The Role of N2A and N2B Titin Isoforms in Muscle Development

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Abstract

The process of myogenesis is essential in life since it is initiated immediately after conception and continues throughout life time. It enables the formation of muscle tissues during embryonic development, and there are three types of muscle tissues that are formed during this process. The three types of muscles are skeletal, cardiac and smooth muscles. Skeletal muscle is the most abundant and is important in the movement of the body, and is formed by the muscle precursor's cells called the myoblasts. Muscle cells consist of sarcomeres that consist of giant protein called titin that are embedded in the sarcomere. Titin is encoded by a single gene *TTN* that has 363 exons that undergoes alternative splicing to produce the two isoforms; N2A which is located in skeletal muscle and N2B isoform is located in the cardiac muscle. The process of muscle cell development is regulated by growth factors that results to a maturation of a myofiber. *In vivo* culturing of muscle cells, the cells are fed with medium that is supplemented with growth factors and is controlled during different stages of myogenesis. We conducted this experiment in different time points using the C2C12 cell line from a mouse myoblast, with our main focus on how these two genes, N2A and N2B are expressed throughout the process of myogenesis. The cells were fed with high growth serum during the first phase of myogenesis and during differentiation, the medium was supplemented with low growth serum. RNA was collected in 6 different time points. Analysis of gene study was made using RT-qPCR, and N2A and N2B genes were normalized against actin gene to bring out which gene is expressed higher than the other. The genes were expressed highly in different time points and this did not agree with our hypothesis and we therefore concluded that low serum medium does not trigger myogenesis.

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Table of Contents

Title page		i
Signature Page		ii
Abstract		iii
Table of Contents		iv
List of Figures		v
List of Tables		vi

Chapters

Chapter		Page
1	**Introduction**	**1**
1.1	Myogenesis	1
1.2	Process of Satellite cells/Stem cell to Muscle Cells	3
1.3	Transcription factors committed to muscle lineages	5
1.4	MicroRNA (miRNA) in muscle development	6
1.5.1	Titin Protein	8
1.5.2	Titin at the Z-Disk	10
1.5.3	Titin Functions	11
1.5.4	Titin Isoforms	12
1.6	Specific Aims	12
1.7	Hypothesis	13
2	**Methods**	**14**
2.1	Mammalian C2C12 Cell Culture	14
2.2	Cell Expansion	14
2.3	Experimental Design	15
2.4	Cell Morphology	17
2.5	Cell Harvesting	17
2.6.1	RNA Isolation	17
2.6.2	RNA Isolation protocol	17
2.7	Nano Drop reading	18
2.8	Primers Sequences	18
2.9.1	RT-qPCR	19
2.9.2	RT-qPCR Protocol	20
3	**Results**	**21**
3.1	Myogenesis Process	21

3.2	Cell Morphology	21
3.3	RNA Quantitation	24
3.4	DNA Gel	26
3.5	Gene Expression	27
4	**Discussion**	**32**
4.1	C2C12 Cell Culture	33
4.2	Changes in Cell Morphology	34
4.3	Cells Harvesting and RNA Isolation	35
4.4	Gene Expression and Analysis	35

Chapters

Chapter		Page
5	References	44
6	Appendices	48-65

List of Figures

Figures		Page
	Introduction	
Figure 1	MicroRNAs (miRNA) in muscle development	6
Figure 2	Titin Protein	9
Figure 3	Experimental Design	16
	Results	
Figure 4	Cell Morphology	22
Figure 5	Cell Morphology	23
Figure 6	DNA Gel	26
Figure 7	Gene Expression	28
Figure 8	Gene Expression	29
Figure 9	Gene Expression	30
Figure 10	Gene Expression	31
Figure 11	Actin Isoforms	36

List of Tables

Table Number		Page
	Methods	
Table 1	Primer Sequences	18
Table 2	RT-qPCR Protocol	20
	Results	
Table 3	RNA Quantitation 1	24
Table 4	RNA Quantitation 2	25
Table 5	RNA Quantitation 3	26

Chapter 1: Introduction

1.1 Myogenesis

The process of myogenesis involves the formation of muscle tissue during embryonic development, which can be studied in several different stages (Bentzinger et al., 2012).There are three types of muscle tissues in a vertebrate body namely: smooth muscle, cardiac muscle and skeletal muscle. Skeletal muscles are formed by the fusion of many muscle precursor cells called the myoblasts, which form multinucleated cells of myotubes and they finally mature into myofibers (Berendse et al., 2003).

All muscles have different features such as anatomical structures, contractile and metabolic properties, fiber composition, blood supply, pattern of innervations and embryonic origin. The process of myogenesis and events such as speciation of the progenitor cells, proliferation and migration cell- cycle exit, differentiation and fusion are common to all the vertebrates (Rossi and Messina, 2014).

There are additional features that differentiate muscles types. For example, skeletal and cardiac muscles have striations, while smooth muscles lack them. Other than striations in skeletal and cardiac muscles, they are also characterized by physiological requirements through assembly and disassembly of the sarcomeres which are organized in parallel arrangement of thick filaments of myosin that slide against the thin filament (Zollner et al., 2012).

Skeletal muscle is the most abundant of the three muscles in vertebrates and has functions such as locomotion, breathing and energy metabolism (Rossi and Messina, 2014) and it constitutes about 40 % of a mature human being (Chen and Goldhamer,2003). Satellite cells are

quiescent muscle precursor cells in adult muscle (Morgan and Partridge, 2003), it has been proven that the number of satellite cells reduces when an individual becomes older and other factors such as accumulation of fats may explain the reason as to why older people have problems to muscle degeneration when severely injured and it takes long to get healed (Chen and Goldhamer, 2003). Muscle cells also called the myocytes or the myofibrils consist of repeating chains of contractile proteins called sarcomeres (Myhre et al., 2014).

Sarcomeres in striated muscles are seen as light and dark bands separated by a dense material called a Z line, when observed under light microscope. The dark band is also known as A-band, which has H-zone that is composed of the thick filament or the myosin proteins located in the middle of the sarcomere where thin and thick filament overlap. The light bands is also called the I-band and are made of thin filaments or the actin proteins that are located at the end of the sarcomere which are also attached to the Z-line. There is a third giant protein in the sarcomere, called titin which extends from the Z-line where it binds the myosin system and has it has been speculated to interact with the thick filament (Myhre et al., 2013).

Obsecurin is another third giant protein expressed in skeletal muscle along with titin which is a multidomain protein composed of tandem adhesion modules and signaling domains. It is concentrated at the peripheries of Z-disks and M-line where it communicates with myoplasm (Kontrogianni-Konstantopoulos and Bloch, 2005). Its function is to assemble the sarcomere and also works together with titin during the integration of myosin thick filaments into periodic A bands (Aikaterini et al., 2005). A mutation in either of the genes that encode the sarcomeric structural proteins, causes skeletal and cardiac myopathies (Myhre et al., 2013).

During embryonic myogenesis, regulatory factors control cell determination, proliferation and differentiation into adult muscle. Once the muscle is damaged, it regenerates again and the

new skeletal muscle is formed where the myoblasts assemble together and form large multinucleated cells known as the myotubes which mature later to form myofibers. The myotubes formed during muscle embryogenesis function in the repair of muscle damage that may have resulted from trauma or disease. The myotubes and the sarcomeric myofibrils are important during the formation of the skeletal muscle (Monique et al., 2003), which is highly complex and multinucleated that serve many functions in the body of an organism (Bentzinger et al., 2012). The alteration of cellular cytoarchtecture during fusion and differentiation of the myoblasts, form the myofibers, and are important during the sarcomere assembly and the formation of the new muscle fibers depend on the satellite cells that are located in between the basal lamina and the plasma membrane of the myofibers (Zhao and Hoffman, 2004). Molecular biology, cell biology and genetics have contributed to a better understanding of muscle regeneration and this have lead to production of therapeutic strategies that deal with pathological conditions associated with poor muscle regeneration especially in individuals with sarcopenia and muscular dystrophy(Yin et al.,2013).

1.2- Process of satellite cells/Stem cell to muscle cells

Eukaryotic cells divide approximately every 24 hours, and there are usually two phases: mitosis and interphase. Mitosis is also called nuclear division, which is the most dramatic stage of the cell cycle, where the separation of daughter chromosomes and usually ends with cell division, called cytokinesis. Cytokinesis and mitosis last only about an hour. Interphase is the period between the mitoses and during this phase, the chromosomes are decondensed and distributed throughout the nucleus, and the nucleus appear morphologically uniform (NCBI). Skeletal muscle satellite cells have been postulated as the main source of the new myonuclei in post-natal muscle (Zammit et al., 2001).These cells are characterized by large mononuclear to cytoplasm

ratio, few organelles and small nuclear and condensed chromatin structures during interphase. Further studies have stated that satellite cells in a healthy and unstressed muscle, are in G_0 phase and the transcription process is also dormant (Yin et al., 2013).

