Changes in gene expression in C2C12 cells in response to changes in culture conditions, the cellular niche.

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Abstract

C2C12 cells are an immortalized mouse myogenic stem cell line, a model for muscle development. Expression of MyoD, Cdk1, and Titin are good genetic markers for cell cycle, myogenesis and sarcomerogenesis, respectively. This study was conducted to evaluate the effects of different growth media on myogenesis and gene expression during myogenesis. The various serum-based media used are Dulbeccos's Modified media base media supplemented with, either Fetal Bovine Serum (FBS) (10% or 1%) or Horse Serum Media (10% or 1%). In addition to these undefined media (serum) we also looked at defined media, PC-1 defined media (transferrin, insulin like growth factor). The effects of the different medias on C2C12 cell morphology and the gene expression of MyoD and the titin isoforms was determined. Cells were grown over a 12-day time course study with imaging, media change, and pelleting cells for mRNA isolation. Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR) analysis was carried out to measure expression of the specific myogenic genes mentioned above and correlated to changes in morphology. In addition to the time course study a switch-back experiment was done on days 2 and 8 where the cells were changed from their experimental media back into the control 10% FBS. Images and pelleting of cells was done 2 days post-switch-back. The results show changing culture conditions leads to an alteration in cell morphology and genetic expression in C2C12 cells. Cells will proliferate and grow into normal myoblasts however; differentiation does not normally proceed. Myotubes are observed to differentiate but do not do so in the normal fashion or density. In normal differentiation myotubes are observed in long dense sheets of muscle fibers. In the switch-back experiments cells in the cultures did not differentiate normally and were at low densities. When switched back into 10% FBS the density increased and myotube development begins to resemble the controls. To confirm that the cells at confluency are no longer in the cell division cycle and have started differentiation, flow cytometry analysis on pre-confluent, confluent, and post-confluent C2C12 cells was done. These results agree with the previous observation of a dramatic decrease in cdk1.

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Chapter 1: Introduction.

1.1 Skeletal Muscle Overview.

The average adult human body is made up of approximately 40-50% muscle tissue. Muscle aids in maintaining homeostasis, enabling body movement, moving substances through the body and maintaining body temperature by producing heat. There are three types of muscle, smooth muscle, cardiac muscle, and skeletal muscle. Smooth muscle, also known as visceral muscle, is found in organs, blood vessels and the iris of the eye. Smooth muscle is the earliest form of muscle, it is made up of sheets of long, mononucleated, spindle-like cells. Smooth muscle contraction is regulated by nerves and they contract as a unit. Cardiac muscle is found in the heart, they are made up of small, interconnected, mononucleated cells and have a striated appearance. Cardiac muscle contraction is spontaneous and is regulated via the nervous system, allowing for synchronous contraction (Perlingeiro, R. R. C., & Maglil, A., 2017 Skeletal muscle is similar to cardiac muscle in that it is also striated and is regulated via the nervous system. Unlike cardiac muscle, it cannot generate electrical impulses spontaneously and it is multi-nucleated.

Skeletal muscles arise during embryonic development from mesodermal cells, termed myoblasts. Formation of myofibers and myocytes involves cell fusion and changes in gene expression. This fusion explains skeletal muscle`s multi-nucleated morphology. Once myoblasts have ceased fusion, the mature myocyte loses its ability to divide. Therefore, the number of muscles an adult has is predetermined early in development.

Muscle mass can increase through life via enlargement and training of existing muscles. Very few myoblasts continue to exist inside of mature muscle cells however those that do, are referred to as satellite cells. These satellite cells obtain the ability to fuse and form muscle, therefore being able to repair damage and be involved in regeneration. However, there is a limited number of satellite cells and extreme damage cannot be repaired (Crawford, G. L., & Horowits, R., 2011).

Skeletal muscles are made up of muscle fibers which contain bundles of myofibrils. Myofibrils are a contractile organelle and extend the entire length of the muscle fiber. Myofibrils are located in the sarcoplasm, which contains other organelles and a significant amount of glycogen and myoglobin (Crist, C., 2016). Each myofibril is encircled by the sarcoplasmic reticulum, a modified version of the endoplasmic reticulum, which, when muscle is relaxed, stores calcium. Myofibrils are further broken down into two types of filaments, the thick filaments and thin filaments. Thick and thin filaments overlap each other to varying degrees depending on what state muscle is in: relaxed or contracted. Filaments are involved in muscle contraction but do not extend the entire muscle fiber. Thin filaments are light in color and thick filaments are dark in color, giving skeletal muscle its striated appearance. Filaments are compartmentalized into sarcomeres, the functional unit of myofibrils. Within the sarcomere are the A-bands and the I-bands. A-bands are positioned in the middle of the sarcomere and are made up primarily of thick filaments, with a small area of overlap where thin and thick filaments lie side by side. Positioned in the middle of the A-band is a lighter region called the H-zone. The H-zone, in relaxed muscle, has no thin filament overlap but thin filaments will be found in this region when muscle contracts. Thick filaments are anchored to the M-

line, the final region of the A-band. The M-line is in the middle of the A-band and houses proteins that help anchor the thick filament. I-bands are positioned on the outside of the sarcomere and contain the majority of the thin filaments. The A-bands and I-bands are separated by a disc of protein called the Z-line. The Z-line is where thin filaments are anchored, it also distinguishes one sarcomere from another (Garcia-Pelagio, K. P., et al., 2011). Figure 1 shows the alignment of a sarcomere at rest.

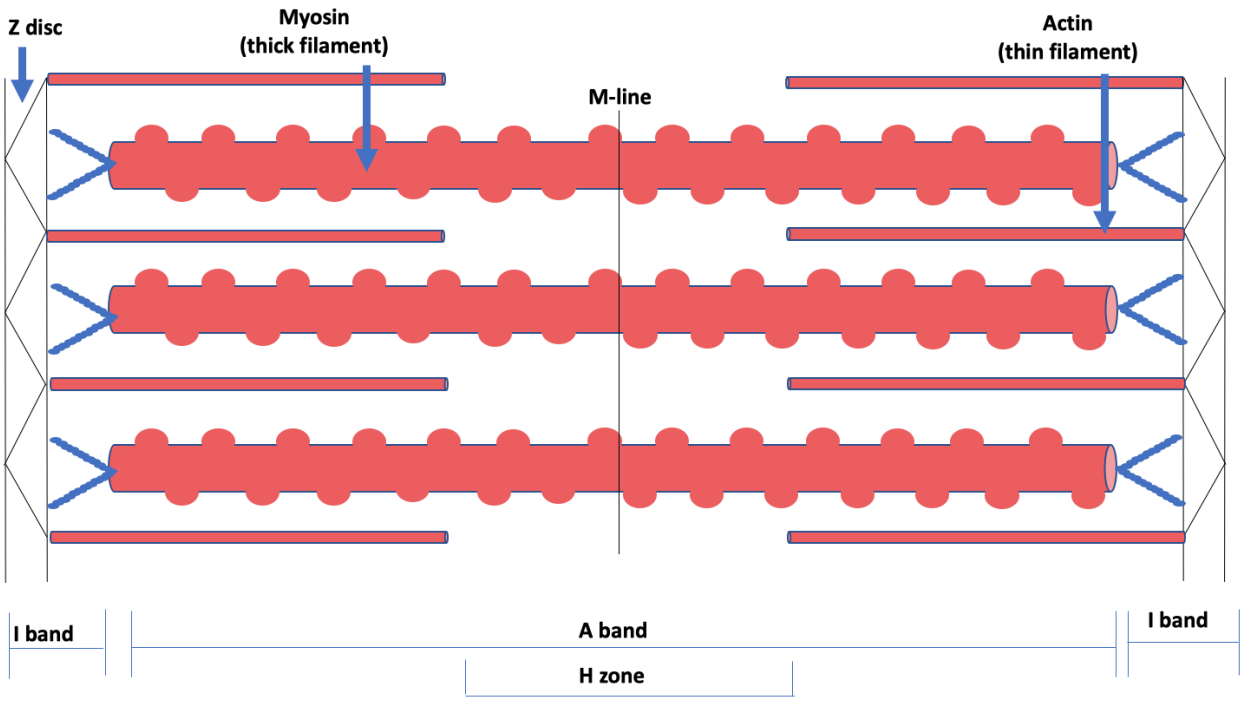


Figure 1: Sarcomere alignment, at rest.

1.2 Titin and its function in skeletal muscle.

Contractile proteins of several types operate during the contraction cycle. There are three types of proteins found in muscles, structural, motor and regulatory, all providing a different function. The motor proteins generate force and movement, in muscle these are the myosins, a super-family of proteins consisting of many isoforms. Myosin is the main component in the thick filament. It is composed of a tail domain and two myosin heads that bind to myosin-binding sites on actin during contraction. Myosin`s main function is

to produce force. Next, the regulatory proteins are responsible for muscle contraction regulation. These proteins include troponin and tropomyosin. Tropomyosin is a component of the thin filament, it blocks myosin-binding sites on actin when muscle is relaxed. Troponin is also a component of the thin filament. When calcium binds, troponin undergoes a conformational change and will move tropomyosin, allowing for myosin to bind to actin. Lastly, the structural proteins, which generate force during contraction and provide stability. Some of the structural proteins found in muscle are actin, desmin, and titin. Actin is the main component of the thin filaments. Actin acts as a static support structure where force is then applied. Titin and desmin are major structural proteins. Desmin functions to reinforce the sarcolemma and integrate the sarcolemma, Z disk, and nuclear membrane in the sarcomere. Titin connects the Z-line to the M-line and helps stabilize the thick filaments position, keeping the thick and thin filaments in alignment. Titin is also responsible for much of the myofibril's elasticity and extensibility (Crawford, G. L., & Horowits, R., 2011).

Titin is the largest known protein, consisting of 364 exons and transcribing over 100kb of mRNA (Nieva-Sousa, M., et al., 2015). Titin works as the architectural protein of muscle, providing attachment points for proteins. Titin also works as a template for thick filament assembly, sarcomeric stability and a sensory and signaling mediator. Many of titin`s functions, however, are still hypothetical or unknown due to its vast size. Titin`s structure is highly repetitive. It is made up of 152 immunoglobulin (Ig) like domains, 132 fibronectine 3 (Fg3) domains, 19 kelch domains, 14 tetratricopeptide repeat domains and 15 solenoid domains (18). Titin is also broken up into bands and regions, the I-band, A-band, Z-disk, and M-line regions make up Titin. The I-band region of titin is made up of

exclusively Ig-like domains. These domains form the proximal tandem Ig segment near the Z-disk and distal tandem Ig segments near the A-band. Those segments, along with the unique N2B sequence and the PEVK region make up the molecular spring segment of titin (Figure 2). The spring region of titin provides the elasticity for the protein which, in turn, enables titin to maintain muscle elasticity during contraction (Frontera, W. R., & Ochala, J., 2015).

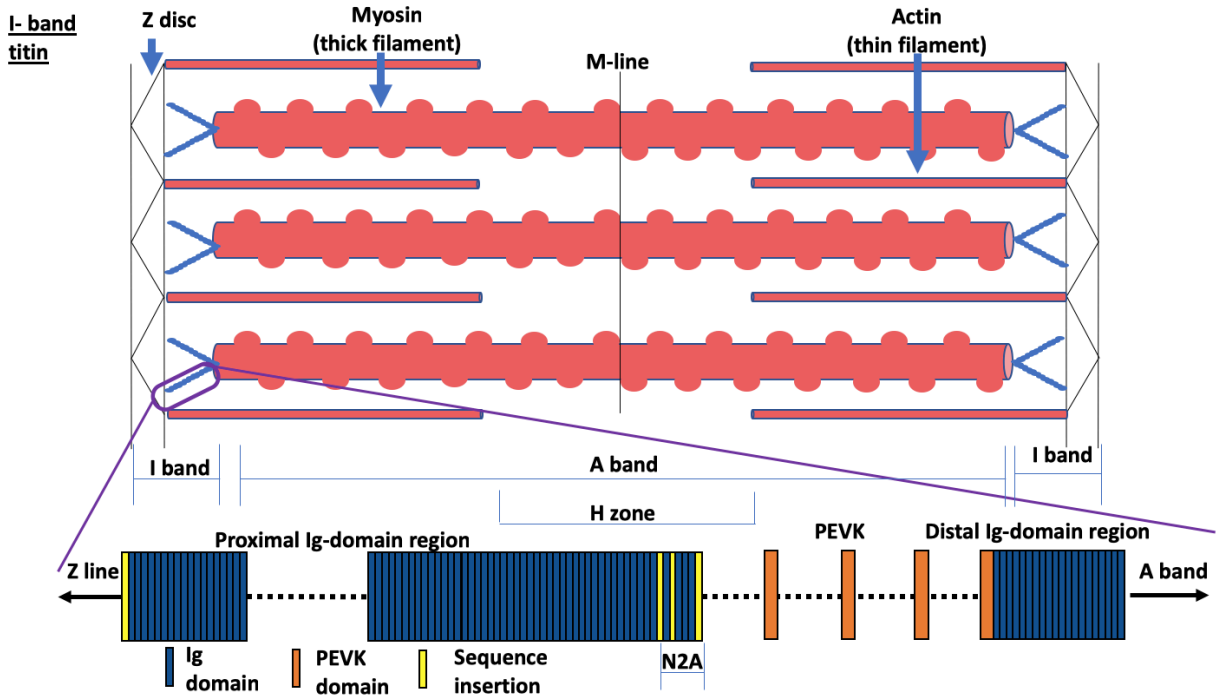


Figure 2: I- band titin

The A-band of titin is the stabilizer (Figure 3). The A-band region is made up of 100 residue Ig-like and Fn3 domains. These domains are super repeated into two types of repeats, a 7-domain stretch occurring 6 times, or an 11-domain stretch occurring 11 times. The muscle ring finger (MURF) protein family, which is expressed throughout muscle development, bind at the end of the A band. MURF 1 and 2 link to other regions of titin however, there are no clear indications that MURF 3 is involved with titin. The z-disk of titin secures titin to the sarcomeric z-disk through protein binding. The z-disk

contains multiple Ig-like domains, interdomain sequences (IS) and up to 7 z-repeats. These z-repeats can vary in number from 2-7 depending on the type of muscle, the age of

