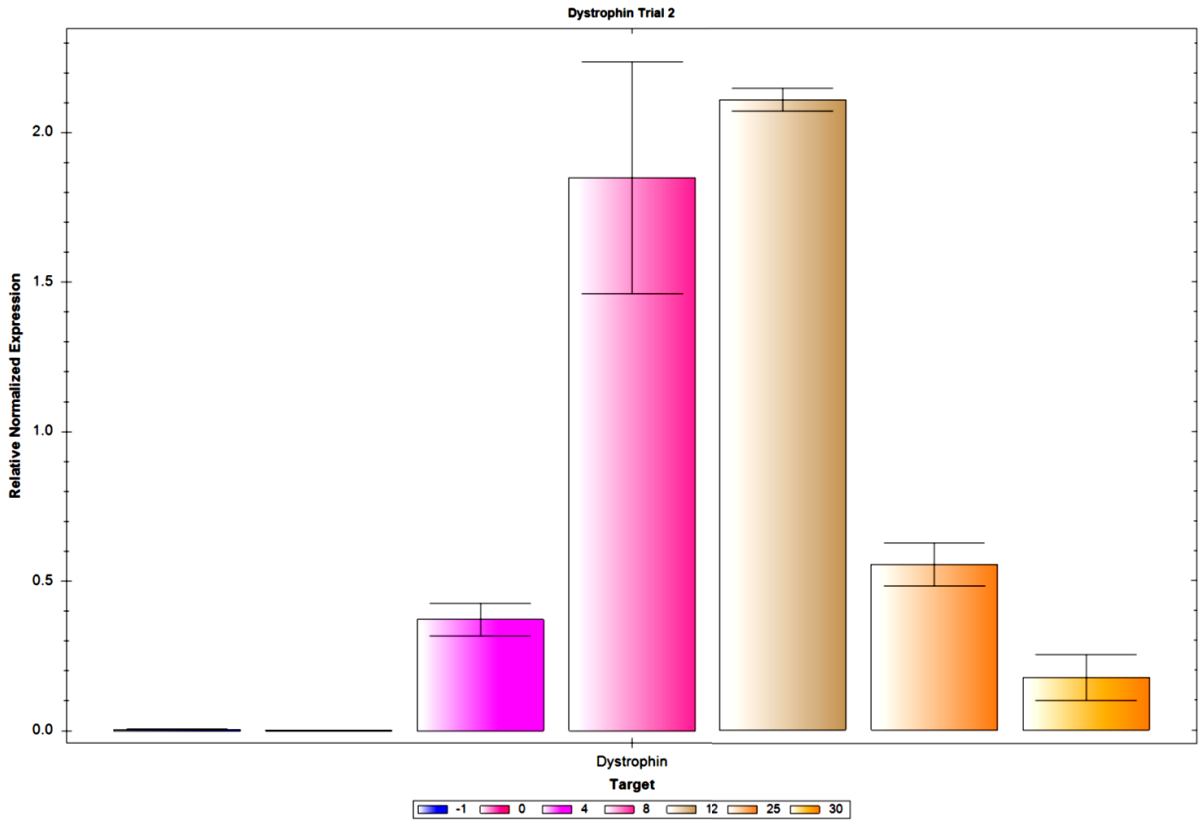

Figure 8: Dystrophin Normalized Expression levels for trial 2

When the mean of trial 1 and trial 2 was calculated (figure 10), it displayed the expected expression profile of low expression levels after cell activation, high expression at the differentiation stage and slight decrease in later aging stage. These expression levels are statistically significant since high expression levels during satellite cell activation helps in asymmetrical cell division thus dystrophin expression level at time points t=-1 and t=0, after cell activation, is low, the cells are already dividing, thus no need for high expression levels. At time points t=4, dystrophin levels start to increase, t=8 and t=12, highest dystrophin expression level, and this is because myotubes and myofibers have formed and dystrophin plays an important role in them. A slight decrease in dystrophin levels during aging is important since it is only required for maintenance.