During embryonic and postnatal life, the satellite cells provides the foundation for stem and progenitor cell maintenance, lineage specification and terminal differentiation. Stem cells play a key role in the maintenance and regeneration of the individual tissue. Skeletal muscles have the capacity to regenerate within a period of two weeks and this is brought about by the mononuclear cell population called the satellite cells that resides in them. Satellite cells are found to be heterogeneous and they differ from one another in terms of their gene expressions, myogenic differentiations and also lineage potentials (Yin et al., 2013). Other studies have shown satellite cells to be intercalated in the myofibers; the basal lamina and the plasma membrane. Studies have shown that during injury, the satellite cells are activated and recruited to enter into the cell cycle process to generate myogenic precursors that are comparable to the embryonic myoblasts (Chen and Goldhamer,2003).There are factors that contribute to the activation of the satellite cells such as growth factors that promote activation and proliferation, the Notch signaling pathway that also respond to any injury and they promote and control satellite cells in a single fiber culture. Other factors such as Basic fibroblast growth factor (bFGF) stimulate satellite cells activation while inhibiting differentiation of the cells. The satellite cells also have a tyrosine kinase receptor c-met ligand, hepatocytes growth factor that promotes the cells during degeneration (Chen and Godhamer, 2003). Studies have also shown that, satellite cells are also considered to be stem cells, although not all of them, and play a key role during severe injuries of a muscle where it helps in muscle regeneration and myotubes formation. Further studies have suggested that stem cells are not only important during muscle

regeneration but also contribute to nonmuscle lineages and this serves as a tool for gene delivery (Xianozahong and Daniel 2006). It has been found that satellite cells are not well associated with this lineage and during cell division, they are found to retain the older DNA template. Satellite cells are also found to undergo asymmetrical division and this lead to stem cell self renewal to replenish the stem cell pool that are capable of producing myogenic progenitors that are essential in skeletal muscle growth and regeneration. It has also been discovered that satellite cells can differentiate into multiple mesenchymal lineages and satellite from a single myofibers is capable of differentiating into myocytes, adipocytes and osteocytes. Other researches have shown that satellite cells resemble bone marrow-derived mesenchymal stem cells in terms of the functions (Xiaozhong and Daniel, 2006).

1.3- Transcription factors committed to muscle lineages

Myogenic regulatory molecules (MRFs) belong to a family of transcription factors that play a major role in initiating a regulatory cascade resulting in the differentiation of somatic cells into myoblasts. (Turnbull et al., 1998)

Studies have indicated, that most of satellite cells in mature skeletal muscle, have the transcription factors Pax7 and Pax 3, myogenic regulatory factor Myf5, MyoD, homeobox transcription factor Barx2, cell adhesion protein M-cadherin, tyrosine receptor kinase c-Met, cell surface attachment receptor α7-intergrin, cluster of differentiation protein CD34, transmembrane heparin sulfate proteoglycans syndecans-3 and 4, chemokine receptor CXCR4, caveolae-forming protein caveolin-1, calicitonin receptor and nuclear envelop proteins lamin A/C and emerin. Pax 7 has only been identified as a canonical biomarker for satellite cells (Yin et al., 2013). MyoD and Myf5 are also responsible in transforming mesoderm cells to a muscle lineage. Transcription factor Mrf4 are found in mammals and teleosts such as the zebra fish, and are important in

research for gene manipulation and they have large number and optical clarity of embryos/larvae that can be harvested and this allow cell movement to be well monitored in real time experiments. In an experiment that was performed in mouse, *Myf or MyoD* does not affect the myoblast, and if one of them is deleted, then muscle development is not affected but in case both are deleted, then they eliminate skeletal muscle lineages. It was also observed that in zebra fish, Myf5 promotes slow muscle formation from adaxial cells and MyoD promote fast muscle differentiation, and MyoD work close with Pbx homodomain protein to promote fast myogenic program. It has also been discovered that pax3 lineage can affect the development of pax7-positive cells, but when pax-7 expressing cells are removed, the effects are not seen in the early stages but later on during the process of development which produces small muscles that have few myofibers during birth. This proves that pax-3 positive cells are the progenitors of the embryonic progenitor of embryonic myoblasts that produce primary fibers in the limb where as pax-7 positive cells form secondary fibers, and this establish the satellite cells pool and aid in maintenance of the terminal differentiation program (Rossi and Messina,2014). In an experiment that was done, it was discovered that regeneration of muscle in *MyoD*-null animals, the mononuclear cells are produced in large numbers but differentiate into few myotubes. Also the satellite cells in *MyoD*-null animals participate fully in self renewal. They further discovered that bone morphogenetic protein (BMP) treatment activates osteogenic marker while down regulating the *MyoD* in C2C12 myoblasts immortalized cell line that were derived from a mouse limb muscle. Also when the cells are treated with thiazolidinediones and fatty acids, they were observed to convert C2C12 to adipogenic cell fate and also cultures for myoblasts from a mature individual was found to respond to C2C12 cells when induced with osteogenic and adipogenic (Chen and Goldhamer, 2003).

1.4-MicroRNAs (miRNA) in muscle development

MicroRNAs (miRNAs) are 22 nucleotides single-stranded RNAs that function in mRNA degradation and also inhibit translation by complementary binding to the 3'untranslated regions (UTRs) of specific target mRNAs (Luo et al., 2013). Williams et al., 2009. Stated that miRNA are transcribed by RNA polymerase II as long pri-miRNAs encoding one or more miRNAs. The pre-miRNAs are then transported to the nucleus by exportin which is processed by another RNase III enzyme, Dicer in order to produce a mature miRNA and also a star strand that is degraded (Gurtan and Sharp, 2013). Myogenin protein activates the expression of miRNA that function during the output of transcription network that result in precise cellular responses to development, physiologic and pathological signals. miRNA are expressed in cardiac and skeletal muscles of an individual and myogenic transcription factors SRF, MEF2 and MyoD control the expression of miR-1 and miR-133a in cardiac and skeletal muscle. miR-206 and miR-133b are expressed specifically in skeletal muscle, and skeletal muscle specific transcription of the *miR-206/133b* has been found to be controlled by an up-stream regulatory region that is enriched for MyoD binding in ChIP-on-chip assay using chromatin from C2C12 muscle cells a shown in figure 1.below.

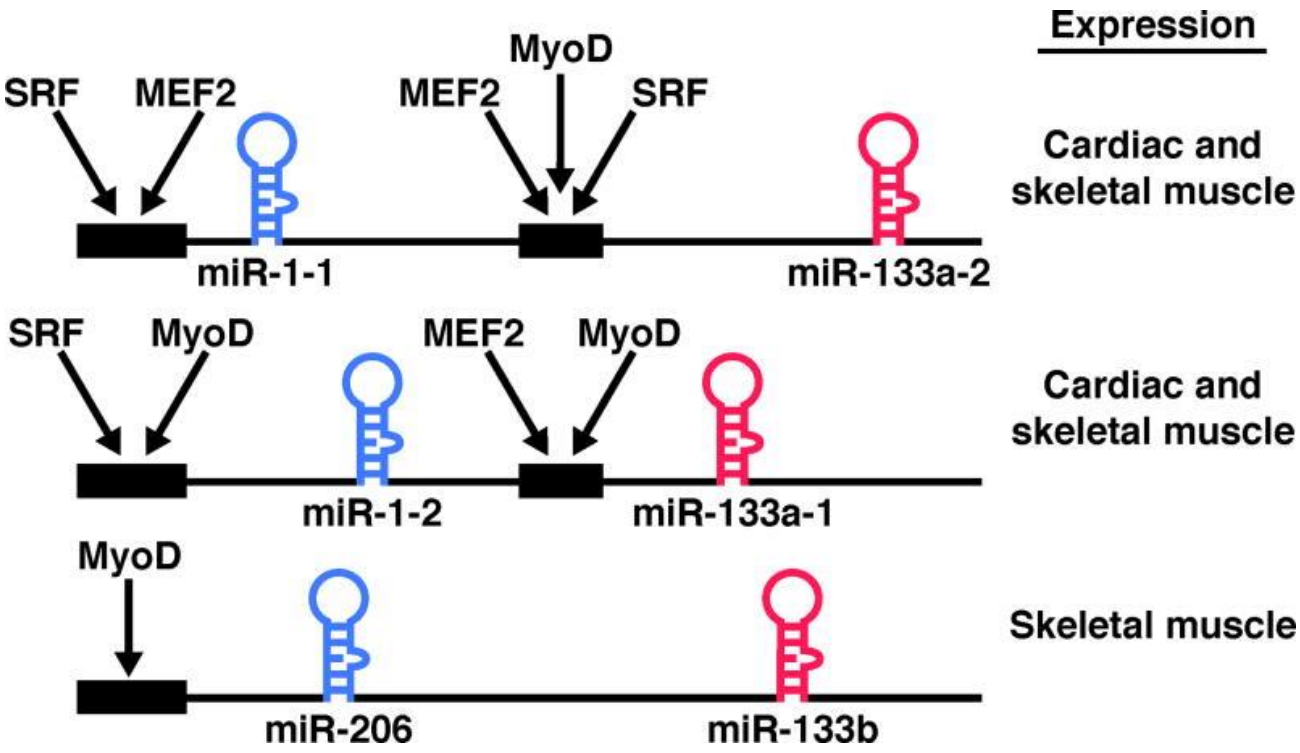


Fig 1. The above figure is a three bicistronic gene clusters each encoding two miRNAs. MiR-1-1, -1-2 and -206 are nearly identical in sequence, as are miR-133a-2, -133-a-1 and -133b. Cis-regulatory elements that direct muscle-specific expression of each locus are indicated by black boxes, and the transcription factors that act through these elements. Blue are members of miR-1/206 that are expressed in cardiac and skeletal muscle .These are miR-206,miR-1-1 and miR-1-2.miR-1-1 and miR-1-2 are identical and they differ from miR-206 by four nucleotides. Blue colours are members of miR-133a-1, miR-33a-2 and miR-133b are from the same families. miR-133a-1 and miR-133a-2 are identical and they differ from miR-133b by 2 nucleotides (Williams et al., 2009)

1.5.1-Titin Protein

Titin is the third giant protein in the striated muscle after myosin and actin protein, and it is encoded by a single *TTN* gene that is located on the long arm of the chromosome 2 in both human and mouse and also has 363 exons that undergo extensive alternative splicing (Alkaterini et al., 2009). In 2010, Machando and Andrew, also identified titin as chromosomal protein in both human cells and Drosophila embryos.

Machando and Andrew, 2009, Labeit et al. stated that single titin molecule span half a sarcomere with their amino and carboxyl terminal that is anchored in the Z-line and M-line as shown below in figure. 2.