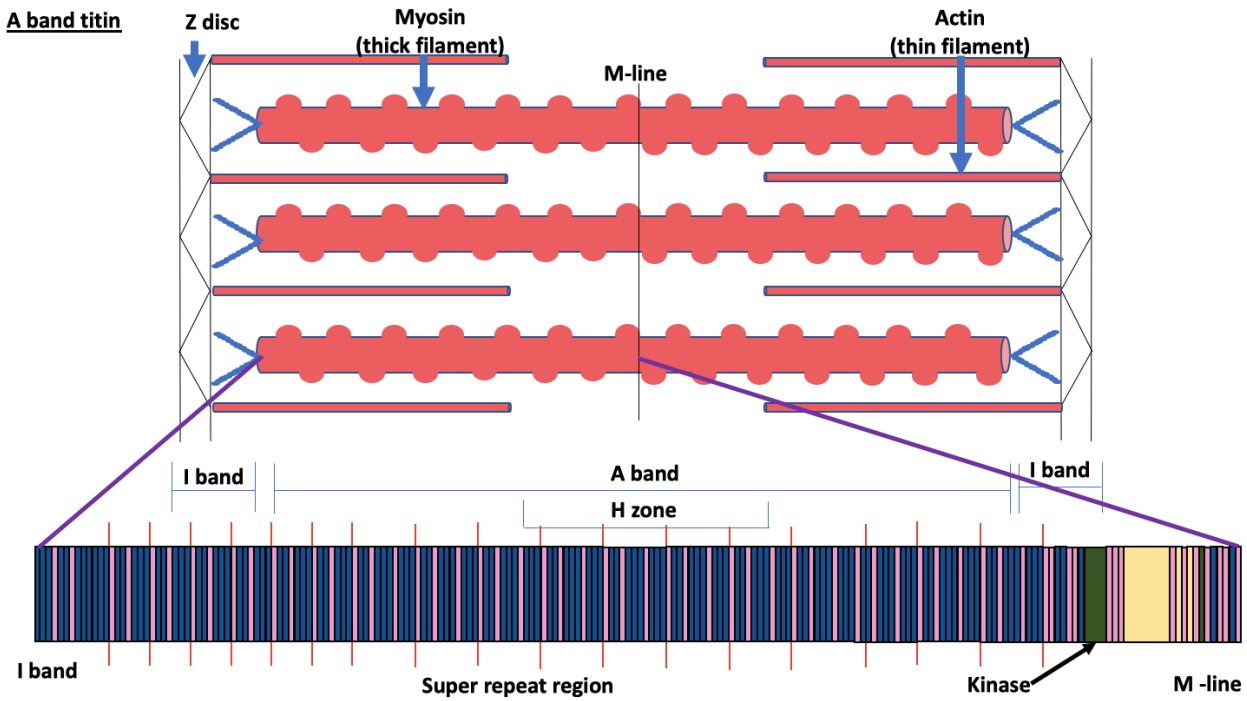


Figure 3: A-band titin

the specimen, and the species being studied (Li, H., et al., 2002).

The role of Titin is one that is widely discussed and studied, but not well understood. There have been many studies on Titin and its functions, however due to its large size and overlapping domains, many of its functions are yet to be well understood. There have been many studies where the focus was to understand titin function and how its many domains regulate sarcomerogenesis, as of yet there have not been any definitive studies to comprehensively explain the roles of titin. For example, Buck, et al. (2014) sought to investigate the effects a 9 Ig region domain deletion had on skeletal muscle (Buckingham, M., & Vincent, S. D., 2009). When knocking out that region of titin the observation within their mouse model showed an increase in whole muscle passive stress

and an altered expression of titin-based signaling proteins. Which further explains the importance of the Ig domains in stiffness and extensibility. In the same year a paper published by Granzier et al. (2014), studied the I/A junction of titin and how its deletion would affect thick filament length. The study deleted this region and found no disruptions in sarcomerogenesis or thick filament length. The singular change in function observed was an increase in diastolic stiffness within the left ventricle of the heart. These two studies demonstrate that titin functions as an important muscle factor yet still leaving its complete functional capabilities not well known (Kruger, M., & Linke, W. A., 2009). Titin is a well-known protein in muscle function, myofibrils also play an essential role in muscle function and contraction.

Titin, obscurin, desmin and nebulin potentially play key roles in the formation of the sarcomere, called sarcomerogenesis. Titin, the largest protein and third most abundant in skeletal muscle, stretches half a sarcomere extending from the M-band to the Z-disk. Titin is suggested to be the molecular ruler of sarcomerogenesis. As Titin has distinct domains that correspond to specific areas of the sarcomere it is suggested that titin forms before sarcomere and myofibers form and it is used a molecular ruler or scaffold for sarcomere formation. It is suggested that titin plays a key role in sarcomere and myofibril formation, but it is unknown whether it is the exact ruler for sarcomere formation. This is unknown due to titin`s size and its other functions within mature muscle. In vivo studies done to knockdown or over express titin have had effects on the viability of subjects, complicating the process of understanding it`s function. Research has been done on titin mutations in attempts to shed light on how this may affect sarcomere organization and function. For example, an article published in Science in 2015 investigated the effects

that titin mutations in induced pluripotent stem cells have on sarcomere organization and function. This study observed that titin mutations in the A- band and two missense mutations in the Z/I junction resulted in sarcomere abnormalities. Sarcomere organization in the mutated cells is reduced compared to wild type as well as sarcomere length being inhibited. Finally, mutated cells had considerable contractile defects. These results agree with the idea that titin is integral in sarcomere alignment and function (Hinson, J. T., et al, 2015). Research is also being done on desmin mutations or losses and also suggests and important role in sarcomerogenesis. Brodehl, A., et al., investigated desmin mutations and how it is related to skeletal myopathies. Abnormalities in actin filaments and cytoskeletal organization were observed (Brodehl, A., et al., 2018). This suggests that desmin is important in cytoskeletal and filamentous organization, diverging from the previous notions that it is involved in sarcomerogenesis. Obscurin and nebulin are suggested to aid titin in formation of the sarcomere in the later stages of sarcomerogenesis but this, as of yet, has not been able to be observed (Kontrogianni-Konstantopoulos, A., et al., 2009).

When a muscle contracts myofibrils contract and shorten, myofilaments do not shorten. Instead filaments slide relative to each other during contraction, which is referred to as the sliding filament model. Thin filaments will slide deeper into the A-Bands and eventually into the H-zone. I-bands will narrow, and the A-bands are brought closer together. Thin and thick filaments overlap and partially touch on each side of the A-band. At rest muscle thin filaments do not continue to the center of the H-zone. The filaments sliding explains the mechanism of contraction based on thick and thin filaments sliding and proteins binding and creating cross bridges. In the mechanism of contraction myosin

must bind to actin. To bind to actin, myosin hydrolyzes ATP and undergoes a conformational change and moves into an energized state. In its energized state myosin binds to actin and forms the cross-bridge. Power stroke then occurs with myosin returning to its original state, releasing ADP and phosphate. ADP and phosphate combine to create ATP which is then hydrolyzed by myosin. This cycle will repeat until the muscle returns to a relaxed state (Irving, M., 2017). ATP is not the only critical component of the cross-bridge cycle, calcium plays a critical role in myosin preparation.

When in a relaxed state myosin is blocked from binding to actin by tropomyosin. Tropomyosin must be moved for the cross-bridge cycle to begin, this requires troponin. Troponin holds actin and tropomyosin together and requires calcium to bind to undergo a conformational change and move tropomyosin from blocking myosin. When a muscle is stimulated to contract muscle fibers depolarizes, this is transmitted deep into the muscle fiber by invaginations in the sarcolemma called the T-tubes. The depolarization of the T-tubes causes calcium channels in the sarcoplasmic reticulum to open. Calcium is then released into the sarcoplasm and diffuses into the myofibrils, binds to troponin, moving the troponin-tropomyosin complex, allowing for myosin and actin to bind and contraction to begin. Calcium`s involvement in contraction is called excitation-contraction coupling, it is one of the regulatory mechanisms of muscle contraction. Calcium is also important to Titin in the way that it binds to actin and plays a role in stiffness. Calcium and ATP are important chemical signals for muscle contraction, there are also nerve impulses regulating muscle.

When muscles are stimulated, they are stimulated by motor neurons. Skeletal muscle is stimulated by somatic motor neurons. The somatic motor neurons nerve fibers,

called axons, extend from the neuron cell body and branches out. These axons form junctions, called synapses, with multiple muscle fibers, called the neuromuscular junction. One axon can stimulate many muscle fibers and a single muscle fiber can be stimulated by more than one motor neuron. A set of muscle fibers innervated by all the axon branches of a motor neuron is called a motor unit. Muscle being categorized by motor units allows for muscle contraction to be graded by the type of movement needed. Fine movement like writing will have smaller motor units, this means there are fewer muscle fibers per neuron. A more powerful muscle contraction that does not require fine movements, such as kicking a ball, will have more muscle fibers per neuron. Motor units can also be selectively activated depending on the type of movement needed, called recruitment. A small movement for instance tapping of toes, will activate a smaller number of motor units. When the body requires a larger movement such as running, more motor units are activated. Muscle contraction is stimulated by a neuron delivering an electrochemical impulse. Once the impulse is sent the motor neuron releases acetylcholine (ACh) at the neuromuscular junction. This influx of ACh binds to receptors on the muscle fiber, opening sodium channels and depolarizing the muscle fiber membrane. Depolarization impulses are then carried through the muscle fiber via the t-tubes which then conduct the sarcoplasmic reticulum to release calcium and the excitation-contraction coupling mechanism begins.

1.3 Gene Regulation of Myogenesis.

The pathway of skeletal muscle embryonic development begins with embryonic mesodermal tissue. Most muscles are developed from the paraxial mesoderm, which produces somites. Somites, embryonic structures that butt off from the unsegmented

paraxial mesoderm, are the source of all skeletal muscle in trunk, limb and, some head muscle (Cao, N., et al., 2016). There is a range of somite stages within the embryo. The somite begins development at the anterior of the embryo and adds posteriorly. This means that anterior somites are older and more advanced than posterior somites. The anterior somites also begin expressing myogenic markers sooner than the posterior somites. Inferior to the new somites is the pre-segmented paraxial mesoderm. The mesoderm continues to segment and give rise to more posterior somites. As somites develop they segment into distinct compartments that give rise to distinct tissues. These compartments include the sclerotome, which produces bone, the syndetome, which produces axial tendons, and the dermomyotome (Mok, G. F., & Sweetman, D., 2011). This is demonstrated in Figure 4. The dermomyotome is where the first myogenic markers PAX 3 and PAX 7 (PAX3/7) can be detected. PAX3/7 are the first molecular markers that label muscle precursors. Their expression is induced by surrounding tissue signals. These signals label precursors of skeletal muscle and with PAX3/7, form a regulatory network to regulate myogenesis. Early muscle cells are formed independent of PAX3/7 but they are important in maintaining the myogenic program. Mutations in

PAX3/7 result in major defects, supporting that they are required for normal muscle development (Jiwlawat, N., et al., 2018).

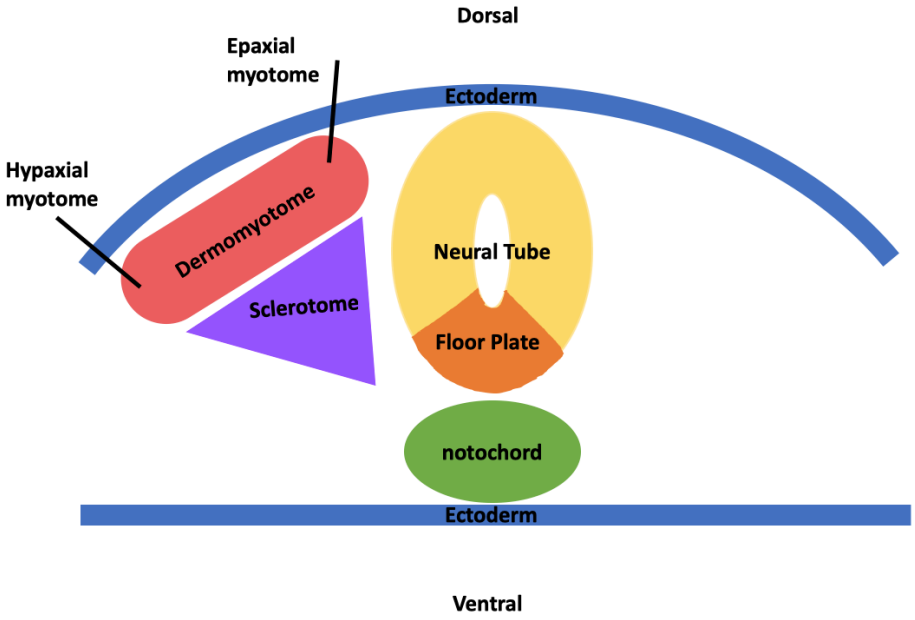


Figure 4: The somite, pre-division of the dermomyotome.