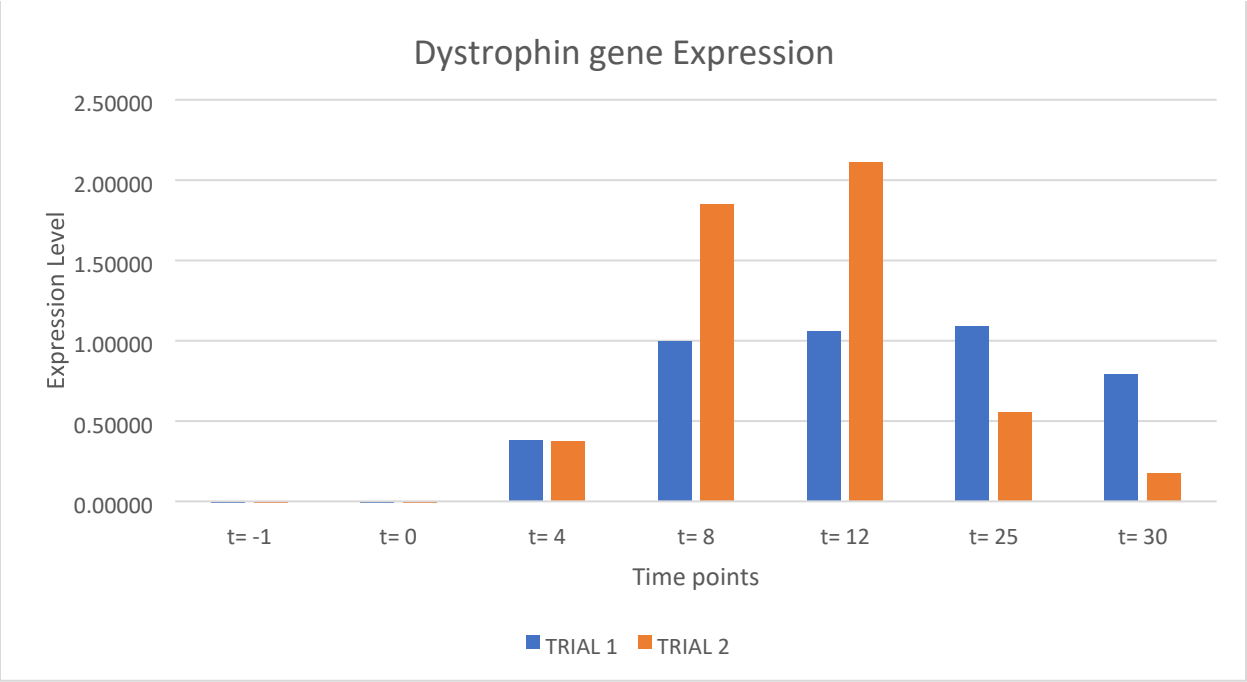

Figure 9: Summary of Expression of dystrophin gene for the two trials

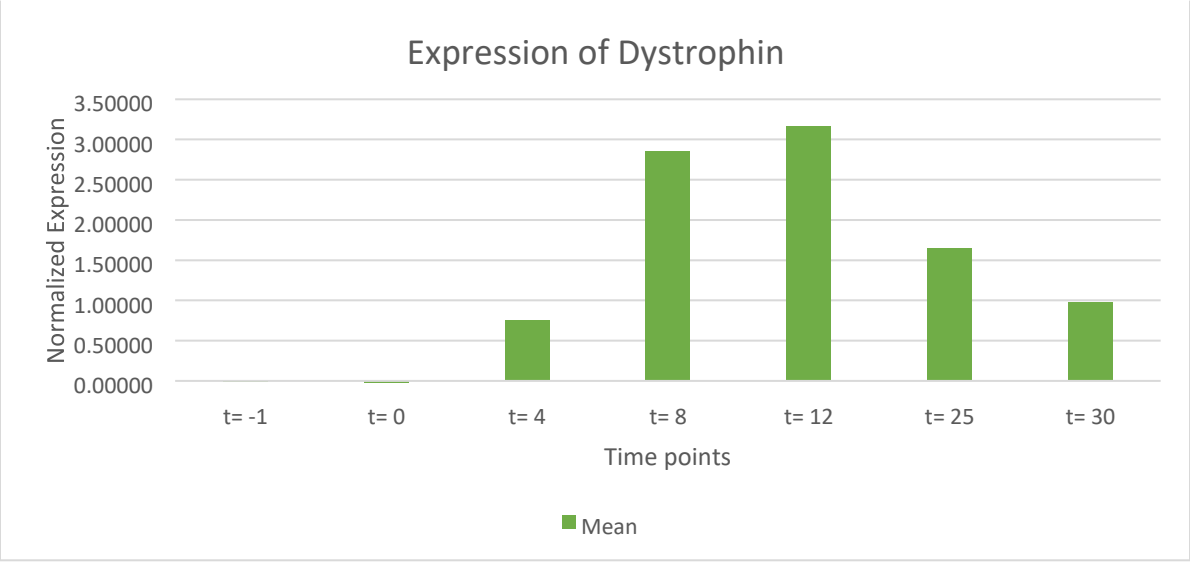

Figure 10: Graph showing the mean for the results of the two trials of dystrophin

In conclusion, high expression levels of dystrophin gene during activation of satellite cells helps to regulate the cell polarity and increase the number of asymmetrical divisions. After the activated satellite cells proliferate to form myogenic progenitors, dystrophin levels go down. After the onset of differentiation, dystrophin levels start to increase and are at the highest level when the myoblasts are fully matured forming myotubes then myofibers. Then during the last two time points, dystrophin levels start dropping to a maintenance level a likely indicator of muscle aging.

CHAPTER 4

4.0 DISCUSSION

4.1 Cell Morphology

C2C12 cells are capable of proliferating and differentiating in vitro, which imitates early embryonic development and muscle regeneration. Also, C2C12 cells grown for long-term represent the older skeletal muscle cells. During myogenesis, myoblasts undergo extensive cell shape change from round to bipolar and afterwards fuse into myotubes. Microtubules are radially distributed in myoblasts and longitudinally distributed in myotubes, which makes them key determinants of the overall shape of the cell (Costa, M. L. (2014). They also help in the localization of the intracellular organelles such as the Golgi apparatus. During pre-differentiation, at time point t=0, cultured myoblasts have reached confluency, they appear star shaped or fusiform showing one central nucleus. Studies conducted by Burattini S, et al, 2004 show that, myoblasts start to express skeletal myosin and sarcomeric and filamentous actin, mainly localized at the cell periphery and focal contacts. Myogenic differentiation is recognizable by a progressive change in cell shape. During differentiation, earlier myotubes, characteristics of muscle tissue, are observed at time point t=8, after the aligning together and fusing of the myoblasts. These myotubes appear elongated and multinucleated. At this time point, structures involved in contraction progressively assemble starting with sarcomeric α-actinin organization (Ojima K, et al, 1999). Fluorescence staining and immunostaining for myosin and alpha-actinin verified assembly of muscle components and continuous fibers as early as the sixth day under differentiation conditions (Rhim C et al, 2007).