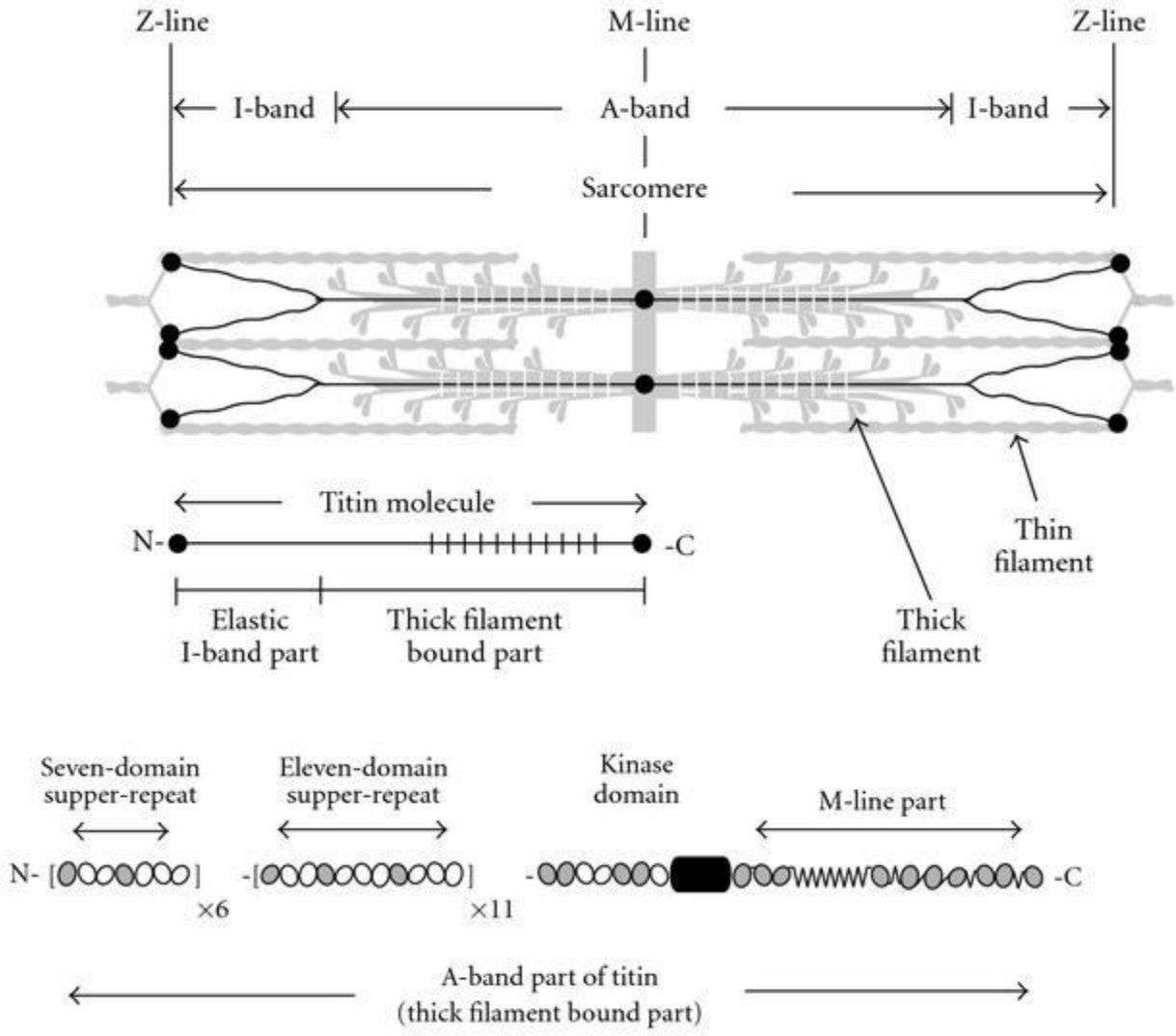


Fig 2. shows a schematic representation of titin layout in the sarcomere (a)domain periodicity in the thick filament bound part and (c)striated zones in the sarcomere and the titin molecule (a) show the location of the large super repeat (Trinick and Tskhovrebova,2010)

In 1993, Kuan et al. stated that, titin molecules are oriented in opposite polarities in the two halves of the same sarcomere and epitopes of titin in the A-band appear to be rigidly attached to the thick filament, whereas epitopes in the I-band behave elastically in response to stretch or contraction and this corresponds to the bipolar orientation of the thick filament.

Titin is highly modular and 90% of its mass consists of repeating immunoglobulin (Ig) and fibronectin type III superfamily. It has 244 β sheet domains and 112 of the β sheets are classified under the immunoglobulin superfamily and 132 are fibronectin type III superfamily. These Fibronectin and Ig domains are known to provide binding sites for proteins such as myofibrillar and membrane components and also enzymes and signaling molecules (Alkaterini et al., 2009) and also they provide the molecular extensibility and passive elasticity (Lu et al., 1998)

Titin has a force that originate from the I-band region and has two segments. The first one contains proline, glutamate, valine and lysine residue and all these are termed as PEVK segment .The second segment is serially linked immunoglobulin (Ig)-like domains and this flank the PEVK segments (Cazorla et al., 1999, Michendo and Andrew, 2010). They went ahead and Michendo and Andrew (2010) stated that titin transcripts are diffentially spliced domain in regions encoding the tandem-Ig and PEVK domain that give rise to many isoforms with different extensible properties.

1.5.2- Titin at the Z-Disk

The NH_2 terminus of titin contain approximately 90kDa of the protein and it includes amino acids 1-826.The first 200 amino acids of titin are found at the periphery of the Z-disk, and is referred to as Z-line titin edge residues, and is marked as the Z-line. Amino acid 201-750 span the entire width of the Z-disk and is referred to as Z-line. Amino acid 751-826 are located at the junction of the Z-disk with the I-band and is referred to as Z/I junction residues (Alkaterin et al., 2011).

In an experiment performed, it was found that the Z-disk of the titin protein showed a complex pattern Ig motif and large interdomain insertions. It was also proposed that the Z-disks structures vary in thickness among different striated muscles, which shows that the number of its constituent proteins are regulated in a way that depend on the fiber type. They also found that fast muscle fiber have thin Z-disks whereas slow muscle fibers have thick Z-disks and this results led them to conclude that narrow Z-disks contain titin isoforms with small number of Z-repeats and fibers with thick Z-disks contain titin isoforms with more Z-disk repeats. Therefore a combination of molecular and biochemical experiments have demonstrated that the Z-disk potion of titin interacts with both myofibrillar and membrane associated proteins. They suggested that it may play an essential role in sarcomeric stability and maintenance as well as contributing to the assembly of the contractile apparatus and associated systems of intracellular membranes (Alkaterin et al., 2009).

1.5.3 Titin Functions

Titin plays a role in regulating the length of thick myosin filaments, by acting as a molecular ruler, it also works as an elastic element in the myofibril that is involved in the regulation and maintenance of muscle elasticity, stiffness and resistance to stretch. It also maintains the position stability of the thick filaments during contraction or stretch and involved and also organization of myofibrils during the de novo assembly of myofibrils in developing muscle cells (Kuan et al., 1993). Titin also influences ventricular filling and maintains the structural integrity of the contracting sarcomere (Freiburg et al., 1999, Tshovrebova and John, 2010). In addition to that, recent studies have indicated that titin is also involved in the modulation of the lattice spring (Fukuda et al., 2009). It has also been speculated that titin acts as a factor in determining the length-dependency of the Ca^{2+}-activated force development, the

molecular basis for the Frank-Starling mechanism (Linke, 2008) which states that cardiac output increases or decreases in response to change in heart rate or stroke volume (Klabundle, 2012) and also aligns structural, regulatory and contractile proteins within the sarcomere (Linke, 2008).

1.5.4 Titin Isoforms

There are only seven described human titin isoforms that have been identified so far by the National Center for Biotechnology (NCBI) database (Chauveau et al., 2014).

Titin undergoes alternative splicing at the I-band and this gives rise to two different isoforms, N2A and N2B (Labeit, 2004). N2B isoforms contains an N2B element and both N2B and N2A are found in the cardiac muscle, and N2A isoforms are found in skeletal muscle. N2B is short and stiffer, while N2BA is longer and more compliant (Freiburg et al., 1999, Labeit, 2004, Lewinter et al., 2004. In addition, N2BA has numerous fetal-neonatal variants but most variation is lost in adult life (LeWinter, 2004). Titins isoforms have PEVK and tandem Ig segments, and it has also 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) (Freiburg et al., 1999). 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 (Labeit,2004).

1.6 Specific Aims

Studies have shown titin as one of the giant protein after Actin and Myosin with many functions in myogenesis, and also important in mature muscles of both skeletal and cardiac. The

aim of this study is to help us understand the role of N2A and N2B titin isoforms as a whole protein in myogenesis with the main focus on the effects of growth factors on myogenesis and also the sequence of genetic event that control myogenesis using C2C12 myoblast in different time points. This research also aims in understanding the role of the titin isoforms as they are expressed during myogenesis process and the specific area of storage during the maturation of the protein.

1.7 Hypothesis

The removal of fetal bovine serum in the Dulbecco's Modified Eagle Media (DMEM) will increase the amount of titin expression as a result of initiation of myogensis. Therefore we expect to see the high expression of N2A and N2B genes in all the experiments that received low growth serum medium.

Chapter 2. Methods

2.1 Mammalian C2C12 Cell Culture

C2C12 cells from mouse myoblast are the cells that were used throughout the experiment. The C2C12 cells which were initially purchased from the American Type Culture Collection (ATCC) Manassas, VA, were obtained from storage in the nitrogen container and thawed out on water bath at 37^0C for a minute and was transferred to a 15ml flask that contained 1 ml of growth medium called Dulbecco's Modified Eagle Medium (DMEM) that was supplemented with 20% Fetal Bovine serum (FBS) and 1% penicillin/streptomycin and it was allowed to sit for 10 mins and then added 2ml of growth medium and was again let to sit for 10 mins and finally added 4 ml of growth medium and allowed to sit for 10 mins.This was transferred to a 50ml conical flask which had 2ml of growth medium and was incubated at 37^0C with 5% CO_2 until the following day and the culture was viewed under the phase-contrast microscope for the confirmation of the attachment of the cells at the flask.