Once myoblasts have been induced, they migrate around the edge of the dermomyotome. The dermomyotome segregates to form the myotome, where myogenic differentiation is first observed, and the dermatome, where the dermis originates from. The segregation of the dermomyotome begins in the dorsomedial lip of the structure (Cao, N., et al., 2016). This is seen in Figure 5.

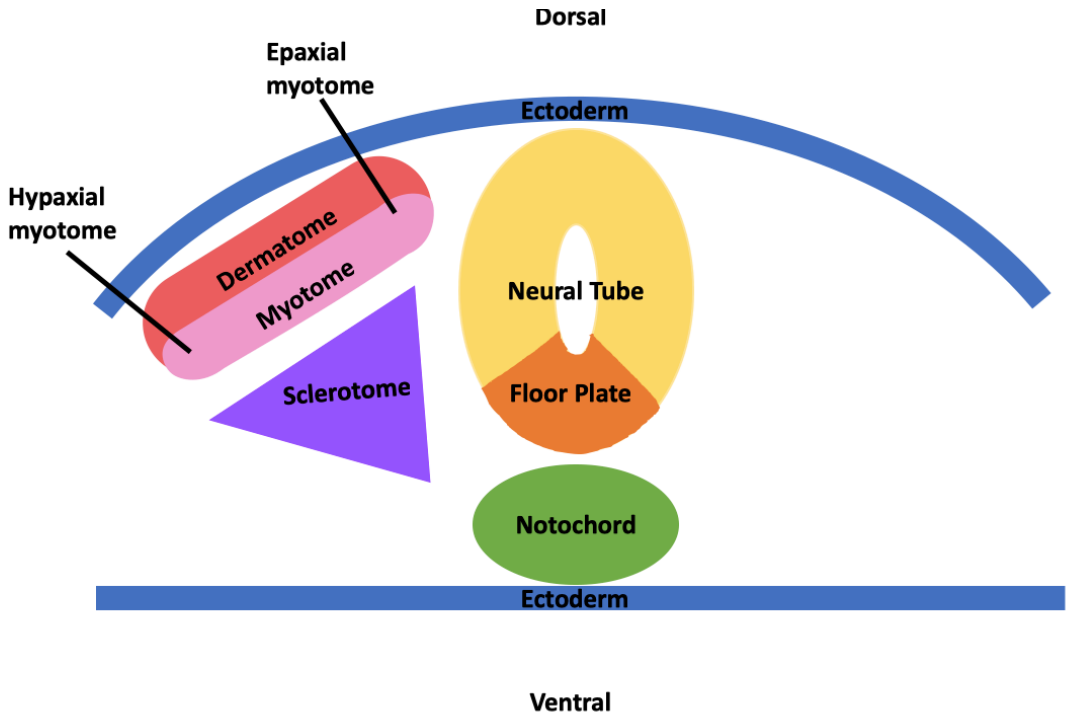


Figure 5: The somite, showing the division of the dermomyotome into the dermatome and myotome.

Myoblasts will migrate ventrolateral from the lip and form the epaxial myotome, a layer of cells lying just under the dermomyotome. Next, myoblasts migrate from the ventrolateral lip and lateral edges of the dermomyotome and produce the primary myotome. Ventrolateral lip myoblasts help form the hypaxial myotome, while lateral edge myoblasts contribute to both the epaxial and hypaxial myotomes. The hypaxial myotome and epaxial myotome is where myogenic differentiation begins, as cells exit the cell cycle, express muscle-specific markers and down-regulate PAX3 (Murphy, M., & Kardon, G., 2011). One of the earliest muscle-specific markers, Myf5, marks muscle-determined cells. Myf5 is closely followed by MyoD. At later stages, central dermomyotome cells move into the myotome, instead of migrating, and form satellite cells (Zammit, P. S., 2017).

The myotome is divided into two regions, the hypaxial myotome and epaxial myotome. The two regions give rise to different muscles of the body. The epaxial myotome, located dorsally in the somite, gives rise to the deep muscles of the back. The hypaxial myotome, located ventrally in the somite, gives rise to the muscles of the body wall and limbs (Li, Y., et al., 2018). These two regions of the myotome have no visible morphological division but can be distinguished by expression of makers like EN1, an epaxial marker, and SIM1, a hypaxial marker. Muscles in the epaxial and hypaxial myotomes are induced via signaling events from nearby tissues such as the neural tube, notochord, overlying ectoderm and lateral mesoderm. For example, signals from the dorsal neural tube, such as WNT1 and WNT3A, and the floor plate of the neural tube and notochord, like Sonic hedgehog protein, initiate myogenesis in the epaxial myotome. There are also inhibitory signals produced from these tissues. Bone morphogenetic protein (BMP), for instance, is produced in dorsal somites but when expressed in the dorsal neural tube, inhibits myogenesis. Hypaxial muscles, however, require BMP and WNT to induce myogenesis (Endo, T., 2015).

Cells differentiating into muscle is activated by myogenic regulatory factors (MRFs), a set of unique transcription factors. The MRFs, a group of four basic helix-loop-helix transcription factors, initiate myogenesis. All four MRFs (Myf5, MyoD, Mrf4 and myogenin) share the ability to initiate myogenesis even when expressed in non-muscle cells (Parada, C., et al., 2012). Each MRF also have a distinct function along with initiating myogenesis, some overlap in function does also occur. Allowing for the up regulation of one factor in the absence or loss of another. MRF expression is seen throughout embryonic muscle, with a time difference in when each MRF is expressed

(Siparsky MD, P. N., et al., 2014). For example, Myf5 is expressed first in myogenesis and all others are induced by this expression and a series of signaling events. Activation of an MRF will often result in the activation of the others, as a way for the myogenic program to lock-in and shut down any other determination pathway. Myf5, is the exception to this. As Myf5 begins myogenesis, it is regulated by enhancers on DNA, each driving expression of a set of myogenic cells (Volpi, M. V., & Hughes, S. M., 2017). The differentiation and determination of muscle precursors is a highly regulated and exacting process, any changes to signaling or cellular conditions can throw off the sequence.

To understand genetic expression of myoblasts and their dependence on cellular environment, known as the cellular niche, a study on the effects of different serum conditions is to be done. Several different types of serum are being utilized to determine how changing the culture conditions *in vitro* can change the morphology and/or genetic expression of myoblasts. C2C12 cells will be used as a model system for muscle development in culture. C2C12 cells are an immortalized mouse muscle cell line. Fetal Bovine Serum (FBS) a complete undefined media, at 10% concentration, is used as a control as it is known to support myogenesis (Nyaboke, R., et al., 2017). Horse Serum (HS) a complete undefined media, at 1% and 10% concentrations, and PC1 and complete, defined media are being used as experimental media. Horse Serum is defined in previous studies as a differential media, meaning it is used to supplement FBS to initiate differentiation of myotubes (Buck, D., et al., 2014). Currently, PC1 has not been used in C2C12 myoblast cultures in an experimental fashion and it is not known whether it will support myogenesis. Frozen myoblasts are thawed and placed in an initial culture of 10% fetal bovine serum, to stimulate growth. The cells are then split and placed into their

respective media. The myoblasts are grown over a 12-day time-course study, being pelleted and fed every two days. Also done when cells are fed and pelleted are micrographs of the culture to document any morphological changes in the cells. The pellets are isolated are frozen and stored at -80ºC until RNA isolation and analysis can be done. Once RNA is isolated from the pellets, qRT-PCR analysis is done to analyze gene expression, specifically titin isoform N2B and MyoD. When analyzing gene expression, a normalizing gene Glyceraldehyde 3-phosphate dehydrogenase is utilized, as it is expressed in all cells. It is expected that Horse Serum and PC1 will not support myogenesis, qRT-PCR will be used to verify this. In addition to the time course study a switch-back experiment was done on days 2 and 8 where the cells were changed from their experimental media back into the control 10% FBS. A visual graphic showing the experimental design of the culture experiments is seen in figure 6 below. Images and pelleting were done after 2 days in these experiments. To complement the culture experiment, flow cytometry analysis on pre-confluent, confluent, and post-confluent C2C12 cells was done to analyze the cell cycle of proliferating and pre-differentiating myotubes.

1.4 Research Aims

The aim of this study is to determine the role of changing cellular environment on myoblast C2C12 differentiation. The more that is understood of myoblast differentiation and control *in vitro* the easier treatments will be to combat satellite cell reduction related to aging muscle *in vivo*.

1.5 Hypothesis

When changing the cellular environment via changing of culture conditions the gene expression of both structural as well as regulatory genes is altered, leading to changes in cellular phenotype.

1.6 Experimental Design

1. Change the C2C12 cell culture conditions
2. Observe changes in C2C12 cell morphology due to the change in culture conditions.
3. Observe changes in C2C12 cell development and differentiation due to changes in culture conditions.
4. Observe changes in gene expression due to changes in culture conditions.
5. Observe the C2C12 cell cycle utilizing flow cytometry

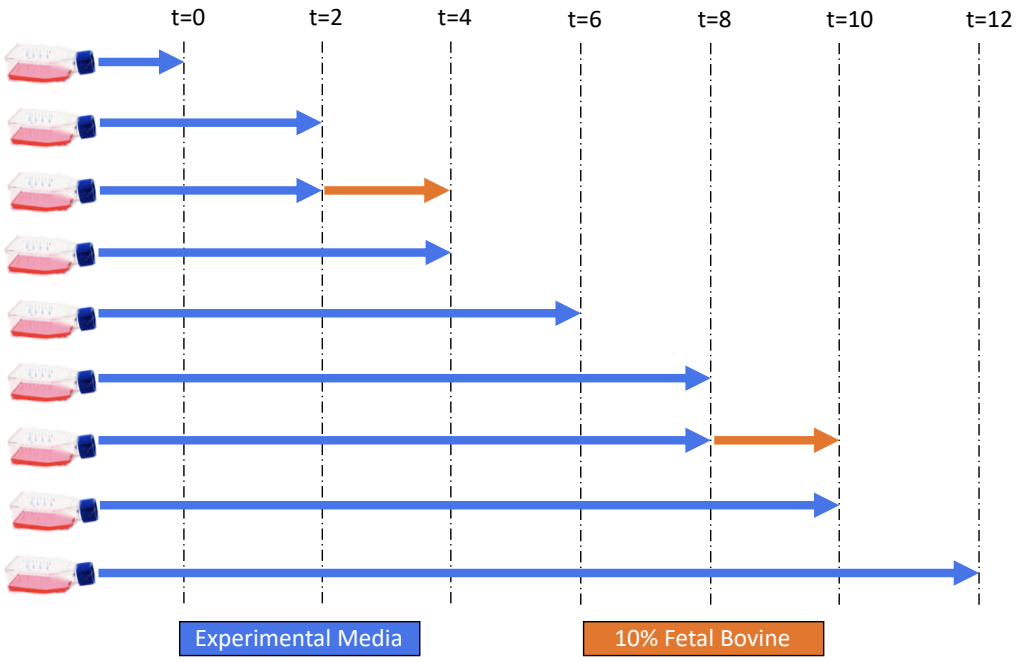


Figure 6: Visual representation of culture experiments done with experimental media as well as switch back experiments.

Chapter 2 Methods

2.1 Cell Culture

2.1.1 Initial Cell Culture

C2C12 mouse myoblast cells, stored in liquid nitrogen in complete growth media containing 5% DMSO cells are thawed quickly in a 37˚ C water bath. They are then transferred to a 15mL sterile conical tube, 1 mL C2C12 growth media (10% FBS) is warmed to 37˚C and then added. Cells are incubated at room temperature (25˚C) for 10 minutes. Growth media is added at a 1:1 ratio and incubation at room temperature for 10 minutes in between until a final volume of 8mL is achieved. This final volume is then transferred to a 75cm^2 flask with 15mL of C2C12 growth media (10% FBS). Cultures are incubated at 37˚C and 5% CO_2 for 24 hours. 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 remained at 37˚C and 5% CO_2.

2.1.2 Sub-culturing of cells

Cells are allowed to reach 80% confluency, averages around 7 days to achieve, and are then split. Media is removed and the cells are washed with 10mL sterile PBS for 10 seconds. PBS is discarded and the cells are treated with 10mL of trypsin and allowed to incubate at 37˚C & 5% CO_2 for 10 minutes. Cells are viewed using the Olympus LH 50A inverted phase contrast microscope to confirm cell detachment. If cell`s detachment is not complete cells are incubated at 37˚C & 5% CO_2 for an additional 5 minutes. Once cell detachment is confirmed, 3mL of the trypsin/cell mixture is transferred to 3 fresh 75cm^2 flasks. 25mL of fresh growth media is added to both the new and original flasks.

Cells are incubated for 24hrs at 37˚C & 5% CO_2. Cells then are viewed using the Olympus LH 50A inverted phase contrast microscope to confirm cell attachment. Every other day following splitting, media is removed and 25mL of CGM is added. Cultures remained at 37˚C and 5% CO_2.

2.1.3 Microscope imaging of cellular morphology

Cells are viewed using the Olympus LH 50A inverted phase contrast microscope at 10x and 1.0x objectives. Cell images are captured using the SPOT imaging software and microscope camera. The microscope is calibrated using a stage micrometer (Baush-Lomb) and images taken at all magnifications.

2.1.4 Time Course Study

Cells are allowed to reach 80% confluency and are split as previously done. Once cell wall detachment is confirmed cells are transferred into 2 sets of new 75cm^2 flasks. Each set of flasks had one flask for each time point (t=0,2,4,6,8,10,12 days) with the exception of t=2 and t=8 days having 2 flasks for the switch-back experiment. 25mL of C2C12 experimental media 1 (10% HS) is added to the first set of flasks. 25mL of C2C12 experimental media 2 (1% HS) is added to the second set of flasks. Each set of flasks had one extra flask for days 2 and 8. All flasks are incubated at 37˚C & 5% CO_2 for 24hrs. Cells are viewed using the Olympus LH 50A inverted phase contrast microscope to confirm cell attachment. Media is changed every other day with either 10% HS or 1% HS with the exception of the extra t=2 and t=8 flasks being changed back to 10% FBS at their respective time points. In conjunction with media change cells are imaged and pelleted at their respective time points. This process is repeated on a single set of flasks for C2C12 experimental media 3, PC1.