This assembly of structures is evident by the increase in dystrophin gene expression levels which forms associations with the other glycoproteins to form an organizing node that provides sarcolemmal membrane integrity where it connects the intracellular actin cytoskeleton with the extracellular matrix protein, laminin, to stabilize the sarcolemma and protect the myofiber from disruption during the physical tensions of continuous excitation-contraction- relaxation cycles. This node also establishes a molecular scaffold and anchoring system for ion channels and enzymes to mediate cellular signaling processes. High dystrophin expression levels also help to organize actin filament attachment to its associated cytoskeletal networks and mediation of lateral force transmission from sarcomeric contraction to the endomysium and its connected layers of the extracellular matrix. At this stage dystrophin also is involved in the organization and stabilization of the costameric microtubules (Dowling P et al, 2021). Dystrophin is basically expressed in all stages of myogenic differentiation. During this process of myoblast fusion to form myotubes, Cav3 is expressed which ensures normal myogenesis and t-tubular structural development (helps in conduction of action potentials within muscle fibers) (Allen G, et al, 2016). Differentiation is accompanied by appearance of M-cadherin, a transmembrane adhesion molecule used to mediate cell-cell interactions playing a role in myoblast fusion and actin and myosin become more concentrated.

Differentiating myoblasts in culture usually express inducible nitric oxide synthase prior to the formation of multinucleated myotubes, which facilitates fusion and terminal differentiation, and from our data during this period dystrophin expression is high to help in the signaling pathways that activate nitric oxide production (Dahlman J, 2009).

As the myotubes mature to form myofibers time points t=12, a striated banding pattern is displayed across the lateral axis of the myofiber as reported by Berendse M, et al, 2003. During this time, calcium ions are critical for development and since dystrophin gene is high at t=12, it helps to signal the pathways for calcium ions entry. Myosin heavy chains are expressed during terminal differentiation where they self-associate to form filaments and to help in the conversion of the chemical energy into mechanical force to drive movement. At t=25 and t=30 time points, the sarcomere is fully formed and functioning, and the muscle cells start to age. Dystrophin gene expression level is decreasing during these time points to a maintenance level, maintain stability of the cell membrane.

4.2 Myogenesis

During earlier embryonic development, Cyclin Kinase 1(cdk1) is a major regulator at the G2-phase transition in proliferating cells. Cdk1 gene is expressed relatively high before confluency and then decreases dramatically after confluency indicating that the cells have left the cell cycle and is then expressed lowly throughout myogenesis (Wagner M,2020). According to our data obtained, once myoblasts are formed, dystrophin levels go low to allow for differentiation to kick off. This is supported by data obtained by Stephanie J et al in 2017, which showed that dystrophin expression levels are low in myoblasts compared to myotubes. This is also supported by studies done by Meng J, et al, 2020 which indicated that dystrophin is switched off when satellite cells become myoblasts and switched on when they differentiate into myofibers. Laminin-1 expression promotes proliferation and migration of myoblast cells (Zhang W, 2021).

Expression of MyoD, the major myogenic determinant factor, increases after confluency, is highly expressed after post confluency and then expression remains significant throughout myogenesis (Nyaboke R, 2016).

This relates to our results of dystrophin expression which increases after differentiation is initiated and continue to be highly expressed throughout the process of myogenesis. Studies done on titin indicate that, it is lowly expressed until past confluency when it starts to increase as the cells leave the cell cycle and induce myogenesis to begin. Titin remains highly expressed as it is important in earlier scaffolding necessary for assembly of actin filaments and order within the sarcomere. Studies done by Patricia Chemutai 2021 also shows an increase in the level of expression of AChR gene at time points t=4, t=8, and t=12 which clearly supports the relationship between dystrophin and Acetylcholine at the NMJ.

Transcriptional factors known as myogenic regulatory factors, MRFs, including MYOD, MYF5, MYF4, and MYOG are responsible for controlling muscle-specific gene expression during myogenesis. These MRFs are expressed both in proliferating mononucleated myoblasts and in cells after differentiation. Also, the MyHC isoforms are expressed in these cells. During differentiation, the cell's myofibrils form mature sarcomeric cross-striated pattern, an indication of titin protein, and all the sarcomeric structures are formed. Desmin expression is also observed in both proliferating and differentiated cells where it maintains the integrity of the myofibrils in myoblast. Myogenin is more expressed in differentiated cells that in proliferating cells. (AbdulHussein, et al, 2012).