We performed another experiment where we took a culture from the above culture and fed with high serum medium for 14 days.

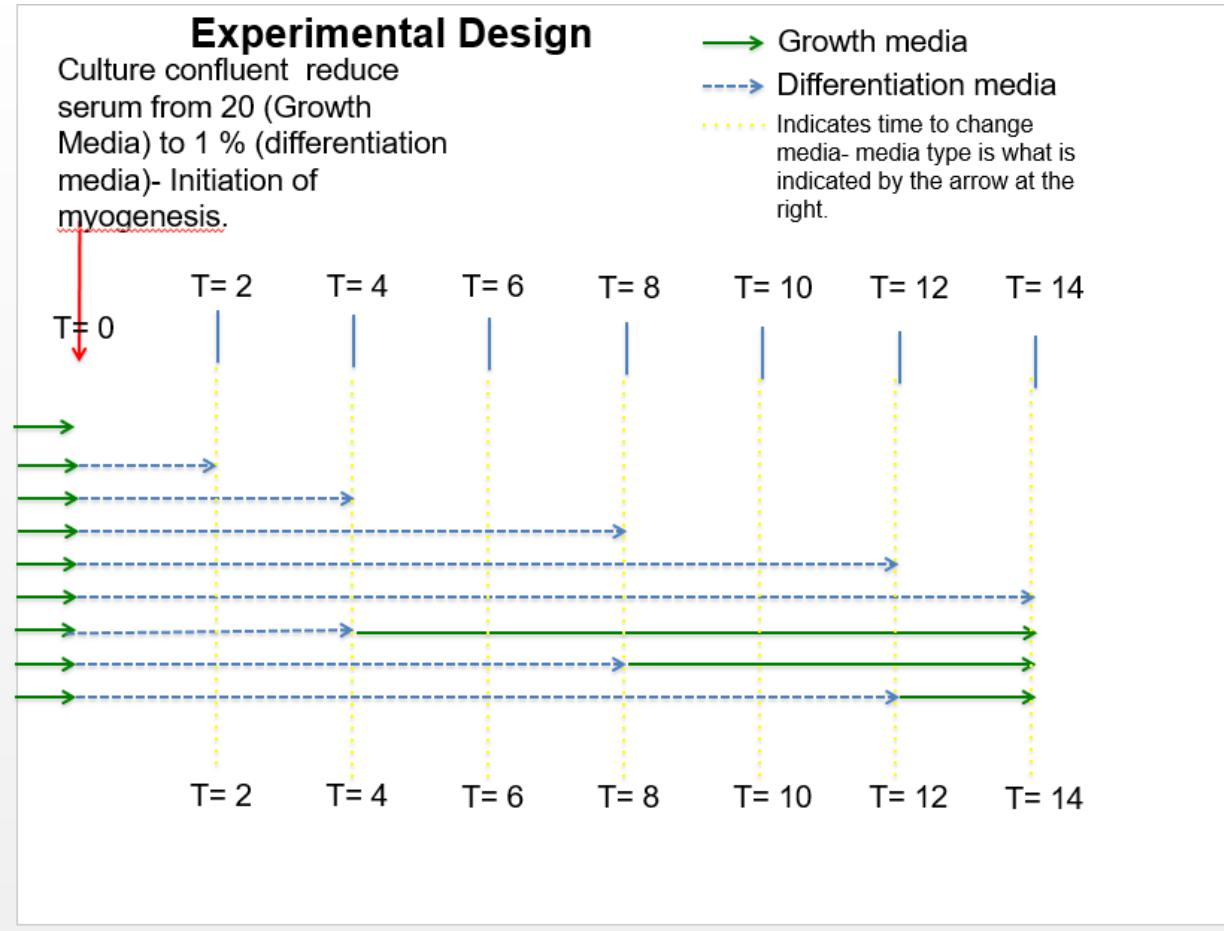
2.2 Cell Expansion

The medium was changed after every 48 hrs. until the cells attained 70-80% confluency. After they achieved the confluency, the cells were washed twice using the sterile DMEM medium to remove the serum proteins from the cells and then treated with trypsin and incubated under 37^0 C with 5% CO_2 for 10 minutes.

2.3 Experimental Design

The cells were transferred to a 15ml flask in the biosafety cabinet, and divided into 9 flasks of 70ml as summarized in figure.3 below. The 9 flasks contained 20ml 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. and pictures taken until the cells were 70-80 % confluent. After the cells attained the confluency, the medium was switched to differentiation medium and the experiment was repeated three times and also in different time points as shown below in the experimental design. The cells were harvested at different times at T=0 Days, T=2 Days, T=4 Days, T=8 Days, T=12 Days and T=14 Days. The days in the experiments shows the appropriate time that the cells were harvested. Add back experiments were AB4, AB8, and AB12. The addback experiments shows the day the medium was switched from differentiation to growth medium and they were all fed up to day 14.

Figure.3



Type of Media (Growth and Differentiation)

Number of days the cells were fed

Fig.3. above shows the type of medium the cells were fed with in different days.The green line indicates the cells that were fed with growth medium, and the blue line shows how the cells were fed with differentiation media and finally the yellow dots indicates the time for switching from one medium to another.

2.4 Cell Morphology

As soon as the culture initated the myogenesis process, the cells were viewed on a phase contrast microscope every day starting with the lowest magnification to the highest. The pictures under 10X on the microscope were taken and saved on the computer to be used as a comparison with all other flasks.

2.5 Cell harvesting

The cells were harvested differently as shown in the experimental design, where we started with T=0, and this was the only flask that was not fed with differentiation medium. The cells in T=0 were collected and pelleted in the centrifuge at 4^0C and 3000 rpm for 10 mins and the pellets were kept under -80^0C. All other cells were fed with medium that had low Fetal Bovine serum and were harvested according to their appropriate time of harvest within a period of 14 days.

2.6.1 RNA Isolation

All the cell pellets were thawed out on ice for 30 mins, and this was followed by the RNA isolation on an RNAse free bench. The isolation of RNA was done following the manufacturer's protocol that was provided by QIAGEN RNeasy ® Mini Kit as shown below.

2.6.2 RNA Isolation Protocol

Following the Qiagen RNeasy protocol, the thawed cell pellets were re-suspended by pipetting up and down with 350 µl of RLT buffer and then transferred in a QIA Shredder column. The cell pellets in a solution were microfuged for 2 mins at 14000 rpm. 350 µl of 70%

ethanol was added to the solution and mixed well and then it was transferred to an RNeasy spin column in a collection tube and was centrifuged for 30 s at 8000 rpm, and the flow through was discarded. 700 µl of RWI buffer was added to the spin column and then microfuged for 30s at 8000rpm and the supernatant was discarded and the 500 µl of RPE buffer was added to the spin column and was microfuged for 30 s at 8000 rpm. The supernatant was discarded and the spin column was transferred to a 2 ml collecting tube and microfuged for 1 min in order to extract residue fluid. The spin column was transferred to 1.5 capped microfuge tube and 60 µl of RNase free water was added to the spin column and microfuged for 1 min at 8000 rpm and RNA was collected.

2.7 Nano Drop Reading

All the RNA samples were quantified using a Nano drop instrument and also for quality identification or if there was any impurity in the RNA, and the results were recorded down. This was also done to help us calculate the amount of RNA that would be needed in every reaction.

2.8 Primer Sequences

The primers were designed and analyzed for our PCR experiments using a free online primer 3 tool, and below is a table of the primers that we used in our experiment. The primers were designed specifically to target the N2A and N2B titin genes against actin gene.

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TABLE 1.

Gene Type	Gene	Forward	Reverse
	Actin3	5'ATCACCATCGGCAATGAGCGTTTC3'	5'TGTTGGCATACAGGTCCTTCCTGA3'
	N2A1	5' TGCTGCATCCATGGTAGTGG3'	5'TCCTGTTGTATGGCAGTCTCTT3'
	N2B1	5'GTGCCCTCAGACGGAAAGTT3'	5'CTCCCCTTCATCCTCCGTTC3'

Primer Sequences

The primers above were tested in a Standard PCR, and DNA gel was run using the agarose buffer in an agarose gel electrophoresis instrument to see if the primers worked, and from the image below.

2.9.1 RT- qPCR

Few RNA samples were prepared and transcribed into cDNA in PCR thermocycler using a reverse transcriptase enzyme, and a DNA gel was also run to determine if our primers were working using a 100 bp ladder to determine the size of our fragments.

2.9.2 RT-qPCR Protocol

The protocol below was used in the three experiments as shown in table 2 below

Table 2. BioRad iCycler qPCR Program

Cycle Number	Number of Repeats	Temperature	Time	Changes
Cycle 1	**1**	**50^0C**	**10 mins**	**Reverse transcriptase RNA to cDNA**
Cycle 2	**1**	**95^0C**	**5 mins**	**Denaturation**
Cycle 3	**45**	**95^0C**	**10 sec**	**Annealing**
		60^0C	**30sec**	**Extension and Elongation**

3. RESULTS

3.1 Myogenic Process

Previous studies have shown that titin protein is highly expressed upon the induction of the differentiation media. In our experiments, upon the initiation process of myogenesis, there was no difference in terms of cell morphology in both the normal flasks and the add backs experiments, however the amount of RNA that was produced before switching of the medium from growth to differentiation media that is T=0, was high and also at T=2 and T=4. The amount of RNA produced reduced gradually as the cells were fed with differentiation medium and this can also be seen in the add backs experiments that do not show any significance difference as compared to T=8,T=12 and T=14. The experiment was carried out three times in order to have a comparable results and draw a better conclusion.