2.2. RNA Isolation & Quantitation

2.2.1 Pelleting of Cells

Cells are pelleted at their respective time points. Media is removed and the cells are washed with 10mL sterile PBS for 10 seconds. PBS is discarded and the cells are treated with 5mL of trypsin and allowed to incubate at 37˚C & 5% CO_2 for 15 minutes. Cells are viewed using the Olympus LH 50A inverted phase contrast microscope to confirm cell wall detachment. If cells detachment is not complete cells are incubated at 37˚C & 5% CO_2 for an additional 5 minutes. Once cell detachment is confirmed the trypsin/cell mixture is transferred to 2mL conical tubes and centrifuged at the highest speed for ≥ 10 minutes. Once a compact pellet is achieved the supernatant is discarded and the pellet is used to isolate RNA. If RNA isolation cannot occur the pellet may be stored in -80˚ C freezer.

2.2.2 RNA Isolation

At each time point T=2,2 switch,4,6,8, 8 switch,10,12 days, cell pellets are utilized for RNA isolation. The isolation of RNA was done on an RNase-free bench. Isolation followed the manufacturer`s protocol provided by QIAGEN as shown below.

2.2.3 RNA Isolation Protocol

RNA extractions are performed at time points 0, 2, 2 switch, 4, 8, 8 switch, 10 and 12-days post differentiation. Pellets are removed from -80˚C storage and thawed. Once thawed, cells are re-suspended in 350-600µL of lysis RLT buffer. The lysate is then vortexed and transferred to a QIAshredder spin column in a 2mL collection tube. The column is then centrifuged at full speed for 2 minutes. 350-600µL (equal volume to the RLT buffer) 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. 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. 30-50µL of RNase free water is added to the column and centrifuged at ≥8,000g for 1 minute. 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 1µL of each RNA sample is analyzed, and the readings are recorded in an excel spreadsheet.

2.3. Analysis of myoblast gene expression

2.3.1 Quantitative Analysis of gene expression (qPCR)

qRT-PCR 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 and start the qRT-PCR run. Protocol for qRT-PCR run is reverse transcription 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.

2.4 Analysis of myoblast cell cycle utilizing flow cytometry

2.4.1 Preparation of cultures

C2C12 cells were grown in T-75 Nunc easYFlasks and allowed a 48-hour period to achieve confluency in 10% FBS. At confluency cells were split into 3 flasks and at 24-, 48- and 36-hour time points were analyzed using flow cytometry.

2.4.2 Cell preparation for flow cytometry

At 24-, 48- and 36-hour time points cells were trypsinized. Media was discarded and cells were washed with PBS and then treated with 1mL of trypsin. Cells were incubated for a 15-minute period and cell detachment was confirmed using phase contrast microscopy. Once cell detachment was confirmed 1mL of growth media was reintroduced to the trypsinized cells acting as a neutralizing agent. Cells were then placed into a dilution series in microfuge tubes with 300, 400 and 500uL of cells being neutralized with 200, 100 and 0uL of media. After cells were diluted, DNA staining using 3uL of Vybrant Dyecycle green was done. Also prepared is a dilution of 400uL cells to

100uL media with no Dyecycle added to the tube. The cells were then incubated at 37˚ C for 30 minutes.

2.4.3 Cell analysis using flow cytometry

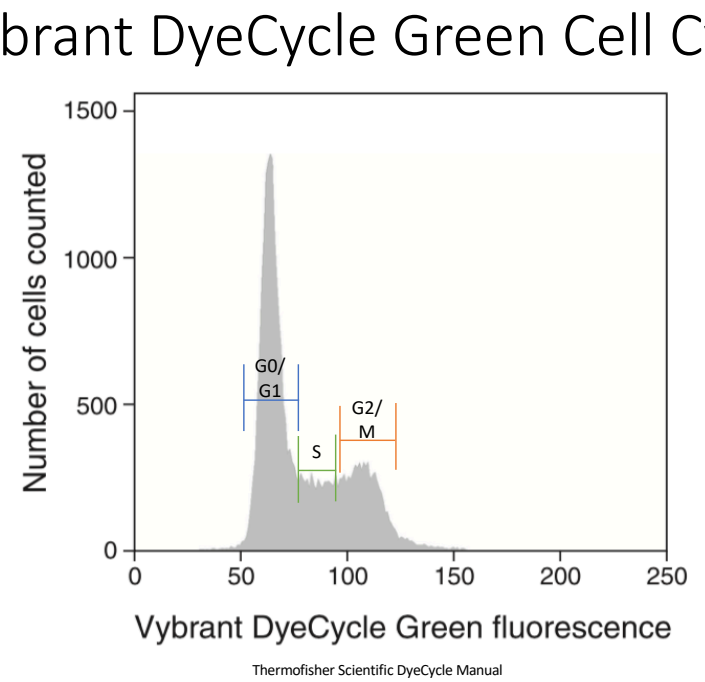
After incubation, cells were analyzed by flow cytometry and compared against unstained cells. All experiments were done on an Attune NxT Flow Cytometer using Attune NxT software.

Chapter 3: Results.

3.1 Cell Cycle Analysis utilizing Flow Cytometry

3.1.1 Vybrant DyeCycle Green comparison chart

Vybrant DyeCycle Green is a DNA dye that permeates the cell membrane to stain the nuclei of living cells. This dye is useful for analysis of cell cycles in living cells due to its precise nature, low cytotoxicity and simple staining protocol. When used for cell cycle analysis the dye has an expected fluorescent pattern for each step in the cell cycle. This expected pattern is what is used to compare samples to be able to visualize what cycle the cells of interest are in. This expected pattern is seen in Figure 6 below.



Thermofisher Scientific DyeCycle Manual

Figure 7: Vybrant DyeCycle Green comparison chart

3.1.2 15% Confluent C2C12 Cell Cycle Analysis

Cells were seeded at post confluent levels in three separate flasks. Cells were allowed to grow for 24, 48, 72 and 96 hours and were analyzed using flow cytometry at

each hour mark. Cells at 24 hours were approximately 15% confluent in the flask. The cells were analyzed, and a live cell count and cell cycle analysis was done. This analysis and image of cells at 15% confluency is seen below in Figure 7.

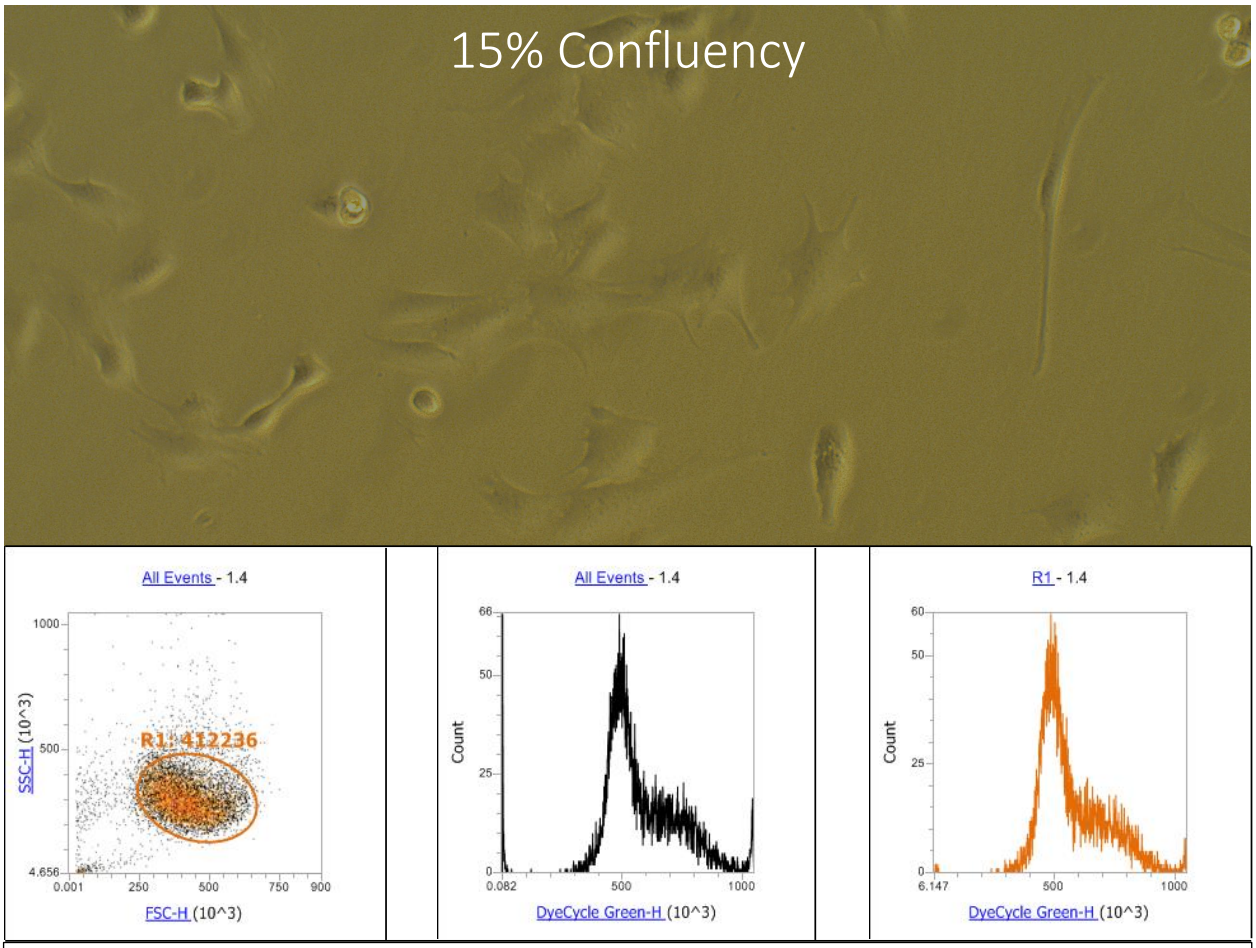


Figure 8: 15% Confluency Cell Cycle Analysis

3.1.3 100% Confluent C2C12 Cell Cycle Analysis

Cells were seeded at post confluent levels in three separate flasks. Cells were allowed to grow for 24, 48, 72 and 96 hours and were analyzed using flow cytometry at each hour mark. At 72 hours cells were approximately 100% confluent. The cells were

analyzed, and a live cell count and cell cycle analysis was done. This analysis and image of cells at 100% confluency is seen below in Figure 8 below.

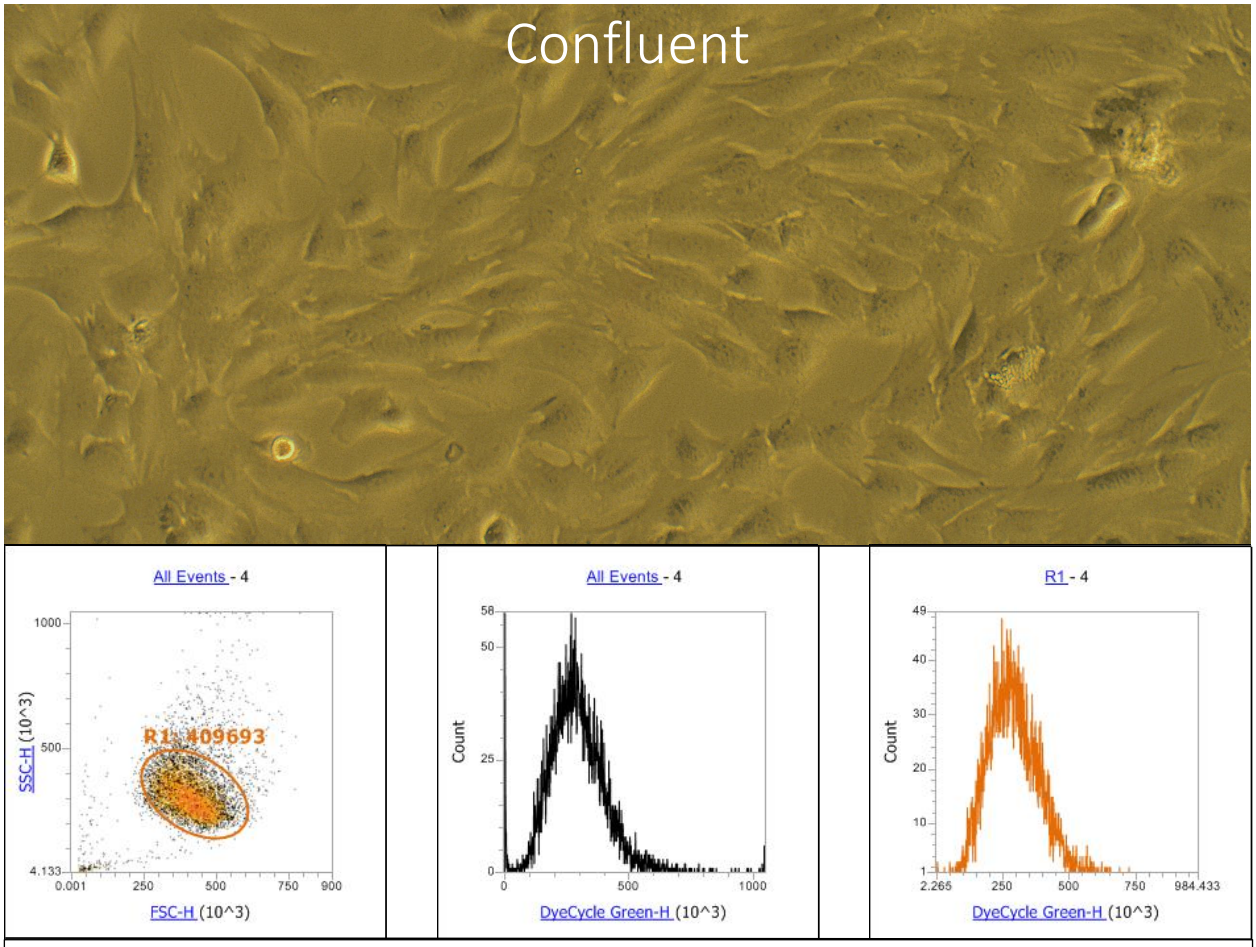


Figure 9: 100% Confluency Cell Cycle Analysis

3.2 Cell Morphology

3.2.1 10% Horse Serum (HS)

In this experiment cells were first grown to confluency in 10% FBS. They were then changed into 10% HS and allowed to grow for a 12-day time course study. A switch-back experiment was also done on days 2 and 8 where cells were switched back into 10% FBS and observed 2 days later. In the early time points t=0 and t=2 there is normal morphology observed in the myoblast cells as seen in Figure 9. At t=4 when

differentiation normally begins and myotubes begin to form, there is a shift in morphology. Throughout the rest of the time course study cells retained their myoblast-like morphology with little to no fusion to form myotubes occurring. The cells appear to attempt to fuse as shown in Figure 9 at t=8 but are unsuccessful, leading to a reduction in cell numbers as shown in t=10 and t=12. The switch-back experiments at t=2 and t=8 show a robust growth change. This is most readily seen in t=8 switch when compared to t=10 in the HS.