Transcription factor Pax7 is expressed in satellite cells that are committed to a myogenic lineage. MyoD, Myf5 and Myf4 are expressed in quiescent muscle stem cells that have entered the cell cycle, where they activate them and stimulate the genes required for muscle stem cell proliferation. These cells are more proliferative during the neonatal period to support the rapid gain in muscle mass (Xin Fu et al 2015).

In activated muscle stem cells, dystrophin is localized, and it interacts with Mark2, which is a polarity protein, and Pard3 polarizing them to opposite sides of the satellite cells. This interaction occurs at the muscle fiber membrane and is mediated by the 8^{th} and 9^{th} spectrin-like repeats of dystrophin. The polarization promotes asymmetric cell divisions leading to generation of satellite cells that lack Myf5 and committed muscle cell precursors (myoblasts). This agrees with our data as at time point t=4, dystrophin start to be expressed again. This suggests that dystrophin is not required when cells are at the myoblast stage at this allows other myogenic proteins to take over their respective functions. At this point, MyoD is expressed to help the activated myoblasts to start differentiating.

At the start of differentiation, t=4, dystrophin levels start to increase, and highest expression levels of dystrophin gene is observed at time points t=8 and t=12. This is also true when the average of the two trials is calculated and graphed. At this phase, dystrophin is very important in sarcolemma integrity. It helps to stabilize the plasma membrane of striated muscle cells during muscle contraction by attaching the actin cytoskeleton to the extracellular matrix through the DGC. Then myogenin will initiate terminal differentiation, where the mononucleated myoblasts fuse to myotubes.

4.3 Muscle aging

Cell morphology comparisons done by Grabowska I et al, 2011 showed no significant differences between satellite cells and C2C12 cells. Both cells' myoblasts were observed to initially adhere to the surface and proliferate and the later align and fuse to form long multinucleated myotubes. Both the myoblasts from the two cells formed typical actin stress fibers and well-organized cytoskeleton. This clearly indicates that C2C12 cells can be used in muscle repair during muscle aging.

Aging leads to a distinct loss in muscle mass and strength, resulting in the depletion of the stem cell population and its proliferative activity. Muscle mass loss is caused by reduced number of muscle fibers and motor units and decline in fiber size. C2C12 cells which are two weeks and older are a good representative of older muscle tissues since from most studies the skeletal muscle specific proteins are lowly expressed during aging phase. Muscle repair is crucial during aging as it is essential for muscle maintenance. It involves formation of new myofibers from activation of muscle specific precursor cells, satellite cells, which are localized between sarcolemma and basal lamina surrounding a single muscle fiber. After severe muscle injury, myofiber necrosis and inflammatory responses leads to activation of the satellite cells, which proliferate and eventually fuse to the damaged myofibers or fuse together to form new myofibers. This is also observed in C2C12 cells which consists of a pure population of myogenic cells that proliferate and can be induced to undergo differentiation in culture, from muscle precursors to myoblasts, then myocytes, and finally form extensive contracting myotubes expressing characteristics muscle proteins.

According to Masakazu Y et al 2018, Myf5 is upregulated in activated muscle stem cells, satellite cells, as an early response to muscle injury. Myf5 regulates muscle differentiation and satellite cells without it show mild differentiation and growth defects and remain stably committed to the myogenic fate. Therefore, the use of muscle stem cells, satellite cells, as sources for tissue repair and renewal shows their potential in tissue regeneration and contribution towards homeostatic maintenance of muscle fibers (Muñoz-Cánoves et al, 2020). Similarly, C2C12 cells can be used to ameliorate the effects of degenerative muscle diseases.

4.4 Future work

Dystrophin has been shown to start functioning early in myogenesis and its association with the various proteins plays a major role during myogenesis. Future work on C2C12 cells should focus on embryonic roles of dystrophin at the different time points to understand embryonic development better.

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