3.2 Cell Morphology

The cells had all the characteristics of the skeletal muscles such as multinuclear and striations which was observed in all of our experiments. In the initial stages of proliferation in the undifferentiated stage, the C2C12 cells looked flat and star-shaped monucleated cells as seen in figure 4A, and this showed the same characteristics observed by Burattini et al., 2004; Zhang et al., 2015. The proliferation of the cells continued and the cells got well spread in the flasks and showed many contacts with each other, as observed in figure 4B. This results are comparable with the results observed by Mermelstein et al., 2003; Burattini et al., 2004). Although Mermelstein et al., 2003 used a normal medium that was not supplemented with Ca^{2+} chelator EGTA for this experimented was treated as a control. The medium was switched from high

serum to low serum and after the second time of feeding, the myotubes were observed as in Fig 4C. The myotubes formed was seen as long corn shape, multinucleated and the myoblasts formed inside the myotubes and was seen in circular form inside the myotubes and they got thickened and they organized to form the sarcomere and myofibrils, and this showed the characteristics of detachment from the mononuclear and these characteristics were also observed by Burattini et al., 2004.

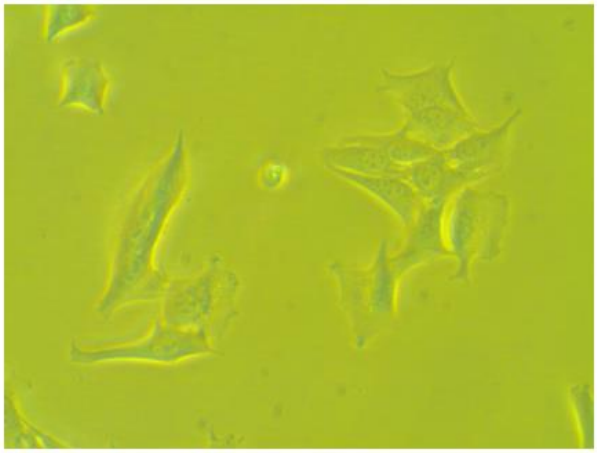


Fig.A 10% confluent under 10X

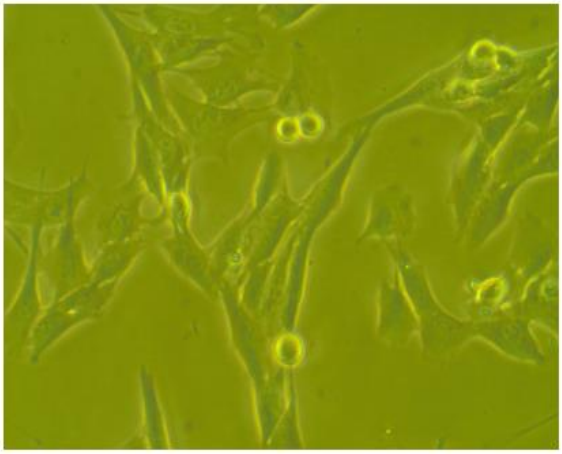


Fig.B 40% confluent under 10X

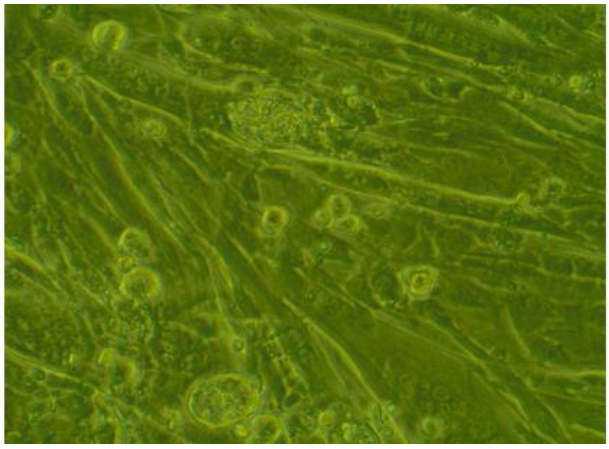


Fig. C Mature Muscle cell under 10X

Figure. 4. Cells were viewed under the phase-contrast microscopes and after 24 hrs. Under the magnification of 10X , the cells were viewed and the pictures were taken using a Macbook camera and saved on the same macbook computer. After myogenesis process initated . Fig. 4A shows 10% confluent which means the cells have started attaching to the flask and the process is in a lag phase. 4B. the cells are 40% confluent and the division of the cells are in rapid growth. Fig. 4C. The cells have matured into a myotube that are seen in long cylindrical forms and the small circular shapes inside the myotubes are the myofibers which contain nuclei.

Figure. 5, the cells were fed with high growth serum throughout the time period. The myotubes formed after 8 days and after 15 days, the myoblast formed and they can be seen in circular form in the myotubes. This experiment shows that myotubes would still form even without cells being fed with low serum medium and this is illustrated below.

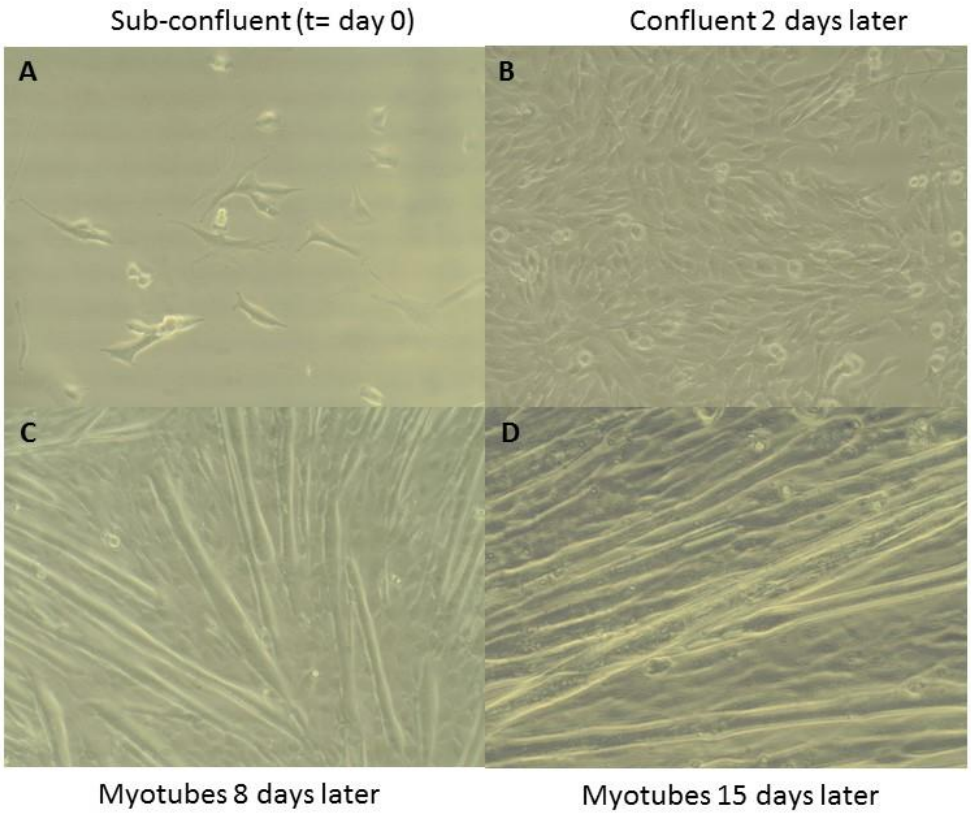


Figure 5A. The first day the cell culture was fed with a high serum medium. 5B. two days later after feeding. 5C. 8 days after feeding and 5D. 15 days after feeding. Figure 5C has mature myotubes and 5D has a mature muscle cell.

3.3 RNA Quantitation

After RNA was successful isolated from the C2C12 cell pellets, it was quantified, and the purity of the samples analyzed using a Nano Drop instrument. The RNA results on the Nano drop reading provided an absorbance ratio of nucleic acid at 260 nm and 280nm of 2.1 for almost all the samples and this was really good because the ratios for RNA needs to be 2.0. Although one sample #AB12 in trial #2 had a ratio of 1.2, it indicated the presence of proteins, phenol or other contaminants that got absorbed at or near 280nm. This is illustrated in three tables.

Table 3. Trial #1

Sample type	RNA Conce ng/µl	A260	A280	260/280	260/230
T=0	729.4	18.2	8.7	2.1	2.2
T=2	259.2	6.5	3.1	2.1	2
T=4	293	9.8	4.8	2.1	2.2
T=8	294.2	7.4	3.5	2.1	0.1
T=12	444.7	11	5.4	2.1	1.5
T=14	176.2	4.4	2.1	2.1	1.06
AB4	283.3	7.1	3.4	2.1	1.4
AB8	280	7.0	3.3	2.1	1.44
AB12	358.3	9	4.2	2.1	1.2