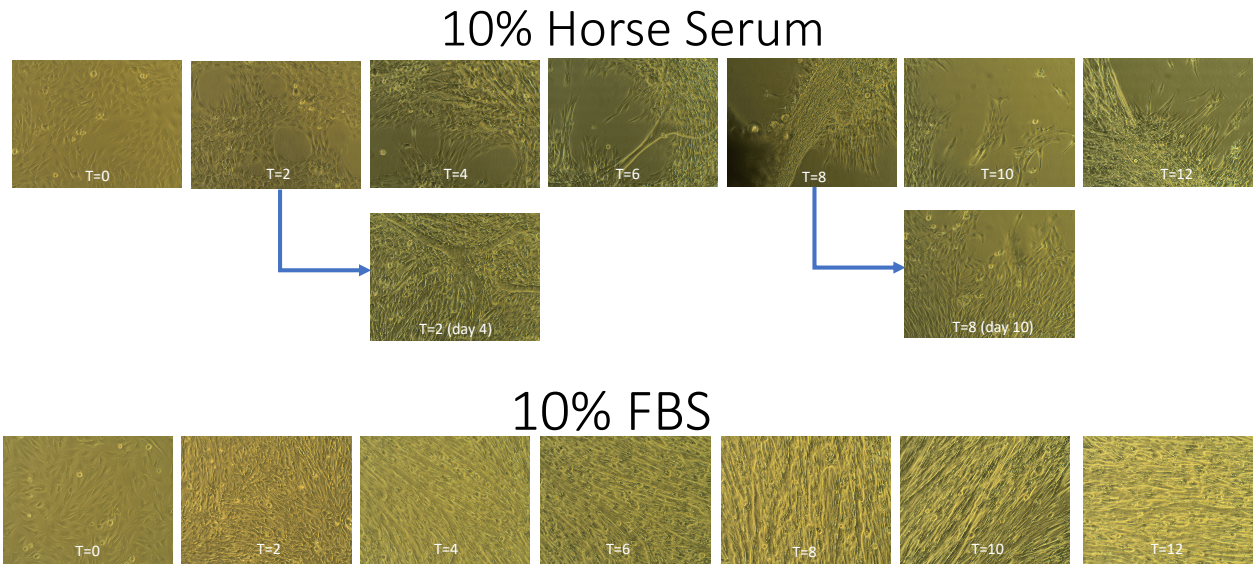


Figure 10: Morphology of 10% Horse Serum culture experiment also showing switch-back into 10% FBS and compared to 10% FBS control.

3.2.2 1% Horse Serum (HS)

In this experiment cells were first grown to confluency in 10% FBS. They were then changed into 1% HS and allowed to grow for a 12-day time course study. A switch-back experiment was also done on days 2 and 8 where cells were switched back into 10% FBS and observed 2 days later. Cells are observed to differentiate normally in the early time points as observed in Figure 10 below. However, differentiation is not observed in the normal robust nature as in 10% FBS as it is in 1% HS. Differentiation occurs in t=4,

as observed in Figure 10, but as the experiment progresses myotube development is seen but not in the robust nature that is expected in these late time points. Comparing the 1% HS at t=12 in Figure 10 to the 10% FBS at t=12 this is more easily seen. When looking at the switch-back experiments a change in morphology is not as apparent when comparing 1% HS t=6 and the t=2 switch images in Figure 10.

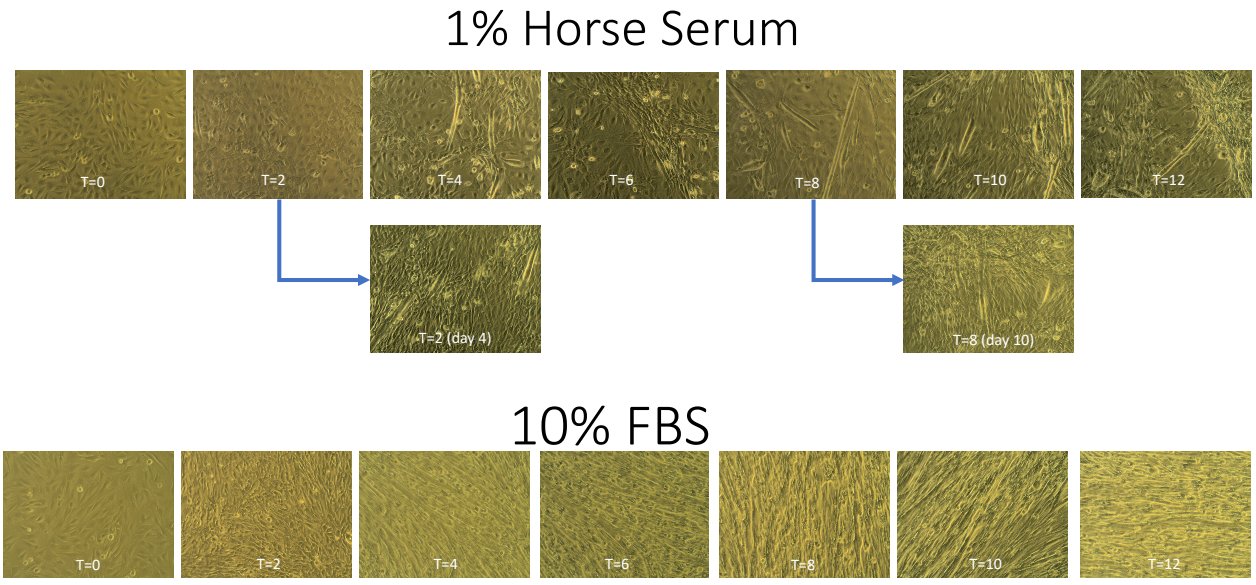


Figure 11: Morphology of 1% Horse Serum culture experiment also showing switch-back into 10% FBS and compared to 10% FBS control.

3.2.3 1% Fetal Bovine Serum (FBS)

In this experiment cells were first grown to confluency in 10% FBS. They were then changed into 1% FBS and allowed to grow for a 12-day time course study. A switch-back experiment was also done on days 2 and 8 where cells were switched back into 10% FBS and observed 2 days later. Normal morphology is observed in early time points as well as in the later time points, just not in the robust nature that is normally observed in 10% FBS. Cells were shown to differentiate beginning at t=4, consistent with

the control which can be seen in Figure 11. Myotube development was observed beginning at t=6 and continuing through the time course study. However, the myotube development was not seen in the normal robust nature as in the 10% control. There is even a layer of myoblasts observed along with the myotubes, seen in Figure 11 at t=10.

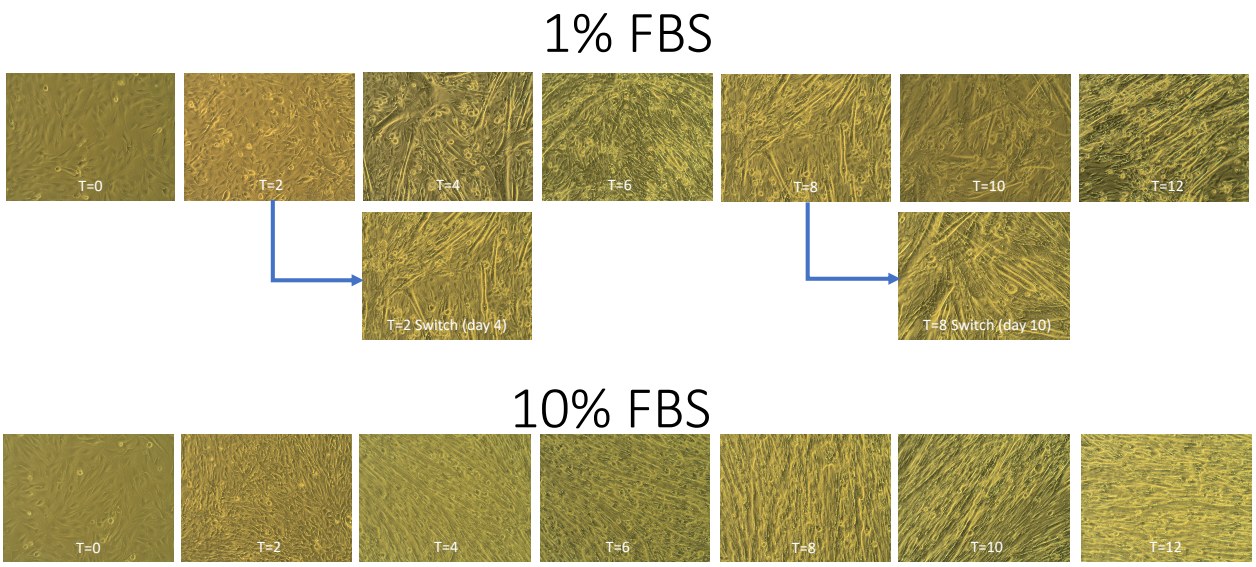


Figure 12: Morphology of 1% FBS culture experiment also showing switch-back into 10% FBS and compared to 10% FBS control.

3.2.4 PC1

In this experiment cells were first grown to confluency in 10% FBS. They were then changed into PC1 and allowed to grow for a 12-day time course study. A switch-back experiment was also done on days 2 and 8 where cells were switched back into 10% FBS and observed 2 days later. Cells were grown in this media to determine whether a complete defined media would be a successful culture environment for myogenesis. Initial morphology at t=0 and t=2, as shown in Figure 12, is consistent with 10% FBS controls. However, at t=4 when differentiation normally begins, there is a shift in morphology. Cells began to grow in a sheet like formation or fuse into abnormal clumps of cells. This morphology is best seen at t=6 and t=10 in Figure 9. In the switch back

experiments cells appear normal in t=2 switch images shown in Figure 12. The t=8 switch back experiment however did not show normal morphology at t=8 and when switched back into 10% FBS recovered the reduction in density but their morphology remained abnormal.

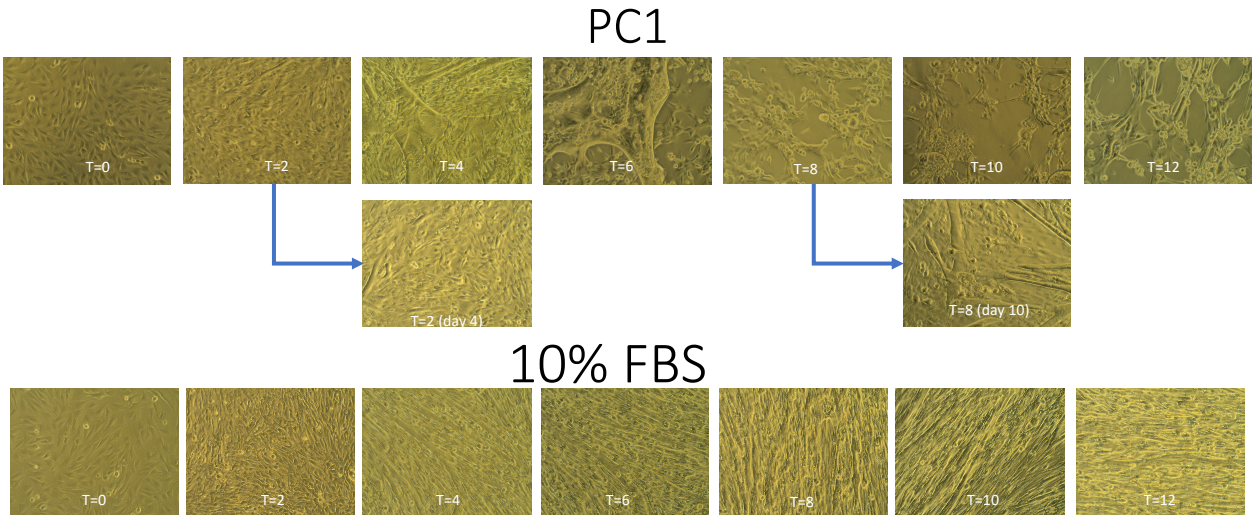


Figure 13: Morphology of PC1 culture experiment also showing switch-back into 10% FBS and compared to 10% FBS control.