Table 4. Trial #2

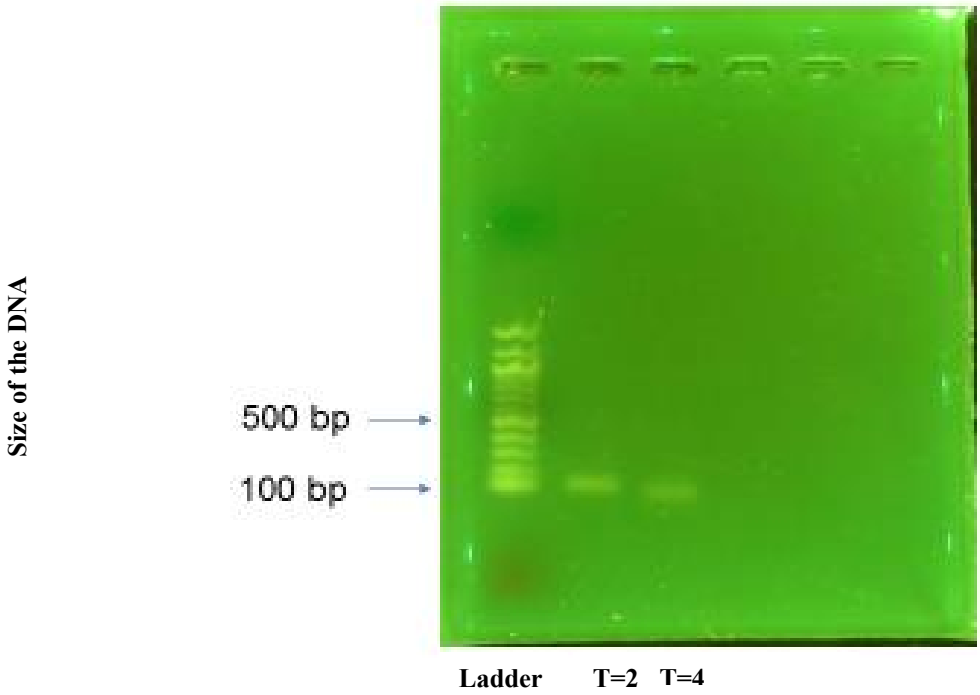
Sample type	RNA Conce ng/µl	A260	A280	260/280	260/230
T=0	396.4	9.9	4.8	2.1	1.9
T=2	492.3	12.3	6.0	2.0	2.1
T=4	221.1	5.5	2.6	2.1	1.9
T=8	263.2	6.0	2.8	2.1	1.4
T=12	229.6	5.7	2.7	2.2	0.5
T=14	220.7	5.5	2.6	2.1	2.5
AB4	90.8	2.3	1.1	2.1	0.26
AB8	65.4	1.6	0.6	2.5	0.6
AB12	97.1	2.4	1.1	1.2	0.17
NYA	94.4	2.4	1.0	2.3	0.2

Table 5. Trial #3

Sample type	RNA Conce	A260	A280	260/280	260/230
T=0	269	6.7	3.3	2.1	1.9
T=2	334.1	8.4	4	2.1	1.9
T=4	259	6.5	3.1	2.1	1.8
T=8	182.6	4.6	2.2	2.1	1.2
T=12	55	1.4	0.7	2.0	1.1
T=14	208.4	5.2	2.6	2.1	1.7
AB4	56.8	1.4	0.6	2.1	0.2
AB8	101	2.5	1.2	2.1	0.5
AB12	228.4	5.7	2.7	2.1	1.8
NYA	227	5.7	2.7	2.1	1.8

3.4 DNA Gel

The gel was run to test whether cDNA was amplified , and also learn if obtained the right size of the fragments using DNA ladder of 100bp.



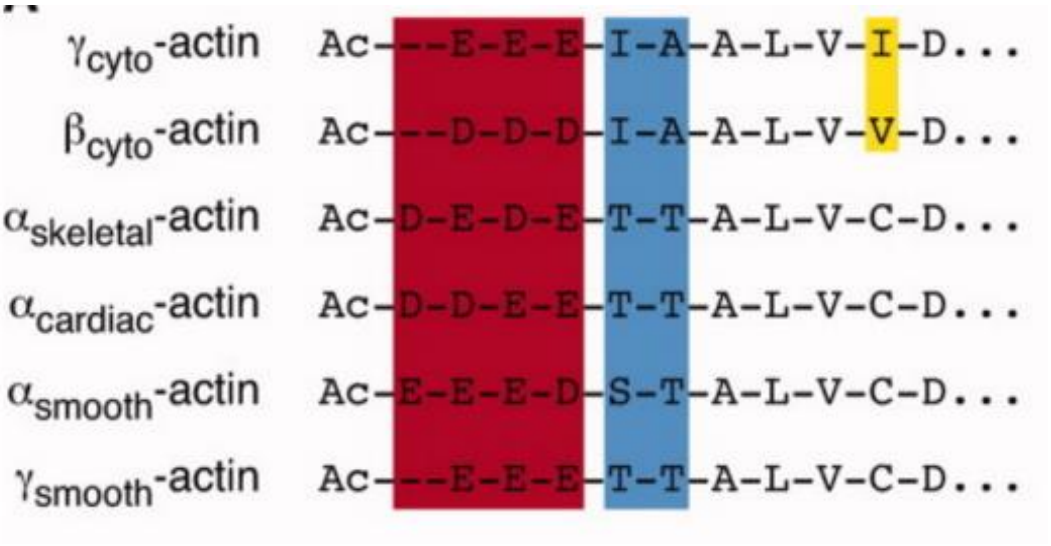
The first lane in figure 5. Show the DNA ladder and lane 2 is one of the sample DNA that was collected at day 2 after being fed with differentiation media and lane 3 indicates the DNA sample that was collected at day of after the cells were fed with differentiation media.

3.5 Gene Expression

Housekeeping genes have been used to control gene expression as a way of normalizing other genes and this can be used in western blotting, Northern blotting and RT-PCR among others (Lin and Redies, 2012). Research has proved actin as a house keeping gene with six isoforms that are similar to each other with slight amino acid sequence variation and this protein carries unique cellular functions and the six isoforms are found in this protein as shown in fig 8.

(Perrin and Ervasti, 2010). Actin can also be classified in three pairs where two isoforms are expressed in the cardiac and the skeletal muscle tissue, two in smooth muscle and two cytoplasmic isoform (Bertola et al., 2008).

Actin isoform Type



Amino Acid Sequences

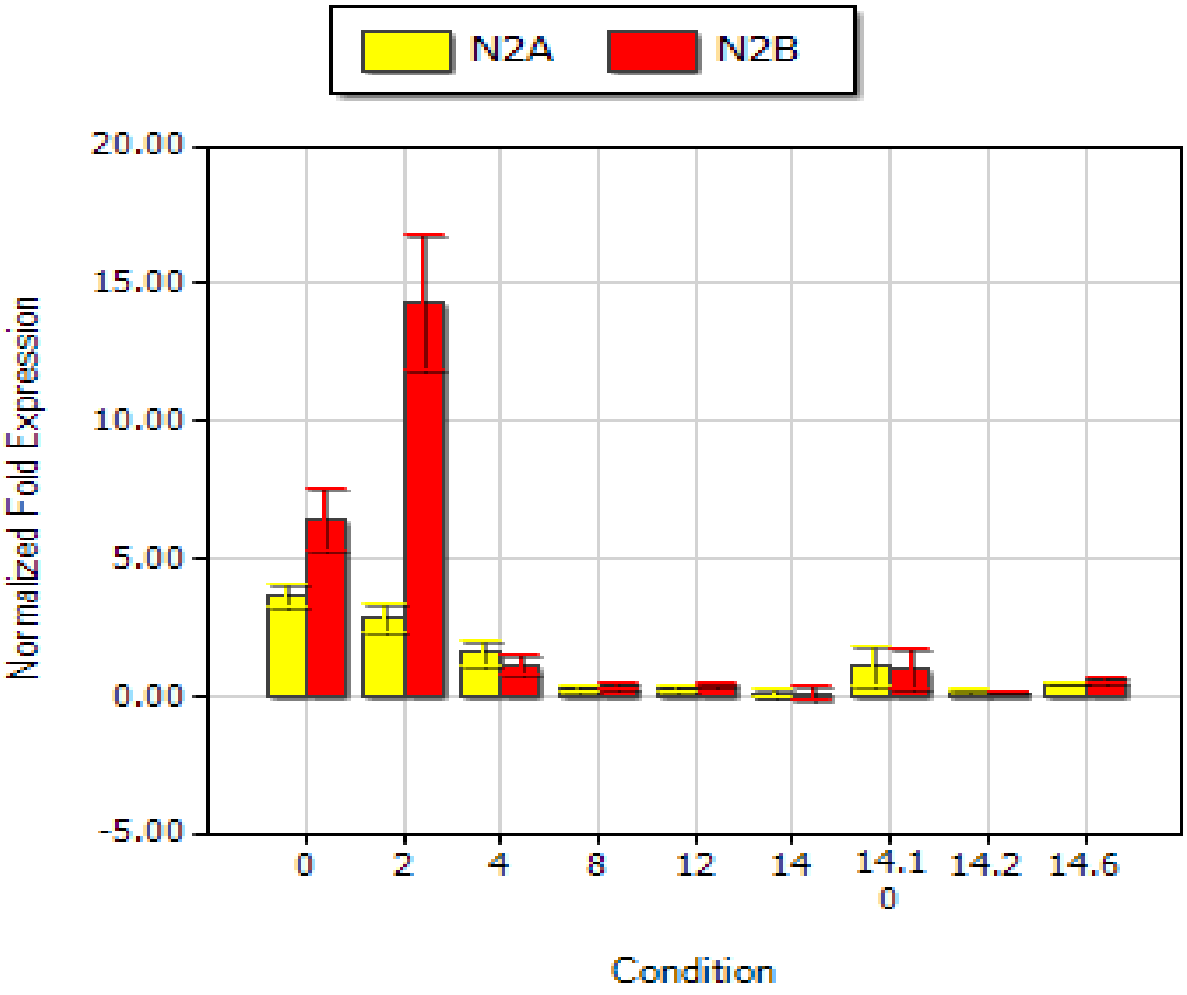
Actin 3 gene was used in our experiment to normalize against N2A and N2B genes, and since it's believed to remain stable at the end of the experiment, and incase it is expressed higher in the experiment, the other genes are also expected to go high and vice versa.

In this study the C2C12 myoblast start out as a single cell mononuclear cells that proliferate and proliferation stops after the culture reach confluency (maximum cell-cell contact). This is consistent with non-transformed plastic cells and at this point the serum in the medium is lowered to enhance the initiation of myogenesis.

We did three trials in our study, we grew cells to confluency and then split them into 9 flasks and again allowed the cells to grow to confluency. The cells were harvested in different

time points in 14 days. T=0 were harvested immediately they became confluent. T=2, T=4, T=8, T=12 and T=14, were fed with low serum medium. In 14.1, 14.2 and 14.6 the cells were fed with both low serum and high serum medium in different days. The cells were pelleted and RNA isolated from them a gene study is done using RT-qPCR and data analyzed. T=2 was highly expressed, followed by T=0. T=4, T=8, T=12 and T=14 were lowly expressed and they do not agree with our hypothesis. 14.1, 14.2 and 14.6, the results were averaged and there was no increase in gene expression. This is illustrated in figure.7

Figure 7. Shows a bar graph of Gene Expression of N2A and N2B Titin Isoforms on the X-axis against Time (no. of days) on the Y-axis

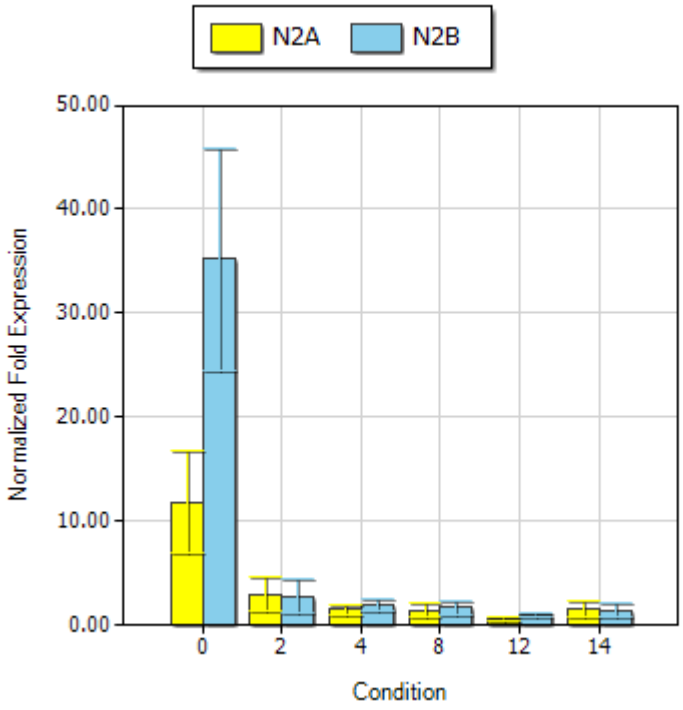


0, 2, 4, 8, 12, and 14 are the normal experiments and they show different no of days the cells were fed with low serum medium and got harvested in 14 different time points.0, the cells were not fed with low serum medium but, instead they were harvested on the first day the other cells were fed. 2, the cells were harvested 2 days after feeding. 4, the cells were harvested 4 days

after feeding. 8, the cells were harvested 8 days after feeding. 12, was harvested after 12 days of feeding and 14, was harvested 14 days after feeding. 14.1, 14.2 and 14.6 are the addback experiments. The medium was switched from low growth serum to high serum. 14.1, the medium was switched on day 4. 14.4, the medium was switched on day 8 and 14.6 the medium was switched on day 12 and all the addbacks cells were harvested on day 14.

Figure .8 Shows trial 2 of gene study. T=2 was expected to go high in terms of gene expression as it was seen in trial 1, while T=0. T=4, T=8, T=12 and T=14 maintained the same level of gene expression.

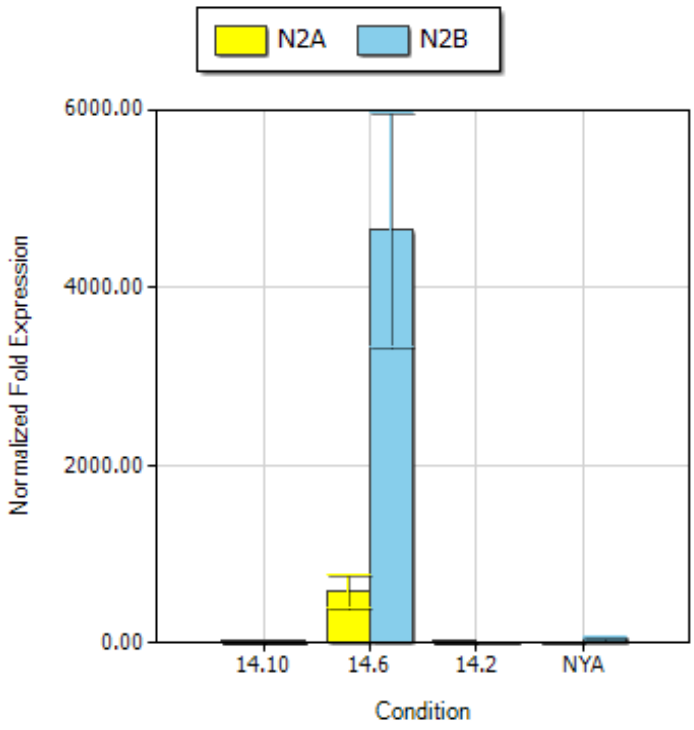
Figure 8. Shows Gene Expression of N2A and N2B Titin genes on the Y-axis against Time (no. of days) on the X-axis



The chart above shows the expression of gene without the addback experiments and cells were harvested at different time points of 14 days of our study. The cells were fed with low growth serum in different days. At 0, the cells were not fed with low serum medium but instead, the pellets got harvested the first day the other cells were with low growth serum medium until their day of harvest. 2, the cells were harvested 2 days after feeding. 4, the cells were harvested 4 days after feeding. 8, the cells were harvested 8 days after feeding. 12, the cells were harvested 12 days after feeding and 14, the cells were harvested on day 14 after feeding with

Figure 9. Shows gene study of the addback experiments. We observed a difference in the level of gene expression. 14.6 was highly expressed among the addbacks, while other experiments maintained the same level.

Figure 9. The Gene Expression of N2A and N2B Titin gene on Y-axis against Time (No. of days) X-axis



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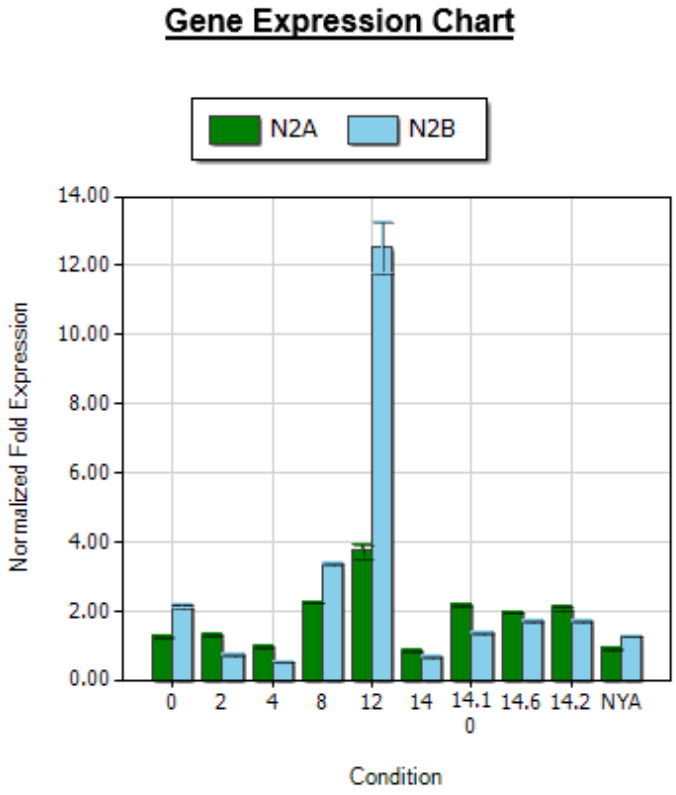
The figure above, 14.10, 14.2, and 14.6, are the addback experiments. NYA is an experiment where the cells were fed with serum free medium on day 10 and 12. 14.10-14.6, the cells were fed with high serum medium. All these cells were harvested on day 14.

Figure 10. Show gene study of our trial #3. T=12 was highly expressed, while the other experiments were averaged since they were lowly expressed.

Figure 10. Shows the gene expression of N2A and N2B Titin genes on Y-axis against Time (No. of days)



The figures above 0-NYA describe different days the cells were fed with low serum medium and low serum medium. 0-14, show the days the cells were harvested after being fed with high growth serum. 14.1, 14.2, 14.3, are the addbacks experiment. This means that the cells were fed back with high growth serum medium and all of them harvested on day 14. NYA was fed on serum free medium on day 10 and day 12, and the cells were harvested on day 14.

CHAPTER 4. Discussion

Our hypothesis states that, the removal of fetal bovine serum in the DMEM medium will increase the amount of titin expression as a result of initiation of myogenesis. Our data do not support our hypothesis.

Titin being a third giant protein after myosin and actin, it has 360 exons that undergo alternative splicing (Alkaterini et al., 2009). The alternative splicing of titin protein takes place at the I-band and it gives the two isoforms N2A and N2B (LeWinter, 2004). The study of these two isoforms is important and it will help us to know how they are formed, where they are located, and also give us a better understanding of their expressions and functions, and above all to understand how this protein as a whole is expressed in skeletal muscles and cardiac muscles.

Studies have indicated that during the perinatal development, the cardiac N2BA/N2B expression ration decreases whereas in chronic cardiac failure, the N2BA/N2B can increase (Kruger and Linke, 2011). Cardiac myocyte have shown to have a larger N2BA isoform and they are expected to produce less force than N2B titin and the main reason why N2BA is longer than N2B is because, N2BA titin isoform has a much longer PEVK segment that reduces the need to extend the stiffer unique N2B sequence. Among the mammalian vertebrates, N2BA isoforms expressed by the cow has been found to be the largest of the various N2BA titins identified so far (Trombitas et al., 2000). Different techniques such as SDS-PAGE, Western blotting have been performed to investigate how titin protein is expressed in different mammalian species and the results have shown on SDS-PAGE, that both small and the large titin isoforms are coexpressed but in widely varying rations, but Western blotting studies with N2A and N2B antibodies

indicate that small isoforms contains the N2B elements, whereas the larger isoforms contains both N2B and N2A elements (Cazorla et al., 1999).