3.3 Genetic Expression

3.3.1 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) of 10% Horse Serum Cultures

Each time point (t=0,2,4, etc) and switch back experiments (t=2&8 switch) cells were harvested and RNA was extracted using Qiagen RNeasy kit and quantified using the NanoDrop spectrophotometric system. Quantified RNA was then utilized in qRT-PCR to analyze gene expression of specific sarcomeric proteins and transcription factors all normalized to the housekeeping gene GAPDH. MyoD was one of these genes of interest, as it is an integral transcription factor for the initiation of myogenesis. In normal expression MyoD is seen at low levels of expression t=2 days past confluency where there is an increase in expression as cells leave the cell cycle and begin differentiation.

Once myotubes have fully developed MyoD is down regulated to homeostatic levels. This is seen in the 10% Horse Serum experiment, suggesting that myogenesis initiated normally. This is readily seen in figure 13 below.

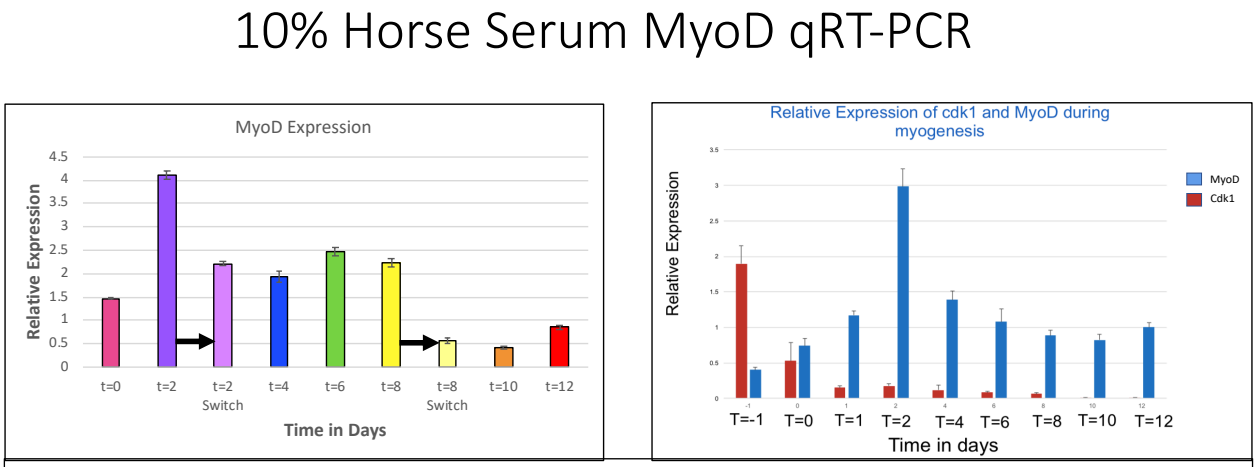


Figure 14: Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) of MyoD in 10% Horse Serum cultures compared to known expression profile (Nyaboke, R., et al., 2017)

Each time point (t=0,2,4, etc) and switch back experiments (t=2&8 switch) cells were harvested and RNA was extracted using Qiagen RNeasy kit and quantified using the NanoDrop spectrophotometric system. Quantified RNA was then utilized in qRT-PCR to analyze gene expression of specific sarcomeric proteins and transcription factors all normalized to the housekeeping gene GAPDH. Titin N2B was one of the genes of interest as titin is an important sarcomeric protein. In normal expression titin is seen at low levels until t=4 days past confluency as cells have since left the cell cycle and induced myogenesis to begin. Titin expression remains at a relatively high plateau throughout mature cultures as it is integral to myotube function. This expression profile is not seen as dramatically in 10% Horse Serum cultures. Suggesting that differentiation and myotube formation did not occur normally, this is easily seen in figure 14 below.

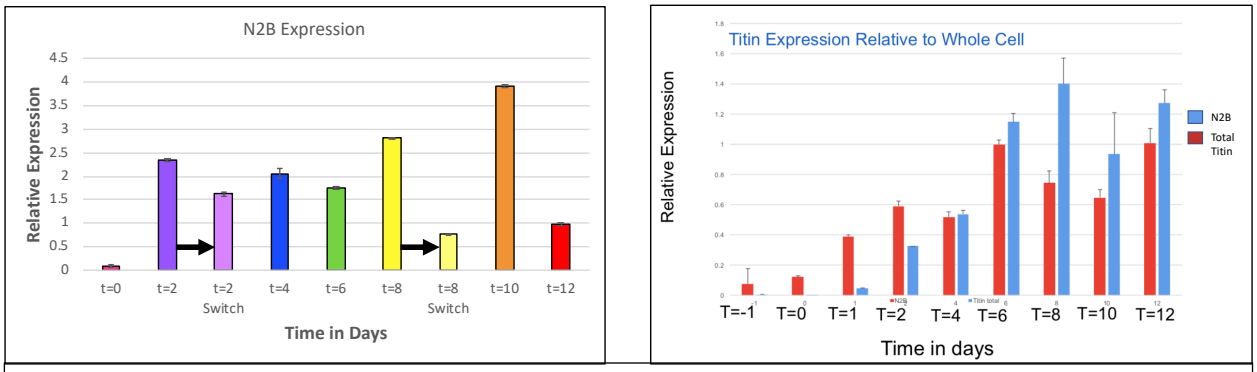


Figure 15: Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) of Titin N2B in 10% Horse Serum cultures compared to known expression profile (Nyaboke, R., et al., 2017)

3.3.2 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) of 1% Horse Serum Cultures

Each time point (t=0,2,4, etc) and switch back experiments (t=2&8 switch) cells were harvested and RNA was extracted using Qiagen RNeasy kit and quantified using the NanoDrop spectrophotometric system. Quantified RNA was then utilized in qRT-PCR to analyze gene expression of specific sarcomeric proteins and transcription factors all normalized to the housekeeping gene GAPDH. MyoD was one of these genes of interest, as it is an integral transcription factor for the initiation of myogenesis. In normal expression MyoD is seen at low levels of expression t=2 days past confluency where there is an increase in expression as cells leave the cell cycle and begin differentiation. Once myotubes have fully developed MyoD is down regulated to homeostatic levels. This expression profile is not seen in 1% Horse Serum cultures. The expression profile increases at t=2, as expected, and reduces at t=4 but instead of remaining at homeostatic levels expression increases again. This data is easily seen in Figure 15 below. This

expression profile suggests that myogenesis was successfully turned on and MyoD was down regulated, however expression increased again after t=4, suggesting a restarting of the myogenic program.

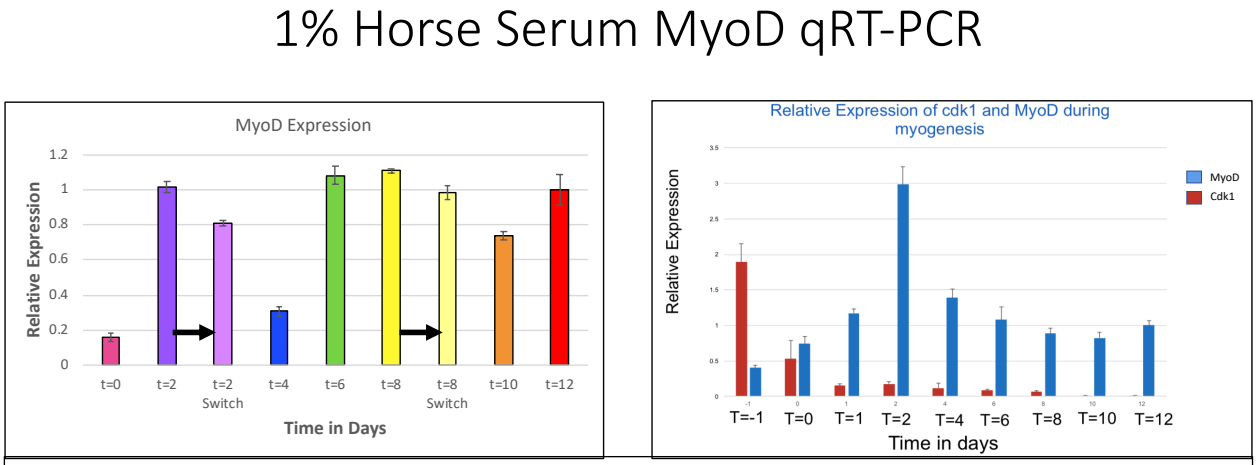


Figure 16: Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) of MyoD in 1% Horse Serum cultures compared to known expression profile (Nyaboke, R., et al., 2017)

Each time point (t=0,2,4, etc.) and switch back experiments (t=2&8 switch) cells were harvested and RNA was extracted using Qiagen RNeasy kit and quantified using the NanoDrop spectrophotometric system. Quantified RNA was then utilized in qRT-PCR to analyze gene expression of specific sarcomeric proteins and transcription factors all normalized to the housekeeping gene GAPDH. Titin N2B was one of the genes of interest as titin is an important sarcomeric protein. In normal expression titin is seen at low levels until t=2/4 days past confluency as cells have since left the cell cycle and induced myogenesis to begin. Titin expression remains at a relatively high plateau throughout mature cultures as it is integral to myotube function. This expression profile is not seen in 1% Horse Serum cultures. Titin expression increases as expected at t=4 but dramatically reduces at t=6 and remains at low expression levels throughout the rest of the time points, seen in Figure 16 below.

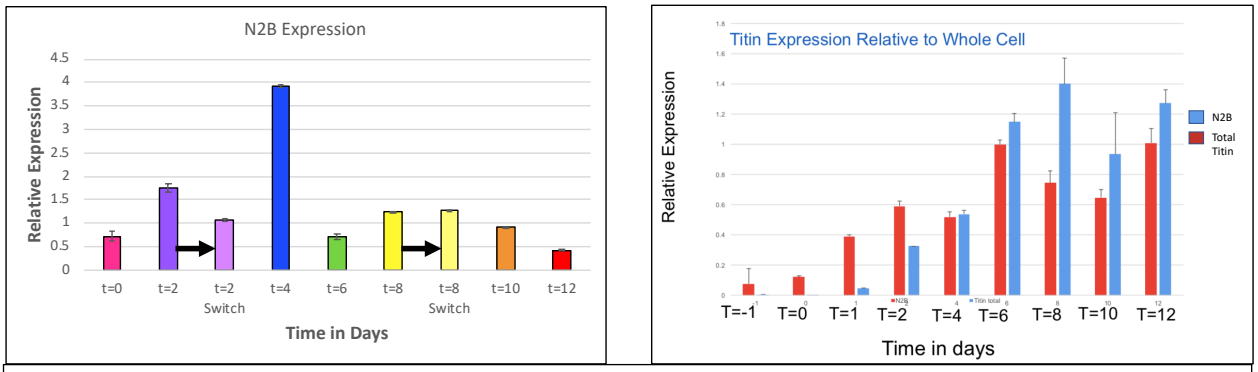


Figure 17: Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) of Titin N2B in 1% Horse Serum cultures compared to known expression profile (Nyaboke, R., et al., 2017)

3.3.3 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) of 1% Fetal Bovine Serum Cultures

Each time point (t=0,2,4, etc) and switch back experiments (t=2&8 switch) cells were harvested and RNA was extracted using Qiagen RNeasy kit and quantified using the NanoDrop spectrophotometric system. Quantified RNA was then utilized in qRT-PCR to analyze gene expression of specific sarcomeric proteins and transcription factors all normalized to the housekeeping gene GAPDH. MyoD was one of these genes of interest, as it is an integral transcription factor for the initiation of myogenesis. In normal expression MyoD is seen at low levels of expression t=2 days past confluency where there is an increase in expression as cells leave the cell cycle and begin differentiation. Once myotubes have fully developed MyoD is down regulated to homeostatic levels. This expression profile is, somewhat, seen in the 1% FBS cultures with the exception of t=0 having a high level of expression. This is seen in Figure 17 below.

1% FBS MyoD qRT-PCR

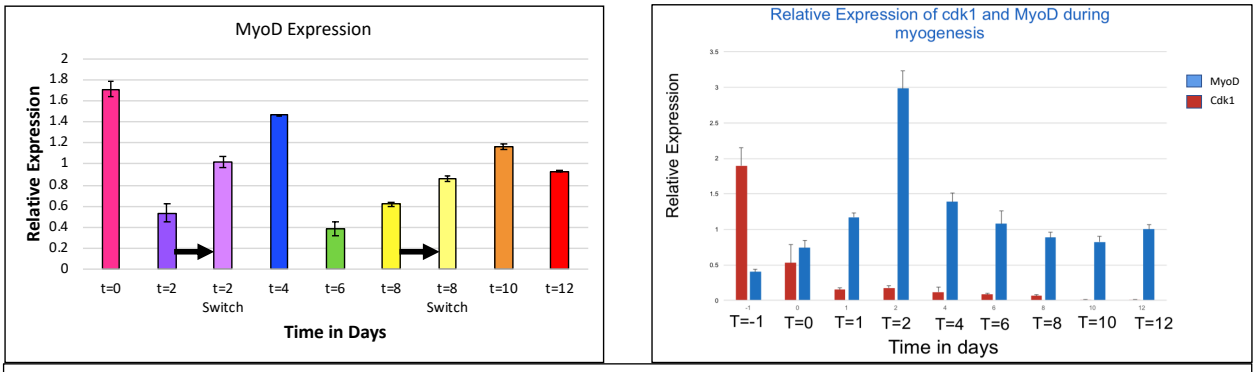
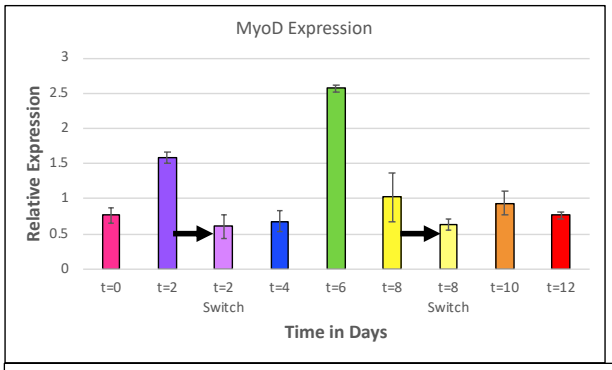


Figure 18: Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) of MyoD in 1% FBS cultures compared to known expression profile (Nyaboke, R., et al., 2017)

Each time point (t=0,2,4, etc.) and switch back experiments (t=2&8 switch) cells were harvested and RNA was extracted using Qiagen RNeasy kit and quantified using the NanoDrop spectrophotometric system. Quantified RNA was then utilized in qRT-PCR to analyze gene expression of specific sarcomeric proteins and transcription factors all normalized to the housekeeping gene GAPDH. Titin N2B was one of the genes of interest as titin is an important sarcomeric protein. In normal expression titin is seen at low levels until t=2/4 days past confluency as cells have since left the cell cycle and induced myogenesis to begin. Titin expression remains at a relatively high plateau throughout mature cultures as it is integral to myotube function. This expression profile is seen in 1% FBS cultures, which can be observed in Figure 18 below.