The main objective of our research was to understand the roles of N2A and N2B titin isoforms, and this study has given us some understanding and one of them being that these isoforms are expressed all over the body before maturation of the muscle cell, and once they mature, they are fixed at a certain part of the body. Therefore this study will give a clear way of understanding the roles in of these isoforms and the expression of titin as a whole. This will also help us employ other techniques related to better our understanding on which isoform between the two is expressed highly than the other and learn the conditions behind them.

4.1. C2C12 Cells Culture

The process of C2C12 cell culturing was established in 1977 and the *in vitro* culture of C2C12 cells, induces myoblast to differentiate and mature into mature myotubes in a medium that contain low growth serum, and this is followed by the expression of other genes such as myosin (Luo et al., 2013). Myoblast are the precursors of contractile muscle cells and this myoblasts fuse to form plurinucleate syncytia, myotubes which further differentiate to acquire the final morpho-functional features of the muscle cells (Burattini et al., 2004).

The culturing of cells was successful since we encountered small problems of cell contamination and therefore we did not lose much of the medium. Also our cells were well cultured since the environment was conducive in the incubator and we grew our cells in 10 flaks and labelled as follows T=0, T=2, T=4, T=8, T=12, T=14, AB4, AB8, AB12 and NYA

4.3 Cells harvesting and RNA Isolation

Same method of harvesting cells was used in all of the three experiments we conducted. The centrifugation was done to obtain a cell pellet, but the pellets did not settle at the bottom of the flasks at the same time. At the same time, the process of centrifugation was repeated more than twice for the pellet to settle at the bottom of the flask and this occurred in all the addbacks experiments. The cell pellets were also hard to dissolve in the RLT buffer. This led us to speculate that during the process of adding back, the cells produced phenol or other contaminant since the production of protein was already in plateau stage, and the cells did not utilize all the nutrients in the medium, some of it formed contaminants. This led us to conclude from the result because all the addbacks had low expression of titin protein. The *in vitro* process of C2C12 cell culturing will help us define in future why it was too difficult for the cells to dissolve in the buffer and also the centrifugation of the cell pellets.

4.4 Gene Expression and Analysis

Cytoskeletal actin protein is ubiquitously expressed in most of the eukaryotic cells and it helps to maintain the cytoskeleton, cell mobility and also muscle contraction. Additionally, actin functions in other cellular processes such as gene transcription and chromosome morphology, control of the cell cycle, modulation of a variety of membrane responses, translation of several mRNA species and modulation of enzyme activity and finally localization within the cell (Bertola et al., 2008). We used actin expression as a measure of normalized gene expression. Comparison of all gene expression to actin express allows us to make specific conclusion of any specific gene level of expression on cell.

The results in all the trials, did not have the same results as expected. In trial #1. Where t=0, we obtained 7 folds of N2B and 3 folds of N2A genes, and t=2, we obtained 14 folds of N2B and 4 folds of N2A. We speculated the difference was that the cells in t=0, the cells were harvested immediately they attained the 80% confluent and that's why there was low expression of the genes as expected. In Trial # 2, where t=0, we obtained 35 folds of N2B and 12 folds of N2A, and t=2, the expression of genes were very low expressed. We speculated that when t=0 in trial #2, the cells had been confluent for long before harvesting and this resulted to high level of gene expression as compared to trial #1.

There was no significant increase in the actin gene expression than N2A and N2B and the expression of N2A and N2B genes were proportional to the actin gene. We expected the amount of RNA to be expressed high upon lowering the serum in growth medium but this wasn't true to our hypothesis stated. We speculated the difference could have resulted due to cells that overstayed to attain 100% confluent before being fed with differentiation medium instead of 70-80% confluent, and this could have led the cells to begin fusing and partially differentiate due to cell to cell contact.

We did not expect to have an increase in the addback experiments due to growth medium that was added back to the cells, but we experienced a higher expression in one of the experiment. We speculated that DNA was being transcribed into mRNA and the protein being synthesized in the early days of feeding with differentiation medium. In the other addbacks experiments, there was no increase in protein synthesis and this caused all the addbacks and the other experiments to have low expression of N2A and N2B genes.

Studies have shown that the expression of titin may point towards contributions during myogenesis as a molecular blueprint for the sarcomere (Person et al., 2000). Therefore we speculate the reason why N2A and N2B genes were lowly expressed in the addbacks and the other experiments is that, there could be degenerative alteration, lack of contractile elements that caused the disorganization and reduction of titin expression (Rui et al., 2010; Person et al., 2000). We also speculated that the cells overstayed in the flask and all the nutrients had been depleted before harvesting and the cells became too mature and started to break off which led to titin protein interference. That's why we observed low amount of titin production in all the addbacks experiments and the other flasks whose pellets were harvested later as compared to the earlier experiments that had higher protein production.

We also expected to have low expression in one of the experiments whereby the flask was fed with growth serum free medium since we thought the fresh medium could interfere with the cells and maybe break them off and disrupt the RNA production in the cells. This was thought that feeding the cells with just free serum medium, could bring contamination and cause RNA to break because the single stranded RNA is very fragile, but this wasn't significant, and therefore this experiment produced the same amount of RNA when compared with other cells that got fed normally and the pellets harvested later and we speculated that titin production had attained the plateau stage when the medium was switched from low growth serum to serum free medium.

C2C12 myoblast cells, only N2A titin isoform is expected to be expressed in skeletal muscle cells but during our study, the expression of both N2A and N2B were observed. N2B is expected to be expressed in the cardiac. N2B was highly expressed in skeletal muscle compared

to N2A unexpectedly. We speculate that during myogenesis, the two isoforms are found all over the body and after maturation they settle at one point of the body.

During our study, we found that our findings correlate with other people's findings. Titin was lowly expressed when the growth serum was lowered and we speculated that this could have occurred due to sarcomerogenesis that got disrupted and the protein formation was poorly expressed. These findings could be compared with Person et al., 2000. His group found that the interference with titin translation by antisense S-ODN, consistently disturbed the incorporation of the myosin filament into the sarcomere. We also observed different peaks at different trials, these results gave us an observation that other cells took long during confluency before the cells got fed with low serum medium and after the switch of growth medium to differentiation medium. We also observed different expression in titin all the three trials. In trial #1, the peak was t=0, and trial #2 was at t=2 and trial #3 the peak was at t=12. We gave an observation that low serum medium does not trigger myogenesis since and while still the cells at high serum medium, they would still differentiate into myotubes. We also speculated that during quantitation, in trial #3, the amount of RNA was used in excess and that's why titin it was highly expressed at t=12. The high expression of titin at different time points gave us another observation that much production of titin gene in myogenesis is as result of the sarcomere being in stable condition and this leads to maturation of the muscle cells without any defects.

Future work

The focus should be on gene study on the cells fed with just high growth serum throughout the experiment because they produced mature muscle cells than expected. The focus should also be on the addbacks experiments and since there was no difference observed in terms of gene expressions with other experiments.

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Chapter 6. Appendices

APPENDIX 1 a-Materials

Mammalian Cell Culture	
Product Name	Manufacturer
C2C12 (ATCCR CRL-1772™)	American Type Culture Collection (ATCC) Manassas, VA
Medium 199	Bio Whittaker Walkersville, MD
Dulbecco's Modified Eagle's Media (DMEM)	American Type Culture Collection (ATCC) Manassas, VA
Fetal Bovine Serum (FBS)	American Type Culture Collection (ATCC) Manassas, VA
EDTA	Fisher Scientific
Penstrep	Corning Life Sciences
Nuclease Free Water	Amresco
Trypsin	Amresco

Appendix 1b

RNA Quantitation	
Equipment Name	Manufacturer
NanoDrop	Thermo fisher Scientific Inc.

Gel Electrophoresis	
Product Name	Manufacturer
25X TAE Buffer	Amresco
Agarose I Tablets, 500mg	Amresco

Gel Analysis	
Equipment	Manufacturer
Pharos FX Plus Molecular Imager	Bio-Rad Laboratories

RNA Extraction	
Product/Reagents	Manufacturer
RNeasy Mini Kit	Qiagen, Inc.
OneStep RT-PCR Kit	Qiagen, Inc. Valencia, CA
iTaq$_{tm}$ Universal SYBER® Green One Step Kit	Bio-Rad Laboratories
iScript™ Select cDNA Synthesis Kit	BioRad
GoTaq®Green Master Mix	Promega Corporation

Appendix 1c

Mammalian Cell Culture	
Media	Chemical Composition
High Serum Growth Medium	500ml DMEM 100ml FBS 5ml Pen-Strep
Low Serum Differentiation Media	500ml DMEM 5ml FBS 5ml Pen-Strep

Appendix 1d Time Course Calendar

Gene Expression Time Course (14 Days)		
Sample Time Point	No.of Days Fed with Low Serum	Harvest Time
T= 0	None	Day 0
T=2	0 Days	Day 2
T=4	2 Days	Day 4
T=8	4 Days	Day 8
T=12	5 Days	Day 12
T=14	6 Days	Day 14

Appendix 1b-Time Course Calendar

Gene Expression Time Course Calendar (14 Days)				
Sample Time Point	No. of Days Fed with Low Growth Serum	No. of Days Fed with High Growth Serum(Add-back)	No. of Days Fed with Serum Free media	Harvest Time
AB4	2 Days	5 Days	None	Day 14
AB8	4 Days	3 Days	None	Day 14
AB12	5 Days	1 Day	None	Day 14
NYA	4 Days	None	2 Days	Day 14

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Review Article

Roles of Titin in the Structure and Elasticity of the Sarcomere

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