1% FBS N2B qRT-PCR



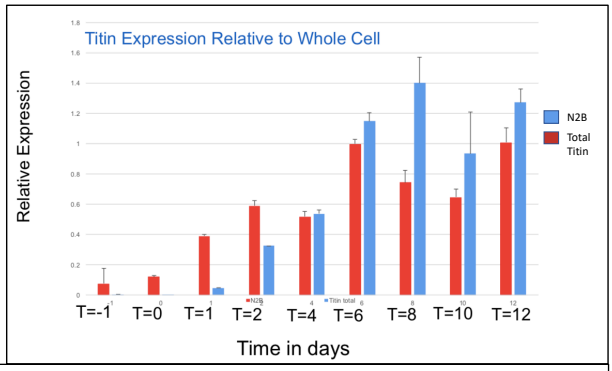


Figure 19: Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) of Titin N2B in 1% FBS cultures compared to known expression profile (Nyaboke, R., et al., 2017)

3.3.4 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) of PC1.

Each time point (t=0,2,4, etc) and switch back experiments (t=2&8 switch) cells were harvested and RNA was extracted using Qiagen RNeasy kit and quantified using the NanoDrop spectrophotometric system. Quantified RNA was then utilized in qRT-PCR to analyze gene expression of specific sarcomeric proteins and transcription factors all normalized to the housekeeping gene GAPDH. MyoD was one of these genes of interest, as it is an integral transcription factor for the initiation of myogenesis. In normal expression MyoD is seen at low levels of expression t=2 days past confluency where there is an increase in expression as cells leave the cell cycle and begin differentiation. Once myotubes have fully developed MyoD is down regulated to homeostatic levels. This expression profile is not seen in PC1 cultures, expression increases at t=2, as expected but fluctuates throughout the entire experiment, this is seen in Figure 19 below.

PC1 RT-qPCR MyoD normalized to GAPDH

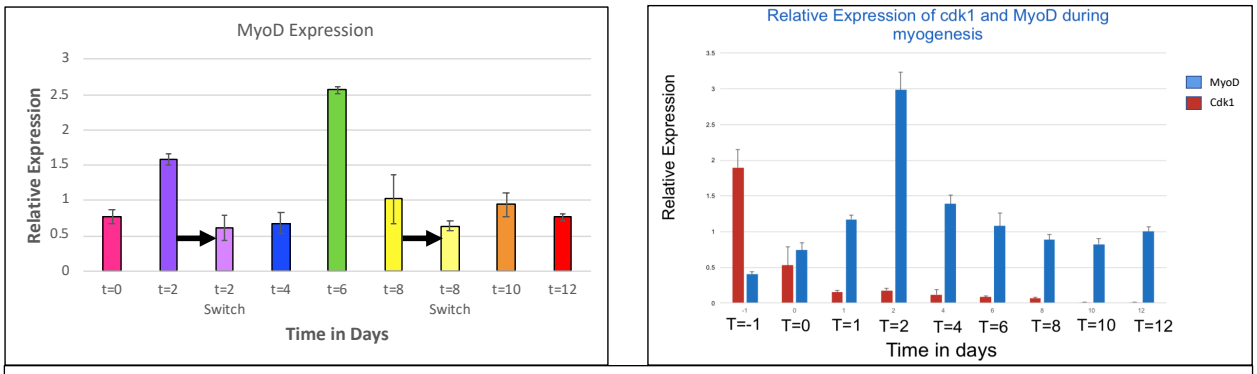


Figure 20: Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) of MyoD in PC1 cultures compared to known expression profile (Nyaboke, R., et al., 2017)

Each time point (t=0,2,4, etc.) and switch back experiments (t=2&8 switch) cells were harvested and RNA was extracted using Qiagen RNeasy kit and quantified using the NanoDrop spectrophotometric system. Quantified RNA was then utilized in qRT-PCR to analyze gene expression of specific sarcomeric proteins and transcription factors all normalized to the housekeeping gene GAPDH. Titin N2B was one of the genes of interest as titin is an important sarcomeric protein. In normal expression titin is seen at low levels until t=2/4 days past confluency as cells have since left the cell cycle and induced myogenesis to begin. Titin expression remains at a relatively high plateau throughout mature cultures as it is integral to myotube function. This expression profile is

not seen at all in PC1 cultures. This expression profile is seen in Figure 20 below.

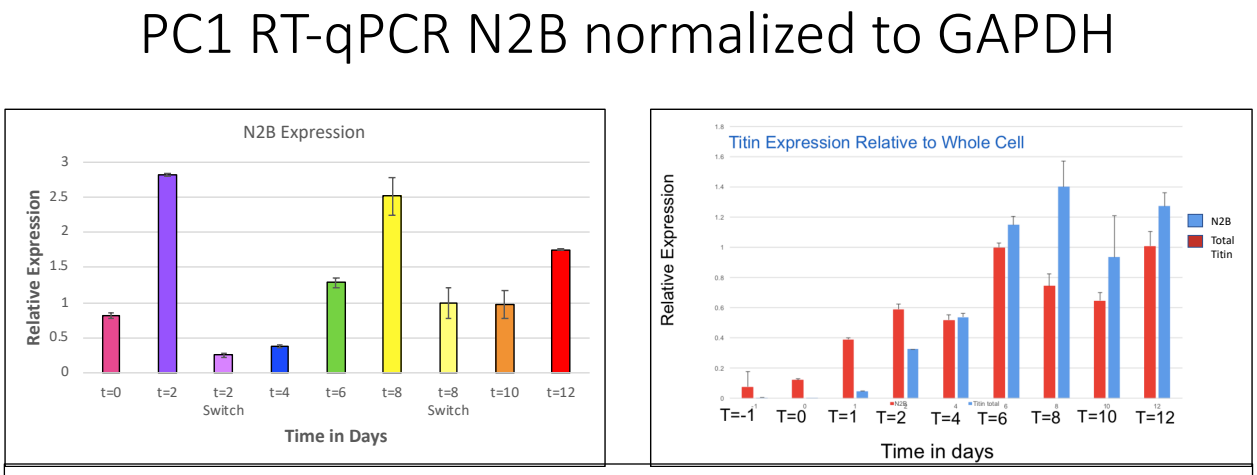


Figure 21: Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) of Titin N2B in PC1 cultures compared to known expression profile (Nyaboke, R., et al., 2017)

Chapter 4 Discussion:

Myogenesis is tightly regulated via transcription factors. An important class of transcription factors regulating myogenesis are the Myogenic Regulatory Factors (MRFs). The first MRF expressed in myogenesis is Myf5, closely followed by MyoD (Volpi, M. V., & Hughes, S. M., 2017). The expression profile of MyoD at the onset of myogenesis will be high and will incrementally decrease as other MRFs take over the myogenic program. Once myogenesis has completed MyoD will be down regulated to homeostatic levels. As myogenesis is completed the differentiation program takes over. One of the hallmarks of muscle is the giant muscle protein titin. As muscle develops genetic expression of titin increases steadily until the muscle has fully developed at which time expression plateaus (Kruger & Linke, 2011).

The purpose of this study was to determine how a changing culture condition affects the myogenic and differentiation programs. The aim was to utilize three new media conditions, fetal bovine serum in low concentrations, horse serum in high and low concentrations and PC1, a serum-free media. The hypothesis was that changing the conditions from our control of 10% fetal bovine serum will alter the morphology and genetic expression of C2C12 cells. The data supports that hypothesis.

4.1 Flow Cytometry Analysis of C2C12 Cell Cycle

Previous work done on C2C12 cells indicates that at early time points, pre differentiation, cells express high levels of cdk1 which is a key factor in cell cycle regulation. Expression of cdk1, after confluency is achieved, is dramatically reduced which indicates the cells leave the cell cycle and begin differentiation. To confirm this analysis of the cell cycle utilizing flow cytometry was done at a pre-confluency, 24 hours

post thaw, and confluency, 72 hours post thaw. At each time point cells were trypsinized and stained with DyeCycle Green, a DNA dye used for cell cycle analysis. A histogram of known cell cycle patterns when utilizing DyeCycle Green is provided by Thermofisher Scientific (Figure 6). This histogram is used to compare C2C12 flow cytometry results to a known pattern.

4.1.1 Flow Cytometry Analysis of C2C12 Cell Cycle: 15% Confluent

C2C12 cells were thawed and allowed to grow for 24 hours. At 24 hours the cells were 15% confluent, meaning that cells covered approximately 15% of the cell culture plate. The histogram produced at 15% confluency shows that cells are mostly in the G0/G1 phase of the cell cycle, indicating the cells are in the growth phase of the cell cycle. There were also a significant number of cells in the S phase, synthesizing DNA, and the G2/M phase of the cell cycle. This further indicates at early time points, before confluency are actively in the cell cycle.

4.1.2 Flow Cytometry Analysis of C2C12 Cell Cycle: 100% Confluent

C2C12 cells were allowed to grow to confluency which is 72 hours after thawing. At confluency the cdk1 expression is seen to decrease indicating the cells have left the cell cycle. Analysis of C2C12 cells at confluency using flow cytometry showed that all cells were in the G0/G1 phase of the cell cycle. This indicates that the cells have paused the cell cycle at G0/G1. This pause indicates the cells have left the cell cycle which confirms the assumption that a decrease in cdk1 expression correlates to cells leaving the cell cycle.

4.2 C2C12 Cell Culture

The protocol for cell culture of C2C12 cells has been established since the late 1970s. The common practice of C2C12 cell line culturing is to culture the cells in 10% fetal bovine serum, as a growth media, and after confluency switching into a serum environment of 1-8% horse serum (Aylor & Williams, 2011). Previous work has shown that a switch into a differential media is not necessary and C2C12 cells grow and differentiate robustly in 10% fetal bovine serum. To confirm this and understand the optimal media conditions for C2C12 cells an experiment using 10% fetal bovine serum as a growth media and 3 experimental, differential, media was done. The experimental media used was 1% fetal bovine serum, 10% horse serum, 1% horse serum and a serum-free media PC1. Cells were cultured in 9 flasks labeled t=0,2,4,6,8,10,12,2 switch, and 8 switch. Cells were grown until confluent in 10% fetal bovine serum and then switched into experimental media. Cell culturing was successful as there was no contamination observed within the media.

4.3 C2C12 Cell Morphology

Cell morphology was analyzed every 2 days utilizing an Olympus inverted phase-contrast microscope. Images were taken using the SPOT imaging microscope camera and imaging software.

4.3.1 C2C12 Cell Morphology: 10% Horse Serum

At the commencement of the time-course study, each time point`s images were compared to the 10% fetal bovine serum control images. 10% horse serum t=0 morphology is similar to 10% fetal bovine serum`s t=0. At t=2 in 10% horse serum a change is seen and a decrease in cell density is observed. This decrease in cell density is enough to see open spaces between clumps of myoblasts on the images. This low density

and open spacing is seen throughout the rest of the time course study. At t=6 in 10% horse serum myotubes are seen, but not in the density expected when compared to 10% fetal bovine serum. Again, this, along with additional cell clumping, is observed throughout the rest of the time course. Sheets of myotubes, as is expected at t=12 are not observed in 10% horse serum. Myotubes are observed to have formed in t=10 and t=12 of 10% horse serum suggesting that the media conditions still support the differentiation of myotubes but not at the optimal levels of 10% fetal bovine serum. This suggests that a high serum environment of horse serum is not the optimum choice of culture conditions.

4.3.2 C2C12 Cell Morphology: 10% Horse Serum Switch

Cells were switched at t=2 and t=8 from experimental media into the control 10% fetal bovine serum and imaged 2 days post switch to asses if there will be recovery from any potential changes in morphology brought on by the experimental media. When compared to t=2 images the t=2 switch (day 4) cultures the number of cells increased in density and the number of myotubes increased. When analyzing the images of these cultures there was no appearance of gaps between clumps of cells. There appears to have been a recovery from the loss of density seen in t=2 when switch back into 10% fetal bovine serum. A similar observation can be made with the t=8 and t=8 switch cultures. There is an increase in density but no myotubes observed in the images taken. These observations further suggest that a high serum environment of horse serum is not the ideal culture condition for C2C12 cells.

4.3.3 C2C12 Cell Morphology: 1% Horse Serum

At the commencement of the time-course study, each time point`s images were compared to the 10% fetal bovine serum control images. At the onset of the experiment

the early time points, t=0 and t=2, do not differ from 10% fetal bovine t=0 and t=2. As the time-course progresses the cells are observed developing into myotubes while retaining myoblast-like cells beneath and around the developed myotubes. The myotubes that are formed are normal in morphology but at low densities. The myoblasts that are observed are at a higher density than the myotubes. These observations indicate that the low horse serum environment is acceptable for myotube formation, but it does not induce differentiation at the robust nature that is seen in the high fetal bovine serum environment.

4.3.4 C2C12 Cell Morphology: 1% Horse Serum Switch

Cells were switched at t=2 and t=8 from experimental media into the control 10% fetal bovine serum and imaged 2 days post switch to asses if there will be recovery from any potential changes in morphology brought on by the experimental media. When comparing 1% horse serum t=2 and the switch t=2(day 4) there is an increase in the number of myotubes seen. There is no change in overall density observed. When comparing the switch t=2 to 1% horse serum t=4 they are similar but myotubes are more easily observed in the t=2 switch images. Comparing 1% horse serum t=8 and switch t=8 (day 10), there is an increase in the number of myotubes observed. When comparing 1% horse serum t=10 and switch t=8 there is, again, more myotubes present. These observations indicate that a switch to 10% fetal bovine serum in both time points induced more myotube fusion. The observations do not suggest that 1% horse serum is an unacceptable serum environment for C2C12 cells.

4.3.5 C2C12 Cell Morphology: 1% Fetal Bovine Serum

At the commencement of the time-course study, each time point`s images were compared to the 10% fetal bovine serum control images. 1% fetal bovine serum t=0 and t=2 are morphologically similar to t=0 and 2 of 10% fetal bovine serum. This indicates that myoblasts successfully achieved confluency. The early short myotubes are observed at t=2 in 1% fetal bovine serum, similar to t=2 10% fetal bovine serum. At t=4 1% fetal bovine serum begins to exhibit more myotubes similar to 10% fetal bovine serum but at a lower density than when compared. The difference in density is seen throughout the rest of the time course. It does not, however, correlate to a decrease in cell density throughout the culture flask. Below the myotubes, there are myoblast-like cells which may be quiescent myoblasts. Morphologically, the 1% fetal bovine serum cultures grew and differentiated into myotubes in a nature similar to the 10% fetal bovine serum but at a slightly diminished density. This suggests that a low serum environment of fetal bovine serum is appropriate for C2C12 culturing. The diminished density is not enough to alter the density of the cultures overall thus maintaining a healthy culture throughout the whole time-course study.

4.3.6 C2C12 Cell Morphology: 1% Fetal Bovine Serum Switch

Cells were switched at t=2 and t=8 from experimental media into the control 10% fetal bovine serum and imaged 2 days post switch to asses if there will be recovery from any potential changes in morphology brought on by the experimental media. For the 1% fetal bovine serum switch samples no change was observed. When comparing t=2 switch to t=4 the morphology is very similar, as is the t=8 switch when compared to t=10. The switch from 1% fetal bovine serum to 10% fetal bovine serum is not a switch that

disrupts the cells further indicating that 1% fetal bovine serum is an appropriate culture environment for C2C12 growth and differentiation.

4.3.7 C2C12 Cell Morphology: PC1

At the commencement of the time-course study, each time point`s images were compared to the 10% fetal bovine serum control images. PC1 is a serum free media used in the culturing of primary and adherent cell lines. Initially this would suggest that this media would be an ideal serum-free environment for C2C12 culturing. However, when observing the images of cultures grown in PC1 they do not retain morphology that resembles myoblasts or myotubes past t=2. T=0 and t=2 cultures appear normal, but the morphology shifts at t=4, when differentiation normally is induced, and sheet-like cells are viewed as well as clumping of myoblast-like cells. A sheet-like morphology was also observed at t=6. Interestingly, the sheet-like morphology seen at t=4 and t=6 was observed twitching. After t=6 the morphology remains abnormal with cells clumping in small groupings but not resembling myoblasts or myotubes. These observations suggest that PC1 is not an optimal environment for C2C12 culturing.

4.3.8 C2C12 Cell Morphology: PC1 Switch

Cells were switched at t=2 and t=8 from experimental media into the control 10% fetal bovine serum and imaged 2 days post switch to asses if there will be recovery from any potential changes in morphology brought on by the experimental media. When comparing PC1 t=2 and t=2 switch (day 4) there is not an apparent difference between the two cultures. However, when comparing PC1 t=4 and t=2 switch the differences are apparent. PC1 t=4 has an abnormal sheet-like morphology with abnormal myotube-like cells around it. T=2 switch retains the expected morphology of a 4-day old culture.

Comparing PC1 t=8 and t=8 switch (day 10) there is an apparent change in the morphology and density of the culture. PC1 t=8 has a reduced density of cells and morphology is abnormal and does not resemble myoblasts or myotubes. The density of t=8 switch increased and there is what appear to be myotubes beginning to form. This suggests there was a recovery from the PC1 morphology change and that myotubes were able to form due to this. This recovery suggests there may be quiescent myoblasts in culture. These observations further indicate that PC1 is not an appropriate culture environment for C2C12 cells.

4.4 Genetic Expression of C2C12 Cells

At each time point cells were pelleted for RNA isolation. The RNA was then used for quantitative real-time polymerase chain reaction (qRT-PCR). The qRT-PCR results were compared to previous work done to characterize gene expression in C2C12 cells using MyoD, a myogenic regulatory factor, and titin, an important structural and regulatory protein in muscle. The normal expression profile for MyoD in C2C12 cells is high levels of expression at early time points with a decrease in expression once confluency has been achieved which turns on the myogenic differentiation program. The normal expression profile for titin in C2C12 cells is the opposite of the MyoD expression profile. Titan expression is low in early time points when differentiation has not yet occurred and is upregulated when confluency is achieved, and differentiation begins. Genetic expression analysis of cultures in experimental conditions was Done to complement the morphology study and to confirm myogenesis completion and successful differentiation. One fallback of gene expression studies is that gene expression does not

necessarily correlate to protein translation in the case of titin. So, while the expression levels may be as expected there may not be a resulting translation of protein.

4.4.1 Genetic Expression of C2C12 Cells: 10% Horse Serum & 10% Horse Serum Switch

Genetic expression utilizing qRT-PCR was done on each time point of the 10% horse serum experiment. Observing the time course overall genetic expression of MyoD the expression is high at early time points and decreases after t=2, when differentiation normally begins. This, in addition to the morphological data, indicates that myogenesis progressed in a normal fashion. Analysis of the titin expression for the 10% horse serum cultures also follows the expected expression pattern fairly closely, with the only discernable difference being an increase in expression at t=2, when it is expected at t=4. This in and of itself does not indicate any abnormalities within the cultures. The gene expression remains normal throughout the rest of the time course which indicates that this datapoint is slightly outside the norm, not abnormal.

Gene expression for the two switch experiments was also done. The t=2 switch (day 4) genetic expression when compared to t=4 expression has no significant difference indicating the switch to 10% fetal bovine serum did not have an effect on gene expression at those time points. When comparing t=8 (day 10) gene expression for MyoD to t=10 there is also no difference between the two time points, indicating that the switch does not have an effect on MyoD expression. When comparing titin expression however, there is a difference between t=8 switch and t=10. The expression at t=10 is significantly higher when compared to t=8 switch. This can be explained by the changing conditions. A switch into a new environment may shock the cells for a period of time and cause them

to exhibit an altered genetic expression profile. This is an avenue that will need to be further investigated in the future. Overall the genetic data does not suggest that 10% horse serum is not an appropriate culture condition for C2C12 cells.

4.4.2 Genetic Expression of C2C12 Cells: 1% Horse Serum & 1% Horse Serum Switch

Genetic expression utilizing qRT-PCR was performed on the 1% horse serum experiment RNA, which was isolated at each time point. MyoD was the first set of qRT-PCR data that was analyzed. The MyoD expression does not follow the known expression pattern. The expected pattern should see a down regulation at the beginning of differentiation, which usually occurs at or near t=4. This data when combined with the morphological study, indicates there are quiescent myoblasts in culture. Confirmation of this will require further studies. Titin expression in 1% horse serum cultures begins to follow the normal expression profile with an increase at t=4, however there is a decrease in expression earlier than is expected but this is not indicative of any serious changes within the culture. The change in expression can be explained by an overexpression in t=4 which is then normalized in later time points. Overall this data does not indicate that 1% horse serum is an un

Analysis on the switch experiment gene analysis was done as well. When comparing t=2 switch (day 4) and t=4 MyoD expression, there is a difference in expression, this is not unexpected as differentiation normally progresses at t=4 so a decrease in MyoD at t=4 is expected. What is unexpected is that the t=2 switch expression did not decrease as well, this could indicate that the change to 10% fetal bovine serum shocked the cells, resulting in a pause in the expected timeline of

differentiation. T=8 switch (day 10) of the MyoD expression does not change appreciably when compared to t=10. Investigation of the t=2 switch (day 4) titin expression, comparing to t=4, shows an obvious difference, again indicating the switch to 10% fetal bovine serum causes a shock to the culture. T=8 switch (day 10) when compared to t=10 does not show much of a change, the expression is almost equal to t=8 expression. A longer analysis of the effects of the switch into 10% fetal bovine serum from experimental media would need to be done to confirm the analyses made and to further understand how the switch effects C2C12 development.

4.4.3 Genetic Expression of C2C12 Cells: 1% Fetal Bovine Serum & 1% Fetal Bovine Serum Switch

Genetic expression studies on 1% fetal bovine serum cultures was done to analyze both MyoD and titin expression which is then compared to the known expression profile of C2C12 cells. When observing the MyoD expression profile of the 1% fetal bovine serum cultures it follows the expected expression profile that was previously seen. Expression is high in early timepoints and as confluency is achieved at t=2, expression is downregulated. When observing the titin N2B expression profile we, again, see the expected expression profile. Titin expression is low in early timepoints and is upregulated as confluency is achieved and the differentiation program is turned on.

Switch cultures were also utilized in gene expression studies. When analyzing t=2 switch (day 4) cultures the genetic expression for both MyoD and titin is similar, indicating the change from 1% fetal bovine serum to 10% fetal bovine serum did not affect genetic expression. This is also seen in the t=8 (day 10) expression when compared to t=10 expression. This genetic expression data confirms the observations from the

morphological study that 1% fetal bovine serum is an adequate substitute for C2C12 cultures.

4.4.4 Genetic Expression of C2C12 Cells: PC1 & PC1 Switch

Genetic expression studies on PC1 cultures of C2C12 cells was done to investigate if there were any changes in gene expression when utilizing experimental media. MyoD expression of PC1 cultures was very different from the expected expression profile. The expression of MyoD remained relatively high throughout the experiment and increased at later time points (t=10). This diversion from the expected expression profile indicates that myoblasts did not successfully leave the developmental program and continued to express MyoD unexpectedly. When analyzing the titin expression of PC1 cultures the expression profile was, again, abnormal. Titin expression remained relatively low with peaks at later timepoints (t=10). This when combined with the abnormal morphology seen further indicates that PC1 induces an abnormal morphology and is not an appropriate culture environment for C2C12 cultures.

Switch cultures were also analyzed for gene expression of MyoD and titin. Given that the expression profile of the PC1 cultures without the switch to 10% fetal bovine serum was abnormal, it is difficult to compare the results. It is unknown whether the change to 10% fetal bovine serum had any shock effect on the cells or if there was any positive effect on the cells based on the gene expression analysis. If the analysis is compared with the morphological data it can be assumed that there was some recovery of cells but this cannot be correlated in the gene expression data.

4.5 Future Work

Future work on C2C12 cells should focus on the effects of aging in culture by extending the time course study past 2 weeks. Additionally, further understanding of how switching serum in early and late time points from experimental sera should be investigated by utilizing additional time points.

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Appendix 1: Materials.

Material	Manufacturer	Catalog Number
C2C12 cells	ATCC	CRL-1772
Fetal Bovine Serum	ATCC	30-2020
Horse Serum	Atlanta Biologicals	s12150
PC1	Lonza	77232
DMEM	ATCC	30-2002
Trypsin	SIGMA-ALDRICH	T4049
Penicillin/Streptomycin	Thermo-Fisher Scientific	15140122
T25 culture flasks	Thermo-Fisher Scientific	156367
T75 culture flasks	Thermo-Fisher Scientific	156499
MilliporeSigma Stericup Quick Release-HV Vaccum Filtration System	Fisher Scientific	S2HVU11RE
50mL graduated serological pipet	Thermo-Fisher Scientific	13-678-36E
25mL graduated serological pipets	Thermo-Fisher Scientific	13-678-36D
10mL graduated serological pipets	Thermo-Fisher Scientific	13-678-36C
5mL graduated serological pipets	Thermo-Fisher Scientific	13-678-36B
1000microL pipet tips	Gene-Mate	P-1237-1250
200microL pipet tips	Thermo-Fisher Scientific	2707419
10microL pipet tips	MULTIMAX	P-3243-10X
Nitrile gloves	Thermo-Fisher Scientific	19-130-1597B

NanoDrop Spectrophotometer	Thermo-Fisher Scientific	ND-ONEC-W
Hot water bath	VWR	
Air-Jacketed CO_2 Incubator 8.5A	Thermo-Fisher Scientific	
LH 50A Inverted Phase Contrast Microscope	Olympus	
Microentrifuge 5430	eppendorf	22620509
One Step RT-PCR Kit	QIAGEN	210210
qRT-PCR machine	BioRad	
QIAGEN RNeasy RNA isolation kit	QIAGEN	74104
QIAGEN shredder columns	QIAGEN	